Université de Montréal

Molecular and Functional studies of human immunodeficiency virus type 1 accessory protein Vpr

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophie Doctoral (Ph.D)
En virologie et immunologie

Dec, 2006

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Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

Molecular and Functional studies of human immunodeficiency virus type 1 accessory protein Vpr

présenté par :

Yong Xiao

A été évaluée par un jury composé des personnes suivantes

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ABSTRACT

The 96-amino acid Vpr protein encoded by HIV-1 performs multiple functions during the retroviral life cycle, including the enhancement of viral replication in macrophages at early stage of viral replication, the induction of G2 cell cycle arrest in proliferating T lymphocytes, and the modulation of HIV-1-induced apoptosis. In addition, extracellular full-length and processed forms of Vpr have been previously detected in the sera and cerebral spinal fluids of HIV-infected patients. However, the mechanism underlying this processing and its implication for HIV-1 pathogenesis remain unknown.

The first goal of this thesis was to investigate the mechanism of Vpr release and processing during HIV-1 infection. Herein, we report that full-length and several cleaved species of Vpr could be detected in the culture media of HIV-1 expressing cells, independently of Vpr virion incorporation. Truncated forms of Vpr were abundant in the extracellular medium from HIV-producing cells but not from cells expressing Vpr alone. Moreover a small portion of cleaved Vpr was found to be associated with the external cell surface of HIV-producing cells through binding with cell surface heparin sulphate proteoglycans. Mutagenesis and mass spectrometry analyses indicated that Vpr was processed at its C-terminus after the highly conserved $R_{85}QRR_{88}$ motif, a putative pair-basic proprotein convertase (PC) cleavage site. Consistently, the PC peptide inhibitor dec-RVKR-cmk and the serine protease inhibitors (α1-PDX and Spn4A) specifically inhibited extracellular Vpr processing. Transient expression of proprotein convertases PC5A and PACE4 and to a lower extent furin increased extracellular Vpr processing, strongly suggesting that Vpr is processed by proprotein convertases. We provide evidence suggesting that Vpr was processed in the extracellular medium through PCs that are cell surface associated. Finally, the truncated Vpr protein was defective for the induction of cell cycle arrest and apoptosis, suggesting that Vpr

proteolytic processing might be a cellular mechanism to control the level of functionally active extracellular Vpr during HIV-1 infection.

The second goal of this thesis was to investigate Vpr-interacting proteins within HIV-1 virion particles and their functional relevance. Vpr early functions are closely related to its specific virion incorporation. Vpr localization within the virion core and its association with the pre-integration complex suggest a role for Vpr in the early phases of HIV infection. However, little is known about Vpr interactions with other viral components in the virion particles and their functional relevance. To address this question, we constructed an infectious molecular clone of HIV-1 expressing HA-tagged Vpr and we isolated purified virions containing HA-Vpr. Analysis of anti-HA co-immunoprecipitated protein complexes by proteomic or western blot approaches revealed that Vpr could form a complex with the matrix protein (MA) within viral particles produced from various human cell lines. Furthermore, the MA-Vpr interaction was shown to occur independently of the presence of RT and IN and could be detected by *in vitro* GST pulldown experiments using recombinant Vpr and purified GST-fused MA proteins. These results indicate that the Vpr-MA association involves a direct interaction. The respective interacting domains were mapped by *in vitro* binding assays. We found that the fifth alpha helix of MA (residues 97-108), and the arginine-rich C-terminal domain of Vpr (residues 86-96) were implicated in the Vpr-MA interaction. Since Vpr and MA are karyophilic proteins, and are both components of the pre-integration complex (PIC), their interaction might have a synergistic effect in the nuclear targeting of PIC and could contribute to the efficiency of viral infection during the early stages of HIV-1 infection.

It is important to investigate how host and viral factors interact to establish HIV-1 infection in human cells. Vpr has been shown to contribute to HIV-1 infection in human cells when it is present as an extracellular species as well as a virion-associated species. Here, we identified a cellular protease that regulates extracellular Vpr activity and characterized Vpr

interacting-proteins within virion particles. The present study might contribute to a better understanding of Vpr early functions during HIV-1 viral replication and might provide new targets for therapeutic intervention.

Key words: extracellular Vpr; proteolytic processing; proprotein convertase; HIV-1; virion; matrix protein; HA-tagged Vpr provirus

RÉSUMÉ

La protéine Vpr codée par le rétrovirus VIH-1 est une protéine de 96 acides aminés qui remplit de multiples fonctions au cours du cycle réplicatif du virus, comme l'augmentation de la réplication virale dans les macrophages primaires aux stades précoces de l'infection, l'arrêt du cycle cellulaire en phase G2 dans les lymphocytes T en division ou encore la régulation de l'apoptose induite par le VIH-1. En plus de la forme sauvage, des formes tronquées de Vpr avaient été précédemment détectées dans le sérum et le fluide cérébro-spinal de patients infectés par le VIH. Toutefois, le mécanisme de ce clivage ainsi que son rôle dans la pathogènèse du VIH-1 restent inconnus.

Le but premier de cette étude était de caractériser le mécanisme par lequel Vpr est modifié et relâché dans le milieu extracellulaire au cours d'une infection par le VIH-1. Nous rapportons ici que la protéine sauvage ainsi que plusieurs formes tronquées de Vpr ont pu être détectées dans le milieu de culture des cellules exprimant VIH-1 indépendamment de l'incorporation de Vpr dans les particules virales. Les formes tronquées de Vpr étaient abondantes exclusivement dans le milieu extracellulaire des cellules exprimant VIH-1 et non de celles exprimant Vpr seul. De plus, une faible fraction de Vpr clivée s'associe à la surface extracellulaire de la membrane plasmique, grâce à la présence de protéoglycanes contenant des chaînes d'héparine sulfate, des cellules exprimant le VIH-1. Des études de mutagènèse dirigée et de spectrométrie de masse ont montré que Vpr est clivé à l'extrémité C-terminale, en aval du motif hautement conservé $R_{85}QRR_{88}$, un site de clivage putatif spécifique des proprotéine convertases (PC). Le peptide dec-RVKR-cmk, un inhibiteur peptidique des protéine convertases, ainsi que les inhibiteurs des sérine protéases α1-PDX et Spn4A inhibent spécifiquement le clivage extracellulaire de Vpr. L'expression transitoire des proprotéines convertases PC5A et

PACE4 et dans une moindre mesure de la furine favorise le clivage extracellulaire de Vpr, suggérant ainsi fortement que Vpr est clivé par les proprotéines convertases. Nous avons apporté la preuve que Vpr était clivé dans le milieu extracellulaire par des proprotéines convertases associées à la surface cellulaire. Enfin, les formes tronquées de Vpr se sont révélées déficientes pour l'arrêt du cycle cellulaire et l'induction de l'apoptose, suggérant ainsi que le clivage protéolytique de Vpr peut être un mécanisme cellulaire destiné à réguler le niveau des formes extracellulaires fonctionnelles de Vpr au cours d'une infection par le VIH-1.

Le second objectif de cette thèse est de caractériser les protéines interagissant avec Vpr au sein des particules virales ainsi que leur fonctionnalité. Le rôle fonctionnel de Vpr est étroitement relié à son incorporation spécifique au sein des particules virales. Sa localisation dans le core viral et son association au complexe de préintégration (PIC) suggèrent un rôle de Vpr dans les étapes précoces de l'infection par le VIH. Toutefois, peu de données sont disponibles sur le mécanisme d'interaction de Vpr avec d'autres partenaires viraux au sein des particules virales ainsi que sur la signification fonctionnelle de telles interactions. Afin de résoudre ces questions, nous avons construit un clone moléculaire infectieux de VIH-1 exprimant Vpr en fusion avec le peptide HA (HA-Vpr) et avons purifié les virions contenant la protéine HA-Vpr. L'analyse par protéomique ou Western blot des complexes protéiques co-immunoprécipités grâce à l'anticorps anti-HA a permis de révéler que Vpr formait un complexe avec la protéine de matrice (MA) au sein de particules virales produites à partir de différentes lignées cellulaires humaines. De plus, l'interaction Vpr-MA est indépendante de la présence de la transcriptase inverse (RT) et de l'intégrase (IN) et a pu être détectée par des expériences de GST pulldown *in vitro* à l'aide de Vpr recombinant et de la protéine GST-MA purifiée. Ces résultats indiquent que Vpr et MA s'associent grâce à une interaction directe. Grâce à des expériences de liaison peptidique *in vitro*, les domaines responsables de l'interaction entre MA et Vpr ont été cartographiés, respectivement, au niveau de la 5ème hélice □ (résidus 97 à 108) et du domaine C-terminal riche en arginines. Étant donné les propriétés karyophiles de MA et de Vpr et qu'elles sont toutes deux membres du

PIC, leur interaction pourrait avoir un effet synergique dans le transport nucléaire du PIC et ainsi optimiser l'efficacité des étapes précoces d'une infection par le VIH-1.

L'étude de l'interaction des facteurs cellulaires et viraux impliqués dans l'infection des cellules humaines par le VIH-1 est d'une importance majeure. Dans ce contexte, il a été démontré que la protéine Vpr contribue à l'infection virale lorsque présente sous sa forme extracellulaire ainsi que lorsque présente à l'intérieur du virion. Dans la présente étude, nous avons identifié une protéase cellulaire qui contrôle l'activité extracellulaire Vpr et avons caractérisé des protéines interagissant avec Vpr à l'intérieur des virions. Ces données pourraient contribuer à une meilleure compréhension des fonctions précoces de la protéine Vpr dans le cycle de réplication viral et pourraient ainsi mener à l'identification de nouvelles cibles thérapeutiques.

Mots-clés : Vpr extracellulaire; clivage protéolytique, proprotéine convertase; VIH-1; virion; protéine de matrice; provirus; HA-Vpr.

DEDICATION

To my wife Xiuying for her love and support.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude and appreciation to my supervisor Dr. Eric A.Cohen for his excellent guidance and support throughout my graduate studies, especially for teaching me how to be vigorous with my experiments, to be a critical thinker, and to be persistent for the sake of producing better science and knowledge of the field. I would like to thank Dr. Ghislaine Duisit and Xiaojian Yao for taking the time to help me and to answer my questions and for their advice and encouragement when experiments did not always go the way that we predicted. I want to thank Dr. Nabil Seidah for the valuable discussions and for providing all the PCs expressor plasmids. I also would like to thank my committee—Dr. Guy Lemay, Ali Ahmad, and Benoit Barbeau—for taking such an active role in the progression and completion of my thesis.

I thank the Cohen lab members, in particular, Dr. Ghislaine Duisit, Jean Phillippe Belize, and Alex Caillaud for their critical review of the manuscript; I thank Nicole Rougeau for her excellent technical support for the Vpr project during the late stage of my PhD work; I thank Nicole Rougeau, Johanne Mercier, and Melanie Welman for their friendship and support; I thank Drs. Jacques Thibodeau, Alexandre Brunet, and Andres Finzi for their collaboration on the MHC-II molecule effect on the HIV-1 assembly, and Dr. Andrew Mouland for his collaboration on the hnRNP A2 project.

Finally, I would like to thank Dr. Hayami Masanori and Shida Hisatoshi for introducing me to this field. I also would like thank my family for being extremely supportive and encouraging me in everything I do; without my wife Xiuying, I would not have made it to the end, and I cannot begin to thank for her for all her encouragement and confidence in me, for her love and support—and mostly I thank her for being my best friend.

PREFACE

This Ph.D. thesis was written in accordance with **the Guild Concerning Thesis Preparation from the Faculté des etudes supérieures at Université de Montréal.** The structure and contents of the thesis conforms to the option, subject to the approval of the department, of including, as part of the thesis, copies of the text of paper(s) submitted for publication, or the clearly-duplicated text of published paper(s). The thesis includes, as separate chapters or sections: (1) a table of contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature of the subject of the study, when the review is appropriate, and (5) a final overall conclusion and /or summary.

I have included, as a chapter of this thesis, two original manuscripts that have been submitted for publication. The papers presented in the thesis are the following:

1) Yong Xiao, Gang Chen, Nicole Rougeau, Hongshan Li, Nabil G. Seidah, Éric A. Cohen

Cell-surface Processing of Extracellular Human Immunodeficiency Virus Type 1 Vpr by Proprotein Convertases. J Virology. Submitted Mar, 2007.

2) Yong Xiao, Pierre-Alexandre Bonicard, Xiao-Jian Yao, Ghislaine duisit and Éric A. Cohen.

Human Retrovirology Unit, IRCM

Direct Association of HIV-1 Vpr and Matrix (MA) Proteins in Virions: Implication for Early Events of HIV-1 Infection. **Manuscript will be submitted to Retrovirology.**

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Publication I: Andrés Finzi1,3, Alexandre Brunet2,3+, Yong Xiao 1,3+,Jacques Thibodeau2,3 and Éric A. Cohen1,3 .MHC-II molecules enhance HIV-1 assembly and budding to late endosomal/Multivesicular bodies compartments. J Virol. 2006 Oct;80(19):9789-97. 200

Publication II: Beriault V, Clement JF, Levesque K, Lebel C, Xiao Y, Chabot B, Cohen EA, Cochrane AW, Rigby WF, Mouland AJ. A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. J Biol Chem. 2004 Oct 15;279(42):44141-53. 200

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LIST OF ABBREVIATIONS

ADCC	:antibody-dependent cellular cytoxicity
AIDS	:acquired immunodeficiency syndrome
ALSV	:Avian Leukosis Sarcoma Virus
APOBEC3G	:apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ARV	:AIDS-associated retrovirus
AT	:ataxia telangiectasia
ATR	:Ataxia-Telangiectasia mutated and Rad3-related
BFA	:brefeldin A
BLV	:Bovine Leukemia Virus
CA	:capsid protein
CAEV	:Caprine Arthritis-Encephalituis Virus
CAF	:CBP-associated factor
CBP	:CREB-binding protein
Cdk9	:cyclin-dependent kinase 9
CMV	:cytomegalovirus
CPE	:cytopathic effect
CRD	:cysteine-rich domain
CREB	:cAMP response element-binding protein
CRM1	:chromosomal region maintenance 1
CsA	:cyclosporin A
CTL	:Cytotoxic T-lymphocyte.
CyPs	:cyclophilins
ECM	:extracellular matrix

EIAV	:Equine Infectious Anemia Virus
ER	:endoplasmic reticulum
ESCRT	:endosomal sorting complex required for transport
FGF	:fibroblast growth factor
FIV	:Feline Immunodeficiency Virus
Env	:envelope glycoprotein
Gag	:group-specific antigen
GFP	:green fluorescent protein
GR	:glucocorticoid receptor
HA	:hemagglutinin
HIV-1	:Human Immunodeficiency Virus-1
HSPG	:heparan sulfate proteoglycans
HTLV	:Human T-cell Leukaemia Virus
IN	:integrase
ICAM	:intracellular adhension molecule
JNK	:c-Jun NH(2)-terminal kinase
LAS	:lymphadenopathy syndrome
LAV	:Lymphadenopathy-Associated Virus
LTNP	:long term non-progressor
LTR	:long terminal repeat
MA	:matrix protein
MHC	:major histocompatibility complex
MVBs	:multivesicular bodies
NC	:nucleocapsid protein
Nef	:negative factor
NES	:nuclear export signal

NF-κB	:nuclear factor-Kappa B
NK	:natural killer cell
NLS	:nuclear localization signal
NPC	:nuclear pore complexes
Nt	:nucleotide
PACE4	:basic amino-acid-cleaving enzyme 4
PAK-2	:p21-activated protein kinase 2
PBL	:peripheral blood lymphocytes
PC	:proprotein convertases
PCD	:programmed cell death
PI	:propidium iodine
PIC	:pre-integration complex
PHA	:phytohemagglutinin
Rev	:regulator of viral protein expression
RSL	:reactive site loop
RT	:reverse transcriptase
RTC	:reverse transcription complex
PTD	:protein transduction domains
RRE	:Rev responsive element
SELDI-TOF-MS	:surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
Serpin	:serine protease inhibitor
SIV	:Simian Immunodeficiency Virus
TAR	:transactivation response element
Tat	:Transactivator of transcription
TCR	:T cell receptor
TGN	:trans-Golgi-network

TSG101	:human tumor susceptibility gene 101
UNAIDS	:United Nation program on HIV/AIDS
VMV	:Visna-Meadi Virus
Vpr	:viral protein R
Vpu	:viral protein U
WHO	:World Health Organization

CHAPTER 1: LITERATURE REVIEW

The Retroviridae family has 7 subfamily, composed of the following virus genera: C-type, B-type, D-type, HTLV-BLV, Spumavirus, Avian Leukosis Sarcoma (ALSV), and Lentivirus. Lentiviruses are further broken down into three categories: human primate lentivirus, nonhuman primate lentivirus, and non-primate lentivirus. Human primate lentiviruses consist of the Human Immunodeficiency Virus-1 and the Human Immunodeficiency Virus-2 (HIV-1 and HIV-2). The former is highly pathogenic and the later demonstrates a similar but less pathogenic etiology. Non-human primate lentiviruses form several distinct lineages named the Simian Immunodeficiency Virus (SIV). They are endemic to their hosts and do not usually cause immune suppression as seen in humans. Simian Immunodeficiency Viruses are distinct primate groups that include strains from mandrills (SIVmnd), mangabees (SIVrcp and SIVsm), l'hoest (SIV l'hoest), and sabaeus (SIVsab) monkeys among others (282). The non-primate lentiviruses include Visna-Maedi Virus (VMV), Caprine Arthritis-Encephalituis Virus (CAEV), Equine Infectious Anemia Virus (EIAV), Bovine Immunodeficiency Virus (BIV), and Feline Immunodeficiency Virus (FIV) (196).

Like other retroviruses, HIV-1 produces Gag, Pol, and Env proteins. HIV-1 produces six additional proteins: Tat, Rev, Nef, Vif, Vpr, and Vpu. While Tat and Rev are required for viral replication, Nef, Vif, Vpr, and Vpu usually are dispensable for viral growth in many *in vitro* systems (112) and hence are known as accessory proteins. However, these proteins are often necessary for viral replication and pathogenesis *in vivo,* and they carry out many essential functions during the viral life cycle. Consequently, the presence or absence of these accessory proteins can significantly change the course and severity of the viral infection (112).

Viral protein R (Vpr) is a small, highly conserved accessory protein that serves many functions in HIV-1 life cycle. These functions include cytoplasmic-nuclear shuttling (146), induction of the cell cycle G2 arrest (144)□and cell killing (337). These three Vpr-specific activities are shown to be functionally

independent of each other (95, 252) and have been demonstrated in a wide variety of eukaryotic cells ranging from human to yeast, indicating that Vpr most likely affects highly conserved cellular processes.

In this review, I describe the background of the HIV virus, the current understanding of Vpr functions in HIV-1 viral infection, and the potential roles of Vpr activities in viral pathogenesis and disease progression.

1.1. The acquired immune deficiency syndrome

1.1.1. Discovery of HIV-1 virus

In the early 1980s, physicians in United States noticed an increasing number of young male homosexuals with a range of opportunistic infections and malignancies which had not been seen before in this age group (133, 156, 331). The most frequently observed symptoms were pneumocystis carinii pneumonia, oesophageal candidiasis toxoplasmosis of the brain, Kaposi sarcoma, and non-Hodgkins lymphoma, as well as other unusual complications. In 1981, Gottlieb *et al.* described these patients as anergic, lymphopenic, later more appropriately renamed acquired immunodeficiency syndrome (AIDS). At first, physicians thought that the origin of these diseases was a possible viral infection from the cytomegalovirus (356). Based on the epidemiological observations, it was proposed that AIDS is caused by an infectious agent transmitted by sex, contaminated blood products, and from mothers to children (277).

When it became apparent that the disease is transmissible, a wide range of known microorganisms was suggested as the agent. The French group from the Pasteur Institute in Paris first reported the isolation of a new human virus as the causative agent for AIDS, which was named LAV for the lymphadenopathy-associated virus. This virus had distinct properties from HTLV-1 because it does not establish a transformed state in CD4+ T helper cells but caused cell death after high level of replication (23). The same French team made the most significant observation that the virus is cytopathic to the helper T4 (CD4) cell, providing for the first time, an explanation of how and why AIDS develops (184).

Several other research groups also were searching for the virus that might be responsible for AIDS. After this description of LAV by Luc Montagnier's laboratory, two other groups reported the isolation of retroviruses from AIDS patients. In a series of papers published in 1984, Gallo and colleagues later reported the discovery of an AIDS-associated retrovirus, which appears distinct from HTLV, named HTLV-III (121, 138). Levy and a coworker isolated a retrovirus from a San Francisco subject and named it AIDS-associated retrovirus (ARV). The discovery of ARV in asymptomatic individuals indicated, for the first time, a carrier state for the AIDS virus (201).

Subsequent research established that the different AIDS virus nucleic acid sequences are extremely polymorphic, making it feasible unequivocally to identify the precise origin of different HIV isolates. This first revealed that HTLV-III is in fact LAV because of its unique identity (280). Actually, the virus had been supplied to Dr Robert Gallo's lab at various times by the French team (233).

The genetic organization and the proteins encoded by HIV-1 were shown to be distinct from those of HTLV; these retroviruses were recognized as members of the same group of retroviruses-Lentivirinae (280). In 1986, the International Committee on the Nomenclature of Virus renamed the AIDS virus the Human Immunodeficiency Virus (HIV) (61). Shortly after the identification of HIV, another human retrovirus was recovered from a West Africa patient with AIDS (59). It was noted to be sufficiently different genetically from HIV-1 (by up to 40%) and was named HIV-2.

1.1.2. Emergence of HIV-1 disease

The origin of HIV-1 has been linked to a virus found in chimpanzees (SIVcpz), originating in the species *Pan troglodyte troglodytes* and *pan troglodytes schweinfurthii* from Central and Western Africa (123). Previous data from the earliest documented case of AIDS in 1959 places HIV-1 within this geographic area, and phylogenetically proximal to the sequences of ancestral origin (412). The mode of transfer of the SIVcpz virus to humans is a continuous debate, but recently theories have been disproved, which suggested that SIV was transferred either by contaminated polio vaccines given to tens of thousands of African workers by French colonialists in the early 1930s, or by reusing needles for

vaccination (387). Direct human contact with primate bush meat during slaughter is still a possibility (139). The appearance of the less pathogenic HIV-2 strain in humans is thought to be caused by multiple transfers of SIV from sooty mangabees, since the sequence similarities between these three viruses group appears to be inseparable by phylogenetic analysis (124).

1.1.3. HIV/AIDS global pandemic: current status

It is well accepted that AIDS began in Africa (172). Not only is the disease widely spread in sub-Saharan Africa but also in Africa, monkey species are naturally infected with lentiviruses related to HIV, as first shown by the Dr. Hayami research team (255). No evidence can be shown for the existence of HIV in Europe, America, or Arabia during the past century or even the first half of twentieth century, strongly suggesting that the widespread HIV infection in Africa is a recent event. The epidemiological evidence thus points to the spread of HIV infection from Africa after the Second World War. The proliferation seems to coincide with the widespread use of syringes and needles from the West, at the time vaccination programs were being promoted and introduced. It also coincides with a post-war period of greatly improved transportation and the extensive migration of African people.

Since the first diagnosis was made in the early 1980s, AIDS has spread through the world, affecting all cultures and ethnic groups. At the end of 1984, the number of AIDS cases in the United States was 7,699 (with 3,665 deaths resulting from AIDS) and in the United Kingdom, 764 cases were reported (50). According to the *2005 AIDS epidemic update report* from the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), the estimated number of people living with AIDS in North America in 2005 is between 650,000 and 1.8 million, and the estimated world population of people living with AIDS is approximately 40.3 million, with 3.1 million death due to HIV-1/AIDS. Seventy percent of all individuals with HIV are living in sub-Saharan Africa. Despite education, testing, and awareness of the disease, the number of newly acquired cases has increased. In the United States, the spread of HIV has disproportionately increased in minorities where AIDS is the leading cause of death among African American women between the ages of 25 to 34. High-risk behaviors, such as

heterosexual and homosexual unprotected sex and IV drug use, account for the increased numbers. The spread of HIV has been most problematic in underdeveloped countries where both treatment and education have not been logistically and financially achievable for these governments. Organizations such as UNAIDS and the WHO have implemented programs involving the support of many Western countries to deliver affordable and simplified treatments.

1.2. HIV viral structure and genomic organization

1.2.1. HIV-1 genomic RNA.

In general, two copies of viral genomic RNA are incorporated into virion particles. The genomic size of HIV is about 9.8 kb with open reading frames coding for at least nine viral proteins (see Figure 1.1).

Like the oncoretroviruses, HIV-1 genomic RNA contains cis-acting elements required for the reverse-transcription in double-stranded DNA, the provirus integration into the host cell genome, the transcription of the different viral RNAs species, and the incorporation of newly synthesized genomic RNAs into nascent particles. HIV expression is flanked by long terminal repeats (LTR). The 5'LTR binds specific and general transcription factors that regulate the initiation of mRNA synthesis, whereas the 3'LTR signals the point at which mRNA synthesis should end, with the addition of a poly A tail. The LTR sequence has three functionally distinct domains (U3, R, and U5) and contains transcriptional promoter elements that regulate basal and inducible transcription functions. U3—the first promoter region—contains a modulatory enhancer region, which is the core promoter region for regulating transcription. The core promoter region is where RNA polymerase II and TATA-box binding proteins form the multiprotein complex that is responsible for initiating transcription and for providing the binding sites for these proteins, as well as three sites for Sp1 (344). The upstream modulatory enhancer region binds NF-κB and the NF-AT transcription factor, and has binding sites for various cellular proteins thought to be important for specificity of replication in cell types such as macrophages (299). These transcription factors include NF-IL-6, the cAMP response element-binding protein (CREB), and nuclear hormone receptors (287). In contrast to the oncoretroviruses, HIV-1 mRNA contains a 5'UTR element

called transactivation response element (TAR), that prevents RNA polymerase II processing in absence of the viral Tat protein (see below) (170). Located between the R/U5 regions in 3'LTR is the site for retroviral 3' end processing and polyadenylation signals. U5—the last promoter region—when located at the 5' end, contains a GT-rich exon that all viral transcripts contain, and encodes putative control elements for 3' end processing when it is located at the 3' LTR. Cellular machinery caps the 5' end of the viral transcript by the addition of methylated G nucleotide, it involves condensation of the trphosphate group of molecule of GT with a diphosphate left at the 5' end of initial transcript. The 5' end cap is necessary for the proper function of mRNAs in protein synthesis; it also seems to protect the growing RNA transcript from degradation.

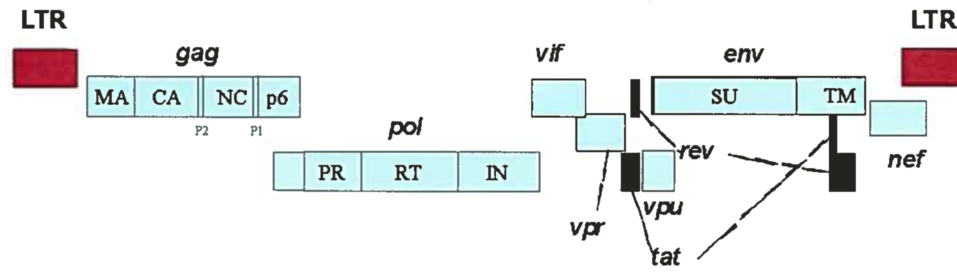

Figure 1.1. Organization of HIV-1 genome. *The relative location of the HIV-1 open reading frame gag, pol, env, vif, vpr, vpu, nef, tat, and rev are indicated.*

1.2.2. The viral proteins.

Like other retroviruses, HIV encodes for Gag, Pol, and Env virion structural and enzymatic proteins. In addition, the lentivirus encodes for two regulatory proteins, Tat and Rev, important for HIV-1 protein expression, and four accessory proteins Vif, Vpr, Vpu, and Nef dispensable for productive infection of transformed T lymphocytes, but often necessary for viral replication and pathogenesis *in vivo.* HIV-1 Tat, Rev and Nef proteins are expressed early during infection from fully spliced messenger RNA. In contrast, Gag-Pol polyproteins, as well as Env, Vpr, Vpu, and Vif are expressed in the late phase of infection from intron-containing viral mRNAs.

HIV-1 Gag precursor p55 gives rise, by proteolytic cleavage, to the smaller proteins, including the capsid protein CA (p24), matrix protein MA (p17), the nucleocapsid protein NC (p9), and the p6 protein (147). Pol is initially made as a Gag-Pol polyprotein Pr160^{gag}. A ribosomal frame shift of –1 in gag allows a ribosomal reading through of gag and the production of Pro-Pol at the ratio of 1:20 within the cell (257). The Pol precursor protein is cleaved into products consisting of the reverse transcriptase (RT), protease (PR), and integrase (IN) proteins, which are essential for viral replication (357).

Env gene encodes a glycoprotein precursor gp160. This precursor is cleaved in the tran-Golgi-network (TGN) by furin-like convertase into a gp120 external surface (SU) envelope protein and gp41 transmembrane (TM) protein, which constitute the membrane glycoprotein of the virus (140).

Tat (transactivator of transcription) is a small transactivating protein (101 amino acids in most clinical HIV-1 isolates, 86 amino acids in the laboratory HIV-1 HXB2 strain). Tat, along with other cellular proteins, interacts with an RNA loop structure formed in the 3' portion of the viral long terminal repeat (LTR) called TAR. Tat potently transactivates LTR-driven transcription, which results in a remarkable increase of viral gene expression (84).

Rev (regulator of viral protein expression) is a 116 amino acid sequence-specific RNA binding phosphoprotein that is expressed during the early stages of HIV-1 replication (216). The protein is necessary for the expression of intron-containing RNAs. In its absence, only the fully spliced class of HIV-1 mRNAs is present in the cytoplasm, while intron-containing RNAs remain nuclear (103). Rev multimers interact with a cis-acting RNA loop structure called the Rev responsive elements (RRE), located within the Env gene (301). This interaction permits the nuclear export of unspliced or partially spliced RNAs (14). The transition from multiple spliced mRNA to unspliced mRNA into the cytoplasm is the marker between early and late stages of the viral replication cycle (388). The export of Rev and single spliced transcript to the cytoplasm is mediated by the cellular transport receptor, CRM1 (Chromosomal region maintenance 1), where the recycling of Rev to the nucleus is mediated by importin α (2, 116).

Vif (viral infectivity factor) gene encodes a 23-kDa protein. Vif is essential for the reproduction of HIV-1 in peripheral blood lymphocytes, macrophages, and certain cell lines (340). Vif suppresses the

antiviral activity of the cellular protein APOBEC 3G found in T cells (143, 323). APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G) is a member of the cytidine deaminase family, which prevents viral cDNA synthesis through deaminating deoxycytidines (dC) in the minus-strand retroviral cDNA replication intermediate (143, 401). Vif binds directly to APOBEC3G and counteracts its anti-HIV activity by promoting its degradation. Vif-mediated APOBEC3G degradation involves the recruitment of a specific E3 ligase complex, which leads to polyubiquitylation and protease-mediated degradation (324, 395).

Vpu (viral protein U) is an accessory protein found only in HIV-1 and SIVcpz strains, and no analogous proteins are present in HIV-2 and other SIVs (64, 222). Vpu is a small (9 kDa) membrane protein that enhances the release of progeny virion from infected cells and induces the degradation of the CD4 protein (34). Vpu expressed in the ER interacts with a membrane-proximal domain of the cytoplasmic tail of CD4 and links it to h-βTrCP (221), a member of the F-box protein family first characterized as components of the ubiquitin-ligase complex (183). The CD4-Vpu-βTrCP ternary complex then recruits SKP1, another member of the ubiquitinated machinery (381). CD4 is ubiquitinated and targeted to proteasomes for degradation after recruiting SKPI. Initially, the ability of Vpu to increase viral release from infected cells had been attributed to ion conductive membrane pore formation characteristic to cells over-expressing Vpu (34). However, a recent report shows that the requirement for Vpu is host cell-dependent, suggesting that Vpu may counteract an inhibitory factor expressed in some, but not in other cells (363). TASK-1, a widely expressed acid-sensitive K+ channel, is structurally homologous to Vpu, suggesting oligomerization as a possible mechanism of inactivation of the ion channel activity of these proteins (154). However, the mechanism by which TASK-1 inhibits virion release is still unclear.

Vpr is a 96-amino-acid, 14-kDa protein which is expressed at the late stage of viral replication and is virion incorporated (62). Vpr is much conserved among the primate lentiviruses HIV-1, HIV-2, and the Simian Immunodeficiency Virus, suggesting that it may play an important role in the viral life cycle *in vivo*. Although Vpr is dispensable *in vitro* viral replication in T cell lines, it plays an important role in

macrophages infection and HIV-1 pathogenesis *in vivo* (65, 131). Details regarding the Vpr protein and its biological function are discussed below.

Nef protein is a 27 kDa myristoylated protein that is abundantly produced during the early phase of viral infection. It is highly conserved in all primate lentiviruses, suggesting that its function is essential for the survival of these pathogens. SIV with Nef mutations quickly reverted to a wild type Nef in infected monkeys or did not progress to AIDS-like symptoms (175). This suggests that Nef is essential for viral pathogenesis *in vivo*. The role of Nef in HIV-1 replication and disease pathogenesis is determined by at least four independent activities of this protein. First, Nef down regulated the cell surface CD4 (125) and the major histocompatibility complex class I (MHC-I) protein (313). Nef-induced CD4 down-regulation has been shown to be the result of rapid internalization and degradation of the CD4 receptor (125, 295). Down-regulation of MHC I protects HIV-1 infected cells from host CTL response, where as down-regulation of CD4 probably limits the adhesion of an expressing T cell to the antigen-presenting cell and prevents the interaction between CD4 and the envelope of a newly produced virion. Second, Nef expression interferes with the cellular signal transduction pathway. Nef myristoylation and its proline-rich SH3-binding domain mediate Nef association with lipid raft, the cholesterol-rich membrane microdomains that concentrate potent signaling mediators (373). Nef was found to complex with and activate the serine/threonine protein kinase PAK-2 (p21-activated protein kinase 2) (283), which may contribute to the activation of infected cells. Third, Nef enhances virion infectivity and viral replication. This effect is mediated by the presence of Nef in the HIV-1 virion and is due, at least in part, to the ability of Nef to induce actin remodeling and to facilitate the movement of the viral core to pass the potentially obstructive cortical actin barrier (48). Fourth, Nef regulates cholesterol trafficking in HIV-infected cells. Cholesterol plays an important role in the HIV life cycle, since HIV assembly and budding—as well as the infection of targeted cells—depends on plasma membrane cholesterol. Nef has been shown to bind cholesterol via a cholesterol-recognition motif at its carboxy-terminus and to transport newly synthesized cholesterol to the site of viral budding (409).

1.2.3. HIV virion structure

The typical mature HIV-1 virion is about 100nm in diameter. It is enveloped by a lipid bilayer that is derived from the membrane of a host cell. The exposed surface glycoprotein SU, (gp120) anchors to the virus *via* interaction with the transmembrane TM protein (gp41). The lipid bilayer also contains several cellular membrane proteins from the host cell, including the major histocompatibility complex antigen, actin, and ubiquitin (15); integral membrane-spanning intracellular adhesion molecules (ICAMs) such as ICAM-1 is also incorporated within the envelopes of HIV-1 virion and can dramatically enhances infectivity of HIV-1 virion (349). A matrix shell comprising the matrix protein (MA, p17) lines the inner surface of viral membrane, and a conical capsid core particle comprising the capsid protein (CA, p24) is located in the center of the virus.

The capsid particle encapsidates two copies of the unspliced dimerized viral RNA genome, which is stabilized as a ribonucleoprotein complex with 2000 copies of the nucleoprotein (NC, p7) and also contains three essential virally encoded enzymes—PR, RT, IN (see Figure1.2). MA has been found in HIV-1 core preparations (187, 380). It is believed that phosphorylated MA locates in the viral core by interacting with integrase (119, 120).

Virus particles also package the accessory protein Vpr via its interaction with p6 in the Gag p55 precursor (186, 266) Vpr is localized within the HIV-1 viral core (1, 380). Muller *et al.* have reported that HIV-1 virus particles contain a lesser amount of Vpr in comparison to Gag (7:1 ratio of Gag to Vpr), i.e. about 275 Vpr molecules per HIV-1 virion particle (239).

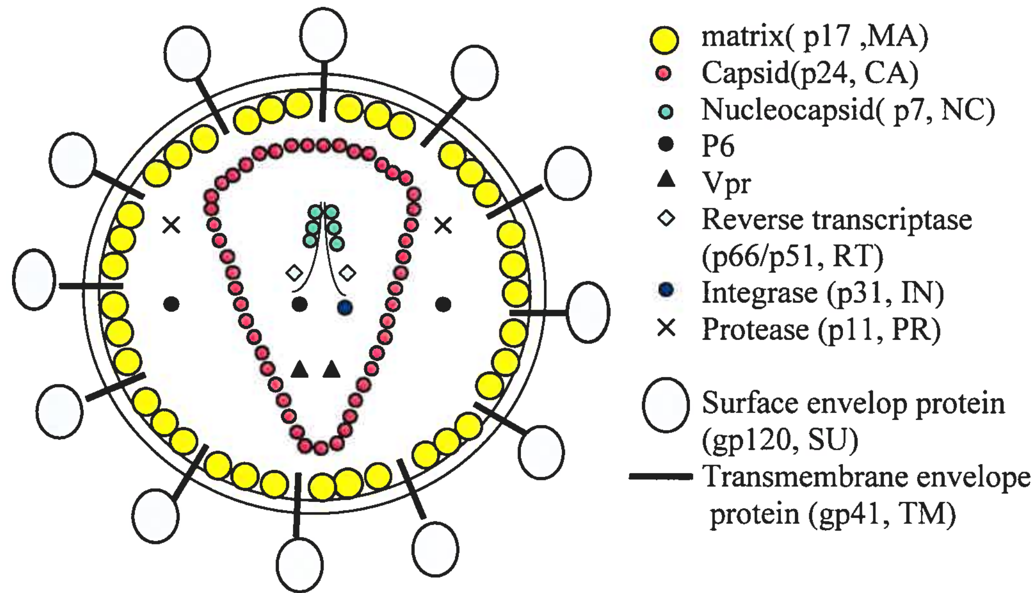

Figure 1.2. HIV-1 virion structure. *Positions of major viral proteins, the lipid bilayer, and the genomic RNA are indicated.*

Recent data shows the association of Nef with the HIV-1 core (187). Vif, another accessory HIV-1 protein, was also found to be associated with the HIV-1 core structure (207). However, whether Vif is a genuine virion component or a contaminant remains controversial because other researchers have reported that Vif is essentially absent from the highly purified HIV-1 particles (81). Three additional accessory proteins Rev, Tat, and Vpu that function in the host cell do not appear to be packaged (353).

1.3. HIV-1 Life cycle

HIV replication cycle closely resembles that of other retroviruses (Figure 1.3). However there are anumber of unique aspects of HIV replication such as HIV target receptor and coreceptors distinct from other retroviruses. Lentivirus like HIV encodes a number of regulatory and accessory proteins not encoded by the genome of the prototypical simple retroviruses. Lentivirus like HIV has the ability to

productively infect certain type of nondividing cells. The basic HIV-1 replication process will be introduced in the following.

1.3.1. Virus entry

Infected host cells. HIV-1 infects CD4+ T helper cells and macrophages of the immune system. Cells of macrophage lineage are among the first cell types to become infected during the process of HIV transmission (379). The ability of primate lentiviruses to infect nondividing cells was first observed in macrophages but now include microglia, mucosal dendritic cells, and epidermal Langerhans cells, all of which are important for establishing a productive infection (127, 124, 193, 281). The successful infection of nondividing cells is attributed to the ability of the virus to transport its viral genome to the nucleus for integration into a host cell. Retroviruses such as MLV require the breakdown of the nuclei envelope for efficient nuclear import and integration of the viral cDNA (297). HIV-1 can replicate in nondividing cells, such as macrophage, which relies on the active transport of the viral PIC through the nuclei pore complex (44). Other cell types, including PBMCs and the activated CD4+ T cell, have also been found to benefit from this function, as mitosis is only a small fraction of the entire cell cycle, and active nuclear import in these cells can enhance their infection (137, 294).

HIV-1 receptors and co-receptors. The primary binding receptor for the HIV-1 envelope is CD4, which is found on lymphocytes and macrophages. Numerous cell types throughout the body are infectable with HIV in the absence of the CD4 receptor. They range from cells in the brain, intestine, and skin to cells in the heart, kidney, as well as other organs (200). Subsequent studies have shown that the CD4 receptor alone is not sufficient, nor is it the only way for HIV to enter cells. Chemokine receptors are found to act as coreceptors for the entry of HIV into cells. The CXCR4 acts as a coreceptor for the HIV-1 T cell-cell tropic strains. Subsequently, other molecules named CCR-5, CCR3, and CCR-2b were found to act as coreceptors for the macrophage tropic HIV-1 strains (29, 58). The CXCR4 coreceptor is expressed on

virtually all lymphocyte subsets, albeit at varying levels. CCR5 is predominantly expressed on primary T cells, macrophages, dendritic cells and microglia (234). Disease progression correlates with coreceptor usage, as viruses from early in the course of infection predominantly use CCR5 for their mode of entry, and later evolve to include the use of CXCR4 (66, 78). It is unclear why these viruses evolve, as cells expressing CCR5 are still present at the late stages of infection. One theory is that the CD4+ T cell is present in larger numbers, and in more tissues, allowing the evolving virus to infect more target cells. The switch from CCR5 to CXCR4 using strains ultimately leads to the increased infection of CD4+ T cells, and the progression to AIDS (66)

Virus entry. HIV-1 replication cycle begins with the attachment of the virus to the target cell. The envelope glycoprotein subunit gp120 initially binds to CD4 (70, 184). The initial contact of CD4 and the envelope leads to conformational changes that expose the surface required for coreceptor binding. A subsequent interaction between gp120 and the corereceptor triggers new conformational shifts in the envelope glycoprotein (190). These sequential conformational changes finally lead to the dissociation of gp120 from gp41, and the transition of gp41 to its fusogenic conformation. The entry of the virions into the cell is achieved by insertion of the gp41 fusion peptide into the target membrane, which results in the fusion of the viral and cellular membranes and the release of the viral core in the cytoplasm (122). HIV-1 entry does not depend on the pH (224).

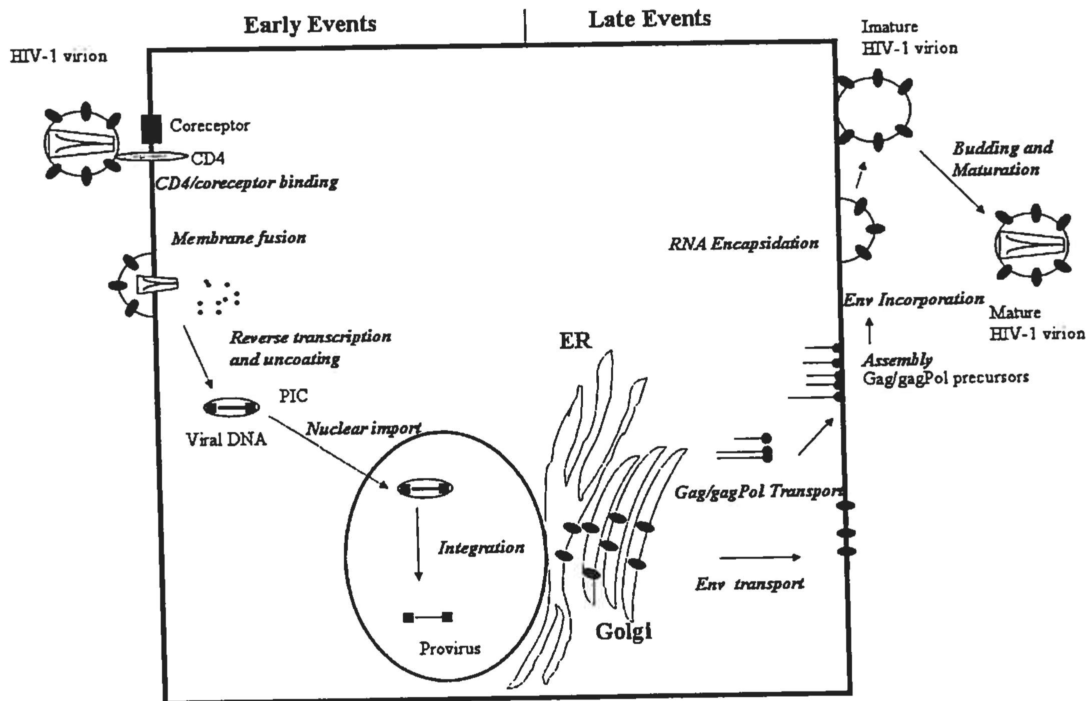

Figure 1.3. Schematic representation of HIV-1 replication cycle. *The major steps in the early and late stages of replication cycle are indicated (described in detail in the text)*

1.3.2. Uncoating.

After the membrane fusion, the viral core is released into the cytoplasm and further rearranged. This process called *uncoating* is not fully understood. Current evidence suggests that both viral and cellular proteins are involved in the process of uncoating. The infectivity defect observed with delta nef viruses occurs after entry and before the completion of reverse transcription, suggesting that nef has a role in the uncoating (312). The second viral accessory protein Vif that may be important for uncoating was described in Section 1.2.2. The most promising cellular factor identified that may contribute to viral uncoating is cyclophilin A (CypA). Cyclophilins are a family of proteins that bind the immunosuppressant cyclosporin A, possess peptidyl-prolyl cis-trans isomerase activity, and assist in the folding of proteins. Human cyclophilins A and B are host cell proteins that bind specifically to the HIV-1 capsid protein proline-rich loop in the virion and are critical for HIV replication in human cells (110). Tripartite motif protein 5α (TRIM5α), a cellular restriction factor for HIV-1 replication, inhibits HIV-1 replication at the

step before reverse transcription by interacting capsid with its CypA domain in owl monkey cell (253). HIV-1 cores undergo a progressive uncoating that leads to the generation of sequential nucleoprotein complexes now referred as *reverse transcription complex* (RTC) and *pre-integration complex* (PIC) (44). HIV-1 proteins RT, IN, NC, MA, Vpr and Nef but not CA remain associated with the viral genome.

1.3.3. Reverse-transcription.

Sequential dsDNA conversion. Reverse-transcription is initiated within the virion and continues shortly after uncoating (402). It should be noted that the distinction between RTCs and PICs is somewhat arbitrary, since uncoating is believed to occur progressively. However, PICs are usually defined as the integration-competent complex, whereas reverse-transcription is incomplete in RTCs.

The biologically relevant form of HIV-1 and HIV-2 RT is an heterodimer consisting of two polypeptides of molecular mass of 66 kDa (p66) and 51 kDa (p51); p51 is derived from p66 by proteolytic cleavage of its C-terminal domain (83). The p51 subunit lacks the RNase H domain. The heterodimer form of the enzyme is found in the infectious virion and represents the biologically relevant and active form of the enzyme, since the isolated subunit is functionally inactive (292). A recent study showed that the interaction between the thumb domain of p51 and the RNase-H domain of p66 plays a major role in an essential conformational change that is required for the proper folding of the primer/template and the tRNA-binding site (for maturation and for activation of heterodimer reverse transcriptase) (237). In addition to providing a strong structural support to the p66 subunit, the functional role of p51 may involve the facilitation of the binding of template-primer to the p66 subunit (142). HIV-1 uses human transfer RNA specific for lysine ($tRNA^{lys}$) to prime negative-strand DNA synthesis. DNA synthesis proceeds to the 5' end of the RNA molecule generating a DNA/RNA hybrid. The RNA portion of this hybrid is degraded by the RNase H activity that is an inherent part of the RT holoenzyme, generating a DNA fragment known as the minus-strand strong stop DNA. By using short regions of homology (the so- called "R" regions), the minus strand strong stop DNA "jump" from the 5' to the 3' end of the genome. This step is refered as the first strand transfer. Minus-strand synthesis occurs, using

the 3' end of the minus-strand strong stop DNA as a primer. Plus-strand synthesis occurs, using as primers fragments of RNA remaining from minus-strand synthesis. The primary site of priming for retroviruses takes place at a purine-rich sequence known as the polypurine tract (PPT). For HIV, priming also occurs efficiently from another site, known as the central PPT. The tRNA bound to the primer binding site is removed by RNase H, thereby allowing second-strand transfer to take place. Plus-strand synthesis proceeds to the end of the minus strand. For HIV, an additional termination site, referred to as the central termination signal (CTS), is located near the center of the genome. Approximately 100 nucleotides of plus-strand DNA is displaced, resulting in the formation of a DNA "flap" (357). Once synthesized linear viral DNA migrate from the cytoplasm to the nucleus of infected cells, where it can integrate in host genome or circularize. The circular DNA is found exclusively in the nucleus as 1-LTR or 2-LTR molecule as a marker of nuclear import detected early after HIV infection.

Fidelity of the RT. It is well-established that HIV mutates or evolves during replication, which allows the virus to escape from both the cellular and hormonal immune response and to develop drug resistance against all licensed anti-retroviral medications. This critical lack of fidelity of HIV has been attributed at least in part to the reverse transcriptase because the enzyme is lacking 3'- to 5'-exonucleolytic proofreading activity and it has been shown to be error-prone in cell-free systems. Multiple factors may also influence HIV fidelity, including cellular DNA deaminases (notably APOBEC3G) and uracil DNA glycosylase 2 (UNG2). The nuclear form of Uracil DNA glycosylase (UNG2) is an enzyme involved in the base excision repair pathway that specifically removes the RNA base uracil from DNA. Uracil can occur in DNA either by misincorporation of dUTP or by cytosine deamination. Interestingly, HIV-1 encodes different proteins able to bind these enzymes. Vif counteracts the mutagenic effect of APOBEC3G by reducing its stability and by incorporating it into the progeny virion (323). Vpr and IN promote UNG2 viral incorporation (32, 384). The interaction of Vpr with uracil DNA glycosylase modulates the Human Immunodeficiency Virus type 1 *in vivo* mutation rate (219) and decrease the mutation rate in cell (166), Priet *et al.* recently showed that RNA interference knockdown of UNG in

macrophages blocked HIV-1 replication (278). They further showed that *in vitro*, on model substrates, UNG and reverse transcriptase acted in concert to remove uracils that had been misincorporated during reverse transcription (278). Another research group had opposite point of opinion, they showed that Vpr interacts with UNG-2 induces UNG-2 proteasomal degradation; their data suggested that removal of UNG by Vpr did not appear to interfere with viral infectivity (310). It is still possible that small amount of UNG are required for HIV-1 replication and these UNG are not removed by Vpr. The exact role of HIV-1 Vpr regulating UNG activity and its impact on RT fidelity during HIV-1 replication need to be confirmed. Although Vpr interacts with another DNA repair protein the human homologue of yeast RAD23 protein (HHR23A), the Vpr-HHR23A interaction does not influence the HIV-1 *in vivo* mutation rate or the Vpr G2 cell cycle arrest function (218).

1.3.4. Nuclear translocation of the PIC.

Nucleoprotein complexe utilize cytoskeletal components to reach the nucleus (41). PICs are composed of the double-stranded linear DNA associated with the viral proteins MA, RT, IN, and Vpr. It has an estimated stoke diameter of 56nm (230). Since the central channel of the nuclear pore has a maximum diameter of 25nm and the pore is known to be able to transport macromolecules up to 39 nm (262), HIV has developed a strategy to pass through these structures. Unlike the oncoretroviruses, lentiviruses including HIV-1 have the ability to infect non-dividing cells without the breakdown of the nuclei envelope and mitosis for viral replication, a feature important to HIV-1 in its establishment of a long-lived infection of the host (379) (44). This property is shared with other lentiviruses and reflects the existence of determinants that govern the active transport of the viral preintegration complex through the nucleopore (44).

Nuclear pore complexes (NPCs) are large supramolecular protein structures that span the nuclear membrane and protrude into both cytoplasm and nucleoplasm. Signal-mediated nuclear import involves the interaction of nuclear localization signals (NLSs) in proteins with nucleocytoplasmic shuttling receptors, belonging to the karypherin β family, also known as importins. NLSs are typically short

stretches of amino acids, the best studied of which are basic amino acid-rich sequences that interact with the adapter importin α (115). Importin β interacts with other classes of NLS by using different adapters, including snurportin, RIP (Rev interacting protein), and importin 7. Recently, importin 7 has been proposed as playing a key role in the nuclear import of HIV-1 PICs in primary macrophages (101). It is believed that multiple factors are involved in the nuclear targeting of the HIV-1 preintegration complex (PIC) in non-dividing cells, such as matrix protein (MA), karyophilic Vpr, integrase protein (IN), and DNA flap (136, 275). Their respective involvement will be discussed later.

1.3.5. Integration

Integrase prepares the viral cDNAs for integration by cleaving their 3' ends. An integrase mediated hydroxyl group oriented attacking occurs on the host cell DNA and this hydroxyl group forms new bonds with the 3' ends of the viral cDNA (309). HIV-1 integration preferentially occurs in genes highly transcribed by the RNA Pol II (309). Several cellular factors have been described to interact with integrase and may therefore constitute good candidates for directing the PIC to its target site. The integrase interactor (Ini1, also called hSNF5), a subunit of the SWI/SNF chromatin-remodeling complex, was initially isolated by yeast two hybrid screen for human proteins interacting with the IN and was proposed to stimulate the *in vitro* DNA-joining activity of the IN and to target the viral genome to active genes in an as yet undetermined manner (167). Equally, high mobility group protein HMG-I(Y), which has been proposed to be important for integration (98), appears to be required for efficient integration *in vitro*, but their respective role in directing the PIC to precise sites of host genome was not evaluated. Two other IN-binding partners were isolated which seem to be critical for directing the PIC to the host chromatin. This is the case for the EED protein which is encoded by the human homologue of the mouse *embryonic ectoderm development (eed)* gene product and of the *Drosphila esc* gene, and which also interacts with the matrix protein of HIV-1 (269, 367) . These genes belong to the family of widely conserved polycomb genes, involved in the maintenance of the silent state of chromatin and reduction of DNA accessibility. An interaction occurring between EED and the viral proteins MA and IN might not

only direct the PIC to the host chromatin but also trigger transcriptional activation (367). Finally, the lens epithelium-derived growth factor (LEDGF/p75), a protein implicated in the regulation of gene expression and in the cellular stress response was found to interact with HIV-1 IN (54). Interestingly, this interaction is not essential for nuclear accumulation of HIV-1 IN, but seems to be absolutely required to dock the PIC to the host chromatin (214).

1.3.6. HIV-1 gene expression

Regulation of the transcriptional activity. After the viral DNA has successfully integrated into the host cell DNA, the process of viral gene expression begins. The cis-regulatory sequences in the LTR promoter allow RNA polymerase II, together with other cellular factors, to bind and initiate transcription (see Figure 1.4). The translation produces the basal amount of Tat, Rev, and Nef (165).

After a sufficient amount of Tat has been produced, Tat controls the transcription of the HIV-1 gene. Tat increases the efficiency of transcription by enhancing the elongation capacity of the RNA Polymerase II complex by 10,000 fold. This increase is accomplished by the ability of Tat to recruit cyclin-dependent kinase 9 (Cdk9) to the HIV-1 LTR by interacting with Cyclin T1 and binding it to the TAR elements, a stem loop structure found at the 5'end of viral transcripts (378). Cyclin T is part of a family of proteins involved in cell cycle regulation that forms a complex with cyclin-dependent kinases (Cdk9)

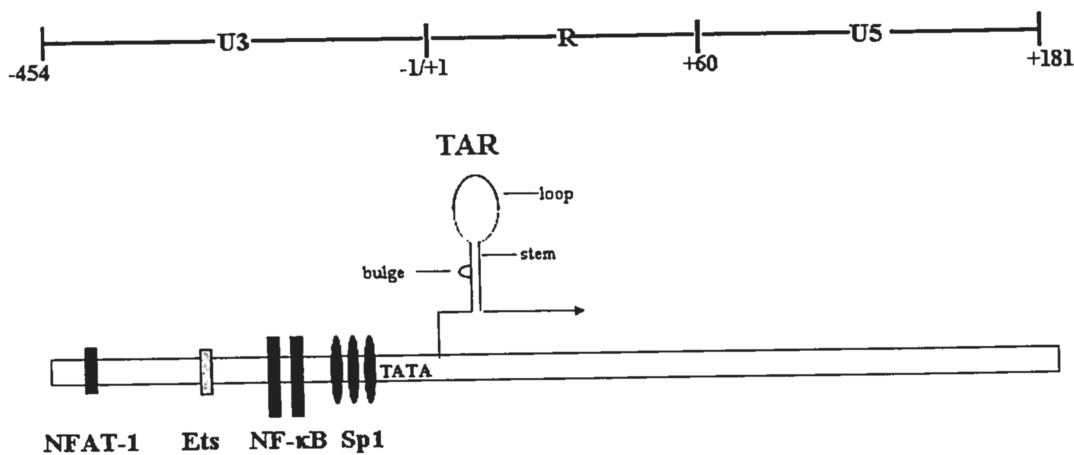

Figure.1.4. Schematic representation of HIV-1 LTR. *The position of binding sites for host factors (LBP-1, NF-κB,LEF,Ets,USF-1, and NFAT-1) are shown at the 5' of the transcription start site. The TAR stem/loop structure, with its bulge, is represented at the 5' end of a nascent mRNA. Numbering below the boxed region is relative to the transcription start site nucleotide +1(here using the HIV-1 HXb2 sequences; accession no. K03459).*

to phosphorylate the C-terminal domain (CTD) of RNA polymerase II and initiate transcription. The binding of both Cyclin T and Tat to the TAR element provides a high affinity complex that binds to the P-TEFb (positive elongation factor-b) complex. Cdk9 phosphorylates the C-terminus of RNA polymerase II and allows efficient elongation to occur. Tat has been found to interact with Sp1 and require Sp1 and NF-κB sites for its function (341, 342). Tat also recruits the chromatin remodeling enzymes (CBP/p300 and pCAF) to the site of transcription to unravel the histone/chromatin structure of the integrated viral DNA, and enhances the elongation process of RNA pol II, resulting in a several hundred fold increase in transcription (260). HIV-1 Vpr interacts with HIV-1 tat which causes synergic effect of Tat transactivation (307). The detailed mechanism of Vpr role on its transactivation LTR is described in section 4.5. Early reports showed that HIV-1 nef had a negative effect on LTR activity (5). The nuclear factor of activated T cells (NFAT) is an important transcription factor in regulation of gene expression in T cell. Together with the activator (AP-1) it promotes transcription of several cellular genes involved in T cell activation, such as interleukin-2. Increased production of IL-2 is a critical step in T cell activation. Since the activation of T cells strongly correlates with the ability of HIV-1 to infect and replicate in these cells. It has been reported that NFAT can also directly bind to and activate HIV-1 LTR (182). Vpr can potentiate Nef-induced activation of nuclear factor of activated T cells (NFAT)-dependent transcription (191). Unlike Nef, which stimulates calcium signaling to activate NFAT, Vpr functions farther downstream via distinct mechanism to cooperate with Nef in NFAT-directed gene expression and promote transactivation by CREB (191).

Post-transcriptional regulation. Regulation of the expression of a large number of structure and regulatory genes within a relatively small genome highlights the complexity of HIV gene regulation compared to other simple retroviruses. This regulation implicates a complex arrangement of genes encoded in overlapping reading frames and the expression of these genes through elaborate splicing of the single mRNA precursor. Through transcription and splicing, HIV-1 produces three classes of mRNA: the multiply spliced ~2kb mRNA species which encode the viral regulatory protein Tat, Rev, and Nef, the unspliced (~9kb) and single spliced (~4kb) transcripts which encode the structure protein (Gag polyprotein precursor, Gag-Pol polyprotein precursor, and Env glycoprotein precursor) and some of the accessory proteins (Vif, Vpr, and Vpu). The expression of unspliced and single spliced mRNA species is tightly controlled by the HIV-1 regulatory protein Rev as described in section 2.2. Full-length unspliced transcripts are exported by Rev and are recruited to the assembling virion by an interaction with the *psi* packaging element located 3' of the 5' LTR.

1.3.7. Assembly and release of HIV-1

Env trafficking. The *env* gene is translated into the precursor protein gp160, which is glycosylated within the endoplasmic reticulum and transported to the plasma membrane via the secretory pathway to areas of high lipid content (sphingolipids and cholesterol), known as lipid rafts. gp160 is cleaved into gp41 and gp120 by the host protease furin during its transport through the Golgi apparatus (140). After translation, the Env proteins migrate and insert into the plasma membrane.

Gag trafficking. The synthesis of the HIV Pr55gag and Pr160 gag-pol precursor polyproteins of HIV occurs on cytosolic polysomes. Unspliced viral RNA is translated by ribosome scanning from the first AUG (+789). At least 90% of all translation events terminate at the UAA stop coden (+2289) and result in the synthesis of the gag polyprotein precursor. An infrequent –1 ribosomal frame shift (approximately 1-5% of gag translation) at a stretch of uridine bases (+2083 to +2089) results in read through to the latter UAA stop code and the synthesis of the protein of these precursors (159). Gag and Gag-Pol polyproteins also migrate to the cellular membrane and start to assemble, directed by a series of basic residues and a

myristoylation sequence present at the N-terminus of MA of the Gag polyprotein (308). At the C-terminal end of Gag, NC recruits two copies of unspliced RNAs, and the p6 late domain mediates the process of budding and detachment by its interaction with the host cellular protein Tsg101.

HIV-1 budding occurs mostly at the plasma membrane of infected T lymphocytes. In macrophage, however, this virus accumulates in an intracellular, vacuole-like compartment (256). Recent ultrastructural studies identified this compartment as late endosome/multivesicular bodies (MVBs) (284). HIV-1 p6 PTAP late domain recruits the cellular protein tumor susceptibility gene 101 (Tsg101) to facilitate virus budding (126, 365). In uninfected cells, Tsg101 functions in the biogenesis of the multivicular body (MVB) (173). Reduction of TSG101 levels by siRNA treatment or introduction of a dominant-negative Tsg101 mutant blocks viral budding and produces tethered structures at the plasma membrane that resemble the phenotype of late-domain (PTAP) mutants (77, 126). All these results suggest that HIV may bind Tsg101 in order to gain access to the downstream machinery that catalyzes MVB vesicle budding. Studies in yeast show that Tsg101 is part of a 350-kDa intracellular complex known as endosomal sorting complex required for transport-I (ESCRT-I), which along with ESCRT-II and ESCRT-III directs monoubiquitinated endocytosed cargo to the MVB (19, 184). Wills *et al.* first noted the topological similarity between budding of vesicles into the lumen of the MVB and budding of viral particles into the extracellular milieu-both processes involve budding away from the cytosol (265). Although ESCRT components are required for HIV-1 particle formation, it is not known if they perform this function at the MVB, with other compartment, at the plasma membrane, or at all three locations.

Particle release and maturation. The process of viral budding or release triggers the activation of the PR that autocatalytically cleaves the Gag and Gag-Pol polyprotein, which releases the structural proteins and enzymes MA (p17), CA (p24), NC (p9), p6, PR (p10), the reverse transcriptase (RT), and integrase (p32) (416). The individual proteins undergo further interaction, with CA and NC forming the conic nucleocapsid, MA remaining associated to the viral envelope (39, 383).

1.4. Biological functions of Vpr.

Vpr is the only accessory protein found within the virion in substantial amounts.In *in vitro* culture systems, Vpr is not required for viral replication in transformed cell lines and primary T lymphocytes; however, it plays an important role in the productive infection of cells, such as macrophages and monocytes (65, 146, 368). HIV-1 replication in macrophages is extremely important for the pathogenesis *in vivo* because terminally differentiated macrophages are natural cell targets for HIV and provide a reservoir of viral production during the asymptomatic stages of disease (20) The most convincing evidence that Vpr plays an important role *in vivo* comes from experiments showing that rhesus monkeys infected with Vpr deleted SIV virus have a low viral burden and slow disease progression compared to those infected with the wild-type virus (157, 192).

1.4.1. Structure of the protein.

Primary sequence. The Vpr gene of HIV-1 encodes a 96-amino-acid 14-kDa protein that is produced late in the viral life cycle (62). The primary Vpr sequence is shown in Fig 1.5. Vpr sequence is well conserved among the primate lentiviruses HIV-1, HIV-2, and SIV (350).

MEQAPEDQGP[10] QREPHNEWTL[20] ELLEELKNEA[30] VRHFPRIWLH[40]
GLGQHIYETY[50] GDTWAGVEAI[60] IRILQQLLFI[70] HFRIGCRHSR[80]
IGVTRQRRAR[90] NGASRS[96]

Figure 1.5. The primary Vpr amino acid sequence. *The negative amino acids are shown in purple, positive charge amino acids in red and phosphorylation site in green.*

3D-structure. Vpr shows well-characterized helical domains with amphipathic properties and γ -turns throughout the protein. NMR analysis of a soluble full length Vpr (1-96) polypeptide was recently performed, and revealed the tertiary structure of the protein, confirming the amphipathic nature of the

three α-helices of HIV-1 Vpr. The helices are connected by loops and are folded around a hydrophobic core surrounded by a flexible N-terminal domain and a C-terminal arginine-rich region that are negatively and positively charged, respectively (Fig 1.6) (235). The flexible and negatively charged N-terminal region (Met1-Glu13) followed by a γ-turn (Pro14-Asn16), then an α-helix of 17 amino-acids, encompassing residues Asp17 to His 33. Vpr first helix has the characteristics of an amphipathic helix. Its hydrophilic face is formed by the amino acid side chains: Asp17, Glu21, Glu24, Glu25, Lys27, Asn28, Glu29 and Arg32, while the hydrophobic face is constituted by the side chains: Trp18, Thr19, Leu20, Leu22, Leu23, Leu26, Ala30, and Val31; an interhelical domain 1 (34-39) followed by a second γ-turn (Phe34-Arg36) and a second α-helix (His40-Glu48). The second helix (residues His40 to Glu48) also has amphipathic properties as the hydrophilic side chains of Gln44 and Glu48 are located on one side of the helix while the hydrophobic side chains of Leu42, Ile46 are on the other; an interhelical domain 2 followed by a γ-turn (Asp52-Trp54). The third–helix is also well defined in the (55–74) region. Gly75 appears to induce a slight curvature in the helix, which is poorly defined in the (78–83) region. The hydrophobic amino acid side chains (Val57, Ile61, Ile63, Leu64, Leu67, Leu68 and Ile74) are located on one face of the helix and form an uninterrupted hydrophobic face, whereas amino acid side chains (Glu58, Arg62, Glu65, Glu66, Cys76 and Arg77) form the hydrophilic face. Vpr C-terminal (Ile84-Ser96) does not have a defined structure; it is an arginine-rich positively charged flexible region (235, 377).

Results from site specific mutagenesis have shown the importance of helix I for Vpr functions such as virion incorporation, stability, and subcellular localization (215, 390). Furthermore, this domain was also implicated in the oligomerization of Vpr.The substitution for the hydrophobic residues in Vpr Helix II severely affected the virion incorporation of Vpr (332).

Amino acids 60-81 of Vpr in Helix III constitute the leucine-isoleucine-rich (LR) domain. It forms a long, well-defined amphiphilic α-helical structure extending from Trp53 to Arg78, one side of the helix offers a stretch of hydrophobic residues that can form a leucine-zipper-like motif (35). This leucine-zipper-like motif forms an extended uninterrupted hydrophobic surface, whereas, polar residues mainly

occupy the other side. This structure may account for the formation of Vpr dimer (311, 375) and /or for the interaction with cellular partners (179, 374, 404). The LR domain is also involved in Vpr-mediated nucleocytoplasmic shuttling (326) which is described in detail in section 4.2

Four conserved prolines (positions 5, 10, 14 and 35) in the N-terminal domain present cis/trans isomerization (40). It was reported that the cellular peptidyl-propyl isomerase cyclophilin A was able to interact with Vpr via prolines at position 14 and 35, which made sure for the correct folding of the viral protein (399).

It was reported that Vpr is phosphorylated in small proportion both in infected cell and virion (239) and the phosphorylation targets four serine residues which are located at S28, S79, S94 and S96 of Vpr (4). The S28 site is less conserved among different HIV isolates. The S79 site is a major phosphorylated site which plays an important role in HIV-1-mediated macrophages infection (4). The Vpr S79A mutation does not have G2 cell cycle arrest. Besides the fact that another report showed that only S79, S94 and S96 triple mutation can block HIV-1 macrophage infection (411), the real contribution of Vpr phosphorylation on HIV-1 macrophage infection needs to be determined.

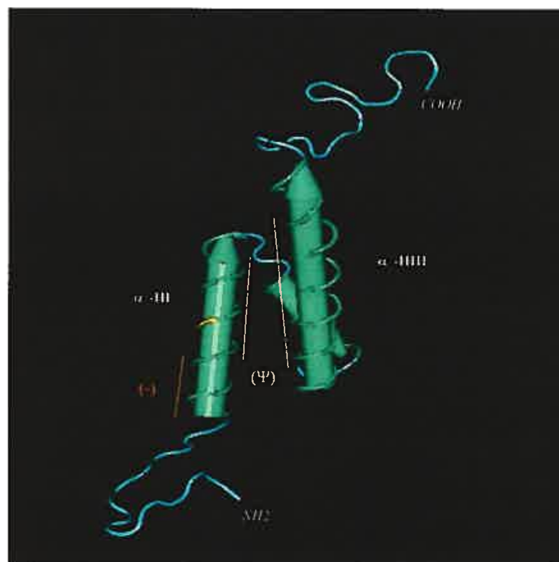

Figure 1.6. 3D structure of Vpr protein. *Vpr has three α-helices. The helices are connected by loops and folded around a hydrophobic core surrounded by a fexible N-terminal domain and a C-terminal*

arginine –rich region that are negatively and positively charged. N Morellet, et al. Pubmed mmdb ID:22329 (http://www.ncbi.nlm.nih.gov/Structure/mmdb)

The carboxy-terminus of Vpr contains six arginines between residues 73 and 96. This domain shows similarity with those of arginine-rich protein transduction domains (PTD), and may explain the transducing properties of Vpr, including its ability to cross the cell membrane lipid bilayers (148, 329).

Oligomerization. The gel filtration experiment using recombination Vpr from bacteria showed that Vpr exists *in vitro* as an oligomer, possibibly as an hexamer. Vpr oligomerization appears to be mediated primarily via the N-terminal domain (aa 1-42) (405). Similar report showed that a small percentage of synthetic Vpr was detected in dimers and trimers. Such candidate oligomers were only detected at a concentration of ≥250 ng of synthetic Vpr but not in preparations from viral lysates (148). The reason could be the sensitivity of detection. However we could readily detect the dimer form of Vpr from infected cells and virion using HA-tagged Vpr, but no other forms of Vpr oligomer could be detected (my unpublished data). Another reason could be that Vpr is associated with other proteins both in the cell and virion which blocks the oligomer formation. Vpr contains a single cysteine at residue 76 that may potentially participate in intermolecular disulfide bond formation About 10% of the molecules exist as disulfide-linked dimers, the formation of which was prevented by the addition of dithiothreitol (DTT) (148). There are three types of protein-protein interactions in which Vpr oligomerization could be involved. First Vpr oligomerization may be important for Vpr interaction with the Gag p6. The tendency of Gag and Vpr to oligomerize could be important for their assembly into mature virion. Second, Vpr oligomerization may enhance Vpr interaction with its cellular target. Third, oligomerized form of Vpr in the HIV-1 pre-integration complex could ensure that Vpr can simultaneously interact with both the viral component in the PIC and the cellular component in the cytoplasm. This may be a prerequisite for Vpr to promote the PIC into nucleus. These possibilities deserve further investigation.

1.4.2. Subcellular localization.

NLS. Vpr localizes in the nucleus when it is expressed alone (210). Two independent nuclear targeting signals have been characterized within the HIV-1 Vpr sequence, one spanning the α-helical domain in the N-terminal part of the protein and the other within the arginine-rich C-terminal region (163, 171). However, within the context of the native Vpr protein, the function of C-terminal nuclear localization domain is controversial. Several other mutagenesis studies have failed to link this basic sequence with nuclear localization (215, 390, 404). Instead, these investigators found that nuclear localization was lost when the first two N-terminal domains of Vpr were mutated. They proposed that the carboxy-domain relates simply to Vpr stability. Detailed description of Vpr NLS and its role in targeting HIV PIC to the nucleus is presented in section 5.1.

NES. In characterizing the NLS in the Vpr N-terminal (1-71), Sherman *et al.* found that the Vpr LR domain ($L_{64}QQLL_{68}$) was also involved in Vpr-mediated nucleo-cytoplasma shuttling (326). The distal leucine-rich helix contains a nuclear exporting signal (NES). This NES utilizes the chromosome maintenance region 1 protein (CRM1), which binds to the leucine-rich NES directly and mediates export through the NPC in a leptomycin B-sensitive manner (249). Jenkins *et al.* reported that the mutated form of Vpr L68A can still be virion-incorporated, suggesting that Vpr nuclear export is not required for virion incorporation (164), while Sherman *et al.* reported that Vpr nuclear export mutant L67A impairs Vpr virion incorporation and subsequently effects viral replication in the macrophage, suggesting that Vpr NES is required for efficient macrophage infection (325). Because Vpr NES $L_{64}QQLL_{68}$ locates in the centre region of Vpr Helix III, and mutation in this motif causes Vpr conformational change which attenuates the incorporation of Vpr in the virion (164, 325), probably it is mutation itself but not Vpr NES mutation disturbing Vpr virion incorporation and HIV-1 macrophage infection. The exact role of Vpr export during HIV-1 infection needs to be further defined.

1.4.3. Viral incorporation of Vpr

Since Vpr is not synthesized as part of the Gag polyprotein precursor, Vpr requires an anchor to associate with the assembled viral proteins that are to be virion-incorporated. It has been clearly demonstrated through deletion analysis that the p6 domain of the p55 Gag precursor constitutes such an anchor for virion incorporation of Vpr (53, 186, 209, 210, 266). A subsequent report mapped the interaction domain in residues 32-46 of p6 that contains a LXXLF motif (185). A predicted putative α-helical domain near the N-terminus plays an important role in the packaging of Vpr into the virion (82, 215, 390). The smallest Vpr fragment that supports the binding is amino acid 1-71 (164). After assembly and proteolytic cleavage of $Pr55^{gag}$ into the Matrix, Capsid, Nucleocapsid, and p6 protein, Vpr is recruited into the mature virion core where p6 is excluded, indicating that Vpr may interact with viral or cellular component(s) other than p6 that are to be localized within the core. It seems that Vpr is less avid for the fully processed p6 protein than for the p6 region in the context of the Pr55gag precursor. Because of differential avidity, Vpr is recruited into the core of the particles where it could interact with nucleic acids (403), NCp7 (73, 74), or the matrix protein (306). It is estimated that Vpr is efficiently incorporated into the viral particle at molar amounts 1:7 to those of Gag that may represent 275 molecules of Vpr per virion; a small part of Vpr is phosphorylated in the virion (239).

1.4.4. Effects of Vpr on HIV-1 replication and pathogenesis *in vivo*

Vpr protein sequences are much conserved throughout different primate lentiviruses; it correlates with conservation of Vpr function. This function associated with HIV-1 Vpr has segregated in the HIV-2/SIVmac/SIVsm lineage, where Vpr induces G2 arrest, and Vpx promotes the nuclear import of PICs (106). The importance of Vpr in viral pathogenesis is addressed in a number of earlier studies of SIV mac219 infection in Rhesus monkeys. In one study, Rhesus monkeys infected with the SIVmac239 defective in Vpr had a low viral burden and no disease progression, whereas monkeys infected with the wild-type virus or the wild-type revertant from Vpr-defective viruses exhibited higher viral burden and rapid disease progression. The reversion of Vpr in three of the five test animals indicates that there is

significant selective pressure for functional forms of Vpr *in vivo* (192). In two other studies, no significant differences in disease progression were found between Vpr-deficient and parental wild-type viruses—all infected monkeys developed AIDS-like symptoms (129, 151). However, in the experiment most relevant to infection by HIV-1 without Vpr, monkeys infected with SIVmac239 defective in both the Vpr and Vpx genes had severe attenuated infection with a much lower viral burden and no evidence of disease progression (80, 129). At least a 100-fold decrease in the pathogenic index was found in the infectivity of these mutant viruses when compared with the wild-type viruses, confirming the role of Vpr in viral pathogenesis (80).

The requirement for Vpr *in vivo* by HIV-1 was further illustrated in chimpanzees and in an accidentally infected laboratory worker who initially was infected with a Vpr-defective HIV-1 laboratory strain IIIB (131). The HIV-1 IIIB strain Vpr gene has frame shift mutation at codon 73, which resulted in a truncated Vpr protein with only the first 72 amino acids. It was reported that this non-functional truncated protein reverts to wild-type Vpr both in the accidentally infected laboratory worker and in the experimentally infected chimpanzees (131). These results clearly demonstrate that Vpr is required for HIV-1 replication *in vivo.*

The Vpr sequence is one of the most conserved regions in the HIV genome, with an estimated similarity of 87% between the different viral strains (199). Usually, mutagenesis is used to predict the functional changes derived from amino acid substitution. A synonymous or non-synonymous amino acid substitution in Vpr could dramatically affect Vpr functions (82, 390). Since Vpr is a small molecule, disruption of any specific domain by insertion of mutation frequently causes major structural changes, and affects the rest of the molecule. Another potential problem associated with these mutagenesis studies is that the Vpr mutants were often artificially created, and therefore, may not represent the profile of naturally occurring mutations. The precise contribution of Vpr to HIV-1 pathogenesis *in vivo* is difficult to determine. The recent discoveries of Vpr mutations in long term non-progressor individuals (LTNPs) have underscored the importance of Vpr in the viral life cycle *in vivo* (211, 334). One Vpr polymorphism Q3R from LTNP contained both Q3R and C-terminal mutations, which significantly impaired the ability

of Vpr to confer cytopathicity, but had no effect on the efficiency of viral replication. Vpr R77Q is reported to associate with HIV patients with slow disease progression (211, 232). Although they have convincingly demonstrated that Vpr R77Q mutation isolated from the HIV-1 viral strain HxBru, impaires the ability of Vpr to induce apoptosis, but it was not proven by other reports using other HIV-1 viral strains (57, 105). Also, Vpr C-terminal mutations are frequently found in LTNP (47, 371, 406). These natural Vpr mutations in LTNP support HIV-1 replication *in vivo*, but these mutations may disturb the other unknown functions of Vpr by eventually compromising HIV-1 pathogenesis *in vivo*. Detailed research needs to be done to address this question. Taken together, these data support the idea that functional Vpr may be one of the viral factors contributing to disease progression.

1.4.5. Biological functions of the HIV-1 Vpr protein

The precise mechanisms by which Vpr influences viral replication are still unclear. Numerous proteins capable to bind Vpr have been identified, suggesting that the viral protein may be implicated in the reverse-transcription, the transcriptional activation of the LTR promoter, the nuclear transport of the preintegration complex in nondividing cells, and HIV-induced growth arrest and cytotoxicity (see summary in Fig 1.7).

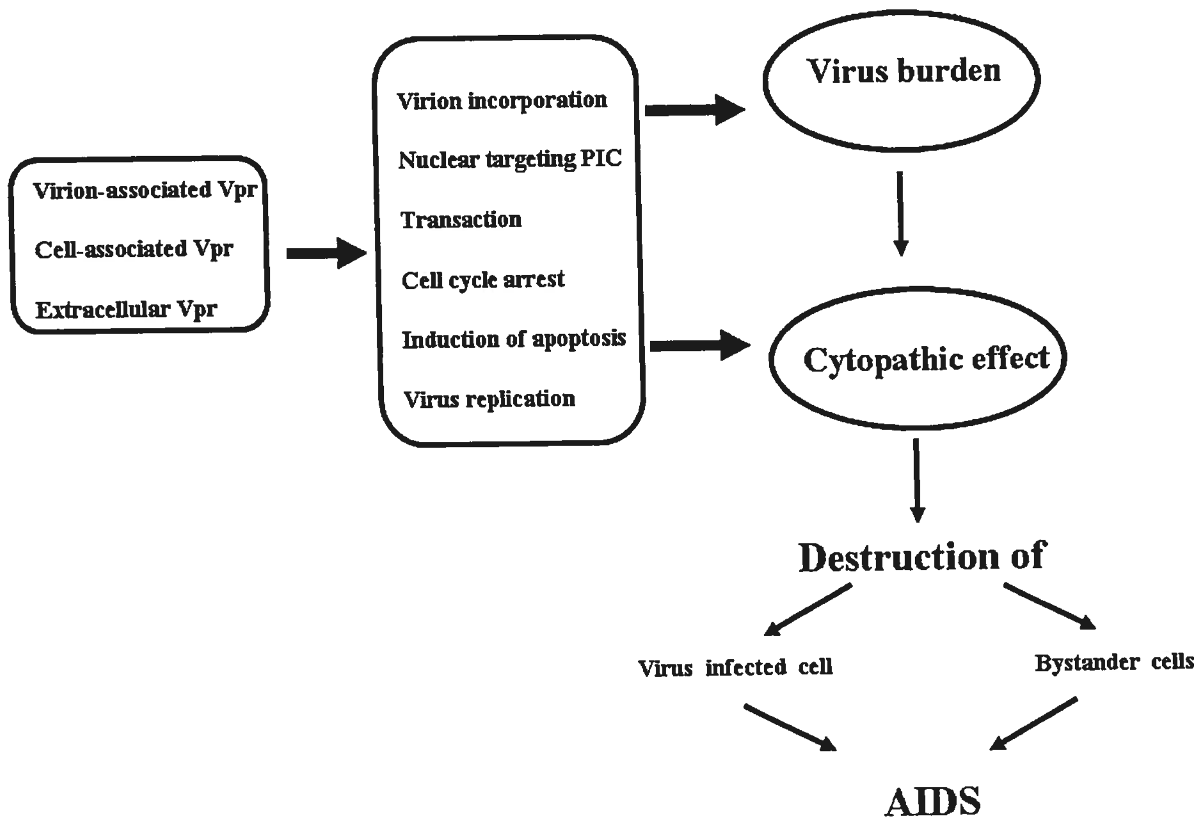

Figure 1.7. Potentil role of Vpr in AIDS pathogenesis.

Implication in the reverse-transcription. The interaction of Vpr with uracil DNA glycosylase modulates the human immunodeficiency virus type 1 *in vivo* mutation rate (219) and decreases the mutation rate in cells (166) that has been described in section 1. 3.3.

Vpr is a general transactivator. In early studies, Vpr was shown to be a moderate transcription activator of several viral and cellular promoters (63, 107). Vpr may provide help in activating HIV-1 LTR direct expression at an early stage to generate the messenger RNA, which produces the early proteins, such as Tat and Rev that are essential regulatory proteins following the integration of viral DNA into the host genome. Consistent with this hypothesis, the virion-associated Vpr stimulates LTR-directed reporter gene expression *in vivo* by increasing the mRNA level (389). The transactivation of HIV-1 induced by Vpr is mediated through *cis*-acting elements, including NF-κB, Sp1, CEBP, and the glucocorticoid

response element (GRE) enhancer sequence found in the LTR promoter (152, 361, 374). Also related to this activity, Vpr regulates the expression of host cell genes such as NF-κB, NF-IL-6, p21waf1, and survivin (56, 303, 414). These studies proposed that Vpr stimulates the HIV-1 LTR promoter by associating with the transcription factor Sp1. Vpr could bind to Sp1 in a gel-shift assay using probes containing the Sp1-responsive elements and in a coimmunoprecipitation assay (374). Direct binding of the two molecules has not been demonstrated, but the second α-helix of Vpr is necessary for stimulation of the HIV-1 LTR. Vpr was also shown to interact with one of the general transcription factors (TF) TFIIB in an *in vitro* GST-pull down assay (3). Subsequent analyses further indicated that Vpr functions as a potent enhancer of Tat-induced activation of HIV LTR (107, 180). Interaction of Vpr with Tat and its partner Cyclin T1 were also found in *in vitro* binding assay (307).

Vpr displays high affinity for nucleic acids, but no specific DNA sequence targeted by Vpr has been identified yet (177, 403). Interestingly, Vpr does not bind to the Sp1 factor or *cis*-elements alone, but it associates with Sp1 in the context of the G/C box array (374), indicating that Vpr might bind to a specific DNA sequence after it associates with a cellular partner, and subsequently drive the expression of both host cell and the viral gene.

It has been reported that Vpr can directly bind to p300 via a LXXLL motif present in the C-terminal α-helix of the protein (180), suggesting that Vpr may act by recruiting the p300/CBP co-activators to the HIV-1 LTR promoter, and thus enhance viral expression. Since p300 is a coactivator of NF-κB, Vpr also can mediate up-regulation of promoters containing the NF-κB and NF-IL-6 enhancer sequence in primary T cells and macrophages. In addition, Vpr markedly potentiates glucocorticoid receptor (GR) action on its responsive promoter (179, 180). The Vpr-induced LTR transcription is inhibited by the addition of the GR antagonist, RU486, in cultured macrophages (288). Vpr-mediated coactivation of the GR is distinct from the G2 arrest and requires both the $LLEEL_{26}$ and $LQQLL_{68}$ motifs from the first and third α-helical domains of HIV-1 Vpr (179, 327).

The last two functions of Vpr (Vpr-mediated nuclear import and cytotoxicity) will be developed in section 1.5 and section 1.6 separately, since they constitute the main topics of my thesis study.

1.4.6. Vpr induced G2 arrest.

To ensure accurate transmission of the genetic information, eukaryotic cells have developed an elaborate network of checkpoints to monitor the successful completion of every cell cycle step and to respond to cellular abnormalities such as DNA damage and replication inhibition as they arise during cell proliferation. Two of the best characterized G2/M checkpoints—DNA damage and DNA replication—were first characterized in detail by genetic analysis of fission yeast. The G2 to M transition is controlled in fission yeast by the phosphorylation status of Tyr15 on Cdc2, the cyclin-dependent kinase that regulates the cell cycle in all eukaryotic cells (236). The Wee1 phosphorylates Tyr15 in Cdc2 and Mik1 kinase to hold the cell in G2, and rapid dephosphorylation by Cdc25 phosphatase triggers the G2 to M transition (188, 236).

One of the major biological activities associated with Vpr is its ability to prevent the passage of the cell through mitosis at the G2 stage of the cell cycle. The HIV-1 Vpr protein induces cell cycle G2 arrest through inhibitory phosphorylation of Cdc2 both in fission yeast and human cells, suggesting that Vpr affects a conserved cellular process. Specifically, this effect is associated with the inactivation of the cyclin-dependent kinase (Cdk) p34cdc2 (144, 285, 407). Vpr exerts its inhibitory effect through phosphorylation site T14 and Y15 of CDK1 and Y15 of Cdc2, as an expression of the nonphosphorylated Cdc mutant, T14A Y15F of CDK1 and Y15F of Cdc2 in the yeast, preventes Vpr-induced G2 arrest (95, 144). Furthermore, Vpr interacts with, and inhibits the activity of the phosphatase Cdc25 (24, 96, 130) and activates Wee1 kinase (96, 397) to promote phosphorylation of Cdc2/Cdk1 during the induction of G2 arrest. Consistent with the role of Wee1 and Cdc25 in Vpr-induced G2 arrest, proteins that are involved in the regulation of Cdc25 or Wee1 have also been identified as either augmenting or alleviating Vpr-induced G2 arrest. A Cdc25 inhibitor *rad25* (208), which is the human 14-3-3 homologue, enhances Vpr-induced G2 arrest when overproduced in fission yeast (96). Recent studies further show that Vpr

binds to 14-3-3 and Cdc25C in human cells (130, 181). The region of Vpr binding to Cdc25C mapped near the catalytic domain of Cdc25C and the Cdc25C phosphatase activity was inhibited. The authors also observed that RNAi knockdown of Cdc25C expression abrogated Vpr-induced G2 arrest (130).

Early research in human cells showed that Vpr does not induce G2 arrest through the DNA damage checkpoint pathway. However, Vpr still induces G2 arrest in cells from patients with ataxia telangiectasia (AT) (24). These AT cells has ATM gene mutation, which is a human homologue of fission yeast Rad3, and they do not arrest in G2 in response to DNA damage (223). However, recent reports show that Vpr activates ATR and a second human homologue of fission yeast Rad3, and other steps in this checkpoint pathway such as Rad17, Hus1, BRCA1, and γ-H2AX, these studies suggest that Vpr induces G2 arrest through either a cellular response to DNA replication stress or to a signal that mimics DNA damage (414, 415). Expression of Vpr does not increase gene mutation frequency (217) or change the radio-sensitivity of the checkpoint defective mutant (96), which goes against the possibility that Vpr actually causes DNA damage. Thus, it is reasonable to think that signals other than actual DNA damage triggers DNA damage-like cellular response. These cellular responses could include the nuclear herniation which is caused by Vpr (72) or cellular stress responses to Vpr gene expression (28, 158). Since ATR and Chk1 are primarily responsive to changes in DNA replication, an alternative possibility is that Vpr may interfere with DNA replication. This possibility is certainly supported by a number of reports showing that Vpr induces genomic instability, formation of micronuclei, and aneuploidy (330, 407). All of these changes in DNA structures could be perceived as replication stresses, which could trigger cell cycle arrest.

Recent findings suggest that Vpr mediated cell cycle arrest at the G2 stage may have advantage for the virus. The viral LTR is highly active in the G2 stage (131, 153, 413). Therefore, Vpr induction of cell-cycle arrest at the G2/M transition can optimize viral gene expression and allows for more efficient viral replication through the up regulation of viral transcription. Brasey and his colleague demonstrated the presence of an IRES element in the 5' leader of HIV-1 DNA, and the HIV-1 IRES showed peak translation activity when cells were arrested in G2 stage (37). It will be interesting to investigate if Vpr-

mediated G2 arrest can result in down-regulation of cap-dependent translation to favor protein translation from internal ribosome entry sites (IRESs), including a putative viral IRES.

1.5. Proprotein convertase and HIV-1 infection

Biologically active proteins and peptides are often generated by intracellular limited proteolysis of inactive precursors. The precursor protein can be cleaved intracellularly, at the cell surface or within the extracellular milieu. This process is accomplished by proprotein convertases (PCs), which are serine proteinases related to bacterial subtilisin and yeast kexin. Today seven mammalian PCs, characterized by their ability to activate precursors at dibasic amino acid residues, have been identified. PCs in this group include PC1 (PC3), PC2, PC4, PACE4, furin, PC5A&B (also known as PC6A&B), and PC7 (LPC) (247, 319). In addition, recently, two other PCs that cleave the precursor at non-basic residues have been characterized. The pyrolysin-like subtilase SKI-1/S1P (305, 321) cleaves proproteins with the consensus motif (R/K)-X-(hydrophobic)-Z↓ (where Z is a variable), whereas the novel neural apoptosis-regulated convertase 1 cleaves its own prosegment at VFAQ↓ (318). SKI and PCSK9 are important regulators in cholesterol and lipoprotein homeostasis (305).

The specificity of PC-precursor interaction is regulated by cellular expression of the precursor and subcellular localization, as well as by the strict requirements of the precursor structure and sequence (319, 346).

1.5.1. Tissue distribution and subcellular localization of proprotein convertases

Furin is a ubiquitous PC of constitutive secretory pathway. Human furin has 794 amino acids. It is a type I membrane protein predominantly localized in the *trans*-Golgi network (TGN).Yet a proportion of furin is also localized at the cell surface (231) and in immature secretory granules of neuroendocrine and endocrine cells (86).

Furin cleaves most efficiently at the R-X-(K/R) R motif: (i) An Arg residue is essential at the P1 position, (ii) In addition to the P1 Arg, at least two out of the three basic residues at P2, P4, and P6 are required for efficient cleavage, and (iii) At the P1' position, an amino acid with a hydrophobic aliphatic side chain is not suitable (247). Three other PCs, PC5/6A, PACE4 and PC7, have the sequence specificity similar to that of furin (247). In accordance with its tissue distribution, a wide range of protein precursors are shown to be substrates for furin, such as the parathryroid hormone-related peptide (PTHRP) (206), the pro-β-nerve growth factor (38), the HIV-1 envelope gp160 (140), and HIV-1 tat (348).

PC1/PC3 and PC2 enzymes exhibit a more restrictive expression pattern than furin. It has been suggested that PC1/PC3 and PC2 are involved primarily in the processing of prehormones within the regulated secretory pathway of cells of the endocrine and neural tissues (359). High levels of expression of PC1 and PC2 mRNA are observed in the neuropeptide-rich regions, such as the hypothalamus, hippocampus, and cerebral cortex. These areas are also characterized by the relatively high expression of carboxypeptidase E (319). Most prohormone processing occurs within the regulated secretory pathway and generates a bioactive hormone that is secreted from mature secretory granules in response to specific stimuli. PC1/PC3 and PC2, which concentrate in mature secretory granules, are the only PCs to enter this pathway (322). The PC1/PC3 and PC2 proteins are shown to possess endoproteolytic activity with specificity for cleavage sites that consist of paired basic amino-acid residues (26, 347).

PC4 exhibits a unique expression pattern, since it is only expressed in testicular germ cells (25). Like PC1/PC3, PC2, and an isoform of PC5A, PC4 belongs to the group of PCs which act within the regulated secretory pathway. Only a few substrate of PC4, like the pituitary adenylate cyclase-activating polypeptide, have been identified so far, which demonstrates the important role of the enzyme in the testicular development system (203). Furthermore PC4 (-/-) transgene mice are infertile.

Northern blot analysis revealed that like the furin gene, the PACE4-encoding gene is broadly expressed. A 4.4-kb PACE4-specific mRNA is found in a wide variety of tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. It is apparent that PACE4 and PC5A are the closest members of the family exhibiting about 74% sequence identity within their catalytic domain. PACE4 seems to be particularly highly expressed in the cerebellum and in the spinal cord (320). Although PACE4 is not expressed in peripheral blood lymphocytes (PBL), it is well expressed in lymphatic tissue, such as the thymus, lymph node, and spleen (141). PACE4 is localized in TGN and the cell surface (345). PACE4 is found in a number of cell lines, such as the human embryonic kidney cell line 293, the hepatoma cell line HEPG2, the monocyte cell line U937 (359), and the Cos-1 cell line (320). PACE4 is also present at the exterior of cells and plays a role in the proteolytic activation of the anthrax toxin PA (132).

PC5 is the only member of the basic amino-acid-specific PC family that exists as two isoforms: soluble PC5A (212) and membrane-bound PC5B (245). PC5/6 undergoes alternative splicing to generate two isoforms (A & B) with distinct C-termini and expression patterns. PC5A/6A lacks the putative transmembrane domain that is found in PC5B/6B and has a much shorter Cys-rich region, whereas PC5B/6B has the largest Cys-rich region of all the PCs (245). PC5A and PC5B are distributed ubiquitously in the tissue (345). The structural differences of these two isoforms govern their sorting to different compartments of the secretory pathway. PC5A is sorted to both the constitutive and regulated secretory pathway, whereas PC5B is localized only within the constitutive secretory pathway (71). PC5A is mostly localized within the dense core secretory granules in regulated cells, and PC5B is localized to the TGN where it communicates with endosomes and the cell surface (71, 345). Recent reports indicate that secreted PC5A and PACE4, but not soluble furin, bind heparin within the extracellular matrix via a cationic stretch of amino acids within cysteine-rich domain (CRD) in both PC5A and PACE4 (254, 351). Previous studies demonstrated that the majority of PC5A cleaved protein precursors are membrane-bound

proteins, such as the adhesion protein, which includes integrin a-chains (336) and the neural adhesion protein L1 (168), as well as TGF β-like precursors (355).

PC7 is the third type membrane-bound protein. Unlike furin and PC5, PC7 has no cysteine-rich region or RGD motif, suggesting it may not associate with external plasma membrane or extracellular matrix (ECM). Phylogenetics analysis suggests that PC7 represents the most divergent enzyme of the PC family and that it is the closest member to the yeast convertase Kexin (319). The mature PC7 is mainly localized to the TGN and cell surface (345), as described for furin that PC7 may recycle between TGN and cell surface. In contrast to furin, PC7 is not shed from the cell (240, 358). PC7 appears to have a redundant, nonessential function, since its absence does not result in any detectable phenotype. Histochemical analysis of internal organs has not revealed any obvious abnormalities. The absence of a phenotype is surprising in light of its ubiquitous expression at all development stages and in adult tissue, including the brain, lung, liver, kidney, spleen, and thymus (67, 228). PC7 is either involved in the processing of a set of nonessential substrates or is active in the cells where sufficient processing redundancy is present.

1.5.2. PC expression in T lymphocytes cells

The Northern blotting analysis of the human immune system showed that PC5A and PACE4 are found in liver, bone marrow, appendix, thymus, lymph node, and spleen, but not in peripheral blood lymphocyte (PBL) (141). Furin and PC7 were found in all tested, tissues whereas PC1 was found only in the appendix. Furin and PC7 were the only enzymes detectable at the mRNA level in primary PBLs, macrophages, and cultured T cells lines (CEM, MOLT-4, TPH-1, HUT-78, H9, Jurkat). THP-1 but not primary macrophage cells were found to have PACE4 expression, suggesting PACE4 is expressed in highly differentiated cell (141).

Semi-quantitative RT-PCR results show that PC7, PC5, and furin are the three main PCs expressed in resting CD4 T lymphocytes, although PC5 expression level is very low. In contrast, no PC1 and PC2 transcripts were found in the isolated cells, and only insignificant levels of PACE4 mRNA were detected. PHA/IL-2 stimulation of PBLs leads to a significant increase in the level of expression of furin and PC7, but not of PC5 (76).

1.5.3. Proprotein convertase Inhibitors.

A considerable effort was made to produce the specific PC-inhibitor because of the potential clinical and pharmacological role of the convertase. The proposed strategies involved the development of either a peptide-based PC-inhibitor or protein-based inhibitors.

PC peptide inhibitor. Garten *et al.* have shown that acylated peptidyl chloromethanes (-CH_2CL, Chloromethylketones) containing a consensus furin cleavage motif—such as decanoyl-Arg-Glu-lys-Arg-CH_2CL—inhibit *in vitro* cleavage of the Influenza-Virus HA protein by furin at a micromolar concentration through covalently modifying the substrate-binding site of convertase (338). Garten *et al.* further showes that this PC peptide inhibitor blockes the cleavage of several viral envelope glycoprotein precursors, such as influenza HA, HIV gp160, cytomegalovirus glycoprotein B, and parainfluenza-virus glycoprotein F0, and subsequently inhibites the formation of infectious viral particles (140, 258, 338, 366). Although these peptidyl-CH_2Cl species are very useful for *in vitro* studies of PCs, they appear to be ineffective in *in vivo* antiviral therapy. One reason is that they are unable to completely abolish the cleavage of these glycoprotein precursors, which is possibly due to their low efficiency for penetrating into a cell and the instability of the chrolomethylketone group. Secondly, they are relatively cytotoxic, possibly due to their irreversible mechanism of inhibition (247). So far, the *in vitro* peptide-based approach has not effectively inhibited the PCs intracellularly, and thus more work is needed to improve the cellular permeability of the designed inhibitors.

Protein based inhibitor for PC. Protein-based furin inhibitors have also been developed, since tissue- or cell-type-specific expression of these inhibitors controlled by a characterized promoter could be therapeutically valuable. In late 1980s, Brennan showed that a variant serpin of α1-antitrypsin, name α1-antitrypsin Pittsburgh (α1-PIT), wth Met to Arg substitution in the reactive-site (AIPM to AIPR) inhibited Kex2p cleavage of proalbumin *in vitro.* α1-PDX is α1-antitrypsin variant serine protease inhibitor (serpin) containing the minimal consensus furin cleavage site (R-I-P-R) in its reaction site loop. α1-PDX inhibits furin by a slow tight-binding mechanism characteristic of the serpin molecule, and functions as a suicide substrate inhibitor (11). α1-PDX inhibits PCs activity by forming the SDS-stable complex with furin, PC5A, PC3 (162), and PACE4 (351). α1-PDX can block the furin-mediated proteolytic processing of HIV-1 gp160 (10). α1-PDX inhibits furin *in vitro* with a $K_{0.5}$ of 30ng/ml (≈0.6 nM) (10). α1-PDX is a potent convertase inhibitor for all tested PCs in the *in vitro* assays (76, 162). However, intracellularly α1-PDX acts primarily within the constitutive secretory pathway, and it strongly inhibits furin, PC5, and PACE4 (27) but is less potent for PC7 (27, 162). Therefore, α1-PDX is a very useful protein-based PCs inhibitor toward process of endogenous growth factors precurssor and the viral envelope glycoproteins. However, α1-PDX may exhibit a limited toxicity to cells *in vivo.*

Spn4A is a previously uncharacterized serpin from *Drosophila melanogaster.* It contains a consensus furin cleavage site, R-R-K-R in its reactive site loop (RSL). Spn4A inhibits human furin and *Drosophila* PC2 by a slow-binding mechanism characteristic of serpin molecules and forms a kinetically trapped SDS-stable complex with each enzyme (296).

1.5.4. HIV-1 env and tat are processed by PCs

The envelope (Env) glycoprotein of HIV-1 is essential for receptor binding and membrane fusion during infection. Env is synthesized as a precursor polypeptide (gp160) that oligomerizes to form a trimer

(87, 93), which is transported through the trans-Golgi network. In the trans-Golgi network, Env is cleaved by the cellular protease into surface (gp120) and transmembrane (gp41) subunits that remain associated by non-covalent interactions. Cleavage of gp160 occurs at a conserved Arg-Glu-Lys-Arg sequence (174, 225). Mutagenesis of the Arg-Glu-Lys-Arg sequence produces noninfectious HIV-1 particles containing unprocessed gp160 (225). Therefore, the cleavage or maturation of the envelope glycoprotein is a crucial step for the propagation of viral infection. Several studies have suggested that several enzymes of proprotein convertase family participate in the maturation process of HIV-1 gp160, furin, PC7 and to the lesser extent PC5A are the PCs expressed in the freshly isolated human CD4 T lymphocyte, the natural host cells of HIV-1 infection. These three PCs were implicated in the intracellular proteolytic processing of the HIV-1 envelope gp160 during HIV-1 infection (75, 238). It is difficult to point which member of the PC family is responsible for HIV Env processing because infected cells may contain several redundant activities, and every cell line that exists contains at least some members of the PC family. The requirements for the participation of an enzyme candidate in the envelope cleavage are: (1) a direct *in vitro* cleavage of the envelope glycoprotein, (2) cleavage of the envelope protein intracellularly using the coexpressing system, (3) colocalization with its substrate in the same intracellular compartment, (4) cleavage inhibition by a specific protease inhibitor, (5) expressed and active in the virus infected cells.

The HIV-1 Tat protein is a transcriptional activator for viral expression. Tat can be secreted through the nonclassic leaderless secretion pathway that shares several features with the acid FGF (fibroblast growth factor) (52). Tat expressed alone can be released in the absence of cell death or permeability changes. Tat release is dependent on the temperature and serum concentration, and it is not blocked by brefeldin A or methylamine. After release, a portion of the protein remains in a soluble form, whereas the rest binds to the extracellular matrix (ECM)-associated heparan sulfate proteoglycans (HSPG), The ability of soluble Tat to attach to membrane heparin sulfate proteoglycan is a step preceeding internalization transducing proteins by endocytosis (229). Extracellular Tat has multiple functions, such as the rescue of virus expression in some latently infected cells (111) and increase of the CXCR4 expression in T cells for HIV infection (316). Tat peptide containing the cysteine-rich domain

can activate monocyte (6) and mediate CD4+T cell killing by inducing tumor necrosis factor-related apoptosis-induced ligand (TRAIL) (408), stimulates cytokine production in T-cells (317) and mediates protein transduction in many cell types (314). Extracellular HIV-1 Tat protein has a highly conserved basic region of HIV-1 Tat protein (amino acids, 48-56). Two putative furin cleavage sites were identified and showed that Tat protein was cleaved *in vitro* at the second site, $RQRR_{56}$. This *in vitro* cleavage was blocked by the furin inhibitor alpha-1 PDX (348). Monocytoid U937 cells rich in surface furin also degraded Tat. Furin processing did not affect the rate of Tat uptake and nuclear accumulation in HeLa or Jurkat cells, but the transactivation activity was greatly reduced. Furin processing is a likely mechanism for inactivating extracellular HIV-1 Tat protein (348).

1.6. Vpr and HIV-1 cytotoxicity

1.6.1. HIV-1-induced cytopathic effect

Direct cytopathicity. HIV infects cells of the immune system, particularly T helper cells, which express the CD4 molecule. HIV infection is characterized by the gradual loss of CD4+ T cells and a progressive immune deficiency that leads to opportunistic infection and ultimately death. 98% of the CD4+ T-lymphocytes reside in the lymphoid tissue. During the course of infection, a steady depletion of lymphocytes occurs, and the structure of the lymphoid tissue is progressively destroyed. In the final stage of HIV infection, when the immune system is severely impaired and non-functional, the lymphoid architecture is totally destroyed with complete loss of cellular tissue and its replacement by fibrotic tissue (261). Inflammatory cytokines and HIV gene products, such as Nef, Tat, and Vpr, induce T cell receptor (TCR)-independent T cell activation and contribute to the efficient production and propagation of the virus from infected cells to adjacent CCR5+CD4+ T cells. It was initially proposed that HIV-associated immunodeficiency results from the direct virus-mediated killing of the CD4+ T cell (200) or indirect cytopathicity and CD8-mediated destruction of the infected CD4 T cells (227). Recent attempts to understand how HIV disrupts T-cell homeostasis suggest that chronic immune activation, due to the

persistent expression of the viral particles, results in high turnover rates of T cells, leading to increased T-cell proliferation that is physiologically controlled by increased apoptosis (89).

HIV-infected patients—with different degrees of immune dysfunction—have similar rates of infected cell clearance, suggesting that direct cytopathicity of the virus determines the life span of the infected cells (150, 267). Increasing attention is being given to apoptosis as a major factor in cell depletion during HIV infection. The HIV structural protein gp120 and accessory proteins Tat, Nef, and Vpr are found to exhibit apoptotic properties (18, 302). Apoptosis has been demonstrated in HIV-infected lymph nodes, the thymus, and other lymphoid tissue (8, 30). One type of HIV-1-induced cytopathic effect (CPE) involves the fusion of HIV-1 infected cells with other infected or uninfected CD4+ cells mediated by HIV-1 Env (393). Another type of HIV-1 induced CPE results in the death of single-cell killing rather than syncytia cell formation, which is the predominant HIV-1-induced CPE in peripheral blood mononuclear cells of HIV-1 patients (334). Cytotoxic T lymphocytes (CTL) could play an important role in killing HIV-infected cells during the early stage of infection (31). A recent study shows that both necrosis and apoptosis contribute to the HIV-induced killing of CD4+ T cells *in vitro*; the predominant population in acutely infected cells present typcal feature of necrosis, whereas less than 12% of cells have the feature of apoptosis (273). Thus, it is likely that the depletion of immune cells during HIV infection occurs through a variety of cell death mechanisms.

Bystander cell killing Increasing evidence exists in favor of the indirect killing of bystander cells by extracellular or cell surface-associated components of HIV-1-infected cells. This could explain why such massive death of CD4+ T lymphocytes occurs during the course of HIV infection when the viral burden is relatively low (12, 200). A number of studies demonstrate that the majority of CD4+ cells undergoing apoptosis during HIV infection are uninfected (104, 241). Coculturing of HIV-infected cells with uninfected cells results in the apoptosis of the uninfected target cell prior to syncytium formation or establishment of productive viral infection (248). Cell death during the course of HIV infection is not limited to CD4+ T lymphocytes. It also has been demonstrated in non-CD4+ cells such as CD8 T cell (30,

49), B cells (49), natural killer cells (286), hematopoietic progenitor cells (22), macrophages, dendritic cells, and endothelial cells (8). Since non-CD4+ cells, which are not infected by HIV, are eliminated, this suggests that their death is mediated by an indirect mechanism.

While contributing to the bystander effect, HIV could also have devised strategies to prevent the death of the cells that it has infected. Inhibition of apoptosis in HIV-infected cells enhances virus production and facilitates persistent infection (13). Finkel *et al.* showed that in HIV-patient lymph nodes, widespread apoptosis occurs in uninfected cells. HIV-infected cells were very rarely apoptotic, and on the basis of these studies, this suggests that a viral protein inside the infected cells may inhibit apoptosis. Both Tat and Vpr proteins has been shown to have a dual effect on apoptosis (68, 69, 400).

1.6.2. Vpr-mediated apoptosis.

Impact in HIV-1 pathogenesis. Apoptosis is a programmed cell death, a mechanism multinuclear organisms to ensure proper development and homeostasis. It is also a biological process necessary to combat the effect of oncogenesis and viral infection. HIV infection causes a depletion of CD4+ T cells in AIDS patients, which results in a weakened immune system that is impaired in its ability to fight infections. The major mechanism for CD4+ T cell depletion is apoptosis, which can be induced by HIV through multiple pathways of both infected cells and non-infected "bystander" cells (7). In recent years, the investigation of Vpr has identified it as a possible causative agent or at least a contributing factor in cell death via the apoptosis pathway. Even though the exact contribution of Vpr as a pro-apoptotic factor responsible for T-cell depletion observed in the natural course of HIV infection is still unknown, evidence has repeatedly shown that Vpr has a cytotoxic potential and is able to induce apoptosis in many *in vitro* systems (391). In addition, transgenic mice expressing Vpr under the control of the CD4 promoter show both CD4 and CD8 T cell depletion associated with thymic atrophy. However, controversial results indicating that Vpr can also act as a negative regulator of T cell apoptosis have been reported (17, 69).

Since apoptosis in response to viral infection is used by many eukaryotic organisms, some viruses have developed strategies to inhibit, or at least delay the process, allowing virus to replicate before the

cell is killed. The dual effect of Vpr on apoptosis (68, 69) may be explained by the following scenario. In the early phase of viral infection, the level of Vpr entering the cell could be insufficient to produce the immediate arrest of the cell cycle, and yet could delay apoptosis and permit integration of the provirus and subsequent replication. The antiapoptotic effect may continue until Vpr is expressed at a level high enough to arrest the cell at the G2+M late stage of viral infection, followed by the eventual progression to apoptosis. The mechanism of induction of apoptosis by Vpr include activation of a transcription factor such as NF-κB, activation of caspase 3 and 9, and the interaction of Vpr with the adenine nucleotide translocator (160, 242, 303).

G2 arrest/apoptosis relationship. Initially, Vpr-mediated apoptosis was proposed as a consequence of the prolonged cell cycle arrest (337, 376, 389). Other investigations have revealed that the Vpr-mediated G2 arrest was not a prerequisite for the induction of apoptosis, suggesting that both G2 arrest and apoptosis functions are separated (250, 251, 370). However, the observation that the activity of the cell cycle regulatory Wee-1 kinase is decreased in Vpr-induced apoptotic cell leads to the hypothesis of a direct correlation between the G2 arrest and the apoptotic properties of Vpr (398). Hence, reduction of Wee-1 activity, probably related to its delocalization provoked by Vpr, results in an inappropriated activation of cdc2, leading to cell death with phenotypical aberrant mitotic features, a process known as mitotic catastrophe (145). By using an established cell line expressing Vpr, it was observed that after the long G2 phase, the cells rounded up with an aberrant M-phase spindle, and multiple poles that resulted from abnormal centrosome duplication (51, 376). The cells stopped prematurely in pro-metaphase and died by subsequent apoptosis.

1.6.3. Extracellular Vpr and its biological function.

Detection of soluble Vpr. The role of extracellular Vpr as an effector of pathogenesis has recently emerged. Extracellular Vpr can be found in the sera and cerebrospinal fluids of HIV-1-positive individuals in quantities that correlate with the level of viremia (197). Additionally, antibodies specific to

Vpr can be detected in HIV-infected patients, indicating that a relevant fraction of Vpr circulating in patients exist in an extravirion state (290, 291, 386). Amounts of extracellular Vpr correlate with the extent of viremia, suggesting that the source of extracellular Vpr likely results from the decay of circulating virion (197). However, a significant source might also be through the release of the cytosolic protein from infected apoptotic cells.

Vpr-transducing properties. A class of proteins with the ability of transducing the plasma membrane, independent of the classical endocytic mechanism, has been identified. These transducing proteins rely on the presence of an arginine-rich protein transduction domain (PTD) (229) and can penetrate lipid plasma membranes in an energy-independent manner (315). Likewise, denaturation of these transducing proteins also enhances their intracellular delivery, presumably through more efficient exposure of the PTD to the cell membrane (315). In fact uptake of exogenous protein very often involves the attachment of the arginine-rich domain of the protein to membrane heparin sulfate proteoglycans (229). Interestingly, it has been shown that synthetic Vpr could also penetrate cells *in vitro* via an energy- and receptor-independent process (148, 329). The Vpr C-terminal arginine-rich region is important for several Vpr biological activities. It is not only critical for protein nuclear localization, G2 arrest and proapoptotic functions (9, 194, 410) but also important for the ability of the protein to transduce into cells (329). Since Vpr contains an arginines-rich domain homologous to the PTDs, it seems logical that the Vpr might belong to this class of proteins (60, 148, 177, 329). The existence of circulating Vpr in an infected individual, and its capacity to enter other cells, further indicate that it is possible that Vpr has an effect on bystander cells.

Addition of purified Vpr to the extracellular medium of cultured cells recapitulates many of the phenotypes ascribed to Vpr synthesized *de novo*. Extracellular Vpr can transduce the cell membrane, localize in the nucleus and cause cell-cycle arrest at the G2 stage (329), which induces cell apoptosis (189, 272). The soluble protein is also able: (i) to enhance HIV-1 replication both in the latently infected leukemia cell line and in the peripheral blood mononuclear cells (PBMCs), (ii) to activate virus replication in a latently infected HIV-1 cell line at concentrations as low as 1ng/ml (198), and (iii) to rescue the replication of the Vpr-defective virus in macrophages (329). On the other hand, extracellular

Vpr inhibits the host inflammatory response by down-regulating pro-inflammatory cytokines (TNFα and IL-12) and beta chemokines (MIP-1α, MIP-1β, and RANTES) in a manner similar to glucocorticoids (17, 243, 288). Vpr additionally suppresses the host inflammatory response by inhibiting NF κB activity through the induction of IκB (17).

Pro-apoptotic function of Vpr C-terminal domain. The C-terminal region of Vpr has a toxic effect and prevents cell proliferation in yeast, as indicated by osmotic sensitivity and gross cell enlargement. These effects depend on the sequence ^{71}HFRIGCRHSRIG^{82}, which contains two H(S/F)RIG motifs (213). Addition of extracellular synthetic Vpr peptides containing the H(F/S)RIG repeat motif has a similar consequence in mammalian cells. When this peptide is added externally to human CD4+ T cells, it induces mitochondrial membrane permeabilization, dissipation of $\Delta\psi m$, morphological changes, formation of apoptotic bodies, and DNA fragmentaton (16). However Vpr C-terminal peptides 83-96 and 80-96 did not display any cell killing or toxic effects on CD4+ T cells (16) or rat neurons (304), respectively.

Implication of Vpr N-terminal domain in the formation of ion channels. In addition, soluble Vpr was found in the sera, as well as in the cerebrospinal fluid of HIV-infected patients, and was proposed to play a role related to its pro-apoptotic activity in AIDS-associated dementia (197, 198). The involvement of Vpr in these neurological disorders has been suggested, since recombinant Vpr has neurocytopathic effects on both rat and human neuronal cells (155, 263, 264, 270, 271). Neurons killed by extracellular Vpr display the typical feature of apoptosis evidenced by the direct activation of the initiator caspase-8 that leads to subsequent activation of the effector caspase. These effects have been linked to the ability of the first amphipathic α-helix of Vpr (1-40) to form cation-selective ion channels in planar lipid bilayers, causing a depolarization of the plasma membrane (264, 270, 271), which may not depend on Vpr transducing ability. These observations indicate that Vpr can trigger apoptotic processes by different alternative pathways, depending on the target cells.

1.7. Vpr modulation of the nuclear translocation of HIV-1 PIC.

1.7.1. Viral components involved in PIC nuclear import.

Multiple factors are involved in the nuclear targeting of HIV-1 preintegration complex in non-dividing cells, such as the matrix protein (MA), Vpr, integrase protein (IN), and the DNA flap (Fig 1.9) (136, 275).

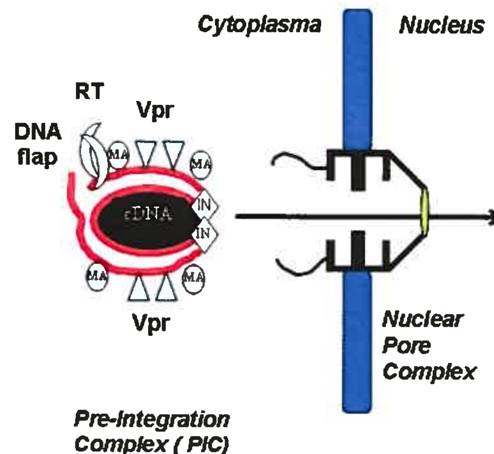

Figure 1.8. Schematic representation of PIC nuclear targeting during the early stage of HIV infection. *The viral protein IN together with MA, Vpr, NC, RT and central DNA flap were involved in active nuclear import of HIV PIC through nuclear pore complex.*

Role of MA matrix protein. MA was the first viral protein implicated in HIV-1 nuclear import (43, 369). MA is a 132-amino acid structural protein that is myristoylated at the N-terminus. The three dimensional structure of MA has been determined by nuclear magnetic resonance as well as by X-ray crystallography (149), and it consists of five α-helices—one to four form a compact globular domain while the C-terminal helix (Helix 5) projects away from the membrane (Fig.1.9). The N-terminal myristyl moiety facilitates the binding of MA to the membrane. MA is required for the incorporation of the envelope glycoprotein into the virion (396). MA is also important in early post entry events of the virus

life cycle. Mutation of a highly conserved Leu at MA amino acid 20 or deletion at the C-terminus causes a significant defect in an early step of the virus life cycle (178, 394). MA phosphorylation has been shown to be a critical regulator of MA functions. Initially, MA was thought to be phosphorylated at Tyr-132 and to regulate the nuclear localization of the PIC (117, 120). However, other research groups were unable to confirm this finding (42, 113). MA is also phosphorylated at serine residues, since protein kinase C was identified as one of the kinase for MA phosphorylation, and Ser-111 was recognized as a putative phosphoacceptor residue (46). However, the role of this modification in virus replication has not been defined. Bukrinskaya and colleagues have reported that at least five serines are phosphorylated during HIV-1 entry into susceptible cells (42). By using kinase inhibitors, they observed that MA-phosphorylated serine or tyrosine residues regulate nuclear targeting the virus nucleic acid, independent of the presence of Vpr. In addition, Nef has been shown to enhance MA phosphorylation through Nef-associated serine-threonine kinase (343). A recent study shows that MA Ser-9, -67, -72 and –77 mutation impairs viral infectivity in dividing and nondividing cells at the early post entry step of virus infection (174).

Most of the matrix in the virion localizes outside the core and forms the layer between the viral capsid and the envelope. Some MA molecules are found in tight association with the HIV core and become part of the PIC after the viral core enters the cell (45, 230). The HIV-1 MA carries two functional, yet rather weak, nuclear localization signals (NLSs) (244), which harbour a short stretch of basic amino acids that introduce a positive charge, which is crucial for the nuclear targeting properties of these sequences (85) (Figure 1.9).

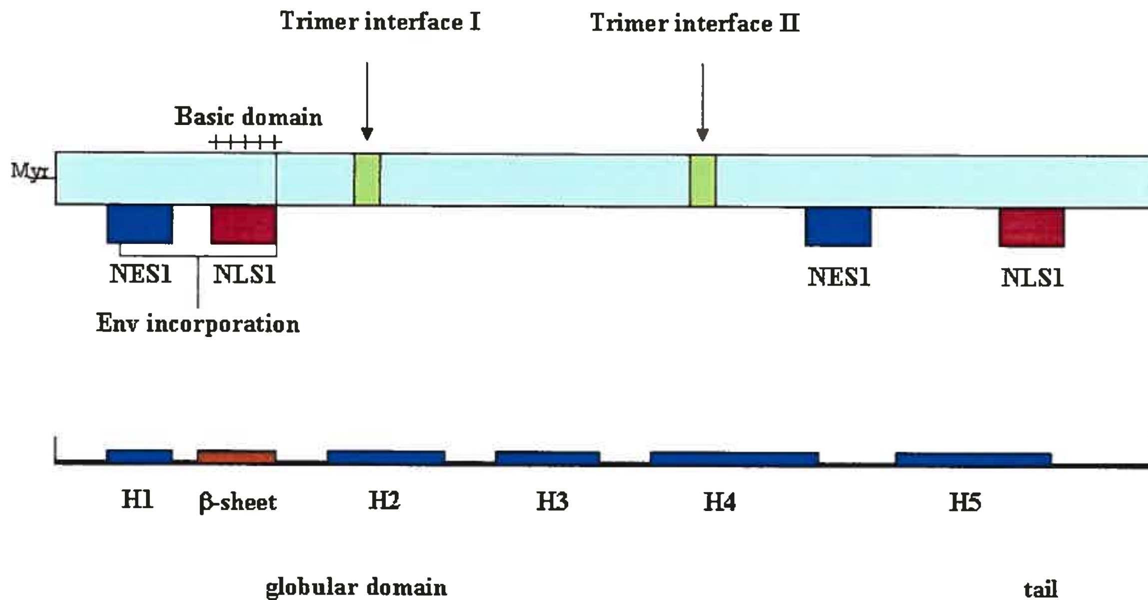

Figure 1.9. HIV-1 MA protein domains. *MA protein consists of five α-helices, one to four forms a compact globular domain while the C-terminal helix (Helix 5) projects away from the membrane. The NES and NLS are indicated.*

Mutations in MA NLSs significantly attenuate HIV-1 replication in non-dividing cells (43, 136, 230). In the case of basic-type NLSs, such as those present in MA, an heterodimer of two importins—importin α and importin β—is involved. Importin α is actually an adapter that ensures the binding of basic-NLS protein complexes to nucleoporins (a collective term for nuclear pore complex proteins) and its translocation through the pore (293). However, several research reports have recently questioned the role of MA in HIV-1 nuclear import (109, 114). The main argument against MA being the principal HIV nuclear targeting protein is the finding that, even though the virus lacked most of the MA, it was still capable of infecting nondividing cells, albeit with a greatly reduced efficiency (289). While MA is clearly important for efficient nuclear import of HIV-1 PIC, it appears to be nonessential and is likely only one of several factors regulating this process (289).

Role of IN protein. The fact that IN is associated with viral DNA supports that IN could be a a good candidate for the nuclear import of viral DNA (99, 230). HIV-1 IN is a 288-amino acid protein composed of three functionally independent domains: an N-terminal domain (which approximately spans the first 50 amino acids), a catalytic core domain (amino acids 51-202), and a C-terminal domain (amino acids 203-288) (97). The first two domains are relatively well conserved among retroviral integrases. The N-terminal domain contains a zinc finger motif (HHCC) that participates in the oligomerization of IN and stimulates its catalytic activity. The core domain is responsible for the catalytic activity of the enzyme. Finally, the less conserved C-terminal region displays unspecific DNA binding properties, similar to those of the full-length integrase. Recent results show that mutations of possible phosphorylation residues on IN have no effect on reverse transcription and nuclear transport of PIC, but have a slight nonessential effect on integration (352).

It has been clearly demonstrated that IN exerts pleiotropic effects on the HIV-1 life cycle (97). Whereas some mutations specifically block the integration step into the host DNA, others block replication at a step prior to integration, indicating that IN may be linked to nuclear import or intranuclear routing of the viral DNA. IN displays a nuclear import function when assayed by microinjection, but unlike Vpr and like MA, IN nuclear import is blocked when the importin α/β pathway is disrupted (117). However, mutations in IN which inactivate the putative NLS. also render HIV-1 replication defective and apparently integration defective It is therefore impossible to separate the nuclear import properties of IN from its integration function using these mutants. Recently, this IN NLS (aa 211-221) has been disputed for its importance in the infection of non-dividing cells (117). However, the nuclear import abilities of IN proteins have been confirmed using microinjection of GFP-IN fusion protein (352). By using truncated fusion proteins, another region of IN with NLS function has been identified by Malim and colleagues (36). This sequence (aa 161-173, IIGQVRDQAEHLK) does not resemble a classical NLS, but the addition of this sequence confers nuclear import to a heterologous substrate. Mutagenesis of this typical NLS prevents the nuclear accumulation of the IN-fusion protein. In the context of viral infection, this IN NLS mutant is replication defective both for dividing and non-dividing cells. The IN NLS stimulates the

efficient nuclear accumulation of viral DNA during initial stages of infection but it is dispensable for catalytic function (36). This NLS is required for infection irrespective of target cell proliferation, suggesting that interaction between uncoated viral nucleoprotein complexes and the host cell nuclear import machinery is critical for HIV-1 infection of all cells (36).

Role of the cPPT. Another factor that has been proposed to regulate HIV nuclear import is the specific structure of the viral cDNA intermediate (36). During the plus-strand synthesis of the HIV cDNA, a 99 nucleotide long "central DNA flap" is produced because of the addition of the plus DNA synthesis at another polypurine tract in the middle of the HIV genome. This short trimeric structure is proposed as a critical determinant of the PIC passage through the nuclear pore (36). This structure has been reported to dramatically increase the efficiency of lentivirus-mediated gene transfer (333, 360). However, more recent research reports demonstrate that the central flap, at least in some cells, is not essential for PIC nuclear import, and its effect is dependent on the selected HIV-1 strain used (92, 205). Most likely, the DNA flap assists the nuclear import function of HIV proteins by providing the optimal conformation to the PIC that is necessary for its interaction with cellular factors and translocation through the nuclear pore.

Karyophilic properties of Vpr. It has been noted that Vpr mutation decreased HIV-1 infection of macrophages (21, 65, 382). Subsequent experiments attributed this result to Vpr nuclear targeting of the PIC in a partially redundant fashion with MA (146). Deletion of Vpr decreased transport of the viral genome to the nucleus (2-LTR circles) and decreased infection of macrophages (146). Again, the attenuation in infection by the Vpr mutant was specific to non-proliferating cells without affecting the infection of proliferating cells. Recent data show that the viral proteins IN and Vpr are both karyophilic and are associated with viral DNA within PIC, indicating that they may cooperatively participate in the nuclear import of PIC (79). Significantly, viruses with deleted Vpr and MA NLS mutation had a more severe phenotype than a single mutant, and caused a decrease in infection of macrophages and growth-

arrested cells. However, no conventional NLS is detectable in Vpr, and Vpr-mediated nuclear import is not disrupted by interference with the importin pathway (118). Vpr binds to importin α, not as an NLS substrate but through a different site on importin α (274). Additionally, Vpr binds to nucleoporins, and it has been proposed that this binding facilitates the docking of PIC to the nuclear pore for nucleus entry viral genome. Moreover, nuclear pore targeting is shown to be necessary for Vpr to positively affect the HIV infection of macrophages (108, 275, 368). Two independent signals within Vpr, one in the amino half and the other in the carboxy half of the protein, have been implicated in Vpr nuclear import (163, 169, 326). These signals seem to function in the absence of additional soluble factors.

A recent report shows that Vpr alters the structure of the nuclear lamina in a manner that leads to formation of nuclear herniation, which intermittent ruptures (72). These ruptures in the nuclear envelope might provide a freely accessible portal for uptake of the large HIV PIC in this situation. However, the precise role of nuclear envelope disruptions (associated with defects in the nuclear lamina) in PIC nuclear import remains uncertain, since Vpr-deficient viruses can efficiently infect non-dividing cells (109, 289). Vpr also has been shown to weakly enhance the nuclear uptake of NLS substrate (276). For HIV-2, the closely related protein, Vpx, can perform a similar function to HIV-1 Vpr in terms of promoting macrophage infection (106, 339). Vpr (or Vpx in HIV-2) is an attractive candidate as a determinant of infection of nondividing cells because it is part of PIC and has been clearly demonstrated to possess the ability for nuclear import or NPC docking. Notewithstanding, the HIV genome clearly contains other means for entry into the nucleus of nondividing cells, since HIV-based gene transfer vectors lacking Vpr effectively transduce cells such as neurons. Vpr maybe a poor candidate for nuclear targeting PIC on the other hand because it is only present in primate lentiviruses; the other lentiviruses could still infect nondividing cells without a Vpr, suggesting other viral components are more important for nuclear targeting PIC.

1.7.2. Interactions of Vpr with other components of the virus particle

Considering the fact that viral DNA is transported into the nucleus within four to six hours post infection (44), viral proteins involved in PIC import should originate directly from the virion. However, functional interactions occurring between the viral components of the core particles are yet to be studied.

Vpr and NC. Nucleocapsid protein p7 (NCp7) is a small basic protein of 72 amino acids and contains two zinc finger domains (CX2CX4HX4C) flanked by basic amino acids. NCp7 is found in tight association with the dimeric RNA genome in the core. *In vivo*, NCp7 is required for the protection of the genome against cellular nucleases and is involved in genomic RNA packaging and morphogenesis of the virus particles (91). Most of these functions are related to the well-demonstrated high affinity of NCp7 for single strand nucleic acid (176) Nuclear magnetic resonance (NMR) studies have demonstrated that the folded CCHC boxes of NCp7 are in spatial proximity, whereas the N- and C-terminal sequence remains flexible (195). The domain encompassing the C-terminal residues of Vpr (52-96) is shown to be involved in the binding of the nucleocapsid protein NCp7 to nucleic acid (60, 74, 177). The distal NCp7 zinc finger is mainly implicated in Vpr-NCp7 interaction, whereas the flexible N- and C-terminal parts of NCp7 are not. Vpr-NCp7 interaction is mainly dependent on hydrophobic contacts and probably on hydrogen bond formation (74). The packaging of the genomic RNA and encapsulation of a large number of Vpr molecules could occur by an interaction of Vpr restricted to the accessible domain of the NC-RNA complex. Accordingly, recent structural studies of complexed NCp7 (12-53) show that a large part of the CCHC boxes remains free for additional interaction (300). Hence Vpr encapsidation does not occur if the protein p6 was not directly linked to a nucleocapsid protein sequence in Pr55gag (185, 209), suggesting that in HIV-1 Gag, NCp7 and p6 link together in a NCp15 form to direct Vpr encapsidation. However, a complex that consists of HIV-1 Vpr and HIV-1 NCp7 has not yet been identified in HIV-1-infected cell (161). Whether Vpr and NCp7 have direct interaction within the virion particles remains to be determined.

Vpr and RT. The direct evidence that Vpr and RT may be associated comes from the analysis of the intracellular reverse transcription complex (100). Evidence also comes from the analysis of the purified

integration complex (120). However, an *in vitro* binding assay showed no direct interaction between RT and Vpr (90). Considering that Vpr can reduce the *in vivo* mutation rate of HIV-1 (219) and decrease the mutation rate in the cell (166), and that NCp7 is shown to reduce nonspecific reverse transcription (135), Vpr may possibly join the NCp7 to influence the RT function. One of the prerequisites for tRNA to act as a primer for reverse transcription is the availability of a free 3-OH group, which is true for deacylated but not for acylated forms of tRNA. Vpr can bind and inhibit Lys-tRNA synthetase, an enzyme involved in the amino-acylation of tRNA. The intracellular levels of deacylated tRNA is lower in a nondividing cell than in proliferating cells. Therefore, it is argued that the inactivation of Lys-tRNA synthetase by Vpr may be more relevant in nondividing cells where it could increase the levels of deacylated tRNA, and thus indirectly support the packaging of deacylated tRNA into the virion and influence the reverse transcription efficiency (335). The recovery of RT in the core preparation appears to be less efficient than that of IN, raising the possibility that not all of the RT present in the virion is located in the core (1). However, by using different core preparation protocols, other researchers show that RT and IN have a similar recovery efficiency (380). Whether Vpr interacts with RT within viral particles remains to be determined.

Vpr and IN. GST-Vpr cannot pull down IN by GST pull-down assay (384). However, the result do not exclude a possible indirect interaction in the core. Vpr has been shown to interact with Uracil DNA Glycosylase (UDG) UNG by using the yeast two-hybrid system (32). Vpr-mediated incorporation of overexpressed UNG modulates *in vivo* HIV mutation rate (219). On the other hand, UNG-2 precursor can be incorporated into the virion in a Vpr-independent way, via its interaction with IN (384). It is noteworthy that residues 1 to 52 of UNG-2 are important for the binding to IN, whereas residues 222 to 225 are reported to be important for the binding to Vpr (33, 384). IN has the ability to preferentially bind the cytoplasmic precursor form of UNG-2 (36kD) (384), Vpr binds the mature form of UNG-2 (28kD) in the cell (32). Vpr has the ability to bind both full length precursor and the mature form of UNG-2 (28kD) in yeast two hybrids and GST-pull down assay (32) (384). It will be interesting to investigate if UNG-2

precursor in the virion acts as a linker between IN and Vpr. The role of UNG association with IN and Vpr, remains to be determined.

Vpr and the matrix protein. MA was initially reported to be absent from several of HIV-1 core preparations (1) but appeares to be depleted to a lesser extent from HIV-1 core preparations as reported by by Kotov *et al.* (187) and Welker *et al.* (380). In the latter study, HIV-1 virions were briefly exposed to detergent, and cores were recovered by rapid centrifugation in a microcentrifuge. Electron microscopy analysis showed that the resulting core preparations were not completely pure (380), which may explain the presence of residual MA. However, Kotov *et al.* observed an enrichment of MA at the expected density of the cores (187), suggesting that under certain isolation conditions, some MA remain associated with HIV-1 cores. Previously, a phosphorylated form of MA was detected in HIV-2 core preparations (120). The phosphorylated form induced the formation of a complex with integrase, triggering the redistribution of some MA to the inner region of the particle, which became part of the viral nucleoprotein complex (120).

By using a special detergent Brij 96 to lyse the virion particle, direct coimmunoprecipitation experiments showed that Vpr associates with the matrix protein p17 in the HIV-1 virion, but not with the capsid protein p24. Protein-protein interactions experiments employing the yeast two-hybrid GAL4 assay also demonstrated a direct association between Vpr and the C-terminal region of the p17 matrix protein (306). However, considering the rare presence of MA in the virion core (1), whether Vpr directly interacts with MA in the virion core remains to be answered.

1.8. The overall objective of the study

The pathogenesis of HIV lies in its special genome structure with 6 accessory genes; among them, Vpr contributes to an important part of the HIV pathogenesis. The Vpr sequence is well conserved among the primate lentiviruses, suggesting that it may play an important role in viral life cycle *in vivo*. Although Vpr is dispensable for HIV replication in T lymphocytes, the protein plays an important role for

macrophages infection (65). Furthermore, several lines of evidence indicate that Vpr is required for HIV replication *in vivo* (131) (192). Although the precise mechanisms by which Vpr influences viral replication are still unclear, increasing data suggesting that Vpr may be implicated in the reverse-transcription, the transcriptional activation of the LTR promoter, the nuclear transport of the preintegration complex in nondividing cells, and HIV-induced growth arrest and cytotoxicity (194).

The goal of my study is to investigate the molecular function of Vpr protein during HIV-1 replication, especially characterization of the extracellular Vpr release and proteolytic processing, determination of Vpr partners within the core. The detail description of objectives as following:

Objective #1: Characterization of the release of extracellular Vpr.

Extending the biology of Vpr beyond the infected cell has important potential consequences for the pathogenesis of HIV *in vivo*. The additional ability of extracellular Vpr to promote productive infection of unstimulated cells may provide an important mechanism for efficient *de novo* infection of resting, nondividing cells (94). Because extracellular Vpr mediates transactivation of latently infected viral reservoirs, it may be responsible for a significant portion of events that currently prevent highly active antiretroviral therapy (HAART) efforts towards disease control. In addition, soluble Vpr can be found in the sera and cerebrospinfluids of HIV-1 positive individuals (197). It has been previously shown that synthetic Vpr could penetrate the cells *in vitro* via an energy- and receptor-independent process (148, 328). Extracellular Vpr causes cell-cycle arrest at the G2 stage, nuclear localization (328), and induces cell apoptosis (189, 272). However, despite these numerous putative functions, mechanisms underlying Vpr release as well as its biological impact on HIV-1 replication have never been addressed.

In the present study I have investigated whether HIV-1 Vpr is released from HIV-1-producing cells. For the first time, I showed that Vpr could be released in the culture media of HIV-1-expressing cells, independent of virion incorporation. Extracellular Vpr was cleaved by the cell surface-associated

proprotein convertase (PC) that recognizes the Vpr C-terminal motif $R_{85}QRR_{88}$. Finally, the truncated Vpr protein was shown to be defective for the induction of cell-cycle arrest and apoptosis, suggesting that Vpr processing by the proprotein convertase might be a cellular mechanism to control the level of functionally active extracellular Vpr during HIV-1 infection.

Objective #2: Determination of Vpr partners within the core.

Human immunodeficiency virus type 1 can replicate in nondividing cells such as macrophages, relying on the active transport of viral DNA into the nucleus of an infected cell (44). Like other retrovirus, it contains the RNA genome that is reverse transcribed and integrated into the host genome in the early event of infection, before viral proteins are expressed. This mode of replication requires the presence of all the necessary components of the replication and integration complex within the virion. Virus particles are highly organized structure built to package and protect the viral genome, to facilitate its entry into a host cell, to control its release and to complete its early function in infection. It is believed that multiple viral factors are involved in nuclear targeting of HIV-1 PIC in nondividing cells, such as the matrix protein (MA), the viral protein R (Vpr) and the integrase protein (IN), as well as the DNA flap. HIV-1 MA and IN proteins are thought to recruit karyopherin alpha through their nuclear localization signal (NLS), while Vpr may modulate the nuclear import by increasing the affinity of karyopherin alpha for the PIC (275).

Vpr possesses karyophilic property when expressed alone (210). Vpr is then incorporated into the nascent virions through its interaction with the $Pr55^{GAG}$ p6 LXXLF domain (185, 209, 210, 266). The early function of Vpr is closely related to the fact that it is specifically incorporated into virion. Although Vpr is dispensable for HIV-1 replication in dividing cells, it is required for efficient replication in nondividing macrophages (146). Its localization within the virion core and functional association with the nuclear complex implies its role in HIV infection. Upon viral particle maturation, Vpr but not p6 localizes

in the virion core (1), suggesting Vpr might interact with other viral or cellular factors for proper redistribution. However, little is known about the mechanism that Vpr uses to enter the virion core or its interaction with other core components. An understanding of these processes will help to provide insight into the early role of Vpr in HIV infection, and to open a new approach to develop drugs to intervene in Vpr core incorporation and its early function in HIV infection of nondividing cells.

In order to facilitate the detection of virion-associated Vpr complexes, we first constructed an isogenic infectious HIV-1 molecular clone expressing HA-tagged Vpr. We found that Vpr was able to co-immunoprecipitate the matrix protein. Interestingly, this interaction occurred independently of the presence of RT and IN proteins and could be detected *in vitro* with recombinant proteins, suggesting a direct MA/Vpr association. GST pull-down assays further demonstrated that the fifth alpha helix of MA and the C-terminal domain of Vpr mediate this association. Involvement of the arginine-rich domain of Vpr in the interaction was finally confirmed *in vivo*. Implication of virion-associated MA/Vpr complexes in the early steps of infection will be discussed.

Authors contribution in article 1: Cell-surface Processing of Extracellular Human Immunodeficiency Virus Type 1 Vpr by Proprotein Convertase

Yong Xiao designed and developed the concept of the project, carried out all the experiments (Figure 2.1 to Figure 2.7, including supplemental figure 2.1and 2.2) except for Figure 2.3D, and wrote the manuscript.

Gang Chen participated in eluting the purified extracellular Vpr protein from immunoprecipated beads. Hongshen Li did the mass spectrometry analysis for purifed extracellular Vpr products from Gang Chen. Their works are presented in Figure 2.3D

Nicole Rougeau provided technique support in constructing 3HA tagged provirus and preparing virus stock used in Figure 2.2.

Nabil G Seidah provided proprotein convertase expressing plasmids, gave helpful suggestions for identifying the protein convertase responsible for processing extracellular Vpr, also participated reviewing the manuscript.

Eric A. Cohen supervised the whole study, participated in designing experiment and interpreting the result, participated in drafting the manuscript.

Chapter 2 (article 1): Cell-surface Processing of Extracellular Human Immunodeficiency Virus Type 1 Vpr by Proprotein Convertase

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Running title: Extracellular Vpr processing by proprotein convertases

Manuscript information: 43 pages, 6 figures, 1 table, 2 supplemental figures

Word and character counts: Abstract: 246 words; Text: 6727 words

Keywords: HIV-1, Vpr, extracellular processing, proprotein convertase, protease, extracellular matrix, G2 arrest, apoptosis

ABSTRACT

Increasing evidence suggests that extracellular Vpr could contribute to human immunodeficiency virus (HIV) pathogenesis through its effect on bystander cells. Soluble forms of Vpr have been detected in the sera and cerebrospinal fluids of HIV-1 infected patients, and *in vitro* studies have implicated extracellular Vpr as an effector of cellular responses, including G2 arrest, apoptosis, induction of cytokines and chemokines production as well as increased viral replication, presumably through its ability to transduce into multiple cell types. However, the mechanism underlying Vpr release from HIV-1 producing cells remains undefined and the biological modifications that the extracellular protein may undergo are largely unknown. We provide evidence indicating that Vpr is released in the extracellular medium of HIV-1-producing cells by a process that is independent of Vpr virion incorporation but requires the expression of viral proteins. Interestingly, extracellular Vpr was found to be cleaved by cell surface-associated proprotein convertases (PC) at a very well conserved site, $R_{85}QRR_{88}\downarrow$, located within the functionally important C-terminal arginine-rich domain of the protein. Consistently, the PC inhibitors α1-PDX, Spn4A and dec-RVKR-cmk inhibited Vpr processing, while expression of PCs that are known to be associated with the extracellular matrix such as, PC5 and PACE4, enhanced Vpr processing. Finally, PC-mediated processing of extracellular Vpr led to the production of a truncated Vpr product that was defective for the induction of cell-cycle arrest and apoptosis, suggesting that PC processing of extracellular Vpr might represent a host cell response to inactivate soluble Vpr during HIV-1 infection.

INTRODUCTION

HIV-1 encodes four accessory gene products -Vif, Vpr, Vpu and Nef- which are thought to collectively manipulate host cell biology in order to promote viral replication, persistence and immune escape (reviewed in reference: (15)). One of these accessory proteins, Vpr, is a 96 amino acid polypeptide that is highly conserved both among the primate lentiviruses HIV-1, HIV-2 and the simian immunodeficiency virus (66), supporting the notion that it plays an important role during viral infection *in vivo*. Indeed, deletion of *vpr* and the related *vpx* genes in Simian Immunodeficiency Virus (SIV) severely compromises virus burden and disease progression in experimentally infected monkeys (18, 25). Despite its small size, Vpr induces multiple effects in host cells in culture that may contribute to the phenotypic effects observed *in vivo* (reviewed in reference: (2, 36, 58)). First, Vpr has been shown to act early in viral infection as a facilitator of HIV-1 preintegration complex (PIC) entry through the limiting nuclear pore (23, 50). This activity is thought to be responsible for Vpr's ability to enhance HIV-1 replication in nondividing cells, most notably in terminally differentiated macrophages (11, 70). Consistent with this function, Vpr is packaged in relatively large amounts into viral particles through an interaction with the carboxy-terminal of the p6 late domain of the $Pr55^{gag}$ polyprotein precursor (40, 48), contains nuclear targeting sequences (30, 56), and is present in PICs (50). Second, expression of Vpr was reported to induce cell cycle arrest in G2 by activating the ATR (for Ataxia-Telangiectasia mutated and Rad3-related) checkpoint signaling pathway, a signaling event that is normally part of the cell response system to DNA damage (53). Such arrested cell, were shown to ultimately die as a result of apoptosis (1, 61, 74). Finally, Vpr was also reported to act as a transcriptional activator of the HIV-1 LTR as well as host cell genes (10, 19, 32, 33, 43, 57).

Interestingly, beside being found in virions and in cells, Vpr and Vpr cleavage products have been shown to exist as free molecules in the serum and the cerebrospinal fluids of HIV-1 infected patients (38), indicating that Vpr may be released extracellularly and may exert its biological function beyond infected

cells. In that regard, extracellular Vpr was shown to transduce cells *in vitro*, apparently via an energy- and receptor-independent process (24). Following cellular uptake, Vpr was shown to retain the ability to localize to the nucleus and to induce G2 cell cycle arrest and apoptosis (24, 59), thus raising the possibility that circulating forms of Vpr observed in HIV-1 infected patients may exert biological effects on a broad range of host cells. Indeed, a large number of studies have reported that treatment of cells with extracellular Vpr or Vpr fragments resulted in apoptosis (5, 28, 47) and cytotoxic effects (27, 49) in a variety of cell types. Furthermore, extracellular Vpr was also shown to activate AP-1, JNK, and NF-κB in promonocytic cells U937 and in primary macrophages (69) and enhance replication in chronically infected cells and in acutely infected primary macrophages (39, 59, 69). However, it has not yet been clearly established how Vpr is released from HIV-1-infected cells. Furthermore, given the cytopathic properties of the protein, it is unclear whether Vpr proteins released in the extracellular milieu remain in a fully functional form.

In the present work, we provide evidence indicating that Vpr is released in the culture media of HIV-1-expressing cells via a process that is independent from its capacity to be packaged into virion but requires expression of viral proteins. Interestingly, extracellular Vpr was cleaved by cell surface-associated PCs at a site, $R_{85}QRR_{88}\downarrow$, located within the functionally important C-terminal arginine-rich motif. The resulting truncated Vpr product was defective for the induction of cell-cycle arrest and apoptosis, suggesting that Vpr processing by PC might be a host cell mechanism to control the level of functionally active extracellular Vpr during HIV-1 infection.

MATERIALS AND METHODS

Plasmids and proviral DNA constructs. The expression plasmid SV CMV 3HA-Vpr was constructed by inserting three consecutive hemagglutinin tags (3HA) to the N-terminus of Vpr in SVCMV-Vpr Wt (72) using a two-step PCR approach. The introduced 3HA tag contains 49 amino acids (aa): MASVS<u>YPYDVPDYA</u> SLGGPSSVS<u>YPYDVPDYA</u>SLGGPSSV<u>YPYDVPDYA</u> (HA epitope sequences are underlined). The single mutant 3HA-Vpr R85Q and the double mutant 3HA-Vpr RR87/88AA were produced by PCR-mutagenesis using SV CMV 3HA-Vpr as template. SV CMV 3HA-Vpr(1-88), (1-86) and (1-78) as well as SV CMV Vpr (1-86) were generated by introducing premature stop codons at aa position 88, 86 or 78 of Vpr using PCR-mutagenesis. The SV CMVexPA, SV CMV Vpr- and R80A constructs as well as the bi-cistronic expressors SV CMV Vpr/GFP, Vpr-/GFP were described previously (72) (26). The SV CMV Vpr (1-86)/GFP plasmid was constructed by inserting a BglII/DraIII fragment from pQB25I (Quantum Biotechnologies, Montreal, Que, Canada) encoding the green fluorescent protein (GFP) coding sequence preceded by the cytomegalovirus (CMV) early promoter into the BamHI sites of SV CMV Vpr (1-86). The pET21c 3HA-Vpr plasmid was generated by subcloning a DNA fragment encoding 3HA-Vpr from SV CMV 3HA-Vpr into pET21c (Novagen, Madison, WI, USA). HxBruBH10.R+ (*gag*$^{+}$-*pol*$^{+}$-*vif*$^{+}$-*vpr*$^{+}$-*tat*$^{+}$-*rev*$^{+}$-*vpu*$^{+}$-*env*$^{+}$-*nef*$^{-}$) and Vpr-defective HxBruBH10.R- proviral constructs were generated by introducing a SalI/KpnI fragment encoding Vpu from HxBH10 (37) into HxBRU or HxBRU R- (73). The HxBruBH10.3HAR+ proviral construct was derived from the HxBruBH10.R+ provirus. In the process of introducing the DNA fragment encoding 3HA-Vpr, a NheI site was created at nucleotide position 5140 (+1= start of BRU transcription initiation site) before the Vpr ATG by PCR-mutagenesis. The creation of the NheI site led to the introduction of a frameshift mutation at aa position 174 in the Vif open reading frame that resulted in a truncated Vif protein. The proviral construct HxBruBH10.3HAR+/p6 (1-17) encodes a truncated p6^{gag} domain (premature stop codon at aa 17). It was generated by replacing an ApaI (nucleotide position 2011)-BstZ17I (nucleotide position 2967) DNA fragment from HxBruBH10.3HAR+ by the corresponding fragment from HxBru.R+.s17stop (8).

The PC-expressing plasmids pIR hfurin, pIR mPC5A+v5 and pIR hPACE4+v5 encoding human furin, murine PC5A and human PACE4, respectively, were described previously (7). Plasmid pIR-PDX-v5 encoding the PC inhibitor α1-PDX fused to a v5 tag was described elsewhere (6), while pShuttle CMV Spn4A encoding the PC inhibitor Spn4A fused to a Flag tag was kindly provided by Dr. François Jean (Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada). All plasmid constructions were analyzed and confirmed by automated DNA sequencing.

Cell lines and reagents. Human embryonic kidney (HEK) 293T, Hela-CCR5 and COS-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Jurkat T-lymphoid cells were maintained in RPMI-1640 medium supplemented with 10% FCS. Anti-HA monoclonal antibodies (mAb) were produced from hybridoma 12CA5, while anti-V5 and anti-Flag mAb (M2) were purchased from Invitrogen (San Diego, CA, USA) and Sigma-Aldrich Canada Ltd (Oakville, Ont, Canada) respectively. The anti-Vpr mAb 9F12 directed against an epitope comprising aa 4-16 was kindly provided by Dr. Jeffrey B. Kopp (Kidney Disease Branch, National Institutes of Health, Bethesda, USA). Rabbit anti-Vpr polyclonal antibodies (pAb) were previously described (73). The viral expression and release from HIV-1-expressing cells are monitored by WB anti-CA. Anti-HIV-1 capsid (CA or p24) protein mAb were produced from hybridoma HB9725 (ATCC; American Type Culture Collection, Manassas, VA, USA) while anti-gp120 mAb recognizing both gp120 and precursor gp160 were obtained from the NIH AIDS Research and Reference Reagents Program. The PC inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-cmk) was purchased from Bachem Bioscience Inc. (King of Prussia, PA, USA). A 10mM stock solution was prepared in DMSO and was further diluted in tissue culture medium to give the required final concentration. Heparin sodium salt was purchased from Sigma-Aldrich Canada Ltd (Oakville, Ont, Canada).

Transfection. 293T and COS-1 cells were transfected by the standard calcium phosphate DNA precipitation method. For detection of extracellular 3HA-Vpr, 10^6 cells were either transfected with 10 μg

of 3HA-Vpr proviral DNA plasmid or co-transfected with 10 μg of Vpr-defective proviral DNA plasmid and 5 μg of 3HA-Vpr expression vector. For native Vpr proteolytic processing detection, 293T ($4x10^6$ cells) were either transfected with 40 μg of proviral DNA plasmid or co-transfected with 40 μg of Vpr-defective proviral DNA plasmid and 20 μg of Vpr expression plasmid vector. Hela-CCR5 cells ($4x10^5$ in 6 wells plate) were transfected with 4 μg of 3HA-Vpr proviral DNA construct and 0.5 μg of PC expression plasmids using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer instructions. Finally, Jurkat cells ($4x10^6$ cells) were transfected with 20 μg of Vpr defective or 3HA-Vpr proviral DNA constructs by the DEAE-dextran method (37). Analysis of 3HA-Vpr processing was conducted 6 days post-transfection as described below.

Analysis of Vpr processing in HIV-1-producing cells, extracellular medium and viral particles. Transfected or infected cells were separated from virion-containing supernatants by low-speed centrifugation and lysed in 1% NP40 lysis buffer (140mM NaCl, 8 mM Na_2HPO_4, 2mM NaH_2PO_4, 1 % NP40, 0.5% SDS, pH7.2) supplemented with a protease inhibitor cocktail (Roche Diagnostics Canada, Laval, Que, Canada). Vpr was immunoprecipitated from cell lysates using anti-HA or anti-Vpr antibodies as described (73). For detection of extracellular Vpr, 16ml of culture supernatants were first centrifuged at 2000 *g* for 10min and then passed through a 0.45 μm filter to eliminate cell debris. Viral particles were separated from the extracellular medium by ultra centrifugation onto a 20% sucrose cushion at 130 000 g for 1.5 h. Eight ml of virus-free extracellular medium treated with 5-fold-concentrated NP-40 lysis buffer was immunoprecipitated with anti-HA or anti-Vpr antibodies as described (73). Pelleted virus was lysed directly in 1% NP40 lysis buffer. Cell and viral lysates as well as immunocomplexes resulting from immunoprecipitations were separated by 14% SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by western blotting using antibodies against HIV-1 capsid p24, HA or Vpr as described previously (37). Bound antibodies were revealed using the 3, 3'-diaminobenzidine detection system, as recommended by the manufacturer (ICN Biomedicals, Irvine, CA, USA). For detection of extracellular

native Vpr, supersensitive Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was used according to the manufacturer instructions. Vpr processing efficiency was analyzed by quantitating the density of each Vpr-related band using an AGFA Duoscan T1200 scanner. Densitometric analysis of WB results was performed with Image Quant 5.0 from Molecular Dynamics.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) analysis. 3HA-Vpr was isolated from virus-free culture supernatant of HxBruBH10.3HAR+-transfected 293T cells by immunoprecipitation using anti-HA antibodies. 3HA-Vpr was eluted from anti-HA antibodies bound to protein A sepharose beads using trifluoroacetic acid (TFA). The 3HA-Vpr-containing eluate was combined with a similar volume of binding buffer (100mM PBS and 0.5 M NaCl pH 7.0), applied onto a NP20 Protein-Chip spot (Ciphergen Biosystems Inc., Fremont, CA, USA) and incubated at room temperature for 20 min to dry. Unbound samples on the chip spot were washed-off following three washes of 5 min with binding buffer. The NP20 chip was then rinsed with 20 µl of distilled H_2O and air dried. A matrix consisting of a saturated solution of 3, 5-dimethoxy-4-hydroxycinnamic acid (SPA) in 50% acetonitrile and 0.5% TFA was added to the chip surface before SELDI-TOF-MS analysis. The SELDI-TOF-MS analysis was performed using a Ciphergen Protein-Chip Reader (Ciphergen Biosystem Inc., Fremont, CA, USA). Cytochrome c (12 kDa) was used as the molecular mass calibrator.

***In vitro* translation.** 3HA-Vpr was *in vitro* translated from pET21c 3HA-Vpr using the Active Pro™ *In vitro* translation kit (Ambion, Austin, TX, USA) according to manufacturer instructions.

Cell cycle profiling and analysis of apoptosis. Cell cycle analysis was performed as described (45). Briefly, 293T cells were co-transfected with 10 µg of Vpr and 0.5µg of GFP-expressing plasmids. Cells were harvested 40 h later, fixed in 1% paraformaldehyde during 10 min and incubated in 70% ethanol for an additional 10 min. Fixed cells were then treated with phosphate-buffered saline (PBS) containing propidium iodine (PI) (50 µg/ml), RNase A (50 µg/ml), and FCS (1%, vol/vol) for 60 min at room

temperature. After gating on GFP positive population, the DNA content was analyzed on a FACScan flow cytometer using the Cell QUEST software (Becton Dickinson, Franklin Lakes, NJ, USA). Relative numbers of cells in the G2/M and G1 phases of the cell cycle (G2/M:G1 ratios) were calculated using the ModFit LT software (Verity Software House, Topsham, ME, USA). Apoptotic cells were detected using the Annexin V-Alexa Fluor 647 assay (Invitrogen, San Diego, CA, USA). Briefly, 40 h post-transfection 0.25×10^6 Hela cells were washed once with PBS and then incubated for 20 min in annexin V binding buffer (2.5 µl of Annexin V-Alexa Fluro 647 per ml, 10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 1 µg/ml of PI). Apoptotic cells, which stained positive for PI and annexin V were detected using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

RESULTS

HIV-1 Vpr and C-terminally cleaved products are detected in the extracellular medium of HIV-1 producing cells

To determine whether Vpr can be detected in the extracellular medium of HIV-1 producing cells, 293T cells were transfected with a proviral construct (HXBruBH10.R+) expressing Vpr in *cis* or with a Vpr-defective provirus (HXBruBH10.R-) complemented in *trans* with an expressor plasmid encoding Vpr. Forty hours post-transfection, cells were isolated by low-speed centrifugation while cell-free culture supernatants were fractionated by ultracentrifugation to separate viral particles from virus-free extracellular medium. The presence of Vpr was analyzed in HIV-1 producing cells, pelleted virions and virus-free extracellular medium as described in material and methods. As shown in Fig.2.1A, Vpr was detected as a single band of approximately 16-kDa in lysates from Vpr+ HIV-1-producing cells (Fig. 2.1A upper panel, lanes 3-4) as well as from HIV-1 virions (Fig. 2.1A, upper panel, lanes 7-8). Interestingly, upon more sensitive immunoprecipitation/western blot (IP/WB) analysis of cell lysates, a discrete band of lower molecular weight reacting specifically with murine anti-Vpr mAb 9F2 was detected in addition to native Vpr regardless of whether Vpr was expressed in *cis* or in *trans* (Fig. 2.1A lower panel, lanes 3-4). Importantly, native Vpr as well as a fast migrating Vpr-related band were detected in the virus-free extracellular medium of Vpr+ HIV-1- producing cells by IP/WB analysis (Fig. 2.1A lower panel, lanes 7-8). In that regard, it is interesting to note that the levels of fast and slow migrating Vpr species were inverted in the extracellular medium as compared to cell lysates (Fig. 2.1A, lower panel, compare lanes 3-4 to lanes 7-8).

To increase the sensitivity of Vpr immunodetection and to analyze further Vpr release and possible processing, we constructed proviral (HxBruBH10.3HAR+) and expression (SV CMV 3HA-Vpr) plasmids encoding a Vpr protein containing three consecutive HA epitope tags fused to the protein N-terminus (3HA-Vpr). The HxBruBH10.3HAR+ provirus was still able to replicate and produce infectious virus in Jurkat T cells, even though the C-terminus of Vif had to be truncated in the process of engineering the construct (data not shown). Experiments similar to the one described in Fig. 2,1A led to

essentially the same observations with, however, a drastic increase in sensitivity of Vpr species immunodetection (Fig. 2.1B). Both slow and fast migrating forms of Vpr were detected in cell and viral lysates as well as in extracellular medium from HxBruBH10.3HAR+-producing cells (Fig. 2.1B, upper and lower panels, lanes 3 and 8). The fact that the fast migrating Vpr band was specifically detected with anti-HA antibodies indeed suggested that full length Vpr undergoes processing at a putative cleavage site located at the Vpr C-terminus since the 3HA tags were fused at the N-terminal-end of the protein. To examine how Vpr products were released extracellularly, we analyzed Vpr release in cell cultures transfected with either the p6-defective proviral construct, HxBruBH10.3HAR+/p6(1-17), which is unable to package Vpr efficiently into virions (8) or with an expression plasmid encoding 3HA-Vpr only (SV CMV 3HA-Vpr). Even though the p6-defective virus was unable to efficiently incorporate Vpr (upper panel, compare lanes 8 and 9), we could still detect large amounts of extracellular forms of Vpr in the extracellular medium (lower panel, compare lanes 8 and 9), thus indicating that extracellular Vpr does not primarily originate from disrupted or decaying viral particles. Interestingly, expression of Vpr alone did not lead to extracellular release of Vpr nor to efficient Vpr processing (upper panel, lane 5 and lower panel lanes 5 and 10). As described for native Vpr in Fig. 2.1A, the ratio of full length 3HA-Vpr/cleaved Vpr was inverted in cell lysates and extracellular medium from HIV-1-producing 293T cells (lower panel, compare lanes 8-9 to lanes 3-4). To examine whether the C-terminally-cleaved Vpr products detected in cell lysates were present intracellularly or were externally associated to cells, we treated HxBruBH10.3HAR+-transfected 293T cells with 0.25% trypsin prior to lysis and Vpr detection by western blot. Fig. 2.1C clearly reveals that proteolytic treatment of cells with trypsin prior to lysis drastically decreased detection of the fast Vpr migrating band while keeping levels of full length Vpr intact (Fig. 2.1C, compare lanes 4 and 3), thus suggesting that full length Vpr undergoes processing at its C-terminus extracellularly, possibly at the cell surface.

Finally, to ensure that these observations were reproducible in the context of infected CD4+ T cells, we analyzed Vpr processing in transfected Jurkat T cells in which viral infection was let to spread over a six day period. As shown in Fig. 2.2, results of these experiments clearly reveal that Vpr as well as Vpr C-

terminal cleaved products are detected in the extracellular medium during HIV-1 infection of T cells (lower panel, lane 6). Furthermore, as shown in HIV-1 producing 293T cells (Fig.2.1), detectable amounts of cleaved products were found associated with cells (lower panel, lane 3).

Taken altogether, these results indicate that Vpr is released in the extracellular medium of HIV-1-producing cells by a process that requires expression of viral proteins but that is independent of Vpr virion incorporation. Furthermore, while Vpr is released, it appears to undergo processing extracellularly rather than intracellularly at a putative cleavage site located within the C-terminal domain of the protein.

Vpr is cleaved at a proprotein convertase processing site located at the protein C-terminus.

Having obtained evidence that Vpr undergoes processing at the protein C-terminus, we next performed deletion analysis in order to map the putative cleavage site. Expression plasmids encoding 3HA-Vpr harboring C-terminal deletions from aa residue 79 to 96 (SV CMV 3HA-Vpr (1-78)) and from residue 89 to 96 (SV CMV 3HA-Vpr (1-88)) were generated and used to *trans*-complement a Vpr-defective HxBruBH10.R- provirus in transfection assays in 293T cells. As shown in Fig. 2.3A, both 3HA-Vpr deletion mutants were expressed at levels comparable to the wild type (Wt) 3HA-Vpr in transfected cells (lanes 3-4) and were efficiently released in the extracellular medium (lanes 9-10), thus suggesting that the C-terminal domain of Vpr is dispensable for extracellular release. Importantly, none of the extracellular Vpr deletion mutants displayed any detectable processing (lanes 9-10). Indeed, processed 3HA-Vpr was found to migrate very closely to 3HA-Vpr (1-88) (compare lanes 11 and 10) but slower than 3HA-Vpr (1-78) (compare lanes 11 and 9) indicating that the putative Vpr processing site most probably lies between residues 78 and 88 and very close to residue 88. Consistently, a putative basic aa-specific PC cleavage site (R/K)-Xn-(R/K)↓ (n=0, 2, 4, 6) (55) was identified at Vpr $R_{85}QRR_{88}$↓ positions using the Prop v.10b Propeptide cleavage site prediction program (14). Alignments of HIV-1 Vpr from different viral isolates and clades show that the putative P1 (R88) and P2 (R87) cleavage positions are very well conserved, while the P4 (R85) position reveals a degree of variation (Fig. 2.3B). To confirm whether this predicted PC processing site was indeed functional, we selectively substituted aa residues

within the putative cleavage site using site-directed mutagenesis. Given that the P4 position is important for processing by furin while the P1 and P2 position are critical for PCs in general (44, 64), we focused our mutagenesis on these positions and generated 3HA-tagged Vpr mutants R85Q (mutated P4 position) and RR87/88 AA (mutated P1 and P2 positions) (Fig. 2.3C). Both mutants were efficiently expressed in HxBruBH10R-co-transfected 293T cells and released in the extracellular medium (lanes 3-4 and 9-10). Interestingly, while extracellular 3HA-VprR85Q mutant exhibited a two-fold decrease in processing as compared to Wt 3HA-Vpr (compare lanes 11 to 9), processing of the double mutant RR87/88 AA was reduced by at least six-fold (lane 10). These results strongly suggest that Vpr contains a PC processing site, **$\underline{R}_{85}Q\underline{RR}_{88}\downarrow$(V/A/G)R**, within the protein C-terminal arginine region that is recognized by a PC that is most probably distinct from furin. Furthermore, the presence of hydrophobic aa residues such as valine, in some viral strains, just following the cleavage site (i.e., at P1') would be indicative of either a PC5 or PACE4-generated cleavage (55).

To further identify the exact Vpr cleavage site, we immunopurified extracellular forms of 3HA-Vpr from virus-free cell culture supernatant of HxBruBH10.3HAR+ provirus-transfected 293T cells by immunoprecipitation using anti-HA mAb and analyzed the molecular mass of proteins eluted from the immunocomplexes by SELDI-TOF (Surface-enhanced laser desorption/ ionization time-of-flight) mass spectrometry (Ciphergen Biosystem Inc., Fremont, CA). Data of Fig. 2.3D reveals the presence of two specific peaks of isolated proteins. The first peak was found to correspond to a protein having a molecular mass 16189.6 dalton, which is indeed very close to the predicted molecular mass of full-length 3HA-Vpr (theoretical molecular mass: 16166.0 dalton). The second peak consisted of a mix of proteins having molecular mass ranging from 15355.6 to 15034.6 dalton), which are indeed very close to the predicted molecular mass for of 3HA-Vpr (1-88) (theoretical molecular mass 15365.6 dalton) and 3HA-Vpr (1-86) (theoretical molecular mass: 15054.4 dalton), respectively.

Taken altogether, the results obtained by mutagenesis and mass spectrometry are consistent with a sequential proteolysis process of Vpr. First, the protein is likely cleaved at a PC processing site located at position 85-88, $R_{85}Q\ RR_{88}\downarrow$, to lead Vpr cleavage product 1-88. Then, the two terminal arginine

residues are most probably removed by a basic-aa specific carboxypeptidases, such as carboxypeptidase D (CPD) (17), thus leading to fully processed Vpr (1-86).

Proprotein convertases mediate extracellular HIV-1 Vpr processing.

To determine if PCs have an exclusive role in extracellular Vpr processing, 293T cells were co-transfected with HxBRUBH10.3HAR+ and expression plasmids encoding the protein-based PC inhibitors α1-PDX or Spn4A (Fig. 2.4A). α1-PDX, which is an α1-antitrypsin variant serine protease inhibitor (serpin) contains the minimal consensus furin cleavage site (R-I-P-R) in its reactive site loop and functions as a suicide substrate inhibitor of PCs, including furin, PC5, PC3 and PACE4 (3, 29, 67). Likewise, Spn4A is a secretory pathway serpin from *Drosophila Melanogaster* that contains a consensus furin cleavage site, R-R-K-R, in its reactive site loop. Spn4A inhibits human furin and Drosophila PC2 by a slow-binding mechanism characteristic of serpin molecules and forms kinetically trapped SDS-stable complex with each enzyme (52). Fig. 2.4A reveals that extracellular Vpr processing was significantly reduced in cells expressing α1-PDX or Spn4A as compared to control cells expressing empty vectors (compare lanes 12 and 11, and lanes 14 and 13). Interestingly, Spn4A appeared to have a more potent inhibitory effect on extracellular Vpr processing than α1-PDX in several reproducible experiments. To ensure that these inhibitory effects were specific, we analyzed the processing of HIV-1 envelope gp160 precursor, a known substrate of furin (21) in cell lysates from the same cell transfectants. As expected, gp160 processing was inhibited in cells expressing α1-PDX or Spn4A, with α1-PDX showing a more potent inhibition of gp160 processing than Spn4A (Fig. S2.1 in the supplemental material). In a second approach, HxBRUBH10.3HAR+ provirus-transfected 293T cells were treated forty hours post-transfection for 7 hours with 10μM of the membrane soluble peptide PC inhibitor dec-RVKR-cmk (29). Processing of extracellular Vpr was specifically inhibited by at least 6-fold in presence of 10μM dec-RVKR-cmk (Fig. 2.4B, right panel, compare lanes 8 and 7; left panel). The fact that both protein-based

(α1-PDX and Spn4A) and peptide-based (dec-RVKR-cmk) PC inhibitors inhibited extracellular Vpr processing strongly suggests that basic aa-specific PCs mediate Vpr processing during HIV-1 infection.

We next investigated which widely expressed PCs associated with the constitutive secretory pathway could mediate processing of extracellular Vpr. Furin, PC5 and PACE4 were selected because of their potential role in proteolytic processing of Env gp160 during HIV-1 infection (12, 71), but more importantly because of their presence at the cell surface as well as in the extracellular medium as enzymatically active shed forms (34, 41, 68). Plasmids expressing human furin, mouse PC5A or human PACE4 were co-transfected with HxBRUBH10.3HAR+ in different cells lines including Hela-CCR5, COS-1 and 293T and 48 h posttransfection, levels of 3HA-Vpr and cleaved products were analyzed in cell lysates and virus-free extracellular medium by western blot or IP/WB (Fig. 2.4C). When PC5A or PACE4 were co-expressed with 3HA-Vpr-expressing virus, the level of fast migrating Vpr-processed forms detected in the extracellular medium of all transfected cell lines was significantly increased (compare lanes 12 and 10 with lane 9). Interestingly, this increased detection of Vpr-processed forms in the extracellular medium was also accompanied by a similar increase in the detection of cleaved product externally associated with cells (compare lanes 6 and 4 with lane 3) as shown in Fig. 2.1C. In contrast, co-expression of furin in HxBRUBH10.3HAR+-producing cell lines had a marginal effect on basal Vpr processing (compare lanes 11 and 9 as well as lanes 5 and 3). Overall, these results suggest that proprotein convertases PC5 and PACE4 can efficiently process extracellular Vpr.

Extracellular Vpr is processed by a PC that is cell surface–associated.

The fact that full length Vpr is detected mainly intracellularly while processed-Vpr is found primarily in the extracellular medium suggests that Vpr proteolytic processing occurs extracellularly - i.e. at the cell surface or in the extracellular medium. To examine these possibilities we first performed a time-course analysis of 3HA-Vpr release from HIV-1-producing 293T cells. Equal amounts of HXBruBH10.3HAR+ provirus-transfected 293T cells plated in 6 wells-plate (10^6 cells/2ml media per well) were extensively washed 40 h post-transfection and incubated with fresh culture medium. Over a 3

h period and at different time intervals, the presence of 3HA-Vpr and cleavage products was analyzed in cell and virus-free extracellular medium by western blot and IP/WB as described in material and methods (Fig. 2.5A). Under these conditions, Vpr was first released out in the extracellular medium mostly as a full-length protein as illustrated by detection of full length 3HA-Vpr as early as 15 min post-culture medium change (lane 3). By 30 min, 3HA-Vpr cleavage products started being clearly detected and by 3 h almost fifty percent of extracellular Vpr consisted of Vpr-processed products (lanes 4-6), thus providing evidence that Vpr processing occurs extracellularly once full length Vpr has been released out of infected cells.

To characterize whether Vpr was processed by a PC at the cell surface or in the extracellular medium, equivalent amounts of exogenous *in vitro* translated 3HA-Vpr was added to 293T cell or to conditioned media for various time intervals. As shown in Fig. 2.5B (upper panel), addition of *in vitro* translated 3HA-Vpr to 293T cell culture led to processing of exogenous 3HA-Vpr in the cell supernatants starting at 6 h and reaching a peak by 12 h post-incubation (compare lanes 2-5 to lane 1). This processing was PC-specific since addition of 10 μM dec-RVKR-cmk to the culture media reduced drastically the accumulation of Vpr cleavage products (compare lanes 6-9 to lanes 2-5). In contrast, only trace amount of Vpr processing was detected upon addition of exogenous 3HA-Vpr to 293 T conditioned medium (Fig. 2.5B, lower panel, compare lanes 2-5 to lane 1). Importantly this residual processing of Vpr in conditioned medium was not affected by addition of PC inhibitor. Taken together, these results suggest that Vpr undergoes proteolytic processing extracellularly upon close contact with cells, most likely by a PC that is predominantly cell-surface associated.

Vpr processing is detrimental for induction of G2 cell cycle arrest and apoptosis.

It was previously reported that extracellular Vpr can transduce cells and cause G2 arrest in HeLa and CD4+ T cells (24, 59). To determine whether fully processed Vpr present in the extracellular medium could induce G2 cell cycle arrest upon transduction of bystander cells, we analyzed the effect of Vpr (1-86) expression on the cell cycle. 293T cells were co-transfected with a GFP-expressing construct as well

as with expression plasmids encoding Vpr (SV CMV Vpr Wt) or fully processed Vpr (1-86) (SV CMV Vpr (1-86) or the G2 arrest-defective mutant R80A (SV CMV Vpr R80A) and the cell cycle profile of the GFP-positive cell population was analyzed by flow cytometry 48 h posttransfection as described in material and methods. As shown in Fig. 2.6A, Vpr (1-86) was unable to induce a G2 cell cycle arrest as compared to full length Vpr (Vpr (1-86) G2/M:G1 ratio = 0.4 versus 3.5 for Wt Vpr) even though both proteins were expressed at similar levels (Fig. 2.6B). Indeed the degree of G2 arrest induced by Vpr (1-86) was similar to that of Vpr R80A, a well characterized G2 arrest-defective Vpr mutant (13, 62). Finally, to determine whether proteolytic processing of Vpr could modulate Vpr-mediated apoptosis, HeLa cells were transfected with plasmids co-expressing GFP and Wt or truncated Vpr, and apoptosis was monitored by PI and annexin V-staining 48 hours post-transfection. Results presented in Table 1 reveal that truncated Vpr (1-86), in contrast to Wt Vpr, was unable to induce apoptosis. In presence of Vpr (1-86), approximately 10% of cells were PI/annexin V-positive while approximately 26% of cells were apoptotic in presence of Wt Vpr. Indeed, the level of apoptotic cells detected in Vpr (1-86)-expressing cells was similar to that obtained in control cells (Vpr-). Overall, these results indicate that processing of Vpr at aa residue 86 abolishes the ability of the protein to induce a G2 cell cycle arrest and mediate apoptosis.

DISCUSSION

In this study, we provide evidence indicating that Vpr as well as Vpr-cleaved products are detected in the extracellular medium of several cell lines, including HEK 293T , HeLa , COS-1 and $CD4^+$ Jurkat T cells, producing HIV-1 *in vitro* (Fig.2.1, 2.2 and 2.4). Interestingly, Vpr expression alone, in the absence of any other viral products, did not lead to efficient release even though the protein has proapoptotic properties (1, 61). Apparently, efficient Vpr release requires co-expression of viral proteins suggesting that other viral components or/and host cell responses may be necessary for extracellular release Vpr. Since Vpr is primarily located in the nucleus when expressed alone (56, 63), it is possible that expression of other nucleocytoplasmic shuttling viral proteins such as the Gag polyprotein precursor might be necessary to transport the protein in the cytosol near the plasma membrane. In that regard, it was previously reported that during viral infection, Vpr was redistributed from the nucleus to the cytosol and membrane compartments by a process that was independent of its interaction with the p6 domain of Gag (30). In addition to nuclear export and transport near the plasma membrane, HIV-1-mediated cytopathicity, including plasma membrane disruptions due to viral egress (16) or the combined proapoptotic function of HIV-1 gene products (20), such as Vpr, Tat, Env and Vpu might be necessary for release of Vpr in the extracellular medium. Nevertheless, our data clearly reveal that Vpr release in the extracellular medium does not rely on the protein ability to be packaged into nascent viral particles; as such, extracellular Vpr originates most likely from HIV-1-producing cells rather than from disrupted or decaying virions, as was previously proposed (38).

Our data also provides evidence that Vpr undergoes processing extracellularly. Deletion mapping analysis is consistent with Vpr processing occurring within the C-terminal arginine-rich domain of the protein and specifically at a very well conserved putative PC motif located at position $R_{85}QRR_{88}$ (Fig. 2.3A). Indeed, site-directed mutagenesis of this putative PC processing site reveals that double mutations of the conserved basic arginine residues located at the P1 and P2 position for alanine (Vpr RR87/88AA) drastically reduced Vpr processing, while substitution of the less conserved arginine residue for a glutamine (R85Q) at the P4 position, attenuated Vpr processing (Fig. 2.3C). These results indicate that the

basic arginine residues in the P1 and P2 position are critical for Vpr processing, while the arginine located in the P4 position is not essential but seems to modulate the extent of Vpr processing. In this regard, it is interesting to note that several Vpr alleles encoded by laboratory-adapted strains such as LAI or HIV-1 primary isolates from different clades contain a glutamine or a proline residue instead of an arginine at that position (Fig. 2.3B), thus raising the possibility that soluble Vpr encoded by these alleles might be less prone to efficient cleavage (35). Evidence that Vpr is undergoing processing at this site is also supported by our mass spectrometry analysis of extracellular Vpr species, which revealed the presence of primarily two Vpr cleavage products with predicted molecular mass corresponding to products undergoing cleavage at positions 88 (Vpr 1-88) and 86 (Vpr 1-86) in addition to full length Vpr (Fig. 2.3D). As it has been previously reported that the last two basic aa residues of the released N-terminal peptide generated upon PC cleavage are generally removed by carboxypeptidases (31) (17), our results are consistent with a Vpr processing occurring at the conserved double arginine residues (R87R88) in the P1 and P2 position, thus generating an 88 aa polypeptide that subsequently undergoes a rapid trimming of the two basic pair residues leading to an 86 aa fully processed Vpr product. The fact that fully processed Vpr was shown to migrate slightly faster than the Vpr (1-88) deletion mutant (Fig. 2.3A) supports such a sequential proteolytic process.

Furthermore, our results reveal that Vpr is initially released as a full-length protein and subsequently processed extracellularly by cell surface PCs (Fig. 2.5). PCs belong to a family of evolutionary conserved dibasic- and monobasic-specific Ca^{2+}-dependent subtilisin-like serine proteinases related to the yeast kexin enzyme that constitute the major endoproteolytic processing enzymes of the secretory pathway in mammals (55, 60). Traditionally, PCs have been shown to cleave their substrates intracellularly. This is particularly true for furin, the best known member of the protease family (64). Furin is known to exist at the cell surface while other PCs, such as PACE4 and PC5, are known to be secreted and anchored in the extracellular matrix (46, 68) and are therefore, presumed to have extracellular substrates. The inhibition of extracellular Vpr processing using PC inhibitors such as dec-RVKR-cmk or serpins α1-PDX and Spn4A strongly suggests that PCs are involved in Vpr processing

(Fig. 2.4A-B). Furthermore, transient co-expression of PCs in several HIV-1 producing cell lines reveal that PC5 and PACE4 efficiently processed extracellular Vpr in 293T, Hela and COS-1 cell lines, while furin expression had only a marginal effect (Fig. 2.5C). These results are consistent with the fact that mutation of the arginine residue at the P4 position of the processing site, a residue critical for processing by furin (64), was not essential for Vpr cleavage. The fact that secreted PC5 and PACE4, but not soluble furin, bind heparin within the extracellular matrix, *via* a cationic stretch of aa within their cysteine-rich (CRD) domain (46, 68), raises the possibility that soluble Vpr, undergoes processing upon attachment to the cell surface extracellular matrix of bystander cells. Indeed, our observations that Vpr protein needs to be in close contact with cells to be efficiently processed as well as our data indicating that treatment of HIV-1 producing cells with soluble heparin (Fig. S2.2 in the supplemental material), a product known to abolish the heparin sulfate-mediated attachment of exogenous protein to cells, promoted the detachment of cell-surface-associated Vpr and cleavage products support such a model.

Our data suggests that PC5 and PACE4 might be involved in extracellular Vpr processing. This is especially relevant since these enzymes can tolerate an aliphatic residue (e.g., Val or Leu) at P1' just following the cleavage site, whereas furin does not (55). Interestingly, PC5 is expressed in freshly isolated human CD4 T-lymphocytes, the natural host cells of HIV-1 (12, 22). Although PACE4 is not expressed in peripheral blood lymphocytes (PBL), it is highly expressed in lymphoid tissues such as thymus, lymph node and spleen (22) and as such, might be present at the cell surface of bystander cells located in close proximity of HIV-1-producing cells *in vivo*. However, we cannot rule out at this point that other PCs displaying similar substrate specificity and cellular location may also participate to Vpr processing *in vivo*, especially for those viruses harboring variants of Vpr sequence at the P1' position where Val is replaced by either Ala or Gly (Fig. 2.3B).

Efficient processing of Vpr by PCs generates a truncated Vpr (1-86) that is defective for G2 cell cycle arrest and is unable to induce apoptosis upon expression in cells (Fig. 2.6 and Table 1). An eight aa C-terminal peptide (^{89}ARNGASRS^{96}) will be presumably generated following Vpr processing. However, it is unlikely that this small peptide displays cytotoxic properties as was reported for other C-terminal

Vpr-derived polypeptides (28, 58), since previous studies have shown that Vpr C-terminal peptide 83-96 or 80-96 did not exhibit any cell killing or cytotoxic effects on CD4+ T cells (4) or rat neurons (54), respectively. These findings raise the possibility that processing of extracellular Vpr by cell-surface PCs, such as PC5 and PACE4, might indeed represent a host cell process to functionally inactivate the cytostatic and cytotoxic activities of extracellular Vpr. In addition to inactivating Vpr-mediated G2 arrest and proapoptotic activities, processing of extracellular Vpr C-terminus may also represent a mechanism to prevent the uptake of the protein by target cells. The carboxy-terminus domain of Vpr contains six highly conserved arginine residues between residues 73 and 96. This domain shows similarity with those of arginine-rich protein transduction domains and may explain the transducing properties of Vpr, including its ability to cross the cell membrane lipid bilayer (9, 24, 59). In fact, transduction of exogeneous protein has been shown to very often involve the attachment of arginine-rich domain of proteins to membrane heparin sulfate proteoglycans (HSPG) (42, 51). Likewise, soluble Vpr may interact with membrane HSPG via its C-terminal arginine-rich domain as a first step prior to internalization as was reported for other transducing proteins (42). Processing of extracellular Vpr by PCs will delete four of the six conserved arginine residues and as such, is likely to interfere with the transduction properties of the protein (59). More studies will now be required to analyze soluble Vpr interactions with cell surface HSPG and its implications in terms of protein cellular uptake. Furthermore, given that the expression profile of PC varies according to tissues and cell types (12, 22), it will be important to assess the level of Vpr processing in any cellular system where the biological activity and function of soluble Vpr is analyzed. Perhaps, inefficient or lack of soluble Vpr biological activity upon contact to certain cell types might be related to Vpr inactivation by cell surface PC. It is interesting that Vpr is not the only HIV-transducing protein undergoing processing by PCs. Extracellular Tat was reported to be cleaved by furin (65). Furin processing did not affect the rates of N-terminal cleavage product uptake and nuclear localization but greatly reduced the protein transactivation activity. It is thought that furin processing is a mechanism to inactivate extracellular Tat protein. Although our results point towards a role of Vpr processing by PCs

as a mean to inactivate Vpr, we cannot entirely rule out that soluble truncated Vpr (1-86) may still have other biological activities and function important for HIV infection *in vivo*.

In conclusion, this study provides evidence that HIV-1 Vpr is released in the extracellular milieu of HIV-1-producing cells where it undergoes processing by cell surface PCs such as PC5 or PACE4. PC processing of extracellular Vpr occurs at a very well conserved processing site located in the C-terminal arginine-rich domain of the protein and leads to the production of a truncated Vpr product that is unable to induce G2 cell cycle arrest and apoptosis. We propose that PC processing of extracellular Vpr represent a cellular process to inactivate soluble Vpr.

ACKNOWLEDGMENTS

We thank Dr. Ghislaine Duisit and Jean-Philippe Belzile for helpful discussions and critical review the manuscript. HIV-1 IIIB gp120 Hybridoma 902 recognizing both gp120 and precursor gp160 was obtained from the NIH AIDS research & reference reagent program while the anti-Vpr mAb 9F12 was kindly provided by Dr. Jeffrey B. Kopp (NIH). EAC and NGS are recipients of the Canada Research Chairs in Human Retrovirology and Precursors Proteolysis, respectively.

This work was supported by grants from the Canadian Institute of Health Research to EAC (NRF-12381) and NGS (CIHR MGP-44363) and from the Fonds de la Recherche en Santé du Québec-AIDS network to EAC.

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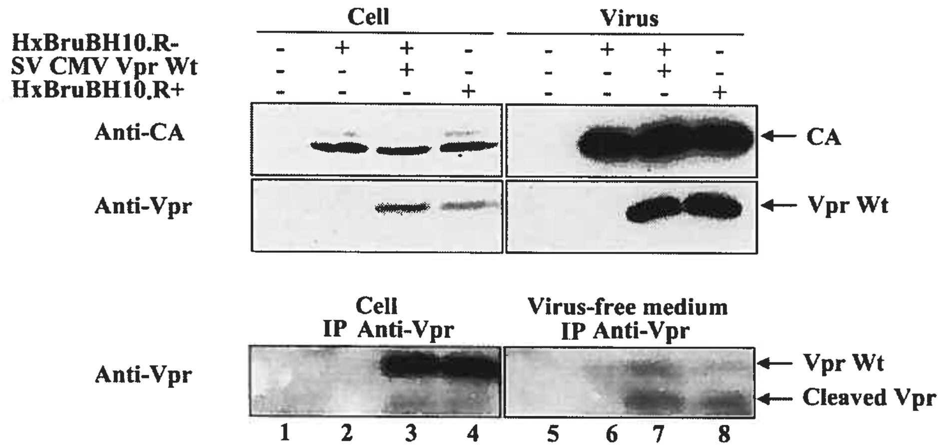
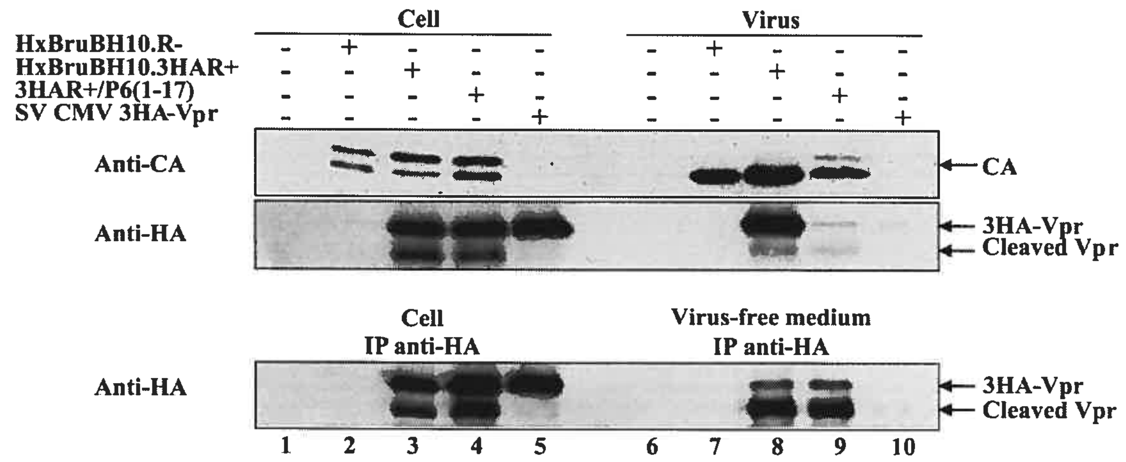
TABLE 1. Effect of Vpr-processing on Vpr-mediated apoptosis.

	PI(+)/Annexin V(-) (%)	PI(+)/Annexin V(+) (%)
SV CMV Vpr-/GFP	2.9± 0.5	9.2± 0.4
SV CMV Vpr(1-86)/GFP	3.2± 0.3	9.6± 1.3
SV CMV Vpr Wt/GFP	7.9± 1.4	25.9± 3.3


A
Cell
Virus
HxBruBH10.R-
SV CMV Vpr Wt
HxBruBH10.R+
Anti-CA
CA
Anti-Vpr
Vpr Wt
Cell
IP Anti-Vpr
Virus-free medium
IP Anti-Vpr
Anti-Vpr
Vpr Wt
Cleaved Vpr
1 2 3 4 5 6 7 8


B
Cell
Virus
HxBruBH10.R-
HxBruBH10.3HAR+
3HAR+/P6(1-17)
SV CMV 3HA-Vpr
Anti-CA
CA
Anti-HA
3HA-Vpr
Cleaved Vpr
Cell
IP anti-HA
Virus-free medium
IP anti-HA
Anti-HA
3HA-Vpr
Cleaved Vpr
1 2 3 4 5 6 7 8 9 10

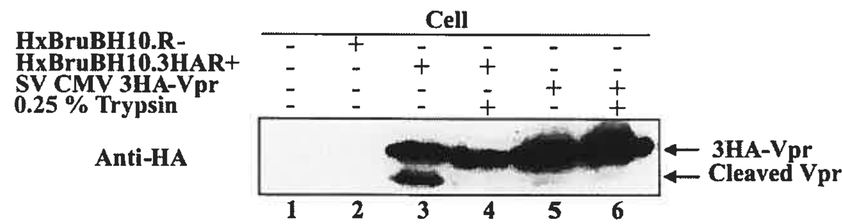

C
Cell
HxBruBH10.R-
HxBruBH10.3HAR+
SV CMV 3HA-Vpr
0.25 % Trypsin
Anti-HA
3HA-Vpr
Cleaved Vpr
1 2 3 4 5 6

FIGURE 2.1. HIV-1 Vpr and cleaved products are found in the extracellular medium of HIV-1-producing cells. (A) Native Vpr and cleaved products are detected in the extracellullar medium of HIV-1 producing 293T cells. 293T cells were mock-transfected (lanes 1 and 5) or transfected with HXBruBH10.R- (lanes 2 and 6) or HXBruBH10.R- with SV CMV Vpr (lanes 3 and 7), or the HxBruBH10.R+ provirus (lanes 4 and 8). Forty hours post-transfection, cells, pelleted virus particles and virus-free extracellular medium were isolated, lysed, and analysed for the presence of native Vpr directly by western blot (upper panel) or by IP/WB (lower panel). Samples corresponding to 5% of the original crude cell (lanes 1-4) and viral (lanes 5-8) lysates were analyzed for the presence of Vpr and CA by western blot using murine anti-Vpr mAb 9F2 and CA mAb respectively (upper panels). In parallel, cell lysates (50 % of total cell lysates) (lanes 1-4) and virus-free extracellular medium (50% of total medium recovered) (lanes 5-8) were immunoprecipitated (IP) with rabbit anti-Vpr pAb and immunocomplexes analyzed by western blot using anti-Vpr mAb 9F2 (lower panel). (B) Extracellular release of Vpr and C-terminally cleaved products requires expression of viral proteins and is independent of Vpr virion incorporation. 293T cells were mock-transfected (lanes 1 and 6) or transfected with HXBruBH10.R- (lanes 2 and 7) or HxBruBH10.3HAR+ (lanes 3 and 8) or HXBruBH10.3HAR+/p6(1-17) (lanes 4 and 9), or SV CMV 3HA-Vpr (lanes 5 and 10). Forty hours post-transfection, cells, pelleted virus particles and virus-free extracellular medium were isolated as described. Levels of HA-tagged-Vpr (3HA-Vpr) and CA were determined in equivalent proportion of cell and viral lysates by direct western blot using anti-CA or anti-HA mAb (upper panel). In addition, the presence of 3HA-Vpr in cell lysates and virus-free extracellular medium was analyzed by IP/WB using anti-HA mAb (lower panel). (C) Cleaved Vpr is associated to the external surface of HIV-1 producing cells. 293 T cells transfected with the indicated proviral constructs or expression plasmids were extensively washed with PBS 40 hours posttransfection prior to a 10 min treatment with 0.25% trypsin. Following addition of 5 ml DMEM supplemented with 10% FCS to stop trypsin digestion, cells were extensively washed with PBS and lysed in NP40 lysis buffer. The presence of 3HA-Vpr and cleaved products in cell lysates was analyzed by western blot using anti-HA mAb.

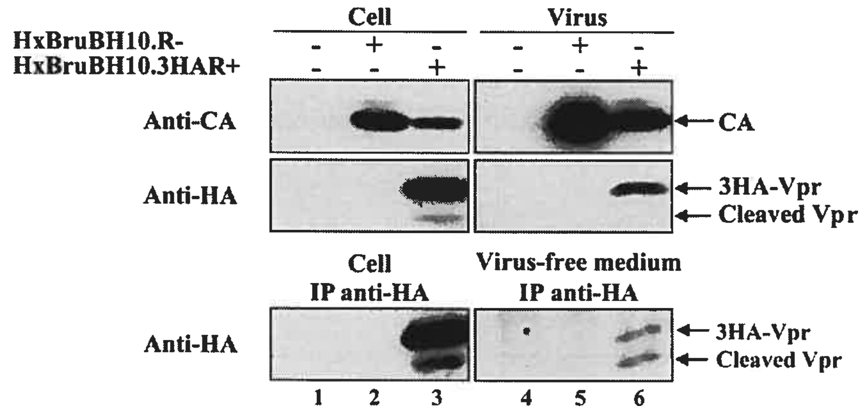
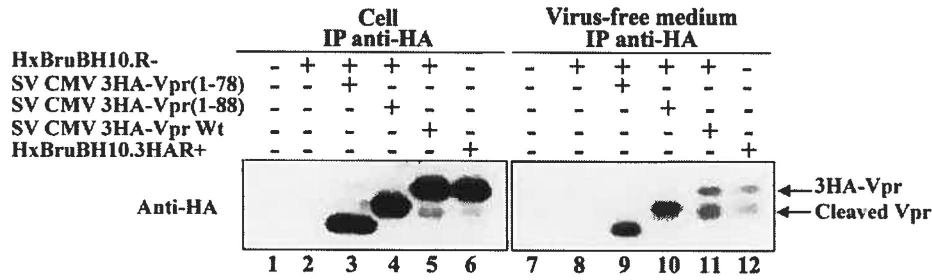


FIGURE 2.2. Vpr and C-terminal cleaved products are detected in the extracellular medium of HIV-1 infected Jurkat T cells. Jurkat T cells were mock-transfected (lanes 1 and 4) or transfected with HxBruBH10.R- (lanes 2 and 5) or HxBruBH10.3HAR+ virus (lanes 3 and 6). The presence of 3HA-Vpr and 3HA-Vpr cleaved products in infected cell and viral lysates as well as in virus-free extracellular medium was determined 6 days post-transfection as indicated.

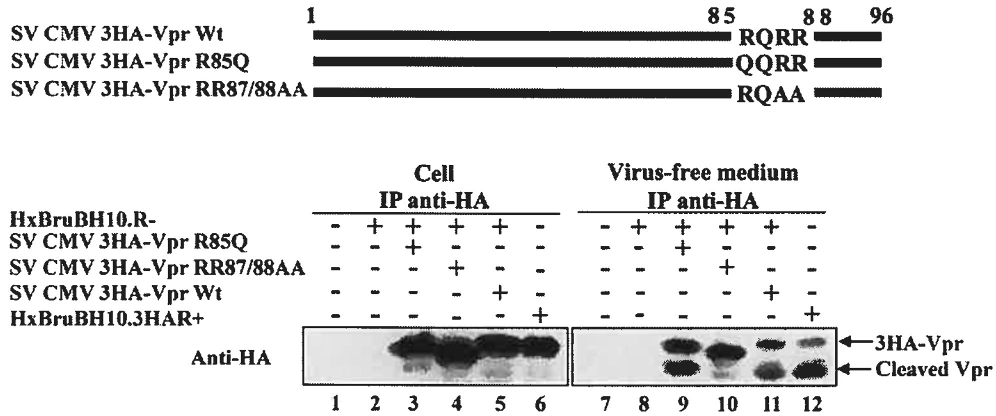
A



B

80 90
P4 P3 P2 P1 P1' P2'
Consensus A RIGI . . . I R G R R V R
Consensus B RIGI . ? ? ? ? Q R R A R
Consensus C RIGI . . . L R Q R R A R
Consensus D RIGI . . . T R Q R R A R
Consensus N RIGI . . . T P Q R R – R
Consensus O RIGINPSNT R G R G R R N G
Consensus cpz RIGI . . ? ? ? R R R ? ?

C



D

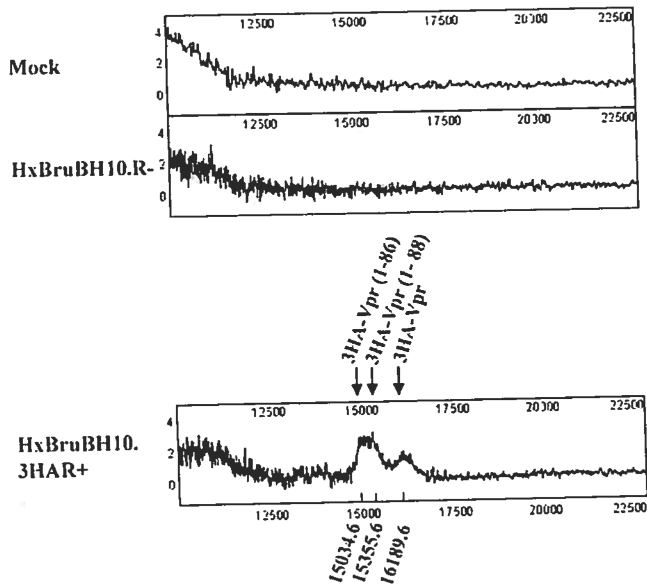
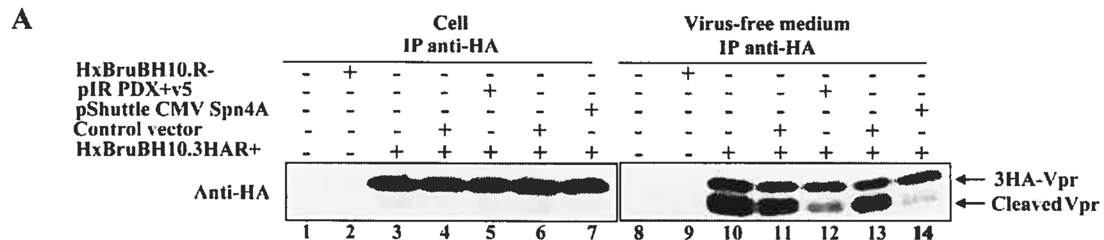


FIGURE 2.3. Vpr is cleaved at a PC processing site located within the arginine-rich C-terminal domain. (A) Deletion mapping of Vpr cleavage site. 293T cells were mock-transfected (lanes 1 and 7) or transfected with HXBruBH10.R- (lanes 2 and 8), or cotransfected with HXBruBH10.R- and SV CMV.3HAVpr (1-78) (lanes 3 and 9), SV CMV.3HAVpr (1-88) (lanes 4 and 10) or SV CMV.3HAVpr (lanes 5 and 11). As an additional control, cells were transfected with HXBruBH10.3HAR+ (lanes 6 and 12). Forty hours post-transfection, the presence of 3HA-Vpr and cleavage products was analyzed in cells and virus-free extracellular medium by IP/WB as indicated. (B) Alignment of HIV-1 Vpr putative proprotein cleavage sites from different HIV-1 subtypes. The consensus Vpr aa sequences between amino acid residue 80 and 90 are derived from the HIV sequence database, Los Alamos National Laboratory (http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). Predicted PC processing site aa residues are underlined and their positions indicated above. (C) Mutagenic analysis of the putative Vpr PC cleavage site. 293T cells were mock-transfected (lanes 1 and 7), or tranfected with HXBruBH10.R- (lanes 2 and 8) or HXBruBH10.3HAR+ (lanes 6 and 12), or co-transfected with HXBruBH10.R- provirus and SV CMV.3HA-VprR85Q (lanes 3 and 9), SV CMV.3HA-Vpr RR87/88AA (lanes 4 and 10) or SV CMV.3HA-Vpr (lanes 5 and 11). Forty hours post-transfection, 3HA-Vpr expression and extracellular release were analyzed in cells and virus-free extracellular medium by IP/WB as indicated. *D,* Analysis of extracellular Vpr products by mass spectrometry. Soluble Vpr and cleavage products from virus-free extracellular medium of mock-transfected or HXBruBH10.R- or HXBruBH10.3HAR+- transfected 293T cell were immunopurified. Eluted proteins with molecular mass ranging from 10 to 22 kDa were then analyzed by SELDI-TOF-MS using the Protein-Chip Reader (Ciphergen Biosystem Inc., Fremont, CA).

A



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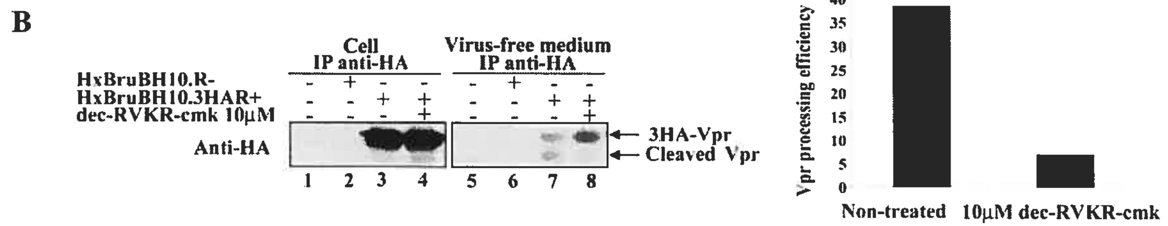

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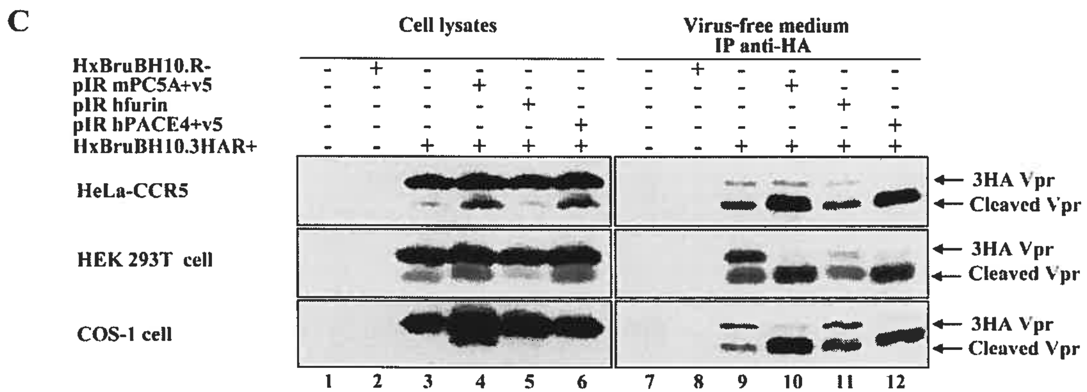


FIGURE 2.4. Proprotein convertases mediate HIV-1 Vpr processing. (A) Extracellular Vpr processing is inhibited by PCs inhibitors α1-PDX and Spn4A. 293T cells were mock-transfected (lanes 1 and 8), or transfected with HXBruBH10.R- (lanes 2 and 9) or HxBruBH10.3HAR+ (lanes 3 and 10), or co-transfected with 10μg of HxBruBH10.3HAR+ and 1 μg of pIR v5 empty vector (lanes 4 and 11) or pIR PDX+v5 (lanes 5 and 12), or SV CMVexPA control vector (lanes 6 and 13), or pShuttle CMV Spn4A (lanes 7 and 14). Forty hours post-transfection, the presence of 3HA-Vpr products was analyzed in cells and virus-free supernatants by IP/WB as indicated. (B) Extracellular Vpr processing is inhibited by the PC inhibitor dec-RVKR-cmk. 293T cells were mock-transfected (lanes 1 and 5), or transfected with either HXBruBH10.R- (lanes 2 and 6) or HXBruBH10.3HAR+ (lanes 3-4 and 7-8). Two days later, culture media were replaced with fresh medium and cells were cultured for 7 hours in presence (lanes 4 and 8) or in absence (lanes 1-3 and 5-7) of 10μM dec-RVKR-cmk. 3HA-Vpr and cleaved products levels were analyzed in cell lysates and virus-free extracellular medium by IP/WB as indicated (left panel). Vpr processing efficiency is expressed as the percentage of cleaved-Vpr over total Vpr products in the extracellular medium. (C) PC5A and PACE4 expression increases Vpr processing in different cell lines. Hela-CCR5, COS-1 and 293T cells were mock-transfected (lanes 1 and 7) or transfected with HXBruBH10.R- (lanes 2 and 8) or co-transfected with HxBruBH10.3HAR+ and pIR v5 empty vector (lanes 3 and 9) or pIR mPC5A+v5 (lane 4 and 10) or pIR hFurin FL (lanes 5 and 11) or pIR hPACE4+v5 (lanes 6 and 12). Forty hours post-transfection, levels of 3HA-Vpr products were analyzed in cells and virus-free supernatants as indicated.

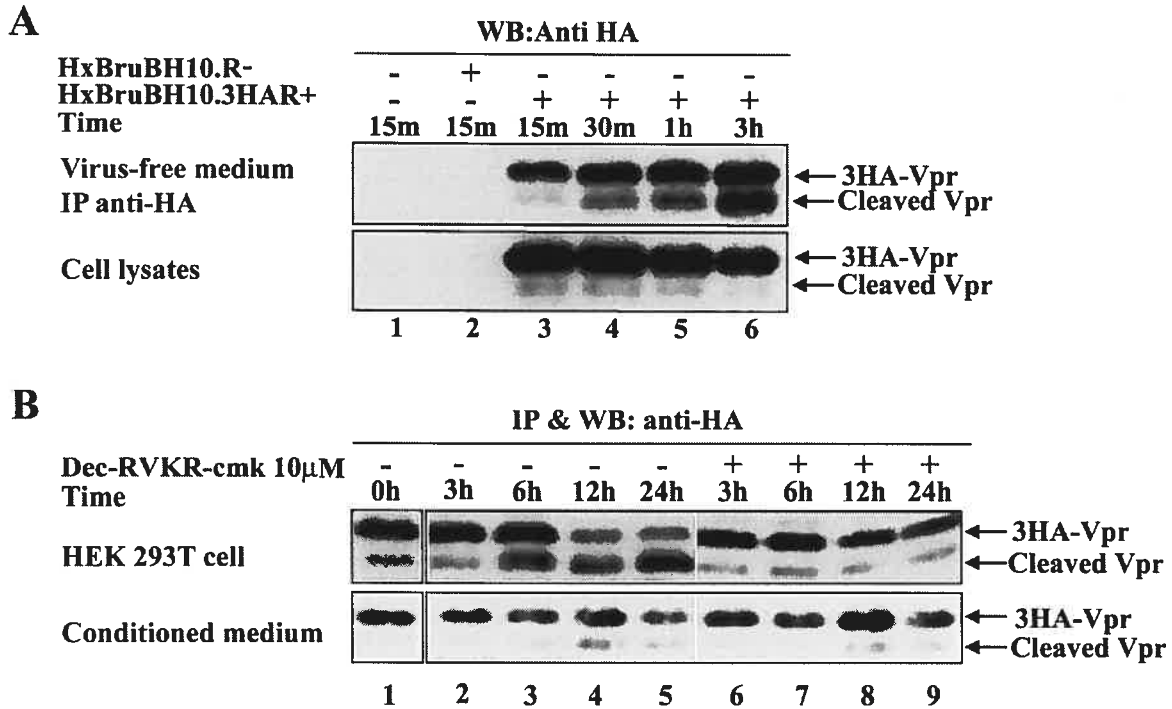
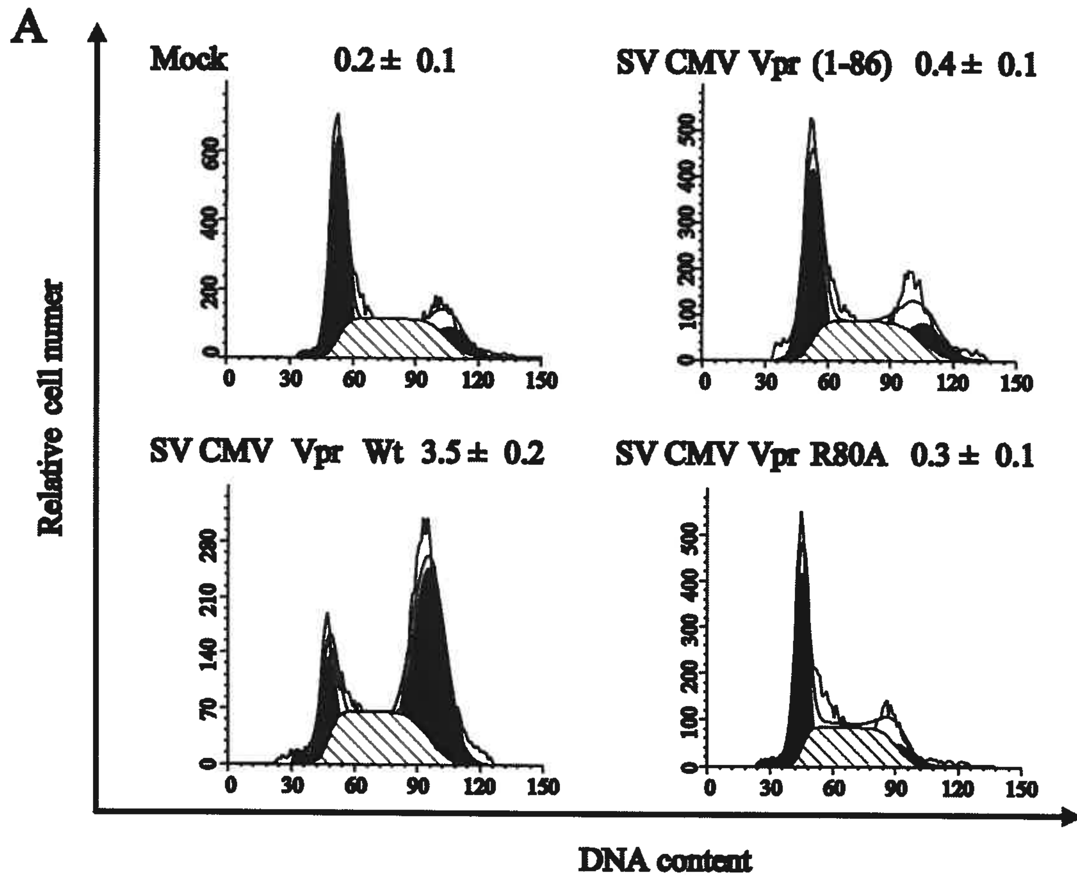


FIGURE 2.5. Vpr is processed extracellularly by a PC that is cell surface-associated. (A) HIV-1 Vpr is processed in the extracellular medium. 293T cells were mock-transfected (lanes 1) or transfected with HXBruBH10.R- (lane 2) or HXBruBH10.3HAR+ (lanes 3-6). Two days later, media were replaced with fresh culture medium and cells were further cultured for different intervals of time as indicated. Cells and virus-free culture media were then harvested at the indicated times and analyzed for the presence of 3HA-Vpr as indicated. (B) Extracellular Vpr is efficiently processed by a cell surface-associated PC. Equivalent amounts (approximately 1μg) of *in vitro* translated Vpr were added to 293T cells ($2X10^5$/2ml of culture media) or to cell-free conditioned media (2ml) collected from 293T cell overnight cultures in presence or absence of 10 μM dec-RVKR-cmk. Cell-free supernatants (upper panel) and conditioned media (lower panel) were collected at the indicated time intervals and analyzed for the presence of 3HA-Vpr and cleavage products by IP/WB.



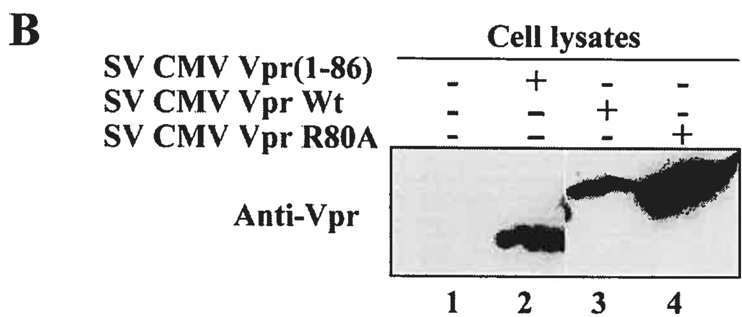
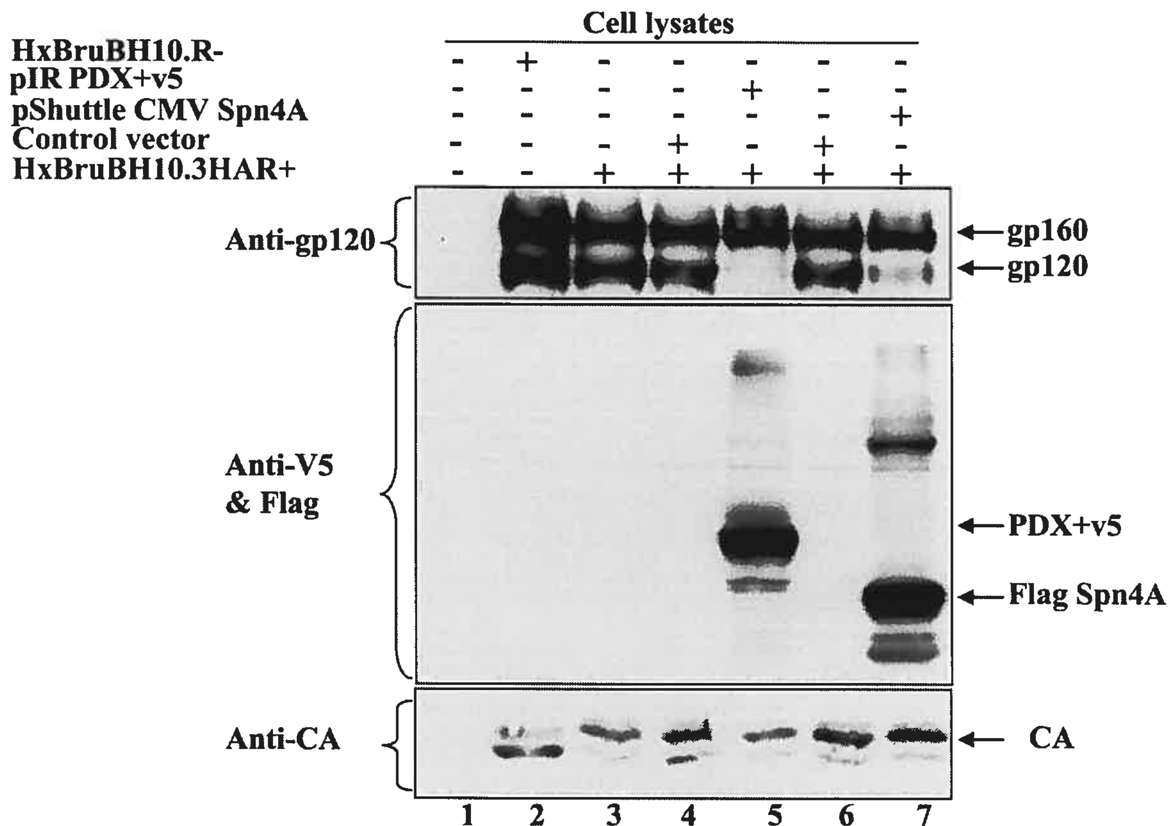
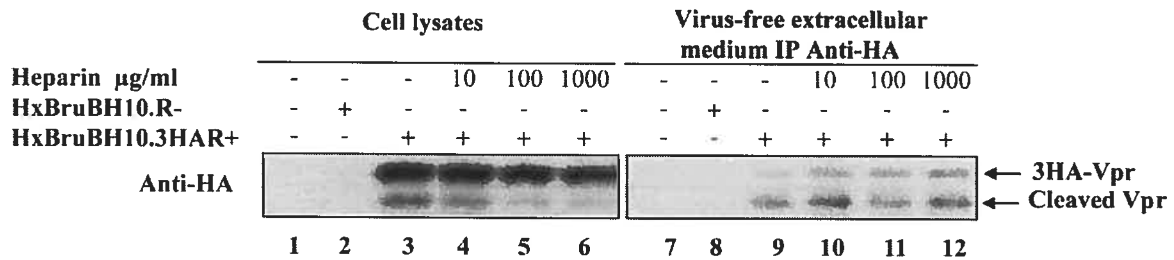


FIGURE 2.6. Effect of processing on Vpr-mediated cell-cycle arrest. (A) 293T cells were co-transfected with GFP-expressing plasmid (pQBI 25) and expression plasmids encoding Wt Vpr, Vpr (1-86)-truncated mutant and Vpr R80A. Forty-eight hours post-transfection, cell-associated DNA content of GFP-expressing cells was analyzed by PI staining and FACS analysis. The ability of Wt Vpr, Vpr (1-86) or Vpr R80A to induce G2 arrest was determined by calculating the G2/M:G1 ratio. Similar results were obtained in 3 independent experiments. (B) Vpr levels in each cell transfectant was analyzed by western blot using murine anti-Vpr mAb 9F2.



SUPPLEMENTAL FIGURE S2.1. Analysis of HIV-1 Env gp160 processing in presence of PCs inhibitors α1-PDX and Spn4A. 293T cells were mock-transfected (lane 1), or transfected with 10μg of HXBruBH10.R- (lane 2) or 10μg HxBruBH10.3HAR+ (lane 3), or co-transfected with 10μg of HxBruBH10.3HAR+ and 1 μg of pIR v5 empty vector (lane 4) or pIR PDX+v5 (lane 5), or SV CMV exPA control vector (lane 6), or pShuttle CMV Spn4A (lane 7). Forty hours post-transfection, levels of precursor gp160 and processed gp120 products were analyzed by western blot using monoclonal anti-gp120 antibodies. Levels of HIV-1 p24 as well as v5-tagged α1-PDX or Flag-tagged-spn4A were determined by western blot analysis using anti-p24, anti-v5 and anti-Flag antibodies respectively. Please note that in addition to bands corresponding to α1-PDX and Spn4A, we also detected the presence of several high molecular weight bands corresponding to typical trapped SDS-stable complexes consisting of the inhibitor and targeted-PC enzymes.



SUPPLEMENTAL FIGURE S2,2. Extracellular Vpr is associated with heparan sulfate proteoglycans. 293T cells were mock-transfected (lanes 1 and 7) or transfected with HXBruBH10.R- (lanes 2 and 8) or HxBruBH10.3HAR+ (lanes 3-6 and lane 9-12). Forty hours post-transfection, 293 T cells were washed 3 times with PBS, and treated with the indicated concentrations of heparin (dissolved in DMEM) 20 min at 37° C. After treatment, cells and extracellular virus-free medium were isolated, lysed, and analyzed for the presence of 3HA-Vpr either directly by western blot (left panel) or by IP/WB (right

Authors contribution in article 2: Direct Association of HIV-1 Vpr and Matrix (MA) Proteins in Virions: Implication for Early Events of HIV-1 Infection

Yong Xiao designed and developed the concept of the project, carried out the experiments (Figure 3.1, 3.2, 3.3, 3.6, 3.7), and wrote the manuscript.

Nicole Rougeau provided technique support in constructing mutant MA provirus with HA-Vpr and analyzed their interaction with HA-Vpr, her work is present in Figure 3.5.

Pierrre-Alexandre Bonicard did the experiment Figure 3.4 under my help.

Ghislaine Duisit participated in developing the concept of project and interpreting the results and drafting the manuscript.

Xiaojian Yao provided the R-/RI- provirus plasmid, and gave helpful discussion in the initial period of the project,

Eric A. Cohen supervised the whole study, participated in designing experiment ,interpreting the results and drafting the manuscript.

CHAPTER 3 (Article 2): Direct Association of HIV-1 Vpr and Matrix (MA) Proteins in Virions: Implication for Early Events of HIV-1 Infection

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Running title: HA tagged Vpr and MA interaction within HIV-1 virion particles

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Abstract

Vpr localization within the virion core and its association with the pre-integration complex suggest the protein plays an important role during HIV-1 early infection. However, little is known on Vpr interactions with other viral components within the virion particles. To address this question, we constructed an infectious molecular clone of HIV-1 expressing an HA-tagged Vpr. Protein analysis of immunoprecipitated HA-Vpr complexes present in purified virions revealed that Vpr could associate with the matrix protein (MA). Furthermore, MA-Vpr interaction was shown to occur independently of the presence of RT and IN and could be detected *in vitro* binding experiments, strongly suggesting the association was direct. The respective interacting domains were mapped by *in vitro* binding assays. We pointed out the implication of the fifth alpha helix of MA (residues 97-108), and the arginine-rich C-terminal domain of Vpr (residues 86-96) in the Vpr-MA interaction. Since Vpr and MA are karyophilic proteins, and are both components of the pre-integration complex (PIC), their interaction might have a synergic effect in the nuclear targeting of PIC and could contribute to the efficiency of viral infection during the early stages of HIV-1 infection.

Introduction

Human immunodeficiency virus type 1 can replicate in non-dividing cells such as macrophages, relying on the active transport of viral DNA into the nucleus of an infected cell (5). It is believed that multiple factors are involved in nuclear targeting of HIV-1 pre-integration complex (PIC) in non-dividing cells, such as matrix protein (MA), viral protein R (Vpr) and integrase protein (IN), as well as the DNA flap. HIV-1 MA and IN proteins are thought to recruit karyopherin alpha through their nuclear localization signal (NLS), Vpr may modulate the nuclear import by increasing the affinity of karyopherin alpha for the PIC (22). Although Vpr is dispensable for HIV-1 replication in dividing cells, it is required for efficient replication in non-dividing macrophages (12).

Vpr possesses karyophilic property when expressed alone (19). Vpr becomes membrane-associated in virus-producing cells (13) and is then incorporated into the nascent virions through its interaction with $Pr55^{GAG}$ p6 LXXLF domain (21) (18, 19) (16). Upon viral particle maturation, Vpr but not p6 localizes in the virion core (1), suggesting Vpr might interact with other viral or cellular factors for proper redistribution. However, nothing is known about the protein interactions occuring in the core and their subsequent role, if any, in the preintegration fate. Understanding of these processes will provide clues on the role of Vpr during the early stages of HIV-1 infection.

In order to facilitate the detection of virions-associated Vpr complexes, we first constructed an isogenic infectious HIV-1 molecular clone expressing HA-tagged Vpr. We found that Vpr was able to co-immunoprecipitate the matrix protein. Interestingly, this interaction occurred independently of the presence of RT and IN proteins and could be detected *in vitro* with recombinant proteins, suggesting a direct MA/Vpr association. GST pull-down assays further demonstrated that the fifth alpha helix of MA and the C-terminal domain of Vpr mediate this association. Involvement of the arginin-rich domain of Vpr in the interaction was finally

confirmed *in vivo*. Implication of virion-associated MA/Vpr complexes in the early steps of infection will be discussed.

Materials & Methods

Plasmids and provirus constructs. Mammalian expression plasmid SVCMV HA-Vpr was constructed by one-step PCR insertion of a hemagglutinin (HA) tag at the N-terminus of Vpr in SVCMV Vpr (28). The SpeI/SacI digested PCR fragment was inserted into the corresponding Xba I/Sac I site of SVCMV Vpr. SVCMV HA-Vpr (1-86) and Vpr (1-78) were constructed by ligating an EcoRI/SacI PCR fragment containing premature stop codons into the same sites of SVCMV HA-Vpr.

For bacterial production of recombinant MA, matrix sequence was first amplified from HxBruBH10.R+ provirus. The pGEX-4T3-MA plasmid was constructed by ligation the digested PCR fragment into the corresponding sites in pGEX-4T3 plasmid (Amersham, Buckinghamshire). Similarly, deletion mutants of MA (GST-MA (1-96), GST-MA(1-108) and GST-MA (1-116)) were made by PCR using different 3' MA primers with premature stop codons, and cloned in pGEX-4T3.

For *in vitro* expression of wild-type and truncated T7-tagged Vpr, pET21C-Vpr and its deletion derivatives were constructed by PCR amplification of Vpr from HxBruBH10.R+ provirus. PCR fragments digested with BamHI/SacI were subcloned in frame into the corresponding sites in pET21C (Novagen).

To introduce HA-Vpr sequence into the provirus, the intermediate plasmid BSSK-HAR+/Apal-SalI was first generated by a two-steps PCR-based method. HA-Vpr was first generated as above, the HA-Vpr sequence linked to the upstream Vif sequence by overlapping PCR and cloned in BSSK plasmid. All PCR fragments have been sequenced. HxBruBH10.HAR+ provirus was finally constructed by replacing the HxBruBH10.R+ ApaI-SalI

fragment with that of BSSK HAR+ Bru /ApaI-SalI. pNL4.3 HAR+ was constructed using a similar approach. In these HA-Vpr provirus constructs, the Vif C-terminal reading frame has an additional 10 amino acids CTHTMFQITL insertion following Vif W174 amino acid position because of HA tag insertion at the C-terminal of Vif which is overlapping with the N-terminus of Vpr. The provirus HxBruBH10.R-/RI- without Vpr, RT and IN expression was described before (2).

The provirus constructs pNL43 K98G and pNL43 E106V containing MA mutation K98G and E106V were kindly provided by Dr Eric Freed, NIH (7). A NL43 provirus containing a L101A mutation in MA was constructed by PCR-mediated site-directed mutagenesis followed by the insertion of the mutant sequence into the BssHII/SphI sites of pNL4.3. pNL43 K98G, pNL43 L101A and pNL43 E106V containing HA-Vpr were constructed by replacing the ApaI/ Sal I fragment containing the native Vpr sequence with the same fragment from pNL43 HAR+. All the constructed plasmids were sequenced to confirm the presence of the desired mutations.

Cell lines and reagents. Human embryonic kidney 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Jurkat and MT4 T-lymphoid cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS). The HIV-1- positive human serum 162 used in this study were previously described (27). The anti-HA antibody was generated from the ascite fluid of mice injected with the anti-HA hybridoma 12CA5. Monoclonal Anti-HIV capsid (p24) protein monoclonal antibodies were harvested from the supernatant of hybridoma HB9725 (ATCC; American Type Culture Collection, Manassas, VA). Polyclonal rabbit antiserum to HIV-1 p17 from Dr Michael Phelan was obtained through the NIH AIDS research and reference reagent program and the monoclonal antibody for HIV-1 p17 was from the supernatant of hybridoma HB9875 from ATCC.

HIV production and immuno-precipitation. Transfection in 293T cells was performed by a standard calcium phosphate DNA precipitation method. 5×10^6 cells were transfected with 20 µg

of provirus and 10 μg of SVCMV-HA-Vpr expressor. At 40 hours post-transfection, cell supernatants were collected, clarified through 0.45 μm-filters (Nalgene), and ultra centrifuged at 35,000 rpm in a Beckman 70 T1 rotor for 1.5 hour to pellet HIV virions. Each viral pellet was resuspended in 800 μl RPMI 1640 medium, lyzed with 200 μl of 5 % NP40 lysis buffer (700mM NaCl, 40mM $NaHPO_4$, 10mM $Na_2H_2PO_4$, 5% NP40, 2.5% SDS, pH7.2) with protease inhibitor cocktail (Roche) and then 90% of viral lysates were immmuno-precipitated with anti-HA antibody (2 hours, at 4 degrees). The remaining 10% of viral lysate was kept for direct Western blot analysis.

Silver staining and Mass spectrometry. IPs were done using the anti-HA 12CA5 antibody. Purified products were separated by 12.5% SDS-PAGE and visualized by silver staining using ProteoSilverTM kit (Sigma). Bands of interest were excised from the gels, washed twice with 50% acetonitrile in water and analyzed by mass spectrometry at Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

Purification of GST-fusion proteins. E. Coli BL21 transformed with pGEX-4T3 MA or derivaties. Expression of GST-fused proteins was induced for 4 hours at 30°C with 0.1 mM isopropyl-β-D thiogalactopyranoside. Recombinant proteins were purified as described in (14).

In vitro translation. Radiolabeled Vpr was synthesized *in vitro* using the TNT-coupled reticulocyte lysate system (Promega, Madison), according to the manufacturer's instructions. Briefly, 1 μg of plasmid DNA was used for transcription/translation in a 50μl-reaction mix in presence of ^{35}S-methionine.

In vitro GST-pull down assays. 30 μl of 50% gluthatione-coupled sepharose 4B slurry were incubated with 5μg of GST-MA in 800μl of PBS pH 7.4 at 4°C for 40 minutes. Beads were washed 3 times with PBS. Five μl of *in vitro*-translated T7-Vpr product in RPMI1640 medium (800μl/sample) were added to the GST- or GST-MA-bound beads and shaken at 4°C for 2 hours. After vigorous washing with PBS, proteins were eluted with 50mM reduced glutathione and

separated by15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed and exposed on films or immunoblotted for detection of bound Vpr.

HIV-1 replication analysis. 5 x 10^6 Jurkat T cells were transfected with 10 μg of proviral DNA plasmids using the DEAE-dextran method (8). Following transfection, the Jurkat T cells were cultured at a density of 0.5 x 10^6 cells/ml. Virus levels in the supernatants at specific time points after transfection were determined by the HIV reverse transcriptase (RT) assay, as previously described (27).

Results

Insertion of a N-terminal HA tag in HIV-1 Vpr. It has been reported that Vpr, IN and phosphorylated MA all localize within viral core. However, the putative interactions occurring between these three karyophilic proteins have not been studied yet. One of the major obstacles to the characterization of Vpr-interacting proteins is the lack of strongly reactive antibodies. We then constructed a HIV-1proviral clone expressing an HA-tagged Vpr (Fig.3.1A). We first ensured the presence of the N-terminal tag did not alter Vpr properties, in terms of protein stability, virus incorporation, cellular distribution and cytostatic abilities (data not shown). Lastly, we investigated the impact of the HA tag on viral replication. MT4 cells were transfected with the proviruses either mutated for Vpr initial coden (ATG to GTG), or expressing the wild-type or HA-tagged proteins (HxBruBH10.R-, HxBruBH10.R+, and HxBruBH10.HAR+ respectively). The viral replication was monitored by quantification of RT activity from the culture supernatants. As shown in Fig.3.1B, all the three viruses replicated well, indicating the introduction of a small foreign HA tag at the N-terminus of Vpr did not interfere with viral replication.

Identification of virion-associated Vpr-complexes. First, we used HIV particles produced from 293T cells cotransfected with Vpr-minus provirus and HA-Vpr expressor. Trans-complementation of HA-Vpr was sought to increase the amount of Vpr incorporated inside virions (24). Released particles were concentrated by ultracentrifugation, lyzed and subjected to immunoprecipitation (IP) using anti-HA antibody. IP products were then separated by 12.5% SDS-PAGE gel, fixed and silver stained. Interestingly, a 18kDa protein was co-immunoprecipitated along with HA-Vpr from the viral lysate containing HA-Vpr (Fig. 3.2A, lane 3), but not from the Vpr-negative viral lysate (lane 2). The 18kDa protein band was cut from the gel and analyzed by mass spectrometry. Three peptide sequences were obtained from the mass spectrometric analysis (Fig. 3.2B). They were corresponding to HIV-1 Matrix protein (MA).

Vpr interacts with MA in HIV-1 particles. To exclude the possibility of an artificial interaction between MA and over expressed tagged Vpr, IPs were performed on virions recovered from 293T cells transfected with Vpr-minus provirus as negative control, or with HxBruBH10.HAR+ provirus, that expressed HA-Vpr in *cis.* Aliquots of the virus lysates were subjected to IP with anti-HIV antiserum, to ensure similar amounts of particles had been concentrated before lysis (Fig. 3.3A, right panel) The rest of the samples were used to pull-down HA-Vpr (left panel). Purified proteins were detected by western blot with specific antibodies against HIV-1 p24 capsid, MA and HA-Vpr. As shown in Fig. 3.3A, right panel, similar amounts of p24 and MA were found when IPs were performed with HIV antiserum, indicating the transfected 293T cells had released equivalent number of particles. In contrast, MA but not the capsid p24 protein could co-immunoprecipitated with HA-Vpr (Fig.3.3A, lane 3), suggesting the binding was specific. Noteworthy, no immunoprecipitation of the matrix could be found in absence of HA-Vpr. Taken together, these results provide evidence that Vpr specifically interacts with MA in virion particles from transfected 293T cells.

Importantly, similar results were obtained with viruses produced by HIV-infected MT-4 cells. MA protein could co-immunoprecipitate with HA-Vpr in virion lysates prepared from MT4 cells infected with HxBruBH10.HAR+ (Fig.3.3B, lane 3) but not HxBruBH10.R- viruses (Fig. 3.3B, lane 2). These results suggest that Vpr and MA are able to interact under the normal physiological conditions in virions produced from HIV-1 infected T-cells. However, the thinness of the band (lanes 3) as opposed to the huge band detected for the inputs (lanes 6) tends to indicate the amount of matrix present in Vpr complexes is weak, but may reflect the small quantity of MA that is actually located inside the core.

The presence of IN and RT is dispensable for the formation of the complexes. In addition to Vpr and phosphorylated MA, mature core particles contain RT and IN.

To determine whether these proteins could also participate to the complex, similar IP assays were performed using a RT/IN/Vpr-deleted provirus Tran complemented or not with HA-

Vpr. Results show that MA could still be co-immunoprecipitated with HA-Vpr in RT/IN-negative viral particles (Fig. 3.3C, lane 3), although to a lesser extent than that of wild type viruses (Fig.3.3C, lane 5 and 6), suggesting that the Vpr and MA interaction was independent of the presence of RT and integrase, but that these proteins might contribute to the stability of this interaction.

Matrix 5th α-Helix is critical for Vpr interaction. To determine whether Vpr and MA interact directly, we used a GST pull-down assay with bacterially produced GST-MA and *in vitro* translated T7-Vpr. We found that GST-MA but not GST alone was able to interact with T7-Vpr (Fig. 3.4B, lane 5) suggesting that Vpr and MA have a direct physical interaction. Moreover, these results confirm that the observed interaction between MA and Vpr was not mediated by the HA tag. Finally, recombinant Vpr could still interact *in vitro* with GST-MA even in the presence of DNase and RNase indicating that this interaction might not mediated by DNA or RNA (data not shown).

In order to map the MA region responsible for Vpr binding, GST-MA (1-96), GST-MA (1-108), and GST-MA (1-118) deletion mutants were produced in bacteria and purified (Fig. 3.4A). We found that GST-MA (1-118) (lane 8) and (1-108) (lane7) were able interact with Vpr as well as wild-type GST-MA (lane 5), whereas the GST-MA (1-96) mutant (lane 6) almost completely lost its ability to bind to Vpr (Fig.3.4B). Amino acids 97-109 of MA corresponds to the fifth α–helix, thus suggesting that this structure might contain the minimal sequence required for Vpr interaction. Several MA mutations (K98G 101A, and E106V) were then introduced into pNL4.3HAR+ provirus and their effects on the Vpr-MA interaction were analyzed in the viral particles (Fig. 3.5A). All the MA mutants tested could bind Vpr at least as efficiently as the wild type matrix (lane 3, 4 and 5). Surprisingly, virus expressing MA L101A exhibited a reduced particle release compared to wild type HIV (lane 10 compared to lane 12), as determined by capsid and MA protein levels in the viral lysate, even though the HA-Vpr and MA L101A

interaction was 14 time stronger than that of wild type MA (Fig. 3.5B, compare pNL4.3 HAR+ L101A with pNL4.3 HAR+). Since the MA 5th α-Helix seems to be involved in the formation of Vpr complexe, other residue(s) within that domain are likely implicated.

Vpr amino acids 86-96 are responsible for MA binding. To further map the region of Vpr responsible for the interaction, a series of Vpr deletion mutants were produced by *in vitro* translation and used for GST pull-down experiments. As shown in Fig. 3.6B. GST-MA was able to interact with full-length Vpr. In contrast, the truncated forms Vpr (1-86) and Vpr(1-78) exhibited similar 50-75% reduction in MA association (lane 5 and 6), suggesting the 86-96 region contains the main binding domain. Further deletion 63-96 completely abolished the interaction. That region appears to participate in the formation of MA complexes, somewhat at lesser extent than the C-terminal part.

Finally, to confirm the involvement of the 86-99 region, HA-Vpr(1-78) and HA-Vpr(1-86) were used to trans complement Vpr-minus provirus in 293T cells. Virions were purified, lyzed and subjected to IP as above. Results presented in Fig.3.7 show that amounts of MA pulled-down by HA-Vpr(1-78) and HA-Vpr(1-86) are both reduced, when compared to that obtained with wild-type protein (compare lanes 3-4 to lane 5). The weaker intensity of the MA band observed for HA-Vpr(1-86) is likely due to a small difference in MA content in the lysates (compare lanes 9 and 10), rather than to distinct affinities of these two Vpr mutants for the matrix.

Discussion

HIV-1 can replicate in non-dividing cells such as macrophages, a process which relies on the active transport of viral DNA into the nucleus of infected cells.

Vpr is packaged into the virions via an interaction with the p6 domain of $Pr55^{GAG}$ precursor (16, 17, 21) and further localized within the viral core (1, 26). After virus entry in host cells, Vpr becomes part of the PIC, suggesting the protein plays a role during the early stages of HIV-1 infection.

We seek to identify Vpr-interacting proteins within the viral particles. Epitope tagging strategy provided a powerful tool to bypass the lack of sensitive anti-Vpr antibodies. HA was fused to the N-terminal part of Vpr, without altering neither the protein stability nor its incorporation into nascent virions. Vpr tagging also had a minor impact on the virus replication. Mass spectrometry analysis of immunopurified Vpr-associated complexes pointed out that Vpr present in the virions was able to interact with HIV MA protein. Importantly, MA binding was observed in particles produced from transfected 293T cells as well as from HIV-infected MT4 cells. Our result is consistent with the interaction previously reported by Sato *et al.*, with distinct IP conditions (23). Since MA appears to play important roles in the nuclear transport of the proviral DNA in non-dividing cells as part of the PIC (4), we further characterized Vpr/MA interaction.

We showed here that Vpr is able to interact with MA in absence of incorporated RT and integrase. Results obtained from *in vitro* GST pull-down strongly suggest the interaction is direct. However, we found that HA-Vpr is also able to pull-down the integrase in virus lysates (data not shown). Since Vpr affinity for MA seems to be decreased in absence of IN, we cannot rule out the possible involvement of integrase in the stabilization of the complex.

By yeast two-hybrid assays, Sato *et al.* have mapped Vpr-binding domain in the C-terminal region of the matrix protein (23). We further demonstrated by *in vitro* GST pull-down that the region encompasses amino acids 97 to 107, which corresponds to the fifth alpha helix α-H5. We then constructed proviruses expressing both HA-Vpr and MA variants K98G, L101A or E106V. Unfortunately, introduction of point mutations had no impact on the complex formation, as determined by IP on the virus lysates. Although the residues targeted by mutagenesis are well-conserved among HIV clades A to D (data not shown), they are dispensable for Vpr binding. Other amino acids in the ^{96}DTKEALDKIEEEQ^{108} should be considered. In regard of MA α-H5 structure and the binding domain we mapped in Vpr (see below), implication of the likely-exposed residues E99, D102 and E105 will be evaluated.

Using *in vitro* binding assays, we demonstrated that MA interacting region of Vpr spans the C-terminal domain 86-96. Deletion of this region decreased matrix binding to 50-75%. That domain shows no evident 3-D structure but it contains several arginine residues. It is thus tempting to speculate an electrostatic interaction between the negatively-charged amino acids of MA α-H5 and the basic C-terminal tail of Vpr. Deletion of the 78-96 had a weak impact, suggesting the phosphorylation site S79 plays a minor role, if any. Importantly, IP performed on viruses expressing HA-Vpr(1-86) or (1-78) forms confirmed the reduced ability of these truncated proteins to interact with the matrix *in vivo*. Finally, the larger C-terminal deletion 63-96 resulted in a complete loss of MA binding. This region is composed of a leucine-rich domain which is involved in the interaction with many cellular proteins (15, 25, 29). That region might form a hydrophobic interphase which could facilitate Vpr/MA interaction. Alternatively, the large deletion Vpr (1-63) may alter the 3-D structure of the protein, as suggested by the high instability of this mutant *in vivo* (data not shown). Precise mapping of the C-terminal domain of Vpr is actually in progress.

Both Vpr and MA are present in the viral core (1, 9, 10) and become components of the preintegration complex following HIV entry into the host cells (6, 12, 20). Thus, one can imagine the interaction between the two proteins plays a role during the early stages of infection. Unfortunately, introduction of a premature TGA stop after Vpr codon 86 appeared to be detrimental for HIV replication preventing us from studying the role of this binding (data not shown). Does one protein recruit the other to the core? Matrix protein mainly forms the outer shell of the viral core (11), but it can also relocate into the viral core, presumably upon additional phosphorylations on MA serine residues (3). Given that bacterially-produced MA is not phosphorylated, our *in vitro* data suggest that Vpr may interact with the matrix proteins present in the virions regardless of their phosphorylation status. However, amount of MA that is effectively pulled-down in virions lysates is weak, as if only a fraction of the protein was susceptible for the IP. It is possible that Vpr interacts with MA only once both proteins have been targeted independently to the core. On the other hand, we cannot rule out the possibility that Vpr might recruit (or be recruited by) the matrix protein near the virus membrane, its interaction with MA occurring concomitantly with the MA phosphorylation of the serine residues. So far, Vpr ability to bind to MA in immature particles, or outside the cores has not been evaluated. Analysis of the core composition of viruses expressing truncated MA and Vpr mutants defective for the binding will be of great interest. It will help in understanding where the interaction takes place and its impact in the recruitment of the diverse components of the preintegration complex, leading to some clues for the comprehension of the PIC fate.

In summary we constructed an infectious molecular clone of HIV-1 expressing an HA tagged Vpr and isolated purified virions containing HA-Vpr. Immunoprecipitation assays revealed that MA and Vpr could form a complex within the virions. Determining whether the binding occurs in the core particles should help us in understanding the respective role of the PIC components during the early stages of HIV-1 infection.

ACKNOWLEDGMENTS

We thank Belzile Jean-Philippe and Alexandre Caillaud for the help in reviewing the manuscript. Thank Dr Eric Freed, NIH for providing provirus constructs pNL43 K98G and pNL43 E106V. Antiserum to HIV-1 p17 from Dr Michael Phelan was obtained through the NIH AIDS research and reference reagent program. EAC is the recipient of the Canada Research Chairs in Human Retrovirology. This work was supported by grants from the Canadian Institute of Health Research to EAC from the Fonds de la Recherche en Santé du Québec - AIDS network to EAC.

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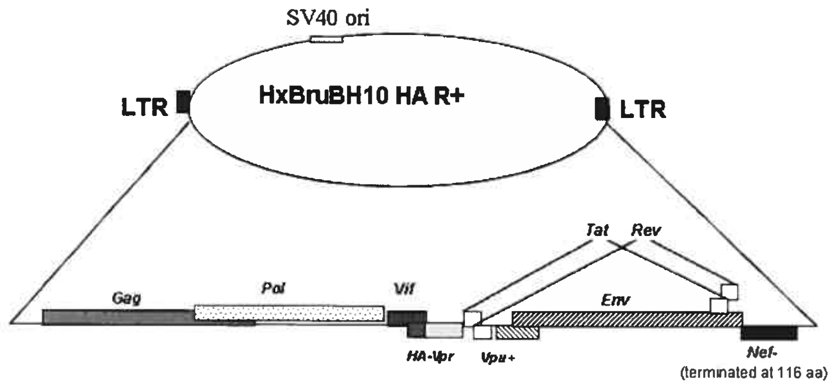
Figures legends

A



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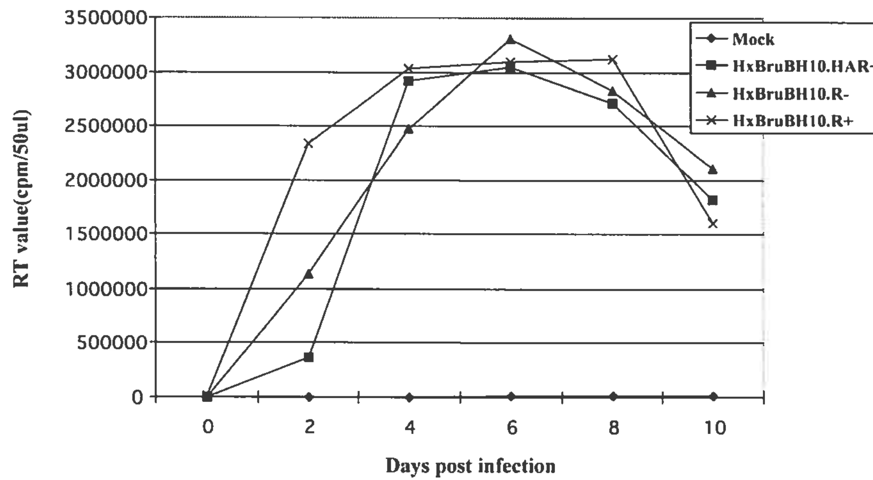


Figure 3.1.Construction of isogenic provirus expressing HA-tagged Vpr *(A) Schematic map of isogenic provirus expressing HA-tagged Vpr.* (B) Kinetics of viral replication. MT4 cells were transfected with HIV-1 proviruses. Levels of RT activity present in culture supernatants were determined for 10 days. Representative experiment (n=2).

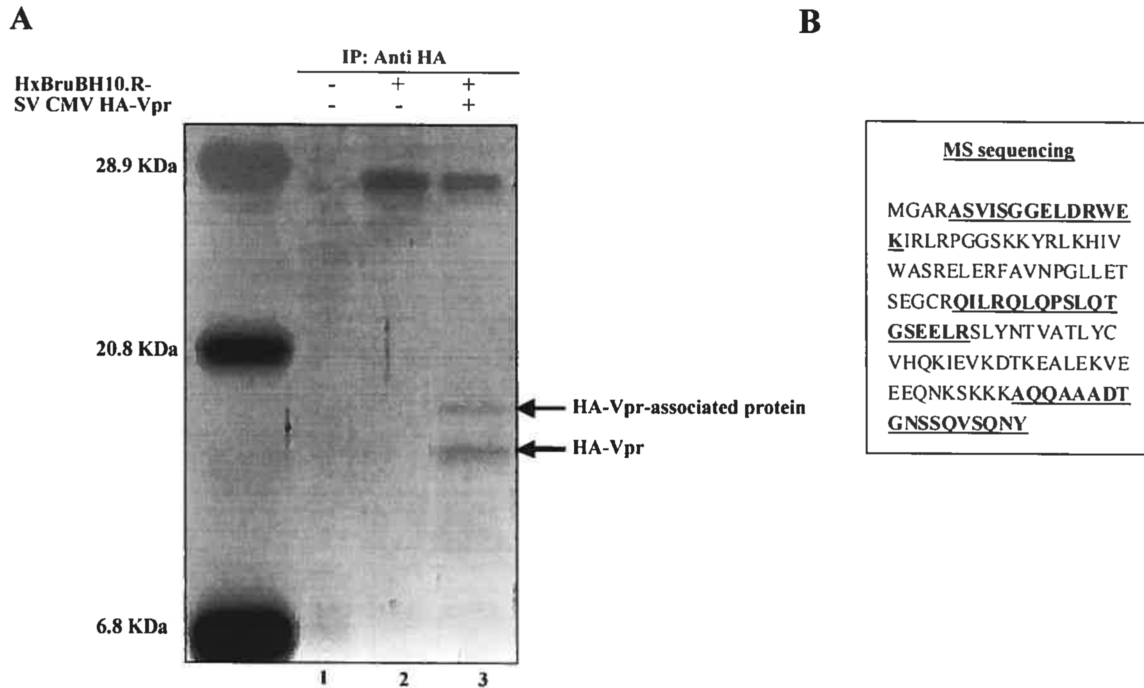
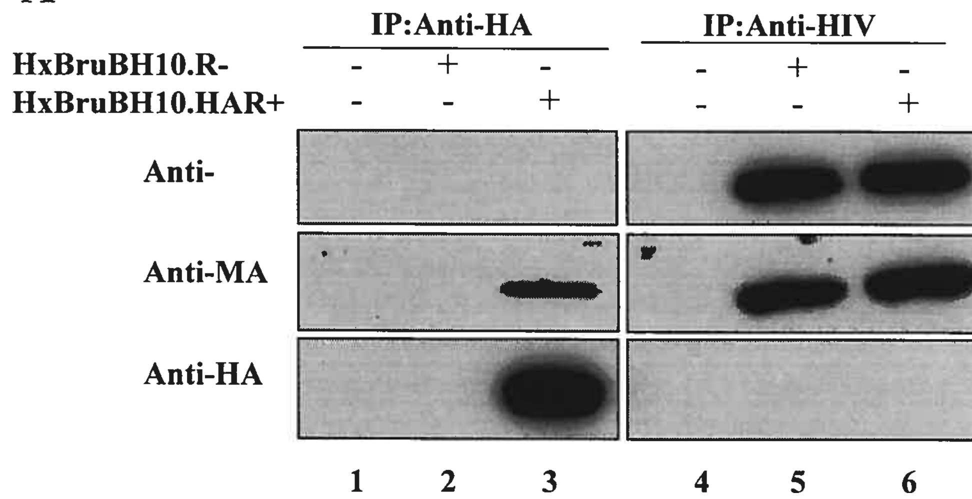


Figure 3.2. Detection of Vpr interacting proteins within virion particles. (A) Immunoprecipitation of Vpr complexes. Culture supernatants from mock 293T cells (lane 1) or cells co-transfected with HXBruBH10.R- provirus (lane 2) or HXBruBH10.R- and SV CMV HA-Vpr (lane 3) were concentrated by ultracentrifugation. Viral pellets were resuspended in lysis buffer and were subjected to Vpr immunoprecipitation using anti-HA antibody. Co-immunoprecipitated proteins were separated by SDS-PAGE and revealed by silver staining. (B) Mass spectrum analysis of the 18 kDa protein. Peptides identified by mass spectrometry analysis are underlined in the MA sequence.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IP:Anti-HIV
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Anti-
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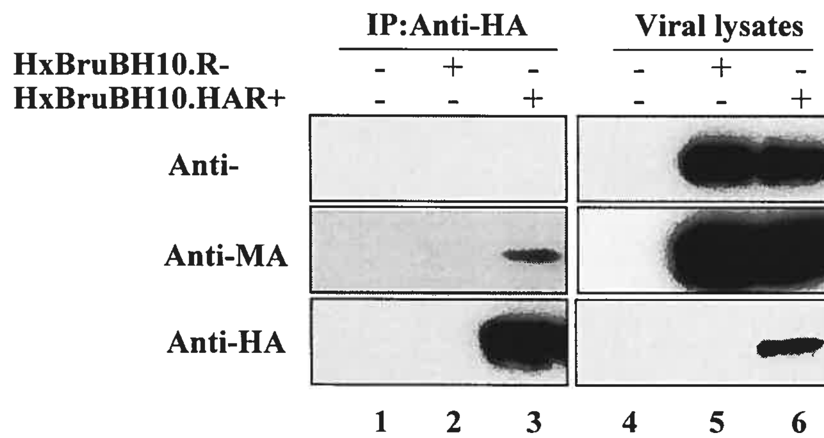
1 2 3 4 5 6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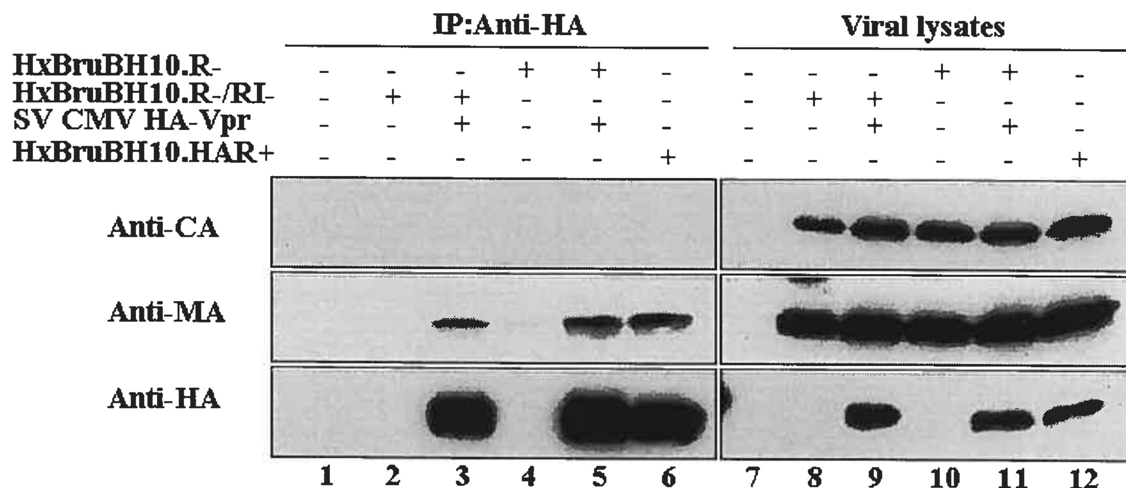


Figure 3.3. Vpr associates with p17 (MA) protein in HIV-1 virions. (A) Culture supernatants recovered from mock-transfected 293T cells (lane 1 and 4) or from cells transfected with HXBruBH10.R- (lane 2 and 5) or HXBruBH10.HAR+ proviruses (lanes 3 and 6) were concentrated by ultracentrifugation. Viral pellets were resuspended in lysis buffer and subjected to anti-HA (left panel) or anti-HIV immunoprecipitation (right panel). Presence of the capsid protein (p24), MA and HA-Vpr was determined by western blot following SDS-PAGE (B) Culture media recovered from uninfected MT4 cells (lane 1 and 3), or MT4 infected with HXBruBH10.R- (lane 2 and 4), or HXBruBH10 .HAR+ (lanes 3 and 6) were concentrated by ultracentrifugation. 90% of the resuspended pellets were used for anti-HA immunoprecipitation (left panel) whereas the remaining fraction (right panel) was analyzed directly by Western blot. (C) 293T cells were transfected with HXBruBH10.R- /RI- (lanes 2-3 and 8-9) or HXBruBH10.R- (lanes 4-5 and 10-11). Alone (lanes 2 and 8, 4 and 10) or complemented with SVCMV-HA-Vpr (lanes 3 and 9, 5 and11). As control, cells were mock-transfected (lane 1 and 7) or transfected with *cis* HA-Vpr- expressing provirus (lane 6 and 12). Culture media recovered 2 days later were concentrated by ultracentrifugation. 90% of the resuspended pellets were used for anti-HA immunoprecipitation whereas the remaining fraction was loaded directly on the acrylamide gel

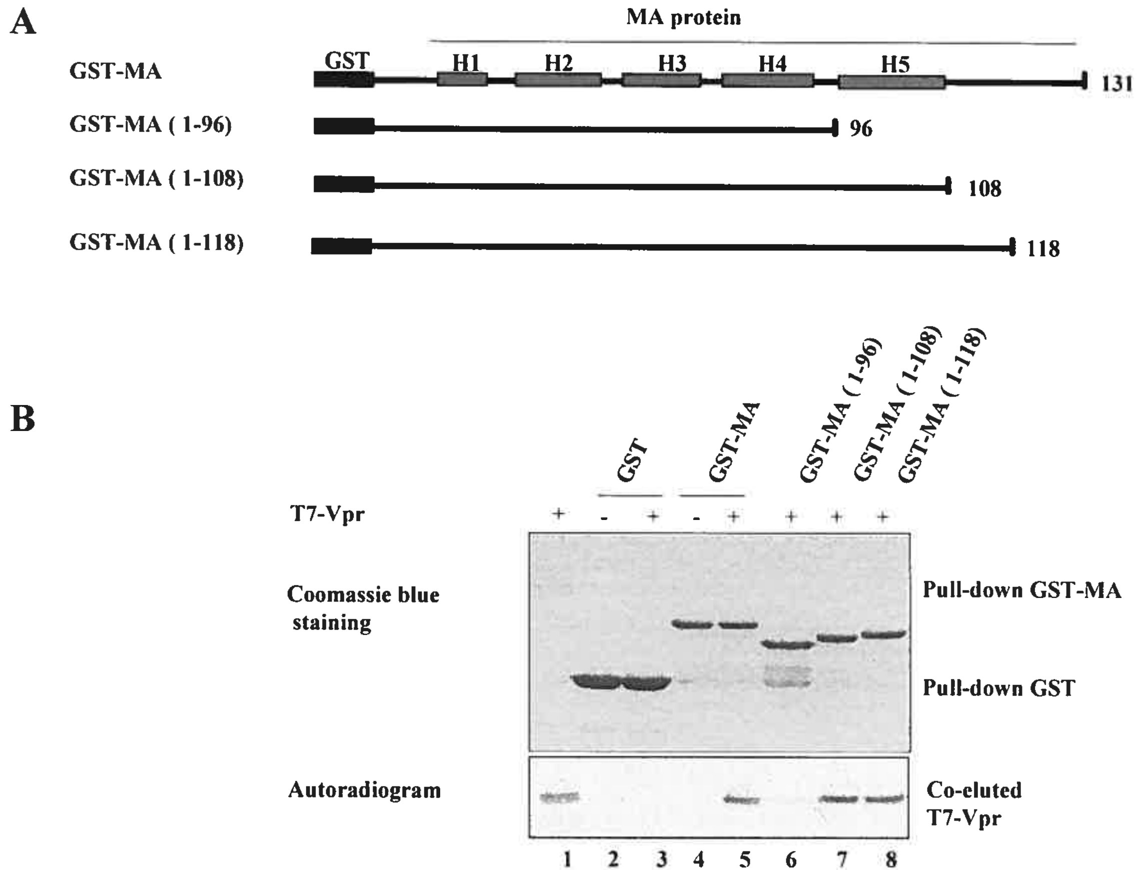
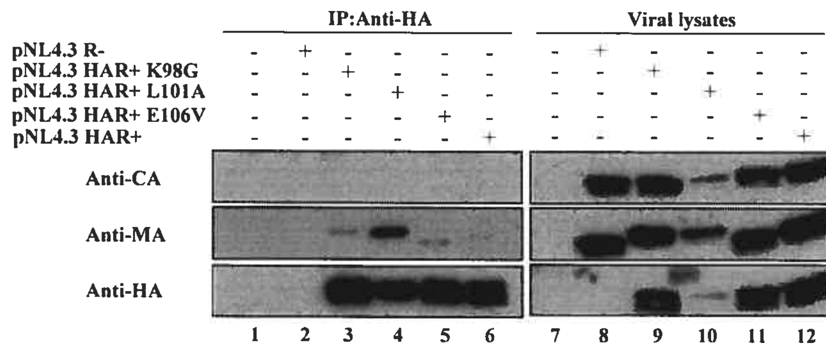


Figure 3.4. The 5th helix of MA protein is involved in the interaction with Vpr. (A) Diagram of GST-MA deletion mutants (B). *In vitro*-translated Vpr was incubated with wild type or truncated GST-fused MA and subjected to GST pull-down assay as described in materials and methods. Sample were loaded and run on 12.5% SDS-PAGE gels and detected by Coomassie blue staining and autoradiography.

A



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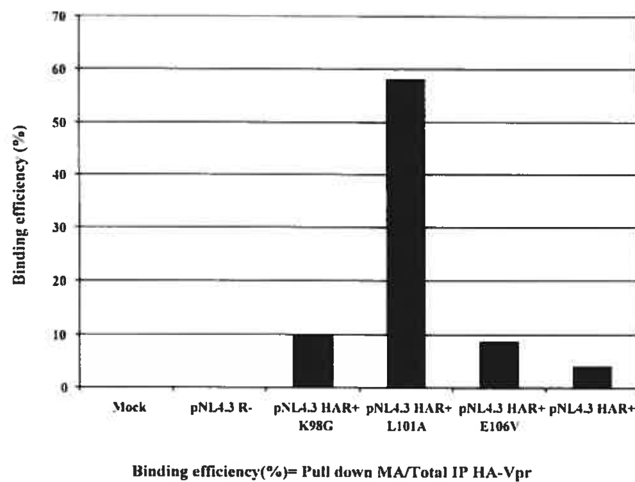
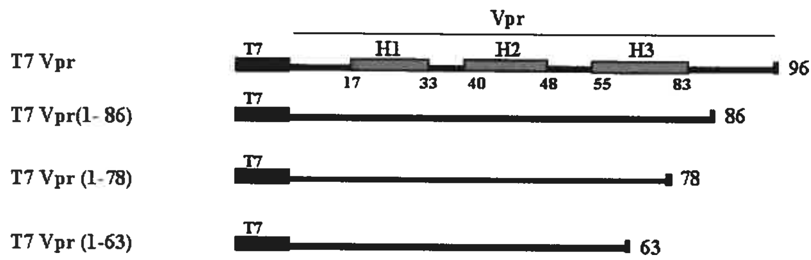


Figure 3.5. Effect of Matrix mutants on Vpr interaction. (A) Culture media recovered from mock transfected 293T cells (lane 1 and 7), or transfected with pNL4.3.R- (lane 2 and 8), pNL4.3.HAR+ K98G (lane 3 and 9), pNL4.3.HAR+ L101A (lane 4 and 10), pNL4.3.HAR+ E106V (lane 5 and 11), pNL4.3.HAR+ (lanes 6 and 12) were concentrated by ultracentrifugation. 90% of the resuspended pellet was used for anti-HA immunoprecipitation whereas the remaining fraction was analyzed directly by Western blot. (B) The binding efficiency of MA with HA-Vpr was determined by the quantification of the density of the band by densitometry analysis. Densitometric analysis of Western blot results was performed using AGFA Duoscan T1200 scanner and Image Quant 5.0 from Molecular Dynamics.

A



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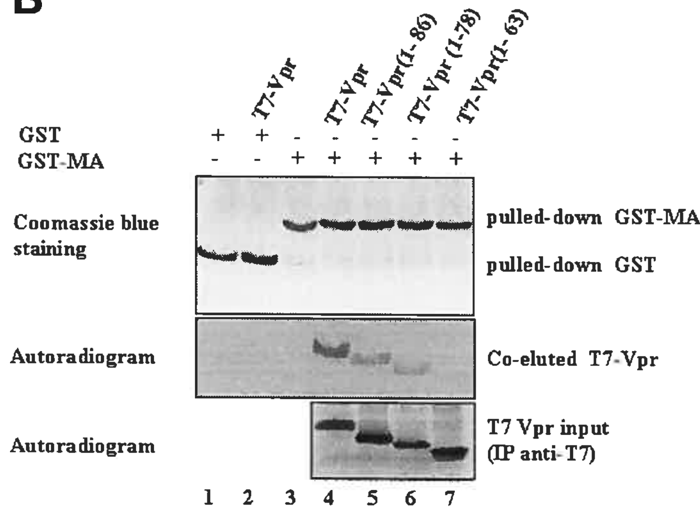

Figure 3.6. Direct interaction of Vpr with MA in GST pull-down assays. (A) Diagram of T7-Vpr deletion mutants. (B) *In vitro*-translated ^{35}S–labeled Vpr mutants were incubated with bacterially produced GST-MA and subjected to GST pull-down assays as described above.

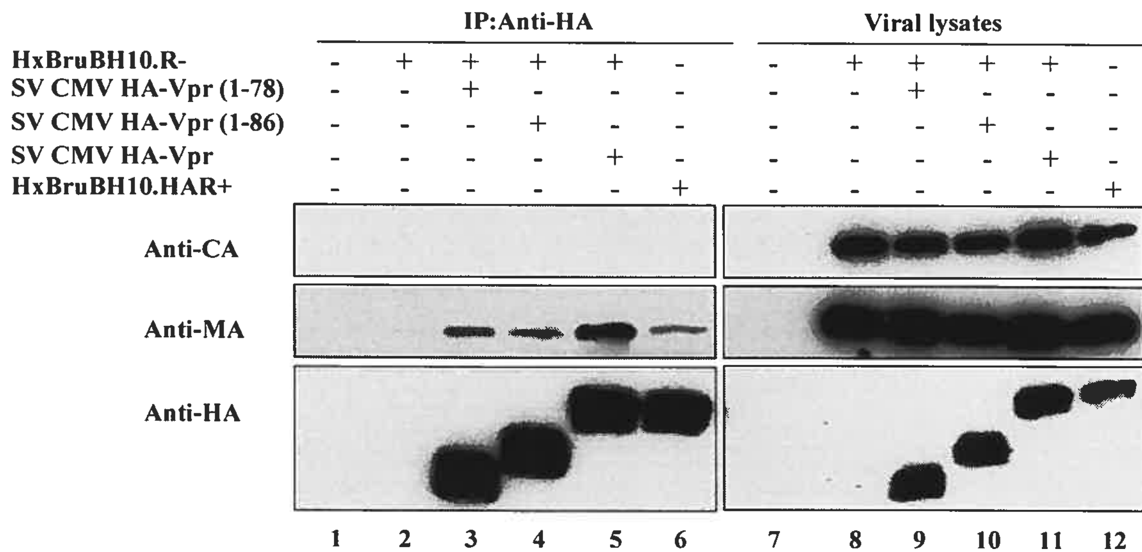

Figure 3.7. Effect of Vpr truncation on MA interaction. 293T cells were mock-transfected (lane 1 and 7), or transfected with HxBruBH10.R- (lanes 2-5 and 9-11) or HxBruBH10.HAR+ (lane 6 and 12). Proviruses were transcomplemented with HA-Vpr(1-78) (lane 3 and 9), HA-Vpr(1-86) (lane 4 and 10) or wild-type HA-Vpr (lane 5 and 11). Two days later, virions were collected, concentrated, lyzed and subjected to IP against HA as previously described.

CHAPTER 4: GENERAL DISCUSSION

Extracellular forms of Vpr have been previously found in the sera and cerebrospinal fluids of HIV-1 positive individuals (196). Extracellular Vpr can transduce the cell membrane (147, 325), suggesting that this protein may have a bystander cell effect. Specifically, it could penetrate bystander latently infected cells or non-infected cells to perform several biological activities, such as a stimulation of HIV replication in latently infected cells (197), an increase of chemokine and TNF-α production (241, 358), or a modulation of apoptosis in T cells (17). However, despite the numerous putative functions described for extracellular Vpr, the mechanisms underlying Vpr release, as well as its biological impact on HIV-1 replication, have never been addressed. In the present work, for the first time, it has been shown that the protein can be released and cleaved in the culture media of HIV-1-expressing cells, and further, characterized the mechanism of the release and processing.

This study shows that soluble Vpr is released from virus-producing cells by a process independent of Vpr incorporation into HIV-1 particles. Since HIV-1 Vpr does not possess a signal peptide for secretion and contains nonclassical NLS, the protein is mainly located in the nucleus when it is expressed alone (208). Most of the Vpr still stays in the nucleus even when the protein is fused with the baculovirus secretory polypeptide, suggesting that Vpr possesses a strong nucleophilic signal (54). Interestingly, the protein is redistributed to the cytoplasm and membrane compartment independent of its interaction with the p6 domain of Gag during viral infection (163). Vpr is released from HIV-1-producing cells but not from cells where Vpr is expressed alone (see Chapter 2), implying that other viral component or some host cell responses may be necessary for Vpr release. As shown in Chapter 2, Vpr can still be released and processed without virion incorporation in the absence of functional p6, suggesting that Vpr does not need to interact with p6 to be released out, and that extracellular forms of Vpr are not derived from decayed virion particles, since no Vpr will be virion-incorporated in the absence of p6. The

provirus HxBruBH10.3HAR+ used in the study does not have a functional nef gene, suggesting that nef does not play a role in releasing extracellular Vpr. Vpr can also be released in the absence of Vpu, Env, and protease protein (data not shown), suggesting that Nef, Vpu, protease, and Env may not contribute directly to the Vpr release during HIV-1 infection. The viral assembly and cytopathic effects resulting from HIV-1 infection—including plasma membrane disruptions due to viral egress (101) or the proapoptotic function of HIV gene products (133)—might be necessary for the redistribution and release of Vpr from the infected cell. Further efforts need to be made to clarify the viral components and activated cellular gene that are responsible for Vpr release.

In this study, we showed that soluble Vpr is predominantly cleaved shortly after extracellular Vpr releases. Extracellular Vpr and cleaved products can be found in HIV-1 patients' sera and cerebrospinal fluid (196), suggesting that Vpr release and proteolytic processing could be a physiological phenomenon during HIV-1 infection. It is unlikely that HIV-1 protease is responsible for Vpr processing, since virion-associated Vpr remains intact in the mature virion particles where HIV-1 protease is active. Deletion mapping analysis revealed that extracellular Vpr was processed at the C-terminus, where a pair basic amino acid proprotein convertase-processing motif $R_{85}QRR_{88}$ is located. Mutagenesis, mass spectrometry, and PC inhibitor experiments strongly suggest that Vpr is cleaved by proprotein convertase (see Chapter 2). Site-directed mutagenesis of this putative PC processing site reveals that double mutations of the conserved basic arginine residues located at the P1 and P2 position for alanine (Vpr RR87/88AA) almost completely abolished Vpr processing, whereas substitution of the less conserved arginine residue for a glutamine (R85Q) at the P4 position attenuated Vpr processing.

The P4 position mutation Vpr R85A has a G2 arrest function (81), which may explain why HIV-1 can tolerate mutation at this site *in vivo*. HIV-1 often has another arginine insertion at position P2' to compensate for the P4 arginine mutation such as HIV-1 YU-2 and SF2 strains

(Gene bank accession number M93258, K02007) and other virus isolates (from QQRRARN to QQRRARRN) (47). This insertion creates another PC processing motif RARR, suggesting that P4 position arginine may be required for efficient Vpr processing *in vivo*. Eventually, HIV-1-infected patients with the restored Vpr processing motif died of AIDS. Yet interestingly, the Cali *et al.* report indicates that the additional Vpr glutamine to proline mutation in the P3 position in this virus—with the restored PC processing motif (from QQRRARRN to QPRRARRN)—correlates with non-symptomatic the HIV-1 patient while no other genes mutations are found in the viral genome (GenBank accession number A4779550-A4779564) (47). Many long term non-progressors (LTNP), as well as mother to child non-transmitter, carry the virus strain that has the Vpr mutation at the P4 position (47, 330, 367, 388). Since the Vpr processing site overlaps with the arginine rich C-terminus, which is important for Vpr stability and the G2 arrest function, it is not clear whether Vpr lost its basic function due to the mutation near the PC processing site or whether the Vpr processing defect itself causes the different *in vivo* phenotype. The exact consequence of Vpr proteolytic processing and its effect on HIV-1 pathogenesis *in vivo* need to be determined.

Among the seven members of the pair-basic proprotein convertases, furin, PC7, and PC5A are expressed in freshly isolated human CD4 T-lymphocytes, the natural host cells of HIV-1, although PC5A expression level is lower than that of furin (only 20% of furin) in activated T cells (75). Notably, these particular PCs were implicated in the intracellular proteolytic processing of the HIV-1 envelope gp160 during HIV-1 infection (75). Even though PACE4 is not expressed in peripheral blood lymphocytes (PBL), it is highly expressed in lymphatic tissues such as thymus, lymph node, and spleen (140). The transient co-expression of PCs and provirus showed that PC5A and PACE4 efficiently processed extracellular Vpr in 293T, HeLa, and Cos-1 cell lines, whereas furin only had a marginal effect in the 293T and Cos-1 cell lines. The presence of a hydrophobic Val just following the cleavage site (for example as seen in HIV-1 isolates A, F, G, H) at P1' is especially relevant and indicates either a PC5 or PACE4-

generated Vpr cleavage, because these enzymes can tolerate an aliphatic residue at P1' just following the cleavage site, whereas furin does not (316). These results suggest that at least PC5A and PACE4 might be involved in the extracellular Vpr processing. As it is the case for HIV-1 envelope gp160 processing, other members of the pair-basic PC family or other cellular proteases may also contribute to the processing of extracellular Vpr, especially for those viruses harboring variants of the Vpr sequence at the P1' position replaced by either Ala or Gly (see Chapter 2) (316). Usually, several PCs are expressed in the same cell, and it will be difficult to knock down all of them to underline the individual role of each in the proteolytic processing of Vpr. The redundancy in the convertase might be useful to ensure the proper processing of Vpr in different host cells.

The predominant presence of Vpr -leaved products in the extracellular medium suggests that Vpr is cleaved extracellularly. Time course experiment results showed that Vpr was first released as a full-length protein and then was processed extracellularly by plasma membrane-associated PCs. Little cleavage was detected when Vpr was exposed to a conditioned medium from normal 293T cells, further implying that: 1) the PC responsible for Vpr processing is not released in the extracellular milieu, and 2) Vpr cleavage is more efficient when extracellular Vpr is in close contact to cells. Although soluble forms of PCs in the extracellular medium have been found from culture supernatant of PC-transfected cells (347, 381), their biological functions remain unknown (245). Vpr is not processed inside the cell; one possible reason is that Vpr may not have access to the secretory machinery where PCs are located.

The proprotein convertase furin and PC7 have a transmembrane domain that may lead to their possible presence at the plasma membrane (316). Indeed, furin cycles between the cell surface and the trans-Golgi network (TGN) (229). The presence of a consensus integrin-binding site (RGD) in PCs (except PC7) further suggests that these enzymes might associate directly with extracellular matrix (ECM) components (245). Secreted PC5A and PACE4, but not soluble furin, bind heparin within the extracellular matrix *via* a cationic stretch of amino acids within

their cysteine-rich (CRD) domain (252, 347). The observation of the present study that Vpr protein needs to be in close proximity with cells to be efficiently processed suggests that cell surface- or extracellular matrix–associated PCs such as PC5A and PACE4 may be responsible for Vpr processing. Indeed, the study found that PC5A and PACE4 expression can efficiently process extracellular Vpr in different cell lines.

Early study showed that Vpr localizes at the plasma membrane and at the surface of intracellular vacuoles during HIV-1 infection (368), suggesting that Vpr could possibly localize at the plasma membrane during HIV-1 infection. Cleaved Vpr is associated with the cell membrane as confirmed by trypsin digestion experiments. Moreover, transient over-expression of PC5A and PACE4 in the provirus transfected 293T, Hela, and Cos-1 cells dramatically increased Vpr processing and consequently cell-associated cleaved Vpr (see Chapter 2). It is interesting to note that extracellular Tat associated with heparin sulfate proteoglycans in the extracellular matrix is protected from protease degradation (52) but also as a step prior to its internalization into cell (350). Cell surface-associated Tat can also modulate HIV-1 infection and spreading by a specific interaction with the gp120 viral envelope protein (218). Interestingly, the data from our study also suggests that extracellular Vpr is also associated with the cell surface heparin sulfate proteoglycans. Further experiments need to be performed to determine the role of Vpr and heparin sulfate proteoglycans interaction during HIV-1 infection. It will be interesting to identify the viral or cellular factor(s) and the structural motifs that mediate the association of cleaved Vpr with the cell membrane, and to determine how this association relates to the function of Vpr during HIV-1 infection, primarily in term of the transduction capacity of Vpr.

Extracellular transducing Vpr can be functional similarly to the intracellular expressed Vpr. Addition of the recombinant protein enhances HIV-1 replication in leukemia T cells, PBMCs, or latently-infected cells (197) and is sufficient to rescue the replication of Vpr-defective virus in macrophages (325). Such an effect may be related to the abilities of synthetic Vpr to alter the production of pro-inflammatory cytokines and β-chemokines, at least in part by

inhibiting the NF-κB pathway (17, 241, 285). Extracellular Vpr still causes G2 cell-cycle arrest (325) and apoptosis (188, 270). Hence, release of the biologically-active protein may be crucial for the progression of the disease. Since full-length and C-terminal Vpr peptides cause membrane permeabilization and apoptosis of a wide range of cells, soluble Vpr may contribute to the killing of bystander cells which is observed in HIV-infected tissues (reviewed in (18)).

Protein transduction relies on the presence of an arginine-rich transduction domain (PTD) (325). However, the Vpr C-terminal domain $R_{85}QRRAR_{90}$ that resembles the PTD present in other transducing proteins eventually becomes $R_{85}Q_{86}$ after being processed by extracellular convertase and carboxypeptidase. Therefore, it is unlikely that cleaved Vpr is still able to transduce the cell membrane through its PTD domain. The data of the present study indicate that truncated Vpr (1-86) was defective in causing G2 cell cycle arrest and had strongly attenuated capabilities to induce apoptosis, suggesting that Vpr is inactivated by PCs. The mutant Vpr (1-84) was reported to be non-functional for G2 arrest in transfected cell (81). However, the possibility cannot be ruled out that extracellular Vpr (1-86) may behave differently compared to intracellularly expressed Vpr (1-86). The processed form of Vpr (1-86) may be active and has other unknown functions that are important for HIV-1 infection *in vivo*. Although the majority of extracellular Vpr was cleaved in *in vitro* culture systems like 293T cells, a certain proportion of full-length Vpr was still present even when the dynamic equilibrium of Vpr processing was reached. Therefore, this remaining amount of full-length Vpr could still be biologically active.

The results of the present study showed that trypsin treatment removed the cell-associated cleaved Vpr in the provirus transfected 293T cell, indicating that cleaved Vpr was located outside of the plasma membrane in HIV-producing cells, no matter if it was 3HA-tagged Vpr or native Vpr (Fig2.1A and 1C). Interestingly, exogenously added recombinant Vpr could be processed by the cells, but the Vpr cleaved form was not associated with the cells, as we found in HIV-1-producing cells (data not shown). The cell-associated cleaved Vpr was only observed in HIV-producing cells, suggesting that viral factor(s) or cellular factors regulated by

HIV-1 infection may contribute to the association of cleaved Vpr with cell surface. Membrane-associated Vpr functions may resemble membrane-associated HIV-1 tat. Extracellular Tat has been shown to be associated to Env at the cell surface, thus promoting HIV-1 infection and cell to cell transmission (218), increasing cell stress and causing apoptosis by forming cell to cell synapsis (266). HIV-1 transmission occurs predominantly in lymphoid tissue where cell-to-cell contact is more essential. It will be interesting to investigate if cell surface associated cleaved Vpr plays a role in promoting HIV-1 replication. No PC5 and PACE4 mRNA could be detected in primary PBL and macrophages by northern blotting analysis, but they were abundant in lymphoid tissue such as thymus, lymph node, and spleen cells (140). PC5 expression level only has 20 % that of furin in activated T cells, and PACE4 has a much lower expression than PC5 in these cells (74). It will be interesting to investigate the presence of PC5A and PACE4 and their influence on Vpr function during HIV-1 infection. Given that cleaved Vpr is also associated outside virion, it would be interesting to test its effect on virion infectivity.

The PC processing motif in the Vpr C-terminal is conserved among different HIV-1 clades, including SIVcpz. In the HIV-1 YU-2 strain, an infectious molecular clone isolated directly from an AIDS patient's brain (202), a natural mutation at the Vpr processing position was compensated by the insertion of another RR site downstream in the C-terminal part, strongly suggesting that this motif is important for HIV-1 *in vivo* replication. Although HIV-2 and SIVsm Vpr do not possess such putative PC processing motifs like HIV-1 Vpr, I found SIVsm Vpr can be partially cleaved within the cell by unknown cellular protease, no cleaved SIVsm Vpr products was not found in the extracellular medium (data not shown). It will be interesting to investigate the Vpr processing in other primate lentivirus, and analyze if this function is conserved and their function relevance. Different Vpr proteolytic processing may not be essential for basic viral replication, but might contribute to different *in vivo* pathogeneses.

During the establishment of cultured cells chronically infected with HIV-1 primary isolates, the Vpr C-terminal region could be selectively deleted or mutated (244, 295),

suggesting that Vpr C-terminus does not have selective advantage for *in vitro* HIV-1 replication. At present, it is difficult to evaluate the Vpr processing effect on viral replication using T cell lines; however, it will be helpful to assess the Vpr processing effect on viral replication by using the HIV-1 primary isolate and primary cells to avoid any possible *in vitro* adaptation of the HIV-1 virus. On the other hand, Vpr from the HIV IIIB isolate, which has a frame-shift mutation at codon 73, reverted to wild-type Vpr both in an accidentally infected laboratory worker and in experimentally infected chimpanzees (130). These results demonstrate the importance of the Vpr C-terminal domain for productive HIV-1 replication *in vivo*. The Vpr C-terminal arginine-rich region that overlaps the PC cleavage motif is extremely important for its nuclear localization and G2 arrest function (406), and thus is less likely to be mutated. In that context, its truncation by an extracellular convertase could constitute a cellular process leading to the inactivation of soluble Vpr proteins.

HIV-1 can establish viral infection in non-dividing cells by using a process based on the active transport of the viral genome into the nucleus of an infected cell (44, 200). Considering that viral DNA is transported into the nuclei within 4-6 h post infection (44), viral proteins involved in PIC import should originate directly from the virion. The other viral proteins RT and IN as well as the genomic RNA are condensed into the core (228). It has been reported that Vpr, IN, and phosphorylatd MA are all localized within the viral core. Vpr is a major virion associated protein that is localized within the core when the virus matures (1), suggesting that Vpr might interact with other viral or cellular factors for proper redistribution. It is known that Vpr, together with these viral proteins, are actively involved in the nuclear transport of viral DNA, but the putative interactions that occur between these three karyophilic proteins within the virion cores and during the PIC nuclear import are yet to be demonstrated. Moreover, the functional implications of these interactions remain to be determined. Understanding these processes will provide clues to the role of Vpr during the early stages of HIV-1 infection.

The second part of my study is to identify Vpr-interacting proteins within viral particles during HIV-1 infection. The epitope tagging strategy provided a powerful tool for dissecting Vpr -interacting proteins, by bypassing the lack of sensitive anti-Vpr antibodies. It has been reported that the fusion of the HA tag to the N-terminal part of Vpr does not alter the stability of protein nor its G2 arrest functions and incorporation into nascent virions (81, 93). In contrast, Vpr C-terminal HA tags were reported to render Vpr non-functional for G2 arrest (81). To facilitate the analysis of the Vpr-interacting protein in continuous virus replication condition, we constructed an HA-tagged Vpr isogenic infectious provirus clone. For the first time we presentdata on isogenic infectious HA-tagged Vpr in the provirus. Although, in that HA-Vpr provirus construct, the Vif C-terminal reading frame has an additional 10 amino acids, i.e., CTHTMFQITL, HA-tagging has only a minor impact on the virus replication in the dividing T cell line (see Chapter 3).

Mass spectrometry analysis of immunopurified Vpr-associated complexes pointed out that Vpr present in the virions was able to interact with the HIV MA protein. Importantly, MA binding was observed in particles produced from transfected 293T cells, as well as from HIV-infected MT4 cells (see Chapter 3). The result of the present study is consistent with the interaction previously reported by Sato *et al.*, with distinct IP conditions (303) . Since MA appears to play important roles in the nuclear transport of the proviral DNA in non-dividing cells as part of the PIC (45), this study further characterized the Vpr/MA interaction.

Although Vpr is able to interact with MA in the absence of incorporated RT and integrase (see Chapter 3), the interaction efficiency is lower than that of wild type viruses, suggesting a possible involvement of RT and integrase. Furthermore, given that RT and integrase are located in the core, these results suggest that Vpr-MA interaction is likely to occur in the core. Matrix proteins form the outer shell of the viral core (127), but it can also be located inside the viral core due to phosphorylated MA interacting with integrase proteins (118, 119). In

the same experiment, the present study found that HA-Vpr is able to pull down not only MA but also the integrase (data not shown). Given that RT, integrase, and Vpr are located in the virion core (1, 376), this suggests that the MA that is associated with Vpr also could be located in the core. The RT and integrase proteins might contribute to the stability of this interaction. Thus, it is possible that Vpr interacts with MA within the core; however, the possibility cannot be ruled out that Vpr might interact with MA outside the viral core. Further experiments using a purified virion core will be necessary to analyze this interaction.

Vpr binds DNA and RNA (72). The Vpr C-terminal basic amino acids rich region was found to be responsible for the interaction with DNA (72, 399). Vpr interacts with NCp7 in *in vitro* binding assay (297). NCp7 is involved in genomic RNA encapsidation during the budding process, suggesting a possible interaction of Vpr with nucleic acids, either directly or via the NCp7 intermediate. However, a complex that consists of HIV-1 Vpr and NCp7 has not yet been identified in HIV-1-infected cells (160). MA has also the ability to bind RNA (257, 276). It was previously shown that Gag assembly relies on the binding of RNA by MA or NC sequences to condense, organize, and stabilize the HIV-1 Gag-Gag interactions that form the virion (300). The results of the present study indicate that the recombinant Vpr could still interact *in vitro* with GST-MA even in the presence of DNase and RNase, which strongly suggests that the virion-associated Vpr-MA interaction is not mediated by DNA or RNA (data not shown).

Together with the results obtained from the *in vitro* GST pull-down experiment (see Chapter 3), this data strongly suggest that the Vpr and MA interaction is likely to be a direct physical interaction. Given that bacterially produced MA were not phosphorylated, this *in vitro* data suggest that Vpr may interact with MA without phosphorylation.

Matrix is the N-terminally myristoylated cleavage product of the Gag polyprotein precursor. This cleavage product is generated by the viral protease following the maturation of the precursor (360). As an essential viral structural protein, MA is involved in the HIV-1 assembly by targeting the poly gag precursor to the plasma membrane. MA also plays an

assembly by targeting the poly gag precursor to the plasma membrane. MA also plays an important role in the incorporation of the envelope protein into the virion (87) and affects the early steps of the viral life cycle that immediately follow viral entry (43). Phosphorylated MA is present in the viral core (118, 119) and becomes a component of the PIC during the early stages of viral infection (45, 145, 228). Moreover, Vpr and MA traffick together within the cell after viral entry (224). All these observations suggest that the Vpr-MA interaction might have a biological role under physiological conditions. Interaction between these two proteins may have a synergic effect to facilitate HIV-1 PIC nuclear import, which is critical for HIV-1 to establish the infection of non-dividing cells.

We further demonstrated that MA fifth alpha helix (α-H5) amino acids 97 to 107 containing the minimal domain required for Vpr interaction. This result is in consistent with previous report that MA C-terminal region is responsible for binding with Vpr (303) Several point mutations within the α-H5 did not decrease the Vpr and MA complex formation, as determined by IP on the virus lysates. MA L101A mutant dramatically increased the affinity with Vpr, although it has less Vpr incorporated and virus produced. If Vpr has increased affinity with MA L101A in the viral particles, it should have same affinity with gag intermediate such as p41 and Pr55gag inside the cell. But no increased affinity of Vpr with gag intermediate in the cell was found (data not shown), suggesting that the increased Vpr and MA L101A affinity is post viral maturation events. It will be interesting to investigate if MA L101A increased affinity with Vpr is due to losing affinity with other viral component such as IN. Other likely exposed amino acids in MA α-H5 helix such as E99, D102 and E105 should be considered.

Given that bacterially produced MA is not phosphorylated, the *in vitro* data of the present study suggest that Vpr may interact with the matrix proteins present in the virions, regardless of their phosphorylation status. It is possible that Vpr interacts with MA only after both proteins have been targeted independently to the core regardless of their phosphorylation

status. On the other hand, the possibility cannot be ruled out that Vpr might be recruited by the matrix protein near the virus membrane.

So far, the ability of Vpr to bind to MA in immature particles, or outside the cores, has not been evaluated. Analysis of the core composition of viruses expressing truncated MA and Vpr mutants defective for the binding will be of great interest. Such an analysis will help to understand where the interaction takes place and its impact on the recruitment of the diverse components of the preintegration complex, perhaps leading to some comprehension of the fate of PIC. We tried to analyze Vpr (1-86) virus replication capacity; Vpr (1-86) has decrease affinity with MA protein as shown in chapter 3 Fig.7. Unfortunately, the introduction of a premature TGA stop after Vpr codon 86 appeared to be detrimental for HIV replication, thus preventing the analysis of the role of this interaction in HIV-1 infection (data not shown). The reason could be that Vpr (1-86) stop mutation itself locates close to a RNA splicing acceptor site in HIV-1 genomic RNA, it may change the viral RNA structure disturb viral splicing. Alternative Vpr truncation mutant or way introducing stop code should be considered.

Deletion of Vpr C-terminal region decreased matrix binding to 50-75% of wild type levels. This domain shows no evident 3-D structure but contains several arginine residues. Thus, it is tempting to speculate that an electrostatic interaction exists between the negatively-charged amino acids of MA α-H5 and the basic C-terminal tail of Vpr. Deletion of the 79-96 had a weak impact compared to the deletion of 87-96 in viral particles, suggesting that the phosphorylation site S79 plays a minor role, if any; however, the phosphorylation sites (S79, S94, S96) cannot be ruled out as a group that can facilitate the interaction *in vivo*. Finally, the larger C-terminal deletion 63-96 resulted in almost complete loss of MA binding as shown in *in vitro* binding assay. The large deletion Vpr (1-63) and mutations in this LR domain may alter the 3-D structure of the protein causing its instability, as suggested by the high instability of Vpr (1-63) and mutants in this region *in vivo* (data not shown). The domain encompassing the C-terminal residues of Vpr was shown to be involved in the binding of the nucleocapsid protein NCp7 and

nucleic acid *in vitro* (59, 73, 176). The Vpr C-terminal arginine-rich region is extremely important for its nuclear localization and G2 arrest function, and so mutation in this region will cause protein instability (406). Vpr C-terminal arginine mutation such as R85A still has G2 arrest (81) and can be tolerated by HIV-1. It is interesting to investigate if Vpr R85Q or RR87/88 mutations decrease Vpr and MA interaction and its effect on HIV-1 macrophages infection.

It is important to investigate how host and viral factors interact to establish HIV-1 infection in human cells. Vpr has been shown to contribute to HIV-1 infection in human cells when it is present as an extracellular species as well as a virion-associated species. Here, we identified a cellular protease that regulates extracellular Vpr activity and characterized Vpr interacting-proteins within virion particles. The present study might contribute to a better understanding of Vpr early functions during HIV-1 viral replication and might provide new targets for therapeutic intervention.

CHAPTER 5: CONCLUSIONS

Works presented in this thesis have focused on the function and mechanism of one of HIV-1 the accessory proteins, Vpr, in the HIV-1 replication cycle. The following novel findings have been demonstrated and are listed according to chapters.

Chapter 2: To investigate the mechanism of HIV-1 Vpr release, the results of the present study first indicate that soluble Vpr is released from HIV-1-producing cells, and Vpr release is independent of its virion incorporation. The study found that Vpr predominantly cleaved at its C-terminus extracellularly during HIV-1 infection shortly after its release; and some of the cleaved Vpr products are associated with the external cell membrane through the binding with the external cell surface molecule heparin sulphate proteoglycan. Mutagenesis and mass spectrometry analyses further showed that Vpr is cleaved at $R_{85}QRR_{88}$, a proprotein convertase-processing motif. This is proven by the fact that the proprotein convertase (PCs) peptide inhibitor dec-RVKR-cmk and serpins (α1-PDX and Spn4A) specifically inhibited extracellular Vpr processing. Moreover, results demonstrated that the transient expression of proprotein convertase PC5A and PACE4 dramatically increased extracellular Vpr processing. Indeed, the provided evidence suggesting that Vpr is processed in the extracellular medium through PCs that are cell-surface associated. Finally, the truncated Vpr protein mimicking the fully processed product was defective for the induction of cell cycle arrest and apoptosis. The Vpr C-terminal arginine-rich region that overlaps the PC cleavage motif is extremely important for its nuclear localization and G2 arrest function, and thus is less likely to be mutated. In that context, its truncation by an extracellular convertase could constitute a cellular process leading to the

inactivation of soluble Vpr proteins. The study concludes that PC5A and PACE4 and related PCs are the essential components of the cellular machinery that controls the level of the functionally active extracellular Vpr during HIV-1 infection.

Chapter 3: To investigate the Vpr-interacting protein within HIV-1 virion particles and the relevance of their function, we first constructed an infectious molecular clone of HIV-1 expressing HA tagged Vpr. The analysis of anti-HA co-immunoprecipitated protein complexes from isolated purified virions by proteomics or western blots revealed that Vpr could form a complex with the matrix protein (MA) within viral particles produced from various human cell lines. Furthermore, the MA-Vpr interaction was shown to occur independently of the presence of RT and IN, and could be detected by *in vitro* GST pull down experiments using recombinant (r) Vpr and purified GST-MA proteins. These results indicate that the Vpr-MA association involves a direct physical interaction. The respective interacting domains were mapped by *in vitro* binding assays. The study pointed out the implication of the fifth alpha helix of MA (residues 97-108) and the arginine-rich C-terminal domain of Vpr (residues 86-96) in the Vpr-MA interaction. Since Vpr and MA are karyophilic proteins, and both are components of the pre-integration complex (PIC), their interaction might have a synergic effect in the nuclear targeting of PIC and could contribute to the efficiency of viral infection during the early stages of HIV-1 infection.

During my thesis study I also participated in another project whose results are not included in my thesis. The first project is: MHC-II molecules enhance HIV-1 assembly and budding to late endosomal/Multivesicular bodies compartments. The result is published in Journal of Virology as shown in APPENDIX I: Publication I.

I also cooperated with Dr. Andrew Mouland on the role of hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. The result has been published in J Biol Chem as shown in APPENDIX I. Publication II.

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APPENDIX I:

Publication I: Andrés Finzi1,3, Alexandre Brunet2,3+, Yong Xiao 1,3+,Jacques Thibodeau2,3 and Éric A. Cohen1,3 .MHC-II molecules enhance HIV-1 assembly and budding to late endosomal/Multivesicular bodies compartments. J Virol. 2006 Oct;80(19):9789-97.

Initially I developed concept of this project together with Alexandre Brunet when we tried to characterize Vpr is released through exosome pathway. By chance I found HIV-1 release decreased in presence of MHC-II DR expression, because we used MHC-II as an exosome marker. Then I confirmed that HIV-1 gag accumulating in the cell in presence of MHC-II DR expression by chemical analysis experiments. I and Alexandre did EM analysis; the EM analysis showed that MHC-II DR enhances HIV-1 assembly and budding intracellularly in MVB like compartment. I did the first immunofluorescence experiment suggesting HIV-1 gag and MHC-II DR colocalized in transfected 293T cell, which suggesting that HIV -1 may assembly in MBV created by MHC-II DR molecule, Andres Finzi helped for the staining process for this experiment

Publication II: Beriault V, Clement JF, Levesque K, Lebel C, Xiao Y, Chabot B, Cohen EA, Cochrane AW, Rigby WF, Mouland AJ. A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. J Biol Chem. 2004 Oct 15;279(42):44141-53.

I did A2RE mutants provirus constructs with HA-Vpr for Dr Mouland AJ. Later I did the binding experiment between Vpr and A2RE mutants proteins, I found the Gag-Vpr interaction is not influenced by A2RE mutations which is presented in discussion section.

JOURNAL OF VIROLOGY, Oct. 2006, p. 9789–9797 Vol. 80, No. 19
0022-538X/06/$08.00+0 doi:10.1128/JVI.01055-06
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Major Histocompatibility Complex Class II Molecules Promote Human Immunodeficiency Virus Type 1 Assembly and Budding to Late Endosomal/Multivesicular Body Compartments

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Received 22 May 2006/Accepted 11 July 2006

Human immunodeficiency virus type 1 (HIV-1) assembly, budding, and release occur mostly at the plasma membrane in T lymphocytes as well as in established nonlymphoid cell lines, while in macrophages these processes occur primarily in intracellular compartments that harbor late endosomal/multivesicular body (LE/MVB) markers, including human leukocyte antigen DR (HLA-DR). Major histocompatibility complex class II molecules (MHC-II), which are expressed in macrophages and activated T cells, have been previously reported to induce the formation of multilaminar and multivesicular endocytic MHC-II-like structures analogous to MVB upon their expression in HEK 293 cells. Here, we have examined the role of MHC-II in HIV-1 Gag targeting as well as in virus assembly and release. Expression of HLA-DR in nonlymphoid cell lines induced a relocation of Gag to intracellular compartments that harbored LE/MVB markers and increased the accumulation of viral particles assembling intracellularly. Consequently, viral production and release from the cell surface was found to be substantially decreased in HLA-DR-expressing cells. This process was specific, since it was not observed with HLA-DR molecules lacking their cytoplasmic tails, nor with structurally related but functionally distinct MHC-II molecules such as HLA-DM or HLA-DO. Importantly, virus released intracellularly in HLA-DR-expressing cells retained infectivity. Overall, these results suggest a role of MHC-II molecules in promoting HIV-1 assembly and budding to LE/MVB and raise the possibility that this activity might be part of a normal pathway of virus production in cell types physiologically expressing MHC-II molecules, such as macrophages.

Production of retrovirus particles is a multistep process that requires the coordinated assembly of viral structural components at a membrane budding site. The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein, $Pr55^{gag}$, plays a central role in viral assembly and release, since Gag expression alone is sufficient for the production of noninfectious virus-like particles (16). $Pr55^{gag}$ is composed of four domains that are cleaved by the viral protease (PR) during the budding process to generate matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6, as well as two spacer peptides, SP1 and SP2 (12, 16). Functional domains that promote Gag binding to membrane and multimerization have been mapped in $Pr55^{gag}$ to the myristoylated N-terminal portion of MA and the region spanning from the C terminus of CA to the N terminus of NC, respectively (12, 16). p6, through its tetrapeptide (PTAP) late motif, plays a central role in the release of viral particles by recruiting Tsg101 and other components of the endosomal sorting complex required for transport involved in the biogenesis of multivesicular bodies (MVB) (14, 30, 49, 51).

HIV-1 has been recently reported to assemble and bud either at the plasma membrane or in late endosomes (LE)/MVB. In cells such as T lymphocytes and transformed human cell lines such as HeLa and HEK 293T, the majority of virus assembly takes place at the plasma membrane (12, 34, 36, 46). In contrast, in primary macrophages, assembly occurs primarily in intracellular compartments that express late endosomal or MVB markers, including major histocompatibility complex class II molecules (MHC-II), such as human leukocyte antigen DR (HLA-DR), CD63, and Lamp1 (33, 38, 40, 42). However, the mechanism governing whether virus release occurs via internal or plasma membranes remains poorly understood. Interestingly, several reports have established that in addition to directing Gag membrane binding, the HIV MA domain regulates the targeting of Gag to the site of virus assembly (7, 9, 13, 18, 37). On the other hand, the cell-type-dependent nature of HIV-1 assembly subcellular location strongly suggests that in addition to viral determinants, host cell factors must also play an active role in determining whether HIV-1 particle assembly and release occurs at the plasma membrane or in LE/MVB. However, the identity of cellular factors promoting HIV-1 targeting to LE/MVB remains to be defined.

Interestingly, MHC-II molecules, which are expressed in macrophages and activated T cells, have been previously reported to induce the formation of CD63/Lamp1-positive multilaminar and multivesicular endocytic structures, reminiscent of MHC-II-enriched compartments (MIIC), upon their ectopic expression in HEK 293 cells (4). Interestingly, the transmembrane and cytoplasmic tails of the class II α and β chains were found necessary for the induction of these prototypical MHC-II endocytic compartments in HEK 293 cells, indicating

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that MHC-II molecules contain information critical for the formation or maturation of MHC-II-like compartments. Since HIV-1 particles preferentially assemble at the plasma membrane in HEK 293T cells (18, 46), we investigated the impact of MHC-II expression on Gag localization as well as on assembly and release of HIV-1 particles. Our results suggest that expression of classical MHC-II molecules promotes assembly and budding of infectious HIV-1 to LE/MVB in a process that implicates the cytoplasmic domain of the α and β chains of MHC-II. These findings shed light on host cell factors governing the cell-type-dependent subcellular location of HIV-1 assembly and budding and reveal a novel effect of MHC-II molecules on HIV-1 replication and persistence.

MATERIALS AND METHODS

Cells and plasmids. HEK 293T, HeLa-CD4-LTR-β-Gal (25), HeLa DR1 (DRα + DRβ0101) (43), and HeLa DRαTM/DRβTM (19) cells were maintained as described elsewhere (25). The HIV-1 molecular clone HxBc2 (24) and the MHC-II expression plasmids, including pBud-DO, pBud-DM (10), and pLNCX-DQ (19), were previously described. For the bicistronic pBud-DR construct, cDNAs encoding the DRα and DRβ chains were cloned into the pBudCE4-amp vector. A BamHI DRβ fragment originating from pBSDRβ was cloned into pBudCE4 (pBudCE4-amp DRβ), and a BamHI fragment encoding the DRα chain was cloned into the Bgl II site of pBudCE4-amp DRβ. For experiments where the TM/TM mutant was included, the following plasmids were used: RSV.5 DRα, RSV.3 DRβ, RSV.5 DRαTM, and RSV.3 DRβTM (19).

Transfections, immunoprecipitation, and viral release. Transfections were performed as described previously (52). Immunoprecipitations were done using a mix of human anti-HIV serum together with a monoclonal anti-p24 antibody (Ab), as described elsewhere (52). For pulse-labeling experiments, transfectants were metabolically labeled with 1 mCi/ml [^{35}S]methionine-cysteine ([^{35}S] protein labeling mix; Perkin-Elmer) in Dulbecco's modified Eagle's medium lacking methionine and cysteine and supplemented with 5% dialyzed fetal bovine serum for 2 h. Viral release was calculated as described elsewhere (35). For pulse-chase experiments, cells were metabolically labeled for 30 min as described above and chased for different time intervals in Dulbecco's modified Eagle's medium containing an excess of unlabeled methionine and cysteine. Viral release was calculated as the amount of virion-associated Gag as a fraction of total (cell plus virion) Gag synthesized during 30 min of the metabolic labeling period (0-h chase).

Antibodies and immunostaining. The following antibodies were used: L243 (immunoglobulin G2a [IgG2a]), a murine monoclonal antibody that binds a specific HLA-DRα conformational determinant dependent on the correct conformation of the α/β heterodimer (39); mouse monoclonal antibody (IgG1), which recognizes p17 but not p55^{gag} (catalog no. HB-8975) and mouse monoclonal anti-p24 (catalog no. HB-9725), isolated from supernatants of cultured hybridoma cells obtained from the American Type Culture Collection (Manassas, VA); rabbit anti-p24 polyclonal antibody (catalog no. 4250; NIH AIDS Research and Reference Reagent Program); the anti-HIV-1 serum (no. 162), obtained from an HIV-1-infected individual whose serum tested positive for the presence of HIV-1 antibodies by enzyme-linked immunosorbent assay (25); mouse anti-Lamp-1 (H5G11; IgG1; Santa Cruz Biotechnology, Santa Cruz, CA); anti-CD63 (H5C6; IgG1; Hybridoma Bank, NICHD, University of Iowa); mouse anti-lysobisphosphatidic acid (LBPA) monoclonal antibody (22), a kind gift from J. Gruenberg (University of Geneva, Geneva, Switzerland); and rabbit polyclonal anti-human class II alpha chain serum (31), a kind gift from J. Neefjes (Netherlands Cancer Institute). Alexa 488-conjugated anti-rabbit IgG, Alexa 594-conjugated anti-mouse IgG, Alexa 488-conjugated anti-mouse IgG1, and Alexa 594-conjugated anti-mouse IgG2 were obtained from Molecular Probes (Burlington, ON, Canada). Immunostaining was performed on 5 × 10^4 HEK 293T or 3 × 10^4 HeLa cells as follows: transfected cells were rinsed once with phosphate-buffered saline (PBS), cytospun for 4 min at 1,100 rpm in a Cytospin 2 (Shandon), and fixed with 4% paraformaldehyde for 30 min. HeLa cells were directly fixed in chambered coverglasses, where they were plated 24 h before. All procedures were carried out at room temperature unless otherwise indicated. Following a wash with PBS, fixed cells were permeabilized with PBS containing 0.2% Triton X-100 for 10 min, followed by an additional washing with PBS. Subsequently, cells were incubated in PBS containing 50 mM ammonium chloride for 10 min and exposed to primary antibodies diluted appropriately in 2% bovine serum albumin in PBS for 2 h at 37°C. Following three washes with PBS, cells were next incubated for 40 min with an appropriate secondary antibody diluted in PBS. Nuclei were then stained with 4′,6′-diamidino-2-phenylindole for 5 min. After extensive washing, cells were mounted with Permount (Fisher Scientific, Ottawa, ON, Canada) and examined by conventional epifluorescence micrographs on a Zeiss Cell Observer system (Zeiss, Toronto, ON, Canada) equipped with an Axiovert 200 M microscope using the 100× oil lens. Images were digitally deconvoluted with the AxioVision 3.1 software using the nearest-neighbor deconvolution method. Flow cytometry analysis was performed as described previously (3).

Cell-associated infectivity and Pr55^{gag} processing. HEK 293T cells (3 × 10^5) were cotransfected with 1.6 μg of HxBc2 provirus together with 0.8 μg of empty or HLA-DR vectors and washed 16 h later. Twenty-four hours posttransfection, indinavir sulfate (IVS; 10 μM; catalog no. 8145; NIH AIDS Research and Reference Reagent Program) was added to the culture medium to inhibit infectivity of newly produced virus. Forty-eight hours posttransfection, cells were extensively washed in PBS and either lysed in RIPA buffer (5% of cells) or homogenized (95% of cells) in homogenization buffer (0.25 M sucrose, 78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 50 mM HEPES-NaOH pH 7.0) during 60 s using a pellet pestle with a cordless motor (Kontes, Vineland, NJ). Homogenates were centrifuged at 1,000 × *g* for 5 min to pellet nuclei and any cell debris. Evaluation of cell lysis efficiency was accomplished by measuring β-hexosaminidase activity in pellets and supernatants using 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosamine (Sigma-Aldrich, Oakville, ON, Canada) as described elsewhere (50). Infectivity of virus present in postnuclear supernatants (PNS) was assessed by MAGI assay (20). Each sample was analyzed in duplicate. Of note, IVS treatment involved not only a 24-h exposure of transfected cells to the drug but also maintenance of IVS during PBS washes, the homogenization step, and infection of MAGI (HeLa-CD4-LTR-β-Gal) cells. Lysed cells were analyzed for Gag polyprotein precursor processing by Western blotting using the mouse monoclonal anti-p24 antibody as described previously (25).

Electron microscopy. Cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Postfixation of cell pellets was performed using 2% OsO_4 in *s*-collidine buffer for 2 h at 21°C. Pellets were dehydrated in an acetone series before embedding and polymerization in SPURR resin. Thin sectioning was done with an ultramicrotome system (Ultrotome 2128, LKB, Sweden), and the sections were placed on copper-Formvar-carbon-coated grids. Cells were stained with 5% uranyl acetate in 50% ethanol and lead citrate (pH 12.0). For immunogold staining, cell pellets were fixed in 0.1% glutaraldehyde–4% paraformaldehyde in 0.1 M cacodylate buffer. Pellet dehydration, polymerization, and thin sectioning were performed as described above, and the sections were placed on nickel-Formvar-carbon-coated grids. Cells were labeled with a rabbit polyclonal anti-human class II serum (31) followed by incubation with a goat anti-rabbit antibody coupled to 12-nm gold beads before staining using 5% uranyl acetate in 50% ethanol and lead citrate (pH 12.0). The grids were examined on a transmission electron microscope (Hitachi 7100; Japan).

RESULTS

HLA-DR expression induces a relocation of HIV-1 Gag to LE/MVB. Expression of the α and β chains of MHC-II molecules in HEK 293 cells was found to be sufficient to induce the formation of multilamellar and multivesicular MIIC (4). To examine whether MHC-II molecules could affect Gag localization, HEK 293T cells were cotransfected with the HIV-1 molecular clone HxBc2 together with expression plasmids encoding the α and β chains of HLA-DR or an empty vector. In the absence of HLA-DR, the majority of Gag, as visualized with an anti-p24 antibody, was detected as diffuse cytoplasmic and cell membrane staining (Fig. 1A). In contrast, Gag-positive cells expressing HLA-DR often displayed a marked modification of Gag localization, with Gag staining accumulating in large intracellular vesicles (Fig. 1B). Interestingly, these intracellular Gag-containing vesicles were also HLA-DR positive (Fig. 1B, merge).

Since we were able to differentiate between diffuse and large punctuate Gag staining (Fig. 1A and B, respectively), we quan-

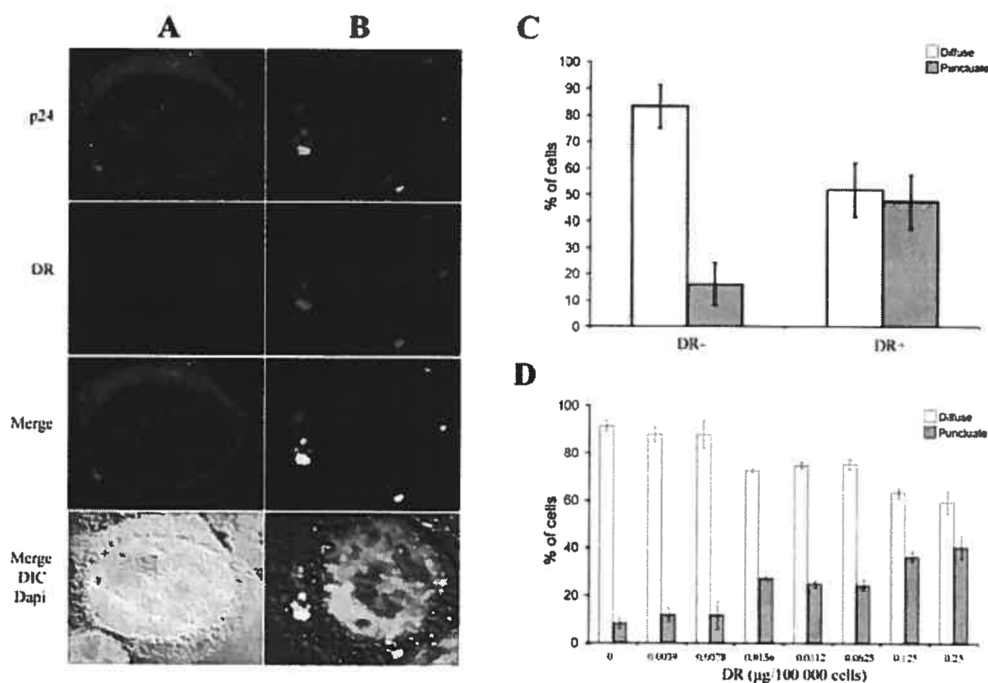

FIG. 1. HLA-DR induces Gag accumulation in intracellular compartments in HEK 293T cells. HEK 293T cells were cotransfected with the HxBc2 provirus together with empty or HLA-DR vectors and analyzed 48 h later by immunofluorescence microscopy using a polyclonal anti-p24 and the monoclonal L243 HLA-DR Abs. (A) In the absence of HLA-DR, Gag displays a diffuse staining. (B) HLA-DR redirects Gag to large intracytoplasmic vesicles (punctuate staining), where it colocalizes with HLA-DR. (C) Quantification of Gag-associated staining. The number of cells displaying a diffuse versus punctuate Gag staining was evaluated in 200 cells per sample. Data shown represent the average of at least 25 independent experiments ± the standard deviation. (D) HLA-DR redirects Gag from a diffuse to a punctuate staining in a dose-dependent fashion. Data are representative of four independent experiments.

tified the extent of Gag relocalization induced by HLA-DR in cell transfectants (Fig. 1C). In the absence of HLA-DR, 80 to 90% of cells showed a diffuse Gag staining, while less than 20% displayed a punctuate Gag staining. Conversely, upon HLA-DR expression, a punctuate Gag staining was detected in approximately 50% of the cells, most probably those expressing higher levels of HLA-DR. Indeed, the effect of HLA-DR expression on Gag localization in intracellular vesicles was dose dependent (Fig. 1D). Importantly, however, levels of HLA-DR expression required to induce Gag relocalization to intracellular vesicles (Fig. 1) were comparable to those detected in activated primary monocyte-derived macrophages (data not shown). Finally, to examine whether the effect of HLA-DR on HIV-1 Gag localization was restricted to the classical MHC-II molecules, such as HLA-DR and -DQ, or was also shared with other structurally related MHC-II proteins, such as HLA-DM and -DO (nonclassical MHC-II molecules), we tested the impact of their expression on Gag localization. HLA-DM is expressed in late endosomal/lysosomal compartments, including MVB and multilamellar compartments (28, 44), while HLA-DO resides in the endoplasmic reticulum when expressed by itself in transfected cells (26); neither HLA-DM nor -DO displayed any effect on Gag localization, while HLA-DQ partially recapitulated the effect of HLA-DR (Fig. 2).

To further characterize the nature of the intracellular compartments where Gag accumulates in the presence of HLA-DR, we performed costaining experiments with antibodies directed against late endocytic markers. These experiments revealed that Gag-containing intracellular vesicles were positive for LE or MVB markers HLA-DR, Lamp1, CD63, and LBPA, a lipid found in the internal membranes of MVB (22) (Fig. 1B and 3A to C, respectively). Because results so far were

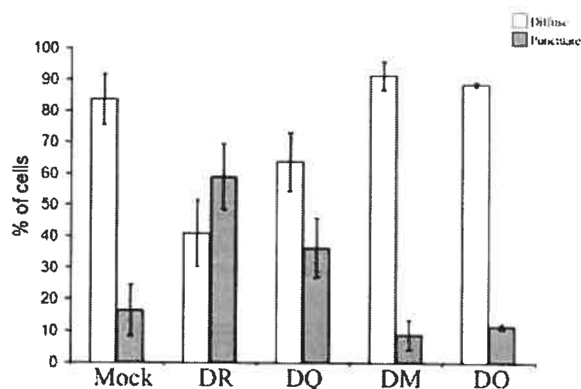

FIG. 2. Classical MHC-II molecules redirect Gag to intracellular compartments. HEK 293T cells were transfected with the proviral construct HxBc2 and plasmids encoding MHC-II-related molecules, including HLA-DR, -DQ, -DM, or -DO, and analyzed for Gag localization by immunostaining and fluorescence microscopy using a rabbit polyclonal anti-p24 antibody. Diffuse or punctuate Gag-associated staining patterns were quantified in 200 cells per sample. Data shown are means ± standard deviations of two independent experiments.

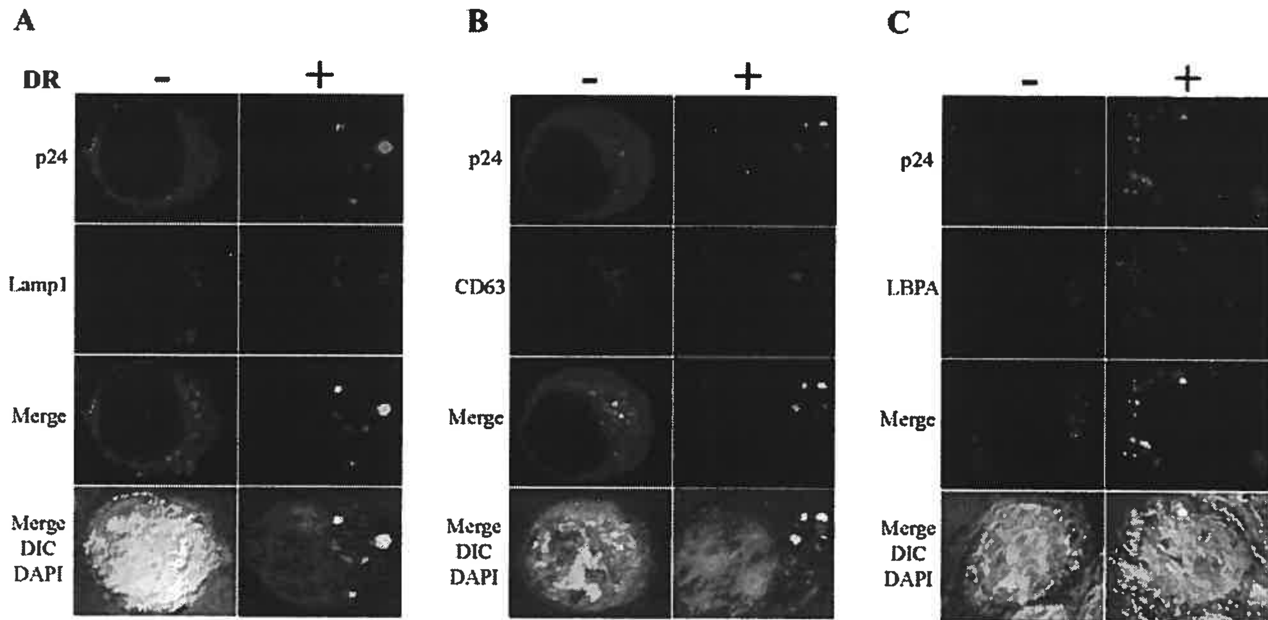

FIG. 3. HLA-DR redirects Gag to LE/MVB in HEK 293T cells. HEK 293T cells were transfected with the proviral construct HxBc2, together with HLA-DR or empty vector. Gag and MVB markers were detected by immunofluorescence microscopy 48 h later using a rabbit polyclonal anti-p24 antibody together with monoclonal antibodies against MVB markers. In the absence of HLA-DR, Gag shows primarily a diffuse staining (left panels of A, B, and C). Upon HLA-DR expression, Gag accumulates into Lamp1-positive (A), CD63-positive (B), and LBPA-positive (C) compartments (right panels).

obtained in a transient expression system where proteins are overexpressed, we also examined the effect of HLA-DR expression on Gag relocalization in HeLa cells stably expressing HLA-DR (Fig. 4A). Given that previous evidence suggested that the cytosolic domain of MHC-II molecules might be implicated in the induction of MHC-II-like compartments (4), we also analyzed Gag localization in HeLa cells stably expressing a truncated form of HLA-DR that lacks the cytoplasmic tails of

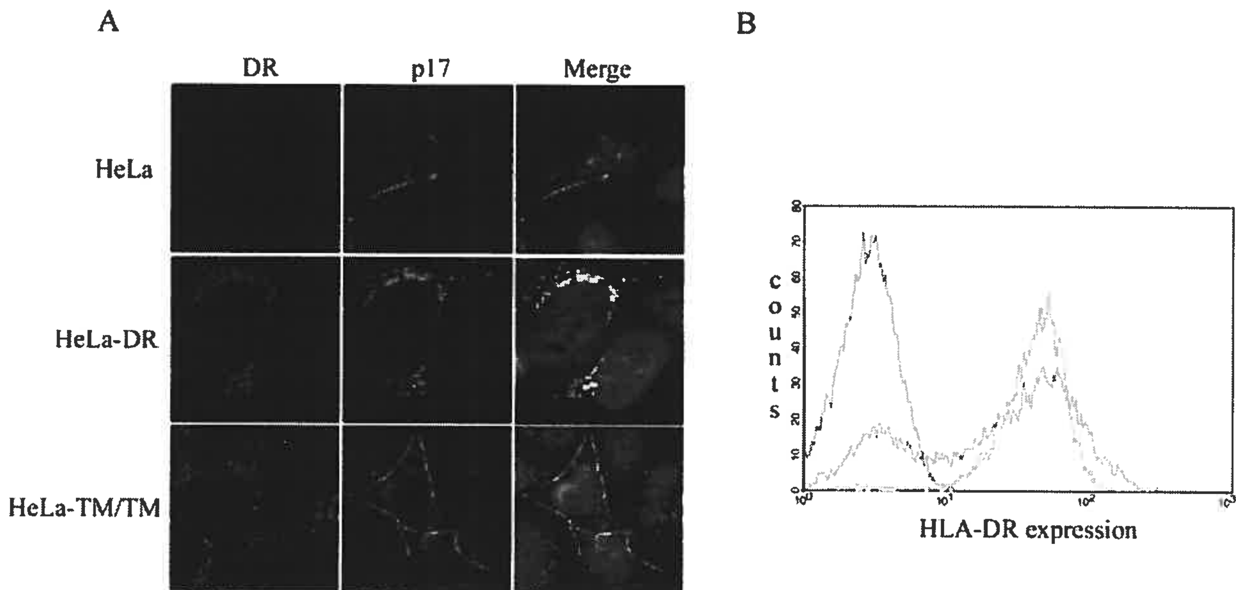

FIG. 4. Stable HLA-DR expression in HeLa cells induces Gag accumulation into HLA-DR-positive intracellular vesicles. Parental, HLA-DR-, or TM/TM-expressing HeLa cells were transfected with the infectious molecular clone HxBc2. Gag and HLA-DR were detected by immunostaining and fluorescence microscopy using mouse monoclonal anti-p17 (MA) and anti-HLA-DR (L243) Abs. (A) In parental HeLa cells, Gag is localized at the plasma membrane, whereas in HLA-DR-expressing cells, Gag is predominantly detected in vesicles at the perinuclear region. Importantly, stable TM/TM expression did not modify Gag localization. (B) Flow cytometry analysis of total HLA-DR expression in cells depicted in panel A using the L243 anti-HLA-DR monoclonal Ab. Black line, HeLa cells; red line, HeLa-DR cells; blue line, HeLa-TM/TM cells.

the α and β chains (TM/TM). HLA-DR-expressing cell lines were transfected with the HxBc2 provirus and analyzed 48 h later by immunofluorescence microscopy, using an antibody that recognizes mature p17 (but not the MA domain in the context of $Pr55^{gag}$) and an anti-HLA-DR antibody. Given that most processed MA is found associated with mature viral particles (16), the MA signal obtained with the anti-p17 antibody, in all likelihood, represents sites at which viral assembly occurs. In parental HeLa cells as well as in TM/TM cells, the majority of the MA signal was observed at the cell periphery on the plasma membrane (Fig. 4A), even though TM/TM molecules have been detected in intracellular vesicles that stained positive for MVB markers (A. Finzi and E. A. Cohen, unpublished data). In contrast, in HLA-DR-expressing cells, we observed a clear redistribution of MA staining to intracellular vesicles, where it colocalized with HLA-DR (Fig. 4A). Importantly, both HeLa-DR and HeLa-TM/TM expressed similar amounts of class II molecules as measured by flow cytometry (Fig. 4B).

Together, these results suggest that ectopic MHC-II expression in human nonlymphoid cell lines induces a redirection of HIV-1 Gag localization and assembly from the plasma membrane to MHC-II-containing LE/MVB. This effect is restricted to classical MHC-II molecules, such as HLA-DR and -DQ and, importantly, appears to involve the cytoplasmic domains of the α and β chains.

HIV-1 particles accumulate into intracellular compartments in MHC-II-expressing HEK 293T cells. To obtain additional evidence that HLA-DR expression promotes HIV-1 particle assembly and budding in intracellular compartments, we performed electron microscopy analysis on HEK 293T cells transfected with HxBc2 alone or cotransfected with vectors encoding HLA-DR or the TM/TM mutant (Fig. 5). In HEK 293T cells transfected with HxBc2 alone or cotransfected with TM/TM, viral particle budding was observed predominantly at the plasma membrane (Fig. 5A and D and 5C and F, respectively). In HLA-DR-expressing cells, mature virions with typical condensed cores were observed in the lumen of large intracellular vesicles (Fig. 5B and E and 5G and H). Viral particles in the process of budding were also seen on the limiting membrane of these enlarged intracellular vesicles (Fig. 5G and H). Furthermore, immunogold staining experiments using an anti-HLA-DR antibody clearly revealed that the internal vesicles containing mature virions and budding viral particles stained positive for HLA-DR (Fig. 5H).

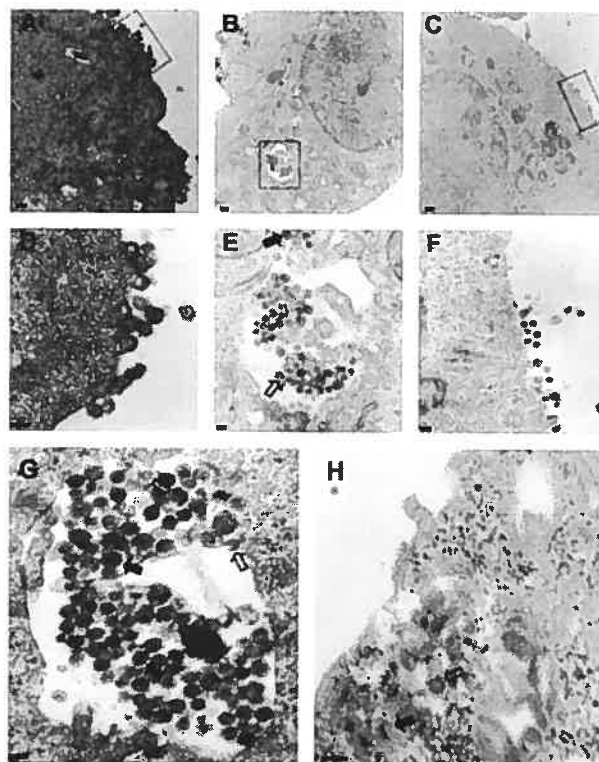

FIG. 5. Mature and budding HIV-1 particles accumulate in intracellular compartments upon HLA-DR expression. HEK 293T cells were cotransfected with HxBc2 and empty, HLA-DR, or TM/TM vectors and observed by transmission electron microscopy (A to G) or processed for immunogold staining with a rabbit polyclonal anti-HLA-DRα Ab (H). In mock- or TM/TM-transfected cells, HIV-1 assembles at the plasma membrane (A and C, respectively). (B) HLA-DR expression induces accumulation of mature and budding HIV-1 particles into large intracellular compartments. (D to F) Magnified views from regions indicated in panels A to C, respectively. (G) Magnified view of intracellular HIV-1-containing compartments in HLA-DR-expressing cells. (H) HIV-1 particles accumulate into HLA-DR-positive compartments. Empty arrows indicate budding virus, whereas solid arrows indicate mature virus. Bar, 300 nm (A), 500 nm (B), 400 nm (C), or 100 nm (D to H).

Effect of HLA-DR expression on HIV-1 production. Having obtained evidence suggesting that HLA-DR expression promotes assembly and budding of HIV-1 particles to LE/MVB in HEK 293T cells, we next examined the impact of this relocation on HIV-1 particle production. HEK 293T cells were singly transfected with HxBc2 or cotransfected with expression vectors encoding HLA-DR or related molecules. Cells were pulse-labeled for 2 h, 48 h posttransfection, and cell and virus Gag-associated proteins were analyzed by immunoprecipitation (Fig. 6A). In transfected cell cultures expressing HLA-DR, virus release was reduced by two- to threefold compared to the mock-transfected control (Fig. 6A and B). In contrast, in TM/TM-, HLA-DM-, or HLA-DO-expressing cells, viral release efficiency was unaffected (Fig. 6A and B) (Finzi and Cohen, unpublished). Importantly, the observed impact of HLA-DR on HIV-1 release was not due to any marked defect at the level of Gag precursor processing, since measurements of Gag precursor cleavage in pulse-chase labeling/immunoprecipitation experiments revealed that the $Pr55^{gag}$ processing kinetics was identical in cells expressing HLA-DR and in the negative control (Fig. 6C and D). These results suggest that expression of MHC-II molecules, such as HLA-DR, can modulate viral release efficiency.

Interestingly, when we analyzed both quantitatively and qualitatively cell- and virus-associated Gag-related products by pulse-chase labeling and immunoprecipitation experiments, we started detecting a reduction of viral release in HLA-DR-expressing cells as early as 1 h postchase. This reduction in viral release efficiency was observed throughout the 24-h chase period, with a peak between 5 and 12 h; during that time interval, HLA-DR-expressing cells were found to release approximately twofold less virus than control cells (Fig. 6C and E). Interestingly, this reduction in viral release efficiency was accompanied by a change in the p24/p25 ratio accumulating in HLA-DR-

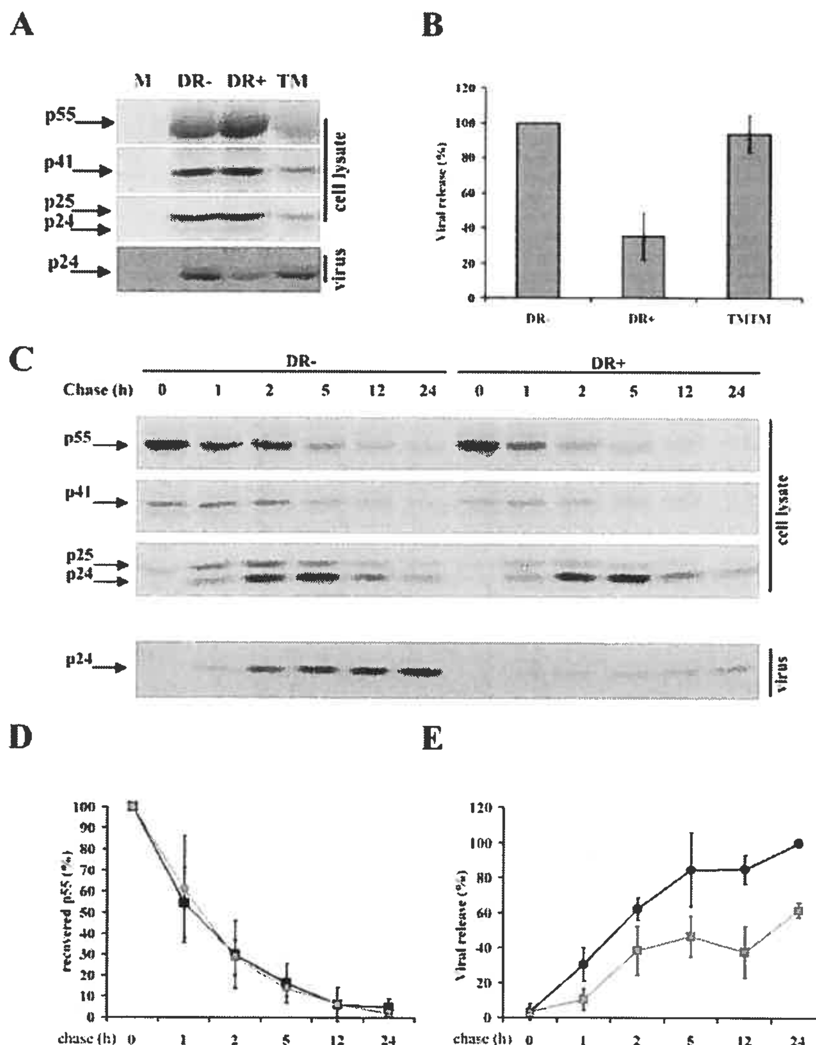

FIG. 6. HLA-DR expression decreases HIV-1 release. (A) HEK 293T cells were mock transfected (M) or cotransfected with plasmids encoding HLA-DR (DR+), TM/TM (TM), or empty vector (DR-) together with the HxBc2 provirus. Two days after transfection, cells were metabolically labeled with [^{35}S]Met-Cys for 2 h, and Gag-associated products in cell and virion lysates were immunoprecipitated using a mix of human anti-HIV serum together with a monoclonal anti-p24 Ab. (B) Quantitation of virus release efficiency. Data shown represent the average of at least four independent experiments ± the standard deviation. (C) Analysis of viral release kinetics by pulse-chase labeling analysis. Cell and virion lysates from HLA-DR$^+$ and HLA-DR$^-$ cells were immunoprecipitated as for panel A after a 30-min metabolic labeling with [^{35}S]Met-Cys or at different chase time intervals. (D) Gag precursor processing is represented as the percentage of p55^{gag}-associated signal recovered from cell lysates after pulse-chase analysis as described for panel C. p55^{gag}-associated signal after 30 min of labeling (0-h chase) was arbitrarily set to 100%. Data shown represent the average of five independent experiments ± the standard deviation. (E) Quantitation of viral release kinetics. Data from two independent experiments were quantified using a PhosphorImager equipped with ImageQuant software 5.0 and are shown as means ± standard deviations. Viral release efficiency was calculated as described in Materials and Methods. Gray lines, HLA-DR-expressing cells; black lines, HLA-DR-negative cells.

expressing cells; quantitative analysis over several experiments revealed that there was 1.5 to 2.0 times more p24 relative to p25 in HLA-DR-expressing cells than control cells (Fig. 6C; compare p24 and p25 levels between HLA-DR$^+$ and -DR$^-$ cells). These results indicate that even though CA is accumulating intracellularly in HLA-DR-expressing cells, it is being processed in a mature form usually found associated with mature viral particles. Overall, these results are consistent with our findings suggesting that expression of MHC-II molecules in HIV-producing cells leads to increased assembly and budding of mature viral particles in LE/MVB.

Infectivity of viral particles assembling intracellularly in the presence of HLA-DR. We next evaluated whether virions assembling intracellularly upon HLA-DR expression retained their infectivity. This is particularly important given that specific endosomal compartments are known to undergo acidification, a process that inactivates HIV infectivity (11), and participate in the degradation pathway that leads to lysosomes (17). To address this question, we adapted a recently described strategy that was used to evaluate the infectious stability of virions that assemble intracellularly in primary macrophages (45). This approach utilizes suprainhibitory concentrations (10

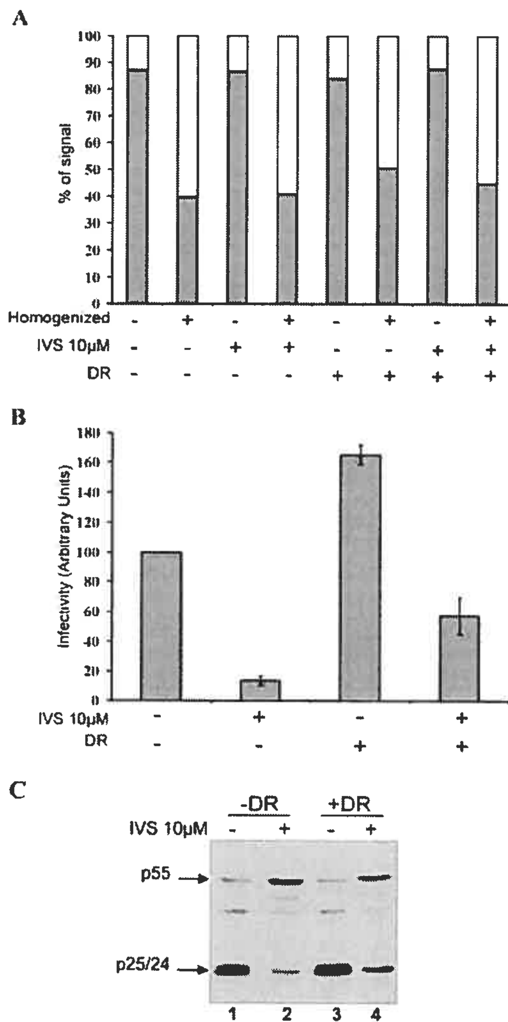

FIG. 7. Virions assembling intracellularly in the presence of HLA-DR remain infectious. HEK 293T cells were cotransfected with HxBc2 provirus and empty or HLA-DR vectors. IVS (10 μM) was added to the culture medium 24 h posttransfection to block production of new infectious virus. Cells were homogenized 24 h later, and cell disruption efficiency was determined by measuring β-hexosaminidase activity in pellets (filled bars) and PNS (empty bars) as described in Materials and Methods (A). Infectious activity was assessed in the PNS by MAGI assay (B). Data shown represent the average of two independent experiments ± the standard deviation. In parallel, Gag processing in each transfectant was analyzed by Western blotting using a monoclonal anti-p24 Ab (C). Data shown are representative of six independent experiments.

μM) of the protease inhibitor IVS to block de novo production of infectious particles. In the presence of 10 μM IVS, processing of Pr55^{gag} is completely inhibited and virus particles that are produced are immature and hence noninfectious (45; Finzi and Cohen, unpublished). Consequently, infectious virus recovered from HIV-1-producing cells following IVS treatment should be formed prior to addition of the inhibitor. HEK 293T cells cotransfected with HxBc2 provirus and HLA-DR or empty vectors were allowed to produce infectious virus for 24 h prior to addition of IVS. Cells were homogenized and fractionated 24 h later to analyze cell-associated infectivity as described in Materials and Methods. Since cell disruption could affect recovery of the intracellular pool of virus, PNS and pellet fractions were analyzed for β-hexosaminidase activity as a marker for cell lysis and endocytic organelle disruption (23) (Fig. 7A). Consistent with the accumulation of intracellular viral particles (Fig. 5 and 6), untreated HLA-DR-expressing cells displayed more infectious activity (1.6-fold increase) in their PNS compared to control cells (Fig. 7B). IVS treatment drastically reduced intracellular infectious activity in control cells, thus suggesting that the bulk of cell-associated infectious activity produced during the first 24 h posttransfection (before adding IVS) was efficiently released in the extracellular medium in absence of HLA-DR. Nevertheless, some infectious activity (14%) was still detectable in PNS and most probably represents the background HIV-1 intracellular assembly detected in HEK 293T cells (Fig. 1 and 7C). Remarkably, IVS-treated HLA-DR-expressing cells retained four times more infectious activity in their PNS than control cells (57% versus 14%) (Fig. 7B). Interestingly, analysis of β-hexosaminidase activity released in PNS was found to be comparable between HLA-DR transfectants and control cells (Fig. 7A), thus indicating that differences observed in intracellular infectious activity cannot be attributed to variations in cell disruption efficiency. Furthermore, analysis of Gag processing in untreated or IVS-treated cells supported our observation that MHC-II molecules enhanced accumulation of mature virus particles into intracellular compartments (Fig. 7C). In the presence of IVS, as expected, there was a clear inhibition of Gag processing, as visualized by the decreased levels of p25/24 cleavage products and increased accumulation of p55^{gag} (Fig. 7C; compare lanes 1 and 2 and lanes 3 and 4). Strikingly, the inhibitory effect of IVS on Gag processing was less efficient in HLA-DR transfectants than in control cells, as evidenced by the marked accumulation of p25/p24 (two- to threefold increase) in HLA-DR$^+$ cells relative to the control (Fig. 7C, compare lanes 4 and 2). These completely processed intracellular Gag products, by definition, had to be produced during the 24-h time interval before addition of the drug.

Altogether, these results provide additional evidence indicating that mature virus particles accumulate more efficiently into intracellular compartments in the presence of HLA-DR and demonstrate that virions released intracellularly within HLA-DR-expressing cells retain their infectivity potential.

DISCUSSION

In this study, we examined the role of MHC-II molecules in HIV-1 Gag targeting as well as in virus assembly and release. Ectopic expression of classical MHC-II molecules, such as HLA-DR and -DQ, in nonlymphoid cell lines was found to promote Gag relocation to intracellular compartments that contained late endosomal and MVB markers (Fig. 1 to 3), in a process that strictly relied on the presence of the cytoplasmic tails of the α and β chains of MHC-II molecules (Fig. 4 and 5). This MHC-II-mediated relocalization of Gag correlated with an increased accumulation of mature viral particles in intracellular compartments (Fig. 5) and, as a consequence, resulted in decreased virus production and release from the cell surface (Fig. 6). Importantly, viral particles assembling intracellularly in HLA-DR-expressing cells retained their infectivity (Fig. 7).

Together, these results provide evidence suggesting that MHC-II molecules promote assembly and budding of infectious HIV-1 virions to LE/MVB and raise the possibility that this process might be part of a normal pathway of virus production in cell types physiologically expressing MHC-II molecules, such as macrophages.

HIV-1 Gag contains motifs that are critical for its transport to the plasma membrane (36) and for interaction with LE/MVB (7, 27). Furthermore, recent evidence indicates that the cell-type-dependent targeting of HIV-1 assembly to the plasma membrane or LE/MVB can also be regulated by host cell factors. For instance, the human ubiquitin ligase POSH, a *trans*-Golgi network-associated protein (1), and more recently phosphatidylinositol (4,5)biphosphate [PI(4,5)P_2], a member of the phosphoinositide family of lipids concentrated primarily on the cytoplasmic leaflet of the plasma membrane (35, 48), were found to regulate HIV-1 Gag targeting to the plasma membrane, such that depleting hPOSH or cellular PI(4,5)P_2 redirected virus assembly from the plasma membrane to LE and inhibited virus release. Interestingly, in the case of MHC-II molecules, it is the expression of a host cell factor physiologically expressed in macrophages that relocalizes Gag and viral assembly to LE/MVB in HeLa or HEK 293T cells. Nevertheless, MHC-II-mediated relocation of viral assembly to LE/MVB in HEK 293T cells resulted in a reduction of virus release, as has been reported for hPOSH or cellular ([PI(4,5)P_2] depletion.

How does expression of class II molecules result in a marked accumulation of HIV-1 into LE/MVB? One possibility is that MHC-II molecules interact with a structural component of HIV and retarget a fraction of viral assembly to intracellular compartments following their transit into the endocytic pathway. MHC-II molecules, such as HLA-DR, have been reported to be present at the surface of the HIV-1 virion (5, 6) and, based on a previous study, Env gp41 appears to be required for efficient insertion of HLA-DR molecules within HIV-1 (41). The mechanism underlying MHC-II-mediated relocalization of HIV-1 assembly and budding does not appear to involve an interaction between the viral envelope and HLA-DR, since the effect of HLA-DR on Gag relocalization and viral particle production was observed with proviral constructs lacking Env (Finzi and Cohen, unpublished). Although this redirection could also result from an interaction of Gag with HLA-DR, we were unable to detect any specific interaction between these molecules in coimmunoprecipitation experiments (Finzi and Cohen, unpublished). However, the lack of physical interaction between HLA-DR and Gag does not preclude the possibility that the two molecules can interact functionally. In fact, it was reported that Gag can affect HLA-DR trafficking (15). In this study, Gag expression was shown to be sufficient to specifically restore defective transport of HLA-DR from intracellular compartments to the cell surface in a subclone of the HUT78 human T-cell line, suggesting that at some point Gag and HLA-DR share the same trafficking pathway. Interestingly, it was reported that a significant pool of MHC-II molecules traffic to endosomal-lysosomal compartments by means of the cell surface (8, 29). Consequently, it is therefore possible that MHC-II-induced relocalization of Gag into LE/MVB and subsequent accumulation of mature viral particles in these intracellular compartments could result from an increased internalization of virions from the plasma membrane mediated by HLA-DR rather than an enhanced targeting of Gag to LE/MVB. Interestingly, a recent report indicates that expression of a dominant negative form of dynamin (K44A), known to inhibit clathrin-mediated endocytosis (47), prevented the accumulation of Gag at intracellular sites in the absence of Vpu in HeLa cells (32). Although our preliminary data indicate that the effect of HLA-DR on viral release was not affected by K44A expression, we did observe a partial reduction of Gag relocalization to intracellular compartments under these conditions (Finzi and Cohen, unpublished), thus suggesting that a pool of Gag retargeted by HLA-DR could be plasma membrane associated. Importantly, however, since Gag relocation to intracellular compartments could not be completely abolished upon K44A expression, this suggests that HLA-DR may also affect the cellular localization of Gag by enhancing its direct targeting to MVB.

Indeed, an alternative but not exclusive model to explain the effect of MHC-II molecules on HIV-1 assembly and budding to intracellular compartments postulates that expression of MHC-II may contribute to the formation or maturation of compartments to which Gag molecules would be targeted. Importantly, expression of MHC-II molecules, such as HLA-DR, in HEK 293 cells was found to be sufficient to induce a MIIC-like structure having a multilamellar and multivesicular morphology and expressing CD63 and Lamp1 (4). Both types of structures were proposed to reflect different maturation states of MIIC (21). One might envision that formation and/or maturation of MIIC-like compartments by MHC-II in HEK 293T cells may provide additional internal membrane platforms toward which Gag can be targeted for assembly and budding. Interestingly, treatment of U1 promonocytic cells with gamma interferon, a strong upregulator of MHC-II expression, was found to significantly increase the redirection of virus assembly from the plasma membrane to intracytoplasmic vesicles (2). More studies are required to fully understand the precise mechanism underlying the effect of MHC-II molecules on HIV-1 assembly and release. Furthermore, experiments aimed at depleting MHC-II molecules are currently in progress to elucidate whether MHC-II influences Gag targeting and assembly to MVB in primary macrophages and as such constitutes a cellular determinant governing HIV-1 production and egress in this cell type.

ACKNOWLEDGMENTS

We thank G. Duisit for helpful discussions, as well as A. Robert and A. Vallée for expertise in electron microscopy analysis. We thank J. Gruenberg and J. Neefjes for providing Abs. The polyclonal anti-p24 Ab, IVS, and HeLa-CD4-LTR-β-Gal cells were obtained through the NIH AIDS Research and Reference Reagent Program.

A.F. is recipient of a studentship from a Canadian Institute of Health Research (CIHR) training program in cancer research. E.A.C. and J.T. are recipients of the Canada Research Chair in Human Retrovirology and a Fonds de la Recherche en Santé du Québec (FRSQ) scholarship award, respectively. This work was supported by grants from CIHR and FRSQ to E.A.C. and J.T. and from the FRSQ-AIDS network to E.A.C.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 279, No. 42, Issue of October 15, pp. 44141–44153, 2004
Printed in U.S.A.

A Late Role for the Association of hnRNP A2 with the HIV-1 hnRNP A2 Response Elements in Genomic RNA, Gag, and Vpr Localization*[S]

Received for publication, April 27, 2004, and in revised form, July 22, 2004
Published, JBC Papers in Press, August 3, 2004, DOI 10.1074/jbc.M404691200

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Two *cis*-acting RNA trafficking sequences (heterogenous ribonucleoprotein A2 (hnRNP A2)-response elements 1 and 2 or A2RE-1 and A2RE-2) have been identified in HIV-1 *vpr* and *gag* mRNAs and were found to confer cytoplasmic RNA trafficking in a murine oligodendrocyte assay. Their activities were assessed during HIV-1 proviral gene expression in COS7 cells. Single point mutations that were shown to severely block RNA trafficking were introduced into each of the A2REs. In both cases, this resulted in a marked decrease in hnRNP A2 binding to HIV-1 genomic RNA in whole cell extracts and hnRNP A2-containing polysomes. This also resulted in an accumulation of HIV-1 genomic RNA in the nucleus and a significant reduction in genomic RNA encapsidation levels. Immunofluorescence analyses revealed altered expression patterns for pr55^{Gag} and particularly that for Vpr. Vpr localization became almost completely nuclear and this was reflected in a significant reduction in virion-associated Vpr levels. These effects coincided with late steps of the viral replication cycle and were not seen at early time points post-transfection. Transcription, splicing, steady state RNA levels, and pr55^{Gag} processing were not affected. On the other hand, viral replication was markedly compromised in A2RE-2 mutant viruses and this correlated with lowered genomic RNA encapsidation levels. These data reveal new insights into the virus-host interactions between hnRNP A2 and the HIV-1 A2REs and their influence on the patterns of HIV-1 gene expression and viral assembly.

Human immunodeficiency virus type 1 (HIV-1)[1] is the cause of acquired immunodeficiency syndrome (AIDS). Transcription of the integrated provirus produces one primary 9-kb transcript that is spliced to produce three size classes of RNA (1). The smallest size class, the 2-kb RNAs, is constitutively exported to the cytosol early in the HIV-1 replication cycle and encodes for the regulatory proteins Tat, Rev, and Nef. Late in the replication cycle, the two other size classes of RNA, the unspliced, 9-kb genomic RNA and the singly spliced, 4-kb RNAs make their way to the cytosol due principally to the activity of Rev, which binds to the Rev responsive element (RRE) present in these RNAs (2). Whereas an abundant amount of information is available about the mechanisms, cellular cofactors, and regulation involved in Rev-mediated RNA nucleocytoplasmic transport (3), very little is understood about HIV-1 RNA trafficking following Rev's disengagement in the cytosol. Recent work demonstrates a role for the cellular human Rev-interacting protein (hRIP) at this step (4). The HIV-1 structural protein, pr55^{Gag} also plays a role at this late step by binding to RNA via it N-terminal matrix (MA) and C-terminal nucleocapsid (NC) domains (5–7). pr55^{Gag} association to molecular motor proteins (8) provides a mechanism by which RNA trafficking is achieved within the cytoplasm. In support of the existence for a trafficking mechanism are data showing that kinesins and microtubules are both necessary for the trafficking of several HIV-1 RNAs (9). Furthermore, recent observations of Moloney murine leukemia virus and HIV-1 indicate that vesicular trafficking on microtubules exists to achieve cytosolic trafficking of retroviral components, including the RNA, to sites of assembly (10–12).

There are only a handful of examples that implicate RNA transport mechanisms in human disease. In particular, expansion of CUG repeats in the myotonic dystrophy protein kinase RNA leads to its nuclear sequestration (13, 14). Other examples include RNAs that are expressed in neural cells to influence memory and plasticity. A defect in myelination for instance is a characteristic of multiple sclerosis and may be the result of aberrant RNA trafficking (15). The Fragile X mental retardation protein (FMRP) is involved in RNA transport and translation (16), and the absence of FMRP in fragile X syn-

* This work was supported by grants from the Canadian Foundation for AIDS Research (to A. J. M.), the Canadian Foundation for Innovation (to A. J. M.), and grants from the Canadian Institutes of Health Research (CIHR) (to A. J. M., A. W. C., B. C., and É. A. C.) and a National Institutes of Health Grant (to W. F. C. R). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplementary Materials.

[c] Supported by studentships from the *Fondation Georges-Phenix* and *Fonds pour la recherche en santé du Québec* (FRSQ).

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RRE, Rev responsive element; nt, nucleotide; RT, reverse transcriptase; PBS, phosphate-buffered saline; FISH, fluorescence *in situ* hybridization; RPA, RNase protection analysis; AIDS, acquired immunodeficiency syndrome; A2RE, A2 response element; hnRNP, heterogenous ribonucleoprotein; NC, nucleocapsid.

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drome could cause mRNAs to be de-repressed at the wrong intracellular address or at an inappropriate time, leading to alterations in neuronal dendritic spines (17).

The link between HIV-1 disease and the HIV-1 RNA localization and the cytoskeletal machinery is also very compelling (18). The use of Rev transdominants for example has underscored the essential nature of Rev-mediated nucleocytoplasmic trafficking of HIV-1 RNA for HIV-1 replication (19), and this pathway also impinges on the cytoskeleton (20). Several Rev cofactors that are critical to Rev function interact with nuclear actin concomitant to RNA transport (21). HIV-1 RNA trafficking is dependent on microtubules and kinesin expression (9, 22), and we have made a link between long-term non-progression to AIDS and the trafficking signals involved based on changes at the A8 nucleotide in the A2RE-2 sequences from three non-progressors (9). HIV-1 $pr55^{Gag}$ and the quintessential RNA trafficking protein, Staufen, physically interact (23), are found in association with kinesins and are both implicated in HIV-1 genomic RNA trafficking into assembling virus supporting a dependence on these for viral assembly (8, 24, 25). Finally, viral entry of the HIV-1 reverse transcription ribonucleoprotein complex depends on an intact cytoskeletal network (26).

In general, the family of hnRNP A/B proteins (A1, A1b, A2, B1) are involved in post-transcriptional gene regulation including splicing, RNA metabolism, transport, and translation (27). They contain several functional domains including RNA recognition motifs and the M9 nuclear import/export signal in the C terminus. A general role of these proteins has been identified in HIV-1 RNA splicing regulation, binding to *cis* sequences on HIV-1 RNA (28, 29). While recombinant hnRNP A2 has been shown to modulate splice site selection in *in vitro* splicing assays (29) several studies demonstrate that members of this family have specialized roles in transcription (30), in RNA trafficking (9, 27, 31, 32) and members of this family also respond differentially to hypoxia and stress (33, 34). Knockdown by siRNA also has differing degrees of effects on splicing suggesting functional differences between related hnRNPs (35). Furthermore, the localization and functions of these proteins are not always confined to the nuclear compartment where RNA processing and maturation occur in eukaryotes (33, 34, 36–38).

We have demonstrated that the association of hnRNP A2 to two *cis*-acting RNA elements is important for cytoplasmic HIV-1 RNA transport in a murine oligodendrocyte RNA trafficking system (9). Because HIV-1 RNA trafficking was found to be dependent on hnRNP A2 expression and selective binding, we named these elements the hnRNP A2 response elements 1 & 2 or A2RE-1 and -2 (9). In our earlier studies using truncated RNAs, the A2RE-1 and A2RE-2 were found to act as RNA transport signals in their respective *gag* and *vpr* RNAs. Both the A2RE-1 and A2RE-2 are selectively bound by hnRNP A2 *in vitro* (9). Furthermore, both A2RE-1- and A2RE-2-containing HIV-1 RNAs were shown to colocalize and co-traffick in RNA transport granules, suggesting that different HIV-1 RNAs are trafficked by the same hnRNP A2-dependent mechanism. However, the A2RE-2-containing *tat* RNA, an mRNA expressed early following infection, was not transported efficiently, but *gag* and *vpr* RNA, RNAs that are expressed late in the replication cycle, were efficiently transported. This suggested that the signals encoded in these RNAs were contextual in the control of cytoplasmic RNA transport by hnRNP A2 (32). To explore the dependence of HIV-1 on the A2REs during HIV-1 replication we examined the relationship between hnRNP A2, the A2REs and the patterns of HIV-1 gene expression. We also explored the impact of wild-type and mutated A2RE sequences on viral replication in human T cells. Our results reveal that the A2REs function in the control of HIV-1 gene expression and have an impact on the export of HIV-1 RNA into the cytosol, the intracellular localization of $pr55^{Gag}$ and Vpr proteins and contribute to Vpr and genomic RNA levels in assembling virions. In addition, we show that hnRNP A2/A2RE-mediated RNA trafficking is important at a late step of the HIV-1 replication cycle.

EXPERIMENTAL PROCEDURES

DNA Proviral Constructs—A2RE-1 and -2 are located at nt 1192–1213 and nt 6157–6178 in HxBc2-based proviral DNA, HxBru (39), respectively. The A2RE proviruses were generated by recombinant PCR using HxBru as template. For A2RE-1, mutations were introduced in internal antisense and sense oligomers that span the A2RE-1 and 5′ SphI (SphI Sense: 5′-TCCAGTGCATGCAGGGCCTAT-3′) and 3′ ApaI (ApaI Antisense: 5′-TTGCAGGGCCCCTAGGAAAAAG-3′) containing flanking oligomers were used for PCR amplification of a 586-bp fragment. The resultant PCR fragments were digested and cloned directionally into the *gag* open reading frame to replace wild-type sequences. The A2RE-2 proviruses were also generated by PCR mutagenesis using a SalI-KpnI fragment in the vector pIIIEx7 (a Tat, Rev, and Nef expressor) as template (40). Following religation into pIIIEx7 and selection for positive clones, a SalI-BamH1 fragment was directionally inserted into HxBru. A SphI-ApaI fragment from a provirus that harbors two silent point mutations in the A2RE-1 (A5G, A8G) was cloned into the provirus harboring A8G, T5C mutations in the A2RE-2 to produce A2RE 4Mut provirus, harboring two point mutations in each A2RE. In some experiments the 4Mut provirus was used (Fig. 1). Transient expression studies using a Tat cDNA expressor construct harboring the A8G, T5C mutations demonstrate that Tat is not expressed because *tat* mRNA is not translated.[2] Because 4Mut harbors these mutations, we supplied Tat *in trans* (41) to make up for deficits in Tat synthesis. The A8G mutations introduced in the A2REs are silent in both *vpr* and *gag* RNAs but the A2RE-2 A8G changes the Tat 2nd amino acid in the overlapping tat open reading frame from Glu^2 to Gly^2. This mutation does not have a repercussion on Tat structure as shown by Rice *et al.* (42), on HIV-1 expression levels, or processing (see Figs. 2A and 7B), or on its ability to transactivate the LTR.[2] The ability of Tat to interact with TAR RNA or cyclin T binding is not influenced by the N-terminal domain as shown previously (43), and Rev expression levels are likewise unaffected (data not shown). The proximity of the A2RE-2 mutations to splicing ESS and ESE does not influence HIV-1 RNA splicing as we show in *in vitro* splicing assays using homologous (HIV-1 sequences) and heterologous (non HIV-1 sequences) splicing substrates (data not shown).

Immunoprecipitations, RT-PCR, and Polysome Isolation—COS7 or 293T cells were transfected with HxBru or A2RE mutant proviruses. 36–40 h after transfection, total cell lysates were prepared by using Nonidet P-40 lysis buffer for 30 min on ice, followed by centrifugation to remove cellular debris. An aliquot representing 25% of the cell lysates was used in a Western blot analysis for Gag, hnRNP A2, or hnRNP A1. Normalized amounts of cellular proteins were immunoprecipitated with either a mouse anti-hnRNP A2 or rabbit anti-hnRNP A1 (44) (or rat anti-hnRNP A3, Ref. 45 and data not shown) and the immunoprecipitations were verified in Western analyses prior to RT-PCR. DNA was digested with DNase I treatment from the remaining of the immunoprecipitates (Invitrogen, Mississauga, ON) followed by proteinase K digestion and subsequent RNA purification by phenol/chloroform extraction and ethanol precipitation as described (25). Extracted RNA was used in RT-PCR analysis for genomic and spliced HIV-1 RNAs essentially as described before (23, 25) using the Thermascript One-Step RT-PCR kit (Invitrogen), using primers to generate a 280-bp fragment (for total spliced and unspliced HIV-1 RNAs) or 450-bp fragment specific to genomic, unspliced RNA. For input control, total RNA was purified from 10% of lysates and used in RT-PCR analysis to amplify genomic HIV-1 or *gapdh* RNA (23). Immunoprecipitation using preimmune mouse or rabbit serum and an RNase A-treated sample were included as negative controls and to monitor DNA contamination in samples and nonspecific immunoprecipitation. Total cellular RNA purified from HIV-1-transfected cells served as a control in amplification and RT reactions. Interactions were calculated by relating the ratio of immunoprecipitated genomic RNA to the total hnRNP A2 or hnRNP A1 signal obtained in the immunoprecipitations in three separate experiments. The deviation from the average was calculated to be no more

[2] V. Bériault and A. Mouland, unpublished data.

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than 5% for hnRNP A2 binding and 12% for hnRNP A1 binding. Only PCR signals that fell within the linear range of this assay were used in the quantitation.

Polysome isolation and immunoprecipitation were performed essentially as described before (44). Polysomes were purified by stepwise ultracentrifugation and an equal amount of polysomes, determined by optical density (OD), were controlled for *gapdh* RNA levels by RT-PCR. Equal quantities of hnRNP A2-containing polysomes were subsequently immunoprecipitated using a mouse hnRNP A2 antiserum (EF67) (44), and the purified RNA was used in RT-PCR analysis for total and genomic HIV-1 RNA, as described above. β-actin mRNA was quantitated in immunoprecipitates by RT-PCR using the following 5′ and 3′ PCR primers: β-Actin (sense): 5′-GTCGTCGACAACGGCTCCG-GCATG; β-Actin (antisense): 5′-CCTTGGGGTTCAGGGGGGCCTCGG, which were designed to amplify a 300-bp fragment in both human and mouse cDNAs. *gapdh* mRNA was also identified in immunoprecipitates using an PCR primer set as described above.

Northern Blotting, Metabolic Labeling, Immunoprecipitation, and Western Analyses—Wild-type and A2RE proviruses were transfected in COS7 or 293T cells. Total RNA was extracted using TRIzol LS Reagent (Invitrogen) from cells at 36–40-h post-transfection, followed by Northern blotting using a [^{32}P]dCTP-labeled cDNA probe to the HIV-1 untranslated region (25, 39, 46). A portion of the cells was starved in methionine-free medium for 2 h and pulsed with 400 μCi/ml Trans-Label (ICN) for 20 min. Cell and viral lysates were sequentially immunoprecipitated using an anti-p24 (ABI Technologies, Inc), an anti-Vif (from the NIH AIDS Research Reference and Reagent Program; kindly provided by Dr. Bryan Cullen), an anti-Vpr (46), a rabbit anti-Rev (raised to recombinant Rev protein, A. W. C.) and a rabbit anti-Tat antiserum (25). For Western blot analysis on viral preparations, rabbit anti-Vpr antiserum R3.7 (46) was used at 1:500 in PBS with 5% dry milk (Carnation).

Immunofluorescence and Fluorescence in Situ Hybridization (FISH) Analyses—COS7 cells were fixed in 4% paraformaldehyde in PBS for 20 min followed by permeabilization with 0.2% Triton X-100 for 10 min at 16–20- or 36–40-h post-transfection depending on the experiment. Cells were washed with PBS, pH 7.2 and blocked with 10% dry milk in PBS. Anti-p24 (to identify pr55^{Gag} and its mature products), anti-Vpr and anti-Vif antisera (see above; and generously provided by Dr. Klaus Strebel, National Institutes of Health for the data presented in Supplemental Fig. S1-B) were used at 1:250. Secondary fluorophore-conjugated antisera (Alexa Fluor 488 and 564) were obtained from Molecular Probes. For FISH/immunofluorescence co-analyses experiments at 16–20- or 36–40-h post-transfection, the FISH analysis was performed first. Following fixation and permeabilization, cells were treated with DNase I (Invitrogen) for 30 min and washed. KS-*pol*Bru was prepared by directional cloning of a 236 bp PCR product encoding the pol region (nt 1724–1960) (5). An antisense RNA probe was prepared by *in vitro* transcription with digoxigenin-labeled UTP as suggested by the manufacturer (Roche Applied Science) and as described (47). The proviral constructs HxB2-M4 (kindly provided by Dr. Michael Green, Ref. 5) and pMRev(-) (from the National Institutes of Health AIDS Research Reference and Reagent Program; kindly provided by Dr. Reza Sedaie, Ref. 48) were used as controls. In some experiments the nucleic acid stain (with a preference for RNA) SYTO14 (Molecular Probes) (49) was used at 1:400 in PBS to stain for total RNA in cells. Protein and RNA localization patterns presented are representative of at least four independent experiments from 100 to 200 cells per experimental condition.

Laser Scanning Confocal Imaging Analyses, and Image Processing—All images were acquired by laser scanning confocal microscopy. Confocal laser microscopy was performed on a Zeiss LSM 410 (Carl-Zeiss) equipped with a Plan-Apochromat 63x oil immersion objective and an Ar/Kr laser. Alexa Fluor 488 and 568 images were obtained by scanning the cells with 488-nm and 568-nm lasers and filtering the emission with 515–540-nm and 575–640-nm bandpasses, respectively. Red and green images were scanned sequentially to minimize cross-talk and then they were merged. The Differential Interference Contrast (DIC) images were obtained by transmitted light using the 543 nm laser and in some experiments shown this is presented as a blue background, the color being artificial but provides for increased resolution of the cell contour. Images were digitized at a resolution of 512 × 512 pixels. The approximate confocal thickness is 1 μm. All images were directly imported Adobe Photoshop version 6, processed to generate monochromatic images representing protein or RNA staining and then imported into Adobe Illustrator version 9 for figure montage shown in the article.

RNase Protection Analysis (RPA)—To quantitate spliced and unspliced RNAs in cellular extracts and purified viral preparations, RPA was performed as we described (50). Following transfection, RNA was isolated from equal quantities of cellular extracts, or for purified virus, RNA was isolated from equal quantities of p24-equivalents quantitated by a p24 ELISA (23) using TRIzol LS reagent. The radiolabeled RNA probe complementary for HxBru was gel purified and prepared exactly as described (50) and designed to identify unspliced, spliced, and total HIV-1 RNA in cellular and viral RNA preparations (51). RPA analyses were performed using the RPAII Kit as suggested by the manufacturer (Ambion). Protected RNA fragments were separated on denaturing 5% polyacrylamide/urea gels and quantitation of the autoradiographic signals obtained was performed by scanning densitometry with the Molecular Analyst software (Bio-Rad). The results presented for genomic RNA encapsidation were related to the signals obtained in HxBru. Student's unpaired t test was used to test for significant differences between the means. $p < 0.05$ was judged significant.

In Vitro Splicing Assays and Analysis of HIV-1 RNA 1.8- and 4-kb Spliced RNA Products—The homologous *in vitro* HIV-1 splicing constructs, pHS1-X and pHS1-X-ESS4 were generously provided by Dr. Marty Stoltzfus (University of Iowa). In order to introduce the A2RE-2 A8G mutation into pHS1-X, recombinant PCR was performed using the sense and antisense oligomers harboring the A8G mutation (in small case), in the A2RE-2: sense: 5′-GAAATGGgGCCAGTAGATCCT and antisense: (5′-AGGATCTACTGGCcCCATTTC). Flanking oligomers haboring a 5′-XbaI restriction site (5′-ATATGCGGCCGCTCTAGAACTA-GTGG) and a 3′-oligomer harboring an XhoI site (5′-ATATGGCCCCC-CCTCGAGTACTACTA) were used in the PCR. PCR products were restricted and then cloned back into the pHS1-X Bluescript SKII (Stratagene) backbone. Clones were verified by DNA sequencing. Splicing activity was calculated as described previously by calculating the uridine content in spliced RNA products (52). Heterologous A2RE splicing constructs were prepared by blunt-end cloning of 21-base pair A2RE-1 or A2RE-2 DNA duplexes in intron sequences. Two blunt-ended ligations were sequentially performed at unique EcoRV and SmaI sites of the parental transcription/splicing vector 68.1. The control splicing vector that contains two copies of the high affinity ABS hnRNP A1 binding elements at these sites was also included in this assay (53). In this case, the inclusion of two ABS in intronic sequences promotes distal 5′-splice site utilization because of the binding hnRNP A1 on these elements. For both *in vitro* assays, radiolabeled pre-mRNAs were prepared by *in vitro* transcription in the presence of tri-methyl cap analogue and [^{32}P]UTP (1000 Ci/mmol; ICN), gel purified, and used in both types of *in vitro* splicing reactions at 15,000 cpm per reaction at 30 °C for 2 h exactly as described (54). Splicing products were separated on 6% denaturing polyacrylamide gels and exposed to film. Identification of the single-spliced (4 kb) and multiple-spliced (1.8 kb) HIV-1 RNAs using RT-PCR was performed exactly as described recently (1, 23). These assays were performed three times.

Viral Replication Analysis, p24, and Reverse Transcription Assays—First round viral replication kinetics was performed by infecting 500,000 MT4 cells with 300,000 cpm of wild-type or A2RE mutant viruses generated in 293T cells as described (39). At 2-day intervals, aliquots were taken for RT or p24 assay as described (25, 55). For second round replication kinetics, equal quantities of MT4 cells were infected with 10 ng of p24 of virus from peak fractions, and aliquots were collected at 2-day intervals. At each time point, cells were washed and replated at 500,000.

For sequencing analysis, RNA was extracted from 250 μl of cell-free viral supernatant collected at the peak of viral production for wild-type and each A2RE mutant using TRIzol LS according to the manufacturer's instructions. The RNA was reverse-transcribed using the Thermascript One-Step RT-PCR kit using an oligonucleotide set (SphI Sense: 5′-TCCAGTGCATGCAGGGCCTAT-3′ and ApaI Antisense: 5′-TTG-CAGGGCCCCTAGGAAAAAG-3′) that amplifies a 586-bp PCR product that encompasses A2RE-1, or an oligonucleotide set (SalI sense: 5′-GTCGACATAGCAGAATAGGC-3′ and SpeI antisense: 5′-GCAATAG-CAGCATTACTAGTTCTC-3′) that amplifies a 318-bp PCR product that encompasses A2RE-2. The amplified fragments were then used in a direct sequencing reaction using the Thermo Sequenase Cycle Sequencing kit (USB) and loaded on a denaturing 5% polyacrylamide gel for analysis.

Real-time PCR to Study Reverse Transcription—Wild-type and A2RE virus were produced in 293T cells and used to infect Hela-CD4-LTR-β-galactosidase cells (P4 cells) (56). Real-Time PCR was performed to identify early minus-strand strong-stop DNA as described (57) with the following modifications. 100 ng of DNase-treated virus from 293T cells was used to infect 1×10^5 P4 cells, and cells were harvested at 8 h post-infection. DNA was isolated using a DNAeasy Tissue Kit (Qiagen), and the DNA was eluted with water. Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Applied

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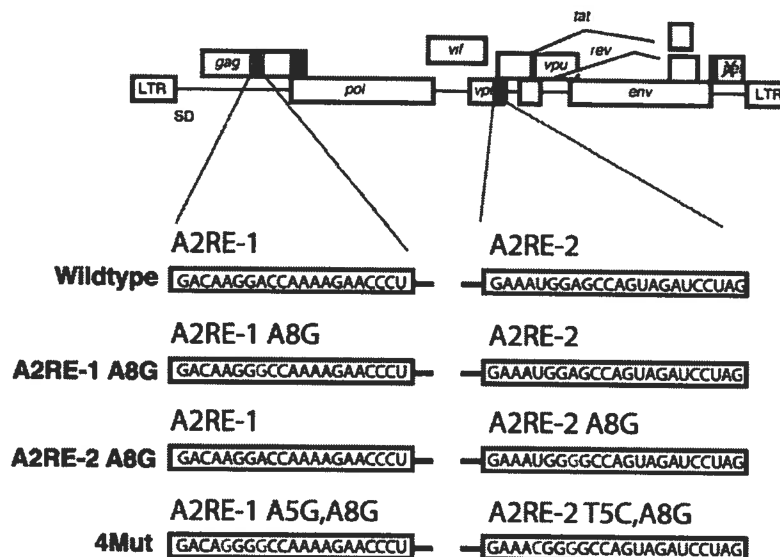

FIG. 1. **Proviral clones used in this study.** Single A8G point mutations were introduced in each A2RE element by recombinant PCR mutagenesis as described under "Experimental Procedures." A2RE-1 A8G (single silent point mutation in A2RE-1); A2RE-2 A8G (single point mutation in A2RE-2); 4Mut contains 2 single point mutations in each A2RE are at the 3rd positions of the codon in *gag* and *vpr* open reading frames. See text for discussion on the consequence on *tat* mRNA and *tat* open reading frame. The locations of the nucleotide substitutions in the A2REs are indicated in *red*: or 4Mut (double point mutations in each A2RE).

Science) according to the manufacturer with 1 ng of genomic DNA in 2 μl, 2.8 mM Mg^{2+} as the final concentration in a final volume of 20 μl. A first denaturation at 95 °C for 10 min was followed by 45 cycles of 95 °C for 10 s, 68 °C for 5 s and 72 °C for 6 s. The standard curve was generated using linearized plasmid DNA, and this assay was linear between 200 and 10^4 DNA copies. Melting curve analysis showed a single PCR product. The PCR products were also verified by gel electrophoresis.

RESULTS

A2RE Mutagenesis Compromises the Interaction of hnRNP A2 to HIV-1 Genomic RNA—The principal notion that hnRNP A2 association to HIV-1 RNA is critical for A2RE function was shown in a murine oligodendrocyte system (9). To test this in a proviral expression context, we introduced mutations in the A2REs in the context of proviral DNA shown in Fig. 1 and expressed these in COS7 cells. Whole cell extracts were prepared from mock- and provirus-transfected cells as described under "Experimental Procedures." The expression levels of $pr55^{Gag}$, hnRNP A1, and hnRNP A2 were assessed prior to immunoprecipitation in cell lysates (Fig. 2*A*). Additional transfections were performed using HxBru for controls in the subsequent immunoprecipitation and RT-PCR analyses (last 3 lanes: HxBru+RNAseA, HxBru preimmune serum, and HxBru Cell RNA). In order to determine if the A2RE mutations in the HIV-1 RNA affected binding of hnRNP A2, it was immunoprecipitated using a specific IgG-purified monoclonal antiserum, EF67 (44) (Fig. 2*B*). This antiserum specifically immunoprecipitates hnRNP A2 and a minor band for hnRNP B1; the use of a preimmune serum did not immunoprecipitate hnRNP A2 (Fig. 2*B*, *last lane*). The specificity of hnRNP A2 interaction with the HIV-1 A2REs was important to address for two reasons. First is the rather promiscuous nature of hnRNPs to bind and modulate HIV-1 RNA splice site selection on adjacent ESS and ESE elements as shown in *in vitro* splicing and interaction assays, for example (28, 29, 59). The second reason is related to the fact that other hnRNPs (hnRNP A1 and hnRNP A3) were shown to bind to a similar, yet not identical, A2RE element in the mouse *mbp* mRNA (60). To verify hnRNP binding specificity, hnRNP A1 was also immunoprecipitated from total cell extracts using a IgG-purified antiserum to hnRNP A1. This antibody immunoprecipitated a principal band corresponding to hnRNP A1 (Fig. 2*B*). Inclusion of a preimmune serum control did not immunoprecipitate hnRNP A1, but resulted in a large background smear. Likewise, hnRNP A3 was immunoprecipitated but we could not efficiently immunoprecipitate this hnRNP using our immunoprecipitation conditions (antibodies were generously provided by Dr. Ross Smith, University of Queensland). We next determined the quantity of genomic RNA that was brought down in the hnRNP immunoprecipitates. First, the quantity of HIV-1 genomic RNA was determined prior to immunoprecipitation (as in Fig. 2*A*) by semi-quantitative RT-PCR analysis in cellular lysates. *gapdh* mRNA was amplified as a cellular RNA control (Fig. 2*C*). Either 25 cycles or 20 cycles was used in the final RT-PCR analyses for *gapdh* mRNA and genomic RNA, respectively, so that the signals obtained would fall within the linear range of this assay. RT-PCR was performed on RNA isolated from equal quantities of cellular lysate (normalized for HIV-1 genomic RNA as in Fig. 2*C*) to determine the quantity of hnRNP A2 associated to genomic RNA in wild-type and A2RE A8G-expressing cells following immunoprecipitation by either anti-hnRNP A1 or anti-hnRNP A2 (Fig. 2*D*). We found a significant reduction in genomic RNA in the hnRNP A2 immunoprecipitate (by 35% in A2RE-1 A8G and by more than 80% in A2RE-2 A8G (Fig. 2*E*); there was no more than a 5% variation in three independent experiments). Approximately equal quantities of genomic RNA were co-precipitated with anti-hnRNP A1 in all proviruses (range 83–112% wild-type levels; Fig. 2*E*). RNase A treatment of the purified RNA prior to RT or the use of a preimmune serum did not yield detectable PCR products and demonstrated that the signals obtained were specific to the co-immunoprecipitation of hnRNP (A1 or A2) and genomic RNA (Fig. 2*D*). These data demonstrate that the specific point mutations introduced in each of the A2REs resulted in lowered hnRNP A2 binding while, in contrast, the association of hnRNP A1 to HIV-1 RNA was not affected by these introduced mutations. Consistently, a 10bp deletion immediately upstream of the *tat* ESS2 that coincides with the A2RE-2 does not affect hnRNP A1 association (59). These observations support the notion that general hnRNP binding is not affected by the introduced A2RE point mutations.

We demonstrate here that the A2RE mutants specifically prevented hnRNP A2 binding in whole cell lysates to the HIV-1 RNA during proviral gene expression. The relative binding efficiencies that we find here in COS7 cells correspond quantitatively to the *in vitro* binding properties of hnRNP A2 to the HIV-1 A2REs that we have shown previously in that mutagenesis of the A2RE-2 resulted in a more dramatic loss of hnRNP A2 than that found for the A2RE-1 (9). Identical hnRNP A2/

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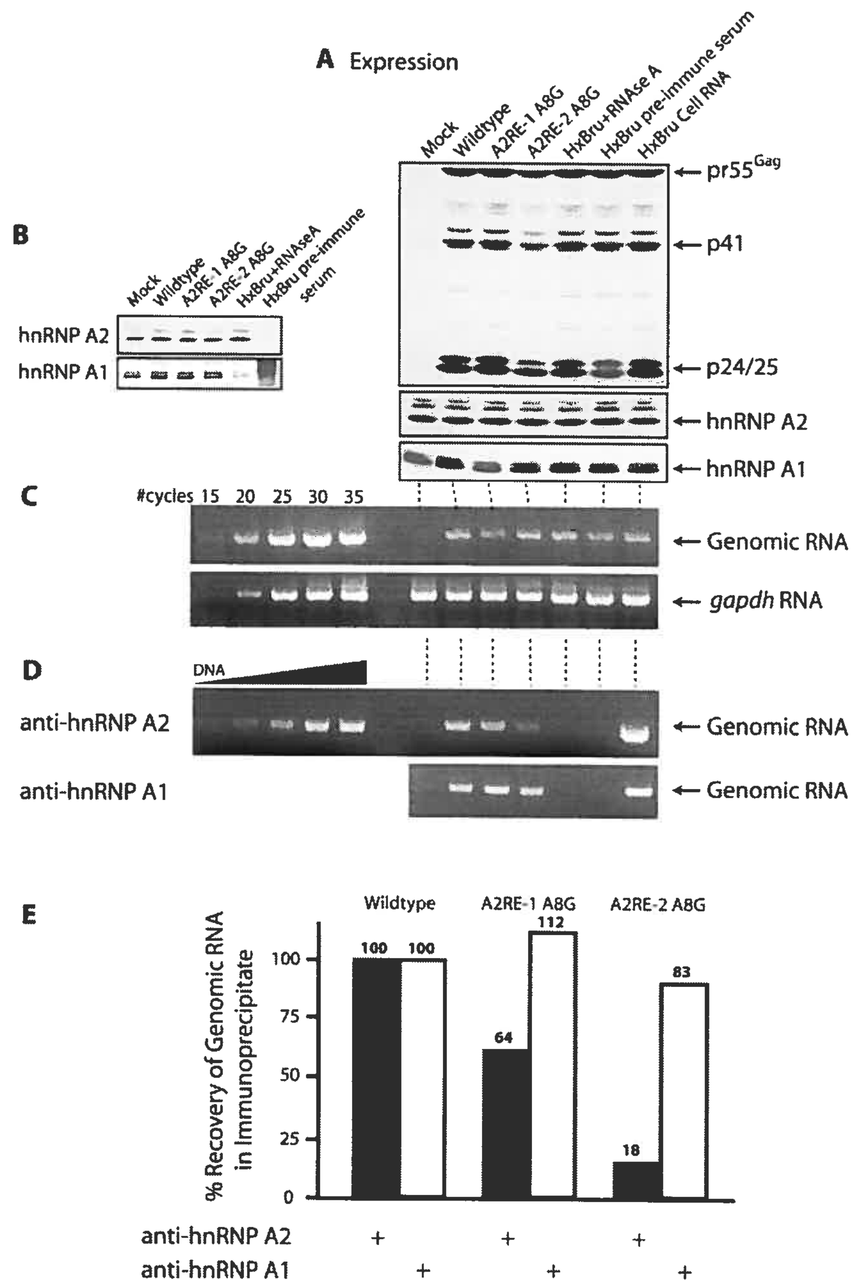

FIG. 2. **A2RE A8G blocks hnRNP A2 association to genomic RNA in whole cell lysates.** *A*, COS7 cells were mock-transfected or transfected with HxBru, A2RE-1 A8G, or A2RE-2 A8G. Expression levels of Gag proteins, hnRNP A2, and hnRNP A1 in equal quantities of whole cell extract (determined by Bradford protein assay) are shown. The last three HxBru lanes are included because lysates derived from these transfections serve as controls in the RT-PCR reactions shown in *C* and *D*. *B*, hnRNP A2 and hnRNP A1 were immunoprecipitated from the cell lysates in *A* and identified by their respective antiserum in Western blot analysis. Approximately equal expression levels of these hnRNPs were observed and the immunoprecipitations were quantitative. The use of a preimmune serum failed to immunoprecipitate either hnRNP. *C*, RNA was extracted from cell lysates in *A*, and a one-step RT-PCR reaction was performed to quantitate input HIV-1 genomic RNA and *gapdh* RNAs in subsequent immunoprecipitation analysis. The PCR cycle number was determined beforehand in order that the signals obtained fell within the linear range of the reaction (25 cycles for HIV-1 RNA and 20 cycles for *gapdh* RNA). *D*, from equal quantities of total cell extracts shown in *C*, either hnRNP A2 or hnRNP A1 (as control) was immunoprecipitated using specific antibodies (anti-hnRNP A2 or anti-hnRNP A1). One-step RT-PCR was performed on the immunoprecipitates to quantitate the amount of bound HIV-1 genomic RNA. Mutagenesis of the A2RE-1 or A2RE-2 blocked genomic RNA association by 36 and 82%, respectively, while this did not affect hnRNP A1 association. This determination was performed in three separate experiments and the average of these experiments is shown in the histogram in *E*. A representative experiment is shown here and there was no more than a 5% deviation in the values determined for hnRNP A2 and no more than a 12% deviation for those calculated for hnRNP A1.

A2RE binding results for both the A2RE-1 and A2RE-2 were obtained at 20-h post-transfection (data not shown). While hnRNP A2 can bind other HIV-1 RNA elements with splicing modulating properties, our data suggest that the association of hnRNP A2 on the A2REs represents a major binding event of hnRNP A2 since we can block this interaction by over 80% with A2RE mutagenesis (Fig. 2*E*). These data also suggest that the A2RE RNA elements synergize to promote hnRNP A2 association to HIV-1 RNA via long range RNA interactions, since mutagenesis of either A2RE results in a loss of the association of hnRNP A2.

A2RE Mutagenesis Results in a Dramatic Change in HIV-1 Genomic RNA Distribution—Abrogation of hnRNP A2 binding directly correlated to its capacity to promote RNA trafficking in an oligodendrocyte system (9). To test if this was the case during proviral gene expression, we determined whether the A2RE sequences had effects on the distribution of HIV-1 RNA. The A2RE proviral mutants were individually transfected in COS7 cells and combined FISH using a *pol*-specific digoxigenin-labeled antisense RNA probe and immunofluorescence analysis on $pr55^{Gag}$ was performed followed by laser scanning confocal microscopy at 40-h post-transfection (Fig. 3, *panels A–R*). Mock-transfected cells did not have any appreciable staining for either genomic RNA of $pr55^{Gag}$ (Fig. 3, *panels A–C*). In wild-type HIV-1, $pr55^{Gag}$ was found in a discrete, punctate pattern throughout the cytosol (Fig. 3, *panel E*). HIV-1 genomic RNA was detected in the nucleus and dispersed throughout the cytoplasm in a discrete, punctate pattern, like the staining pattern obtained for $pr55^{Gag}$, but there was no significant overlap (Fig. 3, *panels D–F*). A minor change in the cytosolic staining of genomic RNA and $pr55^{Gag}$ distribution was found in the A2RE-1 A8G mutant (Fig. 3, *panels G–I*). Markedly less cytosolic RNA staining was consistently observed in this mutant. In contrast, HIV-1 genomic RNA was completely sequestered to the nucleus in the A2RE-2 A8G mutant (Fig. 3, *panel J* and Supplemental Fig. S1-A). The distribution of

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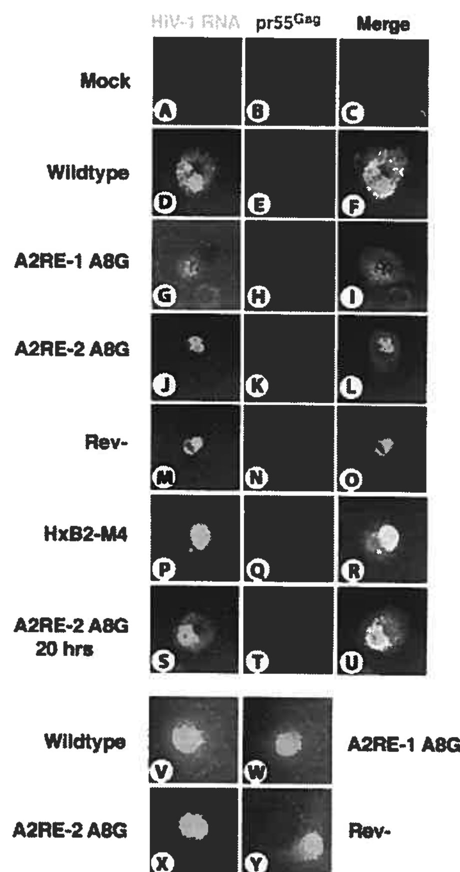

FIG. 3. **A2RE mutations alter HIV-1 genomic RNA localization.** COS7 cells were mock-transfected (*panels A–C*), or transfected with HxBru (wild type) (*panels D–F*), A2RE-1 A8G (*panels G–I*), A2RE-2 A8G (*panels J–L* and *S–U*), pMRev(-) (*panels M–O*) or HxB2-M4 (*panels P–R*) proviruses. Combined FISH and immunofluorescence analysis were performed at 36–40 h (*panels A–R*) or 16–20 h (*panels S–U*) post-transfection. HIV-1 genomic RNA (*green*) and pr55^{Gag} (*red*) were identified by FISH and immunofluorescence analyses as described under "Experimental Procedures." Merged images are shown in *left panels* (*panels C, F, I, L, O, R,* and *U*). *Circles* in *panels A–C* indicate cell nuclei in mock-transfected cells. A2RE-2 A8G expression results in a nuclear localization of genomic RNA at a late replication step only. *Panels V–Y*, SYTO14 nucleic acid staining (*green*) of the indicated proviruses did not show any noticeable changes in the distribution of nucleic acid staining in the nucleus or in the RNA staining pattern found in the cytosol at this time point (40 h) or at earlier time points tested (20 h, not shown). The cell contours are outlined by a *dashed yellow line*. See also Supplemental Fig. S1-A for additional examples of the A2RE-2 A8G phenotype.

genomic RNA in A2RE-2 A8G was found to be virtually identical to that observed in both pMRev(-) and HxB2-M4 (Fig. 3, *panels M* and *P*). However, while pr55^{Gag} expression is absent in pMRev(-) (Fig. 3, *panel N*), the pr55^{Gag} staining pattern in A2RE-2 A8G and HxB2-M4 were found to be similarly localized to the perinuclear region (Fig. 3, *panels K* and *Q*) at this time point (40 h). Strikingly, we did not observe the same pattern of pr55^{Gag} and genomic RNA at an early time point post-transfection for A2RE-2 A8G (Fig. 3, *panels S–U*, see below for discussion). The distribution of pr55^{Gag} and genomic RNA was identical to the gene expression patterns in cells expressing wild-type virus at both 20 h (data not shown) and 40 h (Fig. 3, *panels D–F*). These observations support the idea that genomic RNA is exported to the cytosol for translation and the block that results in genomic RNA nuclear sequestration occurs at a late replication step of the HIV-1 lifecycle. Similar observations were made for HxB2-M4, a proviral mutant that harbors point mutations in the nuclear export signal of MA (5) (compare Fig. 3, *panels J–L* and *P–R*). These data also suggest that the A2RE could represent a dominant signal for HIV-1 RNA localization such that a point mutation within the A2RE-2 sequence appears to interfere with the RNA nucleocytoplasmic export. These altered localization patterns were observed despite equal Rev and pr55^{Gag} expression levels as determined by Western and metabolic labeling experiments (Figs. 2*A* and 7*E*). While pr55^{Gag} localization was more perinuclear in appearance in A2RE-2 A8G, it is nevertheless expressed at near wild-type levels as shown *in situ* (Fig. 3, *panel K*) and in Western blotting experiments (Figs. 2*A* and 7, *A* and *B*). This is in contrast to what we observe using a Rev-defective provirus in which genomic RNA never exits the nucleus and is not translated to produce pr55^{Gag} (Fig. 3, *panel N*). There were no changes in the general pattern of total RNA staining as shown by SYTO14 staining (49) in the A2RE and Rev-defective proviruses at either of the time points tested (Fig. 3, *panels V–Y*).

HnRNP A2-containing Polysomes Contain Reduced Levels of Genomic RNA—In situ and binding assays showed that HIV-1 RNA was sequestered in the nucleus and was not bound by hnRNP A2 as a consequence of A2RE mutagenesis (Figs. 2 and 3). We therefore proceeded to perform a cell fractionation analysis to determine if these observations would be reflected within hnRNP A2-RNA complexes within the cytosol. Although hnRNP A2 is a predominantly nuclear protein, hnRNP A2 has been found in the cytosol of mammalian cells (36) and associated to several mRNAs in an hnRNP A2-containing population of polysomes (44). We proceeded to determine how much HIV-1 genomic RNA was associated to hnRNP A2-containing polysomes using an immunoprecipitation/RT-PCR procedure identical to that described in Brooks and Rigby (44). Cytosolic polysomes were isolated from post-nuclear supernatants and purified by ultracentrifugation from COS7 cells transfected with the A2RE proviruses (Fig. 1). Total HIV-1 RNA (spliced and unspliced) and *gapdh* mRNA levels were first evaluated in polysome extracts by semi-quantitative RT-PCR. Levels of these RNAs were all found to be constant (Fig. 4, *A* and *B*). hnRNP A2 was then specifically immunoprecipitated from equal quantities of polysomes (as determined by OD) as performed above, and levels of HIV-1 genomic RNA were determined by RT-PCR, as described previously (25) (Fig. 4*C*). In the hnRNP A2 immunoprecipitate, markedly reduced levels (50 ± 10%) of genomic RNA were found in hnRNP A2 polysomal fractions prepared from cells expressing A2RE-1 A8G. A more significant reduction of genomic RNA was found in hnRNP A2 polysomal fractions prepared from cells expressing A2RE-2 A8G (70 ± 10%; Fig. 4*C*). RNase A treatment eliminated the RT-PCR signal demonstrating that the signal obtained was due to co-immunoprecipitated RNA. A positive control RNA purified from a wild-type (HxBru)-expressing cellular lysate was also included in this assay. In order to demonstrate specificity, a known hnRNP A2 mRNA substrate in polysomes, β-actin mRNA (44) was quantitated in hnRNP A2 immunoprecipitates by RT-PCR (Fig. 4*D*). Following immunoprecipitation, equal quantities of β-actin mRNA were found to co-immunoprecipitate with hnRNP A2, whereas *gapdh* mRNA was undetectable in the hnRNP A2 immunoprecipitates (not shown) as demonstrated previously (44). While hnRNP A2-containing polysomes contain less HIV-1 genomic RNA when the A2RE A8G proviruses are expressed, the data shown in Fig. 4*A* suggest that A2RE mutagenesis does not result in a general loss in HIV-1 RNA association to polysomes, which would translate into inefficient translation of HIV-1 mRNAs and decreased viral expression levels (see "Discussion" below). While pr55^{Gag} synthesis is not detectably affected when the A2RE mutants are expressed, there was also no general effect on viral protein

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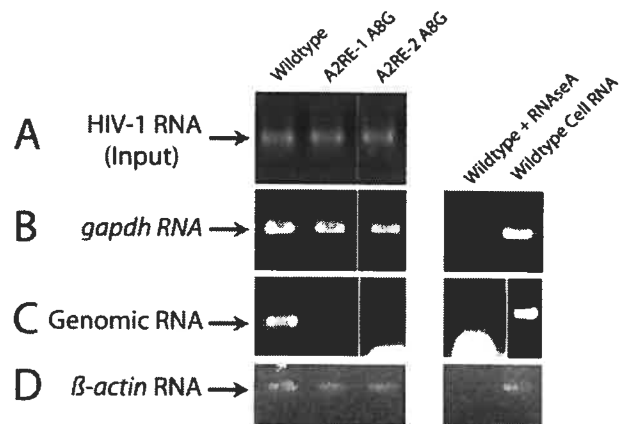
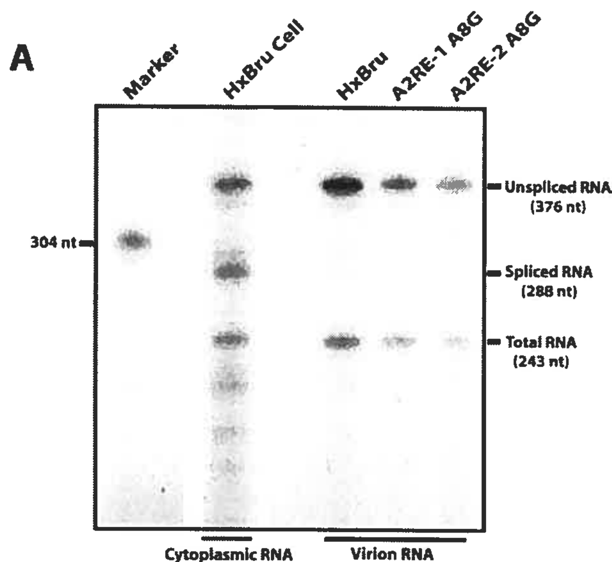


FIG. 4. **A2RE mutagenesis reduces the levels of HIV-1 RNA in the cytoplasmic polysome pool.** Polysome purification and immunoprecipitation were performed on post-nuclear lysates of cells transfected with HxBru (wild type) provirus or the A2RE mutants as described under "Experimental Procedures." *A*, from a corresponding amount of polysome extract (as determined by OD), RNA was purified prior to immunoprecipitation and was used in RT-PCR analysis to amplify HIV-1 RNA (total unspliced and spliced) using an oligomer set to the TAR region and upstream of the major splice donor as described previously (25). *B*, *gapdh* mRNA was concomitantly quantitated by RT-PCR from the same RNA preparation and serves here as a polysome loading control prior to hnRNP A2 immunoprecipitation analyses (23). *C*, equal amount of polysome extract was subsequently immunoprecipitated with the anti-hnRNP A2 antiserum EF67 and RT-PCR was performed using primers specific to unspliced, genomic RNA to determine if A2RE mutagenesis affected genomic RNA association. *D*, amount of the known hnRNP A2 ligand (44), β-actin mRNA, was also identified in the immunoprecipitate, and this was found to be equal in all conditions. Total cellular RNA from HxBru-transfected cells (HxBru Cell RNA) and RNase A treatment of the immunoprecipitate prior to RT-PCR (HxBru + RNase A) served as controls in the amplification and RT reactions.

synthesis (Figs. 2 and 7, and data not shown). Our results that show reduced levels of HIV-1 genomic RNA in hnRNP A2 cytoplasmic polysomes (Fig. 4*C*) are consistent with the levels of genomic RNA that are found in the cytosol as shown in our FISH analyses of the A2RE mutants (Fig. 3, *panels G* and *J*).

A2RE Mutagenesis Results in a Significant Reduction of Genomic RNA Encapsidation in Progeny Virions—We next determined if the nuclear sequestration of genomic RNA was reflected in altered genomic RNA levels in virus particles. To address this question, COS7 cells were transfected with wild type, A2RE-1 A8G, or A2RE-2 A8G DNA. A viral DNA harboring a major deletion (ΔLys^{14}-Thr^{50} (K^{14}-T^{50})) in the NC region of pr55^{Gag} was expressed and included as a negative control for genomic RNA encapsidation (25), and the results are presented in the histogram (Fig. 5*B*). RNA was purified from both cellular and viral extracts as described under "Experimental Procedures." Expression of pr55^{Gag} (or the truncated pr55^{Gag} in the case of NCΔK^{14}-T^{50}) constructs was verified by Western blotting using a rabbit anti-p24 antiserum (data not shown). RNase protection analyses (RPA) were performed to quantitate spliced and unspliced HIV-1 RNAs on equal quantities of cellular RNA and virus (normalized by p24 ELISA as described, Ref. 23) and as we described previously (50). The RPA analysis on equal quantities of virus shown in Fig. 5*A* demonstrates that the nuclear sequestration of HIV-1 genomic RNA observed in A2RE-2 A8G is reflected in a 61% ($p < 0.02$) decrease in genomic RNA in progeny virions generated with A2RE-2 A8G (Fig. 5, *A* and *B*). Genomic RNA encapsidation in the NCΔK^{14}-T^{50} mutant was decreased to 7 (±10%) wild-type levels, as expected (Fig. 5*B*). We can also conclude that the observed 43% reduction in genomic RNA encapsidation of A2RE-1 A8G ($p < 0.02$, Fig. 5*B*) could also reflect the minor, yet detectable changes in the cellular localization patterns of genomic RNA



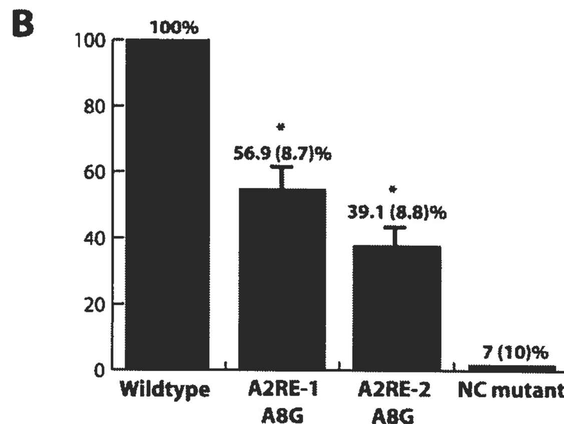


FIG. 5. **A2RE mutagenesis leads to reduced genomic RNA encapsidation in progeny virions.** *A*, RPA of virion-associated HIV-1 genomic RNA was performed using a radiolabeled RNA probe complementary to HxBru *pol* RNA. RNA was isolated from HxBru (wild type)-transfected cells and from equal quantities of virus and analyzed by RPA to show unspliced (376 bp) and spliced HIV-1 RNA (288 bp) species. The total RNA corresponds to the region after the last splice acceptor site and reflects the amount of all spliced and unspliced HIV-1 RNAs (243 bp). In virus, genomic RNA is the predominant form identified in this analysis, and this corresponds to the total amount of RNA in virus. Virions isolated from both A2RE-1 A8G and A2RE-2 A8G contained significantly reduced levels of genomic RNA. *B*, this histogram shows the average levels of RNA encapsidation in five independent assays (±S.E.) with wild-type (*HxBru*) encapsidation levels set to 100%. Genomic RNA encapsidation in the A2RE mutants was significantly reduced (*, $p < 0.02$). There was only 7% RNA encapsidation (compared with HxBru) in NCΔK^{14}-T^{50} as expected (25).

and pr55^{Gag} (Fig. 3, *panels G–I*). This could also reflect the importance of A2RE-1 and its interaction with hnRNP A2 in the assembly of HIV-1 virions, although this does not appear to have any marked effects on viral replication as shown in the replication studies presented later in Fig. 8.

The Cellular Distribution of HIV-1 Proteins Is Mediated by the A2RE—The distribution of candidate proteins that are encoded by A2RE-containing HIV-1 RNAs, including pr55^{Gag}, Vpr and Vif was next examined by indirect immunofluorescence analyses of wild-type and A2RE provirus-expressing cells. COS7 cells were transfected and fixed on glass coverslips. Using antisera to Vpr and p24 (46), Vpr and pr55^{Gag} were

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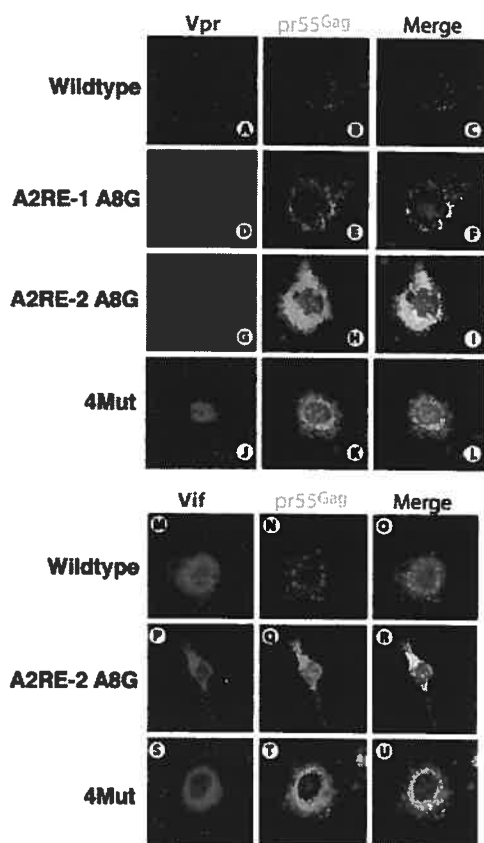

FIG. 6. **Expression of A2RE-2 A8G results in nuclear localization of Vpr during HIV-1 expression.** *Panels A–C*, wild-type-expressing cells (HxBru), Vpr, and pr55^{Gag} co-localize in discrete punctate locations, mostly in the cytosol or at sites of viral assembly as assessed in laser scanning confocal microscopy analysis shown here. *Panels D–F*, A2RE-1 A8G silent mutation did not markedly alter the localization of Vpr or pr55^{Gag} in transfected cells, whereas in the A2RE-2 A8G (*panels G–I*) and 4Mut proviruses (*panels J–L*), Vpr and pr55^{Gag} localization patterns were dramatically altered. pr55^{Gag} distribution appeared more granular and perinuclear and Vpr was found exclusively distributed in the nucleus. Vif distribution was examined in wild-type (*panels M–O*), A2RE-2 A8G- (*panels P–R*), and 4Mut-transfected cells (*panels S–U*). Vif cellular distribution showed diffuse cellular staining and was similar in all conditions (see also Supplemental Fig. S1-B). The staining of pr55^{Gag} appeared more granular and perinuclear similar to that obtained in A2RE-2 A8G (as in *panel K* of Fig. 3). The cell contours are outlined by a *dashed yellow line*.

found to co-localize in punctate staining patterns in wild-type HIV-1-expressing cells as shown by immunofluorescence and confocal microscopy imaging analyses (Fig. 6, *panels A–C*). There was no green or red fluorescence signal when this analysis was performed with a preimmune rabbit serum (data not shown). Mutation of the A2RE-1 modestly affected the localization patterns of pr55^{Gag} (Figs. 6, *panel E* and 3, *panel H*) and Vpr (Fig. 6, *panel D*) when closely compared with wild-type-expressing cells. However, when the localization of Vpr was assessed in cells expressing A2RE-2 A8G, Vpr was found to be almost completely localized to the nucleus (Fig. 6, *panel G*). In A2RE-2 A8G, pr55^{Gag} showed a strong perinuclear staining localization and perinuclear staining (Figs. 6, *panel H* and 3, *panel K* and see Supplemental Fig. S1-A). Of note is that we observed two pr55^{Gag} staining patterns when the A2RE-2 A8G was expressed and one of these is represented in Fig. 6, *panel H*. In the first pr55^{Gag} staining pattern, the more predominant localization pattern was primarily perinuclear (Figs. 3, *panel K* and 6, *panel H* and in Supplemental Fig. S1-A). In this case, pr55^{Gag} appeared to be restricted to the perinuclear space, but was never detected in the nucleus. In ~5% of the cells observed however, we found not only strong pr55^{Gag} staining in the perinuclear space but also intense staining in the nucleolus when A2RE-2 A8G was expressed (data not shown). This is shown by example in Fig. 6, *panels J–L* that shows strong nucleolar expression of pr55^{Gag} when 4Mut was expressed (Fig. 1). The Vpr and pr55^{Gag} expression patterns in 4Mut were nearly identical to those obtained with A2RE-2 A8G and the relative proportion of pr55^{Gag} in (5%) and out of the nucleolus (95%) was quantitatively similar. We do not understand at present the reasons for the nucleolar sequestration of pr55^{Gag}, but this behavior may relate to nuclear NES function of pr55^{Gag} in genomic RNA nuclear export, especially since the A2RE and NES phenotypes are nearly identical with respect to HIV-1 RNA distribution (Fig. 3). The specificity of this effect for Vpr and pr55^{Gag} is underscored by the observation that single or double mutation of the A2RE-2, which is contained in the *vif* mRNA, did not alter the localization of Vif in HIV-1-expressing cells (Fig. 6, *panels P* and *S* and Supplemental Fig. S1-B) when compared with wild type (Fig. 6, *panel M*).

Effects of the A2REs on HIV-1 RNA Splicing—The A2RE-2 A8G mutation falls within a region of the HIV-1 RNA that possesses adjacent exon splicing silencer (ESS) and exon splicing enhancer (ESE) elements. While the A2RE-2 does not coincide with the *tat* ESS2 element (52), it encompasses a recently identified ESE element (59). HnRNPs have also been shown to interact with this region *in vitro* and it was tantamount to rule out any effects of the A2RE-2 A8G mutation on splicing activity. We therefore investigated the effects of the A2RE and the corresponding nucleotide point mutation on splice site selection using three different assays currently used in investigations on HIV-1 RNA splicing. We first tested the A2RE-2 A8G mutation in a well-characterized *in vitro* splicing assay using a homologous, *bona fide* HIV-1 splicing construct pHS1-X and a corresponding ESS2 mutant pHS1-ESS4 (52). These constructs will test for any modulation of ESS function by the introduced A2RE-2 A8G mutation. Their use will allow us to determine if the introduced mutation in the A2RE-2 generates a new ESE element if multiple spliced products are observed on the gels, for example. The A2RE-2 A8G mutation was introduced in pHS1-X by recombinant PCR to generate pHS1-X/A2RE-2 A8G. Nuclear extracts were prepared (61) and uniformly ^{32}P-labeled RNAs were generated from linearized pHS1-X, pHS1-X, and pHS1-X/A2RE-2 A8G DNAs and gel-purified. *In vitro* splicing reactions were carried out as described previously to identify effects on splicing activity (62). The RNA substrate generated by pHS1-X showed few spliced mRNA products as expected, while the RNA substrate from *in vitro* transcription of pHS1-ESS4, which bears 4 point mutations in the ESS2, showed a notable enhancement in the generation of the spliced mRNA, demonstrating the lowered ESS activity (Ref. 63 and data not shown). *In vitro* splicing of pHS1-X/A2RE-2 A8G resulted in a pattern that was identical to pHS1-X demonstrating that the A2RE-2 A8G mutation did not influence ESS2 activity, generate an active ESE element or influence alternate splice acceptor site usage in this pre-mRNA.

To identify each HIV-1 RNA species, we also used an RT-PCR approach followed by gel electrophoresis (1, 23). This analysis, while only semi-quantitative, separates and identifies by molecular weight single- and multiple-spliced HIV-1 RNAs in denaturing polyacrylamide gels. RT-PCR was performed on purified total RNA from cells transfected with wild-type, A2RE-1 A8G, and A2RE-2 A8G DNAs. Genomic RNA and the spliced HIV-1 RNAs were identified by RT-PCR followed by agarose gel electrophoresis as described under "Experimental Procedures." These gel analyses demonstrate that the intro-

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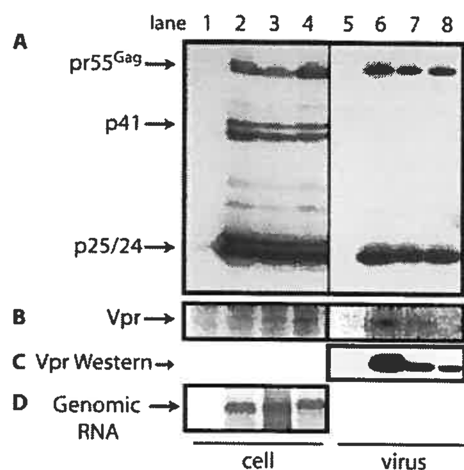

FIG. 7. **Gene expression levels of wild-type and A2RE proviruses.** Cells were mock-transfected (*lanes 1* and *5*), transfected with HxBru (*lanes 2* and *6*), A2RE-1 A8G (*lanes 3* and *7*), or with A2RE-2 A8G (*lanes 4* and *8*) proviruses. Viral protein expression levels were assessed in cellular extracts (*lanes 1–4*) and in purified virus preparations (*lanes 5–8*) following metabolic labeling using radiolabeled amino acids. $pr55^{Gag}$, p25/24, and Vpr, (*A* and *B*) or by Western analyses for Vpr (*C*) as described under "Experimental Procedures." HIV-1 genomic RNA was quantitated by Northern blot analysis (*D*). $pr55^{Gag}$ synthesis and processing were not affected by A2RE mutagenesis (*A* and see Fig. 2*A*). However, Vpr virion incorporation was found to be reduced in both A2RE-1 and A2RE-2 A8G mutants as shown in metabolic labeling and Western experiments (*B* and *C*, respectively). Rev and Vif expression levels were assessed by immunoprecipitation analyses and these were not influenced during A2RE proviral expression (data not shown).

duced A8G mutations in the A2REs did not significantly alter the abundance of the unspliced, 1.8 kb, and 4 kb transcripts during HIV-1 proviral gene expression (data not shown). Further detailed analyses of these transcripts also revealed that there were no marked changes in the abundance or patterns of HIV-1 singly-spliced (4 kb) and multiply spliced (1.8 kb) mRNAs in experiments in which radiolabeled dCTP was included in the last 2 cycles of the PCR reaction (data not shown) (23). There were no general changes in the pattern or quantities of the spliced RNA species.

A third splicing assay shown examined if either of the HIV-1 A2REs behaved like high affinity hnRNP A1-binding sites in alternative splice site selection. The model pre-mRNA used in this last study contains portions of exons 7 or 7B of the hnRNP A1 gene paired with the adenovirus L2 exon (54). While this pre-mRNA is spliced almost exclusively to the proximal 5′-splice site, the inclusion of high-affinity binding sites for hnRNP A1 (ABS) promotes a shift toward the distal 5′-splice site such that it becomes selected predominantly (data not shown). As shown previously, hnRNP A2 also binds to this ABS element to promote distal 5′-splice site utilization (54). Pre-mRNAs carrying either the A2RE-1 or A2RE-2 element were spliced predominantly to the proximal 5′-splice site similar to that obtained with the pre-mRNA 68.1 that contains no ABS insert. These data demonstrate that hnRNP A2 is not bound or is bound in a manner that does not influence splicing modulation, consistent with our RNA and expression analyses (Figs. 2 and 7). In addition, our data demonstrate that while hnRNP A1 can efficiently modulate splice site in this assay when hnRNP A1 high affinity sites (ABS) are present, the A2REs do not possess hnRNP A1 binding capacity, at least in these *in vitro* splicing conditions. Our data also suggest that the binding of additional factors to these elements may prevent hnRNP A2 from modulating 5′-splice site selection. Mutagenesis of the A2REs in this context also has no effect on the *in vitro* splicing reactions. Immunodepletion or add-back type experiments also demonstrated that hnRNP A2 has no influence on the splicing of A2RE-containing pre-mRNAs (data not shown).

Effects of the A2REs on HIV-1 Gene Expression and Virion-incorporated Vpr—Since one of hnRNP A2 functions is to derepress translation of transported A2RE-containing transcripts (64) we determined if viral gene expression levels were influenced by the A2REs. $pr55^{Gag}$ levels appeared to be constant and processing was normal in virions (Figs. 2*A* and 7*A*), confirming our *in situ* analyses of $pr55^{Gag}$ (Figs. 3 and 6). When we examined Vpr incorporation levels in purified virions by metabolic labeling (Fig. 7*B*) or in independent studies by Western analyses (Fig. 7*C*), Vpr virion incorporation was found to be diminished in the A2RE-1 A8G and in a more pronounced manner in the A2RE-2 A8G mutant, while there was a small, yet detectable increase in cellular Vpr levels (Fig. 7*B*, *lane 4*), likely because of decreased incorporation levels in virions. Similar to what we found earlier (Figs. 2 and 4), mutation of each A2RE had graded effects, with the A2RE-2 A8G having the most profound phenotype. Each A2RE element independently influenced Vpr incorporation levels (Fig. 7, *B* and *C*), consistent with the decreased or negligible levels of Vpr in the cytosolic compartment during the expression of the proviral A2RE A8G mutants (Fig. 6, *panels D* and *G*). The effects on Vpr localization (Fig. 6) and incorporation into virions (Fig. 7, *B* and *C*) cannot be attributed to the RNA coding potential of either *gag* or *vpr* RNA since the A2RE mutations are silent in both of these mRNAs (but not in *tat* mRNA; see later). In addition, Vpr incorporation is not influenced by genomic RNA encapsidation levels as shown in earlier studies (65). Cellular Vif synthesis levels corresponded to those of $pr55^{Gag}$ but we could not detect Vif in virions (data not shown). Its incorporation would likely be compromised in the A2RE mutants due to reduced genomic RNA encapsidation levels (66). Finally, equal quantities of steady-state HIV-1 genomic RNA were found in transfected total cell lysates (Fig. 7*D*) and this is reflected in constant Gag expression levels indicating that viral gene transcription or RNA stability were not altered with the introduced A2RE A8G mutations.

The HIV-1 A2RE Influences Viral Replication—Because of the dramatic changes in viral RNA and protein distribution, we investigated the impact of A2RE on viral replication. MT4 lymphocyte cells were infected with either wild-type or A2RE viruses and viral production was measured every 2 days (Fig. 8*A*). At each time point, cells were washed and replated at the same cell density. Wild-type HIV-1 and A2RE-1 A8G had identical replication peaks at about 4 days post-infection, but A2RE-1 A8G showed a diminished peak in several of the kinetics studies performed. The A2RE-2 A8G virus showed a 2–6-day replication delay depending on the experiment (Fig. 8*A*). For second round replication analyses, we isolated peak virus and infected MT4 cells and measured viral replication every 2 days. In the case of A2RE-2 A8G, there was a rapid reversion to wild-type kinetics in the second round of infection (Fig. 8*B*). Moreover, while we observed an even longer initial delay of 6–10 days of 4Mut (in which the *tat* initiation codon is mutated from AUG to ACG), sequence reversion occurred in the 4Mut virus in the second round of infection and showed wild-type kinetics (Table I). In support of the importance of the A8 nucleotide of the A2RE-2 for HIV-1 replication, a G8A reversion occurred in the A2RE-2 A8G virus. There was no evidence for nucleotide reversions in the A2RE-1 viruses. We propose that the replication profiles are due to marked perturbations in viral protein and RNA gene expression patterns in cells and virions (Figs. 3 and 6), similar to what was concluded for a MA RNA binding domain proviral mutant (6).

In order to determine if the observed replication profiles

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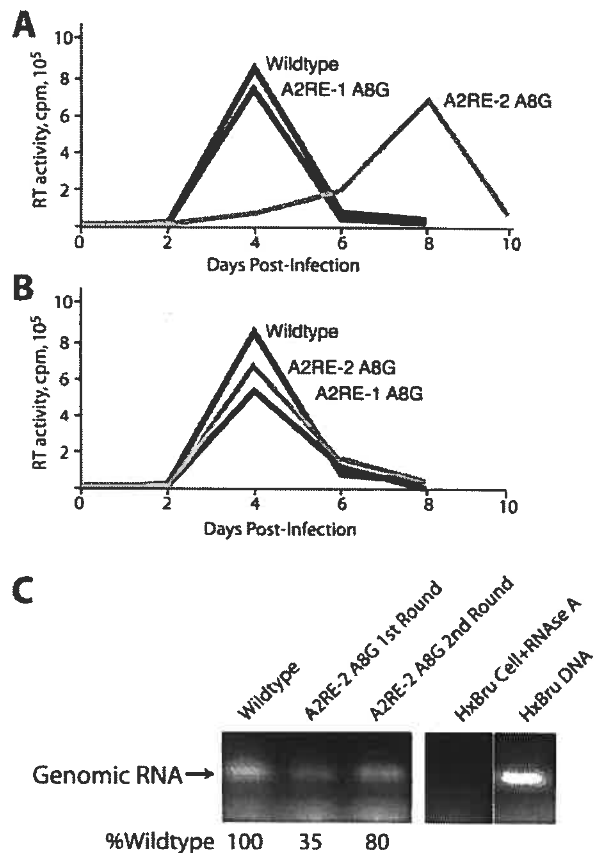

FIG. 8. **Viral replication kinetics analysis of A2RE-1 and A2RE-2 viruses in human T lymphocytes.** Virus wildtype (HxBru), A2RE-1 A8G, or A2RE-2 A8G) was produced in 293T cells and 300,000 cpm virus as determined by exogenous RT assay were used to infect human MT4 cells as described under "Experimental Procedures." *A*, viral production was followed every 1–2 days and assayed for reverse transcriptase activity in the first round of infection. In the second round of infection (*B*), equal amounts of purified cell-free virus from peak fractions derived from experiments presented in *A* (10 ng of p24-equivalents) were used to infect MT4 cells. Virus was harvested every 2 days post-infection and assayed for RT activity. Sequence analysis shows that a reversion to wild type occurs in the second round of kinetics resulting from a G → A reversion in the A2RE-2 A8G sequence (Table I). *D*, viral RNA was purified from equal quantities of peak-minus-1 virus fractions shown in 1st (*A*) and 2nd (*B*) round and used in RT-PCR to quantitate genomic RNA. Numbers *below* gel represent quantity of genomic RNA in virus relative to wild-type content (similar in 1st and 2nd round of infection). An 80% recovery of genomic RNA was observed in the second round of infection in A2RE-2 A8G virus.

were attributable to genomic RNA content in virus, we determined the genomic RNA content in virus derived from the A2RE-2 A8G virus 1 day before peak replication in the first and second round of infection shown in Fig. 8, *A* and *B*. RT-PCR was performed on purified viral RNA to identify unspliced, genomic RNA as described under "Experimental Procedures." In the first round, there was a 65% decrease (average of two determinations) in viral genomic RNA in A2RE-2 A8G virus compared with wild-type levels (Fig. 7*C*; see also Fig. 5), which could partly explain the replication profile observed (Fig. 7*A*). In the second round, RT-PCR analysis of genomic RNA revealed an almost complete recovery of genomic RNA content in the virus to 80% that of wild type, and this correlated with wild-type replication of the A2RE-2 A8G virus and reversion to wild-type sequence at this time. These data support the notion that genomic RNA encapsidation levels of the inoculating virus were a major determinant in producing the replication delay of this virus.

Quantitative Analysis of HIV-1 Reverse Transcription—In order to confirm that genomic RNA content was a major determinant for the replication delay and was not the result of defects in reverse transcription, minus-strand strong-stop DNA (-sssDNA) was quantitated in cells by real-time PCR as described under "Experimental Procedures." P4 cells were infected with wild-type and A2RE virus generated in 293T cells. At 8 h post-infection, genomic DNA was isolated and real-time PCR was performed as described under "Experimental Procedures." These analyses revealed that there was a strong quantitative correlation ($r^2 = 0.99$) between genomic RNA content in the infecting virus and the abundance of -sssDNA. These analyses rule out any major effects of the A2RE mutations at this early step of reverse transcription (data not shown). These data collectively support the idea that the infectivity phenotype is likely attributable to genomic RNA encapsidation levels and virion-associated Vpr (Figs. 7, *B* and *C* and 8*C*).

DISCUSSION

The data presented in this manuscript demonstrate that the hnRNP A2/A2RE association represents a commitment step for HIV-1 RNA trafficking into the cytosol and subsequent downstream trafficking events leading ultimately to RNA encapsidation in progeny virions. Our previous work in which we show that the association of hnRNP A2 to the HIV-1 A2REs is necessary for RNA trafficking clearly supports a role in cytoplasmic RNA trafficking (9) while the present work does not address this role. However, A2RE mutagenesis in both cases blocks hnRNP A2 association to HIV-1 RNA (Fig. 2) and results in dramatically reduced levels of genomic RNA in the cytoplasm (Figs. 3 and 4). As a consequence, this results in significantly reduced levels of genomic RNA in progeny virions (Fig. 5) late in the replication cycle. These data support a role of this interaction in nucleocytoplasmic export of HIV-1 RNA, consistent with the model in which hnRNP A2/A2RE association is proposed to facilitate RNA export from the nucleus (67). We also show that this interaction has a dramatic effect on the cellular localization of $pr55^{Gag}$, and in particular, on that of Vpr (Fig. 6). While there is evidence that the A2RE of mouse *mbp* enhances cap-dependent translation (64), we have ruled out this possibility for the HIV-1 A2REs in several types of *in vitro* translation assays.[3] hnRNP A2 is a predominantly nuclear protein, but it is also found in streaming cytosolic compartments in human cells (36), consistent with its many functions in RNA trafficking and translation.

Several members of hnRNP A/B family of proteins possess both nuclear and cytoplasmic RNA trafficking functions in several different organisms (37, 38, 68, 69). Lall *et al.* (37) reported that *sqd*, a *Drosophila* hnRNP, is required for *ftz* mRNA localization in embryos. The β-actin mRNA zipcode-binding proteins, Zbp2, homologous to hnRNP, is a predominantly nuclear protein that directs the localization of β-actin mRNA (38) and in yeast, an exclusively nuclear protein, Loc1p, binds RNA zipcode sequences of *ASH1* mRNA and is required for efficient cytoplasmic localization to the bud tip (68). The result that the hnRNP A2/A2RE interaction is important for nuclear RNA export was completely unexpected. The data suggest that hnRNP A2 tags the HIV-1 RNA by binding to it (perhaps concomitant to its roles in splicing regulation, see later) and a fraction remains associated during the export and transport in the cytosol. Several recent data support the role of RNA binding proteins, including hnRNPs, that tag RNAs in the nucleus for subsequent post-transcriptional regulation (70–73). In addition, a recent study demonstrates that hnRNP D must first be imported into the nucleus to have its effects on

[3] J.-F. Clément and A. J. Mouland, unpublished results.

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TABLE I
Reversions during replication of A2RE virus

The results of sequencing analysis of the A2RE sequences in the viral genomes are shown. RNA was extracted from viral supernatants at peak fractions in the first and second rounds of infection. The RNA was reverse-transcribed, and the A2RE elements were sequenced as described under "Experimental Procedures." Input represents the mutations in the initial proviral DNAs that were sequenced in parallel.

Proviruses	Input (DNA)		First-round infection		Second-round infection	
	A2RE-1	A2RE-2	A2RE-1	A2RE-2	A2RE-1	A2RE-2
HxBru	—	—	—	—	—	—
A2RE-1 A8G	A8G	—	A8G	—	A8G	—
A2RE-2 A8G	—	A8G	—	A8G	—	**R**[a]
4Mut	A5G, A8G	T5C, A8G	A5G,A8G	**50% R** T5C, A8G	A5G, A8G	**R**

[a] Mutations that have reverted to wild-type phenotype are indicated by **R**.

mRNA turnover in the cytosol (74). Our RNA analyses shown here demonstrate that the A2REs do not influence steady-state HIV-1 mRNA (Figs. 2 and 7*D*) nor do their location in the HIV-1 RNA correspond to any of the previously identified *cis*-repressor or post-transcriptional inhibitory elements that impact on HIV-1 post-transcriptional regulation (75, 76). Cumulatively, hnRNP A2 function is first initiated in the nucleus and this event is important for its role in the cytoplasm, likely playing roles in both nuclear and cytoplasmic trafficking and localization of HIV-1 RNA.

One of our major observations from the data presented in this article is the impact of the A2RE-2 A8G on overriding the nuclear export function of HIV-1 Rev late in replication. One can envisage that the hnRNP A2/A2RE could impinge on the function of Rev to export RRE-containing RNA to the cytosol. This may be achieved in part by interference by unbound hnRNP A2 on the RRE similar to the activity of the hnRNP protein, RREBP49 on Rev function (77) or the interference of hnRNP A1 on the HTLV-1 Rex response element (78). Alternatively, the dependence on the hnRNP A2/A2RE association could also suggest that this protein-RNA complex is a prerequisite for Rev function, perhaps by stabilizing HIV-1 RNA-protein complexes that are competent for nucleocytoplasmic transport. The related hnRNP, hnRNP A1 has also been shown to assemble on HIV-1 RNA to synergize with Rev to promote unspliced RNA nucleocytoplasmic export (79) and to interact with HIV-1 *cis*-acting repressor/inhibitory sequences (INS) that could impact on Rev function (75). Neither of the A2RE elements overlap nor was hnRNP A2 shown to interact with these INS elements (76). Importantly, our data demonstrate that this partial Rev-minus phenotype (partial because pr55[Gag] is expressed) at this late step is not a result of aberrant splicing as we show in the several types of heterologous and homologous splicing assays (data not shown). This partial Rev-minus phenotype in which the genomic RNA is sequestered in the nucleus is also observed when an HxB2-M4 MA NES proviral mutant is expressed (Fig. 3, *panel P* and Ref. 5). MA NES- and hnRNP A2/A2RE-mediated RNA trafficking constitute two trafficking pathways, perhaps overlapping at several levels to play key roles in the nucleocytoplasmic transport of genomic RNA late in the replication cycle.

The activity of the hnRNP A2/A2RE and HIV-1 MA NES RNA localization determinants that promote genomic RNA trafficking to the cytosol and eventual encapsidation can not be completely blocked by a single nucleotide or amino acid point mutation (Fig. 5) (5), suggesting that there are additional signals that contribute to the final quantity of genomic RNA in virions. Consistent with the current model of RNA trafficking mechanisms in which multiple *trans*-acting proteins act in a temporal and spatial manner (27, 80–82), our data favor the idea that the hnRNP A2/genomic RNA association represents one event in a chain of events that promotes the trafficking of HIV-1 genomic RNA from the nucleus to sites of viral assembly and these steps likely involve the activity of a variety of HIV-1 genomic RNA-binding viral and cellular proteins including Rev, MA or pr55[Gag] and hnRNP A2 (9, 25, 47, 82–85). Consistently, recent data point to a role of the cellular protein, hRIP in the trafficking of HIV-1 RNA from a perinuclear space to the cytoplasm (4).

While hnRNP A2 is a *bona fide* nuclear shuttling protein and has multiple roles in RNA processing and transport (27), there is no direct proof -except for the case that is presented in this manuscript- that temporal functions exist for hnRNP A2 in the context of the HIV-1 lifecycle. These functions may be defined, however, by the efficiency of RNA splicing early in infection when multiple-spliced HIV-1 RNAs are rapidly produced when Rev is least abundant (86) and a later role of hnRNP A2 to participate in the inhibition of splicing (when Rev levels are elevated) to promote unspliced, genomic RNA export to the cytosol for assembly. In support of this notion is the coupling that was proposed to exist between negative splicing regulation of HIV-1 RNA and Rev-mediated nuclear export of HIV-1 RNAs late in the replication cycle (83) as well as the effect of Rev on overriding nuclear retention of intron-containing RNAs by the splicing machinery during replication (87–89). A direct link has also been characterized between RNA nucleocytoplasmic transport and splicing inhibition for histone H2a RNA maturation that is, in this case, mediated by an RNA trafficking sequence (90). Consistent with temporal activities of hnRNP A2, its association to HIV-1 RNA is equally affected by A2RE mutagenesis at 20 h post-transfection (data identical to those presented in Fig. 2*D*) yet there is little effect on the distribution of genomic RNA and pr55[Gag] at this early time (Fig. 3, *panels S–U*). Total RNA staining is likewise unaffected by A2REs at either time points (Fig. 3, *panels V–Y* and data not shown). These results suggest that the hnRNP A2/A2RE interaction is functionally relevant but only at a specific time in the HIV-1 lifecycle and it has no effect on general RNA export.

While it is suggested that hnRNPs are functionally redundant proteins, several lines of evidence also support specialized functions for hnRNP proteins in addition to that reported for splicing. The case in point is that for hnRNP A2. It possesses roles in transcription, RNA maturation, splicing, RNA transport, and its localization is differentially affected upon treatment of cells with drugs that affect methylation and oxidative stress (33, 34). HnRNP A1 is not active nor can it replace hnRNP A2 in A2RE-mediated RNA trafficking and there is no available evidence to suggest that hnRNP A3 has such a role except for its localization in mouse neuronal RNA granules (45). While both of these hnRNPs can bind mouse *mbp* A2RE elements *in vitro* (45, 69), this has not been shown formally for the HIV-1 A2REs, which possess several nucleotide differences when compared with the mouse *mbp* mRNA A2RE (9). Furthermore, these studies have been performed with murine or rat proteins, which might not necessarily translate to human or the monkey cells used in this study.

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Nevertheless, we demonstrate here that the association of hnRNP A1 to HIV-1 RNA was not affected in the A2RE single point mutant (Fig. 2, and data not shown at early time points). We were not able to characterize hnRNP A3 binding to HIV-1 RNA because the antibody did not work in our immunoprecipitation procedure (data not shown). Our recent RNAi data also confirm functional differences between hnRNP A1 and A2 during HIV-1 gene expression. Specific targeting of hnRNP A2 gene expression and not that of hnRNP A1 by siRNA demonstrates that HIV-1 RNA trafficking is dependent on hnRNP A2 expression in HIV-1 expressing cells.[4] In support of these data are the noted functional differences in activities between the hnRNP A1 and A2 proteins on *SMN1* mRNA splicing (35) and the lack of effects of the A2REs in our *in vitro* splicing assays (described above).

Our earlier work highlighted the co-trafficking of the *vpr* and *gag* RNAs in RNA transport granules mediated by their respective A2RE (9). As shown in Figs. 6, *panel G* and 7, *B* and *C*, A2RE-2 A8G expression resulted in an almost complete relocalization of Vpr to the nucleus as well as a significant decrease in Vpr virion incorporation levels. While the prevention of the pr55^{Gag}-Vpr interaction alone does not result in nuclear re-import of Vpr during proviral gene expression (91), it is well described to block Vpr incorporation (65, 92, 93) similar to the results we obtain (Fig. 7, *B* and *C*). On the other hand, mutagenesis of the nuclear export signal to cause nuclear retention of Vpr does not prevent the Vpr-Gag interaction in provirus-expressing cells yet this reduces Vpr incorporation significantly as shown recently (94). Because our preliminary studies demonstrate that the Gag-Vpr interaction is not influenced by A2RE mutagenesis (data not shown), the reasons for the re-localization of Vpr to the nucleus and diminished incorporation levels remain to be identified. These phenomena could be related to a loss of coordinated *gag* and *vpr* RNA trafficking and their influence on expression patterns by the hnRNP A2-dependent machinery or on Vpr NES activity. The nuclear localization of Vpr would likely have a negative impact on the function of the HIV-1 pre-integration complex as described recently (94, 95).

Our genotyping analyses reveal the importance of the A2RE-2 sequence, and in particular the A8 nucleotide, in HIV-1 replication (Fig. 8). A rapid reversion to wild-type sequence was found for A2RE-2 A8G (as well as the double A8G,T5C mutant) and this correlated with an almost complete recovery of genomic RNA content in A2RE-2 A8G virions (Fig. 8, *B* and *C* and Table I). This demonstrates that the genomic RNA content in virus contributes significantly to the replication profile found in the first round of replication. Vpr content and localization likely normalized as a consequence of the A2RE-2 sequence reversion at this time because replication delays are characteristic of virus that is deficient in Vpr (96, 97). An A8G polymorphism in the HIV-1 A2RE-2 is extremely rare (32) and we identified the G8 nucleotide of A2RE-2 to be associated with long-term non-progression to AIDS (9, 98). This nucleotide substitution was not maintained in culture by A2RE-2 A8G. While these data do not rule out a contribution of the Tat Glu^2-Gly^2 amino acid change, however, the mutation does not have any marked consequence on protein and RNA expression levels (Figs. 2 and 7) and it is predicted that Tat interaction with cyclin T would not be affected since this interaction is mediated by a distal Tat domain.

The A2RE-1 A8G phenotype deserves mention here because it only had modest effects on genomic RNA localization and modest effects on Vpr and genomic RNA encapsidation levels (Figs. 3, 5, and 7). We consistently observed wild-type replication kinetics in T cells (Fig. 8) and genotyping analysis did not detect any sequence reversions in this element (Table I). Consistently, hnRNP A2 association was shown to be only partially impaired on A2RE-1 A8G RNA *in vitro* (9) and in our study in cells presented in Fig. 2*D*. Attempts to define a more severe RNA trafficking and/or gene expression phenotype could not be achieved even with the introduction of two silent point mutations in the A2RE-1 (using an A5G/A8G mutant; data not shown).[2] This suggests that the A2RE-1 contributes to the total amount of hnRNP A2 associated to HIV-1 RNA, but mutagenesis cannot completely remove it, producing the intermediate phenotype observed. Mutagenesis of each A2RE individually lowers hnRNP A2 binding (Fig. 2*D*) suggesting that these two elements may cooperate in hnRNP A2 binding and could result in RNA conformational changes of HIV-1 RNA or act additively to influence function. This latter mechanism has been shown to exist in a model in which proteins bridge 5′- and 3′-RNA ends to promote efficient translation (99). Such a mechanism has also been put into evidence for hnRNP A1 such that hnRNP A1 bridges two distant regions of the RNA via high affinity binding sites to promote intron excision (53). And in yeast, RNA transport of *ASH1* mRNA is incrementally restored by the one-by-one addition of *ASH1* mRNA localization elements (100). For HIV-1, multiple *cis*-acting RNA elements have been identified to date and their concerted activities are important determinants for total HIV-1 gene expression levels (75, 76, 82, 101). RNA structures or RNA-protein complexes that are formed potentially influence these and RNA conformation could be important for total splicing, translation regulation and RNA encapsidation levels (58, 102). It will be important to determine the interplay between these regulatory elements and further analysis of the contributions of the A2RE-1 to HIV-1 gene expression levels will be required.

There are several reasons why our data provide important new information about virus-host interactions and HIV-1 RNA trafficking. First, the data presented here demonstrate that the hnRNP A2/A2RE interaction represents a distinct determinant for genomic RNA transport in cells expressing replication-competent HIV-1. Furthermore, one of the most striking observations presented in this study is the temporal nature of A2RE activity in the context of the HIV-1 replication cycle such that it is functionally important at a late stage of the replication cycle coinciding with strong splicing inhibition and Rev-mediated RNA export to the cytosol. The data also provide the first evidence that the hnRNP A2/A2RE interaction is functional in non-neuronal cells thus it will be interesting to identify other RNAs that require hnRNP A2 for transport. Finally, it is clear that several mechanisms exist to achieve the cytosolic localization of genomic RNA during HIV-1 gene expression and summed up, these include the activities of a variety of different types of viral and cellular RNA-binding proteins such as Rev, MA, hRIP, and hnRNP A2.

Acknowledgments—We thank Marco Blanchette and Jean-Francois Fisette for advice on *in vitro* splicing assays, Daniela Moisi, Fernando Frankel and Anna Derjuga for help with sequencing and real-time PCR, Stéphane Richard and Marty Stoltzfus for helpful discussions and reagents, Mark Wainberg and Volker Blank for contributions of materials and equipment, Michael Green for proviral constructs, Klaus Strebel and Ross Smith for antisera and Dana Gabuzda, Reza Sadaie, Bryan Cullen, and the National Institutes of Health AIDS Research Reference and Reagent Program for antibodies and genetic clones. We are grateful to Hugo Dilhuydy and Brian Udashkin for help with the laser scanning confocal microscopy imaging analyses and Kimberley Hu for expert technical assistance.

[4] A. J. Mouland, V. Poupon, and K. Lévesque, manuscript in preparation.

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