Involvement of the Mutated M Protein in Altered Budding Polarity of a Pantropic Mutant, F1-R, of Sendai Virus

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Wild-type Sendai virus buds at the apical plasma membrane domain of polarized epithelial MDCK cells, whereas a pantropic mutant, F1-R, buds at both the apical and basolateral domains. In F1-R-infected cells, polarized protein transport and the microtubule network are impaired. It has been suggested that the mutated F and/or M proteins in F1-R are responsible for these changes (M. Tashiro, J. T. Seto, H.-D. Klenk, and R. Rott, *J. Virol.* 67:5902–5910, 1993). To clarify which gene or mutation(s) was responsible for the microtubule disruption which leads to altered budding of F1-R, MDCK cell lines containing the M gene of either the wild type or F1-R were established. When wild-type M protein was expressed at a level corresponding to that synthesized in virus-infected cells, cellular polarity and the integrity of the microtubules were affected to some extent. On the other hand, expression of the mutated F1-R M protein resulted in the formation of giant cells about 40 times larger than normal MDCK cells. Under these conditions, the effects on the microtubule network were enhanced. The microtubules were disrupted and polarized protein transport was impaired as indicated by the nonpolarized secretion of gp80, a host cell glycoprotein normally secreted from the apical domain, and bipolar budding of wild-type and F1-R Sendai viruses. The mutated F glycoprotein of F1-R was transported bipolarly in cells expressing the F1-R M protein, whereas it was transported predominantly to the apical domain when expressed alone or in cells coexpressing the wild-type M protein. These findings indicate that the M protein of F1-R is involved in the disruption of the microtubular network, leading to impairment of cellular polarity, bipolar transport of the F glycoprotein, and bipolar budding of the virus.

Sendai virus, a prototype member of the paramyxovirus family, is an enveloped, negative-strand RNA virus with a genome that encodes the nucleoprotein (NP), phosphoprotein (P), and matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) proteins. The viral envelope consists of a lipid bilayer derived from the host cell plasma membrane, two types of transmembrane glycoproteins, F and HN, and the M protein. In infected cells, the glycoproteins are synthesized in the rough endoplasmic reticulum and transported to the plasma membrane via the Golgi complex, while the other proteins including M are synthesized on free ribosomes and remain free in the cytoplasm. Sendai virus particles are assembled and bud at the plasma membrane. The M protein plays an important role in the assembly process by binding the cytoplasmic tails of the glycoproteins with the nucleocapsid which is composed of the genomic RNA and NP, P, and L proteins (34, 35).

Wild-type Sendai virus is pneumotropic in rodent species (11), and infection is restricted to the respiratory epithelial cells (40, 42, 44). In contrast, a pantropic mutant, F1-R, causes a systemic infection after intranasal inoculation (40). It has been shown that at least two factors are involved in the differences in organ tropism and pathogenicity of the two viruses in vivo. The primary determinant is proteolytic cleavability of the F glycoprotein by host proteases and the organ distribution of the proteases (38, 40, 42, 44). Posttranslational proteolytic

cleavage of the F protein into disulfide-linked subunits, F₁ and F₂, is essential for viral infectivity (10, 36). The cleavage site of the F protein of wild-type virus contains a single arginine residue (2, 37), which is cleavable in vivo only by trypsinase Clara, a trypsin-like host protease secreted by Clara cells that are restricted to the respiratory epithelium (13, 47). The F protein of F1-R has a proline amino acid substitution at the cleavage site which renders the protein cleavable by ubiquitous host protease(s) (39, 40, 43). Therefore, cleavage activation and multiple cycles of replication of the wild-type virus occur exclusively in the lungs, whereas for F1-R they occur in many organs (38, 39, 44, 47).

Differential virus budding at the bronchial epithelium, the primary target of infection, is another determinant of organ tropism and pathogenicity (42, 44). Wild-type virus buds at the apical (luminal) surface of the infected bronchial epithelial cells, thus releasing progeny virus into the lumen of the respiratory tract. In contrast, F1-R buds at both the apical and basolateral domains of the plasma membrane, thereby releasing virus into the basement membrane, where it can spread into subepithelial tissues and gain access to the bloodstream (42, 44). The differential budding behavior of wild-type and F1-R viruses may explain, in part, why infection by wild-type virus is localized in the respiratory tract whereas infection by the F1-R mutant virus becomes systemic, presumably by spreading to distant organs via the bloodstream.

The differential budding phenotypes of wild-type virus and F1-R have also been shown in vitro in polarized epithelial monolayers of MDCK cells (45, 46). The viral glycoproteins, F and HN, of wild-type virus and F1-R are transported to the

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FIG. 1. Construct of the plasmid vector pMEP4. cDNA clones encoding the M or F gene of wild-type (WT) Sendai virus or the F1-R mutant were inserted into the Epstein-Barr virus-derived pMEP4 vector at the position under the control of the human methallothionein promoter. Positions of amino acid substitutions of F and M proteins deduced from nucleotide sequences are shown.

same cell surface from which each respective virus ultimately buds (29, 30, 45). Infection by F1-R causes depolymerization of host cell microtubules into soluble tubulin. This results in an impairment of cellular polarity and polarized protein transport similar to that induced by treatment of cells with colchicine or nocodazole (43). Infection by wild-type virus, on the other hand, has only a marginal effect on the host cell microtubule network and polarized protein transport. By comparing the amino acid changes deduced from nucleotide sequence analyses of the entire genome of wild-type virus, F1-R, and T5, a revertant of F1-R, we have postulated that the mutated F and/or M proteins of F1-R are responsible for the disruption of microtubules leading to an alteration of cellular polarity, bipolar transport of the viral glycoproteins, and bipolar budding of F1-R (43).

In this study, we have established MDCK cell lines transfected with the M and F genes of each virus to directly examine the roles of M and F proteins of the F1-R mutant virus in disrupting the microtubule network, impairment of cellular polarity, bipolar transport of the viral glycoproteins, and altered budding site of the virus.

MATERIALS AND METHODS

Reagents. Mouse monoclonal antibodies (MAbs) against Sendai virus proteins F, HN, and M were provided by H. Tozawa (Department of Immunology, Kitasato University, Sagami-hara, Japan) (F and HN) and A. Portner (St. Jude Children's Research Hospital, Memphis, Tenn.) (M). Rabbit immune serum raised against gp80 (TRPM-2, clusterin), a cellular glycoprotein secreted from the apical surface of MDCK cells (51), was generously donated by C. Koch-Brand (Institut für Biochemie, Johannes Gutenberg-Universität, Mainz, Germa-

ny). Anti- α - and β -tubulin mouse MAbs were from Cederlane Laboratories Ltd., Hornby, Canada. Fluorescein isothiocyanate-conjugated goat immunoglobulins to mouse immunoglobulin G and to rabbit immunoglobulin G were from Organon Teknica Corp., Durham, N.C., and DAKO Immunoglobulins Ltd., Oxford, United Kingdom, respectively. Methyl-(5-[2-thienylcarbonyl]-1H-benzimidazole-2-yl)-carbamate (nocodazole) was from Sigma Chemical Co., St. Louis, Mo.

Virus and cells. Wild-type Sendai virus (Z strain) and a pantropic mutant, F1-R, were propagated in embryonated chicken eggs (40). MDCK cells were subcultured in Dulbecco's minimum essential medium (DME) supplemented with 10% fetal calf serum for 3 days after polarized "tight" monolayers were established on permeable supports with porous collagen films or polycarbonate membrane filters (Transwell; Costar, Cambridge, Mass.) as described previously (45). Uptake of [35 S]methionine and secretion of cellular protein gp80 from the apical and basolateral domains of the monolayer cells were assayed as described elsewhere (45, 51). Electric resistance across the cell monolayers was measured as described previously (45). Cell monolayers were also subcultured on chamber slides (catalog 177402; Nunc, Naperville, Ill.) for the immunofluorescence studies. Plaque assays were done with LLC-MK₂ cells with trypsin in the agar overlay (38).

Construction of plasmids. Detailed procedures were described elsewhere (see Acknowledgments). The wild-type and F1-R genes, F and M, were cloned by reverse transcriptase PCR on viral RNA. After the addition of *Eco*RI linkers, the genes were cloned into the *Eco*RI site of Bluescript KS+ (Stratagene, Inc., San Diego, Calif.) and sequenced, and the results were compared with published sequences (19, 40). Changes from the known sequences were rectified by site-specific mutagenesis. For expression studies, the M genes were cloned into the *Pvu*II site, while the F genes were cloned between the *Not*I and *Xho*I sites in the multiple cloning site of the Epstein-Barr virus-derived expression vector pMEP4 (9) (Invitrogen Corporation, San Diego, Calif.), which contains the Cd²⁺- or Zn²⁺-inducible promoter sequence of the human metallothionein gene upstream of the multiple cloning site and the hygromycin resistance marker for selection (Fig. 1). The plasmids were amplified in *Escherichia coli* MV1190.

Transfection of MDCK cells. MDCK cells were transfected with the M-gene-containing plasmid DNA by the DNA-calcium phosphate precipitate procedure as described elsewhere (31). pMEP4 plasmid DNA without viral genes was used for a negative control. To establish stable transfectant cell lines, clones expressing the hygromycin resistance marker were selected in the presence of hygromycin B (200 to 400 μ g/ml; Sigma Chemical Co.) and subcultured three to eight times. Cell lines passaged 8 to 21 times were used in the present study. To enhance expression of the M gene, 1 to 10 μ M CdCl₂ or ZnCl₂ was added to the culture medium for 12 to 24 h. For transient expression of the F gene in the cell lines, monolayer cells were transfected with the F gene-containing plasmid by using Lipofectamine (Gibco BRL, Gaithersburg, Md.) and incubated for 15 to 30 h in the presence of ZnCl₂ as described above. The proteins of interest expressed in cells were identified and quantified by immunofluorescence and immunoblotting studies.

Quantitative domain-specific immunoprecipitation of the F protein at cell surfaces. The domain-specific immunoprecipitation was done as described previously (43, 45). MDCK cells grown on membrane filters were transfected with the pMEP4 plasmid containing the F gene as described above and incubated at 37°C in DME containing 0.1% fetal calf serum and 1 to 10 μ M ZnCl₂. At 15 to 30 h posttransfection, cells were pulse-labeled at the apical and basal sides for 30 min with [35 S]methionine (740 kBq/ml) and chased for 1 h with DME in the presence of ZnCl₂. The cells were washed with cold phosphate-buffered saline, pH 7.2 (PBS), and incubated with MAb to the F protein at an appropriate dilution at either the apical or basal side for 1 h at 4°C. After extensive washing with cold PBS, the cells were solubilized with radioimmunoprecipitation assay

FIG. 2. Expression of M and F proteins in transfected MDCK cell lines. Monolayers of MDCK cell lines containing the M gene of wild-type virus (WT M 07; lanes 1, 3, and 5) or of F1-R mutant (F1-R M 08; lanes 2, 4, and 6) were transfected with the F gene of wild-type virus (lanes 3 and 4) or F1-R (lanes 5 and 6) and cultured for 24 h in DME in the presence of 6 μ M ZnCl₂. The cells were processed for immunoblotting with a mixture of MAbs against the M and F proteins.

FIG. 3. Growth of MDCK cell lines expressing the M protein of wild-type virus (WT M 07) or F1-R mutant (F1-R M 08). In a 3.5-cm-dish, 10^4 cells were seeded and subcultured in DME in the presence of $1 \mu\text{M}$ ZnCl_2 . Numbers of cells (●) and nuclei (○) were counted daily.

(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 $3,000 \times g$ for 20 min at 4°C . For analysis of the glycoprotein in whole cells, transfected cells were directly solubilized with RIPA buffer, and the resultant supernatant was reacted with antibody. Culture medium at each side was also examined for gp80 as described previously (43). The immune complexes

were precipitated with protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were dried and exposed against Imaging Plate (type BAS III; Fuji Film Co. Ltd., Tokyo, Japan) or X-ray films. Radioactivity of the protein bands was quantified with a Bio-Imaging analyzer (BAS-EWS 3080; Fuji Film).

Quantitative immunoblotting of soluble and polymeric tubulin. Quantitative immunoblotting was done as described previously (43). Cultured cells were treated for 90 s at 30°C with 0.5% Triton-X in tubulin extraction buffer [60 mM piperazine- N,N' -bis(2-ethanesulfonic acid), 25 mM N -(2-hydroxyethyl)piperazine- N' -(2-ethanesulfonic acid), 8 mM EGTA (ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid), 2 mM MgCl_2 , 10 μM taxol (pH 6.3)] (27) and washed twice with PBS at 30°C to remove soluble tubulin. Proteins extracted from the cells and those remaining in the cells were separated by SDS-PAGE. The polypeptides in gels were transferred to a nitrocellulose membrane by using a wet transfer system (model 200/2.0; Bio-Rad Laboratories, Hercules, Calif.) as described elsewhere (43). Tubulin was quantified by the enhanced chemiluminescence Western blotting (immunoblotting) system (Amersham, Buckinghamshire, United Kingdom) using anti- α -tubulin MAb and horseradish peroxidase-labeled anti-mouse immunoglobulin G antibody.

RESULTS

Giant-cell formation after expression of mutant M protein. MDCK cells were transfected with the pMEP4 vector containing the M gene of either wild-type Sendai virus or the F1-R mutant (Fig. 1), and hygromycin-resistant transformed cell lines, 14 for the wild-type M (WT M 01 to 14) and 17 for the F1-R M (F1-R M 01 to 17), were selected as described in Materials and Methods. When M-gene expression was induced in the presence of Cd^{2+} or Zn^{2+} , the wild-type and F1-R M proteins synthesized migrated at different rates in SDS-PAGE

FIG. 4. Microscopy of MDCK cell lines subcultured in the presence of $1 \mu\text{M}$ ZnCl_2 . (A) Control cells; (B) control cells treated with $50 \mu\text{M}$ nocodazole for 16 h; (C) WT M 07 cell line expressing wild-type M protein; (D) F1-R 08 cell line expressing F1-R M protein. The photos were taken at the same magnification just after a confluent monolayer was formed. Bar, $100 \mu\text{m}$.

gels (Fig. 2). This might reflect conformational differences between wild-type and F1-R M proteins, as was previously seen for the proteins found in viral particles (40). Immunofluorescence staining showed that both M proteins were distributed diffusely in the cytoplasm (data not shown). Even in the absence of Cd^{2+} and Zn^{2+} , the M proteins were expressed at a low level (see Fig. 7), probably because the human metallothionein promoter in the vectors was partially inducible by Ca^{2+} present in the culture medium.

Although cell lines expressing the wild-type M protein in the presence of $1 \mu\text{M}$ ZnCl_2 at a level similar to that synthesized in virus-infected cells grew more slowly than did control cells and dome-shaped formation typical for MDCK cells was not seen, cell division occurred normally (Fig. 3 and 4C). The presence of ZnCl_2 affected neither growth nor morphology of control cells (Fig. 4A). In contrast, cell lines expressing the F1-R M protein at a corresponding level grew extremely slowly with restricted mitosis and cell division (Fig. 3). The size of the cells was 40 times larger in diameter than that of control cells when the culture became confluent, and each cell contained one to three nuclei (Fig. 4D). The nuclei were also enlarged to some extent. When such confluent cell cultures were maintained with growth medium in the absence of ZnCl_2 for more than 2 weeks, mitosis and cell division proceeded, though slowly, and the cell size became smaller. The cells could be further subcultured only when the cell size had returned to normal. The growth profile described above was repeated upon further passage. Subculture of control cells in the presence of nocodazole, a microtubule-disrupting drug, did not result in the formation of giant cells, although mitosis and cell division were suppressed (data not shown). Treatment of a control monolayer of MDCK cells with nocodazole caused only marginal morphological changes (Fig. 4B).

Cellular polarity of M-protein-expressing cell lines. We have previously predicted that the mutated M protein of the F1-R mutant is responsible for the alteration of cellular polarity in infected MDCK cells (19, 41, 42, 45). To evaluate this possibility, confluent monolayers of M-protein-expressing cell lines were examined for the impairment of cellular polarity by three methods as described in Materials and Methods. Repre-

FIG. 5. Quantitation of polymeric and soluble tubulin in MDCK cell lines expressing M proteins in the presence of $2 \mu\text{M}$ ZnCl_2 . Cells were treated with microtubule extraction buffer containing 0.5% Triton X-100. Extracted soluble tubulin and insoluble microtubules in the cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins were probed with anti- α -tubulin MAb and quantified by the enhanced chemiluminescence Western blotting method. Open bars, unextracted polymeric tubulin in the cells; hatched bars, extracted soluble tubulin. The data are means of three experiments with standard errors. CONT, control cells without the M genes; NCZ, control cells treated with $50 \mu\text{M}$ nocodazole for 16 h; WT M 07, a cell line expressing wild-type M protein; F1-R M 08, a cell line expressing F1-R M protein.

sentative results are shown in Table 1. For control cells, uptake of [^{35}S]methionine occurred preferentially at the basolateral domain of the plasma membrane, and gp80, a cellular secretory glycoprotein that has been shown to exhibit microtubule-dependent transport (26, 51), was secreted predominantly from the apical domain. Nocodazole treatment of MDCK cells caused an impairment of cellular polarity as judged by the decreased polarized uptake of [^{35}S]methionine at the basolateral side and by the secretion of gp80 at both the apical and basolateral domains as reported previously (26, 43). Cell lines expressing wild-type M protein at a level similar to that synthesized in virus-infected cells were found to retain the polarized phenotype of control MDCK cells, although cellular polarity was altered to some extent. In contrast, the polarity of cell lines expressing the mutated M protein of F1-R was impaired considerably, comparably to the impairment induced by

TABLE 1. Changes in cellular polarity of MDCK cells expressing M proteins

Cells	Uptake of [^{35}S]Met (cpm/ 10^5 cells) from ^a :		Basolateral/ apical ratio	Secretion of gp80 (cpm/ 10^5 cells) to ^b :		Apical/ basolateral ratio	Electric resistance ($\Omega \cdot \text{cm}^{-2}$) ^c
	Apical side	Basolateral side		Apical side	Basolateral side		
Control	167	4,435	26.6	621	132	4.7	202.3 ± 13.1
Nocodazole treated ^d	488	2,763	5.7	268	425	0.6	181.4 ± 11.3
WT ^e M 03	208	3,061	14.7	453	227	2.0	166.5 ± 15.6
WT M 06	191	4,072	21.3	346	145	2.4	158.6 ± 13.6
WT M 07	223	3,482	15.6	415	189	2.2	174.4 ± 12.6
WT M 12	216	3,913	18.1	488	263	1.9	176.8 ± 12.6
F1-R M 04	345	2,438	7.1	326	385	0.9	148.3 ± 14.5
F1-R M 05	286	2,717	9.5	362	287	1.3	171.8 ± 13.6
F1-R M 08	331	2,742	8.3	314	308	1.0	162.5 ± 12.7
F1-R M 15	376	2,607	6.9	295	357	0.8	158.7 ± 12.4

^a Monolayer cells grown on membrane filters in the presence of $1 \mu\text{M}$ ZnCl_2 were incubated with methionine-free DME for 10 min and then with the same medium containing [^{35}S]methionine (370 kBq/ml) at either the apical or basal side for 10 min at 37°C . The cells were dissolved into a scintillation cocktail and counted for radioactivity.

^b Monolayer cells grown on membrane filters were pulse-labeled with [^{35}S]methionine (370 kBq/ml) for 1 h at 37°C . After a chase period of 3 h in DME, the portions of the culture medium at the apical and basal sides as well as the cells were immunoprecipitated with antibody to gp80. After SDS-PAGE under nonreducing conditions, radioactivity of gp80 was measured.

^c Electrical resistances across the cell monolayers were measured between electrodes at the apical and basal sides. The data are means $\pm$ standard deviations ($n = 5$).

^d $50 \mu\text{M}$.

^e WT, wild type.

FIG. 6. Correlation of the expression level of the M protein in cells with disruption of the microtubules and budding site of wild-type (WT) virus. MDCK cell lines transfected with the wild-type M gene (WT M 07) (○) or F1-R M gene (F1-R M 08) (●) were cultured in the presence of various concentrations of ZnCl_2 . M protein was labeled with [^{35}S]methionine, separated by radioimmunoprecipitation and SDS-PAGE, and quantified by a Bio-Imaging analyzer. Disruption of the microtubules was quantified as described in the legend for Fig. 5. The filter membrane-grown cells were infected with wild-type virus at a multiplicity of infection of 5 PFU per cell and incubated for 14 h at 37°C. Virus yield in the apical or basolateral medium was determined by plaque assay.

nocodazole treatment. Electrical resistances across the monolayers were not significantly different among the cell lines, suggesting that tight junctions between cells were not destroyed.

Disruption of microtubules in mutant M-protein-expressing cells. The foregoing study indicated that expression of F1-R M in MDCK cells resulted in an impairment of cellular polarity. A similar effect is seen in infections with the F1-R mutant virus

which has been shown to cause depolymerization of host cell microtubules into soluble tubulin (43). Therefore, microtubule disruption was examined in cell lines expressing either wild-type M or F1-R M protein, and the results were compared with those for nocodazole-treated cells. To quantify the extent of microtubule disruption, extractable and nonextractable microtubules were analyzed by quantitative immunoblotting as described in Materials and Methods. Results presented in Fig. 5 show that control MDCK cells contained predominantly non-extractable tubulin polymers, i.e., microtubules, whereas a significant percentage of the microtubules were depolymerized into soluble tubulin by nocodazole treatment. In cell lines expressing wild-type M, most of the microtubules remained insoluble, supporting the results described above. In contrast, when F1-R M protein was expressed, some of the microtubules were converted into extractable forms. This result is consistent with that of the morphological study, and suggests that the mutant M protein is involved in disrupting the microtubules. The extent of microtubule disruption was correlated with the expression levels of the F1-R M protein (Fig. 6). It should be noted that expression of a large amount of the wild-type M protein also caused the disruption to some extent. Zn^{2+} ions in the culture medium affected neither the integrity of the microtubules nor the cellular polarity of control cells (data not shown).

To ascertain whether the mutated F1-R M protein interacts directly with the microtubule network, binding of the M proteins to the microtubules was examined. Immunofluorescence analysis of cell lines expressing wild-type M or F1-R M protein revealed that both M proteins were distributed diffusely in the cytoplasm whereas the microtubules appeared as a network as described above. Although both M proteins were removed from the cells by the tubulin extraction procedure, immunoprecipitation and immunoblotting of the cell lysates using antibodies against M or against α - and β -tubulin failed to detect any specific binding between the M proteins and tubulin (data not shown). These results suggest that the F1-R M protein depolymerizes the microtubule network through indirect interactions with another host protein(s), although a possible direct interaction between M and tubulin that may have been disrupted under the conditions used for tubulin extraction and immunoprecipitation cannot be ruled out.

Altered budding site of Sendai virus in F1-R M-protein-expressing cells. To test whether apical budding by wild-type

TABLE 2. Virus yield at the apical and basolateral domains of MDCK cells^a

Cell	Wild-type virus (PFU/10 ⁵ cells)		Apical/ basolateral ratio	F1-R (PFU/10 ⁵ cells)		Apical/ basolateral ratio
	Apical domain	Basolateral domain		Apical domain	Basolateral domain	
Control	6.3×10^7	8.2×10^4	768.3	9.6×10^6	7.3×10^5	13.2
Nocodazole treated ^b	7.8×10^5	8.7×10^4	9.0	4.8×10^5	8.6×10^4	5.6
WT ^c M 03	3.9×10^5	3.1×10^3	125.8	6.9×10^5	5.0×10^4	13.8
WT M 06	4.2×10^6	1.7×10^4	247.1	4.7×10^5	3.2×10^4	14.7
WT M 07	6.1×10^6	3.6×10^4	169.4	1.9×10^6	2.1×10^5	9.0
WT M 12	7.9×10^5	8.2×10^3	96.3	6.5×10^5	4.9×10^4	13.9
F1-R M 04	8.9×10^4	5.7×10^3	15.6	2.7×10^4	3.1×10^3	8.7
F1-R M 05	4.6×10^5	3.1×10^4	14.8	7.5×10^4	8.9×10^3	8.4
F1-R M 08	5.6×10^5	2.4×10^4	23.3	3.2×10^5	4.3×10^4	7.4
F1-R M 15	8.6×10^5	4.5×10^4	19.1	6.4×10^4	5.2×10^3	12.3

^a Monolayer cells grown on membrane filters in the presence of 1 μM ZnCl_2 were infected with wild-type or F1-R virus at a multiplicity of infection of 5 PFU per cell and were incubated at 37°C in DME containing 1 μM ZnCl_2 . After 14 h, the portions of the culture medium at the apical and basal sides were assayed for virus yield by plaque assay.

^b 50 μM .

^c WT, wild type.

FIG. 7. Relative distribution of wild-type F (WT F) and F1-R F proteins in MDCK cells expressing M protein. Monolayer cells subcultured on membrane filters were transfected with the F gene of wild-type virus or of the F1-R mutant and incubated with DME in the presence of 4 μ M ZnCl₂. After 24 h, cells were pulse-labeled with [³⁵S]methionine (740 kBq/ml) for 30 min in the presence of Zn²⁺ ions and chased for 1 h with cold DME. Immunoprecipitation with MAbs to F was done differentially for the apical and basolateral cell surfaces and whole cells. After SDS-PAGE, the radioactivity of respective bands was measured to determine the relative distribution of the F protein in cells. The amount of F in the cytoplasm was calculated by subtracting those of the surface membrane domains from that of whole cells. Open bars, apical surface; solid bars, basolateral surface; hatched bars, cytoplasm. CONT, control cells without the M genes; NCZ, control cells treated with 50 μ M nocodazole for 24 h; WT M 07, a cell line expressing wild-type M protein; F1-R M 08, a cell line expressing F1-R M protein. The data are means with standard errors for two or three experiments.

virus in MDCK cells depends upon intact microtubules and whether the impairment of the cellular polarity induced by the mutant M protein is responsible for the bipolar budding of F1-R, cell lines expressing wild-type M or F1-R M were infected with wild-type and F1-R viruses. The budding domains were determined as described in Materials and Methods. The results are summarized in Table 2 and Fig. 6. As reported previously (30, 45), wild-type virus was shown to bud predominantly at the apical membrane domain of polarized control cells. When cellular polarity was destroyed by treatment with nocodazole, viral replication was reduced and apical budding of wild-type virus was significantly decreased, as was the apical to basolateral ratio of virus yield (Table 1). Although there was a decrease in total virus yield and a relative increase in basolateral budding, the predominant apical budding phenotype of wild-type virus was retained in cells expressing the wild-type M protein. On the other hand, in mutated M-protein-expressing cells, the ratio of apical to basolateral budding of wild-type virus was reduced considerably. The relative increase in the basolateral budding was correlated with the amount of F1-R M protein expressed (Fig. 6). Zn²⁺ ions in the culture medium did not affect the budding site of wild-type virus in control cells (data not shown). These results support the concept that the mutated M protein of F1-R is involved in the bipolar budding of the mutant virus. The bipolar budding phenotype of the F1-R mutant in control cells was not altered by treatment with nocodazole or in infected cell lines expressing either wild-type or F1-R M protein. The systematic reduction in virus yield in cells expressing either wild-type or F1-R M protein may be

attributed to the elevated levels of M protein causing a partial inhibition of Sendai virus replication (16).

Altered transport of the F protein in mutant M-protein-expressing cells. The F glycoprotein of the F1-R mutant has six amino acid substitutions, one in the cytoplasmic domain and five in the ectodomain (40). The Sendai virus glycoproteins, F and HN, are colocalized with the M protein before they migrate into the Golgi apparatus (34, 35). The mutated F protein might, therefore, carry a signal responsible for its bipolar transport and thus contribute to the bipolar budding of F1-R. To investigate the possible involvement of the mutant F protein, pMEP4 plasmid vectors containing the F gene of either wild-type virus or the F1-R mutant (Fig. 1) were transfected into MDCK cells for individual transient expression and into the M-protein-expressing cell lines for transient coexpression with M protein (Fig. 2). Wild-type F protein remained as an uncleaved precursor, F₀, whereas F1-R F was partially cleaved into subunits F₁ and F₂, as found in other studies (40, 43, 45).

The distribution of the F glycoproteins in the transfected cells, assayed by domain-specific immunoprecipitation, is presented in Fig. 7. In control cells without any M protein, wild-type F protein was found predominantly at the apical membrane domain. Nocodazole treatment of the cells resulted in a bipolar distribution of the F protein. In the presence of wild-type M protein, the wild-type F protein was transported primarily to the apical domain, although a small fraction was transported to the basolateral domain. In contrast, when the F protein was coexpressed with F1-R M, the wild-type F protein was shown to be distributed in a nonpolarized fashion. On the other hand, when the mutated F protein of F1-R, which was distributed bipolarly in virus-infected cells (41, 44), was expressed alone in control cells, the glycoprotein was found to be localized predominantly at the apical surface. This indicates that the F protein of F1-R, like that of wild-type virus, still carries an apical transport signal. Nocodazole treatment of the cells caused a bidirectional distribution of the protein. In the presence of wild-type M protein, apical expression of F1-R F protein was retained. Predictably, however, when F1-R M protein was coexpressed with F1-R F, the mutant F protein was again found at both the apical and basolateral domains. The same effect was observed when cells not expressing M protein were treated with nocodazole. These results indicate that bipolar transport of the mutated F protein of F1-R is not an indigenous property of the glycoprotein but that the mutated M protein of F1-R is directly responsible for the bipolar transport of wild-type and F1-R F glycoproteins.

DISCUSSION

The present study has provided evidence that the mutated M protein, and not the mutated F glycoprotein, of the pantropic mutant F1-R of Sendai virus is involved in the bipolar budding phenotype of the mutant in polarized epithelial MDCK cells. In infections in mice, altered budding has been shown to be required for F1-R progeny virus in the lungs to spread from the bronchial epithelial cells, the primary focus of infection, to subepithelial tissues and subsequently to distant organs via viremia (42, 43). It is therefore concluded that mutations in F protein resulting in increased cleavability by ubiquitous host protease(s) (39, 40, 43) and mutations in M protein that result in bipolar budding of the virus are both important determinants for the systemic infection caused by F1-R virus in mice (42, 43).

Expression of the mutant M protein had a number of distinct effects on the cytoskeleton of MDCK cells and on virus replication in the cells. These include disintegration of the micro-

tubule network, impairment of cellular polarity, nonpolarized transport of the F glycoprotein, and bipolar budding of wild-type virus as well as the F1-R mutant. Although wild-type M protein expressed at high levels showed a similar effect, it was much less. These results confirm our previous hypothesis that the mutated M protein synthesized in F1-R virus-infected cells interferes with cellular polarity, and thus with polarized virus budding, by depolymerization of microtubules (43).

There has long been a dispute over the question of whether cellular polarity depends upon microtubules and other elements of the cytoskeletal system (1, 3, 5–7, 12, 17, 23–26, 28, 33). Our findings indicate that the microtubular system is involved in the apical transport of the Sendai virus F glycoprotein in MDCK cells. Sorting of glycoproteins destined to the apical surface of MDCK cells into specific transporting vesicles occurs in the trans-Golgi network, which is associated with microtubules (15, 32, 48, 50), and transport of the vesicles to the apical plasma membrane domain may be mediated by microtubule-dependent motor proteins, for which microtubules serve as tracks (18, 52, 53). It has been postulated that sorting and transport of membrane glycoproteins is directed by indigenous signals, although the localization of the signals is a matter of controversy (for reviews, see references 4, 22, and 49). Our data show that the wild-type and mutant F proteins contain such a signal to direct their apical transport but that the transport can be altered by coexpression of the M protein of F1-R. Although the precise mechanism is not clear, it is likely that disruption of the microtubule network indirectly induced by the mutated M protein leads to missorting of the F glycoprotein, and presumably the HN glycoprotein, into basolaterally destined or default transport vesicles at the trans-Golgi network and/or to mistargeting of the apically transporting vesicles to the opposite domain (8, 54). Altered interactions of the mutant M protein with the cytoplasmic tail of the glycoproteins in the trans-Golgi network and the transport vesicles must also be considered (34, 35). Since the site of viral budding corresponds with that of viral glycoprotein expression (29, 30), the F and HN glycoproteins thus expressed at both cell surfaces should direct bipolar budding of the progeny virus.

The mechanism by which the M protein of F1-R destroys the microtubule network is still unknown. When F1-R M protein was expressed in MDCK cells, the microtubules were converted into a soluble form, similar to that induced by treatment with nocodazole. Expression of a large amount of wild-type M protein also resulted in depolymerization of the microtubules to some extent. This may suggest that F1-R M simply causes an enhancement of a marginal effect that expression of normal levels of wild-type M has on the integrity of the microtubule network. Turnover of α/β -tubulin heterodimers, which are depolymerized from and assembled into tubulin polymers (i.e., microtubules) (14, 20), occurs rapidly in a polarized fashion and plays an important role in the establishment and maintenance of cellular polarity (18, 52). Our morphological study with immunofluorescence staining against tubulin indicated that the microtubule network disappeared predominantly in the perinuclear region of F1-R M-expressing cells (data not shown) and F1-R virus-infected cells (43). The results suggest that F1-R M protein might, directly or indirectly, enhance the depolymerization or inhibit the reassembly at the negative (perinuclear) pole of the microtubules. Since M protein did not bind directly to tubulin or microtubules, disruption of the microtubule network may be indirect via an interaction of the mutated M with another host factor(s) such as microtubule-associated proteins (25). The M protein of F1-R possesses two amino acid substitutions, Asp-128 to Gly and Ile-210 to Thr

(19), and a putative conformational change (40, 42). Studies are under way to identify the signal(s) responsible for the enhanced disruption of the microtubules and to determine how the mutated M protein preferentially causes the disruption.

Finally, we would like to point out that cells expressing the mutated M protein showed an enormous increase in volume (Fig. 4D). The formation of abnormally large cells may also be attributed to an impairment of microtubules that are required for mitosis and cell division (21). Since disruption of the microtubule network by nocodazole treatment did not result in such a large increase in cell size, the mutated M protein might have another effect on cell division. It is interesting that these giant cells maintained their size for at least 2 weeks. The observation that, without further expression of the M protein, eventually these cells regained their normal size and the ability to divide indicates that they had regained their normal physiological functions.

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