A Single Amino Acid Change in the Cytoplasmic Domains of Measles Virus Glycoproteins H and F Alters Targeting, Endocytosis, and Cell Fusion in Polarized Madin-Darby Canine Kidney Cells*

Received for publication, November 8, 2000, and in revised form, February 26, 2001

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As we have shown previously, release of measles virus (MV) from polarized epithelial cells is not determined by the viral envelope proteins H and F. Although virus budding is restricted to the apical surfaces, both proteins were abundantly expressed on the basolateral surface of Madin-Darby canine kidney cells. In this report, we provide evidence that the basolateral expression of the viral proteins is of biological importance for the MV infection of polarized epithelial cells. We demonstrate that both MV glycoproteins possess a basolateral targeting signal that is dependent upon the unique tyrosine in the cytoplasmic tails. These tyrosines are shown to be also part of an endocytosis signal. In MV-infected cells, internalization of the glycoproteins was not observed, indicating that recognition of the endocytosis signals is disturbed by viral factors. In contrast, basolateral transport was not substantially hindered, resulting in efficient cell-to-cell fusion of polarized Madin-Darby canine kidney cells. Thus, recognition of the signals for endocytosis and polarized transport is differently regulated in infected cells. Mutation of the basolateral sorting signal in one of the MV glycoproteins prevented fusion of polarized cells. These results suggest that basolateral expression of the MV glycoproteins favors virus spread in epithelia.

Epithelial cells play crucial roles in diverse processes such as water and ion balance, secretion, adsorption of nutrients, and signal transduction. The polarized nature of these cells is central to their function. The plasma membrane of these cells is divided into an apical and a basolateral domain that have different lipid and protein compositions (for review, see Ref. 1). Sorting of membrane proteins was shown to occur in the trans-Golgi network and to depend on special targeting signals. The most thoroughly investigated model for epithelial polarity is the Madin-Darby canine kidney (MDCK)1 continuous cell line. Several viral glycoproteins have been shown to be sorted to either the apical or basolateral surfaces (2). Since glycoproteins of enveloped viruses perform critical functions during viral assembly and serve as main targets of humoral immune responses, polarized expression of these proteins affects the viral life cycle. The viral glycoproteins studied in most detail are the vesicular stomatitis virus G protein and the influenza virus hemagglutinin (HA). Vesicular stomatitis virus G protein was found to contain basolateral sorting information that is critically dependent upon the presence of a tyrosine in the cytoplasmic tail (3). The signal responsible for the apical transport of HA is contained within the extracellular or transmembrane portion of the molecule (4–6). Vesicular stomatitis virus was shown to be released mainly from the basolateral side of infected MDCK cells, whereas influenza virus almost exclusively buds from the apical cell surface (7). These data were the basis for the view that budding of enveloped viruses occurs only at the site at which their envelope proteins are most concentrated (2, 8, 9).

However, recent data on virus release and glycoprotein sorting indicated that this model is not valid for measles virus (10, 11). Measles virus (MV) is a member of the Paramyxoviridae family and possesses a negative-stranded RNA genome encoding for six structural proteins. The nucleoprotein together with the phosphoprotein, the viral polymerase, and the genomic RNA form the ribonucleoprotein complex that assembles in the cytoplasm of infected cells. The matrix protein (M protein), synthesized in the cytoplasm, mediates the contact of the ribonucleoproteins with viral envelope proteins initiating virus budding at the plasma membrane (12, 13). Hemagglutinin (H protein), the envelope protein responsible for the binding of the virus to the cell surface receptor (14–16), is a type II integral membrane protein with a C-terminal ectodomain. The second envelope protein, the fusion protein (F protein), is a type I glycoprotein possessing an N-terminal ectodomain that has to be cleaved into the F1/F2 subunits to mediate pH-independent fusion of virus with the plasma membrane (17). We have previously shown that both proteins are abundantly expressed on the basolateral side of polarized MDCK cells, although virus budding is restricted to the apical cell surfaces (10). This finding was recently confirmed using another polarized cell line, CaCo2 cells (11). Furthermore, we have demonstrated that upon stable expression in MDCK cells, both H and F proteins are targeted almost completely to the basolateral surface (10). Thus, targeting information resides in the proteins itself. Since MV glycoproteins do not determine the site of virus budding, MV must have developed a maturation strategy different from those of viruses such as influenza virus or vesicular stomatitis virus. Most recent work has shown that MV M protein may specify apical virus release because it is predominantly local-

* This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: MDCK, Madin-Darby canine kidney; HA, influenza virus hemagglutinin; MV, measles virus; MAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.
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ized at the apical plasma membrane of polarized MDCK² and CaCo2 cells (11).

The aim of this study was to analyze the signals responsible for the basolateral sorting of H and F proteins to elucidate the importance of the basolateral glycoprotein expression for the pathogenesis of measles virus infection. We show that polarized targeting of both proteins is dependent upon a tyrosine residue in the cytoplasmic tails. The same tyrosines are shown to be part of an endocytosis signal. Since the signals in the F protein cytoplasmic tail appeared to be more efficiently recognized than those of the H protein, they were transferred to another protein. Chimeric proteins from MV F protein and influenza virus HA revealed that the signals residing in MV F protein can only partly override the targeting signals of an apical protein that is not internalized. To analyze the biological importance of the signals residing in the cytoplasmic tails of the MV envelope proteins, the polarized transport and endocytosis of the glycoproteins in infected cells were monitored. Endocytosis, but not polarized transport, appeared to be inhibited. Fusion of polarized MDCK cells was observed only when basolateral sorting signals of both glycoproteins were intact. This result led us to suggest that endocytosis interferes with the assembly of new viruses at the plasma membrane, whereas basolateral expression of the MV glycoproteins favors virus spread in epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells (strain II) were grown in Eagle’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg of streptomycin. For studies of cell polarity, tissue culture-treated 0.4-μm pore size Transwell polycarbonate filters (Costar Corp., Cambridge, MA) were used. Cells were seeded 5 days before experiments (2 × 10⁶ cells/24-mm unit). The polarity was determined by measurement of the transepithelial resistance using a Millipore ERS instrument. MDCK cells formed a tight monolayer with an electrical resistance of 1000–2500 ohms × cm². The different patterns of surface proteins on the apical and basolateral membranes of the polarized cell line were controlled by surface biotinylation.

Plasmid Constructs and Stable Expression in MDCK Cells—Cloning of the viral glycoprotein (H and F protein) genes into the expression vector pcDNA3 (GIBCO/BRL, Gaithersburg, MD) was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The chimeras used in this study are marked by their composition (ectodomain-transmembrane domain-cytoplasmic tail) as H7-H7-F, F7-H7, and H7-F-F. H7 represents the respective domain of the influenza virus hemagglutinin, and F represents the respective domain of the measles virus F protein. The multichain parental proteins F7-H7 and H7-F-F were generated by a recombinant polymerase chain reaction technique (19) using synthetic oligonucleotides and pSG5newHAmut7 and pCG-F as templates. The mutant hemagglutinin HAmut7 of the influenza A virus has been described elsewhere (20). The internal sense primers were 5'-CAGTTGTTTCCGCTGCGACGACATTACCTAACGGAGCGTTCCGTGTTAAACT-3' (sense) and 5'-GCCCGCGCCGCTGCGACGACATTACCTAACGGAGCGTTCCGTGTTAAACT-3' (antisense) and the external primers 5'-ATACCTGTGACATTTACGAGCGTCTGCTGTGAATAAA-3' (sense) and 5'-GCCCGCGCCGCTGCGACGACATTACCTAACGGAGCGTTCCGTGTTAAACT-3' (antisense). The sequences of the plasmid constructs were confirmed by dideoxy sequencing. For stable expression, MDCK cells were cotransformed with either of the expression plasmids and the neomycin resistance-carrying plasmid pG1 at a ratio of 1:10 using the cationic lipid transfection reagent LipofectAMINE 2000 (Life Technologies, Inc.) according to the protocol of the manufacturer. Cells were screened for G418 resistance by addition of 1.0 mg of G418 (Geneticin; Calbiochem)/ml medium. The selected cell clones were screened for expression of foreign proteins by immunofluorescence analysis.

Surface Immunofluorescence Analysis—MDCK cells stably expressing parental proteins (F549Y/A and H12Y/A) or tyrosine mutants (F549Y/A and H12Y/A) were grown on glass coverslips and incubated with the monoclonal antibody (mAb) A504 (directed against MV F protein; kindly provided by J. Schneider-Schaullies) or mAb 8905 (directed against MV H protein; Chemicon, Temecula, CA) for 60 min at 4 °C without prior fixation. The primary antibodies were detected using an FITC-labeled goat anti-mouse IgG (Dako Corp.) for 45 min at 4 °C. The samples were mounted in Mowiol and 10% 1,4-diazabicyclo(2.2.2)octane. The coverslips were viewed and photographed with a Zeiss Axiohot microscope equipped with UV optics.

Surface Biotinylation and Immunoprecipitation—Filter-grown MDCK cells were biotinylated essentially as described by Lissanti et al. (8). The cells were washed three times with cold PBS and incubated with cold N-hydroxysuccinimidobiotin (Pierce) by adding 1 ml of the biotinylating reagent to the respective filter chamber. The same volume of PBS containing 0.1 g/ml streptomycin was placed into the opposite filter chamber. After biotinylation, cells were washed once with cold PBS containing 0.1 g/ml glucose and three times with cold PBS. Cells were lysed in 0.5 ml of radio immunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM EDTA, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 50 units/ml aprotinin, and 20 mM Tris-HCl, pH 8.5), followed by centrifugation for 20 min at 100,000 × g. The supernatant was immunoprecipitated with mAb A504 (directed against MV F protein), mAb 8905 (directed against MV H protein), or mAb 2A11-H7 (directed against the H7 subtype of the influenza A virus hemagglutinin; kindly provided by W. Garten) at a final dilution of 1:100. After addition of 40 μl of a suspension of Protein-A Sepharose CL-4B (Sigma) coated with rabbit anti-mouse IgG (Dako Corp.), immunocomplexes were washed three times with radio immunoprecipitation assay buffer and suspended in reducing (MV H protein) or nonreducing (MV F and recombinant parent proteins) sample buffer for SDS-polyacrylamide gel electrophoresis. Following separation on a 10% polyacrylamide gel, proteins were blotted onto nitrocellulose by the semidyblotted technique. After blocking of nonspecific binding sites by 5% nonfat dry milk in PBS, blots were incubated for 45 min at 4 °C with streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech, Uppsala, Sweden) diluted 1:2000 in PBS containing 0.01% bovine serum albumin. Biotinylated antibodies were detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) by exposure to Eastman Kodak XAR autoradiography film.

Antibody Uptake Assay—MDCK cells were grown on coverslips and transfected with either the parental (pCG-F549Y/A and pCG-H12Y/A) or mutant (pCG-F549Y/A and pCG-H12Y/A) protein-expressing plasmids alone or cotransfected with plasmid containing parental proteins to the transfection reagent LipofectAMINE 2000. A total amount of 1 μg of DNA was used. Since infection of confluent MDCK cells has been shown to be very inefficient (10), virus was added to the cells directly after seeding. At 7 h post-infection, the virus-containing growth medium was replaced by fresh minimal essential medium containing 10% fetal calf serum. At 20 h post-infection or 30 h post-infection, surface-expressed protein was labeled with mAb A504 (directed against MV F protein) or mAb 8905 (directed against MV H protein). After incubation for 60 min on ice, the antibody was removed by washing with PBS, and the cells were incubated with cell culture medium for 15 min at 37 °C to allow endocytosis of the antibody-antibody complexes. Internalization was stopped by rapid cooling on ice. Surface-bound antibodies were detected by incubation with rhodamine-conjugated goat anti-mouse Fab fragments (Dianova) at a dilution of 1:100 in PBS containing 0.1% bovine serum albumin. After washing with PBS, the cells were fixed and permeabilized for 5 min at 20 °C with methanol/acetone (1:1). Internalized antibodies were detected with FITC-labeled goat anti-mouse IgG at a dilution of 1:100. The samples were mounted in Mowiol containing 10% 1,4-diazabicyclo(2.2.2)octane and analyzed using a Zeiss Axiohot microscope equipped with UV optics.
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Biotin Internalization Assay—The experiment was mainly performed as described by Graeve et al. (22). MDCK cells stably expressing either one of the parental or one of the mutant proteins were grown on 6-cm plastic dishes and incubated twice for 20 min at 4 °C with a 2 mg/ml concentration of the non-membrane-permeating, cleavable biotin derivative sulfosucinimidyl 2,3-dihydroxypropionate (Pierce). Following washing with cold PBS containing 0.1 M glycine and several washings with cold PBS, cells were incubated with cell culture medium for 0, 5, 15, or 30 min at either 4 or 37 °C to allow endocytosis of the labeled proteins. After internalization was stopped by rapid cooling on ice, cells were incubated for three times for 20 min at 4 °C with 50 μm 2-mercaptoethanesulfonic acid (Sigma) in 50 μl Tris-HCl, pH 8.7, 100 mM NaCl, and 2.5 mM CaCl2. Biotin exposed at the cell surface was thereby cleaved. After thorough rinsing with PBS containing 20 mM Heps, cells were lysed in radioimmunoprecipitation assay buffer, and proteins were immunoprecipitated as described above. Biotinylated proteins were separated on a 10% SDS-polyacrylamide gel under non-reducing conditions, transferred to nitrocellulose, and detected as described above. The internalization rate was determined by densitometric quantification.

Cell Fusion Assays—MDCK cells were seeded in 35-mm diameter wells to reach 90–95% confluence 1 day after plating. The MV glycoprotein-expressing plasmids pCG-FEdm and pCG-HEdm were transfected in the absence or presence of either the corresponding parental protein-expressing plasmids (pCG-FEdm and pCG-HEdm) or the mutant protein-expressing plasmids (pCG-F549Y/A and pCG-H12Y/A) using the cationic lipid transfection reagent LipofectAMINE 2000. A total amount of 5 μg of DNA was used. At 20 h post-transfection, the transiently expressing cells were fixed with ethanol and stained with 1:10 diluted Giemsa staining solution (Merck, Darmstadt, Germany).

To analyze the biological activity of the MV glycoproteins in polarized MDCK cells, MDCK cells stably expressing either MV FEdm or MV F549Y/A were seeded on coverslips at high density. At 24 h post-seeding, expressing plasmids (pCG-FEdm and pCG-HEdm) or the mutant protein-expressing plasmids (pCG-F549Y/A and pCG-H12Y/A) were transfected with pCG-HEdm or pCG-H12Y/A, respectively. For that can interact with the F protein support virus-to-cell or in combination with H proteins that bind to MV receptors and induce syncytium formation. Only fusion-competent F proteins in combination with H proteins that bind to MV receptors and that can interact with the F protein support virus-to-cell or cell-to-cell fusion (23). To analyze the mutants for syncytium formation, confluent MDCK cells coexpressing either parental HEdm or H12Y/A in combination with parental FEdm or F549Y/A were fixed at 20 h post-transfection and stained with Giemsa staining solution. As shown in Fig. 1C, H or F proteins expressed alone did not induce fusion, whereas syncytium formation was observed in cells expressing different combinations of parental and mutant proteins. This result indicates that

FIG. 1. A, sequences of the cytoplasmic domains of parental and mutant H and F proteins. Protein sequences are shown in single-letter code. The vertical lines separate the transmembrane domains from those predicted to be in the cytoplasm. TM, transmembrane domain; CD, cytoplasmic domain. B, surface expression of parental and mutant glycoproteins. MDCK cells transiently expressing either the parental (HEdm and FEdm) or mutant (H12Y/A and F549Y/A) proteins were incubated with either anti-H or anti-F protein monoclonal antibodies at 4 °C without prior fixation. Surface-bound antibodies were detected with FITC-conjugated anti-mouse immunoglobulins. C, fusion activity of parental and mutant proteins. HEdm and FEdm, were transiently expressed in the absence or presence of either the corresponding parental protein (FEdm and HEdm) or mutant protein (F549Y/A and H12Y/A). At 20 h post-transfection, cells were fixed and stained with Giemsa staining solution.
tyrosine mutants were not affected in their ability to mediate fusion of non-polarized cells.

Both Tyrosine Mutants Are Re-targeted in Polarized Epithelial Cells—To analyze the polarized transport of membrane proteins, stably expressing cell lines have to be established. For this purpose, plasmids expressing either the parental H$_{Edm}$ or F$_{Edm}$ protein or tyrosine mutants were transfected into MDCK cells, and neomycin-resistant transfectants were selected using G418. To monitor the targeting of the expressed proteins, the cells were cultured on permeable filter supports, where they form polarized monolayers. Cells grown on filters were cooled to 4 °C, and either the apical or basolateral surface proteins were labeled by adding the non-membrane-permeating reagent sulfo-N-hydroxysuccinimidobiotin to the respective filter chamber. The cells were lysed, and H or F proteins were immunoprecipitated by specific antibodies (mAb 8905 or A504). After separation on a 10% SDS-polyacrylamide gel (reducing conditions for H proteins and nonreducing conditions for F proteins), proteins were transferred to nitrocellulose. Biotin-labeled proteins were detected with peroxidase-conjugated streptavidin. As shown in Fig. 2 (lanes b), efficient biotinylation of both parental proteins was obtained only after labeling the cells from the basolateral side (H$_{Edm}$; 95%; and F$_{Edm}$; >99%). This confirms our previous results that both MV glycoproteins are predominantly expressed on the basolateral surface of polarized MDCK cells. In contrast, strong labeling of both tyrosine mutants was detected only after apical surface biotinylation (F$_{849;YA}$ and H$_{12Y;YA}$ panels, lanes a). Only a minor band was found after basolateral labeling (lanes b). The protein distribution was further confirmed by surface immunofluorescence of filter-grown cells using a confocal laser scanning microscope (data not shown). The redirection of the mutant proteins to the apical cell membrane indicates that both MV glycoproteins contain a tyrosine-dependent basolateral sorting signal.

The Tyrosine Responsible for Basolateral Transport of H and F Proteins Also Mediates Endocytosis—In certain cases, the cytoplasmic signal responsible for efficient localization to coated pits may be very similar to that responsible for basolateral targeting (24–26). To determine if MV glycoproteins also possess an endocytosis signal, we performed an antibody uptake experiment. Without prior fixation, H or F proteins on the surface of stably expressing cells were labeled with the respective antibodies at 4 °C and warmed to 37 °C for 15 min to allow endocytosis to occur. Surface-bound antibodies were detected by incubation of the living cells with a rhodamine-conjugated secondary antibody at 4 °C. After permeabilization of the cells, internalized antibodies were stained with an FITC-conjugated anti-mouse serum (intracellular panels). B, endocytosis of parental and mutant proteins. MDCK cells stably expressing H$_{Edm}$, H$_{849;YA}$, F$_{Edm}$, or F$_{849;YA}$ were surface-labeled with sulfo-succinimidyl 2-biotinamide methyl-1,3-dithiopropionate at 4 °C. Cells were shifted to 37 °C for the times indicated to allow endocytosis to occur. Subsequently, cell-surface proteins were either reduced with 2-mercaptoethanesulfonic acid (MESNA) at 4 °C (+) or left untreated (−). After cell lysis, proteins were immunoprecipitated with an H or F protein-specific antibody. The precipitates were separated by SDS gel electrophoresis under nonreducing conditions and transferred to nitrocellulose. Biotinylated proteins were detected with peroxidase-conjugated streptavidin. C, rate of internalization of parental and mutant proteins. The percentage of internalized protein measured in the experiment shown in Fig. 3B is plotted as a function of the time that cells were incubated at 37 °C before reduction with 2-mercaptoethanesulfonic acid in the cold.

indicating that both proteins possess an endocytosis signal. Since the antibody uptake experiment is only a qualitative test, we performed a biotin internalization assay (see “Experimental
Construction and Analysis of Chimeric Proteins from MV F Protein and the H7 Protein of Influenza Virus—In infected MDCK cells, the F protein was found to be targeted to the basolateral surface, as found in cells singly expressing FEdm (10). Basolateral transport of the H protein was found to be less stringent in infected cells. Furthermore, endocytosis of MV F protein was more efficient than was internalization of the H protein. Taken together, the F protein cytoplasmic tail appeared to have stronger transport signals. To know whether this signal functions as an autonomous sorting sequence, we wanted to determine whether it is able to redirect an apical membrane protein to the basolateral cell surface. For this purpose, we chose the hemagglutinin (H7) protein of fowl plaque virus, an avian influenza A virus. The apical localization of this protein is well documented (7, 27). Furthermore, it was shown that it is excluded from coated pits (28). Therefore, foreign sequences introduced into the H7 protein can be analyzed not only for basolateral targeting information, but also for their ability to mediate endocytosis through coated pits. For the construction of chimeras, an H7 mutant was used in which the recognition site for proteolytic cleavage by furin-like enzymes was destroyed. As we described previously (29), establishment of cells stably expressing the H7 protein is facilitated when the protein is not cleaved intracellularly and thus is unable to mediate cell-to-cell fusion. The chimeric proteins used in this study are shown in Fig. 4A. In the H7-F-F chimera, the 11-amino acid tail of the H7 protein was replaced with the 33 amino acids of the cytoplasmic portion of MV F protein. In the H7-F-F chimera, the transmembrane and cytoplasmic domains of the H7 protein were exchanged with the respective domains of MV F protein. In the H7-F-H7 chimera, only the transmembrane domain of the H7 protein was replaced with the corresponding portion of MV F protein. For all constructs, stably expressing MDCK cells were subjected to domain-specific biotinylation as described above for the tyrosine mutants. Following immunoprecipitation with mAb 2A11-H7, the proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and biotinylated proteins were detected with peroxidase-conjugated streptavidin. As shown in Fig. 4B, the H7 wild-type protein (>-99%) as well as H7-H7-F (98%) were predominantly expressed on the apical cell surface of MDCK cells. H7-F-H7 was found almost equally distributed on both cell surfaces (60% apical and 40% basolateral). Only when both the cytoplasmic tail and the transmembrane domain of MV F protein were transferred to the luminal domain of the H7 protein (H7-F-F) the protein was re-targeted to the basolateral side (>95%). This result indicates that the cytoplasmic tail containing the tyrosine critical for the basolateral transport of MV F protein is not sufficient to redirect the apical H7 protein. The additional replacement of the transmembrane domain appeared to be required either for the recognition of the basolateral targeting signal in the F protein cytoplasmic tail or to provide a second basolateral targeting signal. To assay how far the endocytosis signal can be transferred from the F to H7 protein, cells stably expressing chimeric molecules were analyzed by a biotin internalization assay in which endocytosis was allowed to occur for 5–25 min. After immunoprecipitation using an H7 protein-specific antibody, the samples were sepa-
rated by SDS gel electrophoresis, and endocytosed biotinylated protein was detected after blotting onto nitrocellulose with peroxidase-conjugated streptavidin. In Fig. 4C, quantification of the endocytosis is shown. As described by Lazarovits et al. (28) for the H2 hemagglutinin, the H7 protein was not internalized during 25 min at 37 °C. In contrast, endocytosis of all chimeras could be measured; but none of the proteins was internalized at a rate significantly higher than that of F549Y/A or the bulk of the plasma membrane. Uptake of the H7 chimera containing both the MV F transmembrane and cytoplasmic domains (H7-F-F) was found to be only slightly enhanced in comparison with H7-F-H7 and H7-H7-F. This result indicates that the endocytosis signal in the F protein cytoplasmic tail is not recognized in the H7 protein context even if the transmembrane domain of the F protein is present at the same time.

Apical Expression of the MV Glycoproteins Prevents Syncytium Formation in Epithelial Cells—To elucidate the importance of the basolateral sorting of glycoproteins H and F for MV spread, we analyzed whether inactivation of the targeting signal affects the biological activity of MV glycoproteins in polarized MDCK cells. In polarized cell monolayers, only lateral cell membranes can fuse. Therefore, fusogenic proteins must be expressed on the basolateral cell surface to induce fusion (30). Apically expressed proteins are unable to mediate fusion. Thus, we examined whether replacement of the tyrosines in the cytoplasmic tails of H and F proteins affects the syncytium formation in polarized cell monolayers. For this purpose, cells stably expressing either HEdm or FEdm were grown to over-confluency. The cells that had already established a polarized phenotype were then transfected either with the parental HEdm protein or with the mutant H12Y/A protein. As a control, MDCK cells were infected with MV. At 7 h post-infection or post-transfection, the culture medium was removed, and cells were grown for 24 h either in normal growth medium or in calcium-depleted medium. In the absence of calcium, tight junctions could not be maintained or formed, resulting in non-polarized cells in which apical and basolateral surfaces were no longer separated. At 30 h post-transfection or post-infection, cells were fixed and stained with an H protein-specific antibody and an FITC-conjugated secondary antibody. Immunofluorescence was used to monitor cell-to-cell fusion since syncytium formation in over-confluent MDCK cell monolayers is difficult to detect by staining with Giemsa staining solution. As expected, all samples demonstrated large syncytia in the absence of calcium (Fig. 5, −Ca panels). In contrast, fusion of polarized cells cultured in normal growth medium (+Ca panels) was observed only when parental HEdm and FEdm proteins were coexpressed either in transfected or infected cells (MV and FEdm + HEdm panels). In polarized MDCK cells expressing a tyrosine mutant in combination with a parental protein (F549Y/A + HEdm, and FEdm + H12Y/A panels), fusion was largely prevented. This result indicates that the presence of a basolateral targeting signal in the cytoplasmic tails of H and F proteins has significant consequences for cell-to-cell fusion in polarized cells and thus for the phenotype of MV-infected cells.

Endocytosis of the MV Glycoproteins Is Inhibited in Virus-infected Cells—The basolateral sorting signals were shown to be functional in virus-infected MDCK cells (10). To analyze whether endocytosis signals function also in infected cells, we analyzed the endocytosis of the MV glycoproteins by an antibody uptake experiment as described above. None of the glycoproteins was found to be endocytosed to a detectable amount (Fig. 6A). This observation indicates that internalization of H and F proteins is prevented in infected cells. In contrast, cells cotransfected with plasmids containing HEdm and FEdm showed internalization of both glycoproteins (Fig. 6B). Since internalization was also observed in coexpressing cells having already formed syncytia, lack of endocytosis in infected cells cannot be explained by general cell damage induced by cell fusion. This is further supported by the observation that neither endocytosis of a fluid-phase marker (FITC-dextran) nor endocytosis of a constitutive cellular membrane protein (CD4613F/Y) (25) is affected in virus-infected cells (data not shown).

**DISCUSSION**

We have shown here that both MV envelope proteins possess targeting signals that critically depend upon a tyrosine residue in the cytoplasmic tails. Stable expression of the MV glycoproteins in MDCK cells showed that the tyrosine residues are involved both in basolateral transport and in endocytosis. In MV-infected cells, recognition of the sorting signals of F and H proteins is differently regulated. Whereas endocytosis appeared to be abolished, basolateral transport was not substantially prevented; and thus, efficient fusion of polarized epithelia was observed. This suggests that the basolateral signals have biological importance for virus replication in epithelia.

**Both MV Glycoproteins Possess Overlapping Signals for En-**
have been shown to function poorly or not at all (3, 34, 36, 37). Thus, signals for polarized transport and endocytosis do not necessarily overlap. Here, we showed that in the MV glycoproteins, they do. Mutation of the critical tyrosines abolished basolateral transport and rapid internalization, suggesting that the sequences around the tyrosines are involved in forming a β-turn.

Sorting Signals in the F Protein Cytoplasmic Tail Lack Functionality in HA-F Protein Chimeras—The properties of influenza virus HA completely differ from those of the MV glycoproteins. HA is preferentially expressed on the apical surface of epithelial cells and is internalized 40 times more slowly than is the bulk of the plasma membrane (33). A mutant that is rapidly endocytosed and targeted to the basolateral cell surface could be generated by replacement of cysteine 543 with a tyrosine residue in the short cytoplasmic tail of HA (HA-Y543) (38, 39). Just as the MV glycoprotein sorting signals, the YRIC motif from the HA-Y543 mutant does not follow the YXXØ or NPXY pattern. MV F protein and HA-Y543 have an additional feature in common. In both proteins, the tyrosine responsible for endocytosis and basolateral transport is located at position −5 from the cytoplasmic end of the proteins. Nevertheless, transfer of the F protein cytoplasmic tail with a tyrosine at position −5 to the HA protein (H7-H7-F) resulted neither in efficient endocytosis nor in re-targeting to the basolateral membrane. The lack of functionality cannot be explained by a more degenerated signal (YXXØ in H7-H7-F versus YXXC in HA-Y543) since an HA mutant (HA-Y543/S546) with a serine instead of cysteine (YXXS) was endocytosed efficiently (40). This indicates that other information than only a tyrosine in the correct position is required to override the intrinsic properties of HA. HA was only re-targeted by MV F protein sequences when both the transmembrane and cytosolic portions of the F protein were transferred to the luminal portion of HA (H7-F-F). From this it follows that the tyrosine-dependent targeting signal in the F protein cytoplasmic tail either is overridden by a counteracting apical signal in the HA transmembrane domain or requires an additional supporting signal in the transmembrane region of the F protein. This view is supported by the observation that the F protein transmembrane domain alone (H7-F-H7) could not redirect HA to the basolateral surface, although it abolished the strict apical localization of the chimera. In contrast to basolateral transport, the F protein cytosolic portion was unable to mediate efficient endocytosis even in the presence of the F protein transmembrane domain. Thus, we conclude that the internalization signal in the F protein cytoplasmic tail functions only in the context of the authentic protein.

Endocytosis, but Not Basolateral Targeting, of MV Glycoproteins Is Prevented in Virus-infected Cells—Given the importance of cell-associated viral glycoproteins in mediating cytopathic effects and as targets of host immune responses, endocytosis of MV envelope proteins may be a regulatory mechanism to prevent extensive expression of viral antigen on the cell surface. A likely explanation for the lack of endocytosis in MV-infected cells is that interaction of the H and F protein cytoplasmic tails with the viral matrix protein initiating assembly of new virions (13, 41) prevents the interaction with cellular adaptor complexes. We propose that MV has developed a system that removes those H and F proteins from the cell surface that are not associated with the M protein and are therefore not destined for incorporation into virions. This view is supported by our observation3 that both MV glycoproteins were efficiently endocytosed in cells infected with recombinant MV lacking the M protein (MVΔM) (12). Similar mechanisms to

3 M. Moll, and A. Maisner, unpublished observation.
regulate the expression of viral envelope proteins by interaction with viral core proteins have been proposed for Sendai virus and the human immunodeficiency virus (42, 43). In contrast, glycoproteins from the simian immunodeficiency virus and simian virus 5 have been found to be internalized even in the presence of viral core proteins (44, 45). This points out that the biological relevance of endocytosis signals in viral envelope proteins cannot be predicted, but has to be analyzed in the context of virus infection.

In contrast to endocytosis, we found the basolateral transport of MV glycoproteins not to be severely impaired in infected cells. We have reported previously that both glycoproteins are expressed on the basolateral surface of infected MDCK cells (10). Here we show that cell-to-cell fusion of polarized MDCK cells occurs both in virus-infected cells and in cells coexpressing H and F proteins in the absence of other viral proteins. The ability to mediate fusion critically depends upon intact targeting signals in both proteins. We propose that the presence of H and F proteins on the basolateral surface promotes spread of virus infection from cell to cell. Although it has been recently reported that the M protein can partially re-target the glycoproteins to the apical cell surface, a sufficient amount of glycoprotein appeared to reach the basolateral membrane to induce fusion.

In summary, MV regulates the recognition of its signals for endocytosis and basolateral targeting by differential interaction with other viral components. In this way, MV glycoproteins can have a tyrosine-dependent endocytosis and sorting signal that permits polarized transport, but prevents internalization of glycoproteins during infection of epithelial cells. As virus release was shown to occur only at the apical cell surface, basolateral glycoprotein expression may be required for MV to spread from the primarily infected epithelia to underlying tissues by direct cell-to-cell fusion. In the absence of viral core proteins, glycoprotein expression on the cell surface is downregulated by endocytosis, thereby preventing the exposure of viral antigens on the cell surface. Although just one component of the complex biology of the virus, it is clear from the experiments described here that the tyrosine-dependent signals contribute to the cytopathic properties of MV in culture and may also be important for the pathogenesis in vivo.

Acknowledgments—We thank J. and S. Schneider-Schaulies, W. Garten, and R. Cattaneo for kindly providing monoclonal antibodies and cloned MV genes.

REFERENCES

A Single Amino Acid Change in the Cytoplasmic Domains of Measles Virus Glycoproteins H and F Alters Targeting, Endocytosis, and Cell Fusion in Polarized Madin-Darby Canine Kidney Cells
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doi: 10.1074/jbc.M010183200 originally published online February 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010183200

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