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A lysine-methionine exchange in a coronavirus surface protein transforms a retention motif into an endocytosis signal

Abstract: Transmissible gastroenteritis virus (TGEV) is an enveloped (+) RNA virus belonging to the family *Coronaviridae*. Among the viral membrane proteins, the spike (S) protein mediates receptor recognition/attachment to the host cell and fusion of viral and cellular membranes. The cytoplasmic tail of the S protein contains a tyrosine-dependent sorting signal with the consensus sequence YXXΦ. In the context of the S protein of TGEV (1440YEPI1443), this motif acts as a retention signal, preventing surface expression of the protein. Here, we show that a chimeric S protein, containing the six C-terminal amino acids of the glycoprotein G of vesicular stomatitis virus (VSV) is no longer retained intracellularly, despite the presence of the tyrosine tetrapeptide motif. Following transport to the cell surface, the chimeric protein was rapidly endocytosed. Analysis of mutant proteins generated by site-directed mutagenesis revealed that a single amino acid exchange (1445K/M, position: +2 downstream of the tyrosine-based motif) was responsible for the altered sorting behavior.

Keywords: protein sorting; protein transport; TGEV S protein; tyrosine-based sorting signal; VSV G protein.

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Introduction

Transmissible gastroenteritis virus (TGEV) is a positive-stranded RNA virus that belongs to the family *Coronaviridae*, genus *Alphacoronavirus*. It causes respiratory and enteric disease in swine of all ages and is exceptionally

severe in newborn animals. Mortality in these animals can reach 100% (Enjuanes and van der Zeijst, 1995). Three membrane proteins have been described for TGEV: the membrane protein (M), the small membrane protein (E) and the spike protein (S). The E protein is required for virus morphogenesis and maturation (Fischer et al., 1998; Ortego et al., 2007). The M protein plays an important role during virus assembly and interacts with the E, S, and nucleocapsid proteins (Opstelten et al., 1995; Vennema et al., 1996; Narayanan et al., 2000; Escors et al., 2001). The S protein is the major inducer of virus-neutralizing antibodies and is crucial for virus entry (Godet et al., 1991, 1994). It mediates virus attachment by interacting with porcine aminopeptidase N, the cellular receptor protein for TGEV, and fusion of the viral membrane with the host cell membrane (Delmas et al., 1992; Gallagher and Buchmeier, 2001; Schwegmann-Wessels et al., 2002, 2011). The budding event during the coronavirus replication cycle takes place at a pre-Golgi compartment, the endoplasmic reticulum Golgi intermediate compartment (ERGIC) (Tooze et al., 1984; Krijnse-Locker et al., 1994). Newly synthesized viral structural proteins accumulate at the site of budding and are incorporated together with the viral genome into viral particles, which are then translocated to the plasma membrane and released via exocytosis. Intracellular sorting of coronavirus genomes and proteins to the site of virus budding requires strict regulatory mechanisms to achieve optimal virus production. The precise regulation of viral and cellular factors involved in these processes is not fully understood.

Tyrosine-based signals are involved at different sites in the cellular sorting machinery. Previously we have demonstrated the importance of an YXXΦ-motif in the S proteins of two representatives of alpha- and gammacoronaviruses, the transmissible gastroenteritis coronavirus (TGEV) of swine and the infectious bronchitis virus (IBV) of chicken. The TGEV S protein as well as the IBV S protein were found to be intracellularly retained because of a tyrosine-based signal (Schwegmann-Wessels et al., 2004; Winter et al., 2008). This motif in the C-terminal part of

the TGEV S protein (1440YEPI1443) corresponds to YXXΦ (X can be any amino acid, Φ denotes a bulky hydrophobic amino acid) motifs that have been described to be responsible for basolateral sorting, lysosomal targeting, localization to the trans-Golgi network or endocytosis in other cellular and viral proteins (Howe et al., 1988; Granger et al., 1990; Letourneur and Klausner, 1992; Thomas and Roth, 1994; Rohrer et al., 1996). In the case of CD3-ε, a tyrosine-containing tetrapeptide has also been described as a signal for intracellular retention (Mallabiabarrena et al., 1992).

The glycoprotein G of vesicular stomatitis virus (VSV), a negative-stranded ssRNA virus, also harbors a carboxy-terminal targeting signal of six amino acids (⁵⁰¹YTDIEM⁵⁰⁶), which is important for efficient export of the G protein from the ER to the Golgi complex (Sevier et al., 2000). This sequence contains the above mentioned YXXΦ consensus sequence as well as a di-acidic DXE motif (Nishimura and Balch, 1997; Nishimura et al., 1999).

The aim of this study was to analyze the requirements that lead to different transport behavior of two distinct viral proteins even though they both contain tyrosine-based sorting signals in similar positions of their cytoplasmic domain. We focused on amino acids downstream of the tyrosine-based sorting signal and their influence on intracellular sorting. Chimeric proteins of S and G were constructed and were analyzed for surface transport or intracellular retention. Our findings show that a Lys/Met1445 exchange in the TGEV S protein abolished intracellular retention and resulted in a protein that is transported to the plasma membrane and is subsequently endocytosed.

Results

We were interested to know whether amino acids other than the tyrosine and the isoleucine in the 1440YEPI1443 motif of the TGEV S protein are important for its localization and transport inside the cell. The G protein of VSV contains a similar tyrosine-based signal that is located at a similar position in the protein close to the carboxy-terminus of the cytoplasmic tail. Nevertheless, the VSV G protein is transported to the cell surface and not retained intracellularly (Wehland et al., 1982; Stephens et al., 1986). The tyrosine-based sorting signal in the VSV G protein is described to be responsible for basolateral transport (Thomas and Roth, 1994). In addition, a di-acidic signal nearby was shown to be important for ER-export (Nishimura and Balch, 1997). We wondered whether the

amino acids in the proximity may affect the function of the tyrosine-based motif as a sorting signal. We constructed different TGEV S protein mutants that contained some of the VSV G protein amino acids downstream of the tyrosine motif. A schematic overview of the generated mutants is shown in Figure 1. The SG6 mutant in which the five carboxyterminal amino acids of the TGEV S protein were replaced by the six carboxyterminal amino acids of the VSV G protein differed from the parental TGEV S protein by a clear surface staining when analyzed by immunofluorescence microscopy (Figure 2A). To narrow down the amino acids responsible for the differential transport behavior of the SG6 mutant, the mutant SG-KVH/MNR was generated in which the VSV G tripeptide Met-Asn-Arg replaced the Lys-Val-His sequence in the TGEV S protein (position 1445 to 1447). In addition, we replaced the last two amino acids from the TGEV S protein (Val-His) by the last three of the VSV G protein (Leu-Gly-Lys), resulting in the mutant SG-VH/LGK. The latter mutant behaved like the parental TGEV S protein and was retained intracellularly. By contrast, the mutant SG-KVH/MNR – like the SG6 mutant – was detected at the plasma membrane (Figure 2A). Finally, point mutants were generated (Figure 1). Among them, only the S-K/M mutant in which

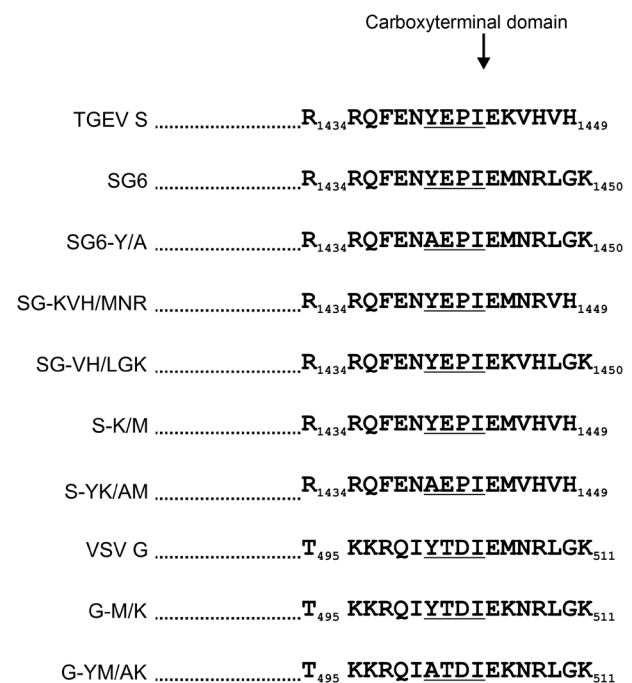


Figure 1 Schematic drawing of TGEV S/VSV G chimeras as well as TGEV S and VSV G parental and mutant proteins. The C-terminal part of the cytoplasmic domain of the TGEV S and the VSV G protein sequence is shown. The tyrosine-based sorting signal is underlined.

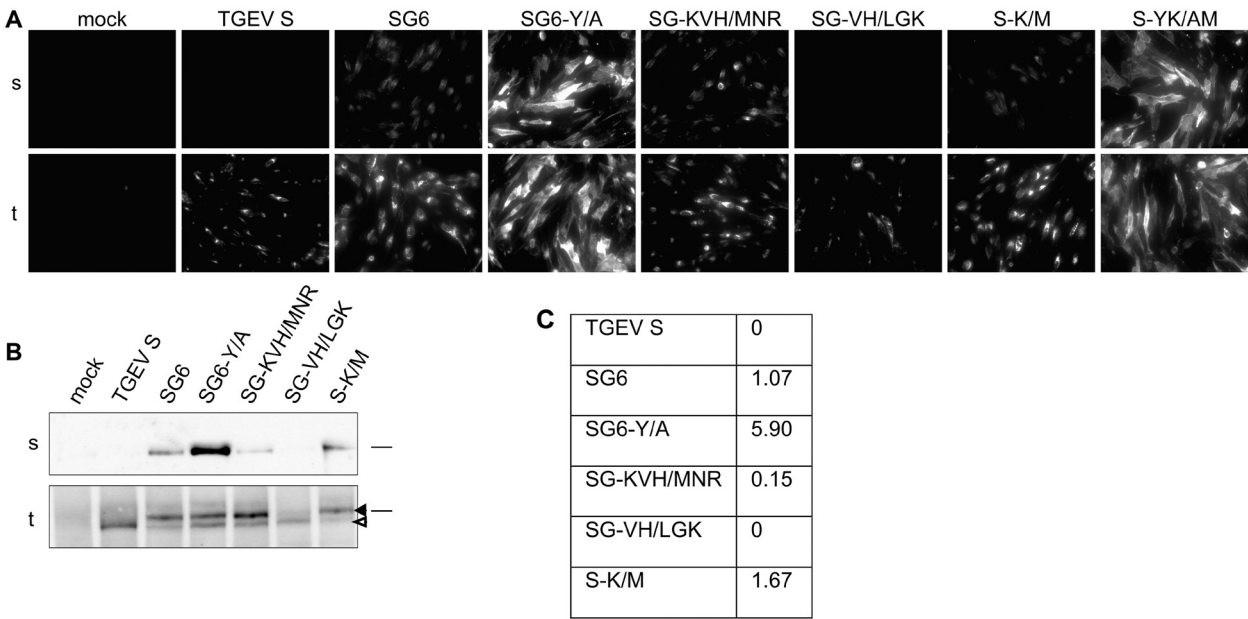


Figure 2 Surface and total protein expression of parental TGEV S, mutant S proteins, and TGEV S/VSV G chimeras. BHK21 cells were transfected, with the pCG1 expression plasmid containing the gene indicated or with no insert (mock). (A) Cells were analyzed for surface (s) and total (t) expression of the indicated proteins at 24 h post-transfection by immunofluorescence microscopy. (B) BHK21 cells were transfected with the pCG1 expression plasmid containing either no insert (mock) or one of the genes indicated. At 24 h post-transfection, cells were prepared for surface biotinylation. One part of the lysates was used for total protein expression analysis in a Western blot (t). The other part of the same samples was used for surface expression analysis (s) after immunoprecipitation of the proteins and detection of the biotin ligand with streptavidin peroxidase. The black arrow indicates the complex glycosylated form whereas the white arrow indicates the mannose-rich form of the S protein. The black bar shows the position of the 250 kDa band of the prestained protein ladder. (C) Relative amounts of surface-expressed proteins shown in Figure 2B.

Lys1445 of the TGEV S protein was replaced by a methionine behaved like the SG6 mutant, i.e., it was transported to the cell surface. In case of the SG6 and the S-K/M mutant protein, we additionally generated a tyrosine to alanine mutation resulting in the mutants SG6-Y/A and S-YK/AM. These mutant proteins showed a bright expression at the cell surface when analyzed by immunofluorescence microscopy (Figure 2A). In this case, antibody distribution and binding seems to be more efficient than in the other analyzed proteins. The result of the immunofluorescence assay was confirmed when the mutant proteins were surface-biotinylated and subsequently analyzed by immunoprecipitation and Western blot (Figure 2B, upper panel). All proteins containing the Lys1445Met mutation were detected on the cell surface. The relative amount of surface-expressed S proteins was determined using Image J (Figure 2C). The SG6-Y/A mutant protein showed a five times higher surface expression than the SG6 protein with an intact tyrosine motif. A Western blot analysis of the total amount of expressed proteins showed that all proteins were properly expressed (Figure 2B, lower panel). The S proteins that were transported to the cell surface

were expressed in two forms as indicated by an upper and a lower band (black and white arrow). With these proteins the upper band was most prominent, whereas the lower band was predominant in intracellularly retained proteins (TGEV S, SG-VH/LGK). The upper band most likely represents proteins with complex N-glycans, whereas proteins containing high-mannose type oligosaccharides are expected in the lower band. With those S protein mutants that are transported to the cell surface, the mannose-rich oligosaccharides that are added in the ER are converted into complex glycans during passage through the Golgi apparatus. The latter type of glycosylation increases the molecular weight and results in a slower migration behavior during SDS-PAGE. A similar transport behavior has been found with chimeric S proteins, where the cytoplasmic tail of TGEV has been replaced by that of the F protein of Sendai virus compared to authentic TGEV S protein; here the identification of the upper and lower bands as proteins with complex or high-mannose glycans, respectively, has been confirmed by analysis of their sensitivity to treatment with endoglycosidase H (Schwegmann-Wessels et al., 2004).

As the Lys1447Met exchange resulted in surface expression of this mutant protein we analyzed whether other amino acids at this position also affect the transport of the S protein. Changes of lysine to alanine (hydrophobic), arginine (basic) or glutamic acid (acidic) did not result in surface expression of the respective mutant proteins. They were all intracellularly retained as determined by immunofluorescence analysis as well as by surface biotinylation experiments (data not shown).

We analyzed the intracellular localization of the S-K/M mutant in more detail. For this purpose, ER and ERGIC markers were co-expressed together with the S protein. Both the parental TGEV S protein as well as the K/M mutant protein barely co-localized with the ER marker, but the parental viral protein co-localized to a large extent with the ERGIC marker (Figure 3A). The mutant S-K/M showed some colocalization with the ERGIC as well, but

the majority of the protein was distributed in a vesicle-like pattern all over the cytoplasm (Figure 3B). This result suggests that the tyrosine-based signal in the cytoplasmic tail of TGEV S that retains the protein in the ERGIC compartment can be suppressed by a methionine at position 1445 in such a way that the S protein is transported in the secretory pathway beyond the ERGIC and Golgi compartments.

As the K/M mutant was distributed in vesicular structures, we investigated whether the tyrosine-based signal in this mutant protein functions as an endocytosis signal. For this purpose, we performed an antibody uptake assay. S protein specific antibodies were bound to S-K/M-expressing cells and incubated for 10 min at 37°C. A substantial amount of the mutant protein was detected in endocytotic vesicles that could be stained after permeabilization of the cells (Figure 4A, second line, green color in the merged image). No endocytosed vesicular structures were observed when the cells were incubated at 4°C, where endocytosis was prevented. The punctuated pattern on the cells at 4°C was detectable by staining the proteins on the cell surface and does therefore not represent endocytotic vesicles. In this study we included the mutant S-YK/AM which in addition to the K1445M mutation contained a Y1440A mutation, which inactivated the Y-X-X-Φ motif. This mutant protein did not show any intracellular vesicles after incubation for 10 min at 37°C. This result suggests that the tyrosine-based retention signal functions as an endocytosis signal when its function as a retention signal is abolished by inserting a methionine at position 1445. To confirm these results, endocytosis was also analyzed by a biotin internalization assay. The SG6-Y/A mutant served as a negative control in this assay (Figure 1). It behaves like the S-YK/AM mutant as far as the transport to the cell surface is concerned (Figure 2A). Figure 4B shows that all three mutants were expressed at the plasma membrane (4°C, -MESNA) and that SG6 and S-K/M were efficiently endocytosed after incubation at 37°C for 10 min (37°C, +MESNA). Endocytosis of S-K/M was already observed at the earliest after 2 min at 37°C and increased over time (data not shown). In contrast, the SG6-Y/A mutant was not endocytosed as it lacks a tyrosine-based internalization motif (Figure 4B, 37°C). The endocytosis rate for SG6 and S-K/M was determined from three independent experiments. The amount of endocytosed protein varied between the different experiments with a peak at an endocytosis time of 10 min. Both proteins showed an average endocytosis rate of about 7%/min (SG6: 7.2%/min; S-K/M: 7.6%/min). This indicated that other VSV G derived amino acids in the SG6 protein (compared to the S-K/M) did not have an enhancing effect on endocytosis.

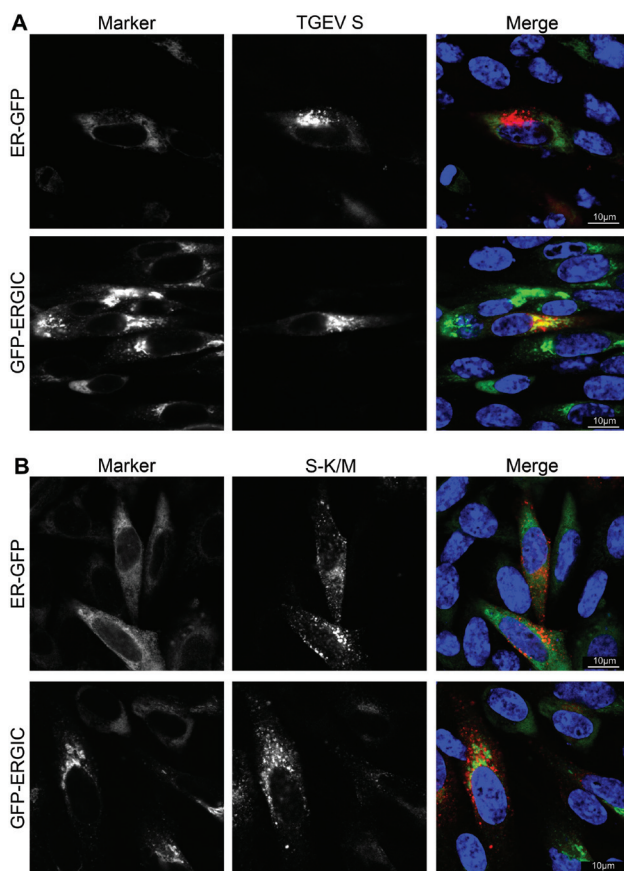


Figure 3 Colocalization analysis of TGEV S protein and S-K/M mutant protein with compartment markers. The S protein (panel A, stained in red) as well as the mutant protein S-K/M (panel B) were cotransfected with GFP-tagged ER, and GFP-tagged ERGIC marker, respectively. Co-localized proteins are shown in yellow (merge).

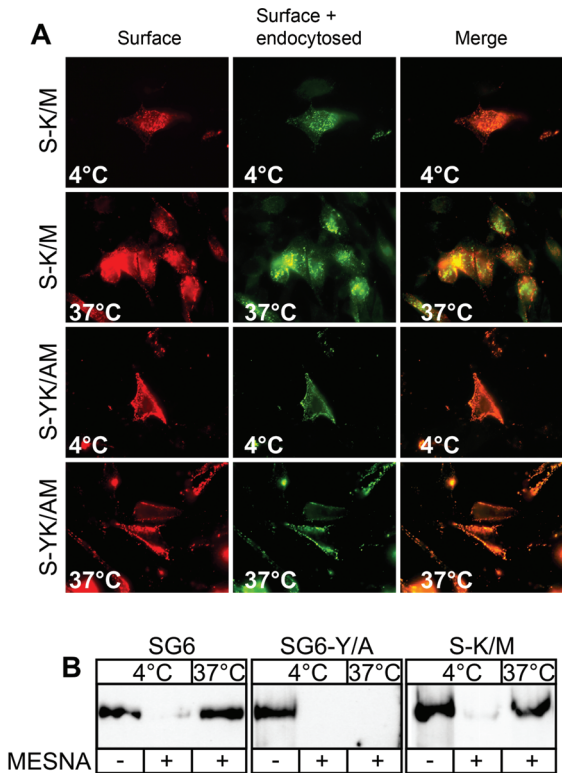


Figure 4 Internalization analysis of TGEV S proteins with mutations in or near the potential endocytosis signal. (A) Surface and intracellular staining of S-K/M and S-YK/AM in an antibody uptake assay. After surface-binding of the anti S protein antibody, cells were incubated at 4°C or 37°C, respectively. Surface protein antibody complexes as well as endocytosed protein antibody complexes could be stained by secondary antibodies conjugated with two different fluorescent dyes (red: surface; green: surface and endocytosed). In the overlays (merge) endocytosed protein is shown in green, whereas surface protein is visible in red or yellow. (B) Biotin internalization assay of SG6, SG6-Y/A, and S-K/M. BHK21 cells were transfected with the pCG1 expression plasmid containing the gene indicated. Cells were analyzed at 24 h post-transfection for endocytosis as described in the experimental procedures section. In order to determine the total amount of surface-biotinylated proteins, the samples were compared with those that were neither warmed to 37°C nor treated with MESNA. To analyze the cleavage efficiency of the biotin disulfide bond under reducing conditions, one part of the cells was kept at 4°C to inhibit endocytosis and then treated with MESNA. After incubation at 37°C the endocytosed protein is seen as a band at ~220 kDa.

These results demonstrate that a single amino acid exchange outside the Y-X-X-Φ motif may result in a completely different transport behavior. Whereas the authentic TGEV S protein is retained in the ERGIC compartment, the mutant protein S-K/M is transported to the cell surface and subsequently endocytosed. Taken together, our results indicate that the function of a tyrosine-based sorting motif as a retention signal can be affected by a

single amino acid downstream of the respective tetrapeptide motif.

As the initial point of our analysis was the comparison of the TGEV S tyrosine-based sorting signal, with the VSV G tyrosine-based sorting signal we proceeded to introduce a lysine in the original VSV G protein at position -6 instead of the methionine. Additionally, we constructed a double mutant with a tyrosine to alanine mutation in addition to the methionine to lysine mutation. In the Western blot analysis of lysates from transfected cells, the parental G proteins as well as both mutant proteins were expressed in comparable amounts (Figure 5A). Both, the G-M/K and the G-YM/AK proteins were expressed at the cell surface but in substantially lower amounts when compared to the parental G protein (Figure 5A, upper panel). The surface-biotinylated samples of the VSV G proteins were taken from the same well as the lysates, i.e., these two results can be directly compared. In an immunofluorescence assay the mutant proteins were expressed in a similar surface distributed pattern as the parental VSV G (data

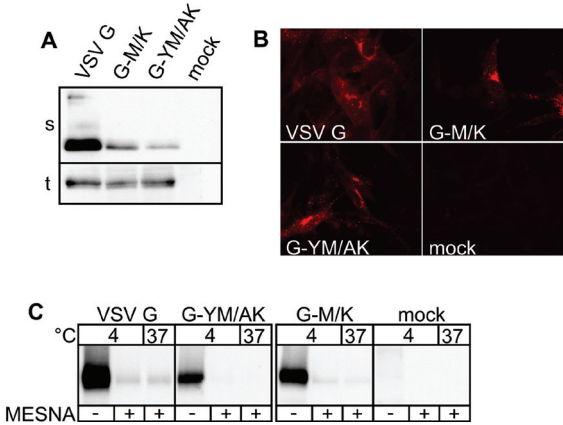


Figure 5 Protein expression and internalization analysis of VSV G parental and mutant proteins. (A) Surface and total protein expression of VSV G parental and mutant proteins. VSV G protein, G-M/K mutant protein, and G-YM/AK mutant protein were expressed in BHK21 cells. Cells were surface-biotinylated at 24 h post-transfection. After lysis, one part of the samples was used for total G protein analysis in a Western blot (t). The other part was immunoprecipitated with monoclonal anti-VSV G antibody and surface expression of the proteins was analyzed after SDS-PAGE and Western blot with streptavidin peroxidase. (B) Intracellular distribution of VSV G parental and mutant proteins. Cells were analyzed for total expression of the indicated proteins at 24 h post-transfection by immunofluorescence microscopy (1000× original magnification). (C) Internalization of VSV G proteins. BHK21 cells were transfected with the pcDNA3.1 expression plasmid containing the gene indicated. Cells were analyzed at 24 h post-transfection for endocytosis as described in the legend for Figure 4B. After incubation at 37°C, the endocytosed protein is seen as a band at ~55 kDa.

not shown). All three VSV G proteins were distributed all over the cell with an accumulation in the Golgi region (Figure 5B). To find out if the reduced surface expression of G-M/K and G-YM/AK is caused by partial endocytosis of these proteins a biotin internalization assay was performed (Figure 5C). All three tested VSV G proteins were not endocytosed. Our data suggest that the methionine in the cytoplasmic tail of the VSV G protein supports the cell surface transport of this protein.

Discussion

Our findings show that the mutation of a single amino acid downstream of a tyrosine-based sorting motif can affect the transport behavior of the S protein. A methionine was introduced at position 1445 of the TGEV S protein, replacing the lysine. Due to this exchange, the protein was transported to the plasma membrane and internalized by clathrin-mediated endocytosis. The authentic TGEV S protein is not endocytosed as it is retained in the ERGIC. Because of this retention it has no possibility to enter the endocytotic pathway. The lysine at position 1445 appears not to be crucial for intracellular retention as mutant proteins with an exchange to alanine, arginine or glutamic acid were still intracellularly retained. This finding is in contrast to the findings for the SARS-CoV S protein that contains – like TGEV S – the C-terminal amino acid sequence KXHXX that was shown to function as an ER retrieval signal (McBride et al., 2007). For TGEV S this retrieval signal may be relevant if the retention mechanism inside the ERGIC is saturated and overexpressed TGEV S protein reaches the Golgi. The tyrosine-based retention signal was affected by the substitution of the lysine at position 1445 only when a methionine was introduced. The K/M exchange resulted in cell surface expression and endocytosis. For the CD3 ϵ subunit of the T cell receptor it has been reported that the signal₁₇₇YXXLXXR₁₈₃ is essential for ER retention (Mallabiabarrena et al., 1995). The side chains of Tyr177, L180, and Arg 183 have been shown to be crucial for the retention in terms of three-dimensional structure. The structure of the ER retention signal was more complex than the β -turn proposed as central element for internalization signals. However, by analyzing protein chimeras, Mallabiabarrena et al. have shown that internalization sequences can at least partially act as retention signals and vice versa in an appropriate context (Mallabiabarrena et al., 1995). It was shown that replacement of Arg 183 by serine or lysine abolishes the intracellular retention of CD3 ϵ resulting in a protein that

is internalized from the plasma membrane by endocytosis (Borrito et al., 1999). For the TGEV S protein we have shown that just a single specific amino acid exchange can transform a retention signal into an internalization signal. Only a methionine at position 1445 but not a lysine, alanine, arginine, or glutamic acid converted the retention signal of the TGEV S protein into an endocytotic signal. Whether this amino acid exchange leads to a conformational change of the protein structure in the sorting signal region is not known. It is possible that a conformational change hinders the mutant S protein to interact with the so far unknown cellular protein that retains the S protein at the ERGIC. Conversely, if the unknown cellular protein that retains the TGEV S protein at the ERGIC would be knocked-down, it is possible that the tyrosine-based sorting signal in the authentic S protein may function as an endocytosis signal. The YXX Φ -motif in the S-K/M mutant worked as a very strong endocytosis signal, which could be demonstrated by the high endocytosis rate. This phenomenon also explains the poor expression of S-K/M and SG6 on the cell surface (Figure 2) compared to the Y/A mutants. The YXX Φ -motif represents the classical motif for interaction with AP2, which leads to clathrin-mediated endocytosis at the plasma membrane (Traub, 2005, 2009). It is likely that this endocytic pathway is used for S-K/M and SG6. Currently, we do not know whether the endosomes are then fused with lysosomes or whether the proteins are transported back to the TGN. A recycling of viral protein back to intracellular compartments could increase the amount of protein present for incorporation into viral particles. So if, by accident, parts of the TGEV S wildtype protein reach the plasma membrane they may be endocytosed via clathrin-mediated endocytosis like S-K/M and recycled back to the Golgi.

The tyrosine-based sorting signal in the VSV G protein is known to be responsible for basolateral transport (Thomas and Roth, 1994). It has been shown that in addition to a di-acidic signal (DXE), the tyrosine as well as the methionine also play a role in ER-export (Nishimura and Balch, 1997; Sevier et al., 2000). Additionally it has been described that the di-acidic signal DXE plays a role in transport of the VSV G protein from the TGN to the plasma membrane by an AP3-dependent mechanism (Nishimura et al., 2002). A mutation of the methionine at position 506 to a lysine in the VSV G cytoplasmic domain decreased the surface expression of the protein. An additional mutation of the tyrosine501 to alanine led to a similar surface expression as for the single point mutant. This reduced surface expression is not due to endocytosis of the protein. Our immunofluorescence results indicated that all analyzed G proteins were accumulated in the Golgi

during their transport through the secretory pathway. This accumulation appeared to be increased for the two VSV G mutant proteins analyzed. At present we cannot distinguish whether these mutations just diminish the export of the VSV G protein or whether they induce a retention mechanism that counteracts the transport of the G protein to the plasma membrane. For the wildtype VSV G protein an efficient transport to the plasma membrane makes sense as it helps to concentrate the protein at the site of VSV budding. The results for the VSV G mutant proteins and the TGEV S K/M mutant indicate that a methionine at position +2 with respect to the tyrosine-based sorting signal inhibits the interaction with cellular proteins that may be required for intracellular retention.

For the S proteins of alphacoronaviruses, the tyrosine-based sequence at the C-terminus is strikingly conserved. It appears that a retention of this protein in the ERGIC helps to concentrate S protein for the assembly and budding process that takes place at the ERGIC. In future studies it will be interesting to find out whether the K/M mutation in the TGEV S protein and thus the altered transport behavior is of any relevance in the context of virus assembly.

Materials and methods

Cells

BHK21 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were propagated in minimal essential medium supplemented with 5% fetal calf serum and non-essential amino acids.

Construction of plasmid

In order to construct a chimeric protein (SG6) containing the ectodomain, membrane anchor and parts of the cytoplasmic domain of the TGEV S and the last six C-terminal amino acids of the VSV G protein (VSV strain Indiana), the corresponding coding regions were amplified from the plasmids pTM1-TGEVS and pTM1-VSVG by standard hybridization PCR technique as previously described (Schwegmann-Wessels et al., 2004). The full length chimeric gene was amplified by PCR and ligated back into the pTM1 vector via *SpeI* and *PstI* restriction sites. Subsequently the wildtype S protein gene of TGEV, strain PUR-46-MAD (Penzes et al., 2001) as well as the full length chimeric SG6 gene were cloned into the plasmid pCG1 by PCR (Cathomen et al., 1995; Schwegmann-Wessels et al., 2006). The pCG1-SG6 was used as a template to substitute the tyrosine at position 1440 to an alanine to generate SG6-YA. The other described mutants were constructed using standard PCR techniques. In the pCG1-Y1440A plasmid, previously described (Schwegmann-Wessels et al., 2004, 2006), an additional mutation was introduced to generate the plasmid pCG1-S-YK/AM. For experiments with VSV G parental and mutant protein

the plasmid pcDNA3.1-VSV G encoding the envelope protein of VSV (strain Indiana) was used (Hanika et al., 2005). The same hybridization technique described above was applied for the construction of pcDNA3.1-G-M/K and pcDNA3.1-G-YM/AK, respectively. The sequence of all plasmids generated by PCR was verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). The different protein mutants that were used in this study are shown in Figure 1.

Immunofluorescence assays

BHK21 cells grown on 12-mm diameter coverslips were transfected with 1 µg of plasmid DNA, 2 µl of Lipofectamine™ 2000 reagent (Invitrogen, Darmstadt, Germany) and incubated at 37°C for 24 h. The assay was performed as described previously (Schwegmann-Wessels et al., 2004). For the detection of VSV G proteins monoclonal anti-VSV G antibodies (I-1, I-14; Hanika et al., 2005) were used followed by incubation with a Cy3-conjugated secondary antibody (sheep anti-mouse, 1:500, Sigma-Aldrich, St. Louis, MO, USA). Fluorescence was visualized with a Nikon Eclipse Ti microscope.

For colocalization experiments, cells were cotransfected with 0.75 µg of pCG1-S or pCG1-S-K/M together with 0.75 µg of an expression plasmid coding for the different compartment markers ER-EGFP (kindly provided by Frank van Kuppeveld, Utrecht, The Netherlands), or GFP-tagged ERGIC-53 (as ERGIC marker), respectively. The ERGIC-53 DNA was kindly provided by Hans-Peter Hauri (Basel, Switzerland). By inserting the GFP open reading frame behind the signal peptide sequence of the ERGIC-53 DNA, GFP-ERGIC was obtained (Winter et al., 2008). After fixation, the cells were permeabilized and stained with the monoclonal antibody against TGEV S as described previously (Schwegmann-Wessels et al., 2004) followed by incubation with a Rhodamine Red-X-conjugated secondary antibody (goat anti-mouse, 1:100, Dianova, Hamburg, Germany). The localization of the viral protein and the respective markers was analyzed with a Leica TCS SP5 confocal microscope.

Western blot analysis, surface biotinylation and immunoprecipitation of proteins

BHK21 cells grown in 35-mm diameter dishes were transfected with 4 µg of plasmid DNA and 8 µl of lipofectamine 2000 Reagent or 4 µg of plasmid DNA and 6 µl of polyethylenimine (PEI, 1 mg/ml, Polysciences, Warrington, PA, USA), respectively. At 24 h post-transfection, cell surface proteins were labeled with an N-hydroxy-succinimide ester of biotin (0.5 mg/ml phosphate-buffered saline, Sulfo-NHS-LC-Biotin, Thermo Scientific, Rockford, IL, USA). The cells were lysed with 100 µl NP-40 lysis buffer and the viral antigens were immunoprecipitated from 80 µl of the cell lysates as described by Zimmer et al. (2001). The monoclonal anti-TGEV S protein antibody (6A.C3; Gebauer et al., 1991) and the monoclonal anti-VSV G antibodies (I-1, I-14; Hanika et al., 2005), respectively were used for immunoprecipitation. Prior to immunoprecipitation, 20 µl of the lysates were kept for Western blot analysis using the mentioned monoclonal antibodies. After incubation with a peroxidase-conjugated secondary antibody (rabbit anti-mouse, 1:1000, DAKO, Hamburg, Germany) bound antibodies were visualized by chemiluminescence (Zimmer et al., 2001). The relative amounts of surface-expressed proteins were quantified by performing a densitometric analysis using Image J software (Rasband 1997–2014).

Antibody uptake assay

The assay was mainly performed as described by Moll et al. (2001). BHK21 cells grown on 12-mm diameter coverslips were transfected with 1 µg of plasmid DNA, 2 µl of Lipofectamine™ 2000 reagent, and incubated at 37°C for 24 h. Cells were incubated on ice with a monoclonal antibody (6A.C3) directed against the ectodomain of TGEV S. After incubation for 10 min at 37°C, cells were fixed and stained with a Rhodamine Red-X-conjugated secondary antibody (goat anti-mouse, 1:100, Dianova, Hamburg, Germany). After permeabilization with 0.2% Triton X-100/phosphate-buffered saline for 5 min internalized TGEV S was stained with a FITC-conjugated secondary antibody (goat anti-mouse, 1:200, Sigma-Aldrich, St. Louis, MO, USA). A parallel control experiment was performed at 4°C. Cells were analyzed by fluorescence microscopy with a Nikon Eclipse Ti.

Biotin internalization assay

BHK21 cells grown in 35-mm diameter dishes were transfected with 4 µg of plasmid DNA and 8 µl of Lipofectamine™ 2000 reagent. Internalization of proteins was analyzed similar to Moll et al. (2001). At 24 h post-transfection, cell surface proteins were labeled twice with a cleavable Sulfo-NHS-SS-biotin (0.5 mg/ml phosphate-buffered saline, Thermo Scientific, Rockford, IL, USA), and endocytosis was initiated at 37°C for 10 min. Cells were then cooled at 4°C, and biotin exposed on the cell surface was cleaved by addition of sodium 2-mercapto-ethane sulfonate (MESNA, 8.2 mg/ml tris-buffered saline, Sigma-Aldrich, St. Louis, MO, USA). Biotinylated S or G proteins protected from MESNA were retrieved by precipitation with monoclonal antibody 6A.C3 and monoclonal anti-VSV G antibodies, respectively,

as described above. Biotin was quantified by immunoblot with streptavidin biotinylated horseradish peroxidase (1:1000, Amersham Biosciences, Freiburg, Germany). Two parallel controls were performed at 4°C to arrest endocytosis. In one control cells were incubated at 4°C and not treated with MESNA and thus demonstrated the surface expression of the proteins. In the other control, cells were also kept at 4°C and subsequently reduced using MESNA, to analyze the efficiency of the Sulfo-NHS-SS-biotin disulfide bond cleavage after MESNA incubation. To calculate the endocytosis rate per min for the mutant proteins SG6 and S-K/M, endocytosis was monitored after 5, 10, 15, 20, and 30 min at 37°C. The amount of endocytosed protein was quantified using Image J and the endocytosis rate per min (%/min) was calculated.

Acknowledgments: Thanks to Sandra Bauer for technical assistance. We thank L. Enjuanes for kindly providing a plasmid containing the TGEVS gene and the monoclonal antibody 6A.C3 against the TGEV S protein. We thank F. van Kuppeveld and H.-P. Hauri for kindly providing plasmids containing the pER-EGFP and ERGIC p53 DNAs, respectively, and R. Cattaneo for providing the pCG1 expression plasmid. C. S.-W. is a recipient of an Emmy-Noether fellowship from Deutsche Forschungsgemeinschaft (DFG). This work was supported by grants from the DFG (SCHW 1408/1-1, SFB 621).

Received November 20, 2013; accepted March 3, 2014; previously published online March 7, 2014

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