Short Communication

Comparison of vesicular stomatitis virus pseudotyped with the S proteins from a porcine and a human coronavirus

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The surface proteins S of severe acute respiratory syndrome coronavirus (SARS-CoV) and transmissible gastroenteritis virus (TGEV) were compared for their ability to mediate infection of viral pseudotypes based on vesicular stomatitis virus (VSV). The cell tropism of the respective pseudotypes corresponded to the tropism of the viruses from which the S protein was derived. Higher infectivity values were obtained with the SARS-CoV S protein than with the TGEV S protein. Differences were observed with respect to the importance of the cytoplasmic tail and the membrane anchor of the S proteins. In the case of the SARS-CoV S protein, truncation of the cytoplasmic tail resulted in increased infectivity. For the TGEV S protein, the inactivation of an intracellular retention signal in the cytoplasmic tail was required. Exchange of the membrane anchor of the S proteins led to a low infection efficiency. Our results indicate that related glycoproteins may show substantial differences in their ability to mediate pseudotype infection.

Surface proteins of enveloped viruses play an important role in the infection of target cells. They mediate the attachment of virions to the cell surface and the subsequent fusion of the viral and the cellular membrane. A convenient way to analyse the first steps during a viral life cycle is the use of viral pseudotypes where the viral surface protein of interest is incorporated into the envelope of an unrelated virus. When the helper virus is defective, allowing virus entry but not the release of infectious virus, pseudoviruses provide a safe tool to analyse viral proteins of highly pathogenic viruses under conventional biosafety conditions. Furthermore, results obtained for a surface protein of an enveloped virus are not affected by other proteins of this virus. Most pseudotype systems are based on lentiviruses or vesicular stomatitis virus (VSV), a member of the family *Rhabdoviridae*. The generation of replication-incompetent VSV in which the open reading frame for the VSV-G glycoprotein is replaced by that for the green fluorescent protein (GFP) facilitates the readout of the VSV system (Takada *et al.*, 1997; Fukushi *et al.*, 2005; Hanika *et al.*, 2005). Other reporter systems depend on enzymes such as luciferase to demonstrate infection of target cells (Chan *et al.*, 2000). The latter enzyme makes the assay very sensitive, although it does not indicate the number of infected cells. Because of the high sensitivity of the pseudotype system it can even be used for viral glycoproteins that are generally not transported to the budding site of helper virus. The E1 and E2 glycoproteins of hepatitis C virus and pestiviruses are intracellularly retained at a pre-Golgi compartment; although VSV matures by a budding event at the plasma membrane, VSV pseudotypes have been used to analyse the entry process mediated by the E proteins (Matsuura *et al.*, 2001; Wang *et al.*, 2004a; Ronecker *et al.*, 2008). However, there is no comparative analysis of how different transport behaviour or other structural features affect the efficiency of pseudotype formation.

In coronaviruses (CoV), glycoprotein S is the major surface protein and is responsible for receptor binding and fusion of the viral membrane with cellular membranes. In addition, it is the main target for neutralizing antibodies elicited in the infected host. SARS-CoV, the aetiological agent of severe acute respiratory syndrome, uses angiotensin-converting enzyme 2 (ACE2) as a receptor to infect

A supplementary table showing the primers used in this study is available with the online version of this paper.

cells (Li *et al.*, 2003; Wang *et al.*, 2004b). Porcine aminopeptidase N is known to serve as a receptor for transmissible gastroenteritis virus (TGEV), a porcine coronavirus (Delmas *et al.*, 1992). In addition, this virus has a sialic acid binding activity which contributes to the enteropathogenicity in swine (Krempl *et al.*, 1997, Schwegmann-Weßels *et al.*, 2002, 2003). Interestingly, the S proteins of TGEV and SARS-CoV differ in their intracellular transport behaviour. Whereas the surface protein of the former virus is intracellularly retained at a pre-Golgi compartment due to a tyrosine-based motif (Tyr-Xxx-Xxx-Ile) in the cytoplasmic tail, the SARS-CoV S protein is transported to the plasma membrane (Schwegmann-Weßels *et al.*, 2004; Simmons *et al.*, 2004).

We and others have analysed the role of the S protein of SARS-CoV in the initial stage of infection using VSV or lentiviral pseudotype systems (Wang *et al.*, 2004b; Giroglou *et al.*, 2004; Ren *et al.*, 2006; Glende *et al.*, 2008; Watanabe *et al.*, 2008).

Here we compared VSV pseudotyped with different coronavirus S proteins. For this purpose, we constructed plasmids for expression of S proteins that lacked the intracellular retention signal either by two-point mutations in the above-mentioned motif [TGEV-S(YI/AA)] or by deletion of the last 14 aa (TGEV-Sdel14). In addition, we generated two chimeric TGEV S proteins bearing a short stem region, membrane anchor and cytoplasmic tail (TGEV-SGG) or only the cytoplasmic tail of the VSV G protein (TGEV-SSG). For comparison, similar constructs were generated for the SARS-CoV S protein (SARS-SGG, SARS-SSG, SARS-Sdel18) as shown in Fig. 1(a). The open reading frames of the S genes of SARS-CoV (strain CUHK-W1) and TGEV (strain PUR-46-MAD) were cloned into vector pCG1 (kindly provided by Roberto Cattaneo, Mayo Clinic Rochester, Rochester, MN, USA) as described previously (Schwegmann-Weßels *et al.*, 2006). Generation of the expression plasmid pcDNA3.1-VSVG encoding the envelope protein of VSV (strain Indiana) has been reported (Hanika *et al.*, 2005). The open reading frames are identical to the published sequences (GenBank accession numbers AY278554, M94101 and NC_001560). The SARS-CoV S deletion mutant SARS-Sdel18 and chimeric proteins (SARS-SSG, SARS-SGG) as well as the TGEV S deletion mutant TGEV-Sdel14, double mutant TGEV-S(YI/AA) and chimeric proteins (TGEV-SSG, TGEV-SGG) were constructed by using the primers listed in Supplementary Table S1, available in JGV Online. For the generation of the double mutant and chimeric proteins an overlapping PCR technique was used as described previously (Schwegmann-Weßels *et al.*, 2004).

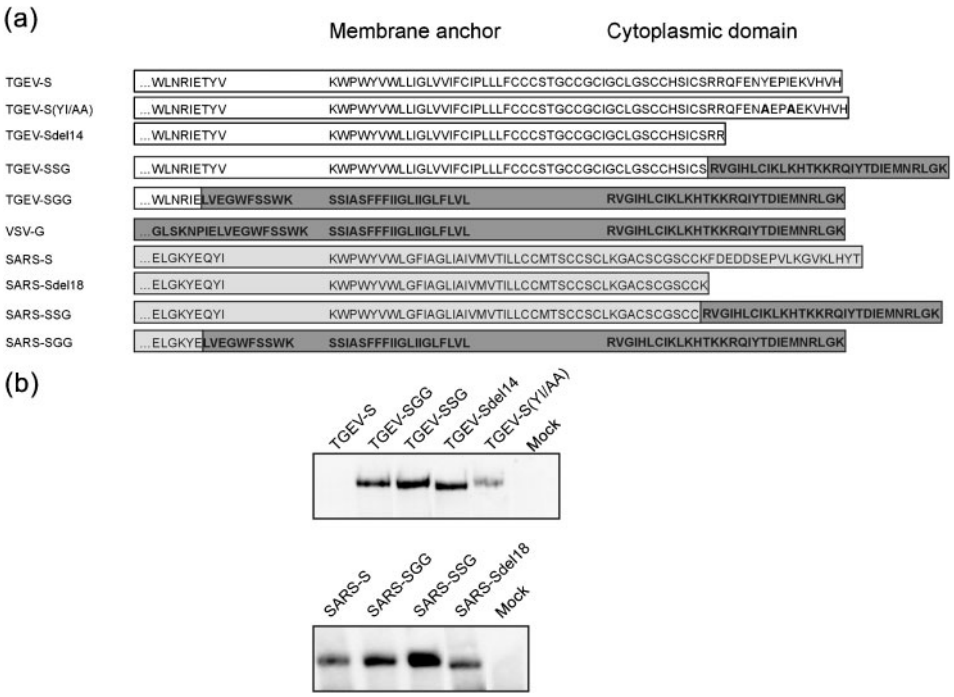

Fig. 1. (a) C-terminal amino acid sequences of parental and chimeric coronavirus S proteins as well as the VSV G protein. Amino acids derived from the TGEV S protein are shown in white boxes. The mutated amino acids in TGEV-S(YI/AA) are indicated in bold letters. The VSV G protein is shown in bold letters within dark grey boxes. The SARS-CoV S protein sequence is shown in light grey boxes. (b) Surface expression of TGEV S protein, chimeras and mutants (upper panel) and SARS-CoV S protein, chimeras and deletion mutant (lower panel). At 24 h post-transfection, cells were subjected to surface biotinylation and immunoprecipitation.

The different S proteins were analysed for intracellular expression and cell surface transport 24 h after transfection of BHK21 cells. For analysis of cell surface expression, the cells were biotinylated and the viral antigens were immunoprecipitated from cell lysates as described previously (Zimmer *et al.*, 2001; Schwegmann-Weßels *et al.*, 2004). For immunoprecipitation of the TGEV S proteins, the monoclonal antibody 6A.C3 (Gebauer *et al.*, 1991), and for immunoprecipitation of the SARS-CoV S proteins, a polyclonal SARS-CoV antiserum (kindly provided by M. Eickmann, Philipps-University, Marburg, Germany), were used.

All S proteins were expressed in transfected cells (data not shown) and, with the exception of the parental TGEV S protein, all were detected on the cell surface (Fig. 1b). The double mutant TGEV-S(YI/AA) was also transported to the cell surface, although the band was weaker when compared with the other TGEV S mutants (Fig. 1b).

The different S proteins were used to create replication-incompetent VSV pseudotypes as described previously (Hanika *et al.*, 2005). For the preparation of SARS-CoV S-pseudotyped VSV (VSV*ΔG-SARSCoV-Sdel18, -S, -SSG, -SGG), TGEV S-pseudotyped VSV [VSV*ΔG-TGEV-Sdel14, -S, -SSG, -SGG, -S(YI/AA)] and the control VSV*ΔG-G, BHK21 cells grown on six-well plates were transfected with 4 μg of the corresponding expression plasmid and 10 μl Lipofectamine 2000 transfection reagent (Invitrogen). The next day, cells were inoculated with VSV*ΔG-G grown on BHK-G43 cells and, after adsorption for 1 h at 37 °C, the inoculum was replaced by medium containing a polyclonal rabbit anti-VSV serum to neutralize the helper virus. Following incubation for 24 h at 37 °C in the presence of 3 % FCS, the cell culture supernatants were harvested and cell debris was removed by low-speed centrifugation.

For the VSV pseudotype infection analysis different cell lines were seeded in 96-well plates. After incubation for 24 h, VSV pseudotypes (50 μl) were added onto the target cells in tenfold dilutions. Cells were fixed with 3 % paraformaldehyde 20–24 h after infection. GFP-expressing cells per well (per 50 μl inoculum) were counted using a fluorescence microscope (Zeiss Axiophot 2). Wells with undiluted virus were photographed for documentation.

As shown in Fig. 2(a) for the deletion mutant Sdel18, the S protein of SARS-CoV mediated infection of VeroE6 cells, whereas the two porcine cell lines, PK15 and LLC-PK1, as well as BHK21 cells were resistant to infection. This finding is consistent with previous reports that human and simian ACE2 can serve as receptors for SARS-CoV (Li *et al.*, 2003; Wang *et al.*, 2004b). The corresponding deletion mutant of the TGEV S protein was able to mediate infection of the two porcine cell lines, but not infection of VeroE6 or BHK21 cells. This result shows that the pseudotype system reflects the tropism of TGEV which recognizes porcine aminopeptidase N as a receptor, but not the corresponding enzyme from other species (Delmas *et al.*, 1992). In contrast to the restricted tropism of the coronavirus S proteins, VSV-G protein mediated infection of all four cell lines analysed. VSV pseudotypes created with the spike proteins of TGEV and SARS-CoV as well as the VSV-G control were neutralized by antibodies directed against the respective surface protein, but not by the heterologous antiserum (data not shown).

When the infectivity was determined by counting the number of infected cells, the SARS-Sdel18 mutant was most efficient among all S proteins analysed in mediating infection. As shown in Table 1, a value of about 5×10^5 infective units (IU) ml^{-1} was determined for the deletion mutant, which was tenfold lower than that obtained with the VSV-G protein. The infectivity of pseudotypes containing the deletion mutant of TGEV-S protein (TGEV-Sdel14) was about 2×10^4 IU ml^{-1} in the two porcine cell lines, which is a 20-fold reduction compared with the value determined for the SARS-Sdel18 mutant in VeroE6 cells. In relation to the infectivity mediated by VSV-G protein, the TGEV-Sdel14 mutant is 40- (LLC-PK1 cells) to 150-fold (PK15 cells) less efficient. This result shows that the S proteins of different coronaviruses may differ in their ability to mediate infection by VSV pseudotypes.

The other mutant proteins (Fig. 1a) were also analysed and the infectivity was expressed in relation to the deletion mutants (100 %). Fig. 2(b) shows the mean values of three different experiments. As reported by several groups, the parental S protein of SARS-CoV is much less efficient than deletion mutants with a truncated cytoplasmic tail in mediating infection of pseudotypes (Giroglou *et al.*, 2004; Fukushi *et al.*, 2005). In our case, the reduction in infectivity was about 98 %. A similar reduction was obtained when chimeric proteins containing the membrane anchor and/or the cytoplasmic tail of the VSV-G protein (SARS-SSG, SARS-SGG) were analysed. In the case of the TGEV S protein, the efficiency of the parental protein is also only about 2 % when compared with the deletion mutant TGEV-Sdel14. However, the difference appears to be mainly due to the intracellular retention of this protein. The mutant proteins TGEV-S(YI/AA) and TGEV-SSG, which lack the intracellular retention signal but retain a cytoplasmic tail, only show a reduction of about 47 and 63 %, respectively. On the other hand, the chimeric protein with both the cytoplasmic tail and membrane anchor derived from the VSV-G protein (TGEV-SGG) is very inefficient in mediating pseudotype infection, with a value lower than 1 % compared with the deletion mutant. These results indicate that the cytoplasmic tail affects the S proteins of TGEV and SARS-CoV to a different extent in their ability to mediate infection by VSV pseudotypes.

Our comparison revealed that glycoproteins from viruses within the same family may differ in their efficiency to mediate infection when analysed in a pseudotype system. To some extent, the variation may depend on the cell type used for the experiment. The differences in the infectivity

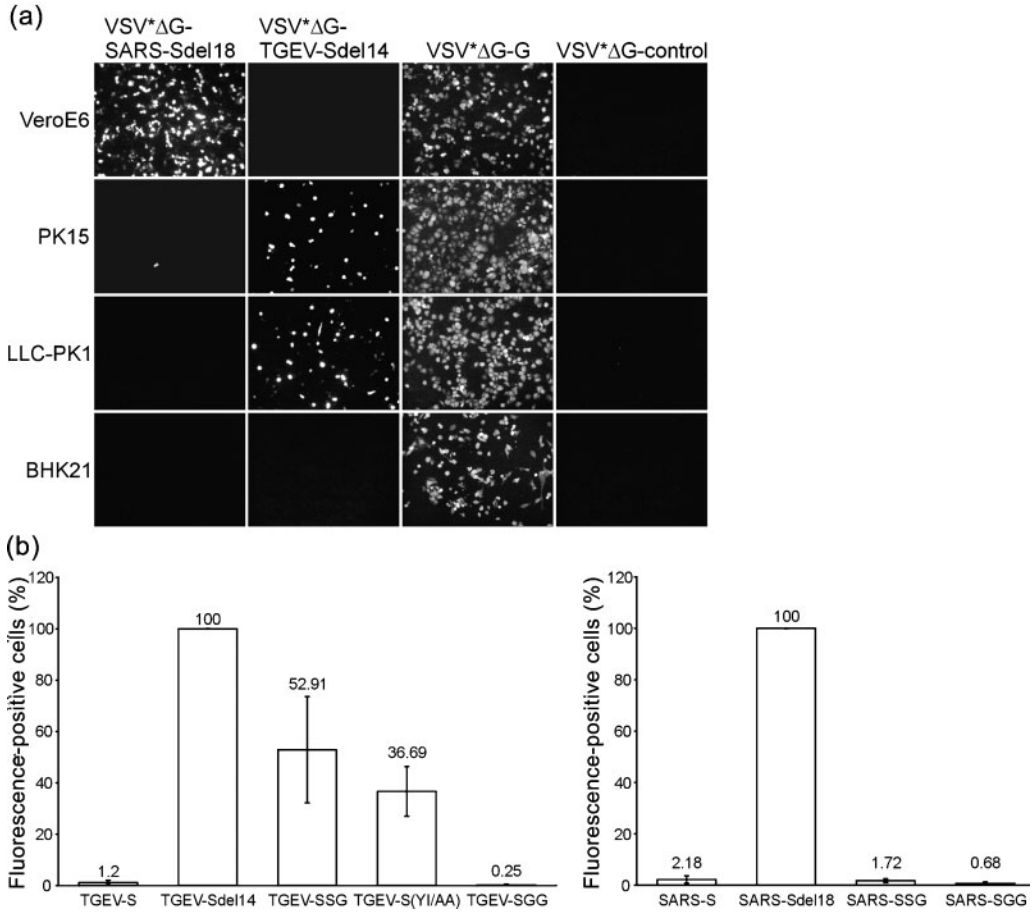

Fig. 2. (a) Different cell lines were infected with VSV pseudotypes bearing the indicated proteins of SARS-CoV (VSV*ΔG-SARS-Sdel18), TGEV (VSV*ΔG-TGEV-Sdel14), VSV (VSV*ΔG-G) or as a negative control with VSV pseudotypes without glycoprotein (VSV*ΔG-control). GFP expression of infected cells was photographed by a fluorescence microscope. (b) Infection of cells by VSV pseudotypes containing coronavirus S proteins. Parental TGEV S, chimeric and mutant proteins were used for the infection of LLC-PK1 cells (left panel). VeroE6 cells were used for the infection experiment with parental SARS-CoV S, chimeric proteins and the deletion mutant (right panel). The different glycoproteins used for VSV pseudotyping are indicated on the *x*-axis. Infected GFP-expressing cells were counted under a fluorescence microscope.

values between the TGEV-Sdel14 mutant and the VSV-G protein were larger in PK15 cells than in LLC-PK1 cells. Structural features of the S proteins also contribute to the different infectivity values. Increased infection efficiency after truncation of the cytoplasmic tail has been observed for both the TGEV and SARS-CoV S proteins. An

Table 1. Infectivity of VSV*ΔG pseudotypes on different cell lines

Titres are mean values of three different titrations (two for PK15 cells).

Cell line	Infectious titre (IU ml^{-1})*		
	VSV*ΔG-TGEV-Sdel14	VSV*ΔG-SARS-Sdel18	VSV*ΔG-G
VeroE6	<40	4.8 ($\pm$1.7) $\times$ 10^5	5.4 ($\pm$2.6) $\times$ 10^6
LLC-PK1	2.5 ($\pm$0.2) $\times$ 10^4	<40	9.9 ($\pm$3.3) $\times$ 10^5
PK15	2.2 ($\pm$1.4) $\times$ 10^4	140 ($\pm$10)	3.8 ($\pm$0.9) $\times$ 10^6
BHK21	<40	<40	6.0 ($\pm$4.5) $\times$ 10^6

*Infectious titres were determined by counting GFP-expressing cells.

explanation for this finding may be that an S protein with a shorter tail is easier to package into VS virions or that truncation of the tail increases the biological activity of the S protein. For other viral glycoproteins, it has been shown that truncation of the cytoplasmic tail may increase fusion activity (Yang & Compans, 1996).

A striking difference between the S proteins of TGEV and SARS-CoV is the intracellular transport behaviour. As the surface protein of the former virus is intracellularly retained, it is not present at the plasma membrane where VSV matures by a budding process. Therefore, it is not surprising that pseudotypes with the parental TGEV-S protein have an infectivity that is 99 % lower than that of the deletion mutant. Pseudotypes have been used also to analyse other viral proteins that are intracellularly retained, e.g. the E proteins of hepatitis C virus or pestiviruses, although the low infectivity required efficient reporter enzymes (Hsu *et al.*, 2003; Wang *et al.*, 2004a; Ronecker *et al.*, 2008). The negative effect of intracellular retention on the ability of the S protein on pseudotype formation is evident most convincingly from the mutants which have lost the intracellular retention motif by point mutations or after replacement of the tail by a heterologous sequence. Infectivity of the pseudotypes containing the TGEV-SSG or the TGEV-S(YI/AA) mutant was reduced only by 47 or 67 %, respectively. This result indicates that the length of the tail is less detrimental for the TGEV S protein than it is for the SARS-CoV-S protein in mediating pseudotype infection. In the case of TGEV-S, the cytoplasmic tail appears to be no major problem for packaging the protein into VSV particles.

The infectivity of S protein-pseudotyped VSV decreased strongly when the SGG mutant of the TGEV S protein and the SGG mutant of the SARS-CoV S protein was used. These results are consistent with the findings by Broer *et al.* (2006). This group reported that the transmembrane domain of the SARS-CoV S protein is essential for the infectivity of SARS-CoV S-pseudotyped retrovirus and important for S trimer stability (Broer *et al.*, 2006).

Taken together, these results show that surface glycoproteins from viruses within the same family may vary to a substantial extent in their efficiency to mediate infection by VSV pseudotypes. These results will help in future studies to analyse the biological activity of the S protein independently of other coronavirus proteins. Our findings should be helpful also for other surface glycoproteins to estimate their suitability for analysis by viral pseudotypes.

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