Altered Budding Site of a Pantropic Mutant of Sendai Virus, F1-R, in Polarized Epithelial Cells

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A protease activation mutant of Sendai virus, F1-R, causes a systemic infection in mice, whereas wild-type virus is exclusively pneumotropic (M. Tashiro, E. Pritzer, M. A. Khoshnan, M. Yamakawa, K. Kuroda, H.-D. Klenk, R. Rott, and J. T. Seto, *Virology* 165:577-583, 1988). Budding of F1-R has been observed bidirectionally at the apical and basolateral surfaces of the bronchial epithelium of mice and of MDCK cells, whereas wild-type virus buds apically (M. Tashiro, M. Yamakawa, K. Tobita, H.-D. Klenk, R. Rott, and J. T. Seto, *J. Virol.* 64:3627-3634, 1990). In this study, wild-type virus was shown to be produced primarily from the apical site of polarized MDCK cells grown on permeable membrane filters. Surface immunofluorescence and immunoprecipitation analyses revealed that transmembrane glycoproteins HN and F were expressed predominantly at the apical domain of the plasma membrane. On the other hand, infectious progeny of F1-R was released from the apical and basolateral surfaces, and HN and F were expressed at both regions of the cells. Since F1-R has amino acid substitutions in F and M proteins but none in HN, the altered budding of the virus and transport of the envelope glycoproteins might be attributed to interactions by F and M proteins. These findings suggest that in addition to proteolytic activation of the F glycoprotein, the differential site of budding, at the primary target of infection, is a determinant for organ tropism of Sendai virus in mice.

Sendai virus, a prototype of the paramyxovirus family, is exclusively pneumotropic in mice (3, 6, 16, 18, 20). Recently, we isolated a protease activation mutant, F1-R, that underwent multiple cycles of replication in several cell lines in the absence of trypsin (18). F1-R virus was also found to be pantropic in mice by causing a generalized infection (18-20). By comparative nucleotide sequence analyses of the genome of the wild type and F1-R, it was suggested that three amino acid substitutions near the cleavage site of the fusion (F) glycoprotein resulted in the increased cleavability of the protein by ubiquitous proteases present in various organs of mice (18, 19). These amino acid substitutions in F were therefore considered prime candidates as determinants for pantropism of F1-R (7a, 18, 20, 21).

Another important observation was made when the bronchial epithelium of infected mice was examined by immunoelectron microscopy. Budding of wild-type virus and expression of the transmembrane glycoproteins were detected exclusively at the apical domain of the epithelial cell membrane, whereas after infection with F1-R, viral antigens and budding were found to be bipolar at the apical and the basolateral surfaces (21). These findings suggested that the progeny virus of F1-R was released from both domains, whereas budding of wild-type virus occurred only at the apical domain. It was also found that infection by wild-type virus was restricted to the bronchial epithelium, whereas F1-R readily invades the subepithelial tissues, leading to a systemic infection via viremia (16, 18-20). Hence, differential budding at the primary target of infection was proposed to be an additional determinant for organ tropism (21).

Enveloped viruses have been reported to mature at distinct membrane domains of monolayers of polarized epithelial cells (12, 15), with budding of paramyxoviruses and orthomyxoviruses at the apical surface and budding of rhabdoviruses, retroviruses, and baculoviruses at the basolateral domain. Several experiments on genetic expression of the viral glycoproteins have suggested that the signals responsible for polarized transport of the proteins are located in their own molecules, although the positions within molecules are controversial and thus determine the site of budding (9, 13-15).

Most of the information on polarized budding of enveloped viruses has been obtained from studies that used MDCK cell cultures. By electron microscopy, wild-type Sendai virus has been reported to bud exclusively at the apical surface of these cells (12). On the other hand, budding of the F1-R mutant was found to occur at the apical and the basolateral domains (21). However, details concerning the assembly of F1-R at the basolateral domain remain unknown. Since amino acid substitutions were found in the F glycoprotein and in the matrix (M) protein of F1-R but none were found in the hemagglutinin-neuraminidase (HN) glycoprotein (7a, 18, 19), it was of particular interest to investigate which of the transmembrane glycoproteins exhibited altered surface expression. In this paper, we demonstrate that HN and F are expressed at the basolateral as well as the apical side of F1-R-infected MDCK cells and that the infectious viral particles are released from both surface domains. These findings are unique for the pantropic mutant of Sendai virus.

MATERIALS AND METHODS

Virus. Wild-type Sendai virus (Z strain) and the pantropic protease activation mutant (F1-R) were propagated in 10-

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day-old embryonated chicken eggs as described previously (18).

Cell culture. Polycarbonate membrane filters (tissue culture treated; pore size, 3.0 μm) and collagen type 1 and 3 membranes (pore size, 0.4 μm), attached to the bottom of plastic cups (6.5-mm diameter), were from Costar (Cambridge, Mass.). They were placed into 24-well tissue culture plates; each well contained 0.6 ml of minimal essential medium (MEM) supplemented with 10% fetal calf serum. A 0.1-ml sample of a single-cell suspension of MDCK cells (10^5 cells per ml) in MEM was seeded onto the membrane and subcultured in a CO_2 incubator at 37°C . In this way, the apical and basal surfaces of the cell monolayers had equal access to the culture medium, bathing each side of the cells. Electrical resistance of cell monolayers was measured as described before (5).

Uptake of [^{35}S]methionine. The procedure with minor modification was carried out as described previously (1). At various time intervals after seeding, the cells on the membranes were incubated at 37°C with methionine-deficient MEM for 10 min and then bathed in the same medium containing [^{35}S]methionine (370 kBq/ml) at the apical or basal side. After a 10-min pulse, the medium from the both sides was assayed for radioactivity to exclude any leakage through the monolayer. The cells on the membrane were washed extensively with cold phosphate-buffered saline (PBS), dissolved into a scintillation cocktail (Aquasol 2; Dupont, NEN Research Products, Boston, Mass.), and measured for radioactivity.

Viral replication in MDCK cells. MDCK cells were subcultured on the membrane filters (pore size, 3.0 μm) for 4 days and infected at the apical side with the viruses at a multiplicity of infection of 10 PFU per cell. After 1 h of adsorption, the cells were washed and incubated in MEM at 37°C . At various time intervals, the medium in each side was harvested separately and assayed for PFU with LLC-MK₂ cells (16).

Surface immunofluorescence of infected cells. Cell monolayers were established on the transparent collagen membranes and infected as described above. After infection for 12 h at 37°C , the monolayers were washed with cold PBS and fixed with 1.75% paraformaldehyde in PBS for 5 h at 4°C . This procedure does not make the plasma membranes permeable to antibodies, nor does it open the tight junctions among the monolayer cells (5, 24). The apical and basal membrane surfaces of the monolayers were stained individually for viral antigens by the indirect immunofluorescence method, using 1,000-fold-diluted monoclonal antibodies specific for Sendai virus glycoproteins and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G goat immunoglobulins (Organon Teknika, Malvern, Pa.). The mouse monoclonal antibodies against HN (22) and F glycoproteins were kindly provided by H. Tozawa and M. Homma.

Immunoprecipitation of viral glycoproteins. Cell monolayers were infected as described above except that MEM containing [^{35}S]methionine (370 kBq/ml) was added to the basal side. After incubation for 12 h at 37°C , the monolayers were washed with cold PBS and incubated at either the apical or basal surface with 2,000-fold-diluted monoclonal antibodies of HN and F for 60 min at 4°C . Cells were washed with PBS, solubilized with RIPA buffer (0.7% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 15 mM Tris hydrochloride, pH 8.0), and centrifuged at 2,500 rpm for 10 min. The immune complexes were precipitated with protein A-Sepharose

TABLE 1. Polarized uptake of [^{35}S]methionine by MDCK monolayer cells

Time (h) ^a	Expt	No. of cells (10^4)	Uptake (cpm/ 10^5 cells) from ^b :		Basolateral/apical ratio
			Apical	Basolateral	
24	1	2.3	3,206	18,383	5.7
	2	2.9	4,328	23,343	5.4
48	1	5.9	1,027	11,260	11.1
	2	6.4	1,123	14,882	13.2
72	1	6.9	327	7,609	24.2
	2	6.9	335	6,865	20.5
96	1	6.9	206	5,380	21.6
	2	7.0	152	4,051	26.7
120	1	7.1	124	3,067	28.0
	2	7.0	141	3,672	26.0

^a MDCK cells (10^4) were plated onto a membrane filter cup (33 mm²) and incubated with MEM containing 10% fetal calf serum at 37°C .

^b At the indicated time, cells were incubated with methionine-free MEM for 10 min and then with the same medium containing [^{35}S]methionine (370 kBq/ml) in either the apical or basal side for 10 min at 37°C . The cells were dissolved into a scintillation cocktail and counted for radioactivity.

CL-4B beads (Pharmacia, Uppsala, Sweden) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (16, 17, 21).

RESULTS

Polarity of MDCK cells on membrane filters. Since apical-basolateral polarity of MDCK cells has been reported to depend on subculture conditions (1, 5, 11, 24), we determined whether the cells, under our experimental conditions, were characteristic of polarized epithelial cells. MDCK cells were subcultured on permeable membrane filters as described in Materials and Methods. The confluent monolayers, established on polycarbonate and collagen membranes 4 days after seeding, showed a dome-shaped formation two to three times larger than that on plastic dishes. The monolayers were similar in morphology to strain I of MDCK cells (11) (data not shown). The electrical resistances of confluent monolayers on the polycarbonate and collagen membranes were 180 and 240 $\Omega \cdot \text{cm}^2$, respectively. The low resistance was comparable to that of epithelial cells of strain II (100 to 150 $\Omega \cdot \text{cm}^2$) rather than strain I (>1,000 $\Omega \cdot \text{cm}^2$) (5, 11).

Since selective uptake of [^{35}S]methionine from the basolateral side was shown to be an indication of the polarity state of MDCK cells (1), the time course of uptake was examined (see Materials and Methods). After tight monolayers were formed on the polycarbonate membranes, [^{35}S]methionine was taken up predominantly from the basolateral side (Table 1), in agreement with published reports (1). In these cells, more than 99.9% of progeny virus of the WSN strain of influenza virus was recovered from the culture medium of the apical side (data not shown). Similar results were obtained when collagen membranes were used (data not shown). It was concluded from these results that the MDCK cell monolayers on the membrane filters were indeed polarized. In the following experiments, therefore, the monolayers subcultured for 96 h were used for viral infections.

Polarity of virus production in MDCK cells. We reported previously that budding of F1-R occurred bipolarly at the apical and basolateral domains, whereas budding of wild-type virus was at the apical surface of mouse bronchial epithelial cells and of MDCK monolayer cells (21). To determine whether infectious progeny of F1-R was released

FIG. 1. Release of Sendai viruses from MDCK cell monolayers from the apical or basolateral domains. MDCK cell monolayers established on the membrane filter cups were infected with wild-type (WT) or F1-R mutant virus and incubated at 37°C. At the indicated time intervals, culture medium facing the apical (○) and basolateral (●) domains of monolayers was collected separately and quantitated by plaque assay.

from the domains and whether the bipolar budding was unique for the protease activation mutant, polarized MDCK cell monolayers were infected with the viruses. The results presented in Fig. 1 illustrate the time course of viral infectivity in the medium from the apical side and from the basal side. No significant difference between wild type and F1-R in the release of progeny virus occurred at the apical surface. At the basal region, wild-type titers were 10^3 to 10^4 times less than those in the medium from the apical side for 18 h after infection. Thereafter, the titer in the basal medium increased significantly, suggesting that the infected cells were no longer polarized or that tight junctions among monolayer cells became leaky. For F1-R, on the other hand, the amount of progeny virus released at the basal region was 10- to 50-fold less than the titer of virus from the apical surface during the replication cycle. Leakage of [35 S]methionine did not occur from the apical medium to the basal region, or conversely, in the monolayers for 15 h after infection (data not shown). These results indicate that release of the F1-R mutant was much less polarized in MDCK cells, whereas release of wild-type virus was primarily, though not absolutely, from the apical domain of the plasma membrane. In addition, electron microscopy revealed that budding of wild-type virus was polarized at the apical domain of the plasma membrane, whereas budding of F1-R was bidirectional at the apical and basolateral domains (21).

Surface expression of viral glycoprotein antigens. Since infectious F1-R was released from the apical and basolateral domains of MDCK cells, it was expected that F and HN glycoproteins would be transported to the basolateral surface, in addition to the apical domain, of infected cells. To verify this prediction, surface immunofluorescence was done. In wild-type-infected cells, F and HN antigen-positive areas were evident throughout the apical surface as granular or small patchy reactions (Fig. 2, 1A), in contrast to the

faintly stained areas at the basolateral domain (Fig. 2, 1B). These findings indicate that transmembrane glycoproteins F and HN of wild-type virus were transported predominantly to the apical surface of the plasma membrane. On the other hand, in F1-R-infected MDCK cells, F and HN proteins were expressed at the apical and basolateral surfaces. At the apical region, the staining patterns of the envelope antigens were similar to those with wild-type virus (Fig. 2, 2A). Fluorescence was clearly evident at the domains facing the lateral spaces, but the basal surfaces were weakly positive compared with the fluorescence at the apical region (Fig. 2, 2B).

The bipolar distribution of the transmembrane glycoproteins in F1-R-infected cells was supported by surface immunoprecipitation analysis. The SDS-PAGE patterns of the immunoprecipitates are presented in Fig. 3, and quantitation of F and HN at the apical and basolateral domains is shown in Table 2. These data confirm that F and HN of wild-type virus were expressed predominantly at the apical domain, whereas transport of F1-R glycoproteins was bidirectional even though apical expression was still predominant. Much of F of F1-R was in the cleaved form, and no selective transport of cleaved versus uncleaved F took place (Fig. 3). Analyses of F1-R particles, released into the medium from the apical and basolateral surfaces, indicate that viral assembly at both regions was normal.

DISCUSSION

Our results demonstrate that infectious progeny of the pantropic variant of Sendai virus, F1-R, was released from the apical and basal surfaces of polarized MDCK cell monolayers, whereas wild-type virus was released predominantly, though not absolutely, from the apical surface. These findings are consistent with our electron microscopic observations made of MDCK cell monolayers and of the mouse bronchial epithelium, both of which are polarized cells (21). Thus, it is evident that the capacity to bud from both surface domains of polarized cells is a genuine trait that differentiates the protease activation mutant from the wild-type virus. This finding gives further support to the concept that infection by wild-type virus remains localized in the bronchial epithelium, whereas F1-R spreads readily from the epithelium into the subepithelial tissues, thereby resulting in a systemic infection (21). The difference in budding domains at the primary target of infection is therefore a likely determinant for pneumotropism of wild-type Sendai virus and pantropism of the F1-R mutant (21), in addition to proteolytic cleavage of the F glycoprotein (4, 6-8, 16, 18).

In accordance with the differential sites of budding, the transmembrane glycoproteins HN and F showed bipolar expression in F1-R-infected cells, as opposed to the polarized expression of these proteins at the apical domain of the plasma membrane after infection with wild-type virus. Nucleotide sequence analyses of the entire genome of the viruses have indicated that F1-R has amino acid substitutions in the F and M proteins but none in HN (7a, 18, 19). Glycoprotein F has one amino acid substitution in the cytoplasmic domain and five in the ectodomain. Three of the latter are located near the site of cleavage, and they are most likely responsible for the enhanced proteolytic cleavability of the protein (7a, 18, 19, 21). It is expected that any of the six exchanges may be associated with bipolar transport of the mutant transmembrane protein, because polarized transport is believed to be determined by signals intrinsic to viral glycoproteins (9, 13, 15). The lack of any amino acid

FIG. 2. Surface immunofluorescence of viral glycoprotein antigens on MDCK cells. MDCK cell monolayers on membrane filters were infected with wild-type (1) or F1-R mutant (2) virus and incubated for 12 h at 37°C. After fixation with 1.75% paraformaldehyde for 5 h, mouse monoclonal antibodies specific for F and HN were reacted with apical (A) or basolateral (B) surface of the monolayers, followed by the secondary reaction with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody.

FIG. 3. SDS-PAGE analysis of immunoprecipitates of viral antigens on MDCK cell surfaces. MDCK cell monolayers on the membrane filters infected with wild-type (WT) or F1-R virus were labeled with [35 S]methionine for 12 h at 37°C. Apical (A) or basolateral (B) surfaces of the monolayers were reacted with monoclonal antibodies against F and HN glycoproteins. The cells were solubilized, after washing, with RIPA buffer, and the immune complexes were precipitated by protein A-Sepharose beads. Viral particles (V) in the medium surrounding the apical or basolateral surface, collected 15 h after infection, are also shown.

substitution in HN suggests, however, that at least in the system described here, other signals may be involved that are not located on the glycoproteins. Since M is important in the assembly and maturation of Sendai virus (23), the role of M in bipolar budding of F1-R must be considered.

As a result of the amino acid exchanges, the M protein of F1-R migrated faster on polyacrylamide gels in the absence of urea than did wild-type M protein, indicating a conformational difference (7a, 18). In preliminary experiments using immunoprecipitation with monoclonal antibodies specific for HN and F, F1-R required more drastic conditions to solubilize HN and F devoid of M, suggesting that M of F1-R is more tightly bound to HN and F than are the transmembrane proteins of wild-type virus (data not shown). It has been shown that Sendai virus M is distributed diffusely in the cytoplasm of infected cells (10), whereas M of vesicular stomatitis virus is transported basolaterally independently of the G glycoprotein (2). It is therefore important to clarify

whether M of F1-R is transported bidirectionally and has an effect on the transport of HN and F. Studies are in progress to answer these questions.

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TABLE 2. Surface immunoprecipitation of viral glycoproteins^a

Virus	Antibody	Radioactivity (cpm) in immunoprecipitates		Basolateral expression (%)
		Apical	Basolateral	
Wild type	F	13,164	865	6.0
	HN	9,823	325	3.2
F1-R	F	12,708	4,965	28.1
	HN	7,428	1,632	18.0

^a MDCK cell monolayers were infected with the viruses and labeled with [35 S]methionine from the basolateral side for 12 h at 37°C. The apical or basolateral surface of the monolayers was reacted with monoclonal antibodies against F and HN. The immune complexes, after solubilization, were precipitated with protein A-Sepharose beads and counted for radioactivity.

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