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Effect of the unfolded protein response on MHC class I antigen presentation

par

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a été évalué par un jury composé des personnes suivantes:

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président-rapporteur

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directeur de recherche

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membre du jury

RÉSUMÉ

L'infection virale et la transformation néoplasique engendrent du stress dans le réticulum endoplasmique (RE). En conséquence, une grande proportion de cellules qui devraient être reconnues par le système immunitaire, sont des cellules stressées. Lors d'un stress du RE, les cellules déclenchent une réponse envers les protéines mal repliées (UPR). L'UPR régule les deux processus clés qui contrôlent la présentation antigénique par les molécules du complexe majeur d'histocompatibilité de classe I (CMH I) : la synthèse et la dégradation des protéines. Nous avons voulu déterminer si l'UPR affecte la présentation antigénique et comment. Tout d'abord, l'impact de l'UPR sur l'expression globale du CMH I ainsi que sur la présentation du peptide SIINFEKL dérivant de la protéine ovalbumine a été évalué. Des cellules EL4 transfectées de façon stable avec des vecteurs codant pour des variantes de la protéine HEL-SIINFEKL ont été stressées à l'aide d'agents pharmacologiques ou encore soumis à une carence en glucose. Nos résultats indiquent que l'UPR diminue l'expression du CMH I à la surface des cellules, mais n'a pas d'effet au niveau de l'ARN messager. Conséquemment, la présentation de SIINFEKL par les molécules $H2K^b$ était diminuée dans les cellules stressées, autant de façon chimique que physiologique. De plus, les cellules stressées présentaient préférentiellement des complexes CMH I-peptide provenant de la variante protéique qui se localise dans le RE par rapport à ceux dérivant de la protéine cytosolique. Par ailleurs, suite à une élution des peptides avec de l'acide, la génération de peptides provenant de la protéine localisée dans le RE était moins affectée que dans le cas de la protéine cytosolique. Nos résultats démontrent que le stress du RE altère la présentation de complexes CMH I-peptide et qu'il régule de façon différentielle l'expression de peptides dérivant du RE ou du cytosol. Ainsi, notre recherche montre que le stress du RE, qui est une caractéristique des cellules infectées ou transformées, peut affecter les signaux nécessaires pour la reconnaissance par le système immunitaire.

Mots clés: CMH de classe I, présentation antigénique, antigène/peptide, stress du RE, réponse envers les protéines mal repliées

SUMMARY

Viral infection and neoplastic transformation trigger endoplasmic reticulum (ER) stress. Thus, a large proportion of the cells that must be recognized by the immune system are stressed cells. Cells respond to ER stress by launching the unfolded protein response (UPR). The UPR regulates the two key processes that control major histocompatibility complex class I (MHC I)-peptide presentation: protein translation and degradation. We therefore asked whether and how the UPR impinges on MHC I-peptide presentation. We evaluated the impact of the UPR on global MHC I expression and on presentation of the Ovalbumin-derived SIINFEKL peptide. EL4 cells stably transfected with vectors coding HEL-SIINFEKL protein variants were stressed with pharmacological agents or exposed to glucose deprivation. UPR decreased surface expression of MHC I at the protein but not the mRNA level. Consequently, presentation of SIINFEKL by H2-K^b molecules was reduced in chemically or physiologically stressed cells. Notably, stressed cells preferentially presented MHC I-peptides derived from an ER-retained as opposed to a cytosol-localized protein variant. Furthermore, generation of new H2K^b-SIINFEKL complexes after acid strip was less affected for ER- than for cytosol-derived SIINFEKL. Our results show that ER stress impairs MHC I-peptide presentation, and that it differentially regulates expression of ER- vs. cytosol-derived peptides. This work indicates how ER stress, a typical feature of infected and malignant cells, can impinge on cues for adaptive immune recognition.

Keywords: MHC class I, antigen presentation, peptide/antigen, ER stress, unfolded protein response

TABLE OF CONTENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
β_2-m	β_2-microglobulin
BiP	Heavy chain binding protein
CHOP	C/EBP homologous protein
CNX	Calnexin
CRT	Calreticulin
CTLs	Cytotoxic T cells
COS	Green African monkey fibroblast
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DRiPs	Defective ribosomal products
DTT	Dithiotheritol
EDEM	ER degradation-enhancing α-mannosidase-like protein
EGFP	Enhanced green fluorescent protein
eIF	Eukaryotic initiation factor
EL4	Mouse thymocytes
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERAAP	Endoplasmic reticulum aminopeptidase associated with antigen processing
ERGIC-53	Endoplasmic reticulum-Golgi intermediate compartment-53
ERSE	Endoplasmic reticulum stress response element
FACS	Fluorescence-activated cell sorting
GPI	Glycophosphatidylinositol
GRP78	78 kDa glucose-regulated protein
HCMV	Human cytomegalovirus
HEK	Human embryonic kidney
HEL	Hen egg lysozyme
HeLa	Human cervix adenocarcinoma
HLA	Human leucocyte antigen
HMGR	3-hydroxy 3-methylglutaryl coenzyme A reductase
HSP	Heat shock protein
IFN-γ	Interferon gamma
IRE1	Inositol-requiring enzyme-1
IRES	Internal ribosomal entry sites

mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex class I
mTOR	Mammalian target of rapamycin
MTS	Mitochondrial targeting sequence
NAC	Nascent polypeptide-associated complex
NK	Natural killer
NLS	Nuclear localization signal
ORF	Open reading frame
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	PKR-related ER kinase
PLC	Peptide-loading comlex
RAC	Ribosome-associated complex
RDP	Rapidly degraded polypeptides
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time reverse transcriptase polymerase chain reaction
SDP	Slowly degraded polypeptides
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRP	Signal recognition particle
TAP	Transporter associated with antigen processing
TCR	T cell antigen receptor
TPPII	Tripeptidyl peptidase II
UGGT	UDP-glucose glycoprotein glucosyltransferase
UPR	Unfolded protein response
UTR	Untranslated region
XBP1	X-box-binding protein 1

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1. INTRODUCTION

1.1. General introduction

A fundamental feature of infected and neoplastic cells is that they are stressed cells (Gleimer & Parham, 2003; Shin et al., 2003; Marciniak & Ron, 2006). Stressed cells express specific antigens that are recognized by T lymphocytes leading to eventual elimination of tumor or infected cells (van der Bruggen & Van den Eynde, 2006; Hammer et al., 2007). The recognition of these antigens depends on the ability of cells to display their intracellular contents in the form of peptides bound to the major histocompatibility complex class I (MHC I) molecules on the surface (Gleimer & Parham, 2003; Shin et al., 2003; Marciniak & Ron, 2006). Generating this complex repertoire of peptides and loading them on the MHC I molecules for export to the cell surface, is accomplished by the antigen processing and presentation pathway.

Peptides suitable for loading the MHC I molecules, as well as peptide-receptive MHC I molecules are generated in the endoplasmic reticulum (ER) (Hammer & Shastri, 2007). The ER stands at the crossroad of another fundamental process: maturation and folding of proteins destined to the secretory pathway. Eventually, the folding of proteins can be perturbed by viral infection and neoplastic transformation, which trigger ER stress (Marciniak & Ron, 2006). Stressed cells respond by launching the unfolded protein response (UPR), an adaptive response that allows survival to limited stress but leads to apoptosis in the presence of overwhelming stress (Rutkowski et al., 2006; Szegezdi et al., 2006). In the following section I will review these fundamental processes that constitute the basis of my research work.

1.2. MHC I-antigen processing and presentation pathway

The end result of the antigen processing pathway is the display of peptide-bound MHC I molecules. These complexes are expressed on the cell surface, where they can be recognized by T cell antigen receptors (TCRs) of $CD8^+$ T cells (Gleimer & Parham, 2003; Shin et al., 2003; Marciniak & Ron, 2006). These peptides derive from the degradation of virtually all proteins inside the cell (Shastri et al., 2002) and represent proteins that are being translated at any given time (Jensen, 2007). In a stepwise manner, the antigen processing pathway generates and protects the proteolytic intermediates until they yield the final peptides that can fit the MHC I in the ER. This leads to MHC I-peptide complexes that are ready to be exported and presented. In the following section, we will see how this highly specialized pathway, operating with essentially conserved components, is nevertheless capable of generating

highly diverse sets of peptides that satisfy a large number of different MHC I molecules.

1.2.1. Bringing the inside out: the immunopeptidome

The repertoire of peptides presented by MHC I molecules, estimated to be composed of hundreds of thousands, is known as the MHC I immunopeptidome (Shastri et al., 2002; Shastri et al., 2005). Under steady-state conditions (in the absence of infection), cell surface MHC I molecules are associated solely with self-peptides. These peptides, collectively referred to as the self-MHC I immunopeptidome (Istrail et al., 2004), play vital roles. They shape the repertoire of developing thymocytes (Huseby et al., 2003; Starr et al., 2003; Baldwin & Hogquist, 2007), transmit survival signals to mature $CD8^+$ T cells (Marrack & Kappler, 2004) and influence mating preferences and other behaviors in mice (Slev et al., 2006). Thus, the self-MHC I immunopeptidome links the intracellular milieu with the surrounding environment of almost all cell types (Shastri et al., 2002).

The self-MHC I immunopeptidome also helps amplify responses against intracellular pathogens (Anikeeva et al., 2006) and allows immunosurveillance of neoplastic cells (Dunn et al., 2004; Zitvogel et al., 2006). It reflects the state of the cell since mutated genes, genes involved in differentiation or genes overexpressed in tumors modify and shape the self-MHC I immunopeptidome (van der Bruggen & Van den Eynde, 2006). Moreover, viral proteins constitute a source of peptides that also molds the MHC I-peptide repertoire (Yewdell, 2007). Altogether, these modifications render the otherwise invisible internal proteome available for surveillance by cytotoxic $CD8^+$ T cells, which have the ability to detect and eliminate cells expressing viral proteins or tumor antigens (Shastri et al., 2002; Jensen, 2007).

The immunopeptidome or MHC I-peptide repertoire is also involved in immunopathology since it can be targeted by autoreactive T cells that initiate autoimmune diseases and alloreactive T cells that cause graft rejection and graft-versus-host disease (Perreault et al., 1990; Liblau et al., 2002). The aforementioned roles of the immunopeptidome highlight its immunotherapeutic potential. In line with this, peptides overexpressed and/or specific to neoplastic cells can be used as targets in cancer immunotherapy (Singh-Jasuja et al., 2004; van der Bruggen & Van den Eynde, 2006; Purcell et al., 2007; Fortier et al., 2008).

The MHC I immunopeptidome is the result of two merging cellular pathways (Figure 1). One pathway generates the peptides suitable for loading the MHC I molecules, whereas the second pathway produces peptide receptive MHC I molecules in the ER (Hammer et al., 2007).

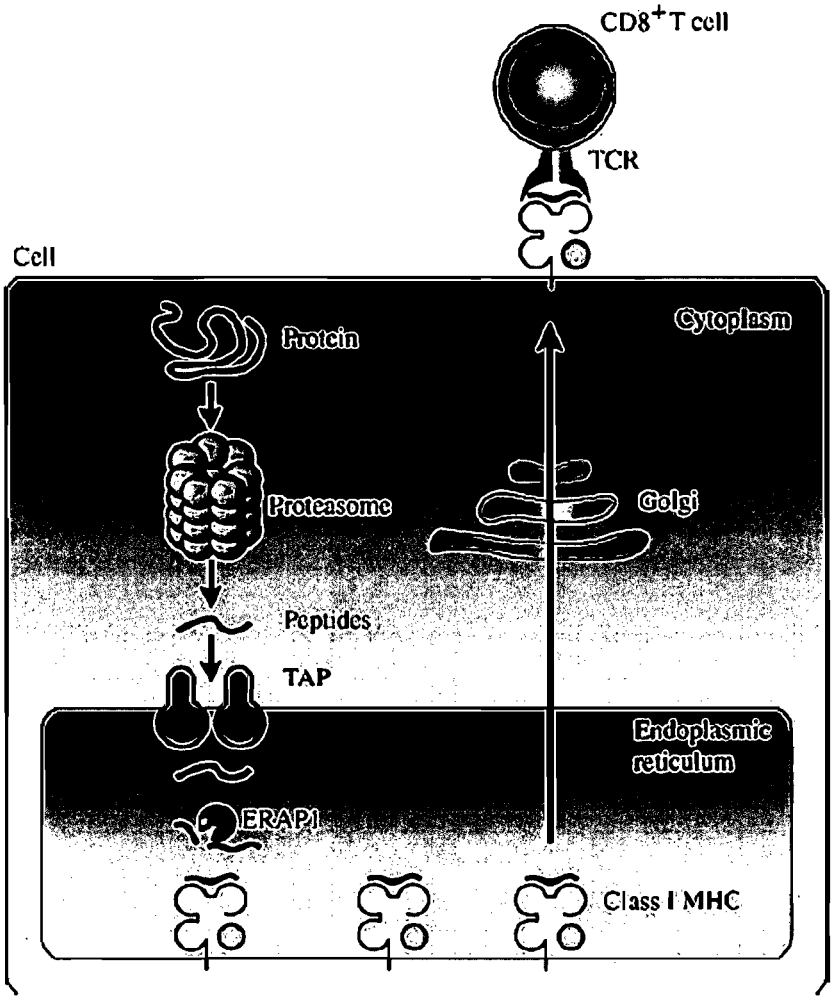

Figure 1. MHC I-antigen presentation pathway. Cytoplasmic proteins are degraded by the proteasome and other proteases. Resultant short peptides are transferred to the lumen of the ER by TAP in an ATP-dependent manner. Long peptides are trimmed by ER aminopeptidases such as ERAP1. Peptides bound to MHC I molecules are exported via the secretory pathway and presented at the cell surface for surveillance by $CD8^+$ T cells. Adapted from (Hanada & Yang, 2005).

1.2.2. Peptide processing in the cytoplasm

Generation of peptides suitable for loading MHC I molecules starts in the cytoplasm. This is the major site of protein degradation, because even ER proteins are retrieved into the cytosol for turnover (Shastri et al., 2005). Thus, this compartment is the logical site for beginning the peptide-processing pathway (the pathway by which peptide substrates for MHC I binding are made). Short peptides of variable lengths (2-25 amino acids) are generated from degradation of endogenous proteins in the cytoplasm through the action of the proteasome and other proteases (Stoltze et al., 2000a; Stoltze et al., 2000b; Jensen, 2007). Treatment of

cells with proteasome inhibitors has revealed that most but not all MHC I-peptide complexes require the proteasome for their generation (Luckey et al., 1998). So, the majority of peptides are produced by the multicatalytic proteasome.

Besides being the site where antigenic peptides are born, the cytoplasm constitutes the site where most peptides are rapidly destroyed (Reits et al., 2003; Jensen, 2007). This rapid degradation by cytoplasmic peptidases limits the availability of peptides and accounts for the inefficiency of the peptide presentation pathway (Shastri et al., 2005). More than 99% of peptides are degraded by cytosolic peptidases before they reach the ER (Yewdell et al., 2003). Cytosolic chaperones, such as Hsp70 (Heat shock protein 70) and Hsp90 (Heat shock protein 90), are thought to protect peptides from exhaustive degradation (Kunisawa & Shastri, 2003).

Proteasomes are thought to generate the final carboxyl-terminal residues of MHC-binding peptides (Cascio et al., 2001), but additional trimming at the amino terminus is required for most peptide epitopes (Jensen, 2007). Various cytosolic aminopeptidases are responsible for cleaving the amino terminus (Rammensee, 2006). Of particular interest is the cytoplasmic tripeptidyl peptidase II (TPPII), involved in trimming of proteasomal products over 15 amino acids in length (York et al., 2006). TPPII has also been shown to participate in proteasome independent pathway for epitope generation (Guil et al., 2006). However, a delicate balance must be maintained because excess trimming by cytosolic peptidases can also destroy MHC I peptides.

At this point, these peptides (usually less than 12 amino acids) constitute precursors of the MHC I-peptide repertoire and are ready to be translocated into the ER. However, as we will see later, these precursors need further trimming in the ER in order to be suitable for loading MHC I molecules.

1.2.3. Generation of peptide-receptive MHC I molecules

As mentioned above, production of the MHC I immunopeptidome requires a concomitant pathway that generates peptide receptive MHC I molecules in the ER. The MHC I molecules are heterodimers of a polymorphic heavy chain (α chain) and β_2-microglobulin (β_2-m) (Zhang & Williams, 2006). Similar to what occurs with the peptide precursors, both polypeptides are cotranslationally translocated into the ER (Hammer et al., 2007).

Once in the ER, early folding and oxidation of the MHC I heavy chain and β_2-m are mediated by the chaperone calnexin (Jensen, 2007). These events are followed by release from

calnexin and assembly with the MHC I peptide-loading complex (PLC) (Jensen, 2007) (Figure 2). The PLC comprises many components, including the soluble chaperone calreticulin (CRT), the transmembrane protein tapasin, the oxidoreductase ERp57, protein disulfide isomerase (PDI) and the heterodimeric transporter associated with antigen processing (TAP) (Zhang & Williams, 2006; Jensen, 2007). The luminal domain of TAP acts as a binding platform for calreticulin and ERp57, supporting the correct folding of MHC I in the PLC (Cresswell, 2005; Cresswell et al., 2005). This multisubunit structure keeps the MHC I molecules in a peptide-receptive state (Hammer & Shastri, 2007). Nevertheless, MHC I heterodimers need to be stabilized by binding to a high-affinity peptide.

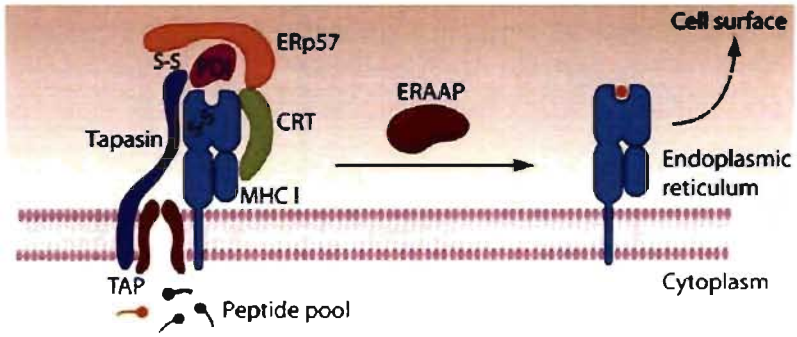

Figure 2. Schematic depiction of the MHC I PLC. The MHC I molecule folds with the assistance of the chaperones calnexin (not shown) and calreticulin (CRT). The multisubunit PLC is centered on TAP. The adaptor molecule tapasin interacts with TAP and the MHC I molecule and forms a covalent disulphide bond (S-S) with ERp57 with the help of PDI. ERAAP trims N-terminal extensions of antigenic precursors, resulting in the generation of the final MHC I-peptide complex that is transported to the cell surface. Adapted from (Hammer et al., 2007).

1.2.4. Peptide processing in the ER

Besides retaining empty MHC I molecules, TAP is responsible for the active transport of peptide precursors into the lumen of the ER (Neefjes et al., 1993). This provides the empty MHC I heterodimers with easier access to the incoming peptide supply (Cresswell et al., 2005). TAP is a heterodimer composed of the TAP1 and TAP2 molecules and shows substrate specificity when selecting peptides for translocation (Seliger et al., 1997; Gromme & Neefjes, 2002). It is important to mention that the import of some peptides appears to be TAP-independent (Lautscham et al., 2003).

Peptide precursors are translocated in a naked form into the ER and can then follow one or more different fates. They can bind MHC I molecules, bind ER chaperones, be trimmed by ER aminopeptidases, be degraded or be retrotranslocated back into the cytosol (Elliott & Neefjes, 2006). Together, these processes keep the concentration of peptides low in the ER such that only the most recent peptides are available for MHC I binding and do not have to compete with those that arrived earlier (Yewdell et al., 2003).

MHC I molecules are promiscuous, and they bind many different peptides with certain amino acid preferences at key positions (anchor residues) which constitute binding motifs (Rammensee et al., 1999). High-quality peptides that confer stability to the MHC I molecules share two important properties: the precise length and amino acid sequence required for a given MHC class I-binding motif (Hammer et al., 2007). Peptides entering the ER via TAP bind to newly synthesized MHC I molecules immediately, as long as the C-terminus is correct and the appropriate motif is present (Rammensee, 2006). At this stage, the length is not yet crucial (Rammensee, 2006). Peptides with a proper MHC I motif, but still too long on their N-terminus, do bind to MHC I molecules. Initial peptide binding is followed by peptide exchange and editing in the ER (Elliott & Williams, 2005) (Figure 3).

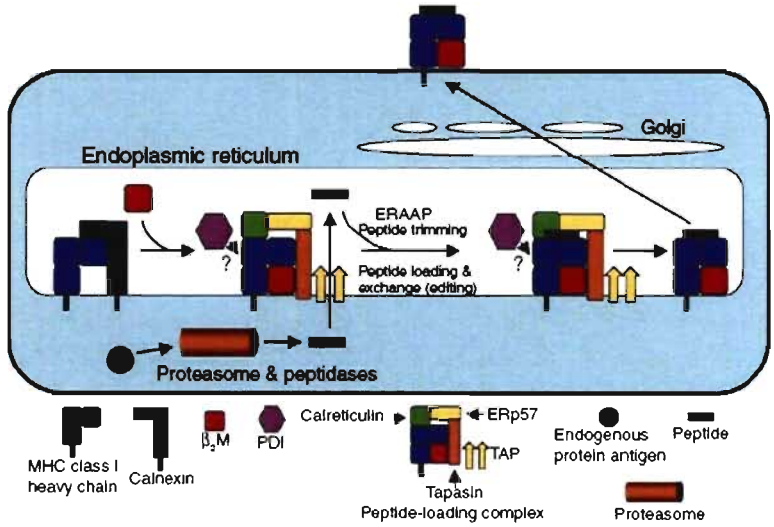

Figure 3. The MHC I processing pathway. MHC I heavy chains initially assemble with β_2-m , followed by recruitment into the PLC in the ER. Endogenous peptides, generated in the cytoplasm through the action of proteasomes and other peptidases are transported into the ER via TAP. ERAAP mediates final N-terminal trimming before or after binding to MHC I molecules. PLC promotes peptides loading and exchange, providing quality control for the preferential export of kinetically stable MHC I-peptide complexes. Adapted from (Jensen, 2007).

Generation of correct MHC I binding peptides sometimes requires trimming by ER

aminopeptidases. The key enzyme responsible for generation of quality peptides and final amino-terminal trimming in the MHC class I pathway is the ubiquitously expressed ER amino peptidase associated with antigen processing, ERAAP in mice (ERAP1/2 in humans) (Serwold et al., 2002). ERAAP recognizes the peptide carboxyl terminus and trims the amino terminus to generate peptides 8-10 residues in length (Chang et al., 2005). This enzyme is induced by interferon gamma (IFN-γ), a proinflammatory cytokine that enhances antigen presentation (Saric et al., 2002; Serwold et al., 2002). Importantly, ERAAP serves a unique function in modifying the MHC I-peptide repertoire and influencing $CD8^+$ T cell responses; ERAAP deficiency leaves some peptides unaffected, whereas others are either absent or dramatically upregulated (Hammer et al., 2006).

Peptide precursors can also bind ER chaperones (Spee et al., 1999). PDI appears to be the most efficient peptide-binding ER chaperone, as it binds to peptides of different length and sequence (Park et al., 2006). Importantly, binding of peptides to PDI protects them from degradation (Elliott & Neefjes, 2006).

Finally, peptides not containing a fitting motif and thus not bound to MHC I molecules, are trimmed and destroyed by ERAAP (Kanaseki et al., 2006), or retrotranslocated back into the cytosol for ER-associated degradation (ERAD) (Elliott & Neefjes, 2006). In this way, they no longer compete for space in the local compartment.

1.2.5. Peptide loading and presentation

The PLC represents the precise point of intersection between the pathways of peptide processing and peptide presentation. The PLC orchestrates the final assembly of MHC I molecules with peptides (now 8-11 amino acids), delivered into the ER by TAP, for generation of stable MHC class I-peptide complexes (Jensen, 2007). Its chief function is to provide 'quality control' by selectively retaining MHC I molecules loaded with suboptimal peptides for replacement by higher-affinity quality peptides (Hammer et al., 2007).

When any of the PLC constituents are missing or are inhibited by viruses, intracellular MHC I molecules can suffer unfolding, degradation and indiscriminate peptide loading, all of which can compromise the stability, expression and function of MHC I-peptide complexes at the cell surface (Hammer et al., 2007).

Finally, after successful peptide loading and customization, MHC class I-peptide complexes are released from the PLC and are transported through the Golgi cisternae and the constitutive secretory pathway to the cell surface (Jensen, 2007). Indeed, only 1-2 out

of every 10,000 peptides generated by the proteasome bind to MHC I molecules (Yewdell et al., 2003).

Different MHC alleles have different motifs and bind different set of peptides. The number of class I MHC molecules per cell is estimated at 50,000-100,000 (Gakamsky et al., 2000). Since as many as $2x10^6$ peptides are estimated to be generated every second, only a small minority of peptide epitopes can be presented on a MHC I at any time (Princiotta et al., 2003). Most peptides are represented at fewer than ten copies per cell. However, it has been estimated that only three copies of the antigenic peptide are sufficient for target cell lysis by cytotoxic T cells (CTLs) (Purbhoo et al., 2004).

1.3. The origin of peptides for display by MHC I molecules

The complexity of the MHC I-immunopepidome reflects the equally complex milieu of intracellular proteins (Shastri et al., 2005). It is widely known that peptides displayed by MHC I molecules derive from degradation of proteins acquired from an exogenous source or from proteins endogenously synthesized, in processes referred to as cross-presentation or direct presentation, respectively (Shastri et al., 2005). When and how intracellular proteins are chosen for entry into the antigen processing pathway? This is an interesting yet not completely solved question. What is now clear, however, is that the MHC I-peptide repertoire is not a random sample of the proteome: many abundant proteins do not generate MHC I-associated peptides while some low abundant proteins have a major contribution to the immunopeptidome (Caron et al., 2005; Milner et al., 2006). In the following section, I will give some examples of generation of peptides from exogenous proteins and describe more in detail the mechanisms by which endogenous proteins can give rise to peptides.

1.3.1. Exogenous proteins as source of peptides

Uptake of exogenous antigens occurs routinely in antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), which represent the sentinels for initiating naïve $CD8^+$ T-cell responses (Mellman & Steinman, 2001). The transferred antigens can take many different forms, ranging from cell debris from apoptotic or necrotic cells to proteins or chaperone-associated peptides (Trombetta & Mellman, 2005). For instance, DCs can ingest infected cells or cancer cells and derive antigens from these sources in a mechanism known as cross-presentation (Jensen, 2007). Also, exogenous protein sources are clearly important in the presentation of peptides derived from intracellular pathogens such as *Listeria monocytogenes* (Pamer et al., 1997).

There are many pathways for cross-presentation, including TAP-dependent and TAP-independent mechanisms, even though the former seem to dominate. Exogenous antigens can be transferred from the endosome to the cytosol, where they are digested by proteasomes and loaded onto MHC I molecules in a TAP-dependent manner (Trombetta & Mellman, 2005). It remains unclear, however, what the mechanisms are by which they traverse the enosomal membrane and reach the cytoplasm. Suggested mechanisms involve transient physical rupture of the endosomal membrane or the action of a specific channel or translocator (Trombetta & Mellman, 2005). Alternatively, antigens might make use of an established retrograde pathway leading from endosomes to the ER via the Golgi. Form the ER, they may reach the cytosol using the translocation channel involved in retrotranslocation during protein degradation (Trombetta & Mellman, 2005). As in the MHC I processing pathway, there is a balance between peptide generation and destruction by proteolytic enzymes in cross-presentation. Mechanisms that reduce the activity of endosomal hydrolases in DCs have recently been shown to enhance the efficiency of cross-presentation (Jensen, 2007).

To some extent, exogenous antigens can also be presented on MHC I molecules in a TAP-independent manner, indicating that they do not require transport to the cytosol and can thus be loaded in the endocytic pathway (Trombetta & Mellman, 2005). This pathway may involve peptide exchange in recycling endosomes or on the cell surface, and a recent study has demonstrated that the lysosomal protease cathepsin S is important in generating peptides presented through this pathway (Jensen, 2007). Recently, it has been demonstrated that peptides can be transferred from virally-infected cells to professional APCs through gap junctions (Neijssen et al., 2005).

1.3.2. Endogenous proteins as source of peptides

Most endogenous proteins destined for presentation on MHC I molecules are fed to the proteasome to initiate fragmentation. However, the source of proteasomal substrate is quite varied. Peptide ligands for MHC I molecules can be derived from cryptic transcription products, defective ribosomal products (DRiPs), "stable" proteins or from retrotranslocation of proteins destined to the secretory pathway from the ER to the cytosol (Figure 4).

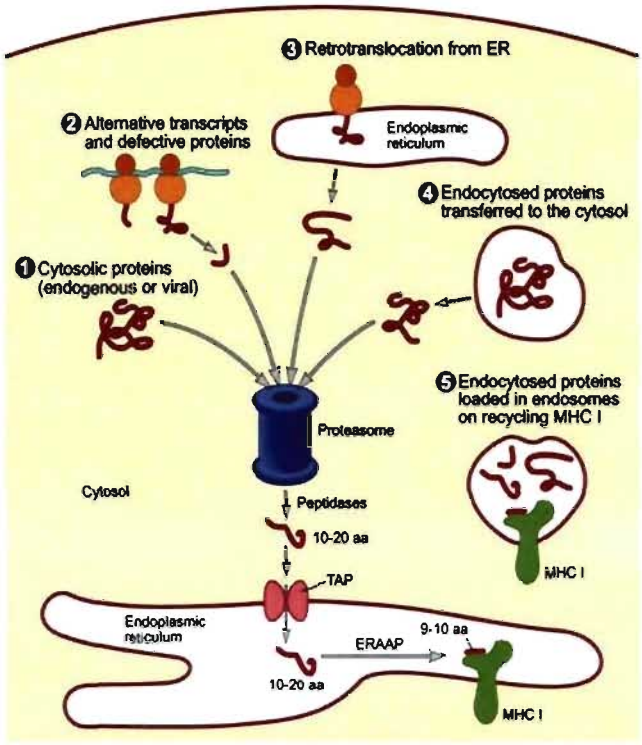

Figure 4. Possible sources of proteins presented on MHC I molecules. MHC I ligands have been shown to derive from various sources, including (1) cytosolic proteins, (2) alternative translation products and DRiPs, (3) proteins retrotranslocated to the cytosol from the ER, and (4) internalized proteins transferred to the cytosol. Adapted from (Trombetta & Mellman, 2005).

1.3.2.1. Peptides derived from stable proteins

MHC I-peptide ligands can be obtained from "stable" proteins, as evidenced by presentation of several species of posttranslationally modified peptides: N-glycosylated peptides, de-aminated peptides (Mosse et al., 1998), phosphopeptides (Zarling et al., 2006) or peptides with modified cysteine residues (Chen et al., 1999).

1.3.2.2. Rapidly versus slowly degraded polypeptides

As mentioned above, most of the peptides that enter the MHC I presentation pathway are generated by proteasome-mediated cleavage of polypeptides (Schubert et al., 2000). Note that the process of fragmenting a protein for generating an antigenic peptide is mutually exclusive from the process of using the same protein molecule to fold into a biologically active form. In principle, proteins can be sampled either at the beginning, the end, or anywhere in the middle of their lifespan. Proteins exhibit a wide range of degradation rates: from minutes to weeks, with an overall half-life of 1-2 days (Yewdell & Nicchitta, 2006). Polypeptides

have been segregated into two general pools: (1) those degraded with an average half-life of ~10 minutes, named rapidly degraded polypeptides (RDPs), and (2) those degraded with an average half-life from hours to weeks (in average ~2000 minutes), referred to as slowly degraded polypeptides (SDPs) (Yewdell & Nicchitta, 2006) (Figure 5).

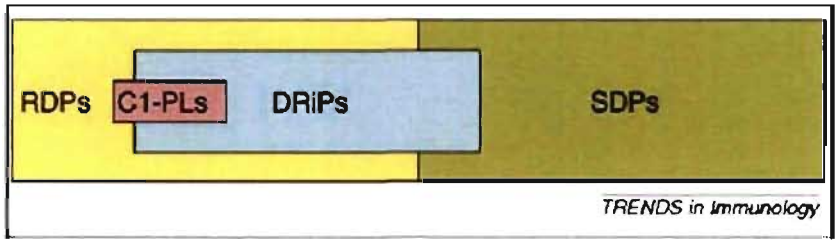

Figure 5. Intracellular polypeptide degradation pools. Polypeptides segregate into two general pools: those degraded with an average half-life of ~10 min (RDP) and those degraded with an average half-life of ~2000 min (SDP). DRiPs belong to the RDP or to the SDP pool, although it seems likely that most naturally generated DRiPs belong to the RDP pool. MHC I-peptide ligands (C1-PLs) seem to be predominantly derived from substrates in the RDP pool. These substrates include DRiPs and cleaved leader sequences. Adapted from (Yewdell & Nicchitta, 2006).

A significant proportion of peptides appears to result from the degradation of newly synthesized, but rapidly degraded polypeptides as opposed to slowly degraded polypeptides (Reits et al., 2000; Schubert et al., 2000). These RDPs are presumably well represented by either misfolded or erroneous proteins, which have been called DRiPs (Yewdell et al., 1996; Princiotta et al., 2003; van der Bruggen & Van den Eynde, 2006). Remarkably, RDPs could constitute 1% or less of newly synthesized proteins, yet still provide all of the peptides presented by MHC I molecules (Yewdell & Nicchitta, 2006). Strong evidence has been published supporting the idea that translation and protein folding must be error prone (Jensen, 2007) and that newly generated polypeptides, including DRiPs, represent the main source of antigens entering the MHC I loading pathway (Yewdell, 2005; Yewdell & Nicchitta, 2006). Thus, it has been suggested that, MHC I molecules preferentially sample what is being translated as opposed to what has been translated (Qian et al., 2006a; Qian et al., 2006b). Accordingly, the MHC I peptide repertoire has been shown to be biased toward peptides derived from highly abundant transcripts (Fortier et al., 2008). To note, long-lived intact proteins can also contribute to the peptide pool although possibly in a lesser extent (Yewdell & Nicchitta, 2006).

1.3.2.3.Driving force for peptide generation: the DRiPs hypothesis

Most proteins, including those that contain MHC I-restricted epitopes, are turned

over very slowly with half-lives of many hours if not days (Eisenlohr et al., 2007). Yewdell *et al.* pointed out that this rate is inconsistent with *in vitro* assays showing that cells become recognizable by CD8+ T cells soon after they are infected and that peptide production must commence very shortly after protein synthesis (Yewdell et al., 1996).

Consequently, Yewdell *et al.* proposed that immediate peptide supply is driven not by senescence of mature proteins but by newly synthesized proteins that are defective, termed DRiPs (Yewdell et al., 1996). These DRiPs, comprising polypeptides that fail to achieve its native structure, owing to imperfections in transcription, splicing, translation, post-translational modifications or protein folding, are flagged by the quality-control machinery and rapidly degraded (Yewdell et al., 1996) (Figure 6).

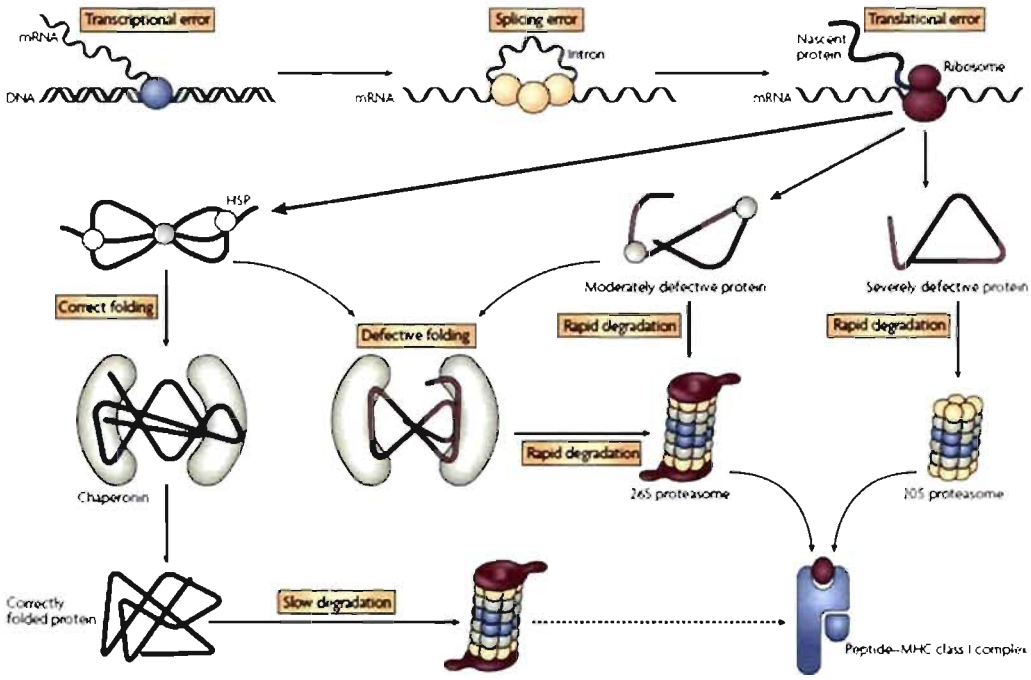

Figure 6. The DRiPs hypothesis. Accumulated errors during protein production (transcription, translation and folding) can lead to defective proteins that are instantly recognized by the quality-control machinery and targeted for rapid degradation, with some products ultimately becoming MHC I-bound epitopes. Misfolded proteins are subdivided into severely misfolded proteins that are degraded by the 20S proteasome and moderately misfolded proteins that are degraded by the 26 proteasome. Adapted from (Eisenlohr et al., 2007).

The DRiPs hypothesis was initially supported by some indirect evidence. First, early studies showed that mutant proteins that cause disease, as well as misfolded proteins induced in the presence of certain compounds, are immediately degraded after their synthesis (Knowles et al., 1975; Rieder et al., 1975). Second, a substantial fraction of newly synthesized proteins is rapidly turned over. This fraction has been estimated to correspond to 40% and

30% per hour (Wheatley et al., 1980; Yewdell et al., 1996; Schubert et al., 2000). Third, increasing the degradation rate of an antigen results in a substantially higher level of epitope production (Townsend et al., 1988). The DRiPs hypothesis was subsequently supported by experimental results showing that peptide production ceased 30 minutes after inhibition of protein synthesis, suggesting that mature protein turnover is too slow to make a meaningful contribution to the peptide pool (Reits et al., 2000; Yewdell, 2005).

Nevertheless, some aspects of the DRiPs hypothesis have been questioned (Eisenlohr et al., 2007). First, a DRiP has not yet been identified nor produced (Yewdell, 2005). Second, a window of 30 minutes for peptide production, implies a very short half-life of 15 minutes or less for the substrates from which the peptides are derived (Princiotta et al., 2003) and a quickly disposal of defective proteins. This seems not to be in line with current concepts of protein production and quality control arguing against the DRiPs model (Eisenlohr et al., 2007). On one side, there is an ever growing list of 'natively' or 'intrinsically' unfolded proteins that bypass the quality control machinery and are not degraded (Eisenlohr et al., 2007). On the other side, many misfolded proteins can be rescued by prolonged interaction with HSPs (Markossian & Kurganov, 2004; True, 2006). Concordantly, increased degradation is not always the fate of misfolded proteins. They can enter aggregates that resolve very slowly or not at all, becoming candidates for ubiquitin-mediated autophagy and not proteasomal degradation (Bukau et al., 2006). The DRiPs model has also been called into question by recent evidence showing that newly synthesized polypeptides are mostly protected from proteasomal degradation during and immediately after translation and that preexisting proteins represent the main proteasome substrates (Vabulas & Hartl, 2005).

A recent alternative model, not excluding the DRiPs hypothesis, proposes that a subset of nascent polypeptides is stochastically delivered to the 20S proteasome owing to neglect by the folding machinery (Eisenlohr et al., 2007) (Figure 7). This subset would presumably correspond to 25% of all synthesized proteins (Qian et al., 2006a). For a given antigen, the basal level of peptide presentation from immediately degraded substrate by the 20S proteasome will be supplemented by the cohort of newly synthesized proteins that is successfully intercepted by the folding machinery (Eisenlohr et al., 2007). The more defective the protein is, the sooner and more intense the presentation of the peptide will be, which is due to more rapid rejection by the quality control machinery.

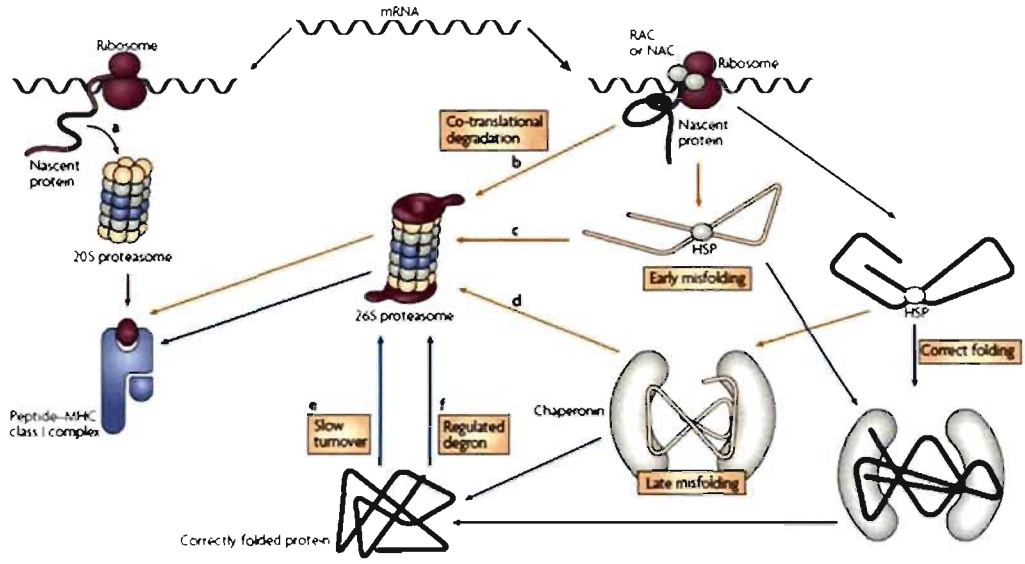

Figure 7. An alternative to the DRiPs model. Most mRNAs are translated by ribosomes associated with HSPs such as RAC and NAC, resulting in partial compaction of the nascent polypeptide and its delivery to downstream chaperones. If the protein carries an overt degradation signal (degron), then degradation starts immediately (b). If it carries a covert degron, then it is rapidly degraded after having achieved the mature state (f). Proteins are identified as defective at different steps towards maturation (a and c). A small fraction of mRNAs is translated by unengaged ribosomes and these unfolded protein species are degraded by the 20S proteasome (a). Adapted from (Eisenlohr et al., 2007).

1.3.2.4. Cryptic translation as a source of naturally processed peptides

In addition to conventional translation products, cells can also generate peptide ligands for MHC I molecules from cryptic translation products. Cryptic translation refers to polypeptides that are synthesized by unconventional translational mechanisms. These include peptides encoded by open reading frames contained within 5' and 3' untranslated regions, alternative open-reading frames, introns, or intron-exon junctions (Bullock & Eisenlohr, 1996; Mayrand et al., 1998; Shastri et al., 2002; Cardinaud et al., 2004). The list of MHC I-peptides derived from cryptic translation has been steadily growing (Ho & Green, 2006). Many examples of peptides of viral origin or in tumor cells have been described (Ho & Green, 2006). Cryptic translation also operates in normal professional as well as non-professional APCs (Schwab et al., 2003). Although cryptic peptides are expressed at low abundance within the MHC I-peptide pool, they have been shown to induce tolerance in transgenic mice that generate cryptic peptides, and elicit $CD8^+$ T-cell responses in normal mice (Schwab et al., 2003). Besides cryptic translation, translational errors, including ribosomal frameshifting, can have biological relevance in generating $CD8^+$ T cell determinants (Zook et al., 2006).

1.3.2.5. Peptides derived from peptide splicing

It has been reported that MHC I ligands can contain sequences that are not contiguous in the original protein but are spliced together from neighboring peptides (Hanada et al., 2004; Vigneron et al., 2004; Hanada & Yang, 2005; Warren et al., 2006). They represent spliced peptides generated by the proteasome. This suggests that the number of MHC I-peptides could be potentially larger than anticipated.

1.3.2.6. Peptides derived from proteins destined to the secretory pathway

Secretory and membrane proteins are also a known source of MHC I-peptides. Nevertheless these proteins have to exit the ER because there are no proteasomes present in the ER lumen (Wojcik & DeMartino, 2003). Proteins destined to the secretory pathway can gain access to the cytosol after being retrotranslocated from the ER, in a process that typically results in ubiquitination and proteasomal degradation, defined as ER-associated degradation (ERAD, see below) (Tsai et al., 2002). Many peptides derived from transmembrane or secretory proteins correspond to sequences derived either from transmembrane regions or signal sequences (Shastri et al., 2005).

1.4. Translation and Protein Folding

Protein synthesis is energetically the most expensive process in the cell. It places heavy demands upon the cell in terms of requirements of both amino acids and metabolic energy (Proud, 2007). Not surprisingly, translation rates are tightly regulated to match the requirements of the cell/tissue with the supply of metabolic energy and amino acids. Regulating the overall rate of protein synthesis is important not only for modulating cell and tissue metabolism and growth, but also for controlling gene expression (Proud, 2007). Cell proliferation also depends upon maintaining an adequate rate of protein synthesis (Scheper et al., 2007).

Protein synthesis is mediated by ribosomes. Ribosomes comprise two subunits: the 'large' subunit (60S in mammals) and the 'small' subunit (40S in mammals) (Scheper et al., 2007). The process of translation itself requires several other proteins, which are known as translation factors (Scheper et al., 2007). mRNA translation is conventionally divided into three stages: initiation, elongation and termination. During initiation, the ribosome and the tRNA for the first amino acid residue (methionyl-tRNA) are positioned at the first codon (AUG) of the mRNA. During elongation, the polypeptide chain is assembled step-by-step as dictated by the ORF of the mRNA. When the ribosome encounters a stop codon, termination occurs, resulting in release of the completed polypeptide and the ribosomal subunits (Scheper

et al., 2007).

Accuracy is essential at all these stages, particularly in locating the start codon. For most mRNAs, this involves a process whereby the 40S ribosomal subunit is recruited to the mRNA's 5' cap structure. Together with the methionyl-tRNA and certain translation initiation factors, the 40S subunit then scans along the 5' untranslated regions (5'UTR or 'leader') of the mRNA to find the start codon. In some cases, features within the 5' UTR allow the 40S subunit to enter downstream of the 5' cap, largely obviating the need for scanning. Such internal ribosomal entry sites (IRES) were first found in certain viral RNAs, but also occur in a subset of human cellular messengers (Jackson, 2005). Nevertheless, in eukaryotes, most mRNAs are translated in a cap-dependent manner.

Both 5' and 3' UTRs can contain other elements that modulate the efficiency of mRNA translation. Furthermore, sequences in the 3' UTR can affect translation through interaction of the poly-A-binding protein with eukaryotic initiation factor (eIF) 4F (Mangus et al., 2003).

It is worth to note that translation also occurs in mitochondria. Nonetheless, the components of the mitochondrial protein synthesis machinery are quite distinct from their cytosolic counterparts, generally being more similar to those of bacteria (Scheper et al., 2007).

After synthesis, proteins must rapidly fold to perform their biological activities. Folding takes place in three main subcellular compartments: cytosol, ER and mitochondria. Each organelle is equipped with a specific set of chaperones and folding enzymes (Anelli & Sitia, 2008). Whereas it is generally accepted that the ER functions uniquely in the biogenesis of secretory and integral membrane proteins, it has been recently shown that the ER membrane also supplies a substantial portion of newly synthesized proteins to the cytosol (Stephens & Nicchitta, 2008). The following section, I will concentrate on the mechanism by which proteins destined to the secretory pathway are produced, since it is the most studied and best understood.

1.4.1. mRNA partitioning and translation in the ER

mRNA partitioning between the cytosol and the ER compartments is an ubiquitous, highly conserved property of eukaryotic cells that serves to create a dramatic compartmentalization of protein synthesis: in general, mRNAs encoding cytosolic proteins undergo translation on free ribosomes, whereas mRNAs encoding secretory and integral membrane proteins are translated on ER-bound ribosomes (Nicchitta et al., 2005) (Figure

8).

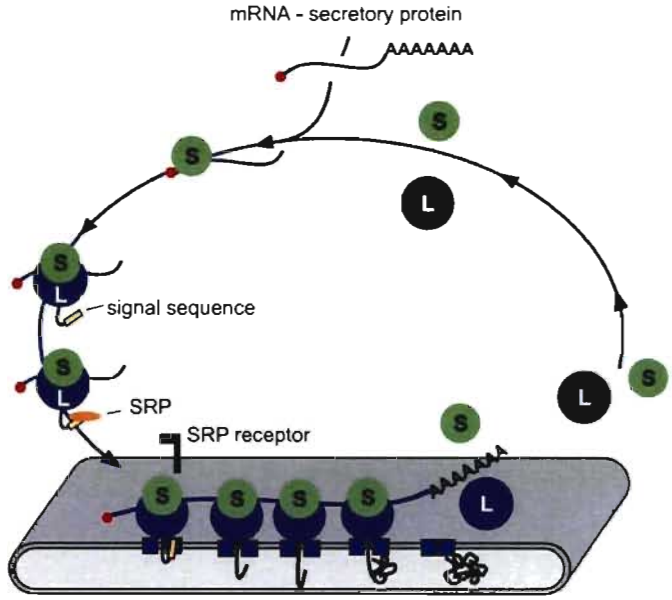

Figure 8. The SRP-ribosome cycle. Cytosolic ribosomes engaged in the translation of mRNAs encoding secretory or membrane proteins, are targeted through the SRP pathway to the ER membrane. At the ER, the signal sequence engages the protein-conducting channel and protein translocation ensues. Termination of the protein synthesis leads to the release of ribosomal subunits from the ER membrane to the cytosol. L: large ribosomal subunit, S: small ribosomal subunit. Adapted from (Nicchitta et al., 2005).

A considerable fraction of the proteome consists of molecules that are either secreted or inserted into membranes, to act as ligands and receptors, respectively (Anelli & Sitia, 2008). Proteins destined to the secretory pathway are directed to the ER through a predominantly hydrophobic signal sequence (Schroder & Kaufman, 2005). They are synthesized on ER-bound ribosomes, and are cotranslationally translocated into the ER lumen where they attain their native conformation, before being transported to the Golgi and downstream compartments (Anelli & Sitia, 2008).

In general, in the absence of an encoded signal sequence, mRNAs undergo continued translation on cytosolic ribosomes (Nicchitta et al., 2005). In the case of mRNAs containing a signal sequence, the mRNA-ribosome-nascent chain complex is relayed to the membrane by the signal recognition particle (SRP) early in translation (Nicchitta et al., 2005). The SRP elicits a suppression of translation and enables the trafficking of complexes to the ER (Meyer & Dobberstein, 1980). Then the protein traverses the ER membrane through an aqueous channel, named the Sec61p complex (Schroder & Kaufman, 2005). Sequential interactions of the mRNA-ribosome-nascent chain complex with the SRP receptor and with Sec61p, results in the release of the SRP, the binding of the ribosome to Sec61p, and a coupled process of protein synthesis and protein translocation (Stephens & Nicchitta, 2008).

The signal peptide is then cleaved off by signal peptidase (Schroder & Kaufman, 2005). Indeed, the signal sequence influences the timing of N-linked glycosylation and signal sequence cleavage (Rutkowski et al., 2003). Also, inefficient cleavage can result in prolonged interaction of the protein with ER chaperones (Stevens & Argon, 1999).

1.4.2. Protein folding in the ER

Initial stages of protein folding, which consist of shielding and compacting hydrophobic domains, are carried out by chaperones of the HSP family (Frydman, 2001). Specialized HSPs, positioned at the exit channel of the ribosome, rapidly intercept nascent polypeptides. In yeasts and higher eukaryotes, this function is carried out by two different complexes: the heterodimeric nascent polypeptide-associated complex (NAC) and the HSP70-associated ribosome-associated complex (RAC) (Wegrzyn & Deuerling, 2005; Wegrzyn et al., 2006). Partially compacted proteins are then transferred to the chaperonins, which shepherd the substrate through the final stages of condensation (Eisenlohr et al., 2007).

Many principles governing protein folding in the cytosol apply to the ER. However, protein folding in the ER is more complex than protein folding in the cytosol because proteins are posttranslationally modified (Schroder & Kaufman, 2005). The ER is unique in sustaining a set of covalent modifications, which include removal of the signal sequence, disulfide bond formation, N-glycosylation and glycophosphatidylinositol (GPI) addition. To note, removal of signal sequence and N-glycosylation are unique to secretory proteins (Anelli & Sitia, 2008). Different chaperones and folding assistants determine different folding pathways, such as the calnexin/calreticulin (CNX/CRT) cycle or the Binding Protein (BiP, also called GRP78) pathway. The choice of one or another is dictated by the localization of the N-glycans: the closer these are to the N-terminus, the higher the tendency to use CNX/CRT as a chaperone system (Molinari & Helenius, 2000). Very rarely glycoproteins are found to bind simultaneously BiP/GRP78 and CNX/CRT (Anelli & Sitia, 2008). Also, certain proteins that are produced in large amounts are assisted by substrate- or tissue-specific chaperones (Anelli & Sitia, 2008).

1.4.2.1. N-glycosylation and the CNX/CRT cycle

N-glycosylation involves binding of a preformed oligosaccharide ($Glc_3Man_9GlcNAc_2$) to consensus Asn-X-Ser/Thr residues (Figure 9). The sugar moieties are then progressively trimmed by resident enzymes. Soon after synthesis, glucosidases I and II sequentially remove the three glucoses from the A branch of the oligosaccharide. UDP-glucose glycoprotein

glucosyltransferase (UGGT) adds back a glucose residue to *N*-glycans (Taylor et al., 2004). The produced monoglucosylated proteins (Glc1Man9GlcNAc2) can then interact with CNX or CRT, two ER chaperones with lectin activity (Williams, 2006). Besides retaining misfolded proteins and preventing their aggregation (see Protein quality control section), CNX and CRT promote oxidative folding via interactions with ERp57. Then, by removing the terminal glucose, glucosidase II dissociates the substrate from CNX/CRT (Anelli & Sitia, 2008). If the protein has attained its native structure, it can now proceed along the secretory pathway by bulky flow or by interaction with specific lectin transporters such as ERGIC-53 (ER-Golgi intermediate compartment-53) or VIPL (Anelli & Sitia, 2008). However, if unfolding persists, the protein enters the CNX/CRT cycle again. On the other side, mannose trimming causes exit of terminally misfolded proteins from the cycle (Anelli & Sitia, 2008). The fate of misfolded proteins will be discussed in further sections.

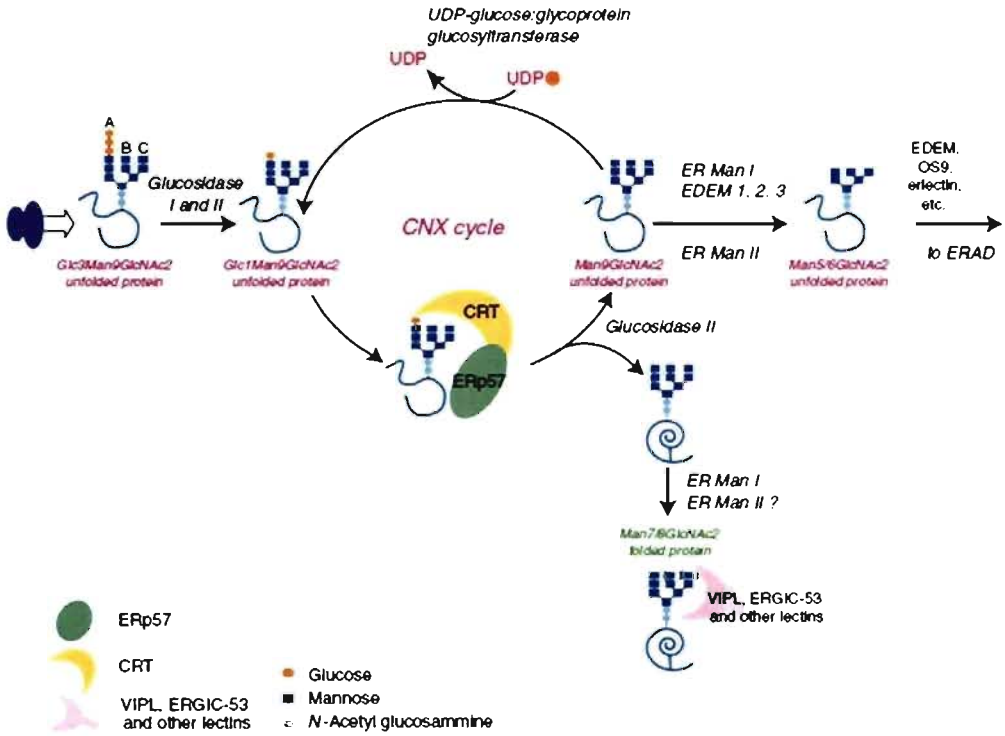

Figure 9. The CNX/CRT cycle. After transfer of the preformed core oligosaccharide onto nascent proteins, glucosidase I and II sequentially remove two terminal glucoses. The resulting monoglucosylated protein interacts with CNX and CRT, which in association with ERp57 prevents aggregation and facilitates folding. Removal of the glucose by glucosidase II liberates the protein form CNX and CRT. If the protein has attaint its native structure, it follows the secretory pathway by bulky flow or by interaction with transporters. If unfolding persists, UGGT1 places a single glucose back onto the protein, causing its entrance into the CNX/CRT cycle. Mannose trimming causes exit of misfolded proteins that can be targeted to ERAD. Adapted from (Anelli & Sitia, 2008)

1.4.2.2. The BiP/GRP78 pathway

The affinity of BiP/GRP78 for substrates depends on ATP. Thus, substrates can undergo cycles of BiP/GRP78 binding and release, depending on ATP hydrolysis (Anelli & Sitia, 2008). This process is regulated by hsp40-like co-chaperones containing a J domain (ERdj) (Shen et al., 2002).

1.4.2.3. Oxidative folding

A hyper-oxidizing environment in the ER lumen may inhibit folding of proteins with multiple disulfides. Therefore, oxidative folding relies primarily on the PDI pathway. PDI or PDI-like proteins catalyze disulfide bond formation in the ER (Anelli & Sitia, 2008). After transferring a disulphide bond to nascent proteins, PDI is re-oxidized by members of the Ero1 flavoprotein family (Anelli & Sitia, 2008).

1.5. Protein quality control in the secretory compartment

As we have seen, the ER provides an environment that facilitates the folding and assembly of newly synthesized secretory and transmembrane proteins and actively participates in the quality control of these proteins. The term 'ER quality control' refers to the processes of conformation-dependent molecular sorting of secretory proteins (Hurtley & Helenius, 1989). By these means, actively folding proteins are retained in the ER and shielded from degradation pathways, folded proteins are destined for export and packaged into transport vesicles, and misfolded proteins are retained and ejected into the cytosol for ubiquitin-dependent degradation (Meusser et al., 2005; Ismail & Ng, 2006).

Quality control in the ER is achieved by two independent mechanisms. The first one is the productive folding mechanism, which is involved in folding of ER-proteins and recognition of misfolding (Oda et al., 2006). The system must be able to distinguish between molecules that are actively folding, fully folded and misfolded (Ismail & Ng, 2006), a process that involves certain chaperones (Carvalho et al., 2006). Nevertheless, detailed information is lacking on how proteins are initially selected for degradation (Denic et al., 2006). The second mechanism, comprising terminal steps of ER quality control, is termed ERAD, retrotranslocation or dislocation (Meusser et al., 2005). Recently, it has been suggested that autophagy could also play a role in protein quality control (Bernales et al., 2007).

1.5.1. ERAD mechanism

The high flux of proteins into the ER together with the complicated multidomain nature of many secreted proteins, inevitably results in some fraction of proteins becoming terminally misfolded (Denic et al., 2006). The ER must specifically identify terminally misfolded proteins in an environment dominated by structurally similar folding intermediates. Proteins transiting the ER can be soluble or membrane bound with significant portions in the lumen, the membrane and the cytosol. To monitor misfolding despite this topological diversity and the misfolded substrate selection problem, the cell has developed distinct ERAD pathways. Substrates are targeted to an appropriate ERAD pathway depending on the site of the misfolded region. For instance, membrane and soluble proteins with luminal lesions are targeted to the ERAD-L pathway, whereas membrane proteins with misfolded cytoplasmic domains use the ERAD-C pathway (Ismail & Ng, 2006). Recently the ERAD-M pathway has been proposed for membrane proteins with misfolded intramembrane domains (Carvalho et al., 2006). These pathways diverge in their components, which are still not completely characterized. In general, the ERAD mechanism occurs in four steps: substrate recognition, translocation across the ER membrane, release in the cytosol and degradation (Figure 10).

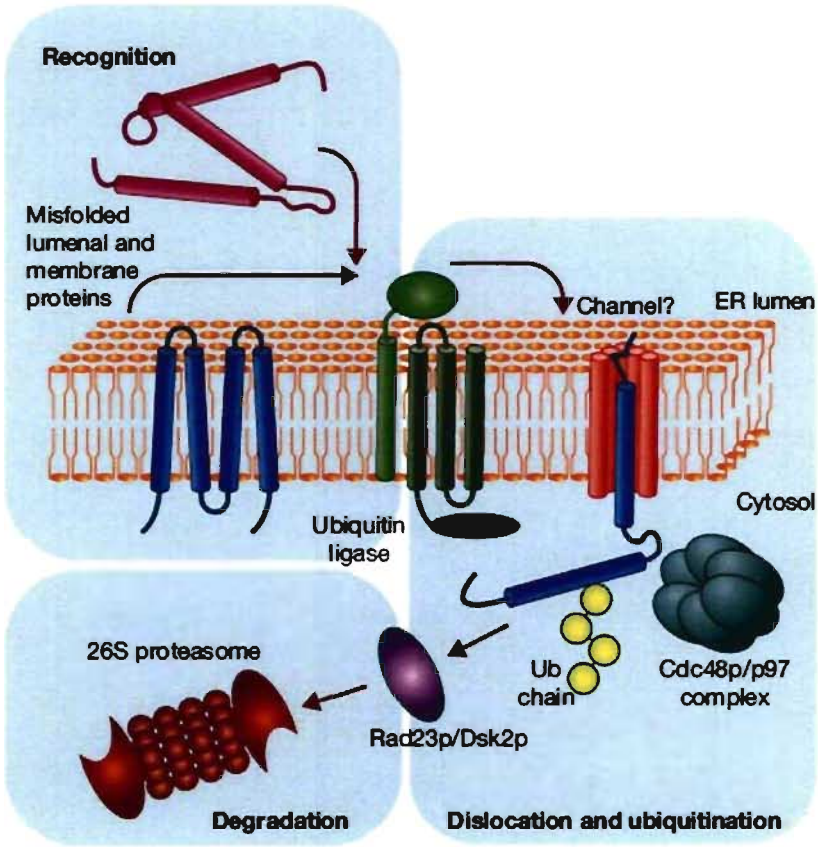

Figure 10. Proteasomal degradation of ERAD targets. Aberrant proteins are recognized within the ER lumen by the quality-control machinery and escorted to a putative channel that facilitates their export to the cytoplasm. Exposed lysine residues are ubiquitinated by ubiquitin ligases. Dislocation is completed with the help of the Cdc48p/p97 and membrane-extracted substrates are conveyed to the proteasome by accessory factors such as Rad23p and Dsk2p. Adapted from (Meusser et al., 2005).

Importantly, misfolded proteins are not the only substrates for ERAD, which is also required for the regulated turnover of ER resident proteins. The regulated breakdown of a key enzyme of the mevalonate pathway, HMG CoA, is controlled by this system (Hampton, 2002). Other examples include the δ-opioid receptor and the MHC I (Shamu et al., 1999; Petaja-Repo et al., 2001). In addition, it is now clear that elements of the ERAD system are exploited by human pathogenic viruses. For example, two membrane-anchored proteins encoded by the human cytomegalovirus (HCMV), US2 and US11, bind to MHC I molecules in the ER and initiate their dislocation into the cytoplasm where they are degraded by the proteasome (Wiertz et al., 1996).

1.5.1.1 Substrate recognition

Polypeptides that do not meet the quality control standards are retained within the ER and delivered to the ERAD ligases. In mammalian cells, the ligase CHIP targets defective proteins whose cytoplasmic domains are recognized by the cytoplasmic chaperones of the HSP70-HSP90 family (Meacham et al., 2001).

Polypeptides with native cytoplasmic domains are then examined for proper folding of the lumenal regions. The secretory pathway recognizes substructures within proteins such as hydrophobic patches, unpaired cysteines and immature glycans (Meusser et al., 2005). Proteins trapped in the CNX/CRT cycle, which normally retain and assist the folding of immature proteins in the ER, are eventually trimmed by ER mannosidase I (Meusser et al., 2005). This leads to their recognition by EDEM, a lectin likely to be specific for Man8GlcNAc2-oligosaccharides. Presumably, EDEM then targets misfolded glycoproteins for degradation (Molinari et al., 2003; Oda et al., 2003). This mechanism allows the selective elimination of proteins that have failed to mature properly even after extensive folding attempts. Much less is known on how terminally misfolded proteins that lack N-glycans are targeted to destruction (Anelli & Sitia, 2008).

Trimming of N-linked glycans is not the only mechansim that targets misfolded proteins for degradation. Aberrant proteins probable undergo partial unfolding and reduction before retrotranslocation (Anelli & Sitia, 2008). Both BiP/GRP78 and PDI have been implicated in this process (Molinari, 2002). BIP/GRP78 and other chaperones associate with hydrophobic surfaces of these proteins, whereas PDI and other oxidoreductases bind free thiols and control the formation of disulphide bonds between correct pairs of cysteine residues (Tsai et al., 2001; Wang & Chang, 2003; Schroder & Kaufman, 2005).

1.5.1.2. Transport across the ER membrane

Following recognition, misfolded proteins are targeted to processing sites on the ER membrane for their extraction. Next, the substrate is transported across the ER membrane, a process that, at least for luminal proteins, probably requires a protein-conducting channel (Carvalho et al., 2006). The nature of this conduit is a particular debated issue. One plausible hypothesis is that retrotranslocation occurs through the Sec61 channel, the one used for the transport of proteins in the "forward" direction, from the cytosol into the ER (Meusser et al., 2005). This has been supported by the fact that nascent polypeptides enter the ER in a largely unfolded conformation, and if export is mechanistically connected to the import machinery, then protein dislocation should also require unfolding of substrates. In agreement with this idea, certain proteins are reduced and unfolded by PDI to prepare them for dislocation (Tsai et al., 2001). In contrast, however, some model ERAD substrates arrive in the cytoplasm in a folded state (Tirosh et al., 2003). N-linked glycans increase the size of the exported molecule even further, because sugar moieties are not removed until the glycoproteins arrive in the cytosol (Meusser et al., 2005). Thus, this implies either some structural flexibility of the pore or alternate translocation mechanisms.

Other candidates for this channel have been proposed including the multispanning membrane protein Derl-1 in mammals (Derp1is the homologue in yeasts) and the ubiquitin ligases Hrd1p and Doa10p (Gauss et al., 2006). Both ligases possess a number of transmembrane domains that could form a pore and could physically link dislocation to ubiquitination (Meusser et al., 2005).

1.5.1.3. Release in the cytosol and degradation

When at least part of the substrate is exposed to the cytosol, E2 ubiquitin-conjugating enzymes and E3 ligases attach polyubiquitin tags to the protein, thus targeting it to destruction (Ismail & Ng, 2006). The substrates are recognized and fully extracted from the membrane by the p97/VCP-ATPase complex in mammals, or the Cdc48p ATPase complex in yeast.

The extracted substrate is finally degraded by the 26S proteasome in the cytosol (Romisch, 2005). In the case of glycosylated proteins, sugar moieties are previously removed by an N-glycanase to facilitate degradation by the proteasome (Anelli & Sitia, 2008). Proteasome inhibitors generally impair dislocation, implying that the dislocation, ubiquitination and degradation steps are tightly coupled (Mancini et al., 2000). Moreover, the association of proteasomes with the ER membranes (Kalies et al., 2005) may be important in coupling substrate extraction and degradation.

1.5.2. Autophagy

It has been recently proposed that in addition to ERAD, cells can dispose of aberrant proteins by autophagy (Bernales et al., 2007). On one hand, inhibition of ERAD stimulates autophagy (Yorimitsu & Klionsky, 2007). On the other hand, blocking autophagy stimulates glycoprotein dislocation and degradation (Anelli & Sitia, 2008).

1.6. ER stress and the Unfolded protein response

The ER is highly sensitive to stresses that perturb cellular energy levels, the redox state or calcium concentration because these factors are necessary for optimum protein folding (Szegezdi et al., 2006). Consequently, multiple physiological or pathological conditions can affect protein folding: hypoxia, glucose starvation, underglycosylation of glycoproteins, calcium flux across the ER membrane, viral infection, elevated protein synthesis and secretion, failure of protein folding, transport or degradation (Ma & Hendershot, 2004; Schroder & Kaufman, 2005; Zhao & Ackerman, 2006). These stress stimuli perturb the normal physiological state of the ER inducing ER stress (Schroder & Kaufman, 2005). Under conditions of ER stress, the quality control is inefficient and/or the client protein load is excessive compared with the reserve of ER chaperones (Marciniak & Ron, 2006; Oda et al., 2006). This imbalance results in accumulation of unfolded proteins in the ER (Oda et al., 2006).

1.6.1. How cells cope with ER stress

Unfolded proteins are prone to aggregation, which is toxic to the cell (Marciniak & Ron, 2006). Therefore, cells respond to ER stress by activating intracellular signal transduction pathways, collectively known as the Unfolded Protein Response (UPR) (Schroder & Kaufman, 2005). The UPR is in principle a homeostatic mechanism developed to maintain the balance between the folding demand and the synthetic capacity of the ER (Schroder & Kaufman, 2005; Marciniak & Ron, 2006). However, if protein misfolding is persistent and the normal ER functioning cannot be restored, signaling switches from pro-survival to pro-apoptotic (Rutkowski et al., 2006). Thus, the level of ER stress encountered by a cell dictates the nature of its UPR (Marciniak & Ron, 2006).

The remedies that the cell uses to restore ER homeostasis are i) to decrease the folding demand and ii) to increase the folding capacity of the ER (Schroder & Kaufman, 2005). On one hand, the folding demand is decreased by attenuating translation, downregulating

transcription of genes encoding secretory proteins, selectively degrading mRNAs encoding polypeptides destined for the ER lumen, and increasing degradation of slowly folding or misfolding proteins through ERAD (Schroder & Kaufman, 2005; Hollien & Weissman, 2006). On the other hand, the folding capacity of the ER is increased by i) promoting the synthesis of ER resident molecular chaperones and foldases involved in folding and ii) increasing the ER size to dilute the increased unfolded protein load (Schroder & Kaufman, 2005).

1.6.2. The UPR acts through three main signal transduction pathways

The UPR is mediated by at least three ER transmembrane sensors, PKR-related ER kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6) (Moenner et al., 2007) (Figure 11). In resting cells, all three ER stress receptors are maintained in an inactive state through their association with BiP/GRP78 (Szegezdi et al., 2006). As explained above, BiP/GRP78 is an abundant chaperone within the ER that binds to folding proteins through interaction with exposed hydrophobic residues (Marciniak & Ron, 2006). Upon accumulation of unfolded proteins, BiP/GRP78 dissociates from the three receptors, which leads to their activation and triggers the UPR (Schroder & Kaufman, 2005). PERK is thought to be the first arm of the UPR to be activated, followed by ATF6 and lastly IRE1 (Szegezdi et al., 2006). The roles of these UPR mediators are explained below.

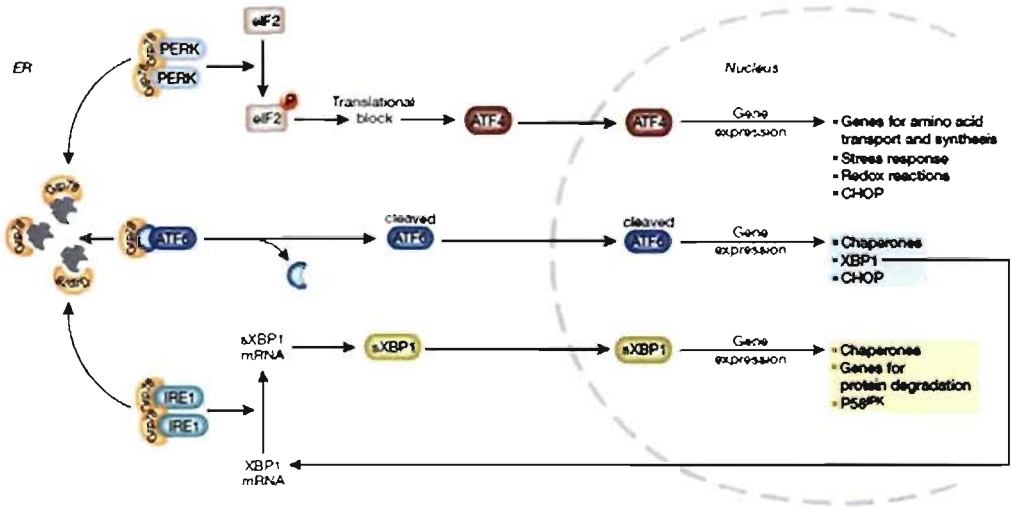

Figure 11. The UPR. Upon accumulation of misfolded proteins, BIP/GRP78 dissociates from PERK, ATF6 and IRE1, leading to activation of the three main signal transduction pathways, collectively referred as the UPR. Adapted from (Szegezdi et al., 2006).

1.6.2.1. PERK initiates the translational arm of the UPR

Dissociation of BiP/GRP78 from PERK initiates the translational arm of the

UPR, which constitutes a short-term adaptation to reduce the load of newly synthesized polypeptides entering the ER lumen (Ron, 2006). This dissociation causes the dimerization and autophosphorylation of the serine threonine-kinase to generate active PERK (Szegezdi et al., 2006). Once activated, PERK phosphorylates eIF2α on Ser^{51} (Moenner et al., 2007). The eIF2 complex is essential in all eukaryotes for new protein synthesis, since it recruits the initiator methionyl-tRNA to ribosomes about to begin translation (Asano & Hinnebusch, 2001). Phosphorylation of eIF2α inhibits this activity and thus globally reduces cap-dependent protein translation (Marciniak & Ron, 2006). Consequently, inhibition of protein translation aids cell survival by decreasing the load of nascent proteins arriving at the ER (Szegezdi et al., 2006).

While the majority or RNA transcripts experience decreased translation during periods of increased eIF2α phosphorylation, a small and still poorly defined subset is translated more efficiently, probably to fulfill basic cellular survival needs (Marciniak & Ron, 2006; Zhao & Ackerman, 2006). This paradoxical effect is due to the presence of multiple upstream non-coding ORFs, that are still translated (Schroder & Kaufman, 2005). Genes carrying certain regulatory sequences in their 5' untranslated regions, such as IRES in the case of BiP/GRP78, can bypass the eIF2α-dependent translational block (Fernandez et al., 2002a). Another well characterized example is the transcription factor ATF4, which promotes cell survival by inducing genes involved in the amino acid metabolism, redox reactions, stress response, and protein secretion (Harding et al., 2003) (Vattem & Wek, 2004). ATF4 induces the transcription factor C/EBP homologous protein (CHOP), also known as GADD153. During ER stress, all three arms of the UPR induce transcription of CHOP (Szegezdi et al., 2006). However, to upregulate CHOP protein expression, the PERK- eIF2α-ATF4 branch is essential (Szegezdi et al., 2006). Thus, the effects of eIF2α phosphorylation are not restricted to attenuation of protein translation, but also include activation of a transcriptional program (Harding et al., 2000).

1.6.2.2. IRE1 activates the transcriptional arm of the UPR

The transcriptional arm of the UPR constitutes a long-term adaptation that increases the capacity of the organelle to handle unfolded proteins (Ron, 2006). This arm is induced in part by IRE1. The IRE1 dependent arm of the UPR is the most ancient in evolutionary terms (Tirasophon et al., 2000). IRE1 is a dual-activity kinase, possessing a serine-threonine kinase domain and an endoribonuclease domain. On activation, the endonuclease activity of IRE1 removes a 26-nucleotide intron from the X-box-binding protein 1 (XBP1) mRNA (previously induced by ATF6) (Szegezdi et al., 2006). The generated frameshift spliced variant (sXBP1) encodes a stable, active transcription factor (Yoshida et al., 2001). sXBP1 has diverse

targets including ER chaperones and the HSP40 family member $P58^{IPK}$ (Lee et al., 2003). Upregulation of the latter is not an immediate event. $P58^{IPK}$ binds and inhibits PERK thereby providing a negative feedback loop that relieves the PERK-mediated translational block (Yan et al., 2002). Also, both XBP1 and ATF6 activate the transcription of genes involved in ERAD (Zhao & Ackerman, 2006).

IRE1 itself but not XBP1 is also involved in posttranscriptional regulation of a specific mRNA subset (Hollien & Weissman, 2006). Probably, engagement of IRE1 with nascent polypeptides encoding secreted and membrane proteins in the luminal side induces degradation of its encoding mRNAs (Ron, 2006). This IRE1-dependent degradation of mRNAs is likely to complement translational repression and further reduce the unfolded protein load on the stressed cells (Ron, 2006).

1.6.2.3. Activation of the transcriptional arm of the UPR by ATF6

The transcriptional arm of the UPR is also activated by ATF6. After dissociation of BiP/GRP78, ATF6 translocates to the Golgi apparatus where it is cleaved into its active form by two proteases, SP1 and SP2 (Szegezdi et al., 2006). The released transcription factor moves to the nucleus and promotes the transcription of genes with an ER stress response element (ERSE) (Schroder & Kaufman, 2005; Szegezdi et al., 2006). So far, the identified targets of ATF6 include ER chaperones such as GRP94, PDI, BiP/GRP78 and the transcription factors CHOP and XBP1 (Szegezdi et al., 2006). Although ATF6 can induce CHOP transcription, no reports have linked ATF6 to ER-stress induced apoptosis. Hence, it seems that ATF6-mediated signals are purely pro-survival and aim to counteract ER stress (Szegezdi et al., 2006).

1.6.2.4. Other effects of the UPR

UPR also incurs profound indirect effects on other cellular processes. For instance, DNA microarray analyses suggest that ER stress upregulates the transcription of many secretory pathways components, including proteins functioning in peptide translocation, glycosylation and folding in the ER, proteins involved in anterograde and retrograde transport between the ER and Golgi, and proteins implicated in secretion from trans Golgi (Travers et al., 2000). Without a functional UPR, protein translocation across the ER membrane is partially impaired (Ng et al., 2000). Moreover, proteins involved in lipid metabolism and heme biosynthesis are also upregulated by the UPR (Travers et al., 2000). This response also inhibits cell cycle at G1 through inhibition of cyclin D1 translation via the PERK/eIF2α branch (Brewer et al., 1999).

Activation of ER stress signaling has been correlated with induction of cell death. Nonetheless, the mechanisms of ER-stress-induced cell death are still not fully understood (Rutkowski et al., 2006; Szegezdi et al., 2006). A strong link between ER stress signaling and apoptotic pathways has been established through the discovery of the physical interaction between the pro-apoptotic BCL-2 family proteins Bak /Bax and the cytosolic domain of IRE1 (Hetz et al., 2006). Interestingly, this interaction is necessary for IRE1 activation (Hetz et al., 2006). IRE1 can also activate the ASK1/JNK mitogen-activated protein kinase or p53 pathways, resulting in commitment of the cell to apoptosis via the classic pathway (Rutkowski et al., 2006; Szegezdi et al., 2006).

ER stress may also cause apoptosis through non-mitochondrial pathways. Capsase 12 is involved in this mechanism (Nakagawa et al., 2000). Cell death due to long-term ER stress may also induce oxidative stress. The gene encoding the oxidoreductase ERO1α is a target of the transcription factor CHOP (Marciniak et al., 2004). ERO1α participates in protein disulfide bond formation during protein refolding to help relief ER stress, but in doing so also transfers electrons to molecular oxygen, forming reactive oxygen species (ROS). ROS are toxic to the cell and may lead to apoptosis (Zhao & Ackerman, 2006).

1.6.3. The UPR in disease and normal physiology

Besides serving to protect cells from normal variations that occur in the cellular environment, the UPR can also be activated in pathological conditions (Ma & Hendershot, 2004). Numerous pathophysiological conditions are associated with ER stress, including viral infection, cancer, neurodegenerative diseases and diabetes (Zhao & Ackerman, 2006).

Expression of mutant, folding-incompetent proteins is the basis for a wide variety of diseases termed ER storage or conformational diseases (Ma & Hendershot, 2004; Schroder & Kaufman, 2005). Accumulation of folding-incompetent proteins resistant to proteasomal degradation in the ER can completely disrupt the ER function and activate apoptotic-signaling pathways. This is the basis for many neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Ma & Hendershot, 2004; Schroder & Kaufman, 2005). Also, mutations in the cytosolic portion of membrane proteins can disturb folding of the ER-luminal part of the protein and cause ER stress, as it is the case for a mutation in the cystic fibrosis transmembrane conductance regulator (Schroder & Kaufman, 2005).

Pancreatic failure associated with autoimmunity that occurs in type I diabetes, can result from misfolded mutant insulin that causes ER stress in pancreatic β-cells (Harding &

Ron, 2002). Mutations or over-activation of the PERK branch of the UPR can also lead to type I diabetes (Zhao & Ackerman, 2006). Furthermore, it has been shown that ER stress is a key player in obesity-induced type II diabetes. For instance, mice heterozygous for a targeted mutation of eIF2α (which can not be phosphorylated) become obese and develop diabetes on a high-fat diet (Zhao & Ackerman, 2006).

ER stress and UPR have also been observed in tumors (Ma & Hendershot, 2004), but how UPR activation contributes to cancer cell survival is not clear. Many ER stress-related proteins such as BiP/GRP78 display an increased expression in cancers (Moenner et al., 2007). In line with this, it has been found that hypoxia and glucose starvation, conditions often found in the core of tumors, can activate the UPR (Ma & Hendershot, 2004; Schroder & Kaufman, 2005). Additionally, the PERK and IRE1 branches of the UPR can upregulate the potent proangiogenic factor VEGF-A (Moenner et al., 2007).

Viral infection can activate, but also modulate the UPR (Schroder & Kaufman, 2005). Some examples include the hepatitis B and C viruses, human HCMV, severe acute respiratory coronavirus, flavivirus and the West Nile virus (Tardif et al., 2005; Chan et al., 2006; Yu et al., 2006; Li et al., 2007; Medigeshi et al., 2007). In general, viral infection leads to eIF2α phosphorylation, as part of an innate antiviral response antagonizing viral protein translation and triggering the UPR (Schroder & Kaufman, 2005; Marciniak & Ron, 2006).

Finally, the UPR is essential to a range of normal physiological and developmental processes (Wu & Kaufman, 2006). These include the regulation of insulin secretion by pancreatic β-islet cells, the differentiation of immunoglobulin-secreting plasma cells and osteoblasts, and the proliferation of hepatocytes (Wu & Kaufman, 2006). Moreover, UPR-signaling pathways respond to the nutritional state of the cell and control regulatory gene clusters involved in metabolism and starvation responses (Schroder & Kaufman, 2005). These examples illustrate how the UPR signaling may extend beyond simply maintaining homeostasis of the ER (Schroder & Kaufman, 2005).

2. RESEARCH PROJECT

2.1. Rationale

Both the diversity and abundance of peptides presented by MHC I molecules at any given moment, determine the response of T lymphocytes and dictate eventual recognition and elimination of infected and neoplastic cells. This highlights the importance of studying conditions that affect the MHC I-antigen processing and presentation pathway that could potentially be applied in immunotherapy. Thereby, we were particularly interested in studying whether and how stressful conditions that affect the homeostasis of the ER regulate presentation of peptides by MHC I molecules.

2.2. Research question and hypothesis

The UPR has pervasive effects on cell protein economy: attenuation of general protein synthesis, increased transcription of UPR-associated genes involved in protein folding, and increased degradation of slowly-folding proteins in the ER. Thus, the UPR alters protein synthesis and degradation in order to reestablish cell homeostasis. However, these effects are mainly seen on proteins destined to the secretory pathway that need to be folded in the ER. Similarly, MHC I-peptide generation depends on protein economy: MHC I-peptides are preferentially generated from rapidly degraded polypeptides relative to slowly degraded proteins. Since UPR depends on protein synthesis and degradation and these processes are crucial for peptide generation, we hypothesized that the UPR should have an impact on peptide presentation by MHC I molecules. Based on this hypothesis, we addressed two main questions in our work:

1) Does the UPR affect MHC I-peptide presentation?
2) Does the UPR differentially regulate expression of MHC I-peptides derived from a protein destined to the ER versus a protein localized in the cytosol?

2.3. Objectives

2.3.1. General objective

The main aim of this study was to determine whether and how the UPR affects presentation of peptides by MHC I molecules.

2.3.2. Specific objectives

Specific objective 1: To assess the effect of the UPR on surface expression of MHC I molecules in different cell lines stressed with pharmacological agents.

Specific objective 2: To measure the effect of the UPR induced by glucose starvation or pharmacological agents, on presentation of the model peptide SIINFEKL by $H2K^b$ molecules in EL4 cells.

Specific objective 3: To determine whether the UPR induced by glucose starvation or chemical ER stress, differentially regulates surface expression of ER- versus cytosol-derived SIINFEKL in EL4 cells.

3. METHODOLOGY

As mentioned above, the main objective of our work was to study the effect of the UPR on MHC I-peptide presentation. For this aim, we used different experimental approaches that I will describe briefly in this section. More details can be found in the "Materials and Methods" section of the manuscript.

3.1. Model for in vitro quantification of MHC I-peptide complexes

It has been suggested that the subcellular localization of a protein may influence MHC I presentation of peptides derived from that specific protein (Golovina et al., 2002; Leifert et al., 2004; Caron et al., 2005). Thus, we used molecular cloning, confocal microscopy and flow cytometry techniques to create a model to quantify MHC I presentation of peptides derived from a source protein located in different subcellular compartments.

3.1.1. DNA constructs

Different variants of the hen egg lysozyme (HEL) were modified by polymerase chain reaction (PCR) (Armstrong et al., 1997; Qi et al., 2000). The constructs encoding HEL targeted to different cellular compartments (nucleus, mitochondria, cytosol, ER or secreted protein) were fused to a minigene coding for the Ovalbumin-derived peptide SIINFEKL and the c-myc tag. PCR amplification products were subcloned into the pPCR-Script Amp vector and then cloned into the bicistronic pIRES-EGFP2 vector.

3.1.2. Localization of HEL protein variants

COS-7K^b and HeLa-K^b cells were transiently transfected with these constructs. Transfected cells were identified by expression of the EGPF protein. Proper localization of the HEL protein variants was verified by confocal microscopy in transiently transfected cells. Antibodies against the c-myc tag, as well as organelle markers (CNX for the ER, mitoTracker for mitochondria and DAPI for the nucleus) were used to analyze the location of the HEL variants.

3.1.3. Assessment of K^b-SIINFEKL surface expression

The SIINFEKL peptide binds to the H2K^b MHC I molecule and cell surface K^b-SIINFEKL complexes are specifically recognized by the 25-D1.16 mAb (Porgador et al.,

1997). We evaluated whether cells transfected with these constructs were able to process the peptide and thus express K^b-SIINFEKL at the cell surface. To this end, we transitory transfected HeLa-K^b cells with the constructs, labeled the cells with the 25-D1.16 mAb and measured K^b-SIINFEKL abundance at the cell surface by flow cytometry.

3.2. Engineering of K^b-SIINFEKL stable transfectant cell lines

The UPR is primarily orchestrated to decrease protein overload in the ER (Schroder & Kaufman, 2005). We therefore wished to determine whether the UPR would differentially affect MHC I presentation of peptides derived from a protein source located in the ER (secretory pathway) versus the cytosol (non-secretory pathway).

3.2.1. Stable transfectants

EL4 thymoma cell line were used to generate stable transfectants because it expresses relatively high levels of MHC I (Fortier et al., 2008). EL4 cells were stably transfected with the described plasmids encoding modified HEL targeted to the ER (HEL-ER-SIINFEKL) or to the cytosol (HEL-Cyto-SIINFEKL). Stable transfectants were selected by repeated cycles of fluorescence-activated cell sorting (FACS) of EGFP-positive cells combined with drug resistance. Processing and presentation of SIINFEKL in these stable transfectants was assessed by staining with the 25-D1.16 mAb. Two of these clones, denoted, EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL, which displayed similar amounts of K^b-SIINFEKL at the cell surface, were used in further experiments.

3.3. Assessment of the effect of UPR on MHC I-peptide presentation

To induce ER stress we used pharmacological agents and glucose starvation (Murray et al., 2004; Ozcan et al., 2008). UPR activation was monitored by upregulation of UPR markers by Western blot and quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) (Yoshida et al., 2001; Lee, 2005; Shang, 2005). MHC I and MHC I-peptide presentation were measured by flow cytometry. MHC I transcript levels were analyzed by RT-qPCR.

3.3.1. Stress treatment

Human (HEK 293 and HeLa) and mouse (EL4) cells were submitted to chemical ER stress. Cells were treated with different concentrations of dithiotheritol (DTT) or tunicamycin for various time periods. DTT rapidly disrupts disulfide bond formation in the ER, whereas tunicamycin prevents N-linked glycosylation. Glucose starvation was induced by culturing EL4 transfectant cell lines in Dulbecco's modified Eagle's medium (DMEM) lacking glucose and sodium piruvate for various time periods.

3.3.2. UPR induction

Activation of the UPR was monitored by RT-qPCR analysis of the UPR markers BiP/ GRP78, XBP-1 and CHOP. In some cases, upregulation of BiP/GRP78 protein level was evaluated by Western blot.

3.3.3. MHC I expression

Surface expression of HLA A/B/C, $H2K^b$ and $H2D^b$ was measured by flow cytometry in human or mouse cell lines submitted to ER stress. Also, MHC I and β_2-m transcript levels were determined by RT-qPCR in these cells.

3.3.4. Measurement of existent and newly generated K^b-SIINFEKL complexes

Cell surface K^b-SIINFEKL complexes were quantified with the 25-D1.16 mAb in cells previously stressed with pharmacological agents or by glucose starvation. We also evaluated the impact of the UPR on generation of new K^b-SIINFEKL complexes. After chemical stress or glucose deprivation, cells were acid stripped to elute existent MHC I-peptide complexes at the cell surface (Sugawara et al., 1987; Storkus et al., 1993; Fortier et al., 2008). Generation of new complexes was measured thereafter by flow cytometry.

4.2. Déclaration de l'étudiant concernant l'article

Ma contribution à cet article s'est fait au niveau de la planification et de la réalisation de toutes les expériences, de l'analyse et l'interprétation des résultats et de la rédaction du manuscrit.

Marie-Pierre Hardy a participé aux expériences impliquant des cinétiques en cytométrie en flux et à la correction finale du manuscrit.

Étienne Caron a participé aux expériences préliminaires en biologie moléculaire et à la correction finale du manuscrit.

Claude Perreault a conçu le projet de recherche, contribué à la planification des expériences et rédigé le manuscrit.

4.3. ER stress affects processing of MHC class I-associated peptides

Running title: *ER stress molds the MHC I peptidome*

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Abstract

Background

Viral infection and neoplastic transformation trigger endoplasmic reticulum (ER) stress. Thus, a large proportion of the cells that must be recognized by the immune system are stressed cells. Cells respond to ER stress of any origin by launching the unfolded protein response (UPR). The UPR regulates the two key processes that control major histocompatibility complex class I (MHC I)-peptide presentation: protein translation and degradation. We therefore asked whether and how the UPR impinges on MHC I-peptide presentation.

Results

We evaluated the impact of the UPR on global MHC I expression and on presentation of the SIINFEKL peptide. EL4 cells stably transfected with vectors coding HEL-SIINFEKL protein variants were stressed with pharmacological agents or exposed to glucose deprivation. UPR decreased surface expression of MHC I at the protein but not the mRNA level. Consequently, presentation of SIINFEKL by H2-K^b molecules was reduced in chemically or physiologically stressed cells. Notably, stressed cells preferentially presented MHC I-peptides derived from an ER-retained as opposed to a cytosol-localized protein variant. Furthermore, generation of new H2K^b-SIINFEKL complexes after acid strip was less affected for ER- than for cytosol-derived SIINFEKL.

Conclusion

Our results show that ER stress impairs MHC I-peptide presentation, and that it differentially regulates expression of ER- vs. cytosol-derived peptides. This work indicates how ER stress, a typical feature of infected and malignant cells, can impinge on cues for adaptive immune recognition.

Background

The ultimate role of the immune system in host defense is to eliminate infected and transformed cells [1, 2]. A fundamental feature of infected and neoplastic cells is that they are stressed cells [3-5]. In line with this, the innate immune system uses receptors such as NKG2D to recognize stressed cells [4, 6, 7]. One key question, however, is whether cellular stress can influence recognition of transformed or infected cells by the adaptive immune system [4, 8].

The single feature uniting different stress stimuli (heat shock, hypoxia, viral replication, abnormal proteins, starvation or transformation) is that they all ultimately lead to accumulation of unfolded or misfolded proteins in the lumen of the ER [4, 5] . Infection and neoplastic transformation increase protein translation and thereby the folding demand on the ER [9, 10]. This is particularly true for cells submitted to hypoxia, nutrient deprivation or low pH in poorly vascularized bulky tumors, metastases and sites of inflammation [11, 12]. Moreover, acquisition of numerous mutations during tumor progression leads to accumulation of abnormal proteins with an increased propensity to misfolding that further increase the ER folding burden [3, 13].

The ER responds to the accumulation of unfolded proteins by activating intracellular signal transduction pathways, collectively called the UPR [14, 15]. The UPR is a highly conserved adaptive response that allows survival to limited stress but leads to apoptosis in the presence of overwhelming stress [16, 17]. Mammalian UPR acts through three main transducers (PERK, ATF6 and IRE1) that are activated by dissociation of the master regulator BiP/GRP78 [5, 15]. Activation of PERK leads to phosphorylation of the translation initiation factor eIF2α and attenuation of cap-dependent translation [18]. The endonuclease activity of IRE1 generates a frameshift splice variant of XBP1 encoding an active transcription factor that activates genes involved in protein degradation and controls the transcription of chaperones [19-21]. Targets of the cleaved active form of ATF6 include the chaperones BiP/ GRP78 and GRP94, and the transcription factors XBP-1 and CHOP [17, 19]. Activation of these UPR transducers has pervasive effects on cellular protein economy: i) attenuation of protein translation, ii) increased degradation of ER proteins by ER-associated degradation (ERAD), iii) transcriptional activation of genes involved in the folding machinery of the ER and iv) increased degradation of ER-localized mRNAs [14, 22].

Presentation of MHC I-associated peptides to CD8 T cells is tightly linked to protein economy. MHC I peptides are preferentially generated from newly synthesized but rapidly degraded polypeptides relative to slowly degraded proteins [23, 24]. Following proteasomal

degradation, peptides are translocated into the ER where they undergo N-terminal trimming, loading onto MHC I molecules and export at the cell surface [25-29]. Since the UPR regulates the two key processes that shape MHC I peptide processing (protein translation and degradation) we reasoned that ER stress should impinge on MHC I peptide presentation. We addressed this question and found that MHC I presentation was impaired during ER stress induced by pharmacological agents or glucose starvation. Moreover, ER stress differentially affected presentation of peptides derived from a protein localized in the ER vs. the cytosol.

Results

UPR activation impairs MHC I surface expression

To induce ER stress we first used dithiotheritol (DTT), a chemical agent known to rapidly disrupt disulfide bond formation in the ER [30, 31]. Human HeLa and HEK 293 cells were treated with a low or high concentration of DTT for 8 and 16 hours. Activation of the UPR was monitored by quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) analysis of BiP and XBP-1 transcripts, which are known to be induced during ER stress [19, 32]. As expected, BiP and XBP-1 mRNA levels were upregulated in DTT-treated cells compared to untreated cells (Figure 1A). Of note, upregulation of BiP was more dramatic in HeLa cells, while that of XBP-1 transcripts was more important in HEK 293 cells.

To evaluate the effect of the UPR on MHC I expression, we quantified surface expression of HLA A/B/C on DTT-treated cells by flow cytometry. Cells in later apoptotic stages were excluded from the analysis by gating on propidium iodide-negative cells. MHC I expression decreased in a DTT dose-dependent manner at the surface of HeLa and HEK 293 cells (Figure 1B). Stressed cells expressed between 60 and 90% of the amount of MHC I expressed by untreated cells. Since one of the UPR transcriptional mechanisms is the degradation of mRNAs encoding secreted or membrane proteins [22], we investigated whether the decreased MHC I surface expression was due to down-regulation of MHC transcripts. Using quantitative RT-qPCR, we found that MHC I mRNA levels were not reduced in DTT-treated cells. In fact, the abundance of MHC I transcripts tended to increase in stressed cells relative to control cells (Figure 1C). These results show that induction of ER stress for 8 to 16 hours significantly decreased MHC I surface expression through posttranscriptional mechanism(s).

Engineering of K^b-SIINFEKL stable transfectant cell lines

Evidence suggests that subcellular localization of a protein (e.g., in the cytosol vs. the secretory pathway) may influence MHC I presentation of peptides derived from that specific protein [33-35]. Moreover, the UPR is primarily orchestrated to decrease protein overload in the ER [14, 15]. We therefore wished to determine whether the UPR would differentially affect MHC I presentation of peptides derived from a source protein located in the cytosol versus the ER. To this end, we created stable EL4 transfectant cell lines expressing a chimeric protein located either in the ER or the cytoplasm (Figure 2A). We selected the EL4 thymoma cell line as a model because it expresses relatively high levels of MHC I [35]

which allows us to assess changes of MHC I abundance over a wider range. To create the chimeric constructs, a minigene coding for the SIINFEKL peptide was fused to previously described plasmids encoding hen egg lysozyme (HEL) targeted to the ER or the cytosol [36, 37]. The Ovalbumin-derived SIINFEKL peptide is presented by $H2K^b$ and cell surface expression of $H2K^b$/SIINFEKL complexes was assessed by staining with the 25-D1.16 monoclonal antibody [38]. As shown in Figure 2B, EL4 stable transfectants, denoted EL4/ HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL, can process and present SIINFEKL derived form the ER-localized or cytosolic chimeric proteins, respectively. These two clones, which display similar amounts of K^b-SIINFEKL at the cell surface, were used in further experiments.

UPR decreases MHC I surface expression in EL4 stable transfectants

In preliminary experiments, we found that concentrations of DTT used for UPR activation caused apoptosis of EL4 cells (data not shown). To circumvent this caveat, we treated EL4 cells with tunicamycin, another UPR-inducing agent that prevents N-linked glycosylation in the ER. Treatment of both EL4 transfectants with tunicamycin for 16 hours did not cause significant apoptosis but resulted in stimulation of the UPR as indicated by up-regulation of BiP, XBP-1 and CHOP transcripts (Figure 3A). Activation of the UPR with tunicamycin reduced cell surface expression of $H2K^b$ and $H2D^b$ by 60-70% in both cell lines (Figure 3B). Notably, tunicamycin had similar effects on the two transfectant cell lines inasmuch as EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL cells showed similar cell surface MHC I down-regulation. RT-qPCR experiments showed that mRNA expression levels of $H2K^b$, $H2D^b$ and β_2-microglobulin were not decreased by treatment with tunicamycin (Figure 3C). In fact, similar to what we found in HeLa or HEK 293 cells treated with DTT (Figure 1C), tunicamycin-treated EL4 transfectants had a tendency to express higher levels of MHC I transcripts relative to non-treated cells (Figure 3C). These results show that UPR induction leads to posttranscriptional attenuation of cell surface MHC I expression in stably transfected mouse cell lines as it did in human cell lines.

UPR differentially affects expression of ER- vs. cytosol-derived SIINFEKL

In the next series of experiments, K^b-SIINFEKL surface expression was quantified in EL4 transfectants grown in the presence of tunicamycin for 16, 21 or 30 hours (Figure 4A). During this time frame, abundance of cell surface K^b-SIINFEKL decreased by more than 70% in treated relative to untreated cells. Furthermore, we found that although K^b-SIINFEKL expression was reduced in both cell lines, it remained higher at all time points in EL4/HEL-ER-SIINFEKL cells than in EL4/HEL-Cyto-SIINFEKL cells. We wish to emphasize that

differences in abundance of K^b-SIINFEKL among the two EL4 transfectant cell lines treated with tunicamycin (Figure 4A) cannot be ascribed to an overall discrepancy in expression of $H2K^b$ at the cell surface, since expression of $H2K^b$ was reduced in the same extent in both cell lines (Figure 3B). Thus, we observed a specific reduction in abundance of K^b-SIINFEKL complexes in stressed cells when SIINFEKL derived from a cytosol-localized as opposed to an ER-retained protein.

Cell surface K^b-SIINFEKL complexes have been shown to be very stable [39]. We therefore postulated that monitoring of K^b-SIINFEKL in the aforementioned experimental conditions might lead us to underestimate the impact of ER stress on exportation of "new" MHC I-peptide complexes at the cell surface. Thus, in the next series of experiments, we took advantage of the fact that cell surface MHC I-peptide complexes can be disrupted by mild acid elution at pH 3.3 [40-42]. EL4 stable transfectants were treated or not with tunicamycin, then existent K^b-SIINFEKL complexes were acid stripped and generation of new complexes was measured at different time points (Figure 4B). We reasoned that in this way we could directly assess the effect of the UPR on the generation of new K^b-SIINFEKL complexes. In the absence of tunicamycin, cells rapidly re-expressed K^b-SIINFEKL and initial levels were reached 9 hours after acid stripping (Figure 4C). EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL cell lines showed similar kinetics. In contrast, generation of new complexes was considerably reduced in tunicamycin-treated cells, as less than 20% of normal levels were regained after 18 hours. Moreover, EL4/HEL-ER-SIINFEKL cells generated significantly more cell surface K^b-SIINFEKL complexes than EL4/HEL-Cyto-SIINFEKL (Figure 4C). We conclude that the UPR decreases presentation of existent and newly generated K^b-SIINFEKL complexes and that it differentially affects abundance of SIINFEKL derived from an ER- vs. cytosol-localized protein.

Decreased MHC I expression during glucose starvation

Though pharmacological agents are widely used to activate the UPR [16, 22], we wished to evaluate the effect of a more physiological ER stress stimulus on MHC I peptide presentation. Glucose is a prototypical and strong inducer of ER stress [43]. We monitored UPR induction in EL4 transfectants grown in high glucose (4.5mg/ml), low glucose (1 mg/ml) or no glucose-containing medium for different time durations (Figure 5A). BiP, XBP-1 and CHOP transcripts were significantly induced in cell deprived of glucose for more than 18 hours, indicating activation of the UPR under these conditions. However, none of these UPR markers were up-regulated in cells grown in low glucose-containing medium.

To evaluate whether UPR induced by glucose deprivation affects MHC I surface

expression, cells were deprived of glucose for 18 hours and MHC I surface levels were measured by flow cytometry. Glucose-deprived EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL cells expressed only 25% and 30% of normal $H2K^b$ and $H2D^b$ levels, respectively (Figure 5B). However, RT-qPCR analyses indicated that $H2K^b$, $H2D^b$ and β_2-m transcript levels did not change in cells deprived of glucose (data not shown). We conclude that alike DTT and tunicamycin, glucose deprivation induced ER stress and reduced cell surface MHC I expression through posttranscriptional mechanism(s).

Differential cell surface expression of ER- vs. cytosol-derived SIINFEKL during glucose starvation

We then asked whether presentation of ER- vs. cytosol-derived SIINFEKL could be differentially regulated during physiological stress as observed in chemically stressed cells. EL4 transfectants were deprived or not of glucose for 18 to 24 hours and K^b-SIINFEKL abundance was measured by flow cytometry using the 25-D1.16 mAb (Figure 6A). Levels of K^b-SIINFEKL were significantly reduced in glucose-deprived cells as compared to cells grown in high glucose-containing media. Whereas both cell lines displayed similar amounts of K^b-SIINFEKL complexes under normal conditions (Figure 2B), EL4/HEL-ER-SIINFEKL cells presented significantly more complexes than EL4/HEL-Cyto-SIINFEKL cells during glucose starvation. Thus, diminution of SIINFEKL presentation by $H2K^b$ was more drastic when the peptide derived from a protein localized in the cytosol than from an ER-retained protein.

We extended these findings to generation of new complexes in glucose-deprived cells. EL4 transfectants were grown under high glucose conditions or deprived of glucose for 18 hours, then acid stripped to remove existing complexes and re-incubated in the appropriate media. Abundance of K^b-SIINFEKL complexes was thereafter estimated at different time points (Figure 6). Cells grown in glucose-containing medium showed a rapid generation of new complexes that reached normal levels by 9 hours (Figure 6B). Again, both transfectant cell lines displayed similar kinetics. By contrast, glucose-starved cells were not able to reach basal amount of K^b-SIINFEKL after acid strip. At the term of the culture period, glucose-deprived EL4 cells expressing HEL-ER-SIINFEKL had generated about 50% of cell surface K^b-SIINFEKL levels relative to cells grown in the presence of glucose. The proportion was significantly lower (30%) in glucose deprived EL4 cells expressing HEL-Cyto-SIINFEKL. Note that it was not possible to measure generation of complexes at time points later than 9 hours after acid strip, since at this time cells were already glucose-starved for 24 hours and cell death became a confounding variable. The differential effect of ER stress on presentation of ER- or cytosol-derived SIINFEKL could be due to differences in the amount of source

protein. We explored this possibility and measured the level of HEL protein by Western blot in cells grown in high glucose-containing media or deprived of glucose for 18 to 24 hours (Figure 7). EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL expressed similar levels of HEL protein both under normal conditions and during glucose starvation. This suggests that the different abundance of SIINFEKL at the surface of both clones during glucose starvation is not due to discrepancies in the amount of source protein. Together, these data indicate that physiological ER stress impairs MHC I-peptide presentation and that this inhibition is more striking for SIINFEKL derived from a cytosolic protein than from an ER-retained protein (Figure 8).

Discussion

The ER stands at the crossroad of two fundamental cellular processes: MHC I antigen presentation and UPR activation during ER stress. The UPR regulates protein synthesis and degradation, chaperoning and decay of ER mRNAs [14, 15]. Thus, it has enormous potential to impinge on MHC I antigen processing which relies on all these processes. Here, we assessed the effect of ER stress on the final outcome of antigen processing and presentation: MHC I-peptide abundance. We demonstrated that ER stress induced by pharmacological agents or glucose deprivation, decreases peptide presentation by MHC I molecules in human HeLa and HEK 293 cells and mouse EL4 thymoma cells. This finding is consistent with prior studies reporting reduced MHC I surface levels in human cells expressing a mutant HFE protein or overexpressing transcriptionally active isoforms of UPR activating ATF-6 and XBP-1 transcription factors [44, 45]. Thus, diminution of MHC I surface expression upon UPR activation appears to be a generalized phenomenon occurring during ER stress induced by a variety of stimuli (chemicals, mutant proteins, glucose starvation).

Since the UPR provokes the degradation of ER-localized mRNAs [22], accelerated decay of MHC I mRNA might have been responsible for the reduction of cell surface MHC I expression. However, the presence of normal or increased levels of MHC I transcripts allowed us to exclude this possibility. A second possibility would be that reduced MHC I-peptide presentation reflects a direct effect of ER stress stimuli (DTT, tunicamycin, glucose starvation) on maturation of MHC I molecules. However, de Almeida et al. showed that overexpressing UPR transducers (ATF-6 and XBP-1) in the absence of genuine stress stimulus resulted in decreased MHC I surface expression [45]. Thus, UPR by itself appears to be sufficient to diminish MHC I expression at the cell surface. Peptide delivery to the ER is the limiting factor in the assembly and presentation of MHC-peptide complexes [28, 46]. Our favorite hypothesis is therefore that decreased MHC I presentation is caused by restriction of peptide availability. During ER stress, transducers of the UPR seek to decrease the ER burden by suppressing translation initiation [14, 15]. Given that MHC I molecules preferentially sample polypeptides that are being actively translated [47], we posit that global attenuation of protein synthesis during the UPR probably limits the amount of peptides available for insertion in MHC I molecules.

A main conclusion of our work is that UPR-induced attenuation of MHC I-peptide presentation is more severe when the source protein is localized in the cytosol than in the ER. Our cell lines expressing HEL-Cyto-SIINFEKL and HEL-ER-SIINFEKL displayed identical responses to tunicamycin treatment or glucose starvation. The two cell lines showed similar up-regulation of UPR markers and equivalent reduction in cell surface levels of $H2K^b$ and

$H2D^b$. The sole difference concerned presentation of K^b-SIINFEKL complexes. This suggests that in stressed cells $H2K^b$ molecules are loaded more effectively with SIINFEKL when the peptide originates from an ER- compared with a cytosol-derived protein.

Only 1-2 out of every 10,000 peptides generated by the proteasome bind to MHC I molecules [28]. Our data therefore beg the question: how would an ER-retained protein generate more peptides than a cytosolic protein? We propose that this results from two discrepancies in the MHC processing of ER vs. cytosolic proteins. The first discrepancy involves ERAD. UPR transducers specifically enhance degradation of proteins in the secretory pathway in order to decrease ER folding load. Proteins destined to the secretory pathway are synthesized on ER-bound ribosomes and are cotranslationally translocated into the ER lumen [48]. During ER stress, cotranslational protein translocation is inhibited and newly-synthesized ER proteins are triaged for degradation [31, 49, 50]. Furthermore, retrotranslocation of ER-resident proteins in the cytosol for proteasomal degradation is enhanced [51]. The second discrepancy concerns peptide binding to the transporter for antigen processing (TAP). Once in the cytoplasm, peptides derived from proteasomal degradation have a very short half-life *in vivo*: around 7 s for 9-mer peptides [28]. More than 99% of peptides are degraded by cytosolic peptidases before they bind TAP (on the cytosolic side of the ER) and thereby enter the MHC I presentation pathway. Thus, the probability that a peptide generated by the proteasome will associate with an MHC I molecule should be maximal when the proteasome is located closest to TAP, that is, on the cytosolic face of the ER [52]. That is precisely where retrotranslocated ER proteins are degraded by the proteasome. In summary, we assume that during ER stress, MHC I peptide presentation is biased in favor of ER proteins because their proteasomal degradation is enhanced and the generated peptides emerge in the vicinity of TAP which facilitates their presentation by MHC I molecules.

What might be the impact of the UPR on immune recognition of infected and neoplastic cells? Paradoxically, if the decreased generation of MHC-peptide complexes results mainly from inhibition of translation, it could facilitate recognition of virus infected cells. UPR-induced attenuation of translation is mediated by PERK-dependent phosphorylation of eIF2α on Ser^{51} [53]. Phosphorylation of eIF2α hampers canonical cap-dependent translation initiation which regulates synthesis of 95-98% of cellular mRNAs. However, viruses can use internal ribosomal entry sites in their 5' noncoding region to initiate cap-independent translation [9, 54]. Thus, by preferentially repressing presentation of self peptides, the UPR could facilitate recognition of viral peptides (the needle in the haystack [55]). On the other hand, by repressing production of MHC I-peptide complexes, the UPR may hinder presentation of tumor antigens to CD8 T cells. Indeed, generation of optimal CD8 T cell responses is promoted by high epitope density on antigen presenting cells [56, 57]. On the other hand, an

elegant study by Schwab et al. has shown that in presence of eIF2α phosphorylation, cells can generate MHC I-associated peptides derived from cryptic translational reading frames [58]. In the future, we anticipate that high-throughput sequencing of the MHC I peptide repertoire [35] will allow us to further evaluate how ER stress molds the peptide repertoire, in terms of both peptide abundance and diversity.

Conclusions

Our work shows that ER stress impinges on the MHC I peptide repertoire in two ways: by decreasing overall MHC I-peptide presentation and by changing the relative contribution ER- vs. cytosol-proteins to the cell surface MHC I peptide repertoire. Since ER stress is a characteristic feature of infection and malignancy, dysregulation of MHC I-peptide presentation could have major implications in the recognition of infected and transformed cells by CD8 T lymphocytes.

Materials and Methods

Cell lines

HEK 293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) and antibiotics. EL4 cells were grown in DMEM medium supplemented with 5% FBS and antibiotics. EL4 stable transfectants were grown in the same medium supplemented with 1000 μg/ml of G418.

DNA constructs

pHYK/HEL-ER/myc and pCMV/HEL-Cyto/myc plasmids encoding ER-retained or cytoplasmic HEL, respectively, were provided by S. Ostrand-Rosenberg (University of Maryland, Baltimore, USA). The pHYR/HEL-ER plasmid contains the HEL gene fused to the ER-retention signal KDEL, whereas pCMV/HEL-Cyto codes for HEL with a modified N-terminus and lacks ER-retention signal. These plasmids have successfully been shown to target HEL to the ER or to the cytosol [36, 37, 59]. pHYK/HEL-ER and pCMV/HEL-Cyto were sequenced to ascertain correct sequence and reading frame. Fragments coding for HEL-ER or HEL-Cyto were fused by PCR to the region coding for the Ovalbumin-derived peptide SIINFEKL, flanked by a sequence of 18 p.b. (LEQLESIINFEKLTEWTS, here referred as SIINFEKL) to assure peptide processing [60]. PCR amplification products were subcloned into the pPCR-Script Amp cloning vector (Stratagene, Cedar Creek, TX, USA). HEL-ER-SIINFEKL or HEL-Cyto-SIINFEKL were excised and cloned into the bicistronic pIRES-EGFP2 vector (Clontech, Mountain View, CA, USA) to generate pIRES-EGFP2/HEL-ER-SIINFEKL and pIRES-EGFP2/HEL-Cyto-SIINFEKL (Figure 2A). Both constructs were sequenced to ascertain correct sequence and reading frame.

Stable transfectants

EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL were generated by transfecting EL4 cells with the appropriate HEL-containing pIRES-EGFP2 plasmid. Transfections were done with Lipofectamine LTX Reagent (Invitrogen, Burlington, ON, Canada) as instructed by the manufacturer. 24 hours after transfection, single cells expressing the brightest signal of EGFP were sorted by fluorescence-activated cell sorting (FACS) on a FACSAria cell sorter (BD Biosciences, Mississauga, ON, Canada). Stable transfected clones were further selected by drug resistance (1000 μg/ml of G418) in combination with repeated cycles of FACS of EGFP-positive cells. Clones expressing similar levels of K^b-SIINFEKL at the cells surface were selected for use in further experiments

Stress treatments

Chemical ER stress was induced by incubating cells in fresh medium containing 2 mM or 10 mM of DTT for 8 or 16 hours, or 2.5μg/ml of tunicamycin for the indicated times. DTT and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose starvation was induced by culturing cells in glucose and sodium piruvate-free DMEM medium (GIBCO, Burlington, ON, Canada) supplemented with 5 % FBS and antibiotics for 18 hours. Control cells were grown in high glucose DMEM medium, containing 4500 mg/L of glucose and 110mg/L sodium pyruvate supplemented with 5% FBS and antibiotics.

Antibodies and flow cytometry

MHC I molecules at the cell surface were stained with anti HLA A/B/C and APC-conjugated anti-mouse IgG_1 (Clone X56), biotin-conjugated anti $H2K^b$ (Clone AF6-88.5) and biotin-conjugated anti $H2D^b$ (Clone KH95), followed by streptavidin PeCy5. All antibodies were purchased from BD Biosciences. K^b-SIINFEKL levels were determined with the 25-D1.16 antibody [38] followed by staining with APC-conjugated anti-mouse IgG_1 (Clone X56). J.W. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) kindly provided the 25-D1.16 hybridoma. Propidium iodide (BD Biosciences) was used to exclude cells in later apoptotic stages from the analysis. Cells were analyzed on a BD LSR II flow cytometer using FACSDiva (BS Biosciences) and FCS Express software (De Novo Software, Los Angeles, CA, USA) [61, 62].

Acid strip assay

MHC I-peptide complexes were eluted with acid treatment as previously described [40-42]. Briefly, cells (~5 x 10^5) were resuspended in 0.2 ml of citrate phosphate buffer at pH 3.3 (0.131 M citric acid/0.066 M Na_2HPO_4, NaCl 150mM) for 1 minute, neutralized with appropriate medium pH 7.4 and either reincubated in fresh medium or stained for flow cytometry.

RNA extraction, reverse transcription and RT-qPCR

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Purified RNA were reverse transcribed using the High Capacity cDNA reverse transcription Kit with random primers (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. A reference RNA (Stratagene, La Jolla, CA,

USA) was also transcribed in cDNA. Expression level of target genes was determined using primer and probe sets from Universal ProbeLibrary (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) or Applied Biosystems (ABI Gene Expression Assays, http://www.appliedbiosystems.com/). Pre-developed TaqMan® assays for GAPDH and β-actin were used as endogenous controls. RT-qPCR analyses were performed as described using a PRISM® 7900HT Sequence Detection System (Applied Biosystems) [63, 64]. The relative quantification of target genes was determined by using the △△CT (threshold cycle) method. Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula $RQ = 2^{-\Delta\Delta CT}$.

Statistical analysis

The means of normally distributed data were compared using the Student *t* test, with a P value of < 0.05 considered significant. Data are presented as mean ± SD. Whenever the results are expressed as a percentage of control, the statistical analysis was performed on the actual value.

Abbreviations

DTT, dithiotheritol; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HEL, hen egg lysozyme; MHC I, major histocompatibility complex class I; RT-qPCR, quantitative real-time reverse transcriptase polymerase chain reaction; TAP, transporter for antigen processing; UPR, unfolded protein response

Authors' contributions

DPG designed the study, carried out experiments and analyzed the data. MPH participated in flow cytometry experiments. EC participated in molecular biology experiments. CP conceived and designed the study. DPG and CP drafted the manuscript, and all authors edited and approved the final manuscript.

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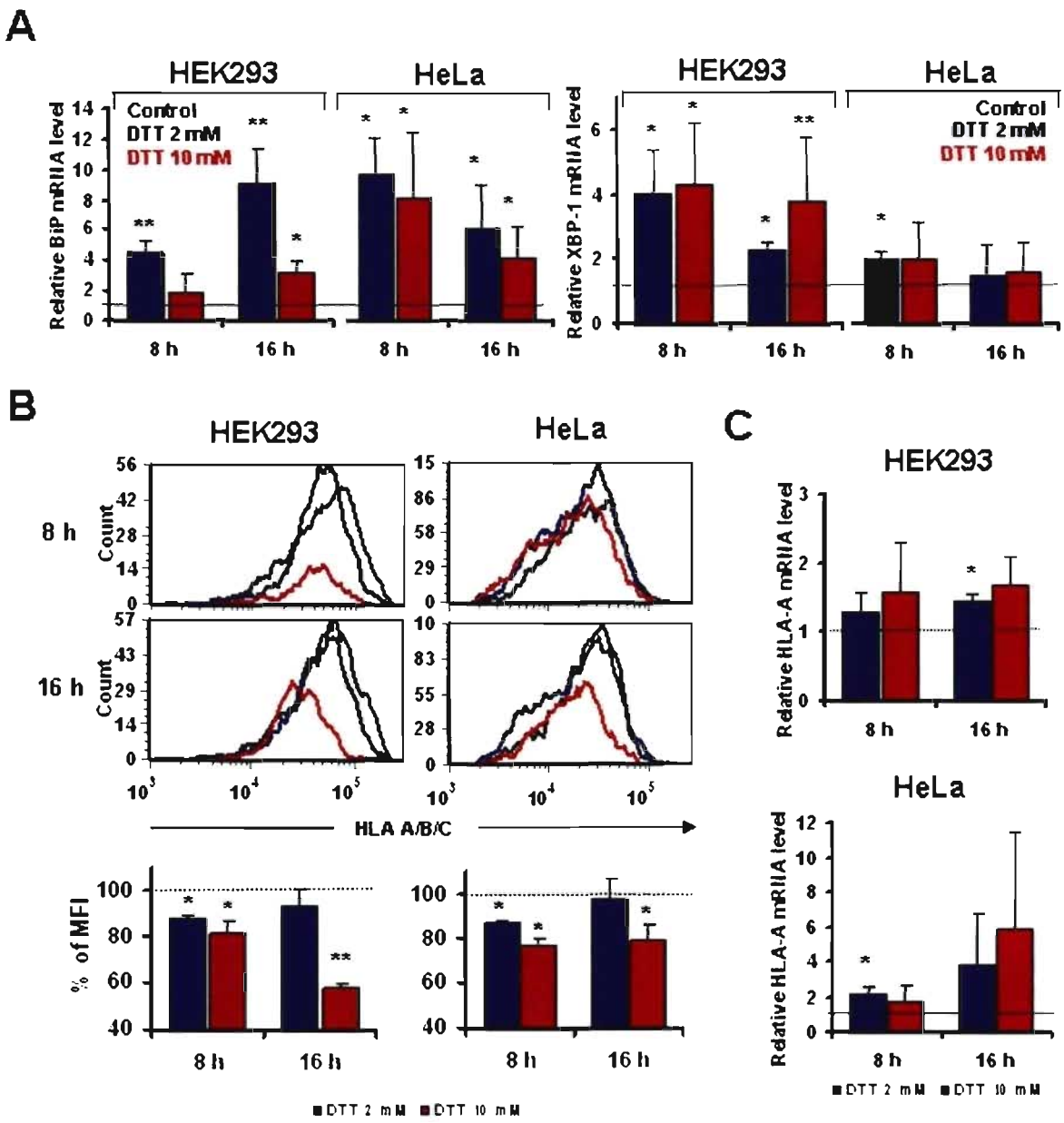

Figure 1. UPR impairs MHC I surface expression but not mRNA level. HEK293 or HeLa cells were either non-treated or treated with 2 mM (*blue bars*) or 10 mM (*red bars*) DTT for 8 and 16 hours. (A) Activation of the UPR. Total RNA was extracted and BiP and XBP-1 mRNA levels were analyzed by RT-qPCR. Expression levels were normalized to the endogenous control gene β-actin. Transcript levels of treated cells were compared with basal mRNA values of untreated cells *(dashed line)*, which were set to 1. (B) UPR decreases MHC-I surface expression. Surface expression of HLA-A/B/C was determined by flow cytometry. Representative histograms of one of three independent experiments show MFI values of untreated cells (*black*), cells treated with 2 mM (*blue*) or 10 mM (*red*) DTT for 8 (*top*) or 16 hours (*bottom*). Graphs on the bottom represent % of MFI intensity in treated cells relative to untreated cells (*dashed line*). (C) UPR does not decrease MHC-I mRNA level. HLA-A mRNA levels were assessed and analyzed as in A. Bars represent mean values ± SD from three independent experiments performed in triplicate. $*P < 0.05$; $**P < 0.01$ when comparing DTT-treated with untreated cells.

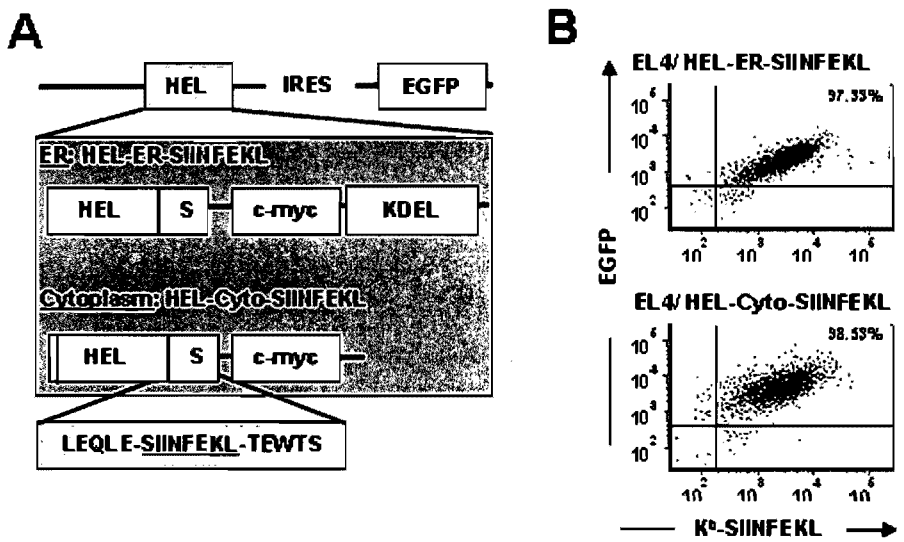

Figure 2. EL4 stable transfectants express the SIINFEKL peptide derived from HEL targeted to the ER or to the cytosol. (A) Schematic representation of the constructs used to generate EL4 stable transfectants. Modified coding sequences of HEL [36, 37] in frame with the region coding for the Ovalbumin-derived peptide SIINFEKL and its flanking region were cloned into the pIRES-EGFP2 vector. HEL-ER-SIINFEKL possess the N-terminal signal peptide and the ER-retention signal KDEL and targets HEL to the ER; HEL-Cyto-SIINFEKL contains a modified N-terminal sequence, lacks the KDEL ER-retention signal and targets HEL to the cytoplasm (See materials and methods). (B) EL4 stable transfectants express Kb-SIINFEKL at the cell surface. EL4 cells were transfected with the pIRES-EGFP2 vector encoding HEL-ER-SIINFEKL or HEL-Cyto-SIINFEKL. Stable transfectants were selected by repeated cycles of fluorescence-activated cell sorting of EGFP-positive cells combined with drug resistance (1000 μg/ml of G418). Cells were stained with 25D1.16 monoclonal antibody, recognizing the K^b-SIINFEKL complex, followed by staining with APC-conjugated anti-mouse IgG1 as secondary antibody. Depicted in the graphs are EGFP and K^b-SIINFEKL MFI values of the two representative clones that were used in further studies: EL4/HEL-ER-SIINFEKL and EL4/HEL-Cyto-SIINFEKL. Percentages represent the proportion of cells expressing EGFP and K^b-SIINFEKL.

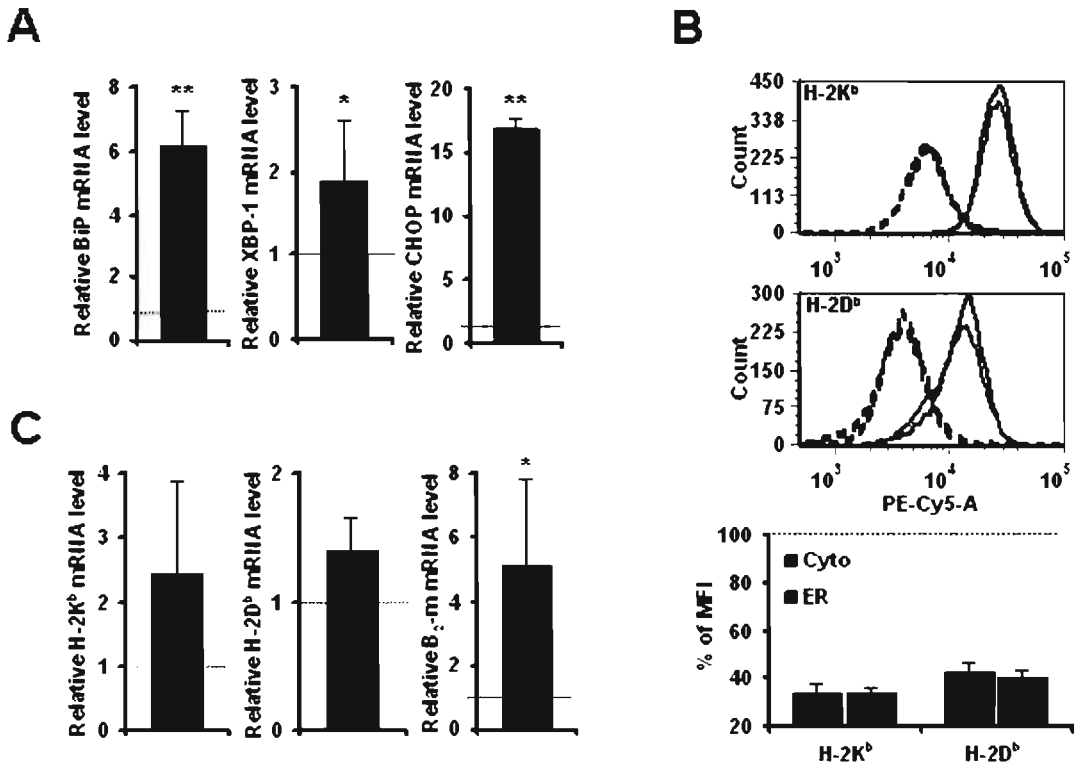

Figure 3. Induction of UPR decreases cell surface levels of H2Kb and H2Db in EL4 stable transfectants. (A) UPR activation in EL4 stable transfectants. EL4/HEL-ER-SIINFEKL cells were either non-treated or treated with 2.5μg/ml of tunicamycin for 16 hours. BiP, XBP-1 and CHOP mRNA levels were analyzed by RT-qPCR. Expression levels were normalized to the endogenous control gene β-actin. Expression levels of treated cells were compared with basal mRNA values of untreated cells (*dashed line*), which were set to 1. Similar results were obtained for EL4/ HEL-Cyto-SIINFEKL (not shown). (B) UPR decreases surface expression of $H2K^b$ and $H2D^b$. Surface expression of $H2K^b$ and $H2D^b$ was determined by flow cytometry in EL4/HEL-Cyto-SIINFEKL *(blue)* and EL4/HEL-ER-SIINFEKL *(green)* cells. Representative histograms of one of three independent experiments showing MFI values of untreated cells (*solid lines*) and tunicamycin-treated cells (*dotted lines*). Bars represent % of MFI intensity in treated cells relative to untreated cells (*dashed line*). (C) UPR does not decrease MHC-I mRNA levels. $H2K^b$, $H2D^b$ and β2-microglobulin (β2-m) mRNA levels were assessed and analyzed as in A. Bars represent mean values ± SD from three independent experiments performed in triplicate. $*P < 0.05$; $**P < 0.01$ when comparing tunicamycin-treated with control cells.

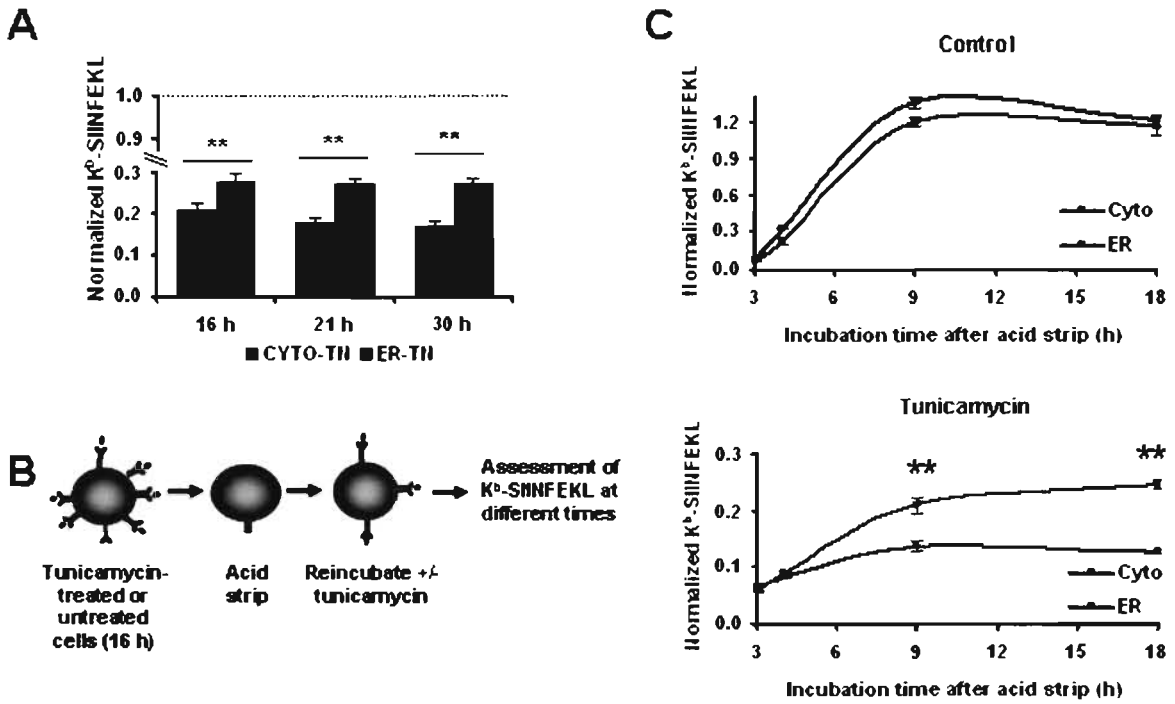

Figure 4. Different peptide abundance in cells expressing ER- or cytosol-localized HEL during ER stress. (A) Effect of UPR on pre-existing Kb-SIINFEKL complexes. EL4/HEL-Cyto-SIINFEKL (*blue*) or EL4/HEL-ER-SIINFEKL (*green*) cells were either non-treated or treated with 2.5μg/ml of tunicamycin for the indicated times. Abundance of K^b-SIINFEKL complexes was assessed at each time point by staining with 25D1.16 monoclonal antibody and APC-conjugated anti-mouse IgG_1 antibody. Graph represents MFI values of tunicamycin-treated cells normalized to values of untreated cells, which were set to 1 *(dashed line)*. (B) Study design to evaluate generation of K^b-SIINFEKL complexes. EL4 stable transfectants were non-treated or treated with tunicamycin for 16 hours. Existent K^b-SIINFEKL complexes were then stripped with acid and cells were reincubated in fresh medium containing or lacking tunicamycin. Expression of new complexes was assessed thereafter as in *A*. (C) Effect of UPR on generation of new K^b-SIINFEKL complexes. EL4/ HEL-Cyto-SIINFEKL (*blue lines*) and EL4/HEL-ER-SIINFEKL (*green lines*) cells were incubated as explained in *B*. K^b-SIINFEKL expression was measured at the indicated times after acid strip. MFI values of unstripped cells grown under normal conditions and representing normal level of Kb-SIINFEKL expression in each clone, were use to normalize MFI values of stripped cells. Bars represent mean values ± SD from three independent experiments performed in triplicate. $*P < 0.05$; $**P < 0.01$.

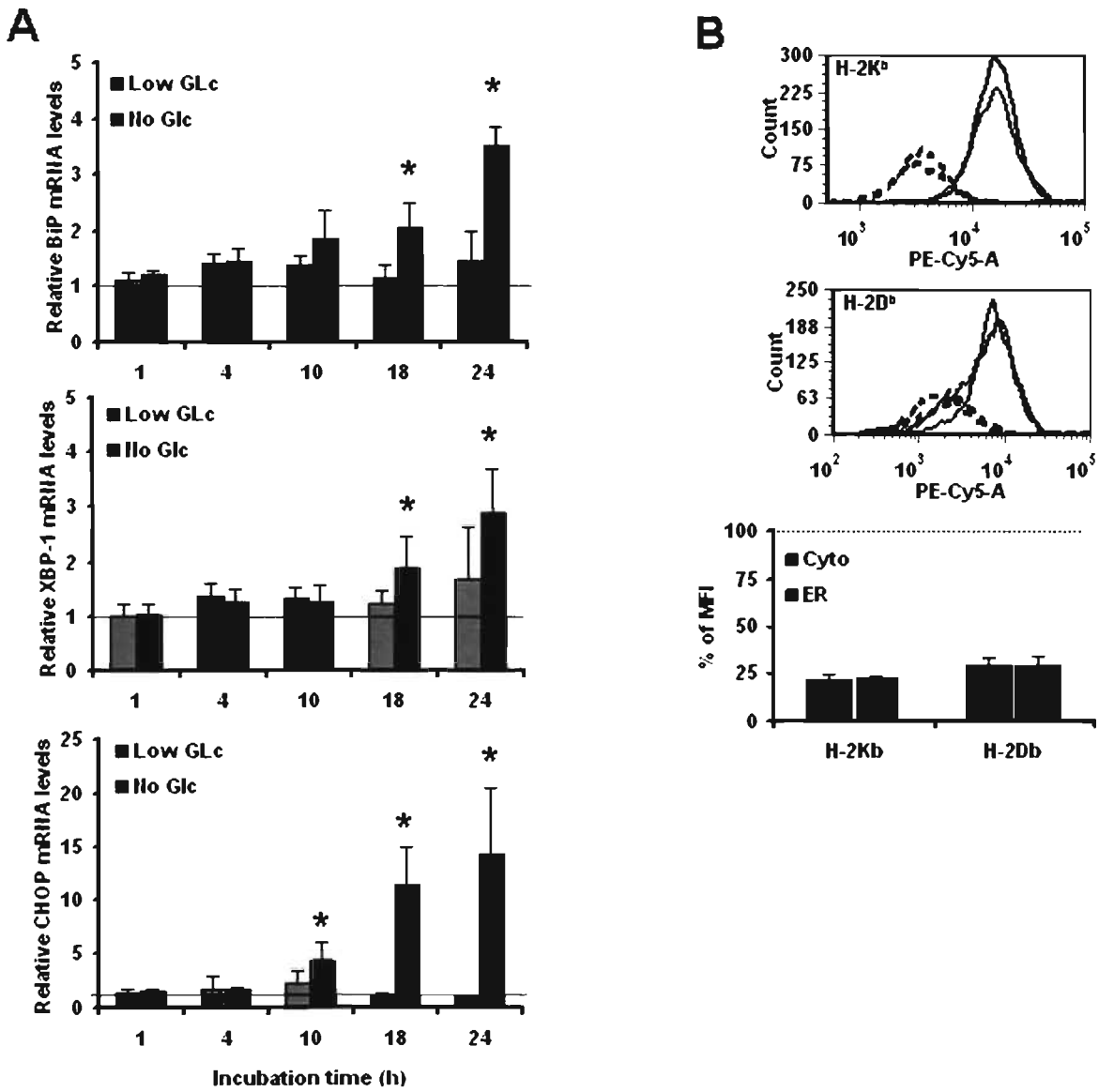

Figure 5. Glucose starvation-induced ER stress impairs H2K^b and H2D^b expression. EL4 stable transfectants were incubated in DMEM medium lacking glucose or containing low glucose (1 mg/ml) or high glucose (4.5 mg/ml) for different durations. (A) Activation of the UPR during glucose starvation. BiP, XBP-1 and CHOP mRNA levels in EL4/HEL-ER-SIINFEKL cells were analyzed by RT-qPCR. Expression levels were normalized to the endogenous control gene β-actin. Transcript levels of cells incubated under low *(grey bars)* or no glucose *(black bars)* were compared to levels of cells grown in high glucose medium *(dashed line)*, which were set to 1. Similar results were obtained with EL4/HEL-Cyto-SIINFEKL cells (not shown). (B) Decreased cell surface expression of H2K^b and H2D^b. EL4/HEL-Cyto-SIINFEKL *(blue)* and EL4/HEL-ER-SIINFEKL *(green)* cells were deprived of glucose for 18 hours. Surface expression of H2K^b and H2D^b was determined by flow cytometry. Representative histograms of one of three independent experiments showing MFI values of untreated cells (*solid lines*) and cells deprived of glucose (*dotted lines*). Bars graph depicts % of MFI intensity of cells deprived of glucose relative to cells grown in high glucose medium (*dashed line*). Bars represent mean values ± SD from three independent experiments performed in triplicate. *$P < 0.05$ when comparing low glucose or no glucose with high glucose conditions.

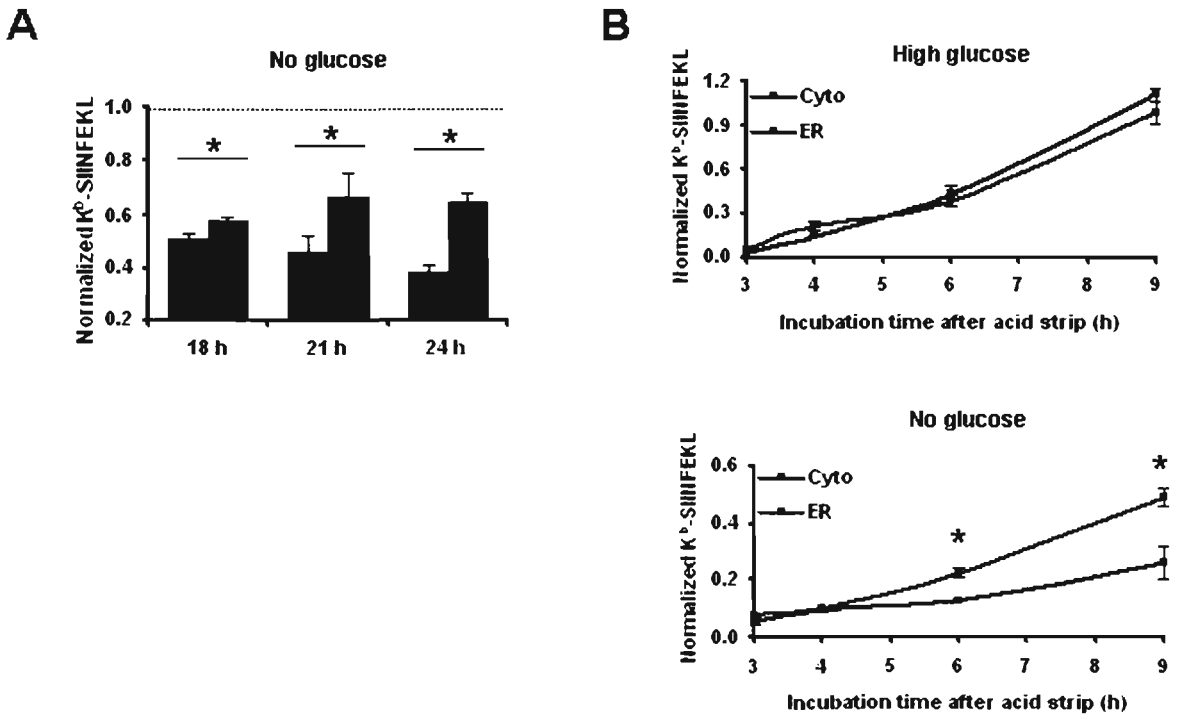

Figure 6. Increased presentation of SIINFEKL derived from ER-localized relative to cytosolic proteins during glucose starvation. (A) Effect of UPR on pre-existing K^b-SIINFEKL complexes. EL4 stable transfectants were deprived of glucose or not for 18, 21 and 24 hours. K^b-SIINFEKL complexes abundance was assessed at each time point with the 25D1.16 monoclonal antibody and APC-conjugated anti-mouse IgG_1 antibody. Graph represents MFI values of glucose-deprived EL4/ HEL-Cyto-SIINFEKL (*blue*) or EL4/HEL-ER-SIINFEKL (*green*) cells normalized to values of control cells, which were set to 1 (*dashed line*). (B) Effect of UPR on generation of new K^b-SIINFEKL complexes. EL4 transfectants were incubated in high glucose-containing medium (*top*) or deprived of glucose (*bottom*) for 18 hours. Cells were submitted to acid strip to elute existent MHC-I complexes and expression of new K^b-SIINFEKL complexes was assessed as in *A* at the indicated times. MFI values of unstripped cells incubated under normal conditions and representing normal level of K^b-SIINFEKL in each clone were use to normalize MFI values of stripped cells. Bars represent mean values ± SD from three independent experiments performed in triplicate. $*P < 0.01$.

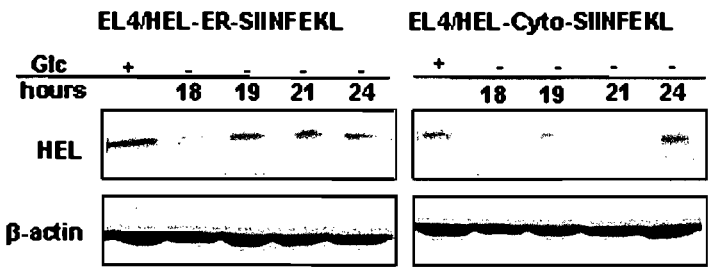

Figure 7. HEL expression during glucose starvation. EL4/HEL-ER-SIINFEKL or EL4/HEL-Cyto-SIINFEKL cells were incubated in glucose-containing media or deprived of glucose for 18-24 hours. HEL from whole cell lysates was detected by Western blot with anti-HEL antibody. β-actin was used as loading control. Representative gel of three independent experiments.

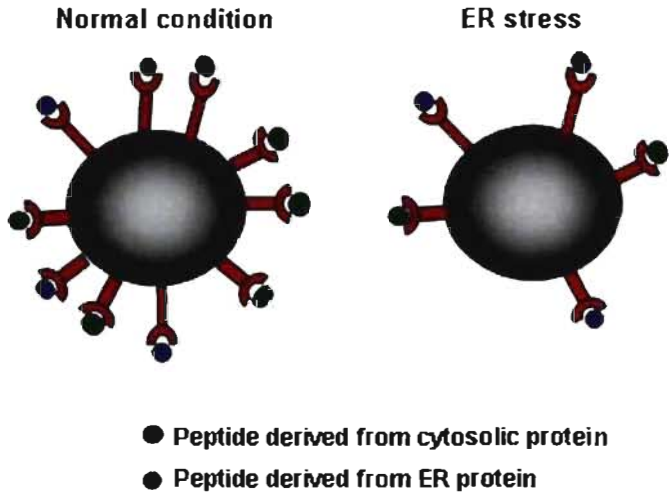

Figure 8. ER stress impinges on the MHC I peptide repertoire. ER stress decreases overall MHC I-peptide presentation and changes the relative contribution ER- vs. cytosol-proteins to the cell surface MHC I peptide repertoire.

5. SUPPLEMENTARY RESULTS

5.1. Model for in vitro quantification of MHC I-peptide complexes

5.1.1. DNA constructs and localization of HEL protein variants

Besides the plasmids encoding HEL retained in the ER or destined to the cytosol, we engineered other plasmids encoding modified HEL targeted to other compartments: nucleus, mitochondria and secreted protein. (Figure S1). The protein variant targeted to the nucleus (HEL-nuc) contains a nuclear localization signal (NLS) in triplicate derived from the simian virus 40 T antigen. The protein variant targeted to the mitochondria (HEL-mito) contains the mitochondrial targeting sequence of the cytochrome c oxidase. The third variant corresponds to secreted HEL (HEL-wt). These constructs also contain the region coding for SIINFEKL flanked by five amino acids of the ovalbumin protein. They were inserted by PCR into a pIRES-EGFP2 vector.

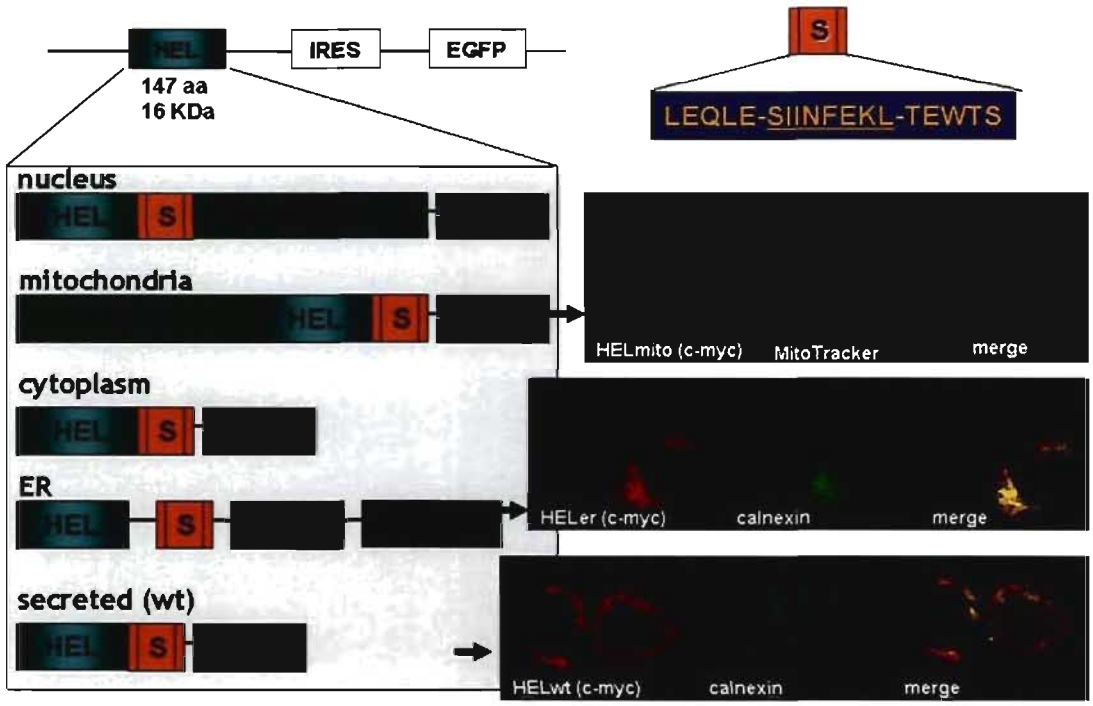

Figure S1. Schematic representation of the HEL constructs and subcellular localization of the protein variants. COS-7K^b cell were transfected with the constructs encoding HEL-SIIFENKL targeted to different cellular compartmens. Subcellular localization of the protein 24 hours after transfection. HEL (Hen egg lysozyme); c-myc: used as tag; IRES (internal ribosomal entry site) contained in the pIRES- EGFP2 vector; S: SIINFEKL; MTS (mitochondrial targeting sequence).

In addition, we verified the proper localization of three of these protein variants

(mitochondrial, ER and secreted) by confocal microscopy (Figure S1). We transiently transfected COS-7K^b cells with the appropriate construct and 24 hours thereafter we identified transfected cells that expressed the EGFP protein. The cells were labeled with antibodies against c-myc (for HEL), calnexin (as ER marker) or MitoTracker (for mitochondria). DAPI staining was used for the nucleus.

As shown in Figure S1, HEL-mito is correctly targeted to the mitochondria. Similarly, there is a perfect colocalization between HEL-ER and calnexin, confirming that this variant is retained in the ER. In the case of HEL-wt, there is a good colocalization with the ER marker, confirming that this protein variant traverses the ER before being secreted.

5.1.2. Presentation of SIINFEKL derived from HEL protein variants

We then verified the correct processing and surface expression of SIINFEKL in HeLa-K^b and COS-7K^b cells transiently transfected with these plasmids (Figure S2). SIINFEKL, derived from HEL targeted to the nucleus, the mitochondria, the cytosol or the ER, or from secreted HEL was correctly processed and presented at the cell surface by H2K^b molecules in both cell lines (see blue gate, EGFP+ and K^b-SIINFEKL+). These results also show a clear correlation between the expression level of the EGFP protein and the amount of peptide presented at the surface. To note, translation of EGFP is cap-independent (IRES-dependent), whereas generation of SIINFEKL relies on the cap-dependent translation of HEL. These results suggest that cells with a high rate of IRES-dependent translation do probably also have a high rate of cap-dependent translation and thus SIINFEKL presentation is high in these cells. This is based on the fact that MHC I molecules preferentially sample what is being actively translated, rather than what has been translated (Qian et al., 2006b). These results also show discrepancies in the amount of peptide presented depending on the localization of the source protein (see blue gate and %). However, these differences could be due to variations in the efficiency of transfection of each plasmid and not to different efficiencies in the processing of the peptide depending on the localization of the source protein. In summary, these results show that proteins localized in different subcellular compartments can be sources of peptides for presentation by MHC I molecules.

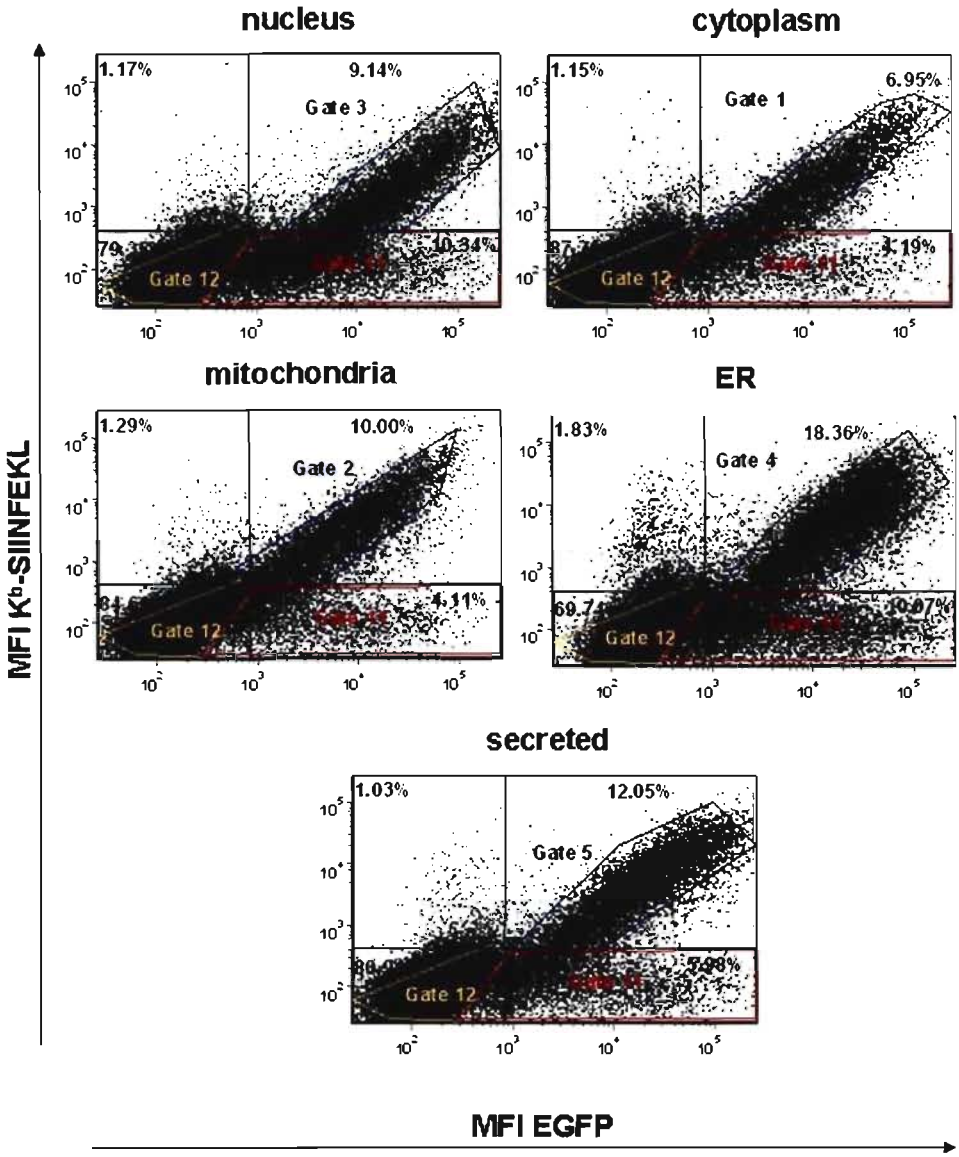

Figure S2. K^b-SIINFEKL presentation in HeLa-K^b cells transfected with the constructs encoding HEL destined to different subcellular comparments. MFI (mean fluorescence intensity); yellow gate: untransfected cells; red gate: EGFP+/ K^b-SIINFEKL- cells; blue gate: EGFP+/K^b-SIINFEKL+ cells). One representative experiment out of three. Numbers indicate % of cells.

The plasmids encoding HEL-mito-SIINFEKL, HEL-nuc-SIINFEKL and HEL-sec-SIINFEKL were not used to create stable EL4 transfectants to study the effect of the UPR on MHC I expression owing to time limitations. These plasmids represent valuable tools that could potentially be used to further corroborate our result showing that the UPR differentially regulates expression of peptides derived from a protein localized in the secretory (ER-retained and secreted HEL variants) versus the non-secretory pathway (HEL located in the mitochondria, the nucleus or the cytoplasm). Furthermore, these plasmid could we used to

create other EL4 transfectants and answer other questions such as whether the intracellular localization of a protein could affect the peptide presentation at the cell surface.

5.1.3. Expression of the BiP/GRP78 protein as marker of UPR activation

Besides monitoring the induction of BiP/GRP78 transcript during ER stress, we also assessed the expression of the protein (Figure S3). EL4 cells were treated or not with DTT (0.5 and 1 mM) for 8 hours or with tunicamycin (0.5 and 2.5 μg/ml) for 16 hours. Then, the protein content of whole cell lysates was determined, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes and immunoblotted with anti BiP/GRP78, anti calnexin and anti β-actin mAbs, and horseradish peroxidase-coupled secondary antibody. The membranes were revealed using the advanced ECL chemiluminiscence system and analysed in a LAS300 imager. It is worth to note that treatment of EL4 cells with high doses of DTT (more than 1 mM) or for prolonged periods (more than 8 hours) caused a considerable rate of apoptosis (not shown).

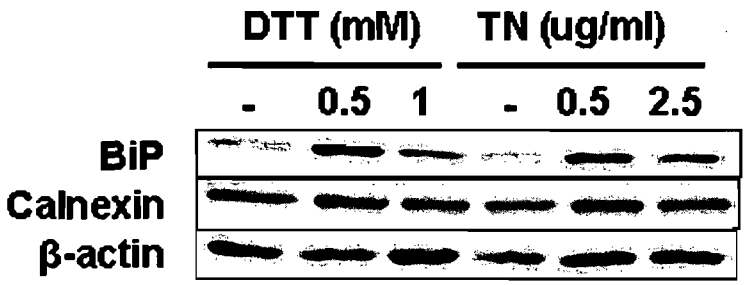

Figure S3. Expression of BiP/GRP78 protein. EL4 cells were treated or not with DTT for 8 hours or with tunicamycin for 16 hours and the expression of BiP/GRP78 protein was analyzed by Wester blot. TN: tunicamycin.

These results show the upregulation of BiP/GRP78 protein in cells treated with low doses of DTT and tunicamycin but not with high doses. As expected, the level of the chaperone calnexin, which is not used as marker of the UPR, did not change. I will comment these results further in the Discussion.

6. DISCUSSION

Our results show that ER stress impinges on the MHC I peptide repertoire in two ways: by decreasing overall MHC I-peptide presentation and by changing the relative contribution of ER- vs. cytosol-proteins to the cell surface MHC I peptide repertoire.

Given that the discussion of the results is included in the manuscript, in this section I will focus on results that were not exposed in the manuscript and deepen the discussion. It is important to note that the numbering of figures which I will refer to through this section corresponds to the figures in the manuscript, otherwise stated.

6.1. Activation of the UPR in different cell lines

In the first set of experiments, we used DTT or tunicamycin to disrupt disulphide bond formation or N-glycosylation in the ER, respectively (Figures 1 and 3). We observed that the UPR induction profile, which was monitored by analyzing the transcript levels of XBP1, BiP/GRP78 and CHOP, varied according to the cell line, the dose and type of chemical agent, as well as the duration of the treatment.

First, these results suggest that different cell lines exhibit different sensitivities to the same stress stimuli. Differences could be due to the fact that EL4 and HeLa but not HEK293 are neoplastic cells. Of note, upregulation of XBP1 was more important in HEK293 cells, while that of BiP/GRP78 transcript was more dramatic in EL4 and HeLa cells. Indeed, it has been found that increased expression of the chaperone BiP/GRP78 occurs in at least 10 different cancers (Lee, 2005; Moenner et al., 2007). In line with this, microarray studies have shown that the rapidity of induction of certain genes by an active UPR differed between fibroblasts and HeLa cells submitted to the same treatment with DTT and this difference was attributed to intrinsic properties of the cells (Murray et al., 2004).

Second, our results show that the nature of the UPR varied according to the dose of stressor used (Figure 1 and S3). Upregulation of XBP1 transcript was similar or higher in HeLa and HEK293 cells treated with a high concentration of DTT (10 mM) than in cells treated with a low dose (2 mM), probably reflecting the dose-dependent extent of ER stress and UPR activation. However, this was not the case for BiP/GRP78 transcripts, a chaperone whose induction is widely used as a marker of ER stress. We observed that upregulation of BiP/GRP78 was greater in HeLa and HEK293 cells treated with a low concentration of DTT (2 mM) than in cells treated with a high concentration of DTT (10 mM) and not the opposite

outcome (Figure 1). The same pattern was observed at the protein level in EL4 cells treated with DTT (0.5 and 1mM) and tunicamycin (0.5 and 2.5µg/ml) (Figure S3). The answer may lie in the ability of BiP/GRP78 to block the apoptotic branch of the UPR through interference with caspase activation (Lee, 2005). Thus, the high induction of BiP/GRP78 mRNA (and its protein) observed in cells treated with a low dose of DTT may represent a major cellular protective mechanism for cells to survive, whereas the low induction observed with a strong dose of DTT could owe to activation of latter pro-apoptotic stages of the response.

Third, we observed variations in the UPR pattern according to the type of stressor used and to the time of treatment. Incubation of EL4 cells in the presence of very low dose of DTT (0.5 mM) for less than 8 hours was sufficient to activate the UPR as detected by an increased level of BiP/GRP78 protein (Figure S3). On the contrary, longer treatments with tunicamycin (16 hours) or of glucose deprivation (18 hours) were required to reach an equivalent activation of the UPR. This is due to the fact that DTT causes immediate accumulation of misfolded proteins in the ER by reducing existing disulphide bonds, whereas tunicamycin and glucose starvation cause a more gradual accumulation of misfolded proteins as old proteins have to be replaced by newly synthesized proteins that have not been properly glycosylated (Murray et al., 2004; Lee, 2005).

Thus, based on our experience, we recommended doing a cell line-specific dosage analysis of more than one UPR marker in studies involving the UPR. Also, the use of more than one UPR inducer agent is highly recommended.

6.2. Activation of the UPR during glucose starvation

Though pharmacological agents are widely used to activate the UPR (Hollien & Weissman, 2006; Rutkowski et al., 2006), we wished to evaluate the effect of a more physiological ER stress stimulus on MHC I-peptide presentation. Thus, we chose glucose starvation on the basis that this stress occurs under a variety of circumstances in the living organism, including cancer (Ma & Hendershot, 2004). As expected, BiP/GRP78, XBP1 and CHOP transcripts were significantly induced in cells deprived of glucose for 18 or 24 hours, indicating activation of the UPR (Figure 5A). This shows that a long-lasting period of glucose starvation is necessary to induce the UPR. Accordingly, previous studies done in chinese hamster ovary cells, have shown that 24 hours of glucose deprivation induces the cleavage of ATF6, which activates the transcription of BiP/GRP78 and CHOP (Nadanaka et al., 2006). It is very probable that effects of glucose starvation are delayed in comparison to those of chemical stressors. This could result in a gradual decrease in the amount of oligosaccharide intermediates used for N-glycosylation and thus less glycosylation of newly-synthesized

proteins, leading to accumulation of unfolded proteins in the ER and activation of the UPR.

On the contrary, none of these UPR markers were upregulated in cells grown in low glucose-containing medium (1 mg/ml). This was surprising since this low amount of glucose, which is almost 4 times less that the amount used in normal culture medium (4.5 mg/ml), was not sufficient to induce these UPR markers. However, cells grown in low glucose-containing medium were indeed stressed because they showed increased apoptosis than cells grown under normal conditions. Furthermore, the amount of K^b-SIINFEKL at the surface of these cells was decreased in comparison with cells grown under normal conditions. This could mean that a reduced MHC I-peptide presentation observed in cells grown under low glucose conditions could be caused not by the UPR itself, but by other consequences of glucose starvation. For instance, glucose deprivation is known to affect the mammalian target of rapamycin (mTOR) pathway (Ozcan et al., 2008), which is the most prominent pathway responsible for regulation of translation (Tee & Blenis, 2005). On the other side, we can not discard the possibility that early events of the UPR that were not tested here, such as the translational repression through the PERK/eIF2α branch, might have been already triggered in cells grown under low glucose conditions. Hence, we hypothesize that various effects of glucose starvation (attenuation of translation and/or mTOR dysregulation) could be responsible for a reduced expression of MHC I-peptide complexes at the cell surface. In other words, we could state that the decreased abundance of peptides is a consequence of ER stress in general.

6.3. Why UPR activation impairs MHC I surface expression?

We demonstrated that ER stress induced by pharmacological agents or glucose deprivation, decreases MHC I surface expression in different cell lines: human HeLa and HEK293 cells and mouse EL4 thymoma cells (Figure 1). This finding is consistent with a recent study reporting reduced MHC I surface levels in 293T human cells or peripheral blood mononuclear cells, in which the UPR was induced by ER stress stimuli different from the ones used in our study (de Almeida et al., 2007). In those studies, carried out by de Almeida et al., expression of a mutant HFE protein, which causes hereditary hemochromatosis, induces UPR activation and impairs MHC I cell surface expression (de Almeida et al., 2007). They also found decreased surface MHC I expression in cells in which the UPR was activated by overexpressing the transcriptionally active isoform of ATF6 and XBP1 (de Almeida et al., 2005; de Almeida et al., 2007). Thus, altogether, our work and the work of de Almeida et al. suggest that diminution of MHC I surface expression upon UPR activation is a generalized phenomenon occurring during ER stress induced by a variety of stimuli (chemicals, mutant proteins, glucose starvation, and overexpression of UPR transducers).

Since the UPR provokes selective degradation of mRNAs encoding secreted and membrane proteins (Hollien & Weissman, 2006), accelerated decay of MHC I mRNA (which encodes a membrane protein) might have been responsible for the reduction of the MHC I at the cell surface. However, the presence of basal or increased levels of MHC I transcripts allowed us to exclude this possibility. In addition, the fact that MHC I mRNA levels were not decreased despite the UPR-dependent degradation of ER-localized mRNAs, could suggest that the MHC I mRNAs contain a signal sequence that allows bypassing this degradation as is the case of BiP/GRP78. Hoollien et al. have shown that the signal sequence determines whether an mRNA is targeted or not to the decay pathway during ER stress. For instance, the signal sequence of BiP/GRP78 allows escape from degradation and this is probably the case of other mRNAs encoding proteins that are important for the UPR or for vital cellular processes (Hollien & Weissman, 2006). In the case of MHC I mRNA, it bypasses this selective degradation but not later unknown steps that lead to a decreased surface expression of the protein.

We showed that induction of ER stress with pharmacological agents or by glucose starvation significantly decreased MHC I surface expression through posttranscriptional unknown mechanisms. One possibility would be that reduced MHC I-peptide presentation reflects a direct effect of ER stress stimuli (DTT, tunicamycin, glucose starvation) on maturation of MHC I molecules. MHC I is a protein destined to the secretory pathway that requires N-linked glycosylation and disulphide bond formation for proper folding inside the ER (Zhang & Williams, 2006). Even though we did not assess the direct effect of the chemical agents or the lack of glucose on the maturation of the MHC I molecules, the results of de Almeida et al. argue against this hypothesis (de Almeida et al., 2007). They showed that overexpression of UPR transducers (ATF6 and XBP1) in the absence of genuine stress stimulus resulted in decreased MHC I surface expression. Furthermore, despite this decrease at the surface level, the total amount of MHC I was not affected (detected in whole cell lysates). Thus, UPR by itself appears to be sufficient to diminish MHC I expression at the cell surface, without affecting its transcription or its translation, as demonstrated in our work and in the work of de Almeida et al., respectively.

Peptide delivery to the ER is the limiting factor in the assembly and presentation of MHC I-peptide complexes (Neefjes et al., 1993; Yewdell et al., 2003). Our favorite hypothesis is therefore that decreased MHC I presentation is caused by restriction of peptide availability. During ER stress, transducers of the UPR seek to decrease the ER burden by suppressing translation initiation (Schroder & Kaufman, 2005; Ron & Walter, 2007). Given that MHC I molecules preferentially sample polypeptides that are being actively translated (Qian et al., 2006b), we posit that global attenuation of protein synthesis during the UPR

probably limits the amount of peptides available for insertion in MHC I molecules. In line with this, a probable dysregulation of the mTOR pathway during glucose starvation (Ozcan et al., 2008; Reiling & Sabatini, 2008) could also contribute to the repression of translation and thus restrict the amount of peptides available.

Besides peptide restriction, malfunctioning of the peptide-loading complex (PLC) could also account to the decreased peptide presentation. Many chaperones involved in the folding of proteins in the ER such as PDI, CRT and ERp57 are also part of the PLC that assembles peptides onto MHC I molecules. Hence, we speculate that accumulation of misfolded proteins in the ER induced by DTT, tunicamycin or lack of glucose, probably keep these chaperones quite busy in retaining misfolding proteins to prevent aggregation. There will be presumably less chaperones available to form the PLC and to load peptides onto MHC I molecules and this could account to the decreased surface expression of MHC I-peptide complexes.

Also, it will be interesting to analyze the stability of MHC I-peptide complexes in stressed cells. It is very likely that not only the peptide supply is restricted and the peptide loading defective, but that the complexes that are produced have decreased stability at the cell surface. Other experiments seeking to determine the peptide-loading kinetics, the rate of endocytosis of MHC I-peptide complexes, as well as their retention time in the ER (which determines the extent of the peptide-loading optimization (Lewis & Elliott, 1998)) will be imperative to test this hypothesis.

6.4. Why does UPR differentially affect expression of ER- versus cytosol-derived peptides?

A main conclusion of our work is that UPR-induced attenuation of MHC I-peptide presentation is more severe when the source protein is localized in the cytosol than in the ER (Figures 4c, 6b and 8). Our cell lines expressing HEL-Cyto-SIINFEKL and HEL-ER-SIINFEKL displayed identical responses to tunicamycin treatment or glucose starvation. Both cell lines showed similar upregulation of UPR markers and equivalent reduction in cell surface levels of $H2K^b$ and $H2D^b$. The sole difference concerned presentation of K^b-SIINFEKL complexes. This suggests that in stressed cells $H2K^b$ molecules are loaded more effectively with SIINFEKL when the peptide originates from an ER-destined protein than from a protein localized in the cytosol.

So, how would an ER-retained protein generate more peptides than a cytosolic

protein? We propose that this results from two discrepancies in the MHC processing of ER versus cytosolic proteins. The first discrepancy involves ERAD. UPR transducers specifically enhance degradation of proteins in the secretory pathway in order to decrease ER folding load. As described in the introduction, proteins destined to the secretory pathway are synthesized on ER-bound ribosomes and are cotranslationally translocated into the ER lumen (Anelli & Sitia, 2008). During ER stress, cotranslational protein translocation is inhibited and newly-synthesized ER proteins are triaged for degradation by the proteasome (Kang et al., 2006; Oyadomari et al., 2006). Furthermore, retrotranslocation of ER-resident proteins in the cytosol for proteasomal degradation is enhanced (Oda et al., 2006). In addition, non proteasomal degradation of ER proteins but not of cytosolic ones induced by ER stress (Shenkman et al., 2007), could increase the contribution of ER- over cytosol-derived peptides to the peptide pool. It has been shown that this alternative nonproteasomal pathway involves a Mn2+/Co2+-dependent metalloprotease or other metalloprotein.

The second discrepancy in the MHC processing of ER versus cytosolic proteins, concerns peptide binding to TAP. More than 99% of peptides are degraded by cytosolic peptidases before they bind TAP and thereby enter the MHC I presentation pathway. Thus, the probability that a peptide generated by the proteasome will associate with an MHC I molecule should be maximal when the proteasome is located closest to TAP, that is, on the cytosolic face of the ER (Caron et al., 2005). That is precisely where retrotranslocated ER proteins are degraded by the proteasome. In accordance, work from our laboratory and other studies have shown that the location of proteins in the ER in close proximity to ER-associated proteasomes can account for their contribution to the peptide repertoire (Leifert et al., 2004; Caron et al., 2005).

Consequently, we decided to analyze the amount of HEL protein in both cell lines during glucose starvation to have a rough estimate about their degradation (Figure 7). Nonetheless, we did not detect major differences in the net cytosolic or ER-retained HEL amount by Western blot of glucose-deprived EL4 transfectants. Nevertheless, more specific techniques to quantify protein translation and degradation rates should be more appropriate to test our degradation hypothesis.

The enhanced degradation characterizing the UPR and that probably favors presentation of ER-derived over cytosol-derived peptides could also be promoted by defective folding and a consequent short half-life of the HEL-ER-SIINFEKL protein during ER stress. Nevertheless, this is not always the case. Some studies have shown that inducing misfolding of model proteins either through amino acid substitutions, substantial deletion or puromycin treatment (causes brief termination of protein synthesis), had no impact on generation of two

epitopes. Only through the addition of a degradation signal (degron) was peptide production consistently enhanced (Golovina et al., 2005). Although we speculate that the folding of HEL-ER-SIINFEKL could be affected by tunicamycin treatment or glucose starvation, this will have to be confirmed. Also, further experiments will be necessary to determine whether the protein is degraded or not during ER stress. In summary, we hypothesize that during ER stress, MHC I peptide presentation is biased in favour of ER proteins because their proteasomal degradation is enhanced and the generated peptides emerge in the vicinity of TAP which facilitates their presentation by MHC I molecules.

6.5. What might be the impact of the UPR on immune recognition of infection and malignancy?

Paradoxically, if the decreased generation of MHC I-peptide complexes results mainly from inhibition of translation, it could facilitate recognition of virus infected cells. As described in the introduction, UPR-induced attenuation of translation is mediated by PERK-dependent phosphorylation of eIF2α on Ser^{51} (Shang et al., 2007). Phosphorylation of eIF2α hampers canonical cap-dependent translation initiation, which regulates synthesis of 95-98% of cellular mRNAs. However, viruses can use internal ribosomal entry sites in their 5' noncoding region to initiate cap-independent (or IRES-dependent) translation (Holcik & Sonenberg, 2005; Tardif et al., 2005). Thus, by preferentially repressing presentation of self peptides, the UPR could facilitate recognition of viral peptides (the needle in the haystack (Yewdell, 2007)). Moreover, some cellular mRNAs also contain IRESs in their 5'-UTRs (Fernandez et al., 2002b). This is the case of BiP/GRP78, whose mRNA is translated under stress conditions via an IRES element found within its 5'-UTR (Fernandez et al., 2002b). Attenuation of cap-dependent translation during the UPR, could lead not only to presentation of viral peptides, but also of peptides derived from some cellular proteins such as BiP/GRP78 and HSP70, whose translation is IRES-dependent under stress conditions (Baird et al., 2006). This could help explain why the expression of peptides derived from HSPs is enhanced under several cellular stresses, including viral infection (Hickman-Miller & Hildebrand, 2004).

Because cancer cells are constantly exposed to ER stress stimuli such as glucose deprivation, hypoxia and low pH in the microenvironment of tumors (Moenner et al., 2007), our results could reflect the in vivo effect of ER stress on MHC I-peptide presentation, specially in bulky tumors. However, by repressing production of MHC I-peptide complexes, the UPR may hinder presentation of tumor antigens to $CD8^+$ T cells. Indeed, generation of optimal $CD8^+$ T cell responses is promoted by high epitope density on antigen presenting cells (Wherry et al., 1999; Henrickson et al., 2008). On the other hand, an elegant study by

Schwab et al. has shown that in the presence of eIF2α phosphorylation, cells can generate MHC I-associated peptides derived from cryptic translational reading frames (Schwab et al., 2004). To note, cryptic peptides can elicit $CD8^+$ T-cell responses in mice (Schwab et al., 2003). On the other side, decreased MHC I-peptide abundance could also be recognized by inhibitory receptors, rendering stressed cells vulnerable to NK-cell (natural killer) mediated lysis (Yokoyama & Kim, 2006). Thus, although MHC I-peptide presentation is impaired during ER stress, modifications in the immunopeptidome could be important in recognition of infection and malignancy through adaptive and innate mechanisms.

7. CONCLUSIONS AND FUTURE PROSPECTS

The intracellular stress response has enormous potential to change the repertoire of peptides in the endogenous antigen presentation pathway. Many aspects of cellular metabolism on which antigen presentation depends, are affected under ER stress in cancer and infected cells. These include regulation of transcription and translation, degradation of proteins and chaperoning. Given all these common processes, we investigated whether and how ER stress affects MHC I presentation. Our results show that ER stress impinges on the MHC I peptide repertoire in two ways: by decreasing overall MHC I-peptide presentation and by changing the relative contribution ER- vs. cytosol-proteins to the cell surface MHC I-peptide repertoire. Since ER stress is a characteristic feature of infection and malignancy, dysregulation of MHC I-peptide presentation could have major implications in the recognition of infected and transformed cells by $CD8^+$ T lymphocytes.

Based on our results obtained with the model peptide SIINFEKL, we expect that the composition of the whole immunopeptidome will change during stress. High-throughput studies aiming to sequence the MHC I peptide repertoire could allow us to further evaluate how ER stress molds the immunopeptidome, not only in terms of peptide abundance but also in terms of diversity. We anticipate that it will modify the repertoire of antigens that are presented and therefore reflect the level of intracellular stress and response mechanisms that the cell activates. For instance, proteins involved in the response to stress such as chaperones that are actively translated, as well as misfolded proteins, which are actively degraded by ERAD, could become major contributors to the MHC I-immunopeptidome. Peptides derived from cryptic translation could also become evident. We expect that by using mass spectrometry techniques, the repertoire of peptides presented by stressed cells, for instance malignant cells, could be characterized. Nevertheless, what might be the overall effect of the UPR on the immunopeptidome is not inherently obvious considering the many ways it affects cell protein economy.

We are confident that the results of the current work and future studies will provide us with unique insights relevant to two areas. First, to the understanding of cancer cell biology. Indeed, the set of peptides presented at the cell surface by MHC I molecules provides a unique perspective on what is being both translated and rapidly degraded in a cell. Second, our work is of prime relevance to cancer immunotherapy. It becomes imperative to determine how ER stress, suffered by many cancer cells, molds the MHC I immunopeptidome and further discover peptides that could be used in cancer immunotherapy.

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