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# Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes

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Influenza C virus contains two envelope glycoproteins: CM2, a putative ion channel protein; and HEF, a unique multifunctional protein that performs receptor-binding, receptor-destroying and fusion activities. Here, it is demonstrated that expression of HEF is sufficient to pseudotype replication-incompetent vesicular stomatitis virus (VSV) that lacks the VSV glycoprotein (G) gene. The pseudotyped virus showed characteristic features of influenza C virus with respect to proteolytic activation, receptor usage and cell tropism. Chimeric glycoproteins composed of HEF ectodomain and VSV-G C-terminal domains were efficiently incorporated into VSV particles and showed receptor-binding and receptor-destroying activities but, unlike authentic HEF, did not mediate efficient infection, probably because of impaired fusion activity. HEF-pseudotyped VSV efficiently infected polarized Madin–Darby canine kidney cells via the apical plasma membrane, whereas entry of VSV-G-complemented virus was restricted to the basolateral membrane. These findings suggest that pseudotyping of viral vectors with HEF might be useful for efficient apical gene transfer into polarized epithelial cells and for targeting cells that express 9-*O*-acetylated sialic acids.

## INTRODUCTION

The major envelope glycoprotein of influenza C virus, HEF, is a multifunctional protein that shows receptor-binding (haemagglutination), receptor-destroying and fusion activities (Herrler & Klenk, 1991). The receptor determinant has been identified as 9-*O*-acetylated sialic acid (Herrler *et al.*, 1985) and the virus has been shown to bind to cellular glycoproteins and glycolipids that contain this type of sialic acid (Zimmer *et al.*, 1992, 1994, 1995). The receptor-destroying enzyme is a serine esterase that hydrolyses the 9-*O*-acetyl group of the receptor determinant (Herrler *et al.*, 1985) and has been shown to play a role in virus entry (Strobl & Vlasak, 1993; Vlasak *et al.*, 1989), as well as in virus spread (Höfling *et al.*, 1996). The fusion activity of HEF requires proteolytic activation of the glycoprotein by trypsin-like proteases that cleave at a single arginine residue preceding the hydrophobic fusion peptide (Herrler *et al.*, 1979). In addition, a low pH is needed to trigger the fusion process (Formanowski *et al.*, 1990) indicating that infection of cells requires uptake of influenza C virus by receptor-mediated endocytosis. In spite of the very low sequence similarity between HEF and the haemagglutinin of influenza A virus, the three-dimensional structures of the two proteins are very similar (Rosenthal *et al.*, 1998). The envelope of influenza C virus also contains a minor membrane protein, CM2, which is probably an ion channel protein (Hongo *et al.*, 2004). Its role in the replication cycle of influenza C virus is not understood, although it might be involved in low-pH-triggered virus disassembly, analogous to the M2 ion channel protein of influenza A virus (Whittaker *et al.*, 1996). However, unlike influenza A virus, influenza C virus uncoating takes place at neutral to alkaline pH (Zhirnov & Grigoriev, 1994). The M2 protein of some influenza A viruses has been shown to prevent the early low-pH-triggered conformational change of furin-cleaved haemagglutinin during transport through compartments with an acidic milieu (Takeuchi & Lamb, 1994). However, there is no evidence for a similar role of CM2, as HEF is proteolytically activated by extracellular proteases.

In this study, the capacity of HEF to mediate infection of a heterologous virus in the absence of any other influenza C virus proteins was investigated. Incorporation of native and chimeric HEF into recombinant replication-incompetent vesicular stomatitis virus (VSV) that lacked the VSV-G glycoprotein gene was studied. With native HEF, infectious VSV pseudotypes were produced that required trypsin for activation and had cell tropism similar to that of influenza

The GenBank/EMBL/DDBJ accession number for the HEF gene sequence of influenza C virus C/Johannesburg/1/66 is AJ872181.

Details of primers used in construction of expression plasmids are available as supplementary material in JGV Online.

C virus. Unlike VSV-G glycoprotein, HEF allowed apical infection of polarized epithelial cells. Pseudotyping of viral vectors with HEF might be advantageous for the selective targeting of polarized or non-polarized cells expressing 9-*O*-acetylated sialic acids. In addition, pseudotyping with HEF will be a powerful tool to investigate HEF functional domains.

## METHODS

**Cells and virus.** Baby hamster kidney cells (BHK-21), human astrocytoma cells (U373), human embryo kidney cells (293T) and Madin–Darby canine kidney (MDCK) cells were maintained as recommended by the ATCC. BSR-T7/5 cells were cultured as previously reported (Buchholz *et al.*, 1999). Human bronchial epithelial cells (16HBE14o) (Cozens *et al.*, 1994) were grown in a mixture of Ham's F12 medium and Eagle's minimal essential medium supplemented with 10 % fetal calf serum (FCS). Influenza C virus (JHB/1/66) was kindly provided by Herbert Meier-Ewert (TU München, Germany) and propagated on MDCK-I cells.

**Antibodies.** A multiple antigenic peptide was synthesized by anchoring residues 1–21 (MSSLKKILGLKGKGKKSKKLG) of the VSV matrix protein onto an immunogenically inert core molecule of radially branching lysine dendrites. The antigen was repeatedly applied to rats by subcutaneous injections at 3-weekly intervals. The animals were bled and serum was prepared by centrifugation of the coagulated blood. Polyclonal antisera were produced by immunization of rabbits with sucrose-gradient-purified influenza C virus or VSV. Hybridomas producing the monoclonal antibodies I-14 and I-1 (Lefrancois & Lyles, 1982a, b) were kindly provided by Volker ter Meulen (Würzburg, Germany).

**Oligonucleotides.** Details of primers used in construction of expression plasmids are available in a supplementary table in JGV Online.

**Construction of expression plasmids.** The G protein gene of VSV (strain Indiana) was excised from the pTM1-VSVG plasmid (Köhl *et al.*, 2004) using *Eco*RI and *Xho*I endonucleases and ligated into plasmid pCDNA3.1 (Invitrogen) to give pCDNA3.1-VSVG. For cloning of the VSV-G gene into the pGeneC vector (Invitrogen), the gene was amplified from pTM1-VSVG by PCR with oligonucleotides VSVG-S and VSVG-AS. Taking advantage of the *Bam*HI and *Eco*RI restriction sites included in the primers, the PCR product was inserted into pGeneC to give pGeneC-VSVG. The total open reading frame (ORF) of G was sequenced and found to be identical to the published sequence (GenBank/EMBL/DDBJ accession no. NC_001560). For cloning of the influenza C virus (JHB/1/66) HEF gene, total RNA was prepared from infected MDCK-I cells and reversed transcribed. The ORF of HEF was amplified from the cDNA by PCR using oligonucleotides HEF-S and HEF-AS. The PCR product was treated with *Kpn*I and *Xho*I endonucleases and ligated into plasmid pCDNA3.1(+) (Invitrogen) to give pCDNA3.1-HEF. The DNA sequence of three clones was determined and compared with the published sequence (GenBank/EMBL/DDBJ accession no. M17868). A new entry in the EMBL nucleotide sequence database was created (GenBank/EMBL/DDBJ accession no. AJ872181) to account for the point mutations detected.

Amino acid exchanges T284I, T284L and T286I were introduced into pCDNA3.1-HEF by an overlapping PCR technique (Schlender *et al.*, 2003; Köhl *et al.*, 2004) using oligonucleotides T284I-S, T284I-AS, T284L/T286I-S and T284L/T286I-AS. The PCR product was digested with *Bam*HI and *Pfl*MI and this fragment was used to replace the corresponding region in pCDNA3.1-HEF. The DNA sequence of the cloned fragment was determined to verify the nucleotide exchanges. An overlapping PCR technique was also used to generate chimeric glycoprotein genes. HEF gene segments were amplified from pCDNA3.1-HEF or from pCDNA3.1-HEF(T284I) using the HEF-S primer in combination with either HEF–G(T)-AS, HEF–G(TM)-AS, HEF–G(STM)-AS or HEF–CD4-AS. To amplify regions of the VSV-G or CD4 cDNA, oligonucleotides HEF–G(T)-S, HEF–G(TM)-S, HEF–G(STM)-S and HEF–CD4-S were used in combination with VSVG-AS(*Xho*I) or CD4-AS, respectively. Hybridization of the PCR fragments was performed as described previously (Schlender *et al.*, 2003; Köhl *et al.*, 2004). The *Pfl*MI–*Xho*I restriction fragments of the hybrid genes were used to replace the corresponding segment in pCDNA3.1-HEF.

**Generation of a stable cell line for conditional expression of VSV-G.** BHK-21 cells were co-transfected with the plasmids pSwitch (Invitrogen) and pGeneC-VSVG and grown for 14 days in selection medium containing hygromycin B (500 μg $ml^{-1}$) and zeocin (1 mg $ml^{-1}$). Cell clones were isolated by limiting dilution and analysed for mifepristone-induced VSV-G expression by immunofluorescence and Western blotting. One cell clone, BHK-G43, efficiently supported replication of VSV*-ΔG-G (the asterisk denotes the eGFP gene; see below) and was used throughout the study.

**Immunofluorescence analysis and flow cytometry.** BHK-21 cells grown on 12-mm-diameter coverslips ($2{\cdot}5 \times 10^5$ cells) were transfected with 1 μg plasmid DNA and 2 μl Lipofectamine 2000 transfection reagent. At 20 h post-transfection, cells were fixed with 3 % paraformaldehyde and permeabilized with 0·2 % Triton X-100 if intracellular antigen was to be detected. Cells were incubated first with rabbit polyclonal anti-influenza C virus serum (1 : 2000) and then with FITC-conjugated goat anti-rabbit IgG serum (1 : 500; Sigma). For flow cytometric analysis, BHK-21 cells grown in 24-well dishes ($3 \times 10^5$ cells per well) were transfected as described above. At 20 h post-transfection, adherent cells were stained at 4 °C with the antibodies described above and finally suspended in PBS. Cells were analysed using a Beckman Coulter Epics XL cytometer equipped with Expo 32 ADC software.

**Generation of VSV pseudotypes.** For generation of replication-incompetent recombinant VSV, a previously published strategy (Takada *et al.*, 1997) was followed. The gene for the enhanced green fluorescent protein (eGFP) was cloned into pVSV-XN2 (Schnell *et al.*, 1996b) taking advantage of the single restriction sites *Mlu*I and *Nhe*I. In this way, the VSV-G gene of the genome was replaced and the resulting vector was therefore designated pVSV*-ΔG (the asterisk denotes the eGFP gene). BSR-T7/5 cells were transfected with pVSV*-ΔG, pCDNA3.1-VSVG and three plasmids driving the expression of the viral polymerase complex (Schnell *et al.*, 1996b). Two days after transfection, VSV*-ΔG-G was recovered from the cell culture supernatant and propagated on mifepristone-treated BHK-G43 cells.

For generation of HEF-pseudotyped VSV (VSV*-ΔG-HEF), BHK-21 cells grown in 35-mm-diameter dishes were transfected with 5 μg HEF or VSV-G expression plasmid and 10 μl Lipofectamine 2000 transfection reagent. At 20 h post-transfection, cells were inoculated with VSV*-ΔG-G (10 p.f.u. per cell) for 1 h at 37 °C and then with medium containing a polyclonal rabbit anti-VSV serum to neutralize the helper virus. Following incubation for 20 h at 37 °C in the absence of FCS, the cell culture supernatant was harvested, clarified by low-speed centrifugation and treated with 5 μg acetylated trypsin (Sigma) $ml^{-1}$ for 1 h at 37 °C to activate the HEF glycoprotein. The reaction was stopped by adding FCS (1 % final concentration). VSV*-ΔG-HEF was titrated on MDCK-I cells grown on 12-mm coverslips by inoculating cells with serial dilutions of the cell culture supernatant for 90 min at 37 °C. At 16 h post-infection, cells were fixed with paraformaldehyde and the number of eGFP-positive cells was determined.

**Pseudotype virus infection.** To analyse the cell tropism of VSV*-ΔG-HEF, cell lines were seeded on 12-mm coverslips ($3\times10^5$ cells) and infected with $10^5$ infectious units of pseudotype virus in 250 µl medium for 90 min at 37 °C. In some experiments, BHK-21 and U373 cells were pretreated with 250 µl affinity-purified neuraminidase from *Clostridium perfringens* (1 U $ml^{-1}$; Sigma) for 60 min at 37 °C, prior to inoculation with 250 µl medium containing $10^5$ infectious units of pseudotype virus for 60 min at 37 °C. Cells were then incubated for 30 min at 37 °C with anti-FluC or anti-VSV serum. At 16 h post-infection, cells were fixed with paraformaldehyde and analysed by fluorescence microscopy to monitor eGFP expression. For analysis of polarized virus entry, MDCK cells were seeded on Falcon cell culture inserts containing porous (1 µm), 6·5-mm-diameter membranes (Becton Dickinson) and maintained for 3 days. Trypsin-activated pseudotype viruses were concentrated by ultracentrifugation and $2\times10^6$ infectious units were inoculated for 5 h at 37 °C with either the apical or the basolateral site of the filter. eGFP reporter expression was detected 16 h post-infection. For virus neutralization, serial dilutions (125 µl) of anti-VSV or anti-FluC serum were incubated for 30 min at room temperature with 125 µl of either VSV*-ΔG-HEF ($10^6$ infectious units $ml^{-1}$) or trypsin-activated VSV*-ΔG-HEF ($4\times10^5$ infectious units $ml^{-1}$), prior to infection of BHK-21 or MDCK-I cells seeded in 24-well dishes ($2\times10^5$ cells per well). The ratio of eGFP-positive cells was determined by flow cytometry 16 h post-infection. Virus that was not pre-incubated with antiserum served as a control; its titre was set to 100 %.

**Analysis of VSV particles.** VSV pseudotypes from the cell culture supernatant of two 35-mm-diameter dishes were pelleted through a 25 % (w/w) sucrose cushion by ultracentrifugation (105 000 ***g***, 60 min, 4 °C). The pelleted particles were dissolved in 100 µl 2× SDS sample buffer. Solubilized proteins (10 µl samples) were separated by 12 % SDS-PAGE under non-reducing conditions and transferred to nitrocellulose by semi-dry blotting. The nitrocellulose membranes were incubated for 60 min at 4 °C with either a rat monospecific antiserum directed to VSV-M (1 : 2000), a rabbit polyclonal anti-influenza C virus serum (1 : 2500) or a mouse monoclonal antibody (I-14) directed towards VSV-G (1 : 100). The blots were washed and subsequently incubated with biotinylated secondary antibodies that were specific for the IgG fraction of the respective species (1 : 1000; Sigma). Finally, the blots were incubated for 60 min with a streptavidin–peroxidase complex (1 : 1000; Amersham Biosciences) and the antigens were visualized by enhanced chemiluminescence (Roche Diagnostics).

**Analysis of HEF esterase activity.** BHK-21 cells grown in 35-mm-diameter dishes ($8\times10^5$ cells per well) were transfected with 5 µg HEF expression plasmid and 10 µl Lipofectamine 2000 transfection reagent. Twenty hours after transfection, cells were lysed and HEF was immunoprecipitated with an antiserum directed to influenza C virus according to published procedures (Zimmer *et al.*, 2001). The immunoprecipitates were run on a 10 % SDS-PAGE gel and blotted to nitrocellulose membranes. Esterase activity was detected as described previously (Döll *et al.*, 1993). For detection of HEF antigen, cell lysates were analysed by Western blotting as described above.

**Haemadsorption.** Freshly prepared chicken erythrocytes were labelled with octadecyl rhodamine B chloride (R18; Molecular Probes) as reported previously (Fischer *et al.*, 1998). The R18-labelled chicken erythrocytes were suspended in ice-cold PBS to obtain a 0·2 % cell suspension and incubated for 60 min at 4 °C with BHK-21 cells grown in 24-well dishes ($2\times10^5$ cells per well) that had been transfected with HEF expression plasmids (1 µg per well). The cells were rinsed several times with ice-cold PBS and bound erythrocytes were visualized by fluorescence microscopy.

## RESULTS

### Cell surface transport of HEF glycoproteins

In previous studies, it has been observed that transfection of HEF cDNA based on the published sequence of influenza C virus JHB/1/66 (Pfeifer & Compans, 1984) did not result in cell surface expression of HEF (Szepanski *et al.*, 1994; Oeffner *et al.*, 1999) (Fig. 1, upper panel, HEFmut). In contrast, HEF was efficiently transported to the cell surface when cDNA derived from influenza C viruses Ann Arbor/1/50, Taylor/1223/47 or Cal/72 was used (Vlasak *et al.*, 1987; Pekosz & Lamb, 1999). Because of this discrepancy, HEF cDNA was recloned using RNA isolated from MDCK cells following infection with influenza C virus JHB/1/66. The amino acid sequence of the resulting HEF clone (GenBank/EMBL/DDBJ accession no. AJ872181) differed from the previously published sequence (GenBank/EMBL/DDBJ accession no. M17868) in four amino acid positions: serines were found instead of phenylalanine residues at positions 96 and 263, leucine substituted for valine at position 457 and threonine-465 was replaced with an alanine. BHK-21 cells transfected with this cDNA expressed HEF at the cell surface, indicating that at least one of the four amino acid residues is critical for the correct folding and transport of the protein (Fig. 1, lower panel, HEF).

In previous studies, influenza C virus mutants have been described that show a higher affinity for the receptor determinant leading to an extended cell tropism (Szepanski *et al.*, 1992; Marschall *et al.*, 1994). The viruses were found to contain characteristic amino acid changes in the HEF

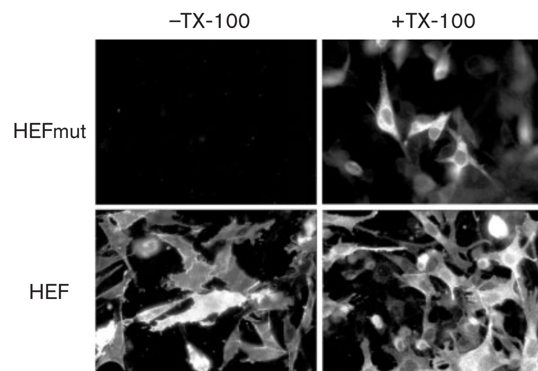


**Fig. 1.** Indirect immunofluorescence analysis of transfected BHK-21 cells. Cells were transfected with either HEFmut (primary sequence as published by Pfeifer & Compans, 1984) or HEF (cDNA cloned in the present study) and fixed with paraformaldehyde 20 h post-transfection. For cell surface staining, cells were directly incubated with rabbit antiserum with specificity for influenza C virus antigens. For intracellular staining, cells were permeabilized with 0·2 % Triton X-100 (+TX-100) prior to antibody staining. FITC-conjugated anti-rabbit IgG serum was used as secondary antibody.

**Table 1.** Flow-cytometric analysis of BHK-21 cells expressing either authentic, mutant or chimeric HEF glycoproteins

BHK-21 cells were transfected with expression plasmids encoding the indicated HEF constructs. At 20 h post-transfection, live cells were stained at 4 °C with a polyclonal antiserum directed to influenza C virus and an FITC-conjugated secondary antibody; 5000 cells of each sample were analysed by flow cytometry. Mean values and standard deviations were calculated from three independent transfections.

| Glycoprotein expressed | Percentage of cells reacting with anti-FluC antibody | Fluorescence intensity (arbitrary units) |
|---|---|---|
| HEFmut | 0·9 ± 0·5 | 29·6 ± 30 |
| HEF | 26·7 ± 2·8 | 118·0 ± 6 |
| HEF(T284I) | 23·3 ± 0·4 | 109 ± 4 |
| HEF(T284L/T286I) | 21·2 ± 0·8 | 103 ± 3 |
| HEF–CD4 | 21·7 ± 1·5 | 102 ± 4 |
| HEF–G(T) | 17·3 ± 0·5 | 117 ± 1 |
| HEF–G(TM) | 18·2 ± 1·2 | 130 ± 4 |
| HEF–G(STM) | 17·9 ± 0·7 | 120 ± 3 |

protein. In one case, a threonine to isoleucine exchange occurred at position 284 (Szepanski *et al.*, 1992). Another mutant had a threonine to leucine exchange at the same position, together with a threonine to isoleucine exchange at position 286 (Marschall *et al.*, 1994). All mutations were close to proline-285, an amino acid directly involved in binding the 9-*O*-acetyl group of the receptor determinant (Rosenthal *et al.*, 1998). It was thought that use of these HEF mutants might improve infectivity of pseudotyped VSV and therefore the respective point mutations were introduced into the cloned HEF gene. Unlike the amino acid exchanges mentioned above, T284I and T284L/T286I had no deleterious effect on HEF cell surface transport (Table 1).

The influenza C virus glycoprotein HEF has a very short cytoplasmic domain, predicted to be composed of only three amino acids (Fig. 2). Since incorporation of glycoprotein G (VSV-G) into VSV particles has been shown to depend on the length rather than the specific sequence of its cytoplasmic domain (Schnell *et al.*, 1998), it is speculated that generation of chimeric HEF proteins containing C-terminal sequences of the VSV-G protein would be beneficial for the generation of VSV pseudotypes; therefore, four different HEF chimeras were constructed (Fig. 2). The HEF–G(T) chimeric protein is composed of the HEF ecto- and transmembrane domains and the VSV-G cytoplasmic domain. As a non-virus control, the cytoplasmic domain of the cellular membrane protein CD4 was linked to the transmembrane region of HEF (HEF–CD4). The HEF–G(TM) chimeric protein combined the HEF ectodomain with the VSV-G transmembrane and cytoplasmic domains. A fourth chimeric HEF glycoprotein, HEF–G(STM), was generated by replacing 15 amino acids of the HEF stem region in HEF–G(TM) with 12 amino acids from the corresponding VSV-G stem domain (Robison & Whitt, 2000). Using flow cytometric analysis, all chimeric HEF glycoproteins were detected at the cell surface of BHK-21 cells (Table 1), thus demonstrating that gross folding defects that interfere with cell surface transport did not occur.

## Receptor-binding and receptor-destroying properties of HEF glycoproteins

For analysis of the esterase function of HEF, the glycoprotein was immunoprecipitated from transfected BHK-21 cell lysates, separated by SDS-PAGE and transferred to nitrocellulose. Esterase activity was detected by applying a chromogenic esterase substrate (Fig. 3a, upper panel). Using this approach, it was found that esterase activity was associated not only with authentic HEF, but also with HEF(T284I) and HEF(T284L/T286I), as well with the chimeric glycoproteins. In contrast, no esterase activity was detected in mock-transfected samples. HEF expression levels were controlled by Western blot analysis of the corresponding cell lysates using a polyclonal antibody directed to influenza C virus (Fig. 3a, lower panel).

To study the receptor-binding activity of parental, mutant and chimeric HEF, a haemadsorption assay was performed using chicken red blood cells that have been shown to contain 9-*O*-acetylated sialic acids (Herrler *et al.*, 1987). Fig. 3(b) shows that BHK-21 cells transfected with any of the constructs were able to bind R18-labelled erythrocytes, whereas mock-transfected cells did not reveal any significant haemadsorption activity.

| | Stem region | Membrane anchor | Cytoplasmic domain |
|---|---|---|---|
| HEF | ...DTKIDLQSDPFYWGSSLG | LAITATISLAALVISGIAIC | RTK |
| HEF-CD4 | ...DTKIDLQSDPFYWGSSLG | LAITATISLAALVISGIAIC | **RCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQKTCSPI** |
| HEF-G(T) | ...DTKIDLQSDPFYWGSSLG | LAITATISLAALVISGIAIC | **RVGIHLCIKLKHTKKRQIYTDIEMNRLGK** |
| HEF-G(TM) | ...DTKIDLQSDPFYWGSSLG | **SSIASFFFIIGLIIGLFLVL** | **RVGIHLCIKLKHTKKRQIYTDIEMNRLGK** |
| HEF-G(STM) | ...DTK---**IELVEGWFSSWK** | **SSIASFFFIIGLIIGLFLVL** | **RVGIHLCIKLKHTKKRQIYTDIEMNRLGK** |
| VSV-G | **...GLSKNPIELVEGWFSSWK** | **SSIASFFFIIGLIIGLFLVL** | **RVGIHLCIKLKHTKKRQIYTDIEMNRLGK** |

**Fig. 2.** C-terminal amino acid sequences of authentic and chimeric HEF glycoproteins. Putative stem region, membrane anchor and cytoplasmic domain are indicated. Heterologous sequences derived from VSV-G and CD4 are shown in bold.

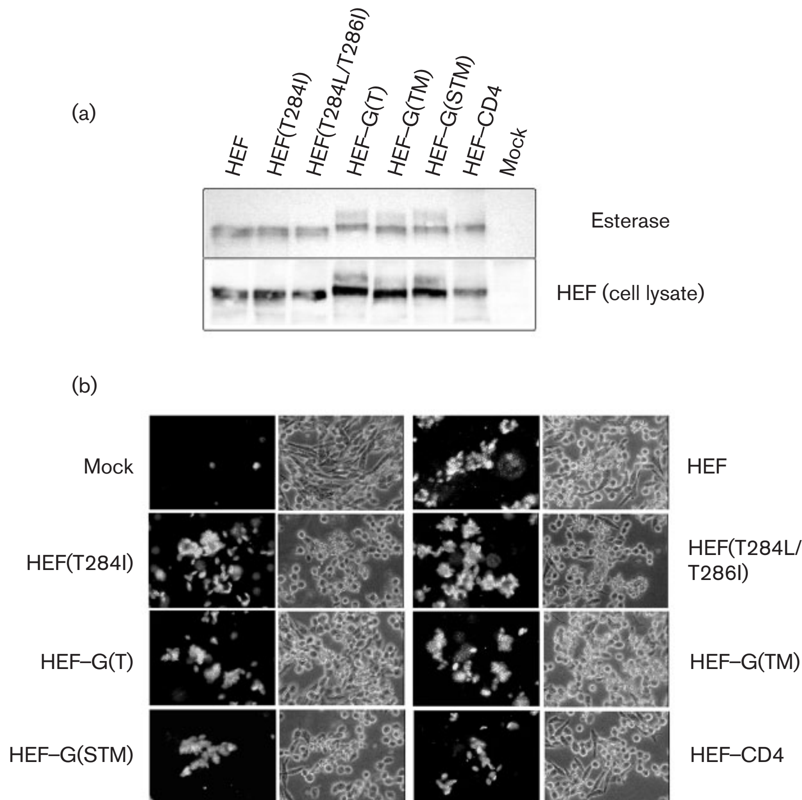


**Fig. 3.** Esterase and haemadsorption activities of BHK-21 cells expressing HEF glycoproteins. (a) BHK-21 cells were either mock-transfected or transfected with authentic HEF, HEF(T284I), HEF(T284L/T286I), HEF–G(T), HEF–G(TM), HEF–G(STM) or HEF–CD4. HEF was immunoprecipitated from cell lysates, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Esterase activity was detected on the blot using α-naphthyl acetate, an esterase substrate that reacts with Fast red TR salt to form an insoluble diazo dye (upper panel). Detection of HEF antigen in cell lysates using an antibody directed to influenza C virus is shown in the lower panel. (b) At 20 h post-transfection, BHK-21 cells expressing the indicated HEF constructs were incubated with R18-labelled chicken erythrocytes at 4 °C. Cells were rinsed several times with PBS and adsorbed erythrocytes were detected by fluorescence microscopy.

## Incorporation of HEF glycoproteins into VSV particles

For generation of VSV pseudotypes, a recombinant replication-incompetent VSV was used with the G gene replaced by the gene encoding eGFP (Takada *et al.*, 1997). VSV-G protein was provided *in trans* and the resulting virus, VSV*-ΔG-G, was propagated on a transgenic helper cell line expressing VSV-G after induction by mifepristone. VSV*-ΔG-G was used to infect BHK-21 cells transiently expressing unmodified or chimeric HEF glycoproteins. The virus particles released into the cell culture supernatant during the following 20 h were pelleted through a sucrose cushion by ultracentrifugation and analysed by Western blotting (Fig. 4a). Infection of mock-transfected BHK-21 cells with VSV*-ΔG-G resulted in the release of virus particles devoid of any viral glycoprotein: the sample reacted with a monospecific antibody directed to the matrix protein of VSV, but not with an antibody directed to influenza C virus or an antibody to VSV-G (Fig. 4a, lane 2). When cells were transfected with HEF cDNA without subsequent infection by VSV*-ΔG-G, release of some HEF antigen into the cell culture supernatant was observed (Fig. 4a, lane 3). However, when HEF-transfected cells were infected with VSV*-ΔG-G, HEF release into the cell culture supernatant was much more efficient, indicating that VSV particles containing HEF had been formed (Fig. 4a, lane 4). Likewise, the three HEF chimeras containing VSV-G sequences at the C terminus were incorporated into VSV particles (Fig. 4a, lanes 5–7). HEF glycoprotein appeared as a 100 kDa band under non-reducing conditions, indicating that the pseudotypes contain predominantly the proteolytically unprocessed precursor $HEF_0$ (Herrler *et al.*, 1979). However, when the cell culture supernatant was treated with trypsin (5 μg $ml^{-1}$) and then collected by ultracentrifugation, Western blot analysis revealed that most $HEF_0$ had been proteolytically cleaved, as indicated by the characteristic 80 kDa band representing $HEF_{1,2}$ (Fig. 4b). Like authentic HEF, the mutant and chimeric HEF glycoproteins were cleaved by trypsin, giving an 80 kDa band. Trypsin-mediated degradation, which is sometimes observed with misfolded proteins (Zimmer *et al.*, 2001), was not detected. The low level of HEF–CD4 found in VSV particles (Fig. 4b) was also observed in untreated samples (data not shown), indicating that this chimeric glycoprotein is not incorporated with the same efficiency as the other constructs.

## Authentic HEF glycoprotein mediates infection of VSV pseudotypes

To analyse whether VSV particles containing HEF glycoprotein are infectious, BHK-21 cells transiently expressing either VSV-G or HEF glycoproteins were infected with VSV*-ΔG-G helper virus. Following infection, cells were incubated with a polyclonal anti-VSV serum to ensure that only progeny virus would be detected in the cell culture supernatant. At 20 h post-transfection, the cell culture medium was harvested, treated with trypsin to activate HEF-pseudotyped virus and titrated on BHK-21 and

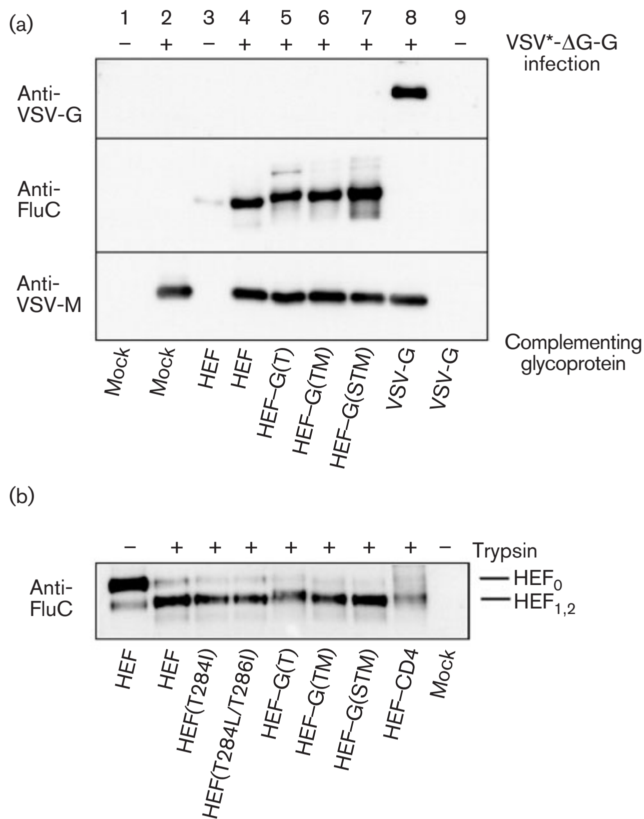


**Fig. 4.** Incorporation of HEF into VSV particles. BHK-21 cells were either mock-transfected or transfected with plasmids driving the expression of the indicated glycoproteins. (a) Transfected cells were either mock-infected or infected with VSV*-ΔG-G as indicated above the figure. Particles released into the cell culture supernatant were analysed by Western blotting using the indicated antibodies. (b) VSV*-ΔG complemented with the indicated glycoproteins was either treated with trypsin or incubated with medium as indicated above the gel. Virions were pelleted by ultracentrifugation and HEF antigen was detected by Western blot analysis using a polyclonal anti-influenza C virus serum. Bands representing the uncleaved precursor $HEF_0$ and the proteolytically processed $HEF_{1,2}$ are indicated.

MDCK-I cells. Infection was monitored by taking advantage of eGFP reporter expression (Table 2). Cells that had been transfected with the VSV-G expression plasmid produced $1{\cdot}2\times10^6$ infectious VSV*-ΔG-G particles per ml unconcentrated cell culture supernatant if titrated on BHK-21 cells. However, on confluent MDCK-I cells, significantly lower titres were obtained, which is probably a consequence of the polarized organization of these cells (see below). A completely different phenotype with respect to infectivity and cell tropism was observed when VSV*-ΔG was pseudotyped with HEF glycoprotein. In contrast to VSV-G, HEF did not mediate efficient infection of BHK-21 cells, probably because these cells produce only very small quantities of the receptor determinant (Herrler & Klenk, 1987). However, when HEF-complemented VSV*-ΔG was titrated on MDCK-I cells, a cell line permissive for influenza C virus, titres of $3\times10^5$ infectious units per ml unconcentrated cell culture supernatant were obtained. When the mutants HEF(T284I) and HEF(T284L/T286I) were used for complementation of VSV*-ΔG, quite similar titres were obtained on this cell line ($4\times10^5$ and $5\times10^5$ infectious units per ml, respectively), whereas titres on BHK-21 cells did not exceed 250 infectious units per ml. In contrast to authentic or mutant HEF, complementation of VSV*-ΔG with any of the chimeric HEF glycoproteins, HEF–G(T), HEF–G(TM), HEF–G(STM) or HEF–CD4, resulted in very low titres regardless of the cell line used for titration.

**Table 2.** Infectivities of G- and HEF-complemented VSV*-ΔG

BHK-21 cells were transfected with expression plasmids encoding the indicated glycoproteins. At 20 h post-transfection, cells were infected with VSV*-ΔG-G and subsequently treated with neutralizing anti-VSV serum. The following day, the pseudotypes released into the cell culture supernatant were treated with acetylated trypsin and titrated on BHK-21 and MDCK-I cells. Twenty hours post-infection, the number of GFP-expressing cells was determined. Mean numbers of infectious units and standard deviations were calculated from three transfection experiments.

| Glycoprotein | Infectious units ($\log_{10}$ ml$^{-1}$) | |
|---|---|---|
| | BHK-21 | MDCK-I |
| None | 0 | 0 |
| G | $6{\cdot}1\pm0{\cdot}3$ | $4{\cdot}1\pm0{\cdot}3$ |
| HEF | $<2{\cdot}4$ | $5{\cdot}5\pm0{\cdot}2$ |
| HEF(T284I) | $<2{\cdot}4$ | $5{\cdot}6\pm0{\cdot}2$ |
| HEF(T284L/T286I) | $<2{\cdot}4$ | $5{\cdot}7\pm0{\cdot}2$ |
| HEF–G(T) | $<2{\cdot}4$ | $2{\cdot}6\pm0{\cdot}3$ |
| HEF–G(TM) | $<2{\cdot}4$ | $<2{\cdot}4$ |
| HEF–G(STM) | $<2{\cdot}4$ | $<2{\cdot}4$ |
| HEF–CD4 | $<2{\cdot}4$ | $3{\cdot}0\pm0{\cdot}3$ |

The receptor determinant 9-*O*-acetylated sialic acid has been reported to be a major factor determining the cell tropism of influenza C virus (Herrler & Klenk, 1987). This prompted the question of whether the cell tropism of HEF-complemented VSV*-ΔG parallels that of influenza C virus. Six different cell lines were analysed. MDCK-I, U373 and 16HBE14o cells were found to be permissive for influenza C virus (data not shown) and these cell lines were also infected by the HEF-pseudotyped virus (Fig. 5a). In contrast, MDCK-II, BHK-21 and 293T cells were not infected by HEF-complemented VSV*-ΔG, which corresponds to our observation that these cell lines are poorly infected by influenza C virus (data not shown) (Herrler & Klenk, 1987). To provide direct evidence that sialic acids are required for infection by HEF pseudotypes, MDCK-I cells were treated with affinity-purified neuraminidase from *Clostridium perfringens* (Fig. 5b). This treatment rendered the cells almost completely resistant towards infection with

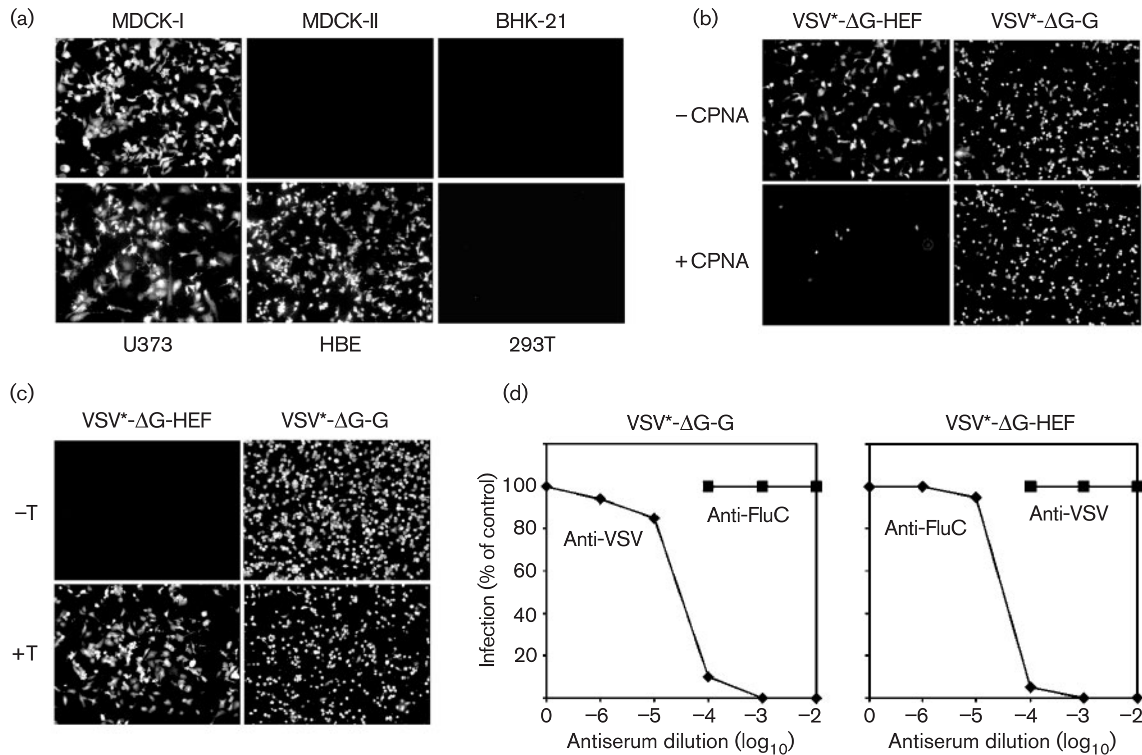


**Fig. 5.** Characteristics of VSV*-ΔG-HEF infection. (a) Cell tropism of HEF-pseudotyped VSV. The indicated cell lines were seeded on coverslips and infected with VSV*-ΔG-HEF. Infected cells were detected by eGFP expression using a fluorescence microscope. (b) VSV*-ΔG-HEF uses sialic acids for infection. U373 cells (left panels) and BHK-21 cells (right panels) were treated with neuraminidase from *Clostridium perfringens* (+CPNA) or were incubated with medium (−CPNA). Cells were infected with either VSV*-ΔG-HEF or VSV*-ΔG-G followed by incubation with neutralizing anti-FluC and anti-VSV serum, respectively. At 16 h post-infection, cells were fixed and monitored for eGFP expression. (c) VSV*-ΔG-HEF is activated by trypsin. VSV*-ΔG-HEF and VSV*-ΔG-G were treated with trypsin (+T) or were left untreated (−T) prior to infection of MDCK-I and BHK-21 cells, respectively. (d) Neutralization of pseudotyped VSV. VSV*-ΔG-G and trypsin-activated VSV*-ΔG-HEF were preincubated with the indicated serial antisera dilutions and then used for infection of BHK-21 and MDCK-I cells, respectively. Virus that was not preincubated with antiserum served as a control; its titre was set to 100 %.

HEF-pseudotyped VSV*-ΔG, but it had no effect on infection by VSV*-ΔG-G. Thus, complementation of VSV*-ΔG with HEF resulted in infectious pseudotype virus that shows a different receptor usage and cell tropism from VSV. In addition, HEF- and VSV-G-complemented VSV*-ΔG differed from each other with respect to proteolytic activation. HEF is known to be proteolytically activated by trypsin-like proteases (Herrler & Klenk, 1991). As many cell lines do not secrete sufficient amounts of these enzymes, VSV*-ΔG-HEF was generally treated with trypsin prior to infection. Indeed, when trypsin treatment was omitted from the protocol, HEF did not mediate infection of the pseudotypes, whereas the function of VSV-G was not affected by trypsin (Fig. 5c). The pseudotypes also differed with respect to neutralization by virus-specific antisera. Fig. 5(d) shows that VSV*-ΔG-HEF, but not VSV*-ΔG-G, was neutralized by antiserum directed to influenza C virus. On the other hand, VSV-specific antiserum neutralized VSV*-ΔG-G, whereas it had no effect on VSV*-ΔG-HEF.

## Infection of polarized epithelial cells

Previous work has shown that infection of polarized epithelial cells by VSV is restricted to the basolateral plasma membrane (Fuller *et al.*, 1984). This property of VSV might explain why infection of confluent MDCK monolayers with VSV*-ΔG-G was very inefficient (Table 2). To test this hypothesis, infection experiments were performed with subconfluent MDCK-II cells. These cells show a characteristic phenotype when seeded at low density on cell culture dishes by forming cell clusters that eventually grow to a tight monolayer. Fig. 6(a) shows that the G-complemented VSV*-ΔG specifically targeted the border cells of these islands, whereas cells in the centre of the clusters that had already become polarized were not infected. A fully polarized monolayer can be established by culturing cells on porous filter supports. When VSV*-ΔG-G was applied to the apical domain of filter-grown MDCK-II cells, infection was not observed (Fig. 6b). However, when the virus was added to the basolateral compartment of the

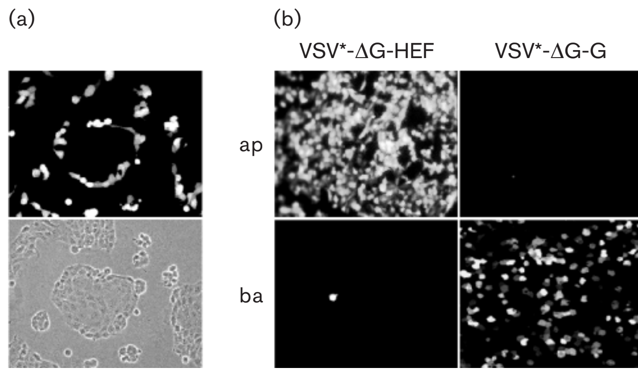


**Fig. 6.** Infection of subconfluent and polarized MDCK cells by VSV pseudotypes. (a) Subconfluent MDCK-II cells grown on coverslips were fixed 6 h post-infection with VSV*-ΔG-G and examined by fluorescence microscopy for eGFP reporter expression (top) and phase-contrast microscopy (bottom), respectively. (b) Polarized MDCK-I cells (left panel) and MDCK-II cells (right panel) grown on porous filter supports were infected via the apical (ap) or the basolateral route (ba) with either VSV*-ΔG-G or VSV*-ΔG-HEF(T284L/T286I). Infected cells were identified by eGFP reporter expression. MDCK-II cells were used for infection by VSV*-ΔG-G because this subline is more permissive for this virus than MDCK-I cells.

filter support, the cells were successfully infected, as indicated by eGFP expression. VSV*-ΔG-HEF showed a different phenotype. This pseudotype virus infected filter-grown MDCK-I cells via the apical membrane, whereas the basolateral route was less efficient. A similar asymmetric infection pattern was reported for influenza C virus and correlated with the polarized distribution of gp40, the major influenza C virus receptor in MDCK-I cells (Zimmer *et al.*, 1995). In summary, these data indicate that a virus that infects polarized epithelial cells from the basolateral site can be modified by pseudotyping to infect cells via the apical plasma membrane.

## DISCUSSION

The envelope glycoproteins HA and NA of influenza A and B viruses, as well as the paramyxovirus glycoproteins HN and F, exert three activities involved in virus entry, namely receptor-binding, receptor-destroying and fusion. Influenza C virus has an exceptional position among these viruses as all three activities are combined in the single spike glycoprotein HEF. In striking contrast to the very rapid antigenic drift observed with influenza A virus haemagglutinin, HEF shows an extraordinarily high sequence conservation (Buonagurio *et al.*, 1985). This low antigenic variation might mirror the limited structural plasticity of the glycoprotein, which could explain why transfection of HEF cDNA derived from the published sequence of the JHB/1/66 strain (Pfeifer & Compans, 1984) did not result in cell surface expression of the glycoprotein (Szepanski *et al.*, 1994; Oeffner *et al.*, 1999). Four amino acid changes at highly conserved positions were identified in this sequence, namely S96F, S263F, L457V and A465T. It is evident that at least one of these mutations is responsible for the HEF misfolding and intracellular retention observed previously.

VSV pseudotypes have proven to be of value for the characterization of envelope glycoproteins from several viruses including Ebola virus (Takada *et al.*, 1997; Ito *et al.*, 2001), measles virus (Tatsuo *et al.*, 2000), hepatitis C virus (Meyer *et al.*, 2000; Matsuura *et al.*, 2001; Burioni *et al.*, 2002; Lagging *et al.*, 1998; Beyene *et al.*, 2004), bunyaviruses (Ogino *et al.*, 2003), human T cell leukaemia virus (Okuma *et al.*, 2001) and Borna disease virus (Perez *et al.*, 2001). In this study, recombinant replication-incompetent VSV were pseudotyped to characterize HEF, the major spike protein of influenza C virus. It was demonstrated that native HEF is efficiently incorporated into VSV particles resulting in infectious pseudotypes. This finding indicates that the shortness of the HEF cytoplasmic domain, which is predicted to consist of only three amino acids, is not critical for glycoprotein uptake. In contrast, the homotypic VSV-G protein has been shown to lose its capacity to complement a VSV mutant with a temperature-sensitive VSV-G protein when the cytoplasmic domain of 29 amino acids was shortened to three amino acids (Whitt *et al.*, 1989). Using VSV-G proteins with truncated cytoplasmic domains and cytoplasmic domains from heterologous proteins, Schnell *et al.* (1998) reasoned that a non-specific glycoprotein cytoplasmic domain sequence of between one and nine amino acids is required to drive efficient budding of VSV. Our study shows that, at least in the case of HEF, a cytoplasmic domain of three amino acids is sufficient for efficient assembly of VSV. Uptake of HEF was not improved if the HEF cytoplasmic domain was replaced by the corresponding VSV-G domain. Likewise, chimeric HEF glycoprotein containing the VSV-G transmembrane and cytoplasmic domains was incorporated into VSV particles with the same efficiency as native HEF. These results confirm previous studies showing that VSV-G does not contain any specific sequences in its C-terminal domain that specify glycoprotein incorporation (Robison & Whitt, 2000; Schnell *et al.*, 1996a). Nevertheless, VSV-G was shown to be incorporated into VSV particles with significantly higher efficiency than any foreign viral glycoprotein. An explanation for this phenomenon was provided by a study showing that the membrane proximal stem region of VSV-G confers efficient virus assembly (Robison & Whitt, 2000). It has been postulated that the VSV-G stem region induces membrane curvature at sites where budding occurs (Robison & Whitt, 2000). However, incorporation of chimeric HEF was not significantly improved when this VSV-G stem domain was introduced. It is speculated that the HEF stem region per se meets the conformational requirements for induction of membrane curvature, so that insertion of the VSV-G stem region did not further improve glycoprotein uptake. Previous work has shown that the cellular protein CD4 or chimeric VSV-G containing the CD4 cytoplasmic domain are efficiently incorporated

into recombinant VSV (Schnell *et al.*, 1996a, 1998). A different result was obtained when the CD4 cytoplasmic domain was linked to HEF (HEF–CD4); this glycoprotein was incorporated into VSV particles with lower efficiency than native HEF or either of the HEF/VSV-G chimeras. The reason for this phenomenon is not exactly clear. It may be that HEF–CD4 adopts a different conformation that is suboptimal for uptake into virus particles.

A striking result of our studies is that chimeric HEF glycoproteins containing C-terminal domains from the VSV-G glycoprotein were efficiently transported to the cell surface and incorporated into VSV particles but, unlike authentic HEF, did not mediate efficient infection of the pseudotypes. Loss of infectivity was also observed when the cytoplasmic domain of the CD4 molecule was fused to HEF indicating that the effect does not depend on the sequence of the cytoplasmic tail. Our finding that the chimeric HEF glycoproteins tested positive for esterase and receptor-binding activities argues for the possibility that the fusion activity of these glycoproteins might not be functional. Indeed, observations pointing in this direction have been made with other viral fusion proteins, in which truncations or elongations of the respective cytoplasmic domains were shown to have profound effects on fusion activity (Bagai & Lamb, 1996; Ohuchi *et al.*, 1998; Tong *et al.*, 2002).

Our finding that native HEF is able to mediate infection of VSV pseudotypes implies that, to perform this function, HEF does not rely on the assistance of CM2, the putative ion channel protein of influenza C virus (Hongo *et al.*, 2004). Since HEF is proteolytically activated by trypsin-like proteases, cleavage does not occur before the glycoprotein reaches the cell surface. Therefore, HEF passes the acidic Golgi compartment without the risk of early low-pH-triggered conformational change. CM2 might be involved in entry of influenza C virus by facilitating dissociation of the matrix protein and the ribonucleoprotein complex. It appears that this activity is not relevant in the context of a VSV pseudotype infection. However, pseudotyping of VSV might be a useful system for further characterization of HEF. Questions which can now be addressed are the role of the esterase activity in virus entry, the function of post-translational modifications and the effects of mutations on receptor affinity and cell tropism.

The present study suggests that the HEF glycoprotein of influenza C virus might be an interesting tool for pseudotyping viral vectors. In particular, the ability of HEF to mediate apical infection of polarized epithelial cells is an important aspect, as many viral vector systems commonly used in gene therapy, including retroviruses (Wang *et al.*, 1998, 2002), adenoviruses (Zabner *et al.*, 1997; Pickles *et al.*, 1998; Kitson *et al.*, 1999; Walters *et al.*, 1999), adeno-associated virus (Duan *et al.*, 1998; Bals *et al.*, 1999) and vaccinia virus (Rodriguez *et al.*, 1991), have been recognized to be rather ineffective in transduction of polarized epithelial cells from the apical side. Future studies will show whether it is feasible to use HEF for pseudotyping of retroviral and lentiviral vectors. The selective binding of HEF to glycoconjugates containing 9-*O*-acetylated sialic acids is also an interesting aspect, as 9-*O*-acetylation has been shown to be a tissue-specific and developmentally regulated modification of sialic acids (Varki, 1992; Herrler *et al.*, 1987). The use of HEF for pseudotyping of viral vectors might therefore allow specific cell subsets to be targeted.

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