



## Cholesterol is important for a post-adsorption step in the entry process of transmissible gastroenteritis virus

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### ABSTRACT

Cholesterol is a major constituent of detergent-resistant membrane microdomains (DRMs). We localized transmissible gastroenteritis virus (TGEV) spike (S) protein in DRMs in the viral envelope. Though S protein was not solubilized by cold non-ionic detergents, this behavior was unchanged when cholesterol was depleted from viral membrane by methyl- $\beta$ -cyclodextrin (M $\beta$ CD) and the protein did not comigrate with cellular DRM marker proteins in flotation analyses. Therefore, the S protein is not anchored in the viral membrane DRMs as they are known to occur in the plasma membrane. Cholesterol depletion from viral membrane may not affect the adsorption process as neither the sialic acid binding activity nor the binding to aminopeptidase N was reduced post-M $\beta$ CD treatment. Reduced infectivity of cholesterol-depleted TGEV was observed only when the adsorption process occurred at 37 °C but not when the virus was applied at 4 °C. Cholesterol is important for a post-adsorption step, allowing membrane rearrangements that facilitate virus entry.

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### 1. Introduction

Transmissible gastroenteritis virus (TGEV) belongs to the family *Coronaviridae* and can cause severe gastroenteritis in young seronegative pigs (Saif and Wesley, 1999; Schwegmann-Wessels et al., 2003). Coronaviruses are enveloped viruses with nonsegmented, single-stranded, positive-sense RNA (Enjuanes et al., 2000, 2008). The spike (S) glycoprotein projects approximately 17–20 nm from the virion surface. Other structural proteins are the integral membrane (M) glycoprotein, a minor envelope (E) protein, and the nucleocapsid (N) protein. Functionally, the S glycoprotein is the major target of neutralizing antibodies (Garwes et al., 1978; Jiménez et al., 1986; Laude et al., 1987; Antón et al., 1996; Sune et al., 1990), and it is also related to cell tropism (Jacobs et al., 1986; Torres et al., 1996; Sánchez et al., 1999; Schwegmann-Wessels et al., 2003), interaction with its cellular receptor (Collins et al., 1982; Delmas et al., 1990; Schwegmann-Wessels et al., 2002, 2003; Liu et al., 2009; Shulla and Gallagher, 2009), pathogenicity (Krempl et al., 1997; Garwes et al., 1978; Tuboly et al., 1994), fusion (Collins et al.,

1982; Spaan et al., 1988; Sune et al., 1990) and hemagglutination activity (Krempl and Herrler, 2001; Krempl et al., 2000).

Detergent-resistant membranes (DRMs) are plasma membrane microdomains characterized by insolubility in cold nonionic detergents such as Triton X-100 or Brij-98 and by enrichment of cholesterol and sphingomyelin (Glebov and Nichols, 2004). Accumulating evidence suggests the involvement of DRMs in virus life cycles (Simons and Ehehalt, 2002). Cholesterol, a major constituent of DRMs is important in the entry of nonenveloped viruses such as simian virus 40 (SV40), rotavirus, enterovirus and rhinovirus (Suzuki and Suzuki, 2006). The binding of enveloped viruses to specific cellular receptors and fusion of the viral membrane with a cellular membrane are indispensable for virus entry. Correspondingly, the initiation of virus infection may require cholesterol in either of the two membranes involved or in both. Previous data show that the infectivity of influenza virus and canine distemper virus is sensitive to cholesterol depletion from the viral membrane (Imhoff et al., 2007; Sun and Whittaker, 2003). In contrast, murine leukemia virus, Ebola virus, and Marburg virus are sensitive to cholesterol depletion from the cellular membrane (Bavari et al., 2002; Lu et al., 2002). Cholesterol in both membranes is required for the infection by human immunodeficiency virus (HIV) and herpes simplex virus (Smith and Helenius, 2004; Nayak and Hui, 2004),

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while vesicular stomatitis virus (VSV) replication is unaffected by cholesterol depletion.

As far as coronaviruses are concerned, the depletion of cellular cholesterol by the drug methyl- $\beta$ -cyclodextrin (M $\beta$ CD), a cholesterol depletion reagent, inhibits virus entry of several coronaviruses: mouse hepatitis virus (Thorp and Gallagher, 2004; Choi et al., 2005), severe acute respiratory syndrome (SARS)-coronavirus (Li et al., 2007; Glende et al., 2008), human coronavirus 229E (Nomura et al., 2004) and avian infectious bronchitis virus (Imhoff et al., 2007). More recently, we showed the importance of cholesterol in both the cellular and viral membranes for TGEV infection (Ren et al., 2008). This finding raises the question whether there are DRMs in the viral envelope.

Here we analyzed whether the S protein of TGEV is located within DRMs in the viral envelope. Though the viral protein was found to be insoluble in non-ionic detergents at low temperature, this solubilization behavior was not abolished by cholesterol depletion. Furthermore, in a flotation analysis the S protein did not appear at the position of DRM marker proteins. Therefore, the S protein in viral membranes does not show the characteristic features of cellular DRM proteins. A functional analysis suggests that cholesterol depletion affects a post-adsorption step in the virus entry process that requires membrane rearrangements.

## 2. Materials and methods

### 2.1. Cells and viruses

Swine testicle (ST) cells and Baby hamster kidney cells (BHK21) were maintained in MEM medium supplemented with 5% newborn bovine serum (Excell Bio) and passaged twice a week, respectively. TGEV strain PUR46-MAD and vesicular stomatitis virus (VSV, strain Indiana) were propagated in ST and BHK21 cells, respectively as previously described (Ren et al., 2008). All viruses used for the experiments were grown in serum-free medium.

### 2.2. Infectivity of cholesterol-depleted viruses

For cholesterol depletion, virus samples ( $2 \times 10^4$  pfu/ml or  $2 \times 10^6$  pfu/ml) were treated with 0–10 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD) at 37 °C for 30 min followed by ultracentrifugation to remove residual M $\beta$ CD. The virus pellets were resuspended and their infectivity was determined with plaque assays in either 6-well or 12-well plates. For cholesterol replenishment, after extraction of viral membrane cholesterol using 10 mM M $\beta$ CD, the virus suspensions were replenished with water-soluble cholesterol (Sigma) by applying final concentrations ranging from 50 to 500  $\mu$ M at 37 °C for 30 min as previously described (Ren et al., 2008). The experiment was performed in triplicate.

### 2.3. Detergent treatment of virions

A clarified cell supernatant of 10 ml TGEV or VSV ( $2 \times 10^6$  pfu/ml) was pelleted at  $100,000 \times g$  at 4 °C for 1 h. The purified virions were homogenized and solubilized with 1 ml 1% Triton X-100 in the cold for 2 h. The viral extract was centrifuged at  $100,000 \times g$  at 4 °C for 1.5 h to yield a supernatant (non-DRMs) and a pellet. The pellet was treated with 1 ml NP 40 buffer overnight at 4 °C followed further ultracentrifugation as above. The resulting supernatant was designated as DRMs (detergent-resistant microdomains). The non-DRM and DRM fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot analysis. A mouse monoclonal antibody against TGEV S protein (6A.C3) and a polyclonal rabbit antiserum against VSV G protein (kindly provided by Dr. Gert Zimmer) were used for detecting the respective proteins. The effect of cholesterol depletion was

analyzed by treating virions with 10 mM M $\beta$ CD at 37 °C for 30 min prior to detergent extraction.

### 2.4. Sucrose density gradient centrifugation

For a flotation analysis, a known lipid raft marker, flotillin-2, and cholesterol-depleted virions (see above) of TGEV and VSV were solubilized at 4 °C for 2 h and then mixed with the same volume of 80% sucrose in PBS (w/v). The mixtures were loaded at the bottom of a gradient tube and overlaid by sucrose-PBS cushions (4 ml each) of 30 and 5%. After centrifugation at  $100,000 \times g$  for 18 h at 4 °C, the fractions were collected and the protein in each fraction was precipitated with water-free ethanol (100%) at –20 °C overnight prior to 10% SDS-PAGE and semi-dry Western blot analysis using mouse monoclonal antibody (6A.C3) against TGEV S protein or rabbit polyclonal antibody against VSV G or flotillin-2 protein (Sigma–Aldrich), respectively, for the detection of the proteins.

### 2.5. Hemagglutination analysis

Haemagglutination-positive virions of TGEV were grown in desialylated cells treated with neuraminidase (NA) at 37 °C for 1 h as described previously (Krempl et al., 1997). Virus ( $2 \times 10^5$  pfu/ml) was treated with M $\beta$ CD (0, 4 and 10 mM) at 37 °C for 30 min. The treated viruses and 1% chicken erythrocytes in physiological salt solution were used for a standard hemagglutination (HA) analysis in micro-titer plates. The experiment was performed in triplicate.

### 2.6. The effect of viral cholesterol depletion on binding of TGEV to cultured cells

After treatment of purified TGEV with 50 mU NA, virions were ultracentrifuged to remove the residual NA. The pellet was resuspended ( $2 \times 10^4$  pfu/ml) and treated with M $\beta$ CD (0, 2, 4, 8 and 10 mM) at 37 °C for 30 min. The virions were ultracentrifuged and resuspended in PBS and the amount of viral protein was determined via photometrical measurement of the UV absorption at 280 nm. ST cell monolayers in micro-titer plates were washed three times with PBS, and each well was incubated for 1 h at 37 °C with 250 mU NA. After washing with PBS, the cells were incubated with the treated viruses (11  $\mu$ g of viral protein per well) at 4 °C for 1 h. The cells were washed three times with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature, and incubated with 0.1 M glycine for 5 min. The fixed cells were incubated with the monoclonal antibody (6A.C3) directed against the S protein and then with a peroxidase-conjugated rabbit anti-mouse antibody. For the detection of bound antibody, the ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate was used. The reaction was stopped with 1% SDS. Extinction was measured at 405 nm (Schwegmann-Wessels et al., 2002). The experiment was performed in triplicate.

### 2.7. The effect of cholesterol depletion on infection by TGEV at 4 °C or 37 °C

TGEV was harvested from NA-treated ST cells as described above. The harvested viruses ( $2 \times 10^6$  pfu/ml) were subjected to mock-treatment or 10 mM M $\beta$ CD treatment. ST cells were treated with 50 mU NA and the desialylated cells were incubated with the viruses at either at 4 °C or 37 °C for 1 h. The cells were washed three times with PBS and overlaid with 1% (w/v) methyl-cellulose in MEM and cultured at 37 °C for 48 h when the plaques were counted. The reduced infectivity of the virus was calculated according to the equation: reduction of virus titer = (the plaque number of cells

infected with drug-treated viruses/plaque number of cells infected with non-drug treated viruses)  $\times$  100.

### 2.8. Statistics

At least three independent experiments were carried out. Each data point was presented as mean  $\pm$  SD. Statistical significance was evaluated using the *t*-test. “\*” means a value of  $P < 0.05$  was considered statistically significant; “\*\*\*” means a value of  $P < 0.01$  was considered statistically highly significant; “\*\*\*\*” means extremely significant in statistics.

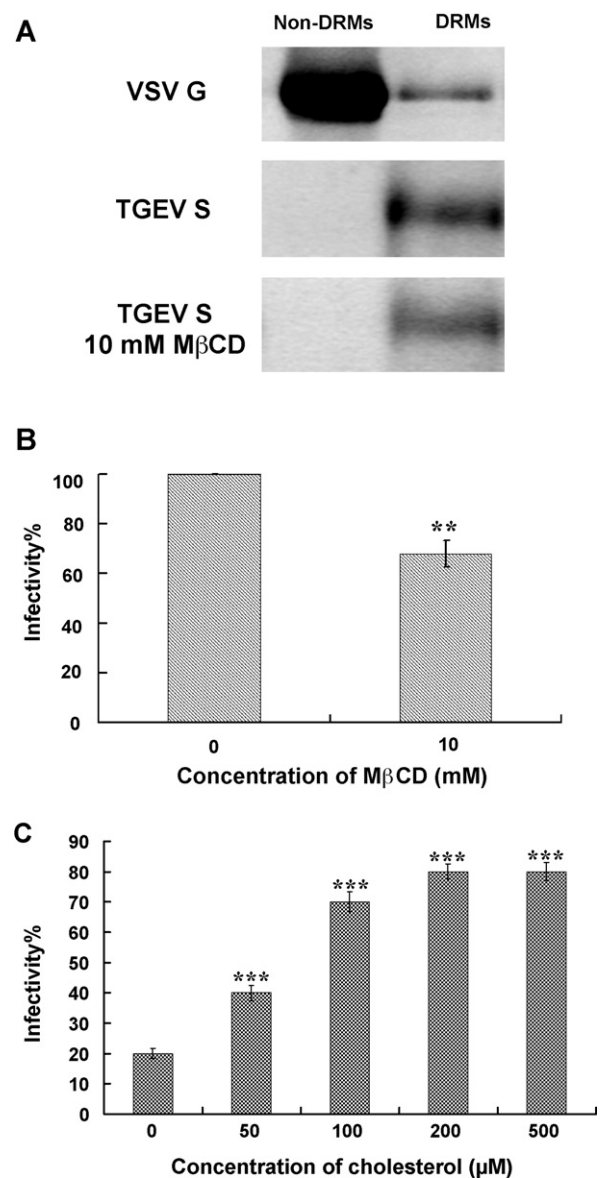
## 3. Results

### 3.1. Detergent solubilization of the TGEV S protein

An increased content of cholesterol is a characteristic feature of DRMs derived from the plasma membrane. As cholesterol depletion from TGE virions results in a reduction of viral infectivity (Ren et al., 2008), we were interested to find out whether the surface protein S that mediates virus entry is localized in DRMs. For this purpose, we analyzed whether the TGEV S protein is resistant to solubilization by Triton X-100 at 4 °C. As shown in Fig. 1A, the S protein was found in the pellet, i.e. it was associated with the Triton X-100-insoluble fraction. For comparison, we used vesicular stomatitis virus that is not affected in its infectivity when cholesterol is depleted from the viral membrane. The surface protein G of this virus was only detected in the soluble fraction. The same result was obtained when two other non-ionic detergents were used, Tween 20 or Brij96 (data not shown). This solubilization behavior would be compatible with the presence of the S protein in detergent-resistant microdomains. To further characterize the membrane association of the S protein, TGEV particles were treated with M $\beta$ CD to deplete cholesterol from the viral membrane. M $\beta$ CD is commonly used to destroy the integrity of DRMs. Application of this drug at a concentration of 10 mM reduced approximately 33% infectivity of TGEV ( $P < 0.01$ ), compared with the infectivity of non-treated viruses (Fig. 1B). To analyze the association of cholesterol with the infectivity of TGEV, a cholesterol replenishment assay was performed. As shown in Fig. 1C, the infectivity of TGEV restored to 80% of the value determined prior to cholesterol depletion, after addition of 200  $\mu$ M exogenous cholesterol to M $\beta$ CD-treated TGEV (Fig. 1C). The data indicate that the viral infectivity is significantly reduced by M $\beta$ CD and can be restored by cholesterol. However, the S protein was still only detected in the detergent-insoluble fraction (Fig. 1A). This result indicates that the surface protein of TGEV is arranged in the viral envelope in a detergent-resistant way that is not affected by cholesterol depletion.

### 3.2. Flotation density of viral proteins

To get more information about the membrane association of the S protein, a flotation analysis was performed. Overlaid by a 5–40% sucrose gradient, DRM proteins are floating to the top fraction upon ultracentrifugation. This approach was applied to the S protein and the VSV G protein after solubilization by 1% Triton X-100 at 4 °C. As shown in Fig. 2, the VSV G protein was detected in fractions 6–10, but not in fractions 1–3 where DRM proteins are expected. The S protein was found to have an even higher buoyant density as it was present mainly in fraction 10. The distribution within the gradient was not affected by prior cholesterol depletion from the viral membrane. In contrast, after the treatment with 10 mM M $\beta$ CD, the lipid raft marker protein, flotillin-2, migrated from fractions 2–3 to a higher density fractions, most of which concentrated in fractions 9–10. These data indicate that the S protein is not localized in typical



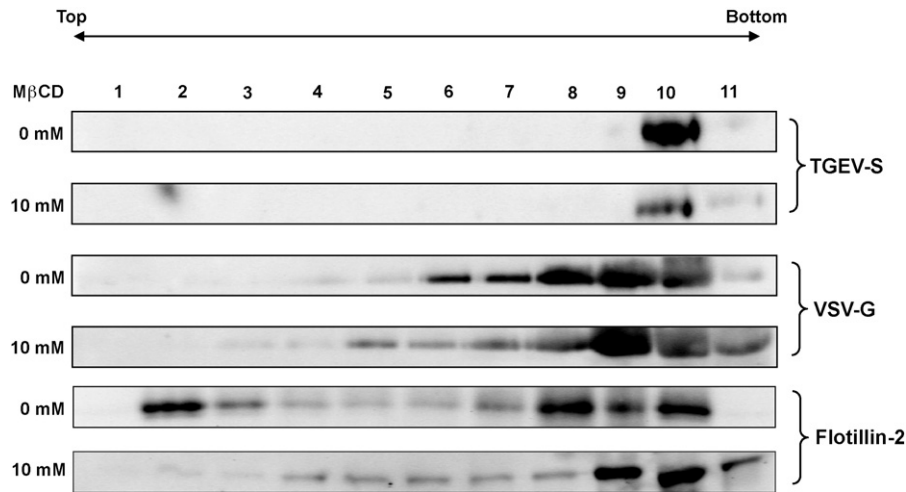
**Fig. 1.** Solubility of viral proteins. The S protein of TGEV treated with or without 10 mM M $\beta$ CD and VSV G protein were extracted with 1% Triton X-100 at 4 °C and were detected by Western blot by corresponding antibody. DRMs is the abbreviation of detergent-resistant microdomains (Panel A); The infectivity of TGEV ( $2 \times 10^6$  pfu/ml) treated with 10 mM M $\beta$ CD was compared with that of mock-treated TGEV (Panel B). Recovery of TGEV infectivity after exogenous cholesterol addition to cells treated with 10 mM M $\beta$ CD is shown (Panel C). Symbol “\*\*\*” means highly significant difference in statistics, “\*\*\*\*” means extremely significant difference in statistics.

DRMs as they have been described to occur in the plasma membrane. Nevertheless, the behavior of the S protein upon detergent solubilization is different from that of the VSV G protein. Possible reasons are discussed below.

### 3.3. Cholesterol depletion does not affect the hemagglutination activity of the S protein

The S protein of TGEV has two binding activities, binding to N-glycolylneuraminic acid on sialoglycoconjugates and binding to porcine aminopeptidase N (pAPN). To analyze the effect of cholesterol depletion on the sialic acid binding activity of TGEV, we applied a hemagglutination assay. Maximum HA titers are obtained with virions that have been enzymatically desialylated to remove





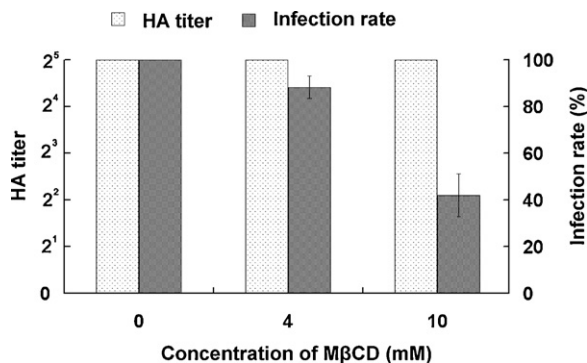
**Fig. 2.** Flotation density of viral proteins by sucrose density gradient centrifugation. The flotation density of either TGEV S protein or VSV G protein (with or without 10 mM MβCD) was analyzed using a 5–40% sucrose gradient after treatment of 1% Triton X-100 at 4 °C. The drug concentrations, viral protein and gradient sequence are indicated.

sialic acids from the viral surface (Krempl et al., 1997, 2000). Therefore, purified virus was treated with neuraminidase prior to cholesterol depletion by MβCD. As shown in Fig. 3, a concentration of 10 mM MβCD significantly reduced the viral infectivity. The same concentration did not affect the HA activity of TGEV. This result indicates that the effect of cholesterol depletion on infectivity is not related to the sialic acid binding activity.

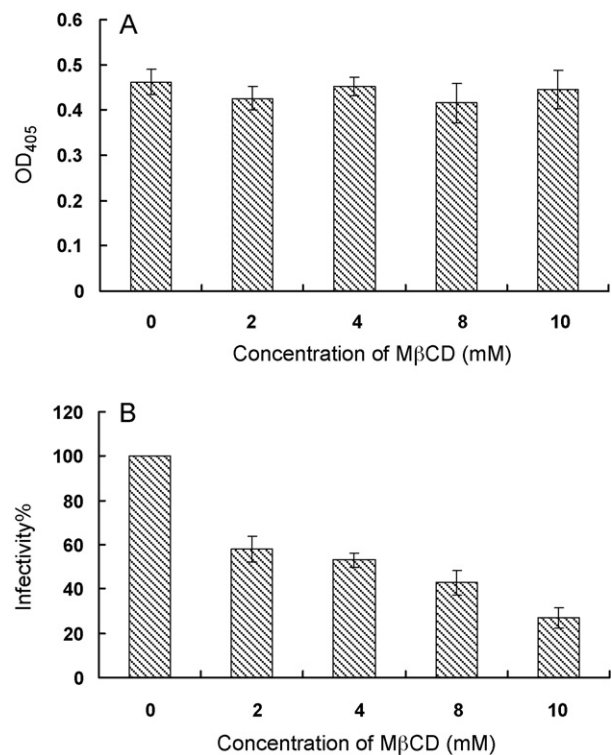
#### 3.4. Cholesterol depletion does not affect the binding of TGEV to pAPN

In order to analyze the importance of cholesterol in the viral membrane for binding to pAPN, the cellular receptor of TGEV, viral cholesterol was depleted as described above. To exclude binding to cellular sialoglycoconjugates, ST cells were enzymatically desialylated to allow only virus binding to pAPN (Schwegmann-Wessels et al., 2002). The neuraminidase-treated cells were incubated with cholesterol-depleted virus or with control virus, respectively, for 1 h at 4 °C. Unbound virions were removed by thorough washing and bound virus was measured by an indirect ELISA. As shown in Fig. 4B, MβCD treatment lowered TGEV infectivity significantly, but had no effect on the binding to desialylated cells. From this result we conclude that cholesterol depletion from the viral membrane does not affect the binding of TGEV to pAPN (Fig. 4A). To characterize a potential postadsorption step affected in the infection by the cholesterol-depleted TGEV we performed an infection

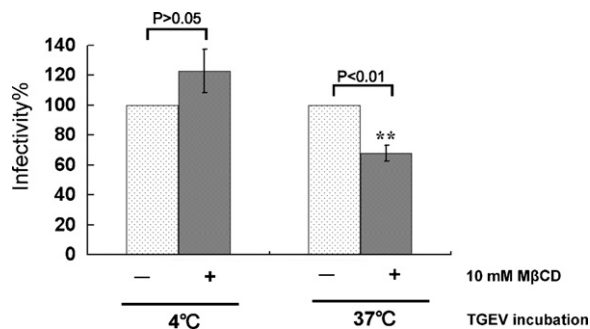
assay where the inoculum was applied for 1 h at either 4 or 37 °C, respectively. The former conditions should allow binding but not concurrent rearrangement of cellular or viral proteins. Following the adsorption period, cells were overlaid by methylcellulose and at 48 h.p.i., the number of plaques was determined. As shown in Fig. 5, when the virus was applied at 37 °C, the infectivity titer of cholesterol-depleted TGEV was significantly lower compared to the control virus. On the other hand, when adsorption occurred at 4 °C, the titer of MβCD-treated virus was not reduced, it was



**Fig. 3.** Effect of MβCD-treatment of virions on infectivity and HA activity of TGEV. TGEV was harvested from neuraminidase-treated ST cells and the resulting viruses ( $2 \times 10^5$  pfu/ml) were treated with MβCD (0, 4 and 10 mM) at 37 °C for 30 min. The treated viruses were used to determine the infectivity and the HA activity.



**Fig. 4.** The effect of viral cholesterol depletion on binding between TGEV S and pAPN. Cholesterol of neuraminidase treated TGEV was depleted with MβCD (0, 2, 4, 8 and 10 mM) at 37 °C for 30 min and then the viruses were used to bind neuraminidase treated ST cells. The binding activity between them was analyzed by indirect ELISA. The OD value was measured at 405 nm wavelength (Panel A); The infectivity of TGEV ( $2 \times 10^4$  pfu/ml) treated with various concentrations of MβCD was reflected by virus plaque-reduction assays (Panel B). The OD<sub>405</sub> is from three independent experiments and each experiment was performed in triplicate.



**Fig. 5.** Binding of TGEV virions. Desialylated ST cells were incubated with untreated or MβCD-treated TGEV ( $2 \times 10^6$  pfu/ml) harvested from NA-treated ST cells for 1 h at 4°C or 37°C prior to further incubation at 37°C for subsequent infection assay. The infectivity of the treated viruses was compared with that of mock-treated viruses at 4°C (A) or 37°C (B), respectively. Each sample was done in triplicate, and bars indicate standard deviation. Symbol “\*\*\*\*” means highly significant difference in statistics.

even slightly increased when compared to the untreated virus. This result indicates that the effect of cholesterol depletion from the viral membrane on the infectivity of TGEV is evident only when infection is initiated at 37°C but not when adsorption occurs at 4°C.

#### 4. Discussion

Lipid rafts have been reported to play important roles in different stages of the virus life cycle such as viral entry, protein transport and targeting, as well as assembly and budding (Nayak and Hui, 2004). Cholesterol, a characteristic structural component of lipid rafts, is thought to function as a dynamic glue that keeps the raft assembly together (Simons and Toomre, 2000). As far as virus entry is concerned most of the information available is related to the role of DRMs in the plasma membrane of the target cell where they may act as platforms for concentration of virus receptors and/or for an efficient penetration process. However, the exact mechanism how membrane microdomains contribute to virus entry is not known. This is also true for coronaviruses which are reduced in their infectivity when the integrity of DRMs is abolished by cholesterol depletion of the target cell membrane (Bavari et al., 2002; Li et al., 2007; Ren et al., 2008; Thorp and Gallagher, 2004). Despite this similarity, coronaviruses differ with respect to the location of the cellular receptor. Aminopeptidase N, the cellular receptor of human coronavirus 229E, TGEV and some other members of this virus family is constitutively present in DRMs. By contrast, the receptor for mouse hepatitis virus, CEACAM, is usually detected in the detergent-soluble fraction but may be recruited to the DRM fraction during the entry process (Choi et al., 2005; Thorp and Gallagher, 2004).

In recent years, it has been reported for a few viruses that cholesterol depletion from the viral membrane also results in a reduction of infectivity: HIV-1 (Ono and Freed, 2001), human herpesvirus 6 (Huang et al., 2006), canine distemper virus (Imhoff et al., 2007), varicella-zoster virus (Hambleton et al., 2007), pseudorabies virus (Ren et al., 2010), duck hepatitis B virus (Funk et al., 2008) as well as influenza virus (Sun and Whittaker, 2003). As these viruses belong to quite diverse families, the importance of cholesterol in the viral membrane may be a more general feature of many enveloped viruses. The importance of cholesterol in the viral membrane may suggest that DRMs are present in the viral envelope and may be important for the function of the viral membrane proteins. The evidence, however, is circumstantial as DRM association of viral proteins has been analyzed only in infected cells. It has not been demonstrated that the association of viral protein with membrane microdomains is maintained in the viral envelope. It gets even

more complicated with viruses like herpesviruses, coronaviruses or hepatitis virus B which mature at intracellular membranes. The concentration of cholesterol and sphingolipids is increasing in the membranes along the secretory pathway, i.e. from the ER to the plasma membrane. Despite budding from intracellular organelles, surface glycoproteins may reach the cell surface which makes it difficult to distinguish the subsets of membrane glycoproteins that enter virus particles.

Membrane microdomains are characterized by light buoyant density, insolubility in cold non-ionic detergents, and relatively high levels of glycosphingolipids and cholesterol, which contribute to their detergent-resistant, liquid-ordered structure. DRMs are typically recovered as low-density material in equilibrium flotation gradients and measuring DRM association provides valuable information regarding the preference for rafts by proteins or lipids of interest (Ono et al., 2005). Sedimentation analysis indicated that the TGEV S protein cannot be solubilized by cold non-ionic detergents. Though this behavior is compatible with the location of a protein in DRMs, other pieces of evidence do not support this conclusion. Cholesterol depletion did not render the S protein soluble by cold non-ionic detergent and in the flotation analysis it was not transferred to fractions where DRM proteins are expected. Therefore, factors other than cholesterol-rich detergent membranes are responsible for the insolubility in cold non-ionic detergents. Interaction with other viral proteins such as the M and/or the E protein may contribute to this behavior. In this respect, it is interesting to note that the VSV G protein behaves different from the TGEV S protein. The former protein may interact with other proteins in the viral envelope not as tightly making it easier to solubilize G by detergent treatment.

Though the effect of cholesterol depletion on virus infectivity cannot be explained by the lipid raft concept, it is evident that cholesterol is important for virus entry. Our analysis did not provide any evidence that the two binding activities of the S protein, binding to sialic acids on sialoglycoconjugates and binding to aminopeptidase N, are reduced in cholesterol-depleted virions. Therefore, a post-adsorption step appears to require cholesterol for optimal infectivity. Stages in the infection cycle following the binding to cellular receptors have also been implicated in the importance of cholesterol for other viruses (Funk et al., 2008; Hambleton et al., 2007). To understand the role of cholesterol, one should keep in mind that cholesterol depletion results in a reduction but not in the abolishment of virus infectivity. Virus entry may occur also at lower cholesterol levels but increased cholesterol makes this process more efficient. In this context it is interesting that cholesterol depletion reduced infectivity only when virus binding occurred at elevated temperatures. When the adsorption step was performed at 4°C, the infectivity was somewhat lower but unaffected by cholesterol depletion. Interestingly Choi and coworkers reported a similar finding when they analyzed the infection by mouse hepatitis virus with cholesterol-depleted cells (Choi et al., 2005). Based on these findings we conclude that virus entry can be optimized when binding of virions to the cell surface can be combined with membrane rearrangements that are possible at 37°C but not at 4°C. Future work has to address the question whether cholesterol facilitates coronavirus entry by affecting the membrane fluidity or whether other molecular interactions depend on an increased content of cholesterol.

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