Addition of exogenous polypeptides on the mammalian reovirus outer capsid using reverse genetics

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ABSTRACT

Addition of exogenous peptide sequences on viral capsids is a powerful approach to study the process of viral infection or to retarget viruses toward defined cell types. Until recently, it was not possible to manipulate the genome of mammalian reovirus and this was an obstacle to the addition of exogenous sequence tags onto the capsid of a replicating virus. This obstacle has now been overcome by the advent of the plasmid-based reverse genetics system. In the present study, reverse genetics was used to introduce different exogenous peptides, up to 40 amino acids long, at the carboxyl-terminal end of the σ1 outer capsid protein. The tagged viruses obtained were infectious, produce plaques of similar size, and could be easily propagated at hight titers. However, attempts to introduce a 750 nucleotides-long sequence failed, even when it was added after the stop codon, suggesting a possible size limitation at the nucleic acid level.

Keywords: Reovirus; Reverse genetics; Tag; Epitope

1. Introduction

Mammalian reovirus under the trademark “Reolysin®” (derived from reovirus serotype 3 Dearing: T3/Human/Ohio/Dearing/55) is presently under study as an “oncolytic” virus in various clinical assays for the treatment of human cancers (recently reviewed in: (Kelly et al., 2009; Lal et al., 2009; Thirukkumaran and Morris, 2009; Harrington et al., 2010). Reovirus, is among the few viruses that can be used as an oncolytic agent without prior genetic engineering. Reovirus is only weakly pathogenic in human adults and its replication is blocked in “normal” cells while it is able to replicate in, propagate, and destroy transformed/cancer cells; this is apparently due to the presence of an activated Ras oncogenic pathway in these cells. Direct or indirect activation of the Ras signalling pathway appears to increase viral uncoating, repress innate immunity and/or augment viral-induced apoptosis, resulting in the natural oncolytic activity of the virus (Alain et al., 2007; Marcato et al., 2007; Rudd and Lemay, 2005; Shmulevitz et al., 2010; Smakman et al., 2005; Strong et al., 1998).

The reovirus capsid is made of two concentric layers of proteins; the trimeric σ1 protein is exposed at the surface of the virion and binds onto target cells; the carboxyl-terminal globular domain being most exposed and responsible for high-affinity binding to the main cellular receptor, JAM-A. Reovirus virions enter cells by endocytosis and are partially uncoated before being released to the cytoplasm; in contrast, extracellular proteases can partially uncoat the virions to infectious subviral particles (ISVPs) that still harbor σ1 at their surface but that directly cross the plasma membrane to enter the cytoplasm where viral replication ensues (Danthi et al., 2010).

Despite its natural oncolytic ability, it is likely that reovirus binding onto normal cells via ubiquitous receptors such as sialic acid, JAM-A, and other unidentified receptor(s) (Antar et al., 2009; Coyne, 2009) will limit efficiency of the virus to reach some target cells in the

organism. There are also some evidence to suggest that the virus receptors on the cell surface are either absent, or “masked” in some in some human cancer cell types, such as colorectal cancer cells (van Houdt et al., 2008). Finally, even though the virus appears to be only weakly pathogenic in humans, it cannot be excluded totally that some individuals, especially younger or immunodeficient patients, could develop disease due to reovirus inoculation, especially after systemic (intravenous) administration. Although its involvement in human biliary atresia of the newborn remains to be proven more conclusively, a significantly higher prevalence of reovirus RNA was detected in hepatic tissues from diseased individuals compared to normal controls (Tyler et al., 1998). Given strains of reovirus were also shown to induce liver pathologies, that are reminiscent of biliary atresia, in a murine experimental model (Parashar, Tarlow, and McCrae, 1992; Forrest and Dermody, 2003). The virus was also shown to induce “black foot syndrome” in immunodeficient SCID/NOD mice (Loken et al., 2004). There are thus possibly important benefits in the development of reovirus strains that are either attenuated (Kim et al., 2011) or targeted to bind specifically onto given cancer cell types, as proposed by others (Van Den Wollenberg et al., 2009). This last approach is currently under study for various oncolytic viruses such as adenovirus, measles virus, and herpesvirus, among others (Blechacz and Russell, 2008; Mathis, Stoff-Khalili, and Curiel, 2005; Campadelli-Fiume et al., 2011).

Until recently, it was not possible to modify the genome of reovirus, using the so-called “reverse genetics” approach that has been so widely used to study viruses belonging to most virus families. Nevertheless, a small 6-amino acids long peptide was added to the extreme carboxyl-terminal end of σ1 using a complex experimental approach in which cells are transfected with the plasmid encoding the tagged protein and then infected with a wild type virus to select recombinant viruses (van den Wollenberg et al., 2008). This strategy appears quite complex and requires an adequate cell line for the selection procedure.

A plasmid-based reverse genetics system was more recently described for mammalian reovirus by the group of Dr Terence Dermody (Kobayashi et al., 2007; reviewed by: Lemay, 2011); this novel approach was used by this group to study various reovirus mutants obtained by site-directed mutagenesis (see for example: (Boehme, Guglielmi, and Dermody, 2009; Danthi et al., 2008a; Danthi et al., 2008b; Kobayashi et al., 2007; Kobayashi et al., 2009)), or to substitute a whole gene segment from one reovirus serotype to the other (Kobayashi et al., 2007; Zurney et al., 2009).

In the present study, the usefulness of the plasmid-based reverse genetics approach was further established by adding different epitope tags at the carboxyl-terminal end of σ1. Viruses harbouring up to a 40 amino acids long peptide were readily recovered. However, infectious viruses could not be recovered when the sequence encoding the yellow fluorescent protein was fused to σ1, even when a stop codon was introduced to avoid the production of a fusion protein. This suggests the possibly of a size limitation of exogenous sequences that can be added at the nucleic acid level, independently of protein structure or function.

2. Materials and methods

2.1. Cells and viruses

L929 cells and Vero cells were originally obtained from the American Type Culture Collection and were used for reovirus propagation and titration; BHK21 cells stably expressing the T7 RNA polymerase (Buchholz, Finke, and Conzelmann, 1999) were a generous gift from the laboratory of Dr John Hiscott (Lady Davis Research Institute, Montréal, Canada). All these cells were grown in minimal Eagle medium (MEM; Wisent) containing 5% fetal bovine serum (FBS Gold, PAA laboratories).

2.2. Antibodies

Hybridoma cell lines producing either anti-σ3 (4F2) or anti-μ1 (10F6) have been described before (Virgin et al., 1991) and were obtained from Dr Kevin Coombs (Department of Microbiology, Manitoba University). These cells were grown in MEM for suspension culture with 10% fetal bovine serum, proline (20μg/ml) and β-mercaptoethanol (50μM); they were fed each 2 or 3 days to obtain cell concentration between 2 X 10^5 and 5 X 10^5 per ml, they were resuspended in fresh medium at a concentration of 5 X 10^5 cells per ml and left until most cells were dead as judged by trypan blue exclusion; cells and debris were removed by centrifugation at low speed and supernatants containing the monoclonal antibodies were kept at 4 ºC with 1mM sodium azide as preservative. The polyclonal antiserum directed against the carboxyl-terminal head domain of σ1 was produced originally in the laboratory of Dr Terence Dermody (Vanderbilt University, Tennessee) and was a generous gift from Dr Earl Brown (University of Ottawa). The mouse anti-polyhistidine monoclonal antibody was obtained from Qiagen (Qiagen, Mississauga, Ont., mouse Penta-His antibody, Catalog #34660). The mouse anti-HA tag (12CA5) was from Berkeley Antibody Company (BabCO) and a generous gift

from the laboratory of Dr Pierre Belhumeur (Département de microbiologie et immunologie, Université de Montréal). The rabbit anti-tubulin was obtained from ICN Biomedicals Inc.

2.3. Plasmid constructs

The plasmids harbouring separately each of the cDNA corresponding to the 10 genes of reovirus serotype 3 Dearing, under the transcriptional control of the T7 promoter, have been described (Kobayashi et al., 2007) and were obtained from the laboratory of Dr Terence Dermody (Vanderbilt University, Nashville, Tennessee). Medium scale plasmid DNA was column-purified using Qiagen plasmid midi kit and endotoxin-free buffers (Catalog #12243 and #19048), as recommended by the manufacturer. Concentration and purity were determined by measuring optical density using a nanodrop microspectrophotometer (ND-1000, Thermo Scientific). To insert sequence encoding for the hexahistidine or single HA tag at the end of the σ1-encoding sequence, the corresponding plasmid was submitted to site-directed mutagenesis using the QuickChange® Lightning site-directed mutagenesis kit (Stratagene, Catalog #200518), as recommended by the manufacturer. In an additional construct, a unique *Spe*I site was introduced following the hexahistidine tag, just before the stop codon, thus adding two amino acids to the protein (a threonine and a serine); this was also done by site-directed mutagenesis using the plasmid encoding hexahistidine-tagged σ1. This same plasmid was used to introduce 3 additional copies of the HA tag. The pMPY-3xHA plasmid (Schneider et al., 1995) was used as a template for PCR amplification of the 3HA-encoding region using oligonucleotides designed to introduce the *Spe*I site at both ends of the PCR product that was cloned thereafter at the *Spe*I site following the hexahistidine tag. The sequence encoding the enhanced yellow fluorescent protein was amplified from the pcDNA.1.eYFP.MCS(MB) plasmid (a generous gift from Dr Jacques Thibodeau) and also subcloned at the *SpeI* site. In a final construct, a stop codon was introduced by site-directed

mutagenesis 3 amino acids after the beginning of the YFP-encoding sequence. The presence of the expected mutations or insertions, and absence of other changes in the adjacent region,was verified by sequencing the final plasmids on small-scale preparation of endotoxin-free plasmid DNA that was column-purified, as recommended by the manufacturer (Zyppy plasmid miniprep kit, Zymo Research). The different oligonucleotides used as primers for mutagenesis, PCR and sequencing are presented in Table I.

2.4. Recovery of infectious viruses by plasmid-based reverse genetics

Recovery of infectious reovirus stocks was done using a modification of the original procedure (Kobayashi et al., 2007). The nine wild-type plasmids, prepared as medium scale preparation, were simultaneously introduced with the plasmid encoding the tagged σ1; transfection was done using Fugene 6 (Roche), as recommended by the manufacturer. Approximately 0.10 μg of each plasmid was used to transfect semi-confluent 35mm-diameter petri dish of BHK21 cells that express the T7 RNA polymerase. The medium was recovered 3-4 days later, cells were trypsinized and plated in a 100mm-diameter petri dish with the medium recovered previously and completed with 9ml of complete medium containing 5% heat-inactivated fetal bovine serum. Two-third of the medium was replaced with fresh medium, 3 days later, and incubated for another 4 days before being frozen at -80 ºC and used as starting virus stocks. Virus clones were recovered from single plaques obtained on Vero cells in the presence of chymotrypsin; plaques were visualized directly by phase contrast microscopy without staining. The clones were amplified on Vero cells in the presence of chymotrypsin, titrated, and further propagated on L929 cells at an approximate MOI of one PFU/cell before being titrated again.

2.5. Plaque assay

Plaque assay was done using a modification of standard procedures. Briefly, Vero cells were plated in 6-wells plates, 24 before infection, in order to obtain confluent monolayers at the time of infection. After medium removal and washing with serum-free medium, serial tenfold dilutions of the virus samples were applied in minimal volume (250µl) in each well. Cells were left at 4 ºC for one hour with gentle agitation each 15 minutes. Plates were briefly warmed at 37 ºC; 2.5ml of 1% agar (bacto-agar, Difco) in serum-free medium 199 (prepared by mixing a 2% sterile agar solution with medium prepared at twice the normal concentration) was added to each well. The sterilized 2% agar solution was melted before in a microwave oven and kept at 45 ºC and mixed with the medium that was incubated at the same temperature before addition of 10µg/ml of chymotrypsin (Sigma Sigma Type I-S from bovine pancreas; prepared at 10mg/ml in 1mM HCl and kept frozen in aliquots). After the agar overlay has hardened (a few minutes at room temperature), plates were incubated for different periods of time (4 to 5 days in routine titration assay). The cells were fixed by adding 2ml of 10% formaldehyde (in PBS) to each of the wells. After one hour at room temperature, the formaldehyde solution and the agar overlay were removed before adding 1ml of 1% crystal violet (in 70% methanol). After 60 minutes, the staining solution was removed and the plates were washed in tap water before being air-dried. Plaque formation for recovery of infectious virus was performed similarly except that the plaques were identified directly by phase contrast microscopy using an inverted microscope. Individual plaques were picked with 1ml sterile pipette tips, transferred in 1ml of sterile medium and virus left to diffuse for at least 24 hours at 4ºC.

2.6. Sequencing of S1 viral genome segment

To verify that the sequences added were actually conserved in the final virus stocks obtained

by reverse genetics, sequencing was done by RT-PCR amplification on the viral genome followed by direct sequencing of the PCR product. Virus stocks were used to infect L929 cells in 10 cm-diameter petri dishes at a MOI of 1 PFU/cell. Cells and medium were recovered at 24-30 hours post-infection, at which time most cells were lysed. Following two cycles of freeze-thaw (-80 ºC 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 15 min of centrifugation at 7,500 rpm at 4 ºC in a Sorvall SS34 rotor (in Corex 15ml tubes), supernatant was recovered and overlayed on a 1ml cesium chloride cushion at a density of 1.3g/ml for ultracentrifugation in a 70.1Ti rotor at 50,000 rpm for one hour at 4 ºC. Virus pellet was recovered in 400µl of TEN buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 250mM NaCl) before addition of 0.25% SDS followed by phenol-chloroform extraction and ethanol precipitation of viral double-stranded RNA. The RNA was denatured at 95 ºC for 5 min and cooled rapidly on ice. Reverse transcription on both strand of the double-stranded RNA was done with MLV reverse transcriptase, as recommended by the manufacturer (Roche) using one-fourth of the RNA and oligonucleotides specific for each strand at the ends of the gene (Table I). One-fifth of the reverse transcriptase reaction was then used in a PCR reaction with the same two oligonucleotides for 35 cycles using the FastStart protocol (Roche). The PCR fragment was then purified using Qiaquick PCR purification kit (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.

2.7. Immunoblotting

Infected cells were recovered by scraping in 500µl of medium and centrifuged in an Eppendorf tube at 1000 rpm for 30 seconds at 4 ºC. Cell pellets corresponding to a 60mm-

diameter petri dish were resuspended in 100µl of Tris-HCl 10mM pH 7.5, 1mM EDTA, 150mM NaCl, 1% Nonidet P-40 and left on ice for 10 minutes before centrifugation at maximum speed for 5 minutes in an Eppendorf centrifuge at 4 ºC. Proteins were analyzed by SDS-PAGE and immunoblotting. Nitrocellulose membrane was blocked with 2% non-fat dry milk (Biorad) in TBS (Tris-HCl 10mM pH 7.5, 150mM NaCl) and sequentially incubated for one hour at room temperature with the primary antibody. When using monoclonal antibodies from tissue culture medium, these 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 1mM sodium azide. Other primary antibodies were diluted in PBS containing 1% bovine serum albumin and sodium azide at either a 1/500 dilution for the anti-σ1 antibody or at the dilution recommended by the manufacturer for the other antibodies. Membranes were washed in TBS containing 1% Tween-20. Revelation was done using peroxydase-conjugated secondary antibody and chemiluminescent substrate as recommended by the manufacturer (Pierce SuperSignal West Dura Extended Duration Substrate). Images were obtained on Kodak BioMax Light Film.

3. Results

3.1 Rationale

As a proof of principle for the utilisation of the plasmid-based reverse genetics approach for addition of exogenous epitope to the reovirus outer capsid, two different small epitopes were first examined for their ability to be added at the carboxyl-terminal end of σ1. This position was chosen since, in one study, an hexahistidine tag was already reported to be functional at this position (van den Wollenberg et al., 2008). Also, when a chimeric protein between the

adenovirus fiber protein and σ1 is added to adenovirus virions, the presence of both hexahistidine and myc tag at its carboxyl-terminal end still allowed cell binding by the σ1 moiety (Mercier et al., 2004; Schagen et al., 2006; Tsuruta et al., 2005; 2007). In the present study, the hexahistidine epitope (6H) was first used and, as a further proof that different epitopes can be accommodated at this position, the more complex HA epitope, YPYDVPDYA (Kolodziej and Young, 1991; Wilson et al., 1984), was examined in parallel (Fig. 1A).

Analysis of the σ1 crystallographic structure (Chappell et al., 2002)(PDB 1KKE) revealed that the peptide epitope should be presented at the external surface of each molecule of the trimeric structure (Fig. 1B), at the basis of the globular structure involved in binding to cellular protein JAM-A, acting as the main receptor for the virus (Fig 1C) (reviewed in: (Schelling et al., 2008)). However, the epitope will be located away from the known region of interaction between σ1 and JAM-A (Kirchner et al., 2008)(PDB 3EOY) and should not preclude this interaction.

3.2. Introduction of an epitope-encoding sequences before the stop codon in the S1 gene

To insert tag-encoding sequence in the σ1-encoding gene, the corresponding plasmid harbouring the type3 Dearing S1 gene under the control of the T7 polymerase was directly submitted to insertional site-directed mutagenesis, as described in Materials and methods. Infectious virus was recovered using transfection of BHK cells expressing the T7 RNA polymerase (Buchholz et al., 1999). These cells were used, as also described by others (Kobayashi et al., 2010), in replacement of a vaccinia virus vector expressing the T7 polymerase originally used for plasmid-based reovirus reverse genetics (Kobayashi et al., 2007). Viruses were recovered from single plaques and propagated, as described in Materials and methods.

The plaque assay procedure, modified to include chymotrypsin in the agar overlay was

adapted to Vero cells. This facilitated the recovery of the plaques that were readily visualized on these cells by direct visual inspection or by phase contrast microscopy. However, the addition of chymotrypsin was necessary to obtain visible plaques (data not shown), consistent with previous report that proteolytic uncoating is a limiting step for reovirus infection in these cells (Golden et al., 2002). The Vero cells could eventually be an useful alternative to L929 cells to recover certain virus mutants since these cells are defective in interferon production (Desmyter et al., 1968; Emeny and Morgan, 1979).

As shown in figure 2, plaque size was comparable at either 2 or 4 days post-infection with either viruses. Only small plaques were observed at 48 hours for all viruses, although foci of infected cells could be detected by phase-contrast microscopy (data not shown). At 96 hours, plaques were readily visualized in all cases, even before staining. Final plaque size was apparently slightly increased for the HA-tagged virus, but the significance of this observation was not further examined.

3.3. Sequencing the S1 gene of viral clones harbouring exogenous epitope

The virus stocks obtained were then used to infect L929 cells and genomic RNA was extracted from viral particles. The nucleotide sequence of the gene was determined by RT-PCR to ensure that the sequence encoding the tag was maintained without any other changes to the coding sequence. Six different clones of the hexahistidine-tagged virus were examined and 5 different clones of the HA-tagged virus. In each case, the sequence encoding the tag was retained by the virus. Except in one virus with the hexahistidine tag, harboring a glutamine to leucine amino acid substitution at position 51, there was no other change compared to the initial sequence of the cloned S1 gene (data not shown).

3.4. Expression of tagged σ1 in infected cells

The virus stocks encoding either wild-type or tagged σ1 were then used to infect L929 cells and proteins were recovered for immunoblotting analysis. The two different tagged σ1 appeared to be accumulated to similar levels as the wild-type protein under these conditions, as judged by immunoblotting using a polyclonal antibody specific to the globular head of the protein (Fig. 3A). When monoclonal antibodies specific to either the hexahistidine or HA tag were used (Fig. 3B and 3C, as indicated), the σ1 protein was easily detected, only in cells infected with the respective virus; a small change in electrophoretic mobility was also noticed for the tagged proteins, as expected. Since there was no apparent secondary band of a mobility corresponding to the untagged protein, when the anti-σ1 polyclonal antibody was used, it is most likely that the tag is stably retained on all copies of the protein and that σ1 moieties incorporated to the viral particles are also harbouring the tag.

Immunoblotting of the same samples to detect the two major outer capsid proteins σ3 and μ1 revealed similar amount of these proteins (Fig. 3D), with a similar ratio of the different σ1 proteins compared to the other major outer capsid proteins.

3.5. Addition of longer peptide sequences at the carboxyl-terminal end of σ1

Having demonstrated that it is possible to introduce exogenous genetic material encoding an exogenous peptide fused to the carboxyl-terminal end of σ1, a plasmid construct was made that encodes the hexahistidine tag followed by a single *Spe*I site, in order to facilitate insertion of additional genetic material to determine the limitations in size and/or complexity that can be added at this position. A 96 nucleotide sequence, corresponding to three consecutive copies of the HA tag, separated by few amino acids (Fig. 1B) was recovered by PCR from an adequate plasmid and subcloned at the unique *Spe*I site, as described in Materials and Methods. Again, virus was easily recovered and forms plaques of similar size as

the virus harbouring a single copy of HA (Fig 2). Immunoblotting analysis confirmed the presence of both the hexahistidine and HA-tag on the protein (Fig. 3B and 3C) and similar levels of the σ1, σ3 and μ1 proteins (Fig. 3A and 3D).

In order to determine if very long sequences can be successfully added at the same position, the coding sequence for the enhanced yellow fluorescent protein (239 amino acids) was amplified by PCR from a plasmid construct and introduced at the *Spe*I site to make a fusion protein with σ1 (Fig. 1A). To verify if the efficiency of virus recovery will be affected if only the nucleotide sequence is present, without actual fusion at the protein level, this construct was then subjected to site-directed mutagenesis to introduce a premature stop codon 3 amino acids after the beginning of the YFP-encoding sequence (Fig. 1A). However, despite numerous attempts, it was not possible to recover viruses with either of these two plasmid constructs.

3.6. Further characterization of tagged viruses

In order to further establish the replicative potential of the tagged viruses, the kinetic of production of infectious virus following infection of L929 cells at a low multiplicity of infection was measured by viral titration (Fig. 4). All three tagged viruses behave similarly and were somewhat delayed compared to wild-type virus, although final titers were only reduced less than tenfold (Fig. 4 and data not shown). When virus binding at the cell surface was compared using a newly developed FACS-based assay, binding was reduced for all three tagged viruses, either as virions or ISVPs, compared to the wild type virus (data not shown). It was not established if this is due to reduced incorporation of tagged σ1 to the virions or reduced affinity of the σ1 for its cellular receptors due to partial interference by the tag.

4. Discussion

Before the present work was undertaken, is has been reported that a small hexahistidine tag could be added to the carboxyl-terminal end of σ1 (van den Wollenberg et al., 2008). In this previous study, "recombinant" viruses were selected for their ability to bind and replicate solely in cells engineered to express an artificial cell surface receptor made of a single-chain antibody against the tag. The mechanism allowing the recovery of the tagged encoding sequence in a viral RNA with authentic 5' and 3' untranslated regions remains unknown. In these viruses, additional amino acids changes were however "selected" in the tagged σ1 protein compared to the original protein but this was not observed in the present study using the more direct plasmid-based reverse genetics approach. The only other amino acid substitution obtained was found in only one randomly-picked plaques and close to the amino-terminal end, and thus far from the carboxy-terminal tag position. The requirement for a co-infecting virus in the previous approach suggest that the virus stock used contained viruses whose sequence differs from the consensus and this is later reflected in the sequence of viruses from randomly picked plaques. Clearly, when direct plasmid-based reverse genetics is used to incorporate the exogenous sequence, there is no requirement for such "second-site" mutations; furthermore, the reverse genetics approach is obviously more direct and does not require a special cell line for selecting the novel viruses.

The carboxyl-terminal end of σ1 thus appears ideally suited to pursue the idea of adding additional peptide at the surface of the reovirus particles. The question is not trivial since other attempts to add exogenous epitopes on other viral proteins had little success (Rouault and Lemay, 2003; Lemay, 2011; and unpublished data). The limits in length that could be accepted at the carboxyl-terminal end of σ1, while maintaining the infectious potential of the viral particle, remains to be determined; the longest tagged σ1 with its triple HA tag has a total of 40 additional amino acids (Fig. 1A). The construct encoding the

hexahistidine epitope followed by a unique *Spe*I restriction will facilitate further work to determine the limitations in size that can be added without affecting viral replication too stongly; the presence of the hexahistidine epitope could play the role of a linker to facilitate insertion of additional amino acids at this position. The addition of even the smallest tag does affect virus binding to a certain extent, although this remains acceptable for most applications; however, it may prove difficult to add much longer and complex peptides without further, and more drastic, consequences on virus infectivity.

In addition to possible consequences of amino acids addition on the protein itself, the importance of nucleotide sequence and structure at both termini are likely of importance in allowing transcription, replication and encapsidation of RNA genomic segments.

In conclusion, additional genetic material could be added to the genome of a replication-competent reovirus using plasmid-based reverse genetics, and up to at least 120 nucleotides can be added before the authentic 3' untranslated region of S1. In addition, the ability to modulate reovirus' ability to replicate in different cell types, by addition of specific microRNA targets, could thus be also envisaged for reovirus. This could add to the specificity of viral infection toward specific cell types and could make safer viruses for use as virotherapy agents, as currently investigated for other oncolytic viruses (reviewed in (Bell and Kirn, 2008; Kelly et al., 2008)). The plasmid-based reverse genetics for the addition of exogenous nucleic acids sequence to the viral genome and/or peptide sequences to viral proteins thus opens novel fields of investigation and applications for mammalian reoviruses as virotherapy agents.

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Figures legends

Fig. 1. Sequence and structure at the carboxyl-terminal end of tagged σ1 proteins. (A) Amino acids sequence at the carboxyl-terminal end of either the wild-type or the different tagged version of σ1 used in this manuscript. (B) Crystallographic structure (from PDB 1KKE) of the σ1 homotrimer viewed from the side with the arrow pointing at the position of the carboxyl-terminal amino acid illustrated as a sphere. (C) Crystallographic structure (from PDB 3EOY) of the complex between the σ1 homotrimer and the JAM-A cellular receptor viewed from the top of the structure with the arrow pointing at the position of the carboxyl-terminal amino acid illustrated as a sphere.

Fig.2. Plaque formation by the tagged viruses. Plaque assay was performed on Vero cells in the presence of chymotrypsin, as described in Materials and methods. A single well presenting well separated plaques are presented for the wild-type (wt), the hexahistidine-tagged (Reo.S1.6H), the HA-tagged (Reo.S1.HA) and the triple-HA tagged (Reo.S1.3HA) viruses. Cell monolayers were fixed and stained at either 48 or 96 hours post-infection, as described in Materials and Methods.

Fig. 3. Immunoblotting of viral proteins in cells infected with the tagged viral particles. Viral stocks of wild-type, 6H-tagged or HA-tagged were prepared and titrated as described in Materials and methods. Murine L929 cells were infected with these viruses at a multiplicity of infection of 2 PFU/cell. Proteins were recovered from infected cells 24 hours later and equivalent amounts were loaded on SDS-PAGE and analyzed by immunoblotting. (A) The polyclonal monospecific anti-σ1 head antibody, the monoclonal anti-HA antibody (B), or the monoclonal anti-hexahistidine antibody (C) were used, as indicated. (D) The membrane

previously probed with the polyclonal monospecific anti- σ1 head antibody was reprobed with monoclonal anti-μ1 antibody. (E) The membrane previously probed with the anti-HA antibody was reprobed with the anti-tubulin antibody.

Fig.4. Replication of tagged viruses. Wild-type or tagged viruses were used to infect L929 cells at a MOI of approximately 0.5 (upper panel) and 0.05 (lower panel) PFU/cell. Petri dishes of infected cells were recovered at 12, 24 or 48 hours post-infection and virus titration was performed by plaque assay, as described in Materials and Methods.

Table I

Use	Virus	Primer	Primer sequence*
Mutagenesis	REO.S1.6H	S1-6HIS	GCGTAGTTTCACGCATCACCATCACCATCACTGAGGATCAGACCAC
		S1-6HIS-AS	GTGGTCTGATCCTCAGTGATGGTGATGGTGATGCGTGAAACTACGC
Mutagenesis	Not determined	S1-SpeI	GCGTAGTTTCACGCATCACCATCACCATCACACTAGTTGAGGATCAGACCAC
		S1-SpeI-AS	GTGGTCTGATCCTCAACTAGTGTGATGGTGATGGTGATGCGTGAAACTACGC
Mutagenesis	Reo.S1.HA	S1-HA	GCGTAGTTTCACGTACCCATACGACGTCCCAGACTACGCTTGAGGATCAGACCAC
		S1-HA-AS	GTGGTCTGATCCTCAAGCGTAGTCTGGGACGTCGTATGGGTACGTGAAACTACGC
Mutagenesis	Negative	S1-YFP-STOP	CACACTAGTATGGTGAGCTAGGGCGAGGAGCTGTTC
		S1-YFP-STOP-AS	GAACAGCTCCTCGCCCTAGCTCACCATACTAGTGTG
PCR (3HA)	Reo.S1.3HA	S1-3HA-5'	CGGCCGCACTAGTTACCCATACG
		S1-3HA-3'	GGCCGCAACTAGTAGCGTAATCTGG
PCR (YFP)	Negative	YFP-Spe-5'	GCGCCGACTAGTATGGTGAGCAAGGGCGAGCTGTTC
		YFP-Spe-3'	CAGGACACTAGTTTACTTGTACAGCTCGTCCATGCCGAG
RT-PCR		S1(1-15)	GCTATTGGTCGGATG
		S1(1416-1394)	AGTGCCGCGGGGTGGTCTGATC
Sequencing	S1(1-15)	S1(1-15)	GCTATTGGTCGGATG
		S1(708-730)	GACTCTCAAGACGACTGTGTTTG
		S1(917-940)	ATGTTAGCGGCGGTATCGGAATGA

*The SpeI site is indicated in red; the sequence encoding the 6His tag is indicated in green; sequences in blue are complementary to YFP-encoding sequence while purple sequences are complementary to sequences encoding the HA epitope.

Figure 1

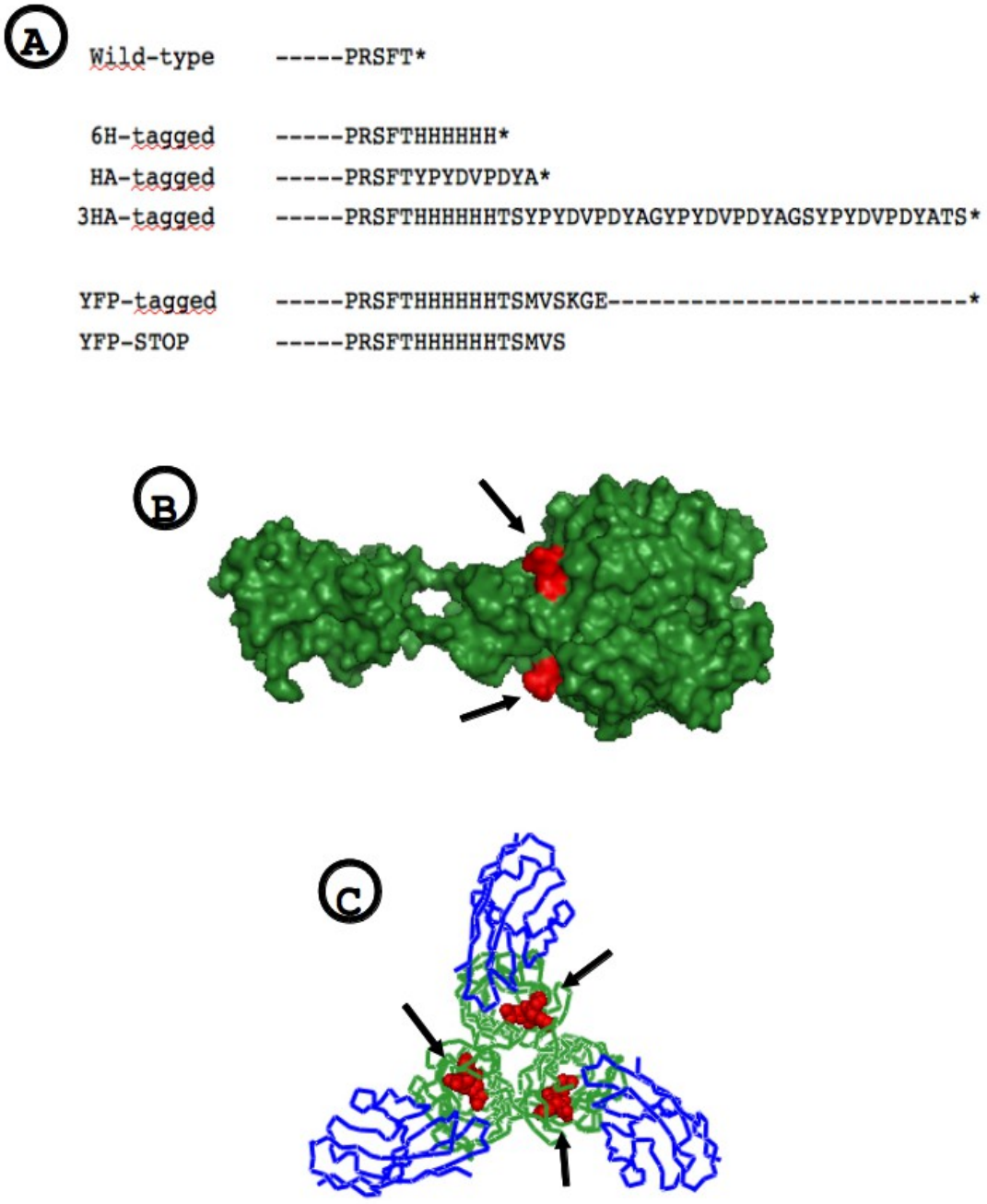

A
Wild-type -----PRSFT*
6H-tagged -----PRSFTHHHHHH*
HA-tagged -----PRSFTYPYDVPDYA*
3HA-tagged -----PRSFTHHHHHHTSYPYDVPDYAGYPYDVPDYAGSYPYDVPDYATS*
YFP-tagged -----PRSFTHHHHHHTSMVSKGE-------------------------*
YFP-STOP -----PRSFTHHHHHHTSMVS
B
C

Figure 2

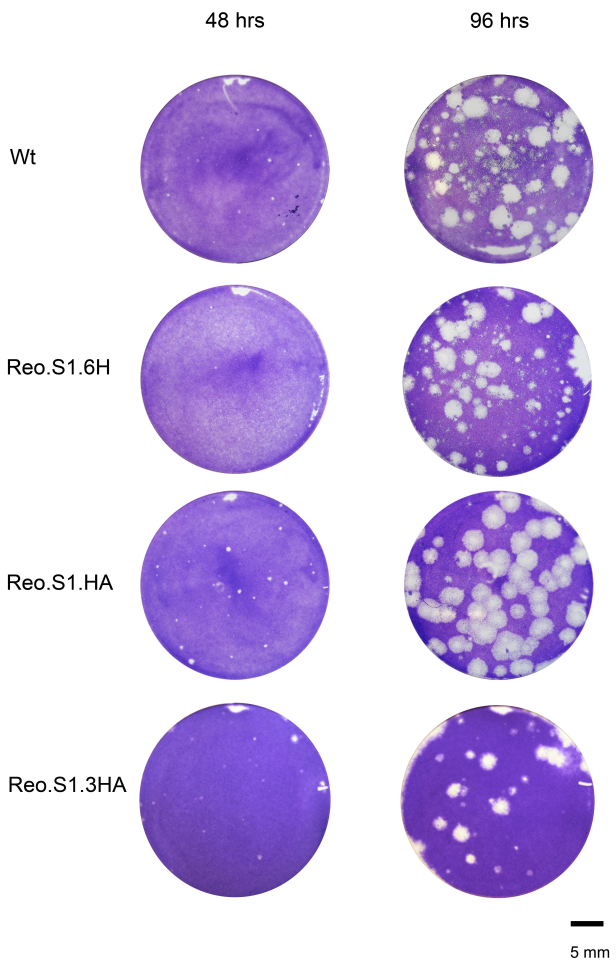
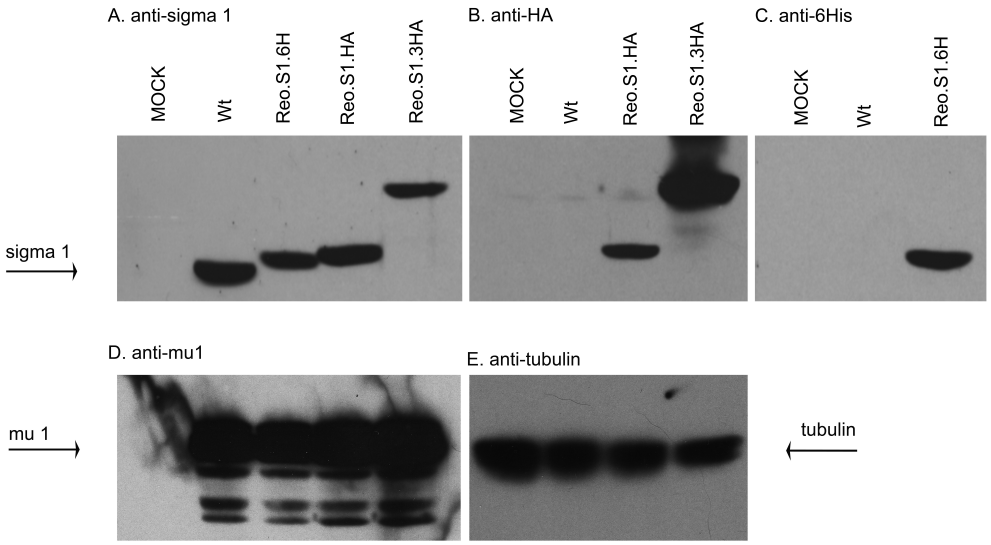


Figure 3


A. anti-sigma 1
MOCK
Wt
Reo.S1.6H
Reo.S1.HA
Reo.S1.3HA
B. anti-HA
MOCK
Wt
Reo.S1.HA
Reo.S1.3HA
C. anti-6His
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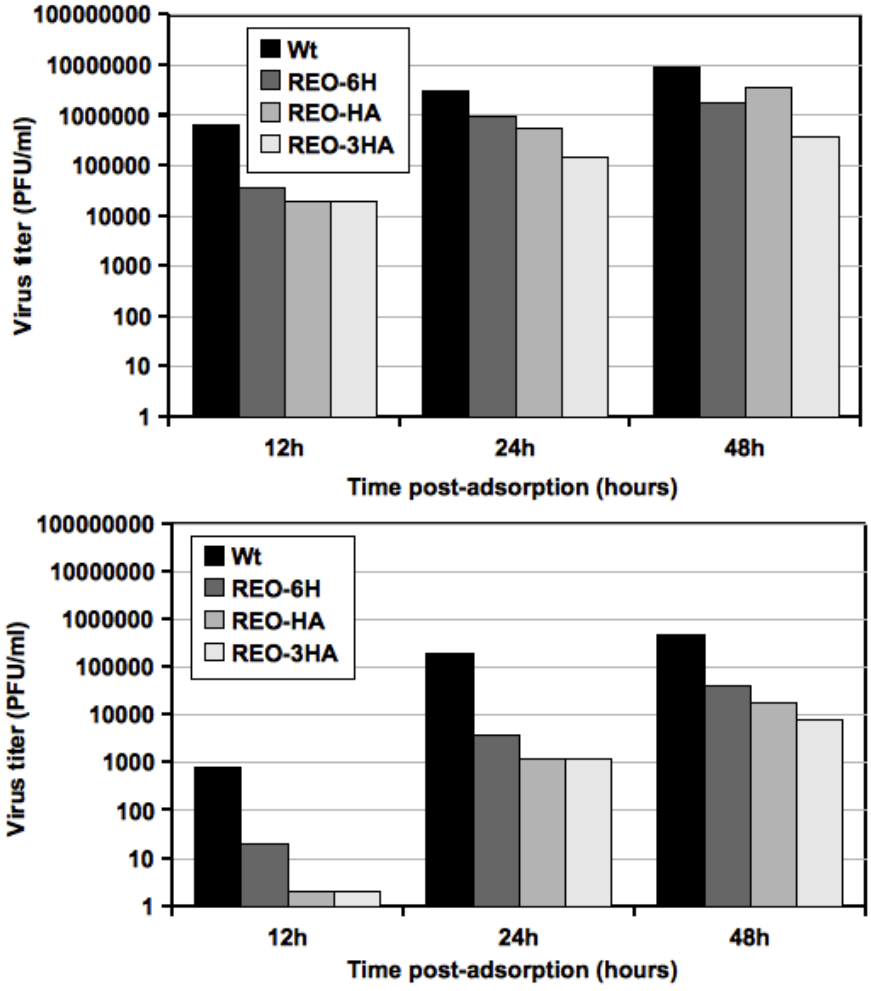
Wt
Reo.S1.6H
sigma 1
D. anti-mu1
E. anti-tubulin
mu 1
tubulin

Figure 4


100000000
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10
1
Virus titer (PFU/ml)
Wt
REO-6H
REO-HA
REO-3HA
12h
24h
48h
Time post-adsorption (hours)
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100000
10000
1000
100
10
1
Virus titer (PFU/ml)
Wt
REO-6H
REO-HA
REO-3HA
12h
24h
48h
Time post-adsorption (hours)