Polarized glycoprotein targeting affects the spread of measles virus \textit{in vitro} and \textit{in vivo}

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We have shown previously that basolateral targeting of plasmid-encoded measles virus (MV) F and H protein is dependent on single tyrosine residues in the cytoplasmic tails of the glycoproteins and is essential for fusion activity in polarized epithelial cells. Here, we present data on the functional importance of polarized glycoprotein expression for the cytopathic properties of infectious MV in culture and for pathogenesis \textit{in vivo}. By the introduction of single point mutations, we generated recombinant viruses in which the basolateral targeting signal of either one or both glycoproteins was destroyed (tyrosine mutants). As a consequence, the mutated glycoproteins were predominantly expressed on the apical membrane of polarized Madin–Darby canine kidney cells. In contrast to parental MV, none of these virus mutants was able to spread by syncytia formation in polarized cells showing that the presence of both MV glycoproteins at the basolateral cell surface is required for cell-to-cell fusion \textit{in vitro}. Using cotton rats as an animal model that allows MV replication in the respiratory tract, we showed that basolateral glycoprotein targeting is also of importance for the spread of infection \textit{in vivo}. Whereas parental MV was able to spread laterally within the respiratory epithelium and from there to cells in the underlying tissue, tyrosine mutants infected only single epithelial and very few subepithelial cells. These data strongly suggest that basolateral targeting of MV glycoproteins helps to overcome the epithelial barrier and thereby facilitates the systemic spread of MV infection \textit{in vivo}.

INTRODUCTION

Mucosal surfaces of the respiratory tract are one of the main sites for viruses to enter the organism. Since respiratory epithelial cells represent primary target cells, replication in these cells has significant implications for virus spread and thus for the pathogenesis of virus infections. Due to the polarized nature of epithelial cells, virus entry as well as virus release can be restricted to either the apical or the basolateral cell surface domain. Whereas virus entry into epithelia has been intensively studied (for review, see Bomsel & Alfsen, 2003), mechanisms underlying virus spread after productive infection of epithelia are less well characterized. For a long time, it has been believed that virus budding polarity plays a key role in virus spread and is a consequence of the polarized expression of the viral envelope glycoproteins. This model was mainly based on earlier studies on influenza and vesicular stomatitis virus and proposes that viruses with apically expressed glycoproteins are released mainly apically and therefore can cause only a restricted local infection of the respiratory tract. In contrast, viruses encoding glycoproteins with basolateral targeting signals are predominantly released from the basolateral cell surface, which is suggestive of facilitating virus spread from epithelia to underlying tissues, thereby establishing a systemic infection (for review, see Tucker & Compans, 1993). Fitting into this model, budding of pneumotropic Sendai virus is restricted to the apical domain of polarized cells, whereas systemic spread of a Sendai virus mutant (F1-R) could be ascribed mainly to bidirectional glycoprotein expression and apical as well as basolateral virus release from bronchial epithelia (Tashiro et al., 1991).

However, recent data on the release of measles virus (MV), a member of the family \textit{Paramyxoviridae}, have indicated that budding polarity does not necessarily play a key role in virus spreading. As for all \textit{Paramyxoviridae}, MV entry and initial replication occur in the respiratory mucosa and the virus is released apically from respiratory epithelial cells (Blau & Compans, 1995). However, in contrast to most other paramyxoviruses, which cause only local respiratory infections, MV disseminates to draining lymph nodes via infected macrophages and replicates in local lymphatic

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tissues followed by systemic spread of infection (Griffin & Bellini, 1996; Menichini et al., 1988; Roscic-Mrkic et al., 2001). This clearly indicates that MV has developed a strategy allowing systemic dissemination without direct virus release from infected respiratory epithelia to underlying tissues. Furthermore, in contrast to the model described above, apical MV release is not the result of an apical expression of the MV envelope proteins but is due to a restricted expression of the viral matrix protein (Naim et al., 2000; Riedel et al., 2002). We have reported previously that, despite apical virus release, the two MV surface glycoproteins, the fusion (F) protein and haemagglutinin (H), are abundantly expressed on the basolateral surface of polarized epithelia. In addition, we have demonstrated that both glycoproteins contain a tyrosine-based sorting signal in their cytoplasmic domains, which is required for basolateral protein expression and for the fusogenic activity of F/H complexes in polarized cell cultures (Maisner et al., 1998; Moll et al., 2001). This led us to suggest a functional role for the glycoproteins in mediating virus spread from epithelia by direct cell-to-cell fusion rather than in virus assembly. The aim of this study was to evaluate the influence of basolateral glycoprotein targeting for the spread of MV infection from epithelial cells. We generated infectious MV from cloned cDNA without basolateral targeting signals in one or both glycoproteins (tyrosine mutants) and characterized their mode of propagation in polarized cell cultures and in vivo. For in vivo studies, we utilized cotton rats (Sigmodon hispidus) as an animal model system because they are susceptible to respiratory infection with different MV wild-type and vaccine strains (Niewiesk et al., 2000; Pfeuffer et al., 2003; Wyde et al., 1992). In infected cotton rats, replication of all cloned MV with replacements of tyrosine residues in the cytoplasmic tails of the glycoproteins appeared to be restricted compared with parental MV. By in situ hybridization analysis, we were able to demonstrate that only parental MV encoding basolaterally expressed F and H proteins could spread laterally within the respiratory epithelium and efficiently to cells in the subepithelial tissue. These data support our concept that, in the absence of basolateral virus budding, the basolateral targeting of both MV glycoproteins allows a more efficient spread of infection from polarized epithelial cells, most likely facilitating the systemic dissemination in vivo.

METHODS

Cell cultures. MDCK II (Madin–Darby canine kidney) cells were grown in Eagle’s minimal essential medium (MEM; Gibco) containing 10% fetal calf serum, 100 units penicillin ml\(^{-1}\) and 0.1 mg streptomycin ml\(^{-1}\). Cells were infected in suspension and seeded on tissue culture-treated permeable membrane filter supports as described previously (Maisner et al., 1998). Cell polarity was determined by measuring the transepithelial resistance using a Millipore ERS instrument. Vero cells, B95a and MV rescue cell line 293-3-46 (human embryonic kidney) were grown in Dulbecco’s modified minimal essential medium (DMEM; Gibco) supplemented with 10% fetal calf serum and antibiotics. Medium for 293-3-46 cells contained 1 mg G418 ml\(^{-1}\).

Primary tracheal cells of cotton rats were isolated by trypsinisation from tracheas removed from CO\(_2\)-asphyxiated cotton rats and were grown in DMEM supplemented with 10% fetal calf serum and antibiotics.

Plasmids and virus rescue. For the generation of recombinant MV (rMV), the plasmid p(+)+MV(Nse) (Singh et al., 1999) carrying the full-length MV Edmonston B-based genome was used. To construct the plasmid p(+)+MVFS\(_{494}Y/A\), the F gene was mutagenized in the shuttle vector peFI (Radecke et al., 1995). A NarI–Pcal fragment containing the altered F gene was subcloned into p(+)+MV(Nse). Plasmids p(+)+MVH\(_{122}Y/A\) and p(+)+MVFS\(_{494}Y/A\) were generated by ligation of a Pcal–SpeI fragment containing the mutated H gene from the plasmid pCG-H\(_{122}Y/A\) (Moll et al., 2001) into p(+)+MV(Nse) and p(+)+MVFS\(_{494}Y/A\), respectively. The sequences of all constructs were confirmed by dideoxy sequencing.

Recombinant MV Edmonston B (rMVEdm) and all mutants were rescued as described previously (Moll et al., 2002). Briefly, 293-3-46 cells mediating both artificial T7 transcription and NPs and P functions were transfected with 3 μg of either p(+)+MV(Nse), p(+)+MVFS\(_{494}Y/A\), p(+)+MVH\(_{122}Y/A\) or p(+)+MVFS\(_{494}Y/A\) plasmid encoding MV, the MV polymerase (pEMC-La). The cultures were monitored daily for appearance of syncytia. Virus stocks were produced following plaque purification. RT-PCR and subsequent dideoxy sequencing confirmed the identity of the rescued viruses.

Syncytium formation and growth analysis. To analyse fusion activity and growth of rMV in polarized cells, MDCK cells were infected as described above and seeded either on coverslips at high density or on 1 μm pore size tissue culture inserts (Falcon). At 48 h post-infection (p.i.), cells on coverslips were immunostained to visualize syncytium formation. After fixation and permeabilization, cells were incubated with monoclonal antibody (mAb) 8905 (directed against MV H protein; Chemicon) and FITC-labelled goat anti-rabbit IgG (Dako). Coverslips were mounted with Mowiol and analysed using an Axiomat fluorescence microscope (Zeiss). Virus release from filter-grown MDCK cells was determined by taking samples of apical supernatants at different times p.i., with the first sample collected immediately after washing of the filter membranes at 24 h p.i. Virus titres were determined by plaque assay.

Domain-selective surface biotinylation and immunoprecipitation. MDCK cells were infected with rMV and seeded on 0-4 μm pore size Transwell polycarbonate filters (Costar). At 48 h p.i., cells were washed three times with PBS and either the apical or the basolateral side of the filter membranes was incubated twice for 20 min at 4°C with PBS containing 2 mg S-NHS-Biotin ml\(^{-1}\) (Calbiochem). Glycine (0-1 M) was added to the opposite membranes. After washing the cells once with 0-1 M glycine and three times with PBS, filter membranes were cut out and lysed in 0-5 ml radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0-1% SDS, 0-15 M NaCl, 10 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF, 50 units aprotinin ml\(^{-1}\) and 20 mM Tris/HCl, pH 8-5). Cell lysates were clarified by centrifugation for 20 min at 100 000 g. Supernatants were immunoprecipitated using F- (mAb A540, kindly provided by J. Schneider-Schaulies) or H-specific (mAb 8905; Chemicon) mAbs and protein A–Sepharose beads (Sigma). Following SDS-PAGE and blotting on to nitrocellulose, biotinylated proteins were detected with streptavidin–biotinylated horseshadish peroxidase complex (Amersham Pharmacia Biotech) and enhanced chemiluminescence (Amersham).

Cotton rats. Cotton rats (inbred strain COTTON/Nico) were obtained from Iffa Credo (Lyon, France). Animals at 6 weeks of age
of both sexes were used. The animals were pathogen free according to the breeder’s specifications and were maintained in a barrier system. They were kept under controlled environmental conditions of 22 ± 1 °C, 55–60% humidity and a 12 h light cycle.

**Animal infections and virus titration.** For intranasal infection, MV was given in PBS in a volume of not more than 100 μl to ether-anesthetized cotton rats. At different times p.i., animals were killed using CO₂ and lungs were removed. Lung tissue was minced with scissors and dounced with a glass homogenizer. Serial tenfold dilutions of virus containing supernatant were assessed for the presence of infectious virus. Infectivity was quantified by the TCID₅₀ method. The TCID₅₀ assay was carried out at the breeder’s specifications and was maintained in a barrier system. The TCID₅₀ was calculated by the method of Reed & Muench (1938). The threshold of detection was 10⁶ TCID₅₀ g⁻¹ lung tissue; <10⁶ signified no virus. Lung lavage was performed using a three-way stopcock and 10 ml PBS/1% EDTA. Lung lavage cells were incubated in tenfold dilutions starting with 10⁶ cells per well of a 96-well plate with 10⁵ B95a cells. Infectivity was calculated per 10⁶ cells using the method of Reed & Muench (1938); thus, the threshold of detection was <10⁵.

**In situ hybridization assay.** The assay was basically performed as described by Mrkic et al. (1998). Briefly, infected cotton rats were killed with CO₂ and skulls and lungs were removed. Skulls were fixed in 4% phosphate-buffered formaldehyde and subsequently decalcified for 7–10 days with Morse’s solution (10% sodium citrate, 22.5% formic acid). Blocks of the nasal passages and sinuses were cut and embedded in paraffin and 4 μm sections were prepared. Lungs were snap frozen in liquid nitrogen and frozen 20 μm tissue sections were prepared in a cryostat. Detection of MV mRNA in situ was performed with a digoxigenin (DIG)-labelled nucleoprotein (N) mRNA-specific probe under appropriate conditions. The hybridization probe was detected with an alkaline phosphatase-conjugated anti-DIG antibody. Sections were counterstained with haematoxylin solution.

**RESULTS**

**Glycoproteins of tyrosine mutants are retargeted in polarized epithelial cells**

MV glycoproteins F and H are abundantly expressed on the basolateral surface of polarized MDCK cells both upon stable expression and infection with MV̄Edm (Maisner et al., 1998). Furthermore, both proteins possess targeting signals that critically depend on a tyrosine residue in their cytoplasmic tails (Moll et al., 2001). Plasmid-encoded mutant glycoproteins with the cytoplasmic tyrosine residues at position 549 in the F protein and position 12 in the H protein replaced with alanine residues were found to be redirected to the apical cell surface. To study the role of polarized glycoprotein expression for the cytopathic properties of infectious MV in culture and for pathogenesis *in vivo*, viruses were generated in which these cytoplasmic tyrosine residues were replaced with alanine residues either in F or H or both (Fig. 1). rMV̄ harbouring glycoproteins with altered basolateral targeting signals (tyrosine mutants) were rescued from cDNA and purified as described previously (Moll et al., 2002). Growth analysis and fusion assays in non-polarized cells showed no significant differences between tyrosine mutants and the parental virus rMV̄ (data not shown).

<table>
<thead>
<tr>
<th>A</th>
<th>TMD</th>
<th>CD</th>
<th>549</th>
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**B | CD | 12 |
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<td>MSPQRDRNAFYYKDNPHPKGSRIVREHLMIDRPYY</td>
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<td>rMV̄FHYA</td>
<td>MSPQRDRNAFYYKDNPHPKGSRIVREHLMIDRPYY</td>
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**Fig. 1.** Amino acid sequences of the cytoplasmic domains of the F (A) and H (B) proteins of rMV̄Edm and the tyrosine mutants. Protein sequences are shown in single letter code. Bold letters indicate residues important for protein sorting and their position in the cytoplasmic domain. The vertical lines separate transmembrane domains (TMD) from cytoplasmic domains (CD).

To analyze polarized expression of the mutant glycoproteins, MDCK cells were infected with rMV̄Edm or the tyrosine mutants, grown on polycarbonate filters and subjected to a domain-specific surface biotinylation assay. Although the H protein distribution was not as polarized as in transfected cells, both glycoproteins were abundantly expressed on the basolateral surface of rMV̄Edm-infected cells (F, > 95%; H, 50%; Fig. 2). In contrast to the parental glycoproteins, the altered F and H proteins were predominantly found on the apical membrane of cells infected

**Fig. 2.** Surface distribution of F and H proteins in infected polarized MDCK cells. MDCK cells were infected with either rMV̄Edm or the tyrosine mutants at an m.o.i. of 1 and cultivated on filters (0.4 µm pore size) for 48 h. Cell surface proteins were labelled with NHS-biotin from either the apical (lane a) or basolateral (lane b) side. After cell lysis, F and H proteins were immunoprecipitated with specific mAbs. Precipitates were analysed by SDS gel electrophoresis under non-reducing (F proteins) or reducing (H proteins) conditions, transferred to nitrocellulose and probed with peroxidase-conjugated streptavidin.

http://vir.sgmjournals.org Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11 On: Sat, 29 Dec 2018 08:24:46
with one of the tyrosine mutants. In MDCK cells infected with rMV_{F549Y/A} or rMV_{FHY/A}, more than 90% of the F protein was expressed apically. In rMV_{H12Y/A} and rMV_{FHY/A} infected cells, the H protein was almost exclusively present at the apical surface. This indicated that, in the context of a virus infection, abundant basolateral expression of the glycoproteins is prevented by mutation of the critical tyrosine residue.

Interestingly, despite its unchanged basolateral targeting signal, the H protein was largely expressed at the apical membrane of MDCK cells infected with rMV_{F549Y/A}. In contrast, the unchanged F protein was basolaterally targeted in rMV_{H12Y/A}-infected MDCK cells, as expected. These data indicated that the polarized expression of the MV H protein in infected cells is influenced by the presence of other viral proteins, whereas the polarized transport of F occurs independently.

**Apical MV glycoprotein expression prevents cell-to-cell fusion in infected polarized epithelial cells**

Disruption of the basolateral targeting signals has significant negative consequences for cell-to-cell fusion in polarized epithelial cells co-expressing plasmid-encoded F and H proteins (Moll et al., 2001). To assess the ability of rMV with apically expressed glycoproteins to induce syncytia formation in polarized epithelial cells, MDCK cells were infected with rMV_{Edm}, rMV_{F549Y/A}, rMV_{H12Y/A}, or rMV_{FHY/A}. Cell-to-cell fusion was monitored by immunostaining. Whereas rMV_{Edm}-infected cells demonstrated large syncytia, cell-to-cell fusion could not be detected in any of the cultures infected with the tyrosine mutants (Fig. 3A). These results confirmed that only cells expressing fusogenic F/H complexes on the basolateral membrane were able to fuse with neighbouring cells. Thus, apical sorting of only one of the glycoproteins completely prevents fusion in virus-infected polarized epithelial cells.

To investigate whether apical expression of one or both glycoproteins and/or the lack of syncytia formation have an influence on the release of infectious MV particles from polarized epithelial cells, virus-infected MDCK cells were seeded on tissue culture inserts, and virus titres in the apical supernatants were determined at different times p.i. As shown in Fig. 3(B), the initial growth of parental rMV_{Edm} as well as the growth of all tyrosine mutants was very similar. At 55 h p.i., maximum titres of \(2 \times 10^4\) p.f.u. ml\(^{-1}\) were reached. Thereafter, the titre of rMV_{Edm} started to decrease and finally dropped to \(4 \times 10^2\) p.f.u. ml\(^{-1}\) at 98 h p.i., whereas the amounts of virus released from cells infected with rMV_{F549Y/A}, rMV_{H12Y/A}, and rMV_{FHY/A} remained basically unchanged. This is probably due to the prevention of fusion and thereby the maintenance of an intact cell monolayer supporting virus replication for longer time periods. Provided that the m.o.i. is high, tyrosine mutants obviously have no disadvantage in replication in polarized cells, though cell-to-cell fusion is inhibited.

![Syncytia formation and virus growth in polarized MDCK cells.](https://example.com/syncytia.png)

**Fig. 3.** Syncytia formation and virus growth in polarized MDCK cells. (A) MDCK cells were infected with either rMV_{Edm} or the tyrosine mutants and seeded on coverslips at high density. At 48 h p.i., syncytia were visualized by indirect immunofluorescence using an anti-H protein mAb and an FITC-conjugated secondary antibody. (B) MDCK cells in suspension were infected at an m.o.i. of 1 with either rMV_{Edm} (●) or the tyrosine mutants (rMV_{F549Y/A} ●; rMV_{H12Y/A} ■; rMV_{FHY/A} △) and cultivated on 1 µm pore size tissue culture inserts at 37°C. Medium was collected at different times p.i. from the apical filter chamber, and infectious virus was quantified by plaque assay. The values plotted represent the means of results from two experiments.

Furthermore, this experiment showed that the predominant apical expression of one or both glycoproteins has no promoting effect on the apical release of MV from polarized cells, indicating that factors other than the amount of F and H proteins on the apical surface are limiting for apical virus budding.

**In vitro infection of cotton rat primary tracheal cells**

To study whether the abundant basolateral expression of the glycoproteins is essential for the spread of MV infection in the respiratory mucosa in vivo, we used cotton rats (S. hispidus) as a rodent model. Since it was unknown which cells of the cotton rat other than lymphocytes and macrophages can be productively infected with MV, epithelial cells from cotton rat tracheas were isolated and cultivated at low or high densities for several days. Subsequently, non-polarized and polarized cell monolayers were infected with a green fluorescent protein (GFP)-expressing rMV (Ehrengruber et al., 2002). As shown in Fig. 4, non-polarized and polarized primary epithelial cells could be successfully infected with MV. In non-polarized tracheal cells, GFP expression in single cells was detected by

![In vitro infection of cotton rat primary tracheal cells](https://example.com/infection.png)
19 h p.i. From then on, infection spread rapidly throughout the monolayer resulting in the formation of large syncytia. Infection of polarized tracheal cells was delayed and GFP-positive syncytia were only found at 88 h p.i. Although syncytia formation was less pronounced than in non-polarized cells, this clearly demonstrated that polarized tracheal cells are potential MV target cells in the cotton rat respiratory tract and that parental rMVEdm can spread from these cells by direct cell-to-cell fusion.

Tyrosine mutants are less virulent and spread of infection is more limited in the respiratory tract

As the tyrosine mutants, in contrast to parental rMVEdm, did not induce cell-to-cell fusion in polarized epithelial cells, we wanted to assess how this affects replication and pathogenesis in vivo. Therefore, groups of eight 6-week-old cotton rats were infected with 10^5 p.f.u. rMVEdm or the tyrosine mutants. Four animals in each group were used to determine the number of cells in lung-draining mediastinal lymph nodes (MDLN), and virus titres in lung lavage cells and in lung tissue. The results in Table 1 show that the number of cells in MDLN was significantly lower in cotton rats infected with rMV_{F549Y/A}, rMV_{H12Y/A} or rMV_{FY/A} (4.6 \times 10^6, 6.0 \times 10^6, 8.0 \times 10^6 cells, respectively) than in those infected with the parental virus rMVEdm (1.4 \times 10^7 cells). The difference in the number of cells in the MDLN indicated a less-pronounced activation of lymphocytes by the tyrosine mutants. Since there is a correlation between activation of lymphocytes and virulence, it can be assumed that the tyrosine mutants are less virulent than rMVEdm. In agreement with this assumption, virus titres in lung lavage cells and lung tissue of infected cotton rats were clearly lower than those found in rMVEdm-infected animals. Whereas virus titres reached 10^3 \pm 0.5 TCID_{50} g^{-1} lung tissue in rMVEdm-infected animals, hardly any virus could be detected in animals exposed to rMV_{F549Y/A}, rMV_{H12Y/A} or rMV_{FY/A}. Thus, rMV-encoding glycoproteins with altered basolateral targeting signals clearly showed a reduced replication in the cotton rat respiratory tract. It did not make any difference whether only one (rMV_{F549Y/A}, rMV_{H12Y/A}) or both (rMV_{FY/A}) glycoproteins were mutated.

To evaluate whether differences in the infection of the respiratory epithelia might be responsible for the differences in virus replication, localization of MV-replicating cells in the respiratory tract of cotton rats was analysed by in situ hybridization. Animals were infected with 10^5 p.f.u. rMVEdm or rMV_{FY/A} and killed at 2 h and 1, 2, 3 and 4 days p.i. Skulls and lungs were removed, tissue sections prepared and MV-infected cells were detected using a DIG-labelled RNA probe complementary to an 851 nt segment.

Table 1. Replication of rMVEdm and the tyrosine mutants in cotton rat lungs

Groups of four animals were infected with 10^5 p.f.u. rMVEdm, rMV_{F549Y/A}, rMV_{H12Y/A}, rMV_{FY/A}, or mock-infected, and the number of lung lymph node cells and the virus titres in lung lavage cells and in lung lymph nodes was assessed on B95a cells on day 4 p.i. Lung lymph node and lung lavage cells were pooled from four animals. Titres of lung tissue were determined for each individual animal and the mean determined. In the group of cotton rats infected with 10^5 p.f.u. rMV_{H12Y/A}, one animal had a titre of 10^{5.5} TCID_{50} (g lung tissue)^{-1}, whereas the other three were negative. However, the difference in virus titre for animals infected with rMVEdm compared with animals infected with either of the three mutants was significant (at least P<0.01; two-tailed t-test).

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of cells in lung lymph nodes</th>
<th>Virus titres in lung lavage cells (TCID_{50} per 10^7 cells)</th>
<th>Virus titres in lung tissue (TCID_{50} g^{-1})</th>
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<tr>
<td>Mock-infected</td>
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<td>&lt;10^3</td>
<td>&lt;10^2</td>
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<td>rMVEdm</td>
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<td>&lt;10^2</td>
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of the MV N mRNA. As expected, no MV-positive cells could be detected in the cotton rats at 2 h p.i. (data not shown), indicating that input virus did not result in any background signal. At day 1 p.i., small groups of cells stained positive for MV N mRNA appeared in the epithelial layer of the nasal cavity of rMV<sub>Edm</sub>-infected animals (Fig. 5A). This showed that rMV<sub>Edm</sub> is transcribed in cotton rat epithelial cells <i>in vivo</i> and is able to spread laterally within the epithelium. Interestingly, the morphology of epithelial cells was unchanged and the integrity of the epithelium appeared to be unaffected. In cotton rats infected with rMV<sub>FHY/A</sub>, only single MV-positive cells were found in the epithelium of the upper respiratory tract (Fig. 5B) suggesting that rMV<sub>FHY/A</sub> has lost its ability to spread directly from an epithelial cell to a neighbouring cell. This view was further supported by the finding that the number of virus-positive cells in the subepithelial tissue in rMV<sub>FHY/A</sub>-infected animals was very low. Whereas numerous infected subepithelial cells could be detected in the nasal cavity of rMV<sub>Edm</sub>-infected cotton rats (Fig. 5C), very few virus-positive cells were found in the same cell population in animals infected with rMV<sub>FHY/A</sub> (Fig. 5D). Similar differences were found in the lower respiratory tract of infected cotton rats. Lungs were free of MV-positive cells until day 2 p.i. (data not shown). At day 3 p.i. in animals infected with rMV<sub>Edm</sub>, limited lateral spread of infection in the epithelium of bronchi and bronchioles was detectable (Fig. 5E). Again, there was no obvious sign of syncytia formation or epithelial damage. In contrast to rMV<sub>Edm</sub>, rMV<sub>FHY/A</sub>-infected cotton rats showed only single infected cells in the pulmonary epithelium and no spread of infection (Fig. 5F). As in the subepithelial tissue of the upper respiratory tract, the total number of virus-positive cells was clearly higher in rMV<sub>Edm</sub>- than in rMV<sub>FHY/A</sub>-infected cotton rats.

In summary, the <i>in vivo</i> experiments showed that although the overall replication of parental rMV<sub>Edm</sub> was not very efficient, it was able to spread laterally within the respiratory epithelium and subsequently to cells in the subepithelial tissue. Destruction of the basolateral targeting signals in the viral glycoproteins resulted in the generation of a rMV that could infect only single epithelial cells and a very small number of subepithelial cells, indicating that the basolateral expression of both glycoproteins facilitates the spread of MV infection, not only <i>in vitro</i> but also <i>in vivo</i>.

**DISCUSSION**

Experimental studies with primates and histopathological findings in humans indicate that MV spreads to lymphoid tissues after local replication in the mucosa of the respiratory tract, thereby establishing a systemic infection (McChesney <i>et al.</i>, 1997; van Binnendijk <i>et al.</i>, 1995). During the systemic phase, the virus mainly replicates in lymphocytes and macrophages but also in endothelial and epithelial cells of numerous organs (Moench <i>et al.</i>, 1988; Norrby & Oxman, 1990). However, it is still largely unknown how

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![Fig. 5](https://example.com/fig5.jpg)

**Fig. 5.** Pathology in the upper respiratory tract (A–D) and lungs (E, F) of intranasally infected cotton rats. Cotton rats were infected with 10<sup>5</sup> p.f.u. rMV<sub>Edm</sub> (A, C, E) or rMV<sub>FHY/A</sub> (B, D, F) and killed at 1 (A, B), 2 (C, D) or 3 (E, F) days p.i. Tissues were collected and processed for MV N mRNA-specific <i>in situ</i> hybridization. Arrows indicate MV-positive cells. Magnification, ×100.
MV enters subepithelial tissues after initial replication in respiratory epithelial cells. So far, it has been assumed that the targeted release of viruses from polarized epithelial cells critically influences the course of infection. According to this model, apically released viruses establish a local infection at their entry site whereas basolaterally released viruses can spread systemically via the lymphatic and vascular system. This theory was mainly supported by the finding that another member of the Paramyxoviridae, a bidirectionally released Sendai virus mutant (F1-R), caused a systemic infection in mice while the apically released parental virus was pneumotropic (Tashiro et al., 1991). Since MV establishes a systemic infection but is predominantly apically released from a variety of epithelial cells, a different mechanism for overcoming the epithelial barrier has to be postulated (Blau & Compans, 1995; Maisner et al., 1998).

We have reported previously that MV envelope proteins F and H are abundantly expressed on the basolateral surface of polarized epithelial cells. Targeting as well as fusion activity in polarized cells has been shown to depend critically on a tyrosine residue in the cytoplasmic tails (Maisner et al., 1998; Moll et al., 2001). Continuing this work, we have described here the successful generation of rMV with mutated basolateral targeting signals in either one or both glycoproteins. While these virus mutants did not show any obvious differences in their fusogenic activity and their growth characteristics in non-polarized cells, their cytopathic properties in polarized cell cultures and, importantly, their pathogenesis in vivo in the cotton rat model were clearly different from that of the parental rMVEdm. The exchange of cytoplasmic tyrosine residues responsible for basolateral targeting resulted in the predominant apical expression of the respective glycoprotein in infected polarized cells. Thus, both glycoproteins contain cryptic apical targeting information that becomes activated after destruction of the original basolateral signal. The finding that the mutant viruses were no longer able to induce cell-to-cell fusion in polarized epithelial cells clearly demonstrates that interaction with neighbouring cells and subsequent initiation of the fusion process strictly depend on the expression of F/H complexes at the basolateral membrane of polarized cells. The fact that tyrosine mutants promoted fusion of non-polarized cells as efficiently as parental MV showed that indeed the retargeting of the glycoproteins, and not a general defect, is responsible for the altered fusion activity in epithelia. By using cotton rats as a small-animal model, we were able to verify that not only fusion but also the spread of infection in vivo is restricted. Although it is not clear yet which cellular receptors are responsible for the infection of cotton rat cells by MV, we clearly demonstrated in vitro that non-polarized and, more importantly, polarized primary tracheal cells of cotton rats are susceptible to MV and allow virus spread by cell-to-cell fusion, thus, very likely contributing to the spread of MV infection in vivo. In agreement with this, rMV expressing apically targeted glycoproteins showed a clearly attenuated phenotype in cotton rats following intranasal infection. These viruses were less virulent and replication was significantly reduced in the respiratory tract of infected animals compared with parental rMVEdm. Even if the total number of virus-positive cells was low, the histological studies led us to suggest that differences in the infection of the respiratory epithelium are responsible for the attenuated phenotype of the virus mutants. Whereas rMVEdm was able to spread from cell to cell within the epithelial layer and from there to cells in the underlying tissue, the virus mutant rMVFHY/A showed virtually no spread of infection. Due to the experimental conditions it was not possible to determine the type of cells infected in the subepithelial tissue, but it seems likely that these cells are macrophages. Macrophages are numerous in the connective tissue of the mucosa and submucosa of the respiratory tract and, furthermore, belong to the main target cells of MV in the organism (Esolen et al., 1993; Roscic-Mrkic et al., 2001).

A striking observation was that, in contrast to cultured epithelial cells, spread of rMVEdm in the respiratory epithelium of cotton rats occurred without apparent formation of syncytia and epithelial damage. This is in full agreement with the observation recently published by Sinn et al. (2002) that the cell layer integrity of MV-infected primary cultures of airway epithelia is maintained. Therefore, it can be assumed that virus dissemination in vivo resulted from the transient formation of microfusion pores induced by the basolaterally expressed F and H proteins rather than from cell-to-cell fusion. This mechanism of virus spread has been already proposed for MV transmission from monocyte–promyelocytic cells (U937) to HeLa cells and between neuronal cells (Ehrengruber et al., 2002; Firsching et al., 1999). Moreover, this mode of propagation is in line with the observation that MV is strongly cell associated in vivo and dissemination is almost always dependent on direct cell-to-cell contact (Mrkic et al., 2000).

Viruses have evolved a variety of mechanisms to accomplish spread from and within the respiratory epithelium. Maybe the most simple mechanism is the production of cell-free virions by budding from the surface of an infected cell and subsequent infection of neighbouring cells as shown for respiratory syncytial virus infections of well-differentiated human airway epithelial cells (Zhang et al., 2002). But this mechanism does not apply to all respiratory viruses. For example, adenoviruses have developed sophisticated mechanisms to disrupt the junctional integrity of epithelial barriers, thereby facilitating escape from the epithelium (Walters et al., 2002). For MV, it has recently been proposed that spread of infection through the respiratory epithelium might involve pathways other than direct binding and entry through the apical surface of airway epithelia (Sinn et al., 2002). In agreement with this, our results indicate that not only the mode of virus release from polarized cells but also the targeted glycoprotein expression has an important impact on the spread of MV within and from airway epithelia. Based on the data presented here, we propose a simplified model for the early steps of MV infection.
Following aerogenous transmission, MV initially replicates in polarized epithelial cells of the upper respiratory tract. Abundant basolateral expression of both MV F and H proteins in these cells promotes the transient formation of microfusion pores between epithelial cells, as well as between epithelial cells and macrophages in the subepithelial tissue. Subsequently, infectious ribonucleoproteins can translocate via microfusion pores, resulting in the infection of neighbouring epithelial cells and subepithelial macrophages without release of infectious particles or syncytia formation. Finally, infected macrophages disseminate MV infection systemically via the lymphatic and vascular system. According to this model, the basolateral expression of MV glycoproteins helps to overcome the epithelial barrier, thereby facilitating the systemic spread of the MV infection in vivo. The fact that in all MV wild-type isolates and vaccine strains known so far the tyrosine-depending sorting motifs are conserved clearly supports this idea of a functional importance for MV replication in vivo.

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REFERENCES


