Mouse hepatitis virus strain A59 is released from opposite sides of different epithelial cell types

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Introduction

Coronaviruses infect humans and animals through epithelial cells of the gastrointestinal and respiratory tracts that serve as their primary target. When studying infections in cultured polarized epithelial cells, we found previously that coronaviruses are released from specific plasma-membrane domains; thus, mouse hepatitis virus (strain A59; MHV-A59) leaves murine epithelial kidney cells from the basolateral surface, whereas release of transmissible gastroenteritis virus from porcine epithelial kidney cells is confined to the apical membrane. This observation begged the question whether a particular coronavirus is consistently shed through the same membrane, irrespective of the nature of the epithelial cell. We therefore extended our studies with MHV-A59 to Madin–Darby canine kidney (MDCK) strain I and human colon carcinoma (Caco-2) cells, both of which are naturally refractory to MHV-A59 but were made susceptible to infection by transfection with recombinant MHV receptor cDNA. The release of MHV-A59 from CacoMHVR cells occurred preferentially from the basolateral side, consistent with our previous observations. In contrast, release from MDCKMHVR cells occurred almost exclusively from the apical surface. Because of this difference, we studied MHV-A59 infection of MDCKMHVR cells in more detail. The virus entered the cells preferentially from the apical side, a situation similar to that in murine epithelial cells, where the highest density of MHV receptor glycoprotein was found. The results from this and previous studies show that targeting of vesicles containing MHV-A59 to a specific side of epithelial cells may vary in different epithelial cell types.
contrast, the basolateral release of MHV-A59 would allow the
virus to infect underlying tissue and to spread by viraemia
throughout the body of the infected animal, leading to hepatitis
and encephalomyelitis.

Studies of coronavirus infections in polarized cells are
hampered by the lack of suitable epithelial cell lines. Ideally,
one would use cells derived from the natural target tissues, e.g.
the respiratory or the intestinal epithelia. These are currently
not available. Though our initial observations with MHV-A59
and TGEV in murine and porcine kidney cell lines, respectively,
were fully compatible with the natural situation with respect to
virus entry and release, we now extend these studies by
analysing the same virus (MHV-A59) in two additional cell
lines derived from canine kidney and human colon. The results
show that while MHV-A59 is secreted at the basolateral
domain of the human cells, as for murine cells, it leaves the
canine cells predominantly through the apical membrane.

Methods

■ Cells, virus and antisera. Madin–Darby canine kidney (MDCK)
strain I and human colon carcinoma (Caco-2) cells were obtained from
the ATCC. The procedure for the stable expression of recombinant MHV
receptor glycoprotein in MDCK strain I cells has been described before
by Gagneten et al. (1995). The same procedure was followed for Caco-2
cells. The resulting cell lines, designated Caco_MHVR and MDCK_MHVR,
were maintained at 37 °C under 5% CO₂ in plastic culture flasks in
Dulbecco’s Modified Eagle’s Medium (Gibco) containing 10% fetal calf
serum and geneticin G418 (500 µg/ml). For the preparation of polarized
cell monolayers, polycarbonate membrane filters attached to the bottom
of plastic cups (Transwell inserts, 0.45 µm, 4.5 cm²; Costar) were placed
in six-well tissue culture plates. Cells from a confluent culture in an
80 cm² culture flask were obtained by trypsinization and were suspended
in 20 ml culture medium of which 1 ml was added per filter. The tightness
of the monolayer was checked routinely by adding medium to the upper
chamber up to a higher level than in the lower chamber (Cerneus
et al., 1994). MHV-A59 was propagated in Sac− cells as described earlier by Spen
et al., (1981), and the production of the rabbit polyclonal antiserum to
MHV receptor glycoprotein (Williams et al., 1990; Dveksler et al., 1993) was kindly provided by Kathryn Holmes (University of Colorado Health Sciences Center, Denver, USA).

■ Virus infections. Caco_MHVR and MDCK_MHVR cells grown on filters
were rinsed once with infection medium (IM; culture medium without
geneticin G418) and inoculated with MHV-A59 diluted in IM at an m.o.i.
of 20 from the apical or basolateral side at different times post-seeding
(p.s.). Basolateral inoculation was done by placing the filter on a 75 µl
droplet of inoculum on Parafilm. Apical inoculation was achieved by
dispersing 450 µl inoculum to the upper compartment. After 1 h incubation
at 37 °C, the inoculum was removed and the filters were rinsed three
times with IM and further incubated in this medium.

■ Metabolic labelling and immunoprecipitation. Filter-grown
MHV-A59-infected Caco_MHVR, and MDCK_MHVR cells were labelled from
4.5–7.5 h and 8–9 h post-infection (p.i.), respectively, with L-35S in vitro
labelling mix (Amersham). Virus proteins were immunoprecipitated from
cell lysates and culture media as described before (Rossen et al., 1995c). In
pulse-chase experiments, cultures were incubated for 30 min with minimal
essential medium lacking methionine before labelling of the cells from
6–6.5 h p.i. with 200 µCi l-35S in vitro labelling mix. The pulse-labelling
period was followed by a chase with culture medium containing 4 mM
methionine and 4 mM-cysteine. In control experiments, MDCK_MHVR
cells were grown on collagen membrane filters (Transwell-COL inserts,
30 µm, 4.5 cm²; Costar) and infected at 15 h p.s. with the San Juan strain
of vesicular stomatitis virus (VSV; m.o.i. of 12) or MHV-A59 (m.o.i. of
20) from the apical or basolateral side. With VSV, all further incubations
were done at 32 °C to slow the cytopathic effects (van Meer & Simons,
1982). Cells were labelled from 5–8 h p.i. with 250 µCi l-35S-labelling mix
and media were collected. VSV and MHV-A59 proteins were immuno-
precipitated as described by Rossen et al. (1995c), using a rabbit anti-VSV
and a rabbit anti-MHV-A59 serum, respectively.

■ Virus titration. Virus infectivity in culture media was determined at
different times p.i. by a quantal assay on L-cells. L-cells grown in 96-well
plates were inoculated with serial dilutions of medium samples from
infected cells made in IM medium. TCID₅₀ values were calculated using
the Spearman–Kärber formula.

■ Localization of the MHV receptor glycoprotein. Filter-grown
MDCK_MHVR cells were fixed at different times p.s. with 3% para-formaldehyde, rinsed and blocked for 30 min with PBS containing
50 mM-glycine and 1% normal goat serum (PBGN). Cells were incubated
for 1 h from both the apical and basolateral side with the MAb CC1
against the MHV receptor glycoprotein (Williams et al., 1990) diluted
1:25 in PBGN. After three washes with PBGN, cells were stained from
the basolateral side with fluorescein isothiocyanate (FITC)-conjugated
goat anti-mouse IgG antibodies (1:200; Cappel) and from the apical side
with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat
anti-mouse IgG antibodies (1:150; Cappel) for 45 min. Finally, cells were
washed three times with PBGN and filters were cut from their holders and
mounted in FluorSave reagent (Calbiochem). Fluorescence was viewed using
a Zeiss Axioscope microscope with a Zeiss ×40 water immersion
lens (numerical aperture = 1.2), in conjunction with a confocal laser
scanning unit (model MRC600; Bio-Rad). Optical sections were taken at
intervals of ~1 µm parallel to the filters. For alternative observation of
MHV receptor glycoprotein distribution, optical sections of ~1 µm
thickness were taken perpendicular to the cell monolayer.

Results

MHV-A59 infection of Caco_MHVR cells

In addition to our studies of MHV-A59 release from kidney
cells, we wanted to analyse its release from a well-established
intestinal cell line, namely the human colon carcinoma cell line
Caco-2. A derivative of this cell line was prepared that was
susceptible to MHV-A59 infection by the stable expression of
the MHV receptor glycoprotein (Caco-MHVR cells). When
grown on filters under our standard conditions, Caco-MHVR
cells established tight monolayers within 34–44 h p.s. The cells
could be infected with the same efficiency from the apical and
basolateral sides at 24 h p.s. (data not shown). However, at
44 h p.s., the virus entered the cells preferentially through the
apical surface as judged from the amounts of progeny virus
produced after inoculation from the apical versus the baso-
larateral side. As is clear from Fig. 1, very little labelled virus was
released into either medium after basolateral inoculation (lanes
3 and 4) as compared with the yields after infection from the
apical side (lanes 1 and 2). The same experiment also revealed
the direction of virus release. Significantly, in both cases, MHV-A59 was preferentially shed from the basolateral surface (lanes 2 and 4). This was further confirmed by titrations of the infective viruses in the same media. These analyses revealed that 90% and 96% of infective virus particles released were shed into the basolateral medium of cells infected from the apical or basolateral side, respectively.

**MHV-A59 infection of MDCK<sub>MHVR</sub> cells**

MDCK cells are without any doubt the best characterized epithelial cells which have been used as the model system of choice for the study of polarized sorting processes. When stably expressing the recombinant MHV receptor glycoprotein (MDCK<sub>MHVR</sub> cells), they can be infected by MHV-A59 (Gagneten et al., 1995). Infected monolayer cultures remained fully intact over the first 24 h p.i., as judged from the transepithelial resistance and from the absence of leakage of apical medium into the basolateral compartment (results not shown).

(i) Release. The polarity in the release of MHV-A59 from MDCK<sub>MHVR</sub> cells was first examined by quantifying the number of infective virus particles appearing in the apical and basolateral media. Surprisingly, unlike all the other cells we studied previously, MDCK<sub>MHVR</sub> cells shed MHV preferentially into the apical medium. At 9 h p.i., on average 99% of infectious virus particles had accumulated in this medium after cells had been infected from the apical side at 20 h p.s. (data not shown).

To confirm these observations in an independent assay and to determine the kinetics of cell polarization, filter-grown MDCK<sub>MHVR</sub> cells were infected apically at different times p.s.
and labelled with \(^{35}\)S-labelling reagent. Apical and basolateral media were then collected and aliquots were taken for endpoint dilution titrations and for immunoprecipitation of virus proteins. The release of radiolabelled virus proteins was polarized from the earliest infection time-point on, i.e. it was already polarized when measured between 10 and 13 h p.s. (Fig. 2). Labelled virus proteins were found predominantly in the apical medium. Note that, in contrast to the membrane (M) and the nucleocapsid (N) proteins, which were detectable almost exclusively in the apical medium, the cleaved form(s) of the spike protein (S/gp90) was found in both the apical and basolateral media. The possible relevance of this observation will be discussed. Quantification of the infectivities in the media revealed that for the four infection time-points (4, 8, 12 and 16 h p.s.) shown in Fig. 2, 99, 92, 98 and 98%, respectively, of the infectious virus particles were shed into the apical medium. As is also clear from the analysis of labelled virus proteins in the culture media (Fig. 2), the total amount of virus release gradually decreased with the age of the cell monolayers.

One might argue that the observed apical release of MHV-A59 might be the result of efficient transcytosis of initially basolaterally released viruses rather than of direct apical secretion. The pulse-chase experiment shown in Fig. 3 does not support this view. In this experiment, the basolateral and apical media were harvested and replaced every 30 min after the 30 min labelling. Virus proteins already started to appear exclusively in the apical culture medium during the first chase period and then increased gradually. There were no indications that radiolabelled virus made a transient basolateral appearance before being redirected to the apical side. We cannot, of course, exclude the possibility that virus particles are released basolaterally but do not detach from the membrane or rebind.

**Fig. 3.** Release of MHV-A59 from MDCK<sub>MHVR</sub> cells: a pulse-chase experiment. MDCK<sub>MHVR</sub> cells were infected apically with MHV-A59 at 20 h p.s. After a 30 min starvation period the cells were pulse-labelled with \(^{35}\)S-labelling reagent from 7–7.5 h p.i. followed by a chase of 2.5 h. The chase media in the apical and basolateral compartments were replaced by new chase medium every 30 min. Subsequently, virus proteins were immunoprecipitated from the apical (A) and basolateral (B) pulse and chase media using a rabbit anti-MHV-A59 serum. The samples were analysed in an SDS–10% polyacrylamide gel. The S/gp90, N and M proteins are indicated.
immediately. Since we did not observe virus particles at the basolateral membrane by electron microscopy (data not shown), we do not consider this option very likely.

(ii) Release of MHV-A59 and VSV from cells grown on 3.0 µm filters. The unexpected apical release of MHV-A59 from MDCK<sub>MHV</sub> cells prompted us to consider that the properties of the MDCK cells might have changed due to the expression of the MHV receptor glycoprotein. We therefore used VSV as a gold standard since it is well established that both the entry and the release of this virus from MDCK cells are restricted to the basolateral surface (Rodríguez-Boulan & Sabatini, 1978; Fuller et al., 1984). Because inoculation of MDCK<sub>MHV</sub> cells with VSV from the basolateral surface was very inefficient when cells were grown on filters with a pore size of 0.45 µm, 3.0 µm filters were used. Several epithelial cell types have been reported to migrate through permeable filters with pore sizes larger than 1.0 µm (Tucker et al., 1992). The penetration of MDCK cells through 3.0 µm pores was, however, small and occurred more slowly than with other cell types. MDCK cells established only some isolated colonies of cells on the lower filter surface by 6 days p.s. (Tucker et al., 1992). In our experiment, MDCK<sub>MHV</sub> cells were grown on the 3.0 µm filters for 15 h before being infected from the apical or basolateral side with MHV-A59 or VSV. At this time, the cells had already formed tight monolayers since no leakage of medium from the apical to the basolateral compartment was observed. The cells could be infected with VSV only from the basolateral side (Fig. 4). In contrast, MHV-A59 could infect the cells through both the apical and the basolateral surface, confirming our observations with the 0.45 µm filters. It further appeared that VSV was preferentially released from basolateral surfaces of the cells, although some VSV proteins were also found in the apical medium. Radiolabelled MHV-A59 proteins, however, were secreted almost exclusively in the apical medium, irrespective of the side from which the cells had been inoculated.

(iii) Entry. In contrast to the difference in the direction of virus release between MDCK<sub>MHV</sub> and all the other cells studied so

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**Fig. 4.** Release of MHV-A59 and VSV from MDCK<sub>MHV</sub> cells grown on 3.0 µm collagen filters. MDCK<sub>MHV</sub> cells, grown on collagen filters with a pore size of 3.0 µm, were infected with MHV-A59 or VSV from the apical (lanes 1, 2, 5 and 6) or basolateral (lanes 3, 4, 7 and 8) side. Cells were labelled with 200 µCi 35S-labelling reagent from 5–8 h p.i. and virus proteins were immunoprecipitated from apical (lanes 1, 3, 5 and 7) and basolateral (lanes 2, 4, 6 and 8) media using a rabbit anti-MHV-A59 or anti-VSV serum. The samples were analysed in an SDS–10% polyacrylamide gel. The structural proteins of MHV-A59 and VSV are indicated on the right- and left-hand side of the figure, respectively. Note that VSV infection was done at a lower m.o.i. than the MHV infection and at 32 °C, both to slow down any cytopathic effects (van Meer & Simons, 1982), which may explain the weak VSV bands.
The polarity of virus entry was similar in all cells, being restricted to the apical surface. Fig. 5 shows that after an initial period in which MDCK<sub>MHV</sub> cells could be infected from either side, entry became gradually restricted to the apical surface. This preference had become very clear at 41 h p.s., though at varying surface densities.

The MHV receptor glycoprotein became gradually restricted to the apical surface. At 72 h p.s., the highest density was found at the apical surface, although some receptor molecules could still be detected at the basolateral surface. The relative abundance of receptor molecules at the apical side compared to the basolateral side was actually even larger than suggested by Fig. 6. From the reverse experiment, in which the FITC- and TRITC-conjugated second antibodies were used apically and basolaterally, respectively, it appeared that FITC gave a two to four times stronger signal than TRITC under the measurement conditions used for the data-processor (results not shown).

**Discussion**

In this study, we extend our analysis of coronavirus infection of polarized epithelial cells. We selected two well-defined epithelial cell lines, Caco-2 and MDCK strain I. Cells were made susceptible to MHV infection by stably expressing the MHV receptor glycoprotein. The experiments show that the human intestinal Caco<sub>MHV</sub> cells infected with MHV-A59 shed progeny viruses through their basolateral membrane, consistent with the direction of MHV release observed in murine kidney cells (Rossen et al., 1995c). It therefore came as a surprise that the same virus was released through the opposite membrane of the MDCK<sub>MHV</sub> cells.

While the polarity of MHV-A59 release from MDCK<sub>MHV</sub> cells was different, the side of entry was similar to that reported for the other epithelial cells. Once cells had become fully polarized, virus could only get access through the apical membrane domain. Young monolayers allowed entry from both sides. Infection was sometimes apparently more efficient from the basal side, but this was simply due to the technical fact far, the polarity of virus entry was similar in all cells, being restricted to the apical surface. This preference had become very clear at 41 h p.s., when infection through the basolateral side was no longer detectable.

The preferential apical entry was confirmed by immunofluorescence analysis (not shown). This approach also revealed that the strong decrease in the amount of overall virus-specific protein synthesis seen after immunoprecipitation (Fig. 5) was due to a reduction of the number of cells that became infected as monolayer cultures grew older, rather than to a general decline in biosynthetic activity.

(iv) Localization of the MHV receptor glycoprotein. The distribution of the MHV receptor glycoprotein on the surface of MDCK<sub>MHV</sub> cells was determined for two reasons. First, we wondered whether the polarized entry of MHV was correlated with a polar distribution of the MHV receptor. Second, we wanted to know whether the observed temporal decrease in susceptibility was caused by the gradual loss of the receptor glycoprotein with time. The plasma-membrane distribution of the MHV receptor glycoprotein was determined by examining filter-grown MDCK<sub>MHV</sub> cells in a confocal laser scanning microscope. This method allows optical sections to be cut either in the horizontal plane (XY section) or in the vertical plane (XZ section). At 24 h p.s., the MHV receptor glycoprotein was present both on the apical side (TRITC channel; Fig. 6) and on the basolateral side (FITC channel). This is consistent with the observation that at this time MDCK<sub>MHV</sub> cells would still be infected from both sides. Remarkably, whereas only 20–30% of the cells could be infected by MHV-A59 at this time, as determined by standard immunofluorescence analysis, the receptor glycoprotein was detectable on almost every cell, though at varying surface densities.

Fig. 5. Entry of MHV-A59 in MDCK<sub>MHV</sub> cells. MDCK<sub>MHV</sub> cells grown on filter supports were infected with MHV-A59 (m.o.i. of 20) from the apical (A) or the basolateral (B) surface at different hours p.s. (HPS). Subsequently, cells were labelled from 6–9 h p.i. with 35S-labelling reagent and intracellular virus proteins were immunoprecipitated from cell lysates using a rabbit anti-MHV-A59 serum. The positions of the S, M and N structural proteins in the gel are indicated on the left-hand side of the figure. S/gp150 refers to the uncleaved form of the MHV-A59 spike protein, whereas S/gp90 represents the cleaved forms. The bands above S/gp150 seen in the 15 HPS lanes represent S complexes not dissociated during sample heating, which was limited to 3 min at 95°C to prevent aggregation of the M protein. The intense high molecular mass band in the 41 and 65 HPS lanes is a cellular protein that is nonspecifically precipitated by the antiserum and becomes more prominent as host shut-off ceases.
that in this case the inoculum virus was presented in a much smaller volume and consequently at a much higher concentration.

Monolayers of MDCK<sub>MHVR</sub> cells became generally less susceptible to MHV infection as a function of time after seeding. This could not be attributed to the lack of receptor molecules; rather, the amount of receptor glycoprotein seemed to increase steadily with time. In addition, receptor molecules were still detectable by confocal microscopy at basolateral surfaces at times when cells had become refractory to infection from this side. For the latter observation one may assume that a critical threshold surface density of the receptor is required for coronavirus entry; at the basolateral surface it may have become too low to initiate infection. This explanation was also suggested for the selective apical entry of measles virus into cells that expressed the virus receptor at the basolateral membrane domain as well (Blau & Compans, 1995). The decrease in susceptibility to MHV infection could not be explained by a low level of receptor glycoprotein expression on cells that were grown for long periods on filters. On the contrary, confocal laser scanning microscopic analysis showed that the level of MHV receptor glycoprotein expression at the apical membranes continued to increase. Our observations are reminiscent of reports showing that some mouse strains are resistant to MHV infection despite the presence of the receptor (Yokomori & Lai, 1992), and that expression of the receptor in MHV-resistant cell lines is not always sufficient to allow infection (Yokomori et al., 1993). These studies suggested that an additional cellular factor may be required for an early step in the infection. Such a factor may be absent in cells that have been grown for longer periods on filters. Alternatively, monolayers may start to produce compounds, such as cytokines, making the cells inimical to virus growth.

Coronaviruses are transported in vesicles along the secretory pathway to the plasma membrane, where they are released by exocytosis (Tooze et al., 1987). Their sorting might thus be subject to the same mechanisms that effect the targeting of secretory proteins. In human intestinal Caco-2 (Traber et al., 1987; Rindler & Traber, 1988) and LLC-PK1 cells (De Almeida & Stow, 1991; Low et al., 1994), most of the secreted polypeptides are found in the basolateral medium. It has therefore been proposed that basolateral secretion in these cells is signal-independent and that the route to the basolateral membrane domain constitutes the default pathway. If coronaviruses were sorted similarly, then the basolateral release of MHV-A59 from Caco-2 cells might just be the consequence of ‘passive’ transport. The apical release of TGEV from LLC-PK1 cells (Rossen et al., 1994), however, would then occur not by default but by active sorting mediated by specific signals.

It is well established that in MDCK cells the default pathway for secretory proteins is non-directional, i.e. specific secretion requires the presence of specific targeting information in a protein’s structure (Brown et al., 1984; Kondor-Koch et al., 1987).
lateral sorting signal in MHV particles might be overruled by another. Accordingly, in MDCK cells (Zurzolo et al., 1992). Finally, a hierarchy in sorting signals might exist where one signal dominates another. Accordingly, in MDCK cells a possible basolateral sorting signal in MHV particles might be overruled by an apical one or vice versa, in mTAL and Caco\textsubscript{MHVR} cells an apical sorting signal might lose the competition with a basolateral one.

Assuming that directional release of coronaviruses is at least sometimes the result of specific sorting, then the question is which of the virus structural protein(s) carries the sorting signals. One obvious candidate is the spike (S) protein, the most prominently exposed moiety at the virion surface and which is also involved in receptor binding and cell fusion. It is known that this protein is not only incorporated into virosomes, but that it also appears independently at the plasma membrane of infected cells. Furthermore, the most N-terminal subunit of the cleaved form of the S protein can easily dissociate from its C-terminal subunit into the culture media (for references, see Cavanagh, 1995). It was therefore important to observe that the cleaved form(s) of the MHV-A59 S protein was not only shed with virus into the apical culture fluid of MDCK\textsubscript{MHVR} but that it was also significantly released into the basolateral compartment (Fig. 2). This observation strongly suggests that the S protein does not carry the sorting signal(s) of the virus. Indeed, the fraction of the S protein that is not incorporated into virosomes is either sorted directionally to the basolateral side or transported by default to both sides.

The deviant release of MHV from MDCK cells as compared to that from other epithelial cells reveals that virus sorting is cell-type dependent. This is not the first report showing this phenomenon; another example is the budding of the togavirus Sindbis virus and Semliki Forest virus, which appeared to occur from the apical membrane of FRT cells but from the basolateral membrane of Caco-2 cells (Zurzolo et al., 1992). A peculiar illustration is the infection by VSV of its sand fly vector Lutzomyia shannoni. While budding of this virus was found to occur exclusively at the basolateral plasma membrane in the midgut epithelial cells, this process took place apically in salivary gland cells (Weaver et al., 1992). Previously, we have correlated the basolateral release of MHV and the apical release of TGEV from certain epithelial cell lines with the phenotypes of the natural infections by these viruses (Rossen et al., 1994, 1995 a, b, c). The results of the present work again demonstrate that one should be cautious when drawing general conclusions from data obtained with these in vitro model systems and when extending such conclusions to in vivo situations.

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References


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