Development of measles virus-based shielded oncolytic vectors: suitability of other paramyxovirus glycoproteins

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INTRODUCTION

Several viruses, including Adeno-, Herpes-, Pox-, Reo- and Paramyxoviridae, are being tested in clinical trials for the treatment of various tumors,1,2 and an oncolytic adenovirus is currently an approved cancer therapeutic in China.3 However, the high prevalence of pre-existing antibodies against many vectors may reduce or eliminate their oncolytic efficacy. This issue has been addressed by exchanging the envelope, or capsid, of different viruses with those of non-crossreactive serotypes.4,5 Measles virus (MV), one of the most promising oncolytic viruses, is currently used in phase I clinical trials to treat glioblastoma multiforme, multiple myeloma and ovarian cancer.6–8 Unlike other viruses, such as vesicular stomatitis virus and adenoviruses, MV has a single serotype.9 Therefore, a novel serotype was engineered based on the glycoprotein complex (the fusion (F) and hemagglutinin (H) proteins) from a related but non-immunologically crossreactive Morbillivirus.10 This shielded MV remained oncolytic in an immunocompetent mouse model even in the presence of anti-MV neutralizing antibodies.10 However, antibodies against the new envelope of the shielded MV will eventually be generated.

To allow for sequential cycles of virotherapy, additional shielding envelopes will need to be developed. Here, we investigated the suitability of the envelope glycoproteins from the Tupaia paramyxovirus (TPMV),11,12 a close relative of the Morbillivirus genus. Although a direct exchange of the canine distemper virus and MV glycoproteins was possible,10 our initial attempts to rescue a hybrid MV with the TPMV glycoproteins were unsuccessful (C Springfield and R Cattaneo, unpublished). Knowing that efficient MV particle assembly depends on the interaction between the matrix protein and the cytoplasmic tails of the glycoproteins,13,14 and that the TPMV glycoprotein cytoplasmic tails are not homologous to the MV glycoprotein cytoplasmic tails, we then sought to generate hybrid glycoproteins. As the cytoplasmic tails of the TPMV glycoproteins are not characterized, we first generated truncation mutants and assessed fusion function. Based on these data, we generated hybrid glycoproteins with a TPMV ectodomain and the corresponding MV cytoplasmic tail. When associated with the wild-type partner, these hybrid glycoproteins retained high levels of fusion competency. However, in combination, hybrid F and H proteins no longer supported fusion.

On the other hand, the combination of a hybrid F protein and a cytoplasmic tail-truncated TPMV H protein sustained fusion function. A hybrid virus with these two proteins in place of MV F and H spread through cell–cell fusion, but failed to efficiently produce particles. We therefore sought to determine the factors influencing hybrid virus assembly by determining glycoprotein surface expression, transport and processing kinetics. We show that reduced hybrid F-protein processing and suboptimal transport of glycoproteins in the virus producer cell line contribute to inefficient particle formation.

MATERIALS AND METHODS

Antibodies

Rabbit anti-TPMV-Firts was raised against the peptide NH2-CELEMDKTQ-KALDRSNKLCOOH corresponding to amino acids 463–480 of the TPMV F protein (courtesy of C Springfield).15 Rabbit anti-TPMV-Hirts was raised against the keyhole limpet hemocyanin (KLH)-conjugated peptide.

References

1. Tupaia paramyxovirus...
NH₂-CSESTDHGDPGVGTEGTRNKHKG-COOH corresponding to amino acids 215–237 of the TPMV H protein. MV F and H proteins were detected using rabbit antibodies against parts of their cytoplasmic tails: F₃₄₁₀¹₆ and H₅₉.¹⁸ The anti-H606 antibody recognizes the KLH-conjugated peptide NH₂-CTVTREDGTNSR-COOH corresponding to the terminal 12 residues of the MV H protein. AlexaFluor 647 goat anti-mouse and AlexaFluor 546 goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) secondary antibodies were used for immunofluorescence. Penta-His AlexaFluor 647-conjugated mouse monoclonal antibody (Qiagen, Valencia, CA, USA) was used for the detection of six-histidine-tagged (6×His-tag) H proteins in confocal and fluorescence-activated cell sorter (FACS).

Plasmids

The plasmid pCG-RES2eo was used for the expression of all deletion and chimeric glycoproteins.¹⁵ The TPMV F and H cDNA were cloned from infected tupaia baby fibroblast cells using the following primers: BamHI-F (5′-AGCAGATCGCAGTATATCAGATCCGACTGCTA-3′) and PacI-TR (5′-AGCAGACGTATGTTAATATACTTATCTTGTA-3′). BamHI-H (5′-AGACGA ATGCAGATCCGACTGCTAATTCACACAC-3′) and H6-PacI-H (5′-AGCA GCATGTTAATATACTTATCTTGTA-3′) were used to replace the F and H open reading frames in the full-length MV F and H cDNA (Invitrogen). DNAs coding for hybrid glycoproteins were generated by inverse PCR (restriction sites are underlined) using the reverse transcriptase Superscript II (Invitrogen). DNAs, 2 µg per each, were labeled with Trans35S-label (100 µCi ml⁻¹) (Perkin-Elmer) in DMEM -cys/-met (1% HEPES, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8)) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem, Rockland, MA, USA). Samples were stained with the anti-GFP antibody, F-protein samples were denatured at 99 °C for 5 min, followed by boiling for 5 min. The samples were washed and dissociated by boiling for 5 min in 50 mM sodium citrate (pH 5.5). F-proteins or 500 µl RIPA buffer (for H proteins) supplemented with protease inhibitor (1 µg phenylmethylsulfonylfluoride and Protease Inhibitor Cocktail Set I). To expose the antigen recognized by the TPMV-F₆₃₀ antibody, F-protein samples were denatured at 99 °C for 5 min, followed by the addition of 900 µl of non-denaturing lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.5), 5 mM EDTA (pH 8)) for 1 h at 4 °C in 50 µl. Samples were then clarified by centrifugation at 21 000 g for 10 min at 4 °C. The lysates were freed of debris at 21 000 g for 15 min at 4 °C, and then cleared by centrifugation at 21 000 g for 20 min at 4 °C. The lysates were mixed with 2× SDS-PAGE loading buffer, boiled for 10 min, resolved by 10% SDS-PAGE, then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in 5% non-fat milk in TBST (100 mM Tris (pH 8), 150 mM NaCl, 0.05% Tween-20) and subjected to an antibody that recognized the N-terminus of the TPMV F protein. After washing, the membrane was incubated in the primary antibody, anti-mouse IgG (1:1000) (Santa Cruz, Santa Cruz, CA, USA) diluted in TBST, for 1 h at room temperature. The membrane was then washed and incubated in horse anti-mouse HRP secondary antibody (1:20 000) (Santa Cruz, Santa Cruz, CA, USA) diluted in TBST, for 1 h at room temperature. The membrane was washed and the bands were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). All filters were exposed to X-ray film (Kodak, Rochester, NY, USA) and the images were quantified using the Typhoon FLA 7000 phosphor imaging system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The results were analyzed by ImageQuant software (GE Healthcare, Waukesha, WI, USA).

FACS analysis

CHO-K1 cells transfected with pCG-MV-H₆₁₇ or pCG-TPMV-H₃₉₀ or pCG-TPMV-H₃₉₀ or the negative control plasmid pEGFP-N1. At 24 h post-transfection, the cells were washed once with PBS and detached in 500 µl Versene (Gibco, Grand Island, NY, USA) and washed with FACS buffer (PBS supplemented with 5% FCS and 0.1% NaN₃). Samples were stained with the anti-H-protein specific antibody Penta-His AlexaFluor 647 (Qiagen) for 1 h at 4 °C, washed with FACS buffer and fixed with 2% paraformaldehyde in PBS. Samples were then subjected to the suppression of auto-fluorescence using the antibodies indicated (GE Healthcare, Waukesha, WI, USA).

Metabolic labeling, immunoprecipitation and glycosidase digestion

CHO-K1 cells transfected with pCG-MV-H₆₁₇ or pCG-TPMV-H₃₉₀ or the negative control plasmid pEGFP-N1. At 24 h post-transfection, the cells were starved in cysteine- and methionine-deficient DMEM (Invitrogen; catalog number 21013-024) supplemented with sodium pyruvate, sodium borate and 1-glutamate (DMEM (–cys/–met) for 30 min, then labeled with Trans³⁵S (100 µCi ml⁻¹) (Perkin-Elmer) DMEM -cys/-met for 1 h at 37 °C and chased with unlabeled DMEM-10 for the times indicated. Cells were then washed with PBS and lysed with 100 µl denaturing lysis buffer (1% SDS, 50 mM Tris (pH 7.5), 5 mM EDTA (pH 8)) for 1 h at 4 °C. The lysates were centrifuged at 21 000 g for 20 min at 4 °C. The samples were then clarified by centrifugation at 21 000 g for 20 min at 4 °C. The lysates were mixed with 2× SDS-PAGE loading buffer, boiled for 10 min, resolved by 10% SDS-PAGE, then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in 5% non-fat milk in TBST (100 mM Tris (pH 8), 150 mM NaCl, 0.05% Tween-20) and subjected to an antibody that recognized the N-terminus of the TPMV F protein. After washing, the membrane was incubated in the primary antibody, anti-mouse IgG (1:1000) (Santa Cruz, Santa Cruz, CA, USA) diluted in TBST, for 1 h at room temperature. The membrane was washed and the bands were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) for 1 h at 4 °C. The lysates were then clarified by centrifugation at 21 000 g for 20 min at 4 °C. The lysates were mixed with 2× SDS-PAGE loading buffer, boiled for 10 min, resolved by 10% SDS-PAGE, then transferred to a nitrocellulose membrane.
proteins, we extended the TPMV H ectodomain with a 6 x His-tag. This His-tag allows us to utilize the Vero-zHis cell line, which expresses a pseudoreceptor recognizing it.23,28 These cells also support the rescue of retroretaped MV-based vectors.23

TPMV F protein with six amino-acid cytoplasmic tail maintains wild-type fusion function

The TPMV F-protein cytoplasmic tail is 38 amino acids in length, 5 amino acids longer than the MV F-tail, with no homology shared between the two F-tails. Towards generating a chimeric F glycoprotein, we first sought to define the minimal sequence necessary for TPMV-F fusion function. We coexpressed the F and H glycoproteins in Vero-zHis cells and observed syncytia formation (Figure 1a).28–30

When the TPMV F-tail was shortened in 10 amino-acid increments (Figure 1b; FΔ10–FΔ40), we observed that deletion of up to 30 amino acids had no effect on fusion function (Figure 1b; FΔ10, FΔ20 and FΔ30, fusion score, + + +). When 40 amino acids were removed from the F-tail, we observed complete loss of fusion function (Figure 1a; FΔ40, fusion score, –). The protein expression levels of the F-tail truncation mutants were analyzed by separating cell lysates from transfected Vero-zHis cells and immunoblotting with a TPMV F-ectodomain-specific antibody (Figure 2a; Endo H lanes). These analyses indicated that all the mutants were expressed at or near wild-type levels. Moreover, all mutant proteins retained proteolytic activation of the Fp precursor to the fusion competent Fp+ reduces Fp+ complex.

To define the minimal functional F-tail, we then shortened the F-tail in two amino-acid increments (Figure 1b; FΔ32, FΔ34, FΔ36 and FΔ38). As FΔ32 was the largest truncation to still maintain wild-type fusion function, we selected this mutant for the generation of a hybrid F protein. This protein consists of the TPMV ectodomain, transmembrane domain and six amino acids of the TPMV F-tail fused to the 33 amino-acid MV F-tail (Figure 2b; FΔ32/mvcyt). When this hybrid F protein was coexpressed with the standard TPMV H protein, it supported a slightly reduced level of fusion compared with the parental FΔ32 mutant (Figure 2b; fusion score, + + +).

TPMV H protein with four-residue cytoplasmic tail maintains wild-type levels of fusion helper function

The TPMV H cytoplasmic tail is predicted to be 94 residues in length, ~60 residues longer than the MV H-tail. We made progressive truncations of the TPMV H-tail to define the minimal segment necessary for fusion-support function (Figure 3a; HΔ10–HΔ100). Up to 60 residues could be deleted without reducing fusion-support function (Figure 3a; mutants HΔ10–HΔ60, fusion scores, + + +). Further truncations of 70 or 80 residues inhibited fusion-support function (Figure 3a; HΔ70 and HΔ80, fusion score, + + +). However, truncation of 90 residues restored normal function (Figure 3a; HΔ90, fusion score, + + +), whereas a deletion of 100 residues completely inhibited function (Figure 3a; HΔ100, fusion score, –). The second round of truncations to further define the minimal functional H-tail thus included mutants HΔ92–HΔ98 (Figure 3a). With the exception of the HΔ92 mutant that had strongly reduced fusion-support function (Figure 3a; HΔ92, fusion score, +), none of the additional mutants were fusion competent (Figure 3a; HΔ94–HΔ98). Therefore, the HΔ60 and HΔ90 mutants were considered the most promising for the generation of hybrid protein.

Next, we documented H-protein expression by immunoblot analysis of cell lysates from Vero-zHis cells transfected with the respective H-protein constructs (Figure 3b). Comparable levels of H-protein expression were documented for HΔ20, HΔ30, HΔ40 and HΔ60 and wild-type H. In contrast, HΔ10 and HΔ50 were expressed at much lower levels while retaining full fusion support, an observation indicating that high expression levels are not required for full function. As HΔ90 was the largest truncation to have maintained wild-type function and protein expression, we selected this mutant for the generation of a hybrid H protein.

We constructed a hybrid H protein comprised of the ectodomain, transmembrane segment and three residues of the TPMV H-tail (HΔ90) fused to the 34 amino-acid MV H-tail. The hybrid protein, which was named HΔ90/mvcyt, supported fusion with slightly reduced efficiency when coexpressed with the wild-type TPMV F protein (fusion score, + + +, data not shown). However, when HΔ90/mvcyt was coexpressed with the hybrid F protein, FΔ32/mvcyt, fusion was below detection levels, indicating that the combination of two-hybrid glycoproteins with slightly reduced fusion efficiency is not a viable option for virus rescue.
To promote assembly while mitigating potential interference from the TPMV H protein’s bulky cytoplasmic segment, we paired the chimeric FΔ32/mvcyt with the truncated HΔ90, and found that this combination supported fusion with only slightly reduced efficiency (Figure 3c). We did not attempt to make a virus with the HΔ60 deletion.

A TPMV-shielded hybrid MV propagates poorly

We then transferred the genes for the TPMV-MV F-protein hybrid and the truncated TPMV H protein to the MV infectious cDNA, substituting the endogenous genes. We used the vaccine strain vector backbone p(+)/MVvac2(GFP)H, which expresses GFP as a marker protein, and named the hybrid virus MVvac2(FΔ32/mvcyt-HΔ90)GFP. This virus was recovered using the helper cell line 293-3-46, followed by propagation on Vero-αHis cells. Green fluorescent infectious centers were observed in multiple experiments, and propagated for several passages. Following each passage cytopathic effect increased, until up to 80% of the cells were in syncytia. At this point, virus stocks were harvested, but found to have low titers usually ranging from 10^3 to 10^4 TCID50 per ml during early passages. Virus titer did not increase at late passages.

Deletion of the TPMV H-protein cytoplasmic tail does not affect intracellular transport

As truncation of the cytoplasmic tail may affect intracellular transport, and thus particle assembly, we next characterized the efficiency of glycoprotein transport and processing. We first compared surface expression of wild-type TPMV-H and HΔ90, and found low but comparable expression of the wild-type and the

Figure 2. Biochemical and functional characterization of fusion (F)-protein mutants. (a) Protein expression analysis. Cell lysates from transfected Vero-αHis cells used to assess fusion function were digested without or with endo-β-N-acetylglucosaminidase H (EndoH) (-/+), fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a Tupaia paramyxovirus (TPMV) F-specific antibody (F ecto, upper panel). F0: precursor protein; F1: larger portion of the active F molecule (upper panel); and actin: cellular actin (lower panel). (b) Schematic representation of the FΔ32 deletion mutant and the hybrid glycoprotein FΔ32/mvcyt (measles virus [MV] amino acids are bold typeface and underlined). Fusion scores are indicated with the same convention as in Figure 1. CT, cytoplasmic tail; TM, transmembrane.

Figure 3. Functional and biochemical characterization of H-protein cytoplasmic tail truncations. (a) Sequence of the predicted 94-residue cytoplasmic tail (CT) and part of the transmembrane (TM) region; the two regions are separated by a vertical line. Hemagglutinin (H)-protein deletions were named for the extent of their truncation, such that an H protein with a CT truncation of 10 amino acids was named HΔ10. The fusion efficiency of each construct when coexpressed with the full-length F protein in Vero-αHis cells is indicated on the right by the same convention as in Figure 1. (b) H-protein expression analysis. Cell lysates were collected and fractionated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with the Tupaia paramyxovirus (TPMV) H-specific antibody H ecto (upper panel) or for cellular actin (lower panel). (c) Fusion of Vero-αHis cells after co-transfection of plasmids expressing FΔ32/mvcyt and HΔ90. A co-transfected plasmid expressing green fluorescent protein (GFP) allows for visualization of transfected cells. Cells were observed 24 h after the beginning of transfection. His, histidine.
truncated H proteins on the surface of Vero-αHis cells (Figure 5; Vero-αHis column). A control cell line known to have optimal vesicular transport, CHO-K1, efficiently expressed both wild-type H and the truncated HΔ90 proteins at the cell surface (Figure 5, CHO-K1 column).

To more precisely compare intracellular transport of the wild-type and truncated H proteins, we performed pulse-chase experiments to measure the acquisition of EndoH-resistant glycans by the H protein over time. The acquisition of EndoH resistance indicates conversion of high mannose glycans to hybrid and complex oligosaccharides following processing through the medial-Golgi apparatus. We determined that 50% of the H proteins acquired EndoH resistance within 3 h, reflecting transport through the medial-Golgi (Figures 6a and c; diamonds). A similar rate of transport was observed for HΔ90 (Figures 6b and c; squares). Thus, the truncation of the H-protein cytoplasmic tail does not hinder intracellular transport.

Modification of the TPMV F-protein tail affects proteolytic activation

We compared proteolytic activation of the wild-type and chimeric TPMV F proteins by measuring the kinetics of F0 cleavage by pulse-chase experiments. The efficiency of protein activation was determined by comparing the intensities of the F0 and F1 bands at different time points after adjusting for differences in signal intensities resulting from differential radiolabeled incorporation between F0 and F1. We found that about 50% of the wild-type F protein was activated in 6 h (Figures 7a and c), whereas approximately 25% of FΔ32/mvcyt was activated during the same time period (Figures 7b and c). Thus, slow proteolytic processing of the hybrid F protein may contribute to inefficient particle production.

**DISCUSSION**

Antibody-mediated neutralization interferes with the efficacy of systemic virotherapy. To avoid MV-neutralizing antibodies, we previously shielded the MV nucleocapsid with the envelope glycoproteins from the related Morbillivirus canine distemper virus, and showed that this hybrid virus retains oncolytic efficacy. To avoid the neutralizing response against the hybrid virus, and allow for sequential cycles of therapy, shielded viruses that are not crossreactive with either the MV or canine distemper virus glycoproteins are required. As all of the well-characterized

Figure 4. Time course of cell-associated and cell-free virus production. Growth analysis of (a) Tupaia paramyxovirus (TPMV) in tupaia baby fibroblast cells, (b) MVvac2(GFP)N and MVvac2/FΔ32/mvcyt-HΔ90/GFP in Vero-αHis cells. All viruses were inoculated at a multiplicity of infection (MOI) of ~0.03. Titers were determined by collection of cellular (circles) and supernatant (squares) fractions every 12 h over a 60-h time period for TPMV and measles virus (MV) (filled symbols), and every 24 h over a 120-h time period for the hybrid virus (open symbols). Vertical axis: titer by 50% tissue culture infective dose (TCID50) per ml; horizontal axis: time postinfection. GFP, green fluorescent protein.

Figure 5. Fluorescence-activated cell sorter (FACS) analysis of surface expression of measles virus (MV) and Tupaia paramyxovirus (TPMV) glycoproteins in CHO-K1 and Vero-αHis cells. Cells were mock-transfected (negative) or transfected with the plasmids encoding MV-H617, TPMV-H His-tag or TPMV-HΔ90. After 24 h, cells were stained with anti-Penta-His AlexaFluor 647-conjugated antibody. His, histidine.
members of the Morbillivirus genus are crossreactive with one of these envelopes, we explored the suitability of the glycoproteins from the related TPMV for particle assembly. While a simple exchange of the MV and TPMV glycoproteins did not support virus rescue, but the combination of a hybrid F protein, including the MV F cytoplasmic tail and a TPMV H protein with a truncated cytoplasmic tail, did allow rescue. However, infectivity production was minimal: while we occasionally observed titers in the 10^5–10^6 TCID_{50} per ml in fresh supernatants, these titers dropped to 10^3 or lower after freeze–thawing. In practice, infectivity could be propagated only by cell fusion, limiting applications for oncolytic therapy.

We think that improper particle assembly is the limiting factor for the generation of stable particles of shielded MV: viral particles may not form properly, or be unstable and fall apart after release from the cell. This implies that detailed information about the interactions required for MV particle assembly is necessary to design hybrid glycoproteins that better retain their fusion or fusion-support function. While the feasibility of envelope exchanges between members of the same Paramyxovirus genus is established,33–35 and in one case glycoprotein exchange between viruses of different genera was successful when based on hybrid glycoproteins,36 recent systematic studies have documented significant losses of fusion function for many hybrids' paramyxovirus F proteins.37 Analogously, we observed here that the TPMV-MV hybrid F protein lost some fusion function.

Consistently with proposed multiple interactions between F and H at cell fusion, this negative effect was amplified when the hybrid F protein was expressed in combination with an H protein with a truncated cytoplasmic tail.

A promising strategy to adapt new shielding envelopes on oncolytic MV relies on the glycoproteins of recently characterized viruses38,39 and potential viruses.40 Those kinds are more closely related to Morbilliviruses than TPMV, and their glycoproteins may be more easily adaptable to the MV nucleocapsid. In particular, we are exploring whether the glycoproteins of feline morbillivirus, a very recently characterized member of the Morbillivirus genus,41 retain efficient function and particle assembly, while at the same time not crossreacting.

Having observed inefficient glycoprotein transport in Vero-2His cells, we have tested other cell lines expressing anti-His-tag pseudoreceptors as candidate hosts for virus rescue. These cell lines include 293 H6,42 U118MG-HissFv.rec43,44 and CHO-2His,23 which have been used for the rescue of other viruses. Both U118MG-HissFv.rec43,44 and CHO-2His23 transport MV and TPMV H...
with approximately three times greater efficiency than Vero-οΗης (data not shown). As the former cells are of human origin, they may be better hosts for the recovery of shielded recombinant MV than Vero-οΗης cells.

Finally, truncated TPMV glycoproteins have recently been utilized for the shielding of lentivirus-based vectors, specifically FA32- and HA80-truncated mutants to package effectively lentiviral capsids. We note that the lentiviral gag-protein contains sequences that promote budding referred to as late domains, and that these capsids do not depend on specific interactions for budding. This mechanism may mitigate the effects of the reduced fusion phenotype on infectivity and particle production. In contrast to the lentiviral system, late domains have not been identified in MV proteins.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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