Amino acid substitutions in σ1 and μ1 outer capsid proteins are selected during mammalian reovirus adaptation to Vero cells

Roland Jabre, Véronique Sandekian and Guy Lemay*

Département de microbiologie et immunologie, Université de Montréal, Montréal (Qué.), Canada H3C 3J7

*Corresponding author. Fax: +1 514 343 5701

Email address: guy.lemay@umontreal.ca (G. Lemay)

Département de microbiologie et immunologie

Pavillon Roger-Gaudry

Université de Montréal

C.P. 6128, Succ. Centre-ville

Montréal (Québec)

H3C 3J7

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Abstract:

Establishment of viral persistence in cell culture has previously led to the selection of mammalian reovirus mutants, although very few of those have been characterized in details. In the present study, reovirus was adapted to Vero cells that, in contrast to classically-used L929 cells, are inefficient in supporting the early steps of reovirus uncoating and are also unable to produce interferon as an antiviral response once infection occurs. The Vero cell-adapted reovirus exhibits amino acids substitutions in both the σ1 and μ1 proteins. This contrasts with uncoating mutants from persistently-infected L929 cells, and various other cell types, that generally harbor amino acids substitutions in the σ3 outer capsid protein. The Vero cell-adapted virus remained sensitive to an inhibitor of lysosomal proteases; furthermore, in the absence of selective pressure for its maintenance, t he virus has partially lost its ability to resist interferon. The positions of the amino acids substitutions on the known protein structures suggest an effect on binding of the viral σ1 protein to the cell surface and on μ1 disassembly from the outer capsid.

1. Introduction

Mammalian reovirus is currently under clinical study as an oncolytic virus for the treatment of various cancers in humans (recently reviewed and discussed by: **Black and Morris, 2012; Harrington et al., 2010; Kelly et al., 2009; Lal et al., 2009**). The rationale behind this approach is the ability of reovirus to preferentially infect and destroy Ras-transformed/cancer cells compared to parental or so-called "normal" cells (**Coffey et al., 1998; Roner and Mutsoli, 2007; Smakman et al., 2005; Strong et al., 1996, 1998**). However, the exact mechanism underlying this preferential replication is still incompletely understood. Various observations initially suggested that inhibition of the interferon response by activation of the Ras signaling pathway is responsible for increased efficiency of reovirus infection (**Park et al., 2010; Rudd and Lemay, 2005; Shmulevitz et al., 2010; Strong et al., 1998**). However, it is likely that better viral uncoating is also involved in increased efficiency of early steps of the viral multiplication cycle in transformed cells (**Alain et al., 2007; Lemay et al., 2007; Marcato et al., 2007**).

Reovirus capsid is made of two concentric protein layers and is incompletely uncoated upon viral entry by endocytosis (reviewed by: **Danthi et al., 2010; Guglielmi et al., 2006**). The outer capsid proteins are partially removed by cellular proteases such as lysosomal cathepsins (L, B or S) (**Ebert et al., 2002; Golden et al., 2004; Johnson et al., 2009**), elastase (**Golden and Schiff, 2005**) or transmembrane serine proteases (**Nygaard et al., 2012**). Partial removal of outer capsid proteins generates infectious subviral particles (ISVPs) that are able to cross endosomal membranes allowing the release of viral particles in the cytoplasm. As an alternative mode of penetration, ISVPs could be generated by extracellular proteases, apparently allowing direct penetration of viral particles through the plasma membrane. In fact, this is probably the major mode of infection in the gastrointestinal tract where proteases are abundant (**Amerongen et al., 1994; Bass et al., 1990; Bodkin et al., 1989; reviewed by: Schiff et al., 2007**).

Although this may not be sufficient to completely explain the preferential infection of transformed cells, an increased level of proteases, protease activity, or changes in subcellular localization of these enzymes, is a known effect of cellular transformation. A correlation between Ras transformation and the level or activity of cathepsins was reported in the past, as well as a redistribution of the enzyme, allowing either secretion or increased presence at the membrane surface (**Cavallo-Medved et al., 2003; Chambers et al., 1992; Collette et al., 2004; Dilakyan et al., 2001; Hiwasa**

and Kominami, 1995; Joseph et al., 1987; Kim et al., 1998; Urbanelli et al., 2010). The level or localization of proteases is thus an important factor to determine host-cell permissivity to reovirus and it appears that viral uncoating is often a limiting factor in different cell lines or cell types such as MEF cells (primary murine embryo fibroblasts: **Golden et al., 2002; Nygaard et al., 2012**), MEL cells (murine erythroleukemia cells: **Wetzel et al., 1997a**), NIH-3T3 cells (immortalized murine embryo fibroblasts: **Alain et al., 2007; Marcato et al., 2007**), SC1 cells (murine feral embryo fibroblasts: **Lemay, unpublished data**), U118 cells (human glioblastoma cells: **Alain et al., 2007**) or Vero cells (african green monkey kidney cells: **Golden et al., 2002**).

Despite the fact that reovirus is naturally "oncolytic" and able to discriminate between parental and transformed/cancer cells, without prior genetic manipulation, it is generally believed that it could be adapted to further optimize its oncolytic ability (**Kim et al., 2007; Rudd and Lemay, 2005; Shmulevitz et al., 2012; van den Wollenberg et al., 2009, 2012**). It has been shown that the establishment of persistent reovirus infection leads to a gradual virus-cell co-evolution, resulting in an increased resistance of the cells to the wild-type parental virus while the virus develops an increased ability to infect these cells; this viral adaptation allows maintenance of the persistent state by continuous re-infection (reviewed by: **Dermody, 1998**). However, the nature of mutations present on the viruses resulting from this co-evolution was clearly documented only in murine L929 fibroblasts and results from amino acids substitution(s) in the surface-exposed lobe of the σ3 outer capsid protein, thus increasing its sensitivity to proteases and favoring viral uncoating under conditions where proteases are present in limited amount (**Baer and Dermody, 1997; Wetzel et al., 1997b**). It remains to be determined if viruses selected in different cell types will be similarly altered in their ability to be uncoated and if similar amino acids substitution(s) will be selected independently of cell types. It was recently suggested that it is the case, although amino acids substitutions in σ1 were also observed (**Kim et al, 2011**). However, the exact role of these amino acids substitutions was not directly established nor further examined. Furthermore, a deletion in σ1, the outer capsid cell binding protein (reviewed by: **Danthi et al., 2010**), selected during persistence in one cell line, was also shown to attenuate the virus while having a limited effect on infection of cancer cells, supporting the idea that viral persistence could be used to select for viruses that are better adapted as oncolytic agent (**Kim et al., 2011**).

In the present study, the reovirus serotype 3 Dearing was adapted to Vero cells by establishment of viral persistence. These cells were chosen since they differ from L929 cells by their lack of interferon production (**Desmyter et al., 1968; Emeny and Morgan, 1979**) and were also reported to be

inefficient at uncoating the virus, likely due to reduced levels of lysosomal uncoating proteases (**Golden et al., 2002**). The Vero cell-adapted virus (VeroAV) infects Vero cells better than the original wild-type virus in the absence of chymotrypsin and forms plaques under these conditions, in contrast to the wild-type virus, while remaining sensitive to an inhibitor of lysosomal cathepsins; this contrasts with uncoating mutants selected during viral persistence in L929 cells (**Baer and Dermody, 1997; Wilson et al., 2002**). Furthermore, in the absence of selective pressure for the maintenance of a certain level of resistance, the virus has apparently lost part of its ability to resist interferon. Sequencing of the genes encoding the three outer capsid proteins revealed the absence of amino acids substitution in the σ3 protein while two amino acids changes were found in the σ1 protein, including one at a location consistent with an increased binding to host cell-surface sugar moieties (**Reiter et al., 2011**). In addition, two amino acids substitution were also found in μ1 at positions that could affect outer capsid structure or disassembly (**Zhang et al., 2005**) and possibly indirectly affect interferon resistance. Altogether these results support the idea that adapting the virus to different cell types could generate novel viruses to be used as alternatives to the wild-type virus in future oncolytic applications.

2. Materials and methods

2.1. Cell lines and viruses

L929 mouse fibroblasts and Vero cells (African green monkey kidney cells) were originally obtained from the American type culture collection (ATCC); all cells were grown in minimal Eagle medium (MEM) with 5% fetal bovine serum, 1% L-glutamine and 1% P/S from commercial stock solutions (Wisent Bioproducts).

Wild-type reovirus laboratory stock was derived from a pure plaque of reovirus type 3 Dearing (T3/Human/Ohio/Dearing/55). The original inoculum was obtained from the American Type Culture Collection (ATCC). A high-passage stock of the virus was obtained by first infecting a semi-confluent 100 mm petri dish of L929 at a MOI of 80; after complete cell lysis, one-twentieth of the cell lysate was used to infect a similar dish; this procedure was repeated each 48 h up to ten passages of the virus.

2.2. Antibodies

Hybridoma cell lines producing either anti-σ3 (4F2) or anti-μ1 (10F6) have been described

(**Virgin et al., 1991**) and were obtained from Kevin Coombs (University of Manitoba). Hybridoma cells were grown in MEM for suspension culture with 10% fetal bovine serum, proline (20 μg/ml) and β-mercaptoethanol (50 μM) and antibodies were recovered as previously described (**Brochu-Lafontaine and Lemay, 2012**). The FITC-conjugated goat antireovirus antibody was obtained from Accurate Chemical & Scientific Corporation (catalog # YV0031-10).

2.3. Determination of virus titer.

Virus titers were determined by plaque assay on Vero cells in the presence of chymotrypsin (Sigma Type I-S from bovine pancreas) at 10 μg/ml, as previously described (**Brochu-Lafontaine and Lemay, 2012**).

2.4. Quantitation of reovirus-infected cells by FACS

To compare the percentage of infected cells, cells were infected at the same multiplicity of infection and analyzed by FACS. Cells from a 6-wells plate were collected by treatment with 6 mM EGTA (Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid) at 37°C for 10 min, 24 h post-infection, and resuspended in 1 ml of EMEM containing 5% fetal bovine serum before recovery by centrifugation at 4°C for 5 min at 1500 x *g*. The pellet was resuspended in 0.25 ml of Cytofix/Cytoperm (Becton Dickinson) on ice with gentle agitation and left 20 min before mild centrifugation in microplates at 350 x *g*. Cells were then resuspended in the 0.25 ml Perm Wash buffer (Becton Dickinson) centrifuged again and resuspended in 0.035 ml of buffer to which 0.015 ml of FITC-conjugated antireovirus antibody was added. Following 30 min on ice with occasional gentle agitation, cells were pelleted, washed twice in buffer, and fixed with 4% paraformaldehyde before being analyzed on a BD FACSCalibur cytofluorometer (Becton Dickinson).

2.5. Immunoblotting

Infected cells were recovered by scraping in small volume of medium and centrifuged in an Eppendorf tube at 13 000 x *g* for 5 min at 4°C. Cell pellets corresponding to a 60 mm-diameter petri dish were resuspended in 45 μl of permeabilization buffer (Tris-HCl 10 mM pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40) and left on ice for 5 min before centrifugation at 13 000 x *g* for 1 min in an Eppendorf centrifuge at 4°C. Proteins were analyzed by SDS-PAGE and immunoblotting. Nitrocellulose membrane (Whatman Protran BA85) was blocked with 2% non-fat dry milk dissolved in

TBS (Tris-HCl 10 mM pH 7.5, 150 mM NaCl) and incubated for 1 h at room temperature with the anti-σ3 and anti-μ1 monoclonal antibodies. Antibodies in tissue culture medium were diluted with an equal volume of TBS containing the blocking agent and directly used. The diluted antibody solution was recovered and kept at 4ºC with 1 mM sodium azide to be used up to 10 times. Membranes were washed in TBS containing 1% Tween-20. Revelation was done using peroxydase-conjugated secondary anti-mouse IgG antibody and chemiluminescent substrate, as recommended by the manufacturer (Pierce SuperSignal West Dura Extended Duration Substrate). Images were obtained using a Typhoon Trio™ imager (GE Healthcare Life Sciences).

2.6. Determination of viral sensitivity to uncoating inhibitors

To determine the sensitivity of wild-type and VeroAV to either an inhibitor of endosomal acidification or of lysosomal protease, L929 cells were treated with either 5 mM of ammonium chloride (at 5 mM) or E64 (L-transepoxysuccinyl-leucylamido-[4-guanidino]butane; Sigma-Aldrich) at 50 or 100 μM; control cells were left untreated. Control or treated cells were then infected at a multiplicity of infection of 2 PFU/cell and incubated for 24 h in the presence of the inhibitor. Petri dishes (cells and medium) were frozen at -80°C, 24 h post-infection, and subjected to three cycles of freeze-thaw before virus titration by plaque assay on chymotrypsin-treated Vero cells.

2.7. Induction of and sensitivity to interferon.

For the detection of induced interferon, L929 cells were infected with either wild-type or VeroAV at a multiplicity of infection of 5 and the supernatant was recovered 12 h post-infection. Recovered supernatant was then passed through a Vivaspin 100 000 molecular weight cutoff filter (Sartorius) to remove infectious reovirus. Virus-free supernatant was then used in encephalomyocarditis titration assay by determination of the “tissue culture infectious dose 50%” ($TCID_{50}$) on L929 cells, as previously described (**Sandekian et al., 2013**).

To determine virus' sensitivity to interferon, L929 cells in 96-wells plates were pre-treated with 500 or 100 IU/ml of murine interferon-β (PBL interferon source) and used in $TCID_{50}$ assay of either wild-type or VeroAV, as previously described (**Danis et al., 1997**). Number of infected wells was determined by direct examination using phase-contrast microscopy.

2.8. Sequencing of viral genome segments encoding outer capsid proteins

Virus stocks were used to infect L929 cells in 10 cm-diameter petri dishes at a MOI of 10 PFU/cell. Cells and medium were recovered at 24-30 h post-infection, at which time most cells were lysed. Following two cycles of freeze-thaw (-80ºC to room temperature), the lysate was extracted once with one-fourth volume of freon (1,1,2-Trichloro-1,2,2,-trifluoroethane, Mallinckrodt Chemicals). After 10 min of centrifugation at 7 000 g in a Sorvall SS-34 rotor at 4ºC (in Corex 15 ml tubes), supernatant was recovered and overlayed on a 1ml cesium chloride cushion at a density of 1.3 g/ml for ultracentrifugation in a 70Ti rotor at 250 000 g for 1 h at 4ºC. Virus pellet was recovered in 400 µl of TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) before addition of 0.2% SDS followed by phenol-chloroform extraction and ethanol precipitation of viral double-stranded RNA. The RNA was denatured at 95ºC for 5 min and cooled rapidly on ice. Reverse transcription was done on both strands using one-fourth of the RNA and oligonucleotides specific for each strand at both ends of the gene to be sequenced. These reverse transcription reactions were done with MLV reverse transcriptase was for 1 h at 37ºC, as recommended by the manufacturer (Roche). One-fifth of the reverse transcriptase reaction was then used in a PCR reaction with the same two oligonucleotides for 40 cycles using FastStart protocol, as recommended by the manufacturer (Roche). The PCR fragment was then purified using the Qiaquick PCR purification kit as recommended by the manufacturer (Qiagen) and sequenced by automated DNA sequencing (Applied Biosystems 3730 DNA Analyzer) in the sequencing service of Institut de recherche en immunologie et cancérologie (IRIC) of Université de Montréal.

The oligonucleotides used for S4 RT-PCR were GCTATTTTTGCCTCTTCCCAGACG (nucleotide 1 to 24) and GATGAATGAAGCCTGTCCCACGTC (nucleotide 1173 to 1196 on the complementary strand); sequencing was done using the same two primers as well as CAAGTTGGACAGATCCTTTGCAG (nucleotide 472 to 494) and GGGTATCAAGTCGGATGAGTCGA (nucleotide 553 to 575 on the complementary strand).

For the M2 gene two separate RT-PCR fragments were used to cover the whole gene: oligonucleotides GCTAATCTGCTGAGCGTTACTCTG (nucleotide 1 to 24) and CCGATCTGTGAAGCAGCAGTCC (nucleotide 1267 to 1288 on the complementary strand) were used together to amplify the 5' portion of the gene while oligonucleotide CTGGACCAAATCGCTCCGATGCGG (nucleotide 1137 to 1160) and GATGATTTGCCTGCATCCCTTAACCCCG (nucleotide 2176 to 2203 on the complementary strand) were used to amplify the 3' portion. Sequencing was done with the same primers and with primers CTGCTGGATGATCAGCTGCCAG (nucleotide 681 to 702), CTGGACCAAATCGCTCCGATGCGG

(nucleotide 1137 to 1160), GGTCAGCTTGCTATCTCGCAACTCG (nucleotide 1737 to 1761) and CTAAGGGTGGGGCTGATGCTGAA (nucleotide 845 to 867 on the complementary strand).

For sequencing of the S1 gene two PCR fragments were obtained and sequencing was performed with five different primers. RT-PCR products were thus obtained using either GCTATTGGTCGGATG (nucleotide 1 to 15) and ATTCCGATACCGCCGCTAACATCA (nucleotide 915 to 938 on the complementary strand) or a combination of GACTCTCAAGACGACTGTGTTTG (nucleotide 708 to 730) and AGTGCCGCGGGGTGGTCTGATC (nucleotide1382 to 1403 on the complementary strand). Sequencing was performed with primers GCTATTGGTCGGATG (nucleotide 1 to 15), GACTCTCAAGACGACTGTGTTTG (nucleotide 708 to 730), ATGTTAGCGGCGGTATCGGAATGA (nucleotide 917 to 940), AGCTCTGCAAGTCCTGTCTCAAGT (nucleotide 345 to 368 on the complementary strand) and AGTGCCGCGGGGTGGTCTGATC (nucleotide 1382 to 1403 on the complementary strand).

Sequences used for comparisons were obtained from the NCBI database. Accession numbers of the reverse genetics clones used as reference sequence were ABP48922 (for σ3), ABP48917 (for μ1) and ABP48919 (for σ1).

3. Results

3.1. Production of a Vero cell-adapted virus (VeroAV)

Vero cells were infected with a laboratory stock of wild-type reovirus serotype 3 Dearing (T3/Human/Ohio/Dearing/55). Viral stocks used were first propagated in standard conditions of low multiplicity of infection (1 PFU/cell), or at a high multiplicity of infection, as described in Section 2. Such high-passage reovirus stocks resulting from serial propagation at a high multiplicity of infections are known to harbor a mixture of mutants and defective viruses and were used by various groups to facilitate establishment of viral persistence (**Ahmed and Fields, 1982; Ahmed and Graham, 1977; Ahmed et al., 1980; Dermody et al., 1993**). Using either of the two virus stocks, gradual cell death was observed in infected Vero cells with the presence of large cytoplasmic inclusions typical of reovirus infection (data not shown). However, a significant proportion of the cells remained alive in the culture despite obvious viral-induced cytopathic effects. Cells infected with the high-passage stock of virus were maintained and propagated with occasional passage with trypsin. Cell death transiently increased each time the cells were passaged but a significant proportion of infected cells remained alive

and gradually adapted until a cell culture presenting minimal cell death and cell growth indistinguishable from that of parental cells, was finally obtained. Analysis of virus produced by these cells was undertaken 21 weeks after the initial infection.

Cell-free supernatant was recovered and readily destroyed L929 cells, indicating the presence and release of infectious virus in the culture of Vero cells despite absence of apparent cytopathic effect at this time. Since this was not the purpose of the present study, the "persistently infected" Vero cells themselves were not further examined.

The Vero cell-adapted virus (VeroAV) was further propagated on L929 cells and viral titer was determined by plaque assay on Vero cells in the presence of chymotrypsin. VeroAV reaches titers similar to that of the wild-type virus under similar propagation conditions. The limiting dilution, $TCID_{50}$ method was also used to determine virus titers on L929 cells using 96-multiwell plates (**Danis and Lemay, 1993**), The relative titers were between 1.3-fold lower to 1.9-fold higher by plaque assay for VeroAV compared to the wild-type virus. This small difference between the two methods could be explained, at least in part, by different plaque size for the two viruses as discussed in the following section.

3.2. Infectivity of VeroAV

Titration of infectious virus on Vero cells was initially performed in the presence of chymotrypsin, since wild-type virions are unable to form plaques on these cells in the absence of exogenous proteases (**Brochu-Lafontaine and Lemay, 2012**) (**Fig. 1**A); even under the microscope foci were very small in the absence of chymotrypsin and consisted of only few cells with incomplete lysis (**Fig. 1**B, panel a). This is consistent with the previous report showing that infection of Vero cells is relatively inefficient but that this blockage can be overcome by prior treatment with a protease, such as chymotrypsin, to generate ISVPs (**Golden et al., 2002; Nygaard et al., 2012**). Accordingly, large plaques were readily formed when chymotrypsin was added to the agar overlay used for viral titration (**Fig. 1**A and B), as previously described (**Brochu-Lafontaine and Lemay, 2012**). In contrast, VeroAV is able to bypass the restriction normally observed in Vero cells and forms plaques in the absence of chymotrypsin (**Fig. 1**A and B), while plaques formed by VeroAV in the presence of chymotrypsin were smaller than that observed with the wild-type virus (**Fig. 1**A and B).

The infection by the two viruses was then compared on a single infection cycle using detection of intracellular viral antigens by FACS analysis of permeabilized cells, essentially as described by others

(**Kim et al., 2010; Marcato et al., 2007**); a commercially available antireovirus antibody directly conjugated to FITC, was used to facilitate the procedure, as previously used (**Sandekian et al., 2013**) and described in Section 2. In preliminary assays, the proportion of L929 cells infected with the wild-type virus at a multiplicity of infection of 1 PFU/cell was approximately 50% compared to a theoretical value of 63%, according to the Poisson distribution (data not shown). It thus appears that the procedure does adequately reflect the efficiency of viral infection. The efficiency of infection of VeroAV was then compared with that of the wild-type virus; a representative experiment is presented on **Fig.2**. On Vero cells, in the absence of chymotrypsin, infection by the wild-type virus was very low compared to that on L929, the average of two different experiments indicated that infection was approximately fourfold more efficient in L929 cells while for VeroAV the difference was less than twofold between the two cell lines. Addition of chymotrypsin on Vero cells increased infection of the wild-type virus by at least sixfold on average, consistent with results obtained by plaque assay. In contrast the effect of chymotrypsin on efficiency of VeroAV infection was at most twofold, indicating that the infection of Vero cells by VeroAV is largely independent of prior uncoating.

These results were further confirmed by immunoblotting analysis of the viral proteins synthesized during viral replication (**Fig. 3**). As expected, the addition of chymotrypsin to the medium allowed the wild-type virus to bypass the restriction while there was essentially no effect, under the same conditions, with VeroAV. In contrast, VeroAV was already able to efficiently infect Vero cells in the absence of chymotrypsin, as observed in both plaque assay and FACS-based analysis. On L929 cells, VeroAV and wild-type virus were similar or even slightly better for VeroAV, as also previously observed by FACS.

3.3. Replicative ability of VeroAV

Virus titers produced upon one-cycle of replication in Vero cells were next examined (**Fig. 4**A). In the absence of chymotrypsin, VeroAV produced slightly more infectious virus than the wild-type virus. As expected, there was an increase in virus titer in the presence of chymotrypsin with the wild-type confirming that infection by the wild-type virus is increased by prior uncoating of the virions. However, in the same conditions, VeroAV titer obtained was actually decreased, an observation that is reminiscent of the small plaques produced by this virus in the presence of chymotrypsin (**Fig.1**). The protocol was then modified by removal of the chymotrypsin 4 h post-infection (**Fig.4**B), allowing proteolytic uncoating of the initial virus inoculum but avoiding the action of chymotrypsin on released

viral particles. Under these conditions, wild-type virus titer was more than 100-fold higher when chymotrypsin was used, further indicating the positive effect of prior uncoating and indicating that the chymotrypsin had a negative effect on the infectivity of the released viral particles under these conditions of one-cycle viral replication. VeroAV titer remained higher than that of wild-type but was only slightly increased by chymotrypsin treatment, indicating that it is less dependent on prior uncoating for infection but more sensitive to the negative effects of chymotrypsin on released virions. When the experiment was repeated with a concentration of chymotrypsin reduced from 20 to 5 μg/ml, virus titer of the wild-type virus was significantly increased (more than 500-fold) while VeroAV titer was only increased fivefold (data not shown). Under these conditions, removal of chymotrypsin after 4 h did not change the results. This further supports the idea that chymotrypsin increased infectivity of the wild-type virus but has only a limited, and even negative, effect on VeroAV. Again, this is probably reflected by the smaller plaque size observed with this virus in the presence of chymotrypsin (as shown on **Fig. 1**).

Replicative ability of VeroAV also correlated with cytopathic effects observed upon infection (data not shown). In the absence of chymotrypsin, Vero cells were quite resistant to viral-induced cytopathic effect with the wild-type virus at a multiplicity of infection of 2 PFU/cell. In contrast, cytopathic effects were observed as early as 24 h post-infection with VeroAV and essentially all cells were destroyed by 48 h post-infection. When chymotrypsin was present, cytopathic effect was evidenced for both wild-type virus and VeroAV; cytopathic effect was already observed at 24 h post-infection and essentially all cells were killed by 48 h.

3.4. Characterization of early events during infection by VeroAV

Since infection of Vero cells with the wild-type virus seems to rely on prior *in vitro* protease treatment, it was first suspected that the increased infectivity of VeroAV could be due to enhanced uncoating. This is normally accompanied by a reduced requirement for intracellular proteases during infection by virions and thus a reduced sensitivity to both inhibitors of lysosomal acidification, such as ammonium chloride (NH_4Cl), or inhibitors of lysosomal uncoating proteases, such as E64 (**Clark et al., 2006; Ebert et al., 2001**). This was examined in L929 cells that have been extensively used in the literature for such studies. VeroAV and wild-type viruses exhibited a similar sensitivity to 5 mM NH_4Cl, titers being reduced approximately 10-fold at this concentration (data not shown).This was further studied using E64 as a more specific inhibitor (**Fig. 5**). Again, both virus titers were similarly

reduced 5 to 10-fold at the 50 μM concentration and between 30 to 50-fold at the 100 μM concentration, VeroAV thus being at least as sensitive as the wild-type virus. This suggests that the replication of VeroAV in the absence of chymotrypsin is not due to a reduced requirement for cysteine protease in the lysosomal compartment and that VeroAV is not an uncoating mutant.

3.5. Induction of, and resistance to, interferon by VeroAV

As mentioned in Section 1, Vero cells were chosen since, in addition to their limited ability to uncoat reovirus, they are also unable to produce interferon. Viruses could thus potentially lose their ability to control the interferon response, if this allows to gain other properties beneficial to viral maintenance and/or replication in Vero cells. Such viruses will be either more prone to induce interferon in interferon-competent cell lines, such as L929 mouse fibroblasts cells, or more sensitive to the antiviral action of interferon in these cells.

Induction of interferon was examined by measuring the antiviral effect of the supernatant from L929 cells infected with either the wild-type or VeroAV reovirus; the antiviral activity was measured against the unrelated encephalomyocarditis virus (EMCV), as previously described (**Sandekian et al., 2013**). Supernatant from cells infected with either wild-type or VeroAV were both protective against EMCV. The undiluted supernatant recovered at 12 hours post-infection with each virus could decrease apparent EMCV titer from 5.4×10^9 PFU/ml to 8.4×10^8 for the wild-type virus and 7.4×10^8 for VeroAV. Therefore, it does not appear that VeroAV could induce more interferon than the wild-type, parental, reovirus. This is also consistent with the presence of a proline at amino acid 208 of μ2 on both the wild-type and VeroAV (data not shown), an amino acid position known to be responsible for differences in interferon induction between different reovirus strains (**Irvin et al., 2012**).

Sensitivity of VeroAV to interferon was next determined by $TCID_{50}$ titration of parental wild-type virus or VeroAV on murine L929 fibroblasts in the presence of 500 international units(IU)/ml of mouse β-interferon. At this high interferon concentration, virus titer was decreased by approximately 6000-fold for the wild-type virus and more than 24,000-fold for VeroAV (data not shown), suggesting an increased sensitivity of the latter. The experiment was repeated at a lower interferon concentration (100 IU/ml) and, under these conditions, VeroAV was clearly more sensitive, its titer being decreased by more than a thousandfold, while the wild-type virus titer was decreased only 15-fold (**Fig. 6**); VeroAV is thus essentially as sensitive as the "interferon-hypersensitive" P4L-12 mutant that was previously described (**Rudd and Lemay, 2005**).

3.6. Sequencing of viral genes encoding outer capsid proteins of VeroAV

Altogether, the three outer capsid proteins, σ1, σ3 and μ1, are responsible for virus binding and entry into host cells (most recently reviewed by: **Danthi et al., 2010**). In view of the previous results, indicating a decreased requirement of prior outer capsid removal for VeroAV infection of Vero cells, while the virus remained sensitive to inhibitors of intracellular outer capsid removal, the sequence of the genes encoding the three outer capsid proteins of VeroAV was determined. The original wild-type laboratory virus stocks, as well as the high-passage virus stock used to establish VeroAV-infected cells, were sequenced in parallel to better assess the significance of eventual amino acids substitutions in VeroAV. Although there are certainly numerous mutants in the high-passage stock, there was no apparent differences with the original wild-type stock when PCR products were directly sequenced, reflecting the lack of selection of a new virus under these conditions. The sequences were also compared with the sequences reported for the genes of reovirus serotype 3 Dearing in the plasmids used for the generation of infectious virus by reverse genetics (**Kobayashi et al., 2007**).

The σ3 protein of VeroAV has no amino acid substitutions compared to the original virus that is also identical with the sequence of the reverse genetics clone; this absence of substitution in Vero AV contrasts with all known uncoating mutants but is consistent with the fact that VeroAV behaves differently from such mutants. In contrast, two amino acids substitutions were found in the σ1 protein of VeroAV compared with both the original wild-type virus and the reverse genetics virus, the Q78P and N198K substitutions. Three other substitutions (T249I, T408A and Y253S) were also found between wild-type and VeroAV on one hand, and the reverse genetics virus on the other hand. Comparisons with sequences in database are difficult due to extensive differences in σ1 protein when different serotypes are compared. Nevertheless, the T249I and T408A substitutions, compared to the sequence of the reverse genetics virus, were also found to be present in more than half the sequences of type 3 viruses in the NCBI database; however, the Y253S substitution appears to be unique to the viruses used in the laboratory at the present time.

The μ1 protein of VeroAV also exhibits two different amino acids substitutions compared to the original wild-type virus and to the reverse genetics virus, the E89G and A114V substitutions. These two amino acids are conserved between different mammalian reoviruses of all four serotypes present in the NCBI database; two other differences between both the wild-type virus and VeroAV compared to the reverse genetics virus were also noted, the A305V and A449T substitution. While a valine is, in

fact, observed more frequently than alanine at position 305 in sequences of all serotypes, the alanine at position 449 is conserved and A449T represents a novel variant of μ1 in the virus' laboratory stock.

4. Discussion

As with most viruses, reovirus' ability to infect different cell types and the final outcome resulting from this infection, cell death or persistence establishment, has been mostly studied in one cell type, namely the L929 mouse fibroblasts. In the last few years, there has been a renewed interest to study reovirus replication and virus-host cell interactions. This is certainly largely due to current efforts to introduce the virus as an oncolytic agent. A better understanding of the virus' replication in different cell types could allow to better adapt the virus to specifically infect, replicate in, and kill cancer cells (**Kim et al., 2007, 2011**; **Rudd and Lemay, 2005**; **Shmulevitz et al., 2012; van den Wollenberg et al., 2009, 2012**).

As mentioned in Section 1, there has been relatively few detailed characterization of viruses adapted through persistence establishment; in the most-studied L929 cells model, there were many different amino acid substitutions on σ1, depending on the virus, and a single amino acid substitution on σ3 (Y354H). This last substitution was later shown to be most important and results in an increased ability of the virus to be uncoated (**Dermody, 1998**). Amino acids substitution in σ3 was most often observed in viruses recovered from other persistently infected cell lines although σ1 substitutions were also frequent, but the exact importance of these amino acids substitutions was not clearly established. (**Kim et al., 2011**). It should be mentioned that the sequence of the other genes, including that of the segment encoding the third outer-capsid protein, the μ1 protein, was not examined in this study. Virus persistence was also reported in CHO cells (**Taber et al., 1976**), MDCK cells (**Montgomery et al., 1991**), SC1 feral mouse embryo fibroblasts (**Danis et al., 1993**) and Balb-3T3 mouse fibroblasts (**Verdin et al., 1986**) but detailed characterization of the resulting viruses is still lacking.

Vero cells chosen in the present study appeared relatively resistant to reovirus while efficiency was increased by prior chymotrypsin treatment resulting in uncoating of the virion. However, this could be due to different factors in addition to a defect of uncoating in these cells that could be bypassed by prior removal of outer capsid protein σ3 and proteolytic cleavage of outer capsid μ1. An increased ability to bind onto sialic acid at the cell surface upon virion to ISVP proteolytic conversion could also be responsible for the increased infectivity of ISVPs (**Chappell et al., 1998; Nibert et al., 1995;**

reviewed by: **Danthi et al., 2010; Schelling et al., 2008**). Finally, a change in the efficiency of final disassembly steps could lead to increased infectivity due to increased ability of the viral particles to cross the cellular, or endosomal, membrane.

Despite their relative resistance to reovirus, infected Vero cells gradually developed cytopathic effects and a significant cell death was observed. This was maintained for a long time before a balance was apparently established and cell death became inapparent in the culture of "persistently-infected" cells. Evolution of the cells was not studied further in this manuscript but the evolution of the virus was evidenced by the apparent lack of remaining wild type sequence for either the σ1 or μ1 encoding gene in VeroAV. However, when individual clones were obtained from the PCR products and individually sequenced, amino acids substitutions in σ1 were consistently observed while the exact nature of amino acids changes in μ1 was variable (**Table 2**), although the two substitutions E89G and A114V were by far the most frequent. Interestingly, in a previous attempt to adapt the wild-type virus to Vero cells, the virus was collected after only 8 weeks; in this virus, the N198K substitution on σ1 was already found but not the Q78P while there was no substitution on μ1. This suggests that the N198K could be most important in the adaptation to Vero cells.

As previously mentioned, Vero cells possess two known interesting properties that may affect the nature of the virus recovered upon adaption to these cells. Vero cells lack the ability to produce interferon (**Desmyter, et al., 1968; Emeny and Morgan, 1979**), thus likely making them more susceptible to reovirus replication and propagation once they are infected. They also exhibit a reduced ability to uncoat the virus, thus probably limiting initial entry steps. It is thus expected that the virus will evolve toward an increased ability to initiate viral infection. Surprisingly, in contrast to classical uncoating mutants, VeroAV was sensitive to the inhibitor of cysteine protease and, accordingly, the σ3 protein did not show an altered sequence. This contrast with the situation observed with viruses recovered from persistently-infected murine SC1 cells; although these cells are also deficient in interferon response (**Danis et al., 1997**) and ability to uncoat the virus (unpublished data), an amino acids substitution in the outer lobe of the σ3 protein was observed in that case (**Table 1**). In all other reports of viral isolates from persistently-infected cells, an amino acid substitution in σ3 was also observed, although substitutions in σ1 were also often found (**Table 1**).

The available crystal structure of the σ3-μ1 heterohexamer that forms the bulk of the outer capsid (**Liemann et al., 2002**) and the high-resolution structure of the virion by electron cryomicroscopy (**Zhang et al., 2005**) permit some prediction. The E89 residue is positioned in the predicted contact

zone between neighboring heterohexamers at their basis in the most inner part of the outer capsid (**Fig. 6**) while the A114 position is located at a position suggesting a possible effect on the interaction between the three μ1 molecules of each heterohexamer (**Fig. 7**). Either one, or both, of these changes could alter outer capsid stability and/or disassembly; interestingly, amino acids substitutions that affect the stability of the σ3-μ1 heterohexamer were previously observed in these regions (**Agosto et al., 2007; Middleton et al., 2007**), including one of E89 that appears to render the virus thermolabile and thus potentially destabilizes the capsid and favors virus entry and disassembly under normal temperature conditions. The amino acids substitutions in μ1 are thus likely responsible for the changes in sensitivity of VeroAV to chymotrypsin, as reflected in reduced plaque size. The interaction between σ3 and μ1 appears to be essential in the control of the function of these two proteins when they are under their free form in the cytoplasm (reviewed by: **Schiff, 1998; 2008**). The σ3 protein is believed to inhibit the dsRNA dependent protein kinase (PKR) activation due to its ability to bind the double-stranded RNA (dsRNA) activator of PKR (**Beattie et al., 1995; Giantini and Shatkin, 1989; Imani and Jacobs, 1988; Lloyd and Shatkin, 1992; Yue and Shatkin, 1997**), an ability that is lost upon its interaction with μ1 (**Huismans and Joklik, 1976; Lemieux et al., 1987**); this is likely one important factor that could affect virus' sensitivity to interferon (reviewed by: **Samuel, 1998; Sherry, 2009**). Accordingly, the PKR inhibition and resulting effect on host translation is likely dependent on the relative level of free σ3 compared to μ1-bound σ3 (**Schmechel et al., 1997**). Reciprocally, the μ1-induced apoptosis is increased in absence of σ3 (**Coffey et al., 2006**). Numerous strands of evidence indicate that dsRNA and μ1 binding by σ3 are mutually exclusive (**Bergeron et al., 1998; Huismans and Joklik, 1976; Lemieux et al., 1987; Liemann et al., 2002; Shepard et al., 1996**) Increased sensitivity of VeroAV to interferon, in absence of any change in interferon induction, is thus consistent with the idea that a change in the efficiency of heterohexamers formation, resulting from amino acids substitutions in μ1, can indirectly affect the control of PKR by σ3 and, therefore, virus' sensitivity to interferon.

The contact regions between μ1 in neighboring heteroxamers are also similar to the contact regions between μ1 and λ2 forming the turret through which σ1 trimers are anchored and exposed at the virion's surface (**Liemann et al., 2002; Middleton et al., 2007; Zhang et al., 2005;**). The nature of the μ1 protein could thus indirectly affect the association and exposure of σ1 at the virion's surface. Interestingly, it was recently suggested that amino acids changes in λ2 itself could affect the function of σ1, probably by modulating its association to the virion or exposure at the virion's surface (**Shmulevitz**

et al., 2012). It should also be remembered that σ1 and μ1 are involved in binding and entry steps in viral infection, and it is thus somehow expected that these two phenomena need to be well adapted to each other. Accordingly, a role of μ1 in a σ1-determined phenotype was also previously observed in different contexts, such as apoptosis, neurovirulence or development of oily fur syndrome (**Clarke et al., 2001; Derrien et al., 2003; Hrdy et al., 1982; Rodgers et al., 1997; Tyler et al., 1996**). Interestingly, in a Vero cell-adapted avian reovirus, gene reassortment experiments assigned the adaptive changes to both the σC cell-binding moieties homologous to σ1 and the μB major outer capsid protein homologous to μ1 (**Meanger et al., 1999**). This further supports the idea that these two proteins functionally interact and are essential in virus' adaption to Vero cells.

Interestingly, although the asparagine-198 of σ1 is not directly involved in the binding to sialic acid, it was previously shown that its substitution by an acidic amino acid (N198E) does prevent binding (**Chappell et al., 1997; Dermody et al., 1990; Reiter et al., 2011**). It is thus possible that substitution by a basic amino acid, as in VeroAV (N198K), could rather positively influence this binding and could be responsible for better infectivity on Vero cells.

In the last few years, a powerful plasmid-based reverse genetics approach has been introduced for the study of mammalian reovirus (**Kobayashi et al., 2007;** reviewed by: **Boehme et al., 2011; van den Hengel et al., 2013; Lemay, 2011**). This approach could be envisaged in the future to determine the respective roles of σ1 and μ1 amino acids substitution of VeroAV in increased infectivity in Vero cells and increased sensitivity to interferon. However, it cannot be excluded that these properties cannot be separately attributed to one of the two genes and that single amino acids substitutions will not be compatible in the wild type virus genetic background, especially that encoded by the plasmids of the reverse genetics systems.

Altogether, the work described herein is a good illustration that reovirus infection and replication need to be further investigated in cell types that differ from the traditionally used models. Clearly, selection of novel viruses during viral persistence is more complex than selecting for σ3 proteins that are more susceptible to uncoating proteases. The use of heterogeneous viral populations that can be adapted to different cells or different conditions thus remains a powerful approach to identify novel viral adaptations that could be of interest in the development of reovirus as a virotherapy agent. Although we do not have evidence that this particular virus (VeroAV) has a superior oncolytic activity, it was recently shown (**Shmulevitz et al., 2012**) that selection of large-plaque variants on L929 cells, as observed with VeroAV on Vero cells, could generate better oncolytic viruses. It will thus be of interest

to pursue the characterization of VeroAV in cancer cell lines. In the future, long-term adaptations of reovirus to cells harboring well-defined genetic alterations in cellular immortalization/transformation pathways could become a powerful approach to better evaluate critical host-cell properties for optimal reovirus replication, propagation, and host-cell killing. In addition to such well-defined cellular models, human cancer cell lines could be used to select novel reovirus for an optimal oncolytic effect in different tumor cell types.

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Figure Legends

Fig. 1. Plaque formation by VeroAV. Plaque assay was performed on Vero cells in the presence (Cht.) or absence (Cont.) of chymotrypsin, as described in Section 2. (A) A single well presenting well-separated plaques, in the presence or absence of chymotrypsin using the same amount of virus inoculum, are presented for the wild-type virus and VeroAV. Cell monolayers were fixed and stained at either 5 days post-infection in the absence of chymotrypsin or 4 days post-infection when chymotrypsin was present. Arrows point to representative plaques observed for the wild-type virus in the presence of chymotrypsin and for VeroAV in absence and presence of chymotrypsin. (B) Closer examination of stained plaques was done under an inverted microscope using a low magnification, 4X, objective; arrows point to representative plaques in panel a and d. Bar is 0.5 mm.

Fig.2. Efficiency of infection by VeroAV determined by FACS analysis. Vero cells (panel A and B) or L929 cells (panel C) were infected at a MOI of 5 with either wild-type virus (WT) or VeroAV, as indicated. The cells were recovered 24 h post-infection and analyzed by FACS for detection of intracellular reovirus antigens, as described in Section 2. Mock-infected cells were similarly processed and incubated with the fluorescent antibody (filled in pale grey). In panel B, chymotrypsin was added for infection of Vero cells, as described in Section 2.

Fig.3. Synthesis of viral protein in VeroAV-infected cells. Vero cells or L929 cells were infected with either wild-type virus (WT) or VeroAV at a MOI of 5, as indicated. Proteins were recovered at 24 h post-infection and analyzed for the presence of viral proteins by immunoblotting, as described in Section 2; positions of the two major outer capsid proteins, σ3 and μ1C, are indicated.

Fig.4. Production of infectious virus in VeroAV-infected cells. Vero cells were infected with either wild-type or VeroAV at a MOI of 5 in the presence (dark bar) or absence of 20 μg/ml of chymotrypsin, frozen 24 hours post-infection and virus titers were determined (panel A). In panel B, the same experiment was performed except that medium was changed 4 hours post-adsorption to remove chymotrypsin and heat-inactivated fetal bovine serum was added.

Fig.5. Sensitivity of VeroAV to lysosomal protease inhibitor. L929 cells were infected with either wild-

type or VeroAV at a MOI of 2 in the absence or presence of E64 at a concentration of 50 or 100 μM, as indicated. Infected cells were frozen 24 hours post-infection and virus titers were determined. Results are presented as the average of two separate experiments, error bars represent standard error of the mean.

Fig.6. Sensitivity of VeroAV to interferon. L929 cells were used to titer the wild-type parental reovirus, VeroAV or the interferon-hypersensitive P4L-12 isolate, using serial tenfold dilution in the $TCID_{50}$ method, in the absence or presence of murine β-interferon at 100 international units(IU)/ml, as described in Section 2.

Fig.7. Positions of amino acids substitutions on μ1 structure. The structure the two adjacent μ1 trimers is presented from below with arrows pointing at the position of E89 and A114 amino acids harboring substitutions on VeroAV; figure adapted from **Zhang et al., 2005**, with permission from Elsevier.

Table 1

Amino acids substitutions in the three outer capsid proteins observed in viruses obtained from different persistently-infected cell lines.

	σ1	σ3	μ1
Vero[1]	Q78P; N198K	None	E89G; A114V
L929[2]	Many	Y354H	Not determined
HT1080[3]	L116P; V127A; Q251stop	S177F; H251L	Not determined
Raji[3]	None	H251L	Not determined
CA46[3]	K26T	H230Y; S233L; N353Q; Y354H	Not determined
SC1[4]	None	S177F	None
CT26[4]	K26T	None	E73D; L457F

Amino acids substitutions observed in viruses recovered from different persistently infected cell lines as described in the text. [1]This study. [2]Dermody, 1998. [3]Kim et al., 2011. [4]Unpublished data.

Table 2

Amino acids substitutions in the µ1 protein of Vero-AV and ten independent clones obtained from PCR-amplified fragment

	81	87	89	114	120	21
Wild-type	N	P	E	A	F	M
Vero-AV	N	P	G	V	F	M
Clone 1	N	P	G	V	F	M
Clone 2	N	S	E	V	F	T
Clone 3	D	P	G	A	F	M
Clone 4	N	P	G	V	S	M
Clone 5	N	P	G	V	F	M
Clone 6	N	P	G	V	F	M
Clone 7	N	S	E	A	F	M
Clone 8	N	P	G	V	F	M
Clone 9	N	P	G	V	F	M
Clone 10	N	P	G	V	F	M

Figure 1

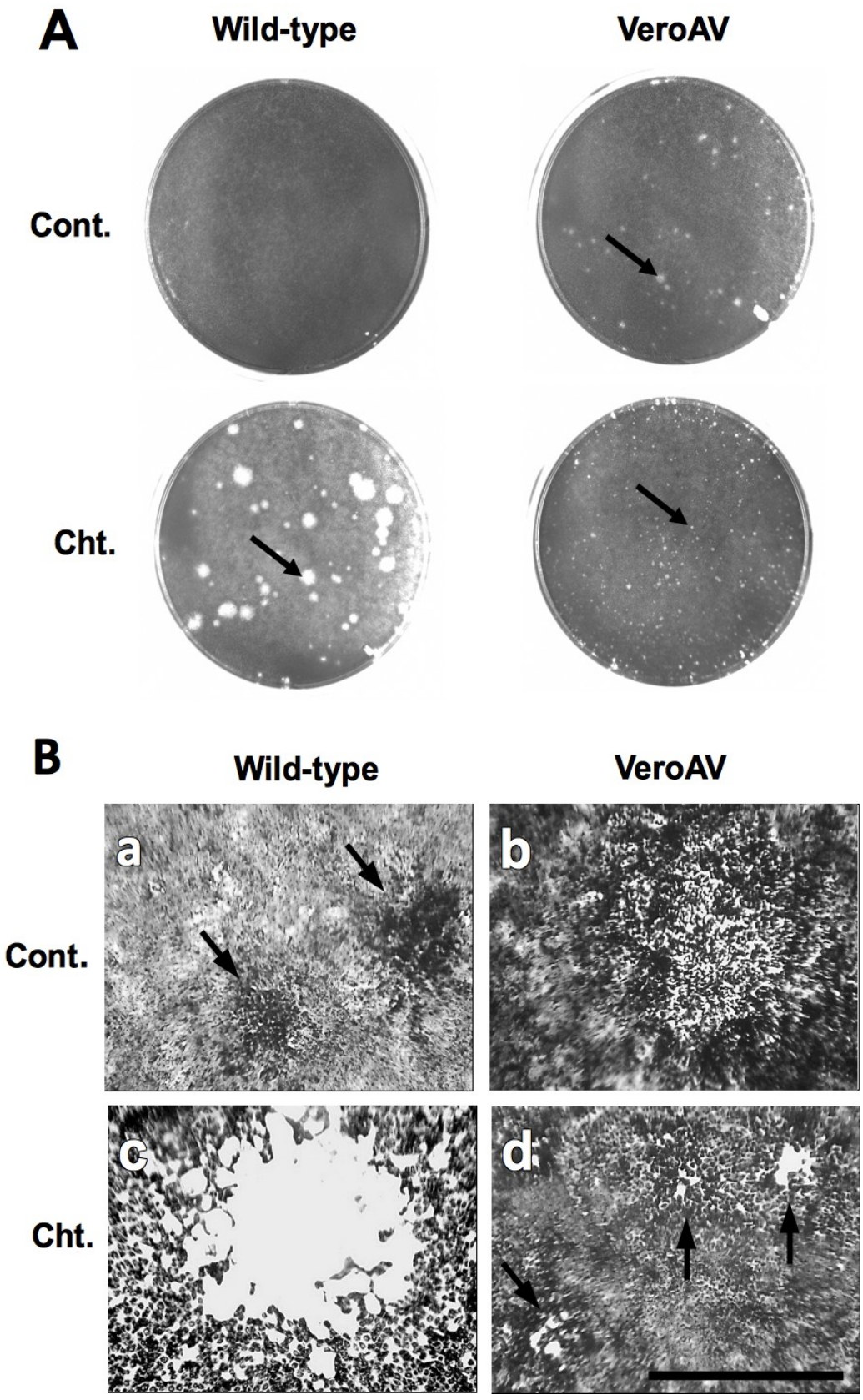

Figure 2

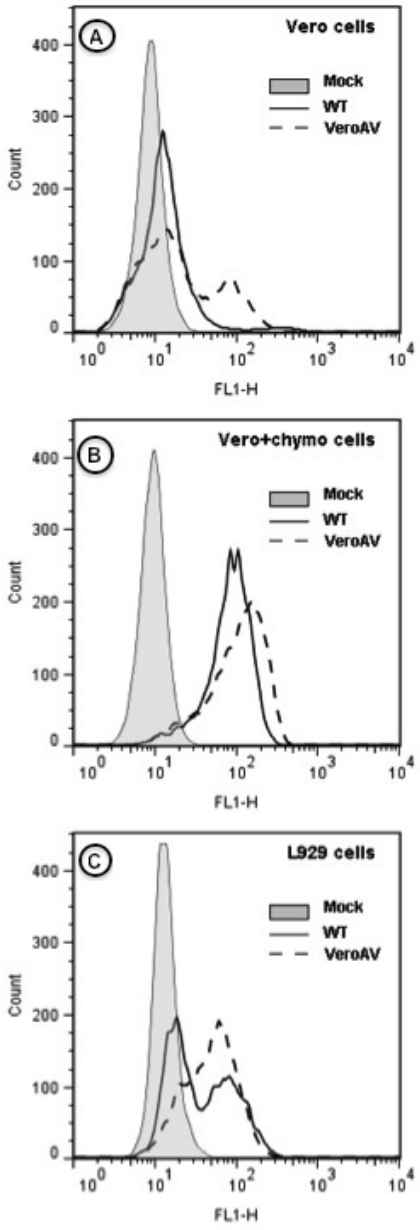
A
Vero cells
Mock
WT
VeroAV
Count
FL1-H
B
Vero+chymo cells
Mock
WT
VeroAV
Count
FL1-H
C
L929 cells
Mock
WT
VeroAV
Count
FL1-H

Figure 3

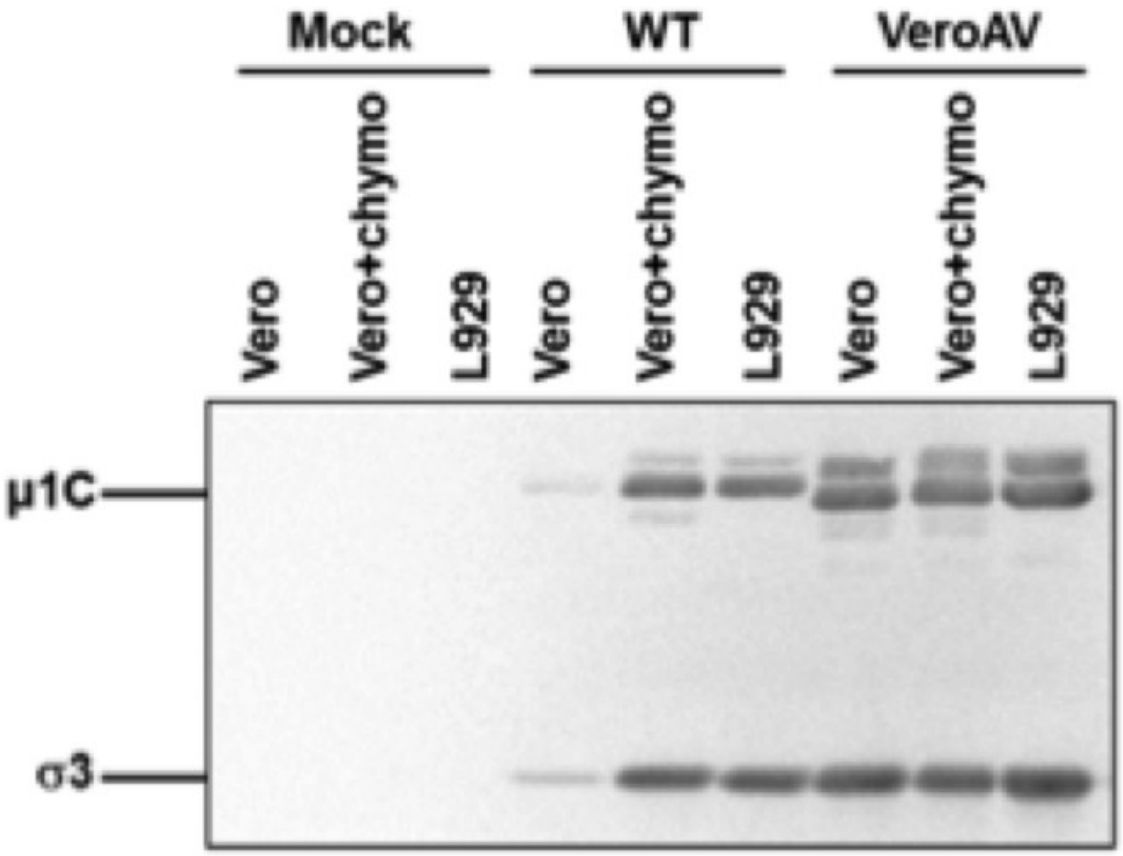
Mock
WT
VeroAV
Vero
Vero+chymo
L929
Vero
Vero+chymo
L929
Vero
Vero+chymo
L929
μ1C
σ3

Figure 4

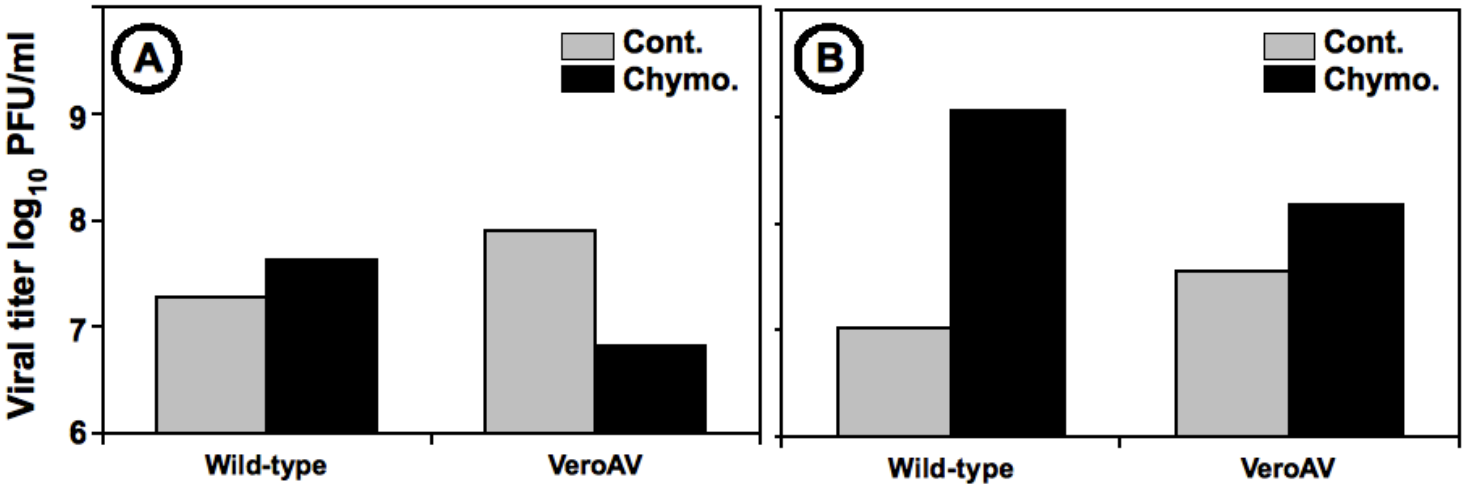
A
Cont.
Chymo.
Viral titer log10 PFU/ml
9
8
7
6
Wild-type
VeroAV
B
Cont.
Chymo.
Wild-type
VeroAV

Figure 5

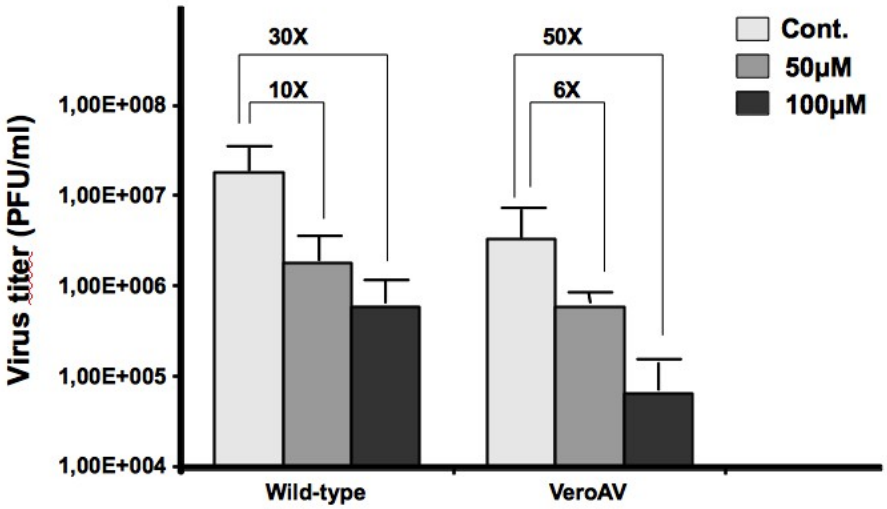
30X
10X
50X
6X
Cont.
50μM
100μM
Virus titer (PFU/ml)
1,00E+008
1,00E+007
1,00E+006
1,00E+005
1,00E+004
Wild-type
VeroAV

Figure 6

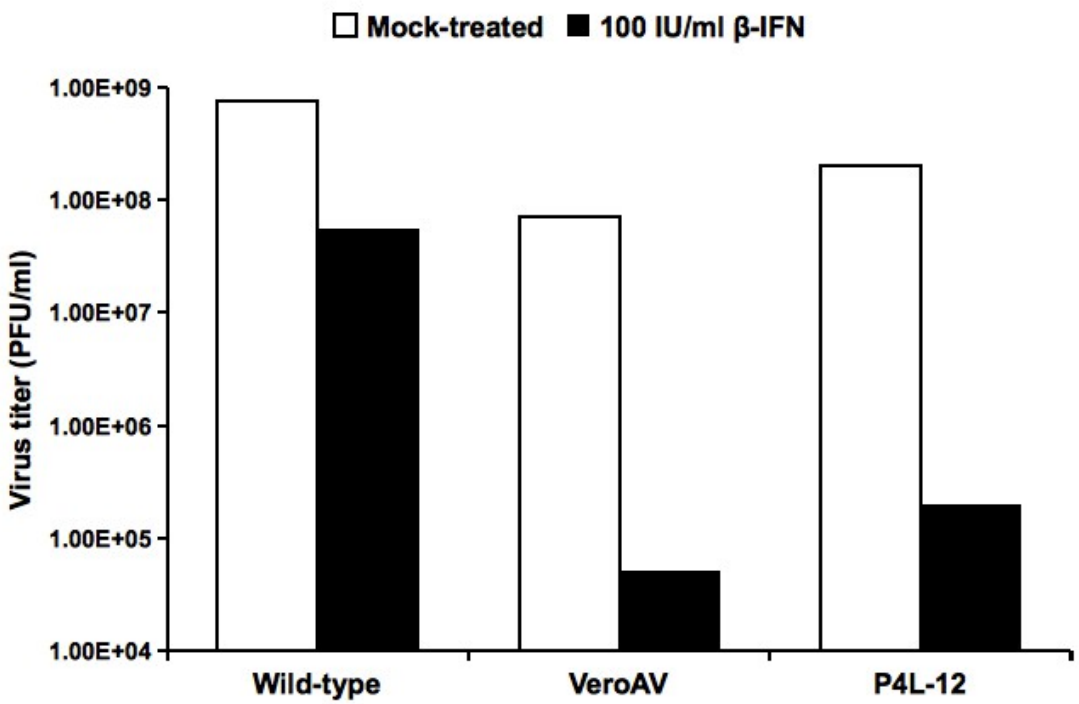
Mock-treated
100 IU/ml β-IFN
Virus titer (PFU/ml)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Wild-type
VeroAV
P4L-12

Figure 7