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of the Same Epithelial Cells

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Epithelial cells are important target cells for coronavirus infection. Earlier we have shown that transmissible gastroenteritis coronavirus (TGEV) and mouse hepatitis coronavirus (MHV) are released from different sides of porcine and murine epithelial cells, respectively. To study the release of these viruses from the same cells, we constructed a porcine LLC-PK1 cell line stably expressing the recombinant MHV receptor cDNA (LMR cells). The MHV and TGEV receptor glycoproteins were shown by immunofluorescence to appear at the surface of the cells and to be functional so that the cells were susceptible to both MHV and TGEV infection. Both coronaviruses entered polarized LMR cells only through the apical surface. Remarkably, while the cells remained susceptible to TGEV for long periods, infectability by MHV decreased with time after plating of the cells onto filters. This was not due to a lack of expression of the MHV receptor, since this glycoprotein was still abundant on the apical surface of these cells. TGEV and MHV appeared to exit LMR cells from opposite sides. Whereas TGEV was released preferentially at the apical membrane, MHV was released preferentially at the basolateral surface. These results show that vesicles containing the two coronaviruses are targeted differently in LMR cells. We propose that the viruses are sorted at the Golgi complex into different transport vesicles that carry information directing them to one of the two surface domains. The apical release of TGEV and the basolateral release of MHV might be factors contributing to the difference in virus spread found between TGEV and MHV in their respective natural hosts, the former causing mainly a localized enteric infection, the latter spreading through the body to other organs. © 1996 Academic Press, Inc.

Coronaviruses are enveloped, positive-strand RNA viruses that infect humans and animals. Each virus has a narrow host range. The course of infection ranges from subclinical to lethal, and the symptomatology from respiratory and enteric disease (most commonly) to hepatitis, peritonitis, encephalomyelitis, and other syndromes. Primary replication is often limited to epithelial cells of the respiratory or gastrointestinal tracts (*1*).

The plasma membrane of epithelial cells is divided into an apical domain, directed to the external milieu, and a basolateral domain, facing the internal milieu. Many viruses enter these cells from a specific side and also virus release is often vectorial (for a review, see Ref. *2*). The polarity of virus release may differ in epithelial cells depending on their origin. Whereas influenza virus and vesicular stomatitis virus are released from the apical and basolateral surfaces, respectively, in both Fischer rat thyroid (FRT) and Caco-2 cells, two togaviruses, Sindbis and Semliki Forest virus, bud from the apical membrane of FRT cells, but from the basolateral membrane of Caco-2 cells (*3*). In all these cases budding appeared to take place at the plasma membrane domain where the respective viral membrane proteins were found to accumulate.

Unlike the viruses mentioned above which mature by budding from plasma membranes, coronaviruses mature by budding from intracytoplasmic membranes in a pre-Golgi compartment called the budding or intermediate compartment (*4–6*). From there the viral particles are transported in vesicles through the secretory pathway to the plasma membrane, where they are released by exocytosis (*7*). Recently, we have shown that the mouse hepatitis coronavirus (MHV) is preferentially secreted through the basolateral side of polarized murine mTAL cells, but that the porcine transmissible gastroenteritis coronavirus (TGEV) is secreted from apical surfaces of

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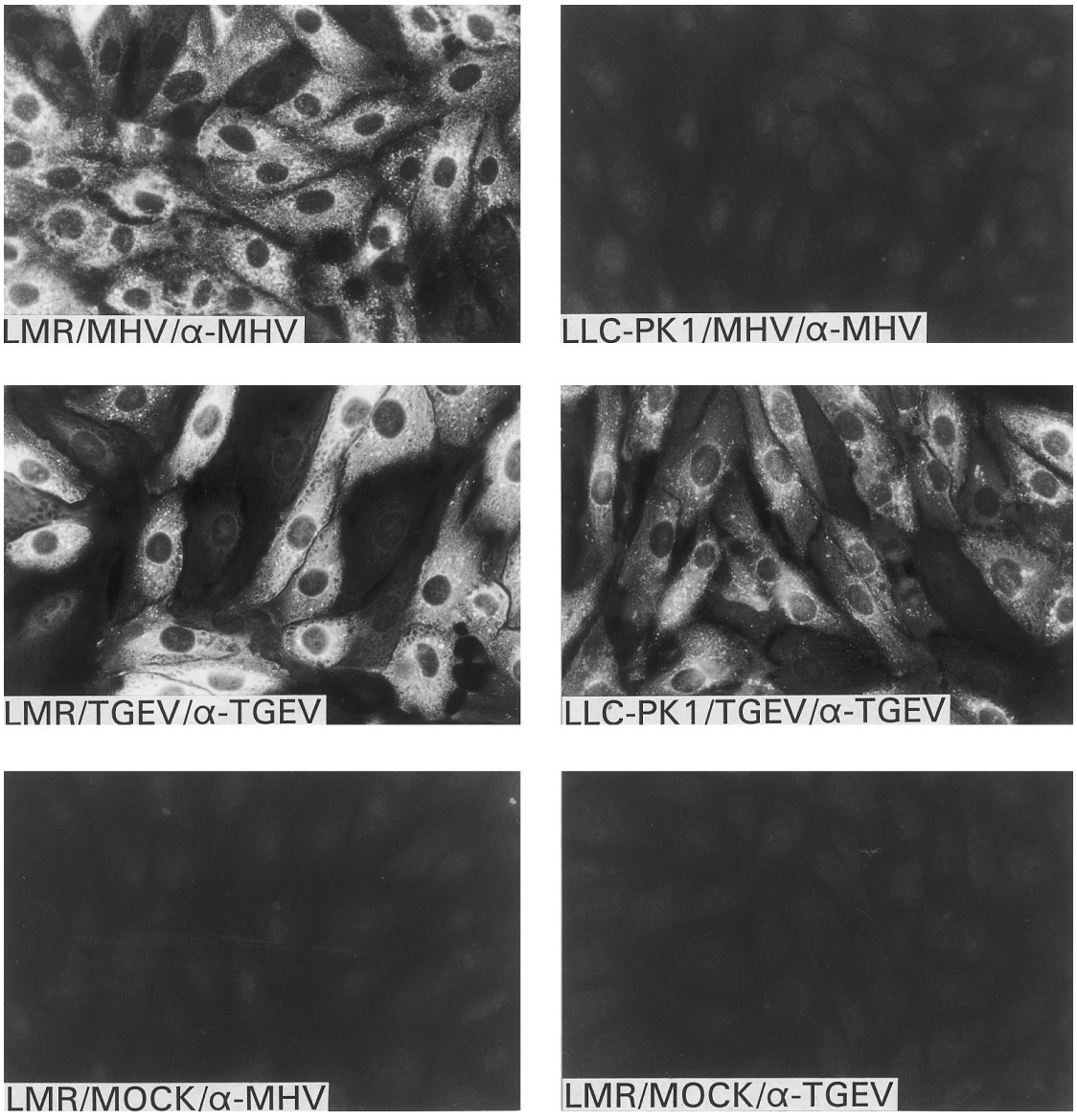

FIG. 1. Susceptibility of LLC-PK1 and LMR cells to MHV and TGEV infection. To prepare an LLC-PK1 cell line stably expressing the MHV receptor glycoprotein, we constructed the expression plasmid pMHVR2 as described before (*11*). pMHVR2 was transfected into LLC-PK1 cells using the calcium phosphate precipitation technique (*12*). G418-resistant cells were cloned by three rounds of limiting dilution. Cells from a selected clone (named LMR cells) and LLC-PK1 cells were grown on coverslips and infected with MHV or TGEV or mock infected. At 6 hr p.i. cells were fixed and processed for immunofluorescence analysis using an anti-TGEV spike (α-TGEV) monoclonal antibody or an anti-MHV (α-MHV) serum as described previously (*10*).

polarized porcine LLC-PK1 cells (*8–10*). To establish whether these differences were due to the use of different cells or caused by differential sorting of the two viruses, we investigated the release of the two viruses from the same cells. To accomplish this aim we prepared a derivative of the porcine cell line. LLC-PK1 cells, which are not susceptible to MHV-infection (Fig. 1), were transfected with plasmid pMHVR2 containing the MHV receptor (MHVR) cDNA and the neomycin resistance cDNA (*11*). After selection of the cells with the neomycin-derivative Geneticin (G418), resistant cells were cloned by three rounds of limiting dilution. One of these cloned cell lines was selected for further experimentation and was named LMR. The immunofluorescence observations of Fig. 1 show that the LMR cells supported MHV infection while remaining susceptible also to TGEV infection. From this experiment, and others where the m.o.i. was varied, it appeared that up to 95% of the LMR cells could be infected by MHV. Since the monolayer started to lose its integrity from around $8\frac{1}{2}$ hr postinfection (p.i.), all experiments were performed between 4 and 8 hr p.i.

Using an immunofluorescence assay we observed

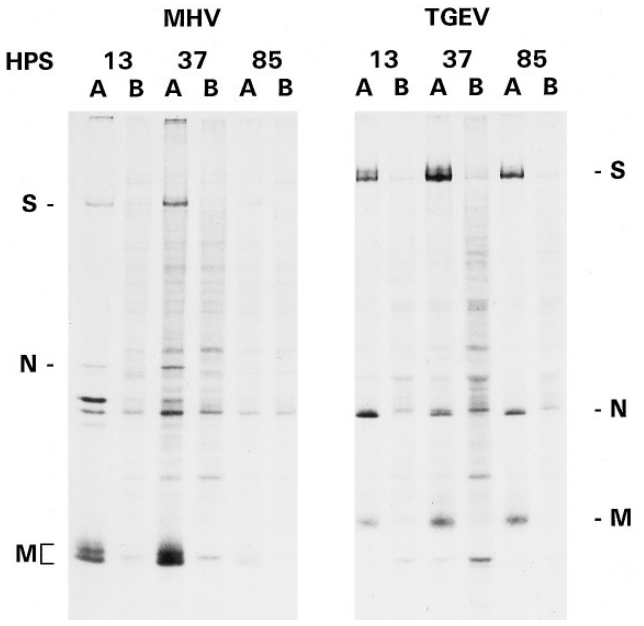

FIG. 2. Entry of MHV and TGEV into LMR cells: a radioimmunoprecipitation assay. LMR cells were cultured on filter supports and inoculated with TGEV or MHV (m.o.i. 10) from the apical (A) or basolateral (B) side at different hours p.s. (HPS). Cells were labeled with 200 μCi ^{35}S labeling mix (Amersham) from $4\frac{1}{2}$–$7\frac{1}{2}$ hr p.i. and viral proteins were immunoprecipitated from the cell lysates either with an anti-MHV or with an anti-TGEV serum as described earlier (*8, 10*). Note that the analysis of the media of these cells is shown in Fig. 5. Indicated are the spike (S), nucleocapsid (N), and membrane (M) proteins of MHV and TGEV.

that, depending on the experimental conditions, up to 50% of the cells could be doubly infected by both TGEV and MHV (data not shown). Furthermore, the two viruses appeared to interfere significantly with each other's replication. The amounts of MHV and TGEV proteins synthesized in doubly infected cells were much lower than those in singly infected cells. A reflection of this effect was observed when the culture media were analyzed. Using immunoisolation, a clear reduction was found in the amounts of each of the two viruses after double inoculation compared to single infections. This was especially the case for the release of MHV (data not shown). In view of the strong interference between the two viruses, the polarity of their release in this double-infection system was not further pursued.

To study the entry of TGEV and MHV into LMR cells, monolayers of the cells were grown on filters as described before (*8, 10*). This allowed inoculation either from the apical or from the basolateral side. Cells were infected at different times postseeding (p.s.) from either side and labeled with ^{35}S labeling mix. Lysates were prepared and viral proteins were immunoprecipitated with an antiserum against TGEV or MHV. As seen from the induction of virus-specific protein synthesis (Fig. 2), both TGEV and MHV entered LMR cells preferentially from the apical side. This polarity of entry was already evident when cells were infected at 13 hr p.s., the earliest time point in this experiment. For TGEV these results are in agreement with our earlier observations in the parental cell line, LLC-PK1 (*8*). The amount of MHV proteins synthesized in the LMR cells decreased when monolayers of increasing age were used. Thus, monolayers infected at 85 hr p.s. hardly produced any MHV proteins. This observation was not due to the cells becoming generally less metabolically active: no such time-dependent decrease in virus-specific protein synthesis was seen after TGEV infection (Fig. 2). The apical entry of the viruses was confirmed in an immunofluorescence experiment in which filter-grown LMR cells were again infected with MHV or TGEV from the apical or basolateral side at different times p.s. and fixed for staining 6 hr later. Figure 3 shows that the entry of MHV into the cells was not yet polarized when cells were infected at 4 hr p.s. From 8 hr p.s. on, virus entry became restricted to the apical membrane domain. The same results were found for the entry of TGEV (results not shown). From about 48 hr p.s. the percentage of LMR cells that became infected by MHV started to decrease (data not shown) until at 73 hr p.s. only a few cells could be infected with MHV, and only from the apical side. In contrast, the cells were still fully infectable with TGEV from the apical side at this time, showing again that the observed phenomenon was specific for MHV. To rule out that the preferential apical entry of both viruses was due to their inability to pass the filters and reach the cells at later time points postseeding, cells grown for 73 hr on filters were treated with 30 m*M* EGTA for 30 min at 37°. This disrupts the tight junctions and allows virus to pass between the cells. EGTA treatment immediately before inoculation allowed the cells to become infected with TGEV and MHV from the basolateral side (data not shown). Notably, however, this treatment did not change the percentage of cells that could be infected with MHV from the apical side.

The distribution of the MHVR glycoprotein on the surface of LMR cells was determined for two reasons. First, we wondered whether the polarized entry of MHV was correlated with a polar distribution of the MHVR. Second, we wanted to know whether the observed temporal decrease in susceptibility was caused by the gradual loss of the receptor glycoprotein with time. Using confocal laser scanning microscopy it was found that at 14 hr p.s., the MHVR glycoprotein was detected both on the apical side (TRITC channel; Fig. 4) and on the basolateral side (FITC channel). In the reverse experiment, in which the FITC- and TRITC-conjugated second antibodies were used apically and basolaterally, respectively, similar results were found (data not shown). However, it was noticed that FITC gave a two to four times stronger signal than TRITC with the settings used for the data processor. Considering this, we can conclude that at 14 hr p.s. the receptor glyco-

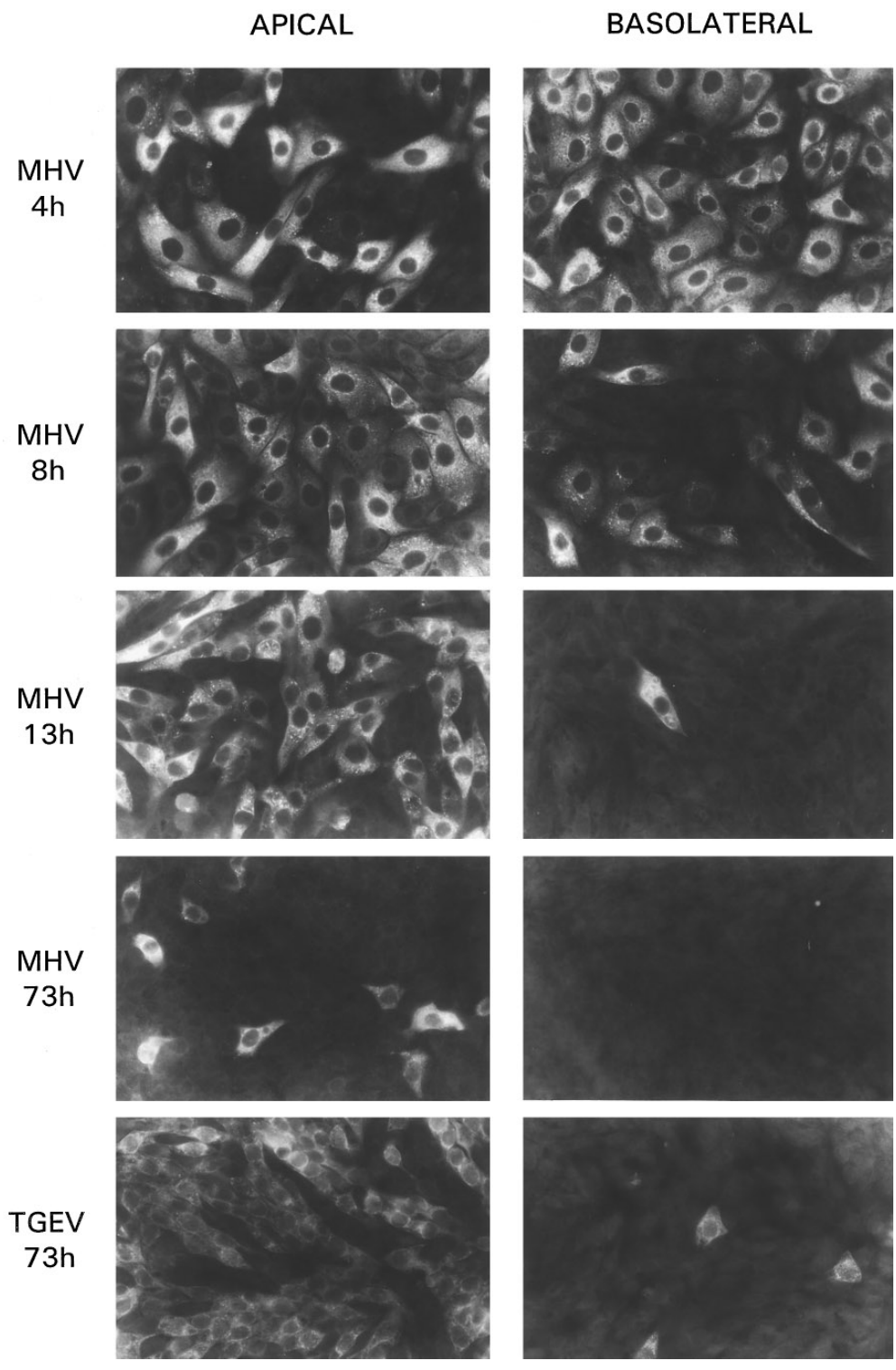

FIG. 3. Entry of MHV and TGEV into LMR cells as shown by immunofluorescence. LMR cells cultured on filter supports were infected with MHV or TGEV from the apical or basolateral side at different hours p.s. (h). Cells were fixed at 6 hr p.i. and processed for immunofluorescence analysis using an anti-TGEV S monoclonal antibody for TGEV-infected cells and an anti-MHV serum for MHV-infected cells as described before (*10*).

protein density at the apical plasma membrane was some two to four times higher than at the basolateral plasma membrane. The presence of the MHVR at the basolateral side at a time point at which MHV entry had already become restricted largely to the apical side was surprising. One may assume, however, that

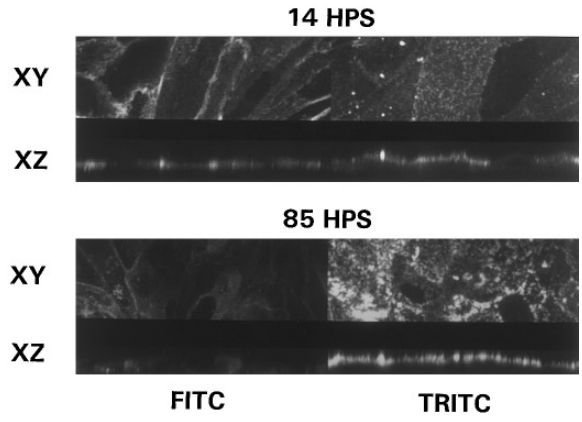

FIG. 4. Localization of the MHV receptor on the plasma membrane of polarized LMR cells by confocal microscopy. Filter-grown LMR cells were fixed at 14 and 85 hr p.s. (HPS) and incubated from the apical and basolateral side with the monoclonal antibody CC1 against the receptor of MHV (*13*). Subsequently, cells were incubated from the apical side with a TRITC-conjugated second antibody (TRITC) and from the basolateral side with a FITC-conjugated second antibody (FITC) and processed for fluorescence microscopy as described previously (*8*). Shown are XY sections through the cells, i.e., parallel to the filter, and XZ sections through the cells, i.e., perpendicular to the XY sections. The apical region of the cells is above. Note that the XY and XZ images of each time point are taken from the same cells.

a critical threshold surface density of the receptor is required for coronavirus entry; at the basolateral surface this density may have become too low to initiate infection. This explanation has also been suggested for the selective apical entry of measles virus into cells that expressed the viral receptor at the basolateral membrane domain as well (*14*). At 85 hr p.s. the receptor glycoprotein was still abundantly present. It was found almost exclusively on the apical side, though a small amount of receptor protein could be detected on the basolateral side of the cells as well (Fig. 4). Hence, the low level of infection by MHV from the apical side at 85 hr p.s. cannot be due to a lack of receptor glycoprotein. Possibly, an additional cellular factor may be required for an early step in the infection as has also been suggested by others (*15, 16*). The plasma membrane distribution of the TGEV receptor glycoprotein in LMR cells was found to be similar to that in LLC-PK1 cells (*8;* data not shown).

The main purpose of this study was to investigate the direction of release of TGEV and MHV from cells of the same line. Therefore, parallel cultures of LMR cells were grown on filters and infected with either virus from the apical side. Apical and basolateral media were analyzed for the appearance of viral proteins and infectious viral particles. In the experiment shown in Fig. 5A infected cells were radiolabeled for 3 hr after which viral proteins were immunoprecipitated from the media. In agreement with the results obtained with LLC-PK1 cells (*8*), TGEV viral structural proteins were observed predominantly in the apical medium. In contrast, MHV viral structural proteins were released from the basolateral side of the cells similar to their release from murine epithelial cells (*10*). Similar results were found when viral particles were affinity purified from the media by adding monoclonal antibodies against the spike proteins of TGEV and MHV in the absence of detergent (data not shown). The ratio between MHV viruses released into the basolateral and apical medium decreased in older monolayers. Possible reasons for this observation are discussed elsewhere (*10*).

The apparent preferential release of MHV into the basolateral medium of LMR cells was confirmed by analyzing the infectivity accumulating on opposite sides of the monolayer. In the experiment shown in Fig. 5B, 17-fold more $TCID_{50}$ units had accumulated in the basolateral medium than in the apical medium after 8 hr of infection. This corresponds to about 5% of the infective MHV particles having been released into the apical medium, whereas 95% appeared in the basolateral medium. This may even be an underestimate because viral particles become trapped and accumulate between cells and between the cells and the filter (*10*). In contrast, more than 99% of infective TGEV particles were released into the apical medium of LMR cells similar to their release from LLC-PK1 cells (*8*).

In conclusion, our studies show that, similar to our earlier observations in mTAL (*10*) and LLC-PK1 (*8*) cells, MHV and TGEV are released from opposite membrane domains, i.e., from basolateral and apical surfaces, respectively. Hence, the vesicles containing the two coronaviruses are sorted differently in cells from the same line. Somehow the cellular sorting machinery can obviously distinguish between vesicles containing MHV and those containing TGEV. Sorting most likely occurs when the viruses exit from the Golgi complex. How this sorting occurs and what determinants are important to effect the selection is unknown. For viruses exiting via the plasma membrane, polarized budding was found to be a consequence of the directional transport of viral membrane proteins to a specific surface (for a review, see Ref. *2*). Although coronaviruses do not bud at the plasma membrane, the viral structural proteins may still play a role in intracellular sorting of virions. One obvious candidate is the spike protein, the most prominently exposed moiety at the virion surface, which is also involved in receptor binding and cell fusion (for references, see Ref. *17*). It is conceivable that the TGEV spike protein harbors signals that direct viral particles into vesicles destined for apical release; accordingly, the MHV spike may contain domains that direct virions to the basolateral pathway.

Another candidate to explain the difference in sorting is the M protein which is N-glycosylated in TGEV but exclusively O-glycosylated in MHV (for references, see

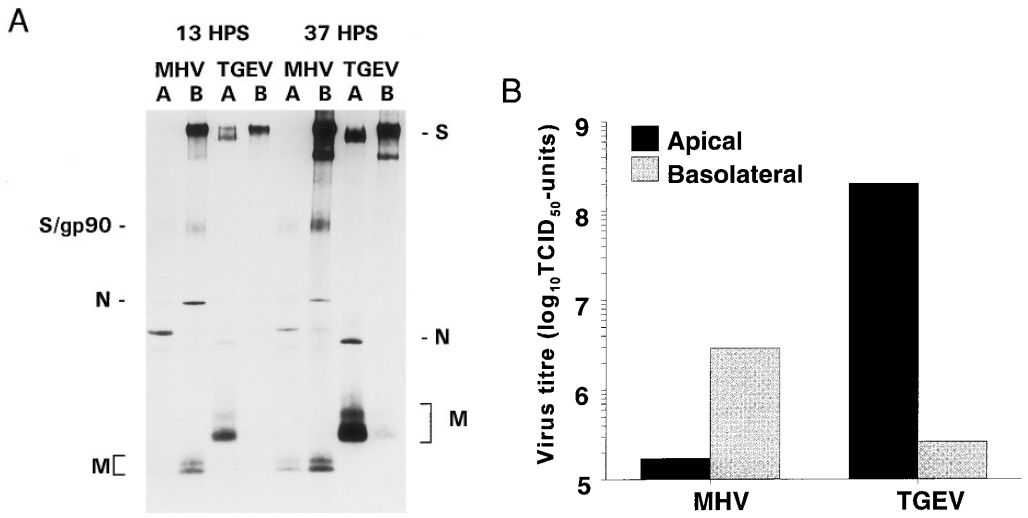

FIG. 5. Release of TGEV and MHV from polarized LMR cells. (A) Filter-grown LMR cells were infected with MHV or TGEV from the apical side at 13 and 37 hr p.s. (HPS). Cells were labeled from $4\frac{1}{2}$ to $7\frac{1}{2}$ hr p.i. with 200 μCi ^{35}S labeling mix. Viral proteins present in the apical (A) and basolateral (B) media were immunoprecipitated with an anti-MHV or anti-TGEV serum as described earlier (*8, 10*). Note that the analysis of the cell lysates of these cells is shown in Fig. 2. The positions of the MHV and TGEV spike (S), membrane (M), and nucleocapsid (N) structural proteins in the gel are indicated on the left and right sides, respectively. S/gp90 refers to the cleaved form of the MHV spike protein. Note that the protein present at the top of the gel in the basolateral samples is an unidentified cellular protein (M_r ~250 kDa) nonspecifically coimmunoprecipitated only from basolateral media of LMR cells. (B) Quantitation of infective virus particles in apical and basolateral media of MHV-infected LMR cells. Filter-grown LMR cells were infected with MHV or TGEV from the apical side at 13 hr postseeding as described previously (*8, 10*). Viral infectivity in the culture media was determined at 8 hr p.i. by limiting dilution assays as described before (*8, 10*).

Ref. *18*). *N*-Glycans are used as apical sorting signals in some secretory proteins (*19, 20*); because coronavirions are transported via the exocytic pathway to the plasma membrane — as are secretory proteins — their sorting may be quite similar. The difference in glycosylation found between the M proteins of the two viruses may thus result in their sorting to opposite surfaces of epithelial cells. The M proteins of TGEV and MHV may also determine budding of the viruses into different, yet unidentified, domains of the intermediate compartment that are already destined for either basolateral or apical release.

As epithelial cells are the initial target cells for most virus infections, their pivotal role in the pathogenesis of viral infections is evident. Many viruses are released from epithelial cells in a polarized way (*2*). Indications of asymmetrical release have also been observed *in vivo* for a number of coronaviruses (for a recent review, see Ref. *21*). Because a virus that is specifically released from the apical surface is targeted to the lumen, the resulting infection is more likely to be restricted to the epithelial surface. In contrast, basolateral release should provide access to the blood and lymph vessels, thereby facilitating the establishment of a systemic infection. The apical release of TGEV and the basolateral release of MHV might be factors contributing to the difference in virus spread found between TGEV and MHV in their respective natural hosts, the former causing mainly a localized enteric infection, the latter spreading through the body to other organs.

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REFERENCES

1. Holmes, K. V., *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, Eds.), Chap. 29, pp. 841–856. Raven Press, New York, 1990.
2. Tucker, S. P., and Compans, R. W., *Adv. Virus Res.* 42, 187–247 (1993).
3. Zurzolo, C., Polistina, C., Saini, M., Gentile, R., Aloj, L., Migliaccio, G., Bonatti, S., and Nitsch, L., *J. Cell Biol.* 117, 551–564 (1992).
4. Tooze, J., Tooze, S. A., and Warren, G., *Eur. J. Cell Biol.* 33, 281–293 (1984).
5. Klumperman, J., Krijnse Locker, J., Meijer, A., Horzinek, M. C., Geuze, H. J., and Rottier, P. J. M., *J. Virol.* 68, 6523–6534 (1994).
6. Krijnse Locker, J., Ericsson, M., Rottier, P. J. M., and Griffiths, G., *J. Cell Biol.* 124, 55–70 (1994).
7. Tooze, J., Tooze, S. A., and Fuller, S. D., *J. Cell Biol.* 105, 1215–1226 (1987).
8. Rossen, J. W. A., Bekker, C. P. J., Voorhout, W. F., Strous, G. J. A. M., Van der Ende, A., and Rottier, P. J. M., *J. Virol.* 68, 7966–7973 (1994).
9. Rossen, J. W. A., Bekker, C. P. J., Voorhout, W. F., Horzinek, M. C., Strous, G. J. A. M., Van der Ende, A., and Rottier, P. J. M., *Adv. Exp. Med. Biol.* 380, 135–138 (1995).

10. Rossen, J. W. A., Voorhout, W. F., Horzinek, M. C., Van der Ende, A., Strous, G. J. A. M., and Rottier, P. J. M., *Virology* 210, 54–66 (1995).
11. Gagneten, S., Gout, O., Dubois-Dalcq, M., Rottier, P. J. M., Rossen, J. W. A., and Holmes, K. V., *J. Virol.* 69, 889–895 (1995).
12. Graham, F. L., and van der Eb, A. J., *Virology* 52, 456–461 (1973).
13. Williams, R. K., Jiang, G.-S., Snyder, S. W., Frana, M. F., and Holmes, K. V., *J. Virol.* 64, 3817–3823 (1990).
14. Blau, D. M., and Compans, R. W., *Virology* 210, 91–99 (1990).
15. Yokomori, K., and Lai, M. M. C., *J. Virol.* 66, 6931–6938 (1992).
16. Yokomori, K., Assanaka, M., Stohlman, S. A., and Lai, M. M. C., *Virology* 196, 45–56 (1993).
17. Cavanagh, D., *In* "The Coronaviridae" (S. G. Siddell, Ed.), pp. 73–113. Plenum, New York, 1995.
18. Rottier, P. J. M., *In* "The Coronaviridae" (S. G. Siddell, Ed.), pp. 115–139. Plenum, New York, 1995.
19. Kitagawa, Y., Sano, Y., Ueda, M., Higashio, K., Narita, H., Okano, M., Matsumoto, S.-I., and Sasaki, R., *Exp. Cell Res.* 213, 449–457 (1994).
20. Scheiffele, P., Peränen, J., and Simons, K., *Nature* 378, 96–98 (1995).
21. Rossen, J. W. A., Horzinek, M. C., and Rottier, P. J. M., *Trends Microbiol.* 3, 486–490 (1995).