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Cell Fusion by the Envelope Glycoproteins of Persistent Measles Viruses Which Caused Lethal Human Brain Disease

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Measles virus (MV) rarely induces lethal diseases of the human central nervous system characterized by reduced expression of the viral envelope proteins and by lack of viral budding. The MV envelope contains two integral membrane proteins, termed fusion (F) protein and hemagglutinin (H) protein, and a membrane-associated matrix (M) protein. Previously, analysis of MV genes from autopsy material indicated that the M protein and the F protein intracellular domain are often drastically altered by mutations. Here, we present evidence that truncation of the F protein intracellular domain does not impair fusion function, and we suggest that this alteration interferes with viral budding. Unexpectedly, certain combinations of functional F and H proteins were unable to induce syncytium formation, an observation suggesting that specific F-H protein interactions are required for cell fusion. We also found that three of four H proteins of persistent MVs are defective in intracellular transport, oligosaccharide modification, dimerization, and fusion helper function. Thus, MVs replicating in the brain at the terminal stage of infection are typically defective in M protein and in the two integral membrane proteins. Whereas the M protein appears dispensable altogether, partial preservation of F-protein function and H-protein function seems to be required, presumably to allow local cell fusion. Certain subtle alterations of the F and H proteins may be instrumental for disease development.

Measles virus (MV) is one of the primary causes of infant death in Third World countries (5), and a recent MV epidemic has caused more than 100 deaths in North American inner cities (46). On rare occasions, MV persistence induces lethal syndromes of the central nervous system known as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE) (57). SSPE, which occurs 5 to 10 years after an acute MV infection, is one of the most thoroughly studied persistent viral infections of the human central nervous system and serves as a model for the study of persistent infections by RNA viruses suspected to cause other human syndromes (26). MIBE shares many characteristics with SSPE but occurs in immunocompromised patients and has a shorter incubation time.

MVs replicating in the brains of SSPE and MIBE patients are characterized by defective expression of their envelope proteins. Infectious virus is not detectable in the brains of SSPE or MIBE victims, but occasionally cell-associated defective viruses can be isolated by cocultivation of brain cells with fibroblasts (62). Most of these cell-associated MVs are defective in expression of the matrix (M) protein, which lines the inner surface of the viral envelope (18, 61). Moreover, the M protein, as well as the two viral integral membrane proteins, termed fusion (F) protein and hemagglutinin (H) protein, often cannot be detected in brain autopsy material (2, 35). Reduced production of the viral envelope proteins in human brain infections can be ascribed partly to a steep gradient of transcription resulting in low-level expression of the distally located genes for M, F, and H proteins (9).

On the other hand, in MVs replicating in brains of SSPE and MIBE patients, numerous mutational alterations of the envelope genes have been identified (3). Characteristically, certain mutations alter drastically the entire M protein and the F protein intracellular domain (11, 48), whereas other, more-subtle mutations distinguish the extracellular domains of the F and H proteins (12). Here, we studied the expression and function of F and H proteins in cultured cells. These proteins were produced from cDNAs of MV mRNAs obtained from brain autopsy material of two SSPE patients and a MIBE patient and from a cell line derived from brain cells of another SSPE patient. We present evidence that, in one instance, aberrant processing of the F protein extracellular domain results in strong fusion inhibition. We also found that three of four H proteins of persistent MVs are significantly or completely defective in intracellular transport, oligosaccharide modification, dimerization, and fusion-helper function and that specific F-H protein interactions are required for efficient cell fusion.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were obtained from Ari Helenius (Yale University, New Haven, Conn.), and HeLa T4 cells were obtained from the American Type Culture Collection (Rockville, Md.). Both types of cells were passaged in Dulbecco's modified Eagle's medium with 5% fetal calf serum. MV Edmonston (MV strain E) was obtained from Stephen A. Udem (University of Medicine and Dentistry of New Jersey, Newark) and propagated as described elsewhere (58). Propagation and purification of the recombinant vaccinia virus-encoding T7 polymerase (vTF7-3) (16) was performed with HeLa cells essentially as described previously (29), except that the final step of sucrose gradient centrifugation was omitted.

Plasmids and expression system. Full-length cDNAs of MV genes were produced according to the method of Schmid et al. (47). Clones of MV strain E (15), from SSPE patients A and B and MIBE patient C (11), and of SSPE cell line

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IP-3-Ca (cell line I) (10) were described previously. These clones were in vector pBluescriptKS+. For expression in a T7 polymerase-driven system, the inserts were subcloned here in the *Hin*dIII site downstream of the T7 polymerase promoter of vector pBluescriptSK+. In preliminary experiments, F protein expression in HeLa cells was more efficient when a vector (peΔ5F1 [54]) retaining only 108 of the 573 nucleotides of the F protein gene 5′ untranslated region was used. Thus, the almost complete F-coding region of the other four F protein genes used in this study (from plasmids paF1, pbF11, pcF5, and pi62F1) was substituted for the strain E insert of this vector by using the *Hpa*I site situated 52 nucleotides downstream of the first F protein gene AUG and a *Sac*II site situated in the polylinker, downstream of the MV insert. The subclones were named paF, pbF, pcF, and piF. For expression of H proteins, full-length H protein genes were subcloned in plasmids named peH1-SK, paH2, pbH4-SK, pcH4-SK, and pi62H1-SK. The vaccinia virus T7 RNA polymerase system (16) was used by following the protocol established by Whitt et al. (63). Plasmid transfection in HeLa or HeLa T4 cells was performed with the cationic lipid TransfectACE (Life Sciences, Inc.), as detailed by Rose et al. (44).

Antibodies and immunofluorescence staining. Polyclonal goat antiserum raised against total MV was a kind gift of Stephen A. Udem (University of Medicine and Dentistry of New Jersey, Newark). Monoclonal antibody I29, recognizing an H protein epitope common to many different MV strains (52), was a kind gift of Erling Norrby (Karolinska Institute, Stockholm, Sweden). Polyclonal rabbit antiserum raised against a fragment of the F_1 protein expressed in *Escherichia coli* was a kind gift of Timothy C. Wong (University of Washington, Seattle). The procedures for indirect immunofluorescence staining were performed as described previously (42) with a 1:200 dilution of monoclonal antibody I29. Fluorescein-conjugated goat anti-mouse immunoglobulin G antibody (Cappel Laboratories, Cochranville, Pa.) was used in a 1:100 dilution as a secondary antibody.

Fusion assays. Fusion assays were performed initially with HeLa cells and then with HeLa T4 cells. Fusion assays performed by using the same F and H proteins with the two cell lines yielded similar results, but formation of large syncytia on HeLa cells was often inhibited by cytopathic effects and cell rounding. Vaccinia virus infection of HeLa T4 cells produced only limited cytopathic effects and cell rounding, and thus, fusion assays were more reproducible (38). Initially, different ratios of F to H plasmid DNA were tested for their fusion effects. Ratios ranging from 1:1 to 1:4 were found to induce similar fusion levels, and a 3:7 ratio was chosen for subsequent fusion assays. Confluent 3.5-cm dishes of HeLa T4 cells were cotransfected with 1.5 μg of DNA of a plasmid expressing an F protein and 3.5 μg of DNA of a plasmid expressing an H protein.

Metabolic labeling and immunoprecipitation. Labeling with [^{35}S]methionine was achieved by using Trans ^{35}S label (ICN Biomedicals, Inc.) in methionine-free, serum-free Dulbecco's modified Eagle's medium for the times indicated in the figure legends. Chases were in Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum and 2 mM methionine. Immunoprecipitation and digestion with endo-β-*N*-acetylglucosaminidase H (endo H) were performed as described previously (43). Surface immunoprecipitations were performed as described by Machamer and Rose (28) by using either monoclonal antibody I29 or anti-MV goat antiserum. Labeled proteins were resolved on polyacrylamide gels containing sodium dodecyl sulfate as described elsewhere (27) and visualized by fluorography (6). For analysis of nonreduced proteins, β-mercaptoethanol was omitted from the loading buffer. Quantification of signals on gels was done with a PhosphorImager (Molecular Dynamics).

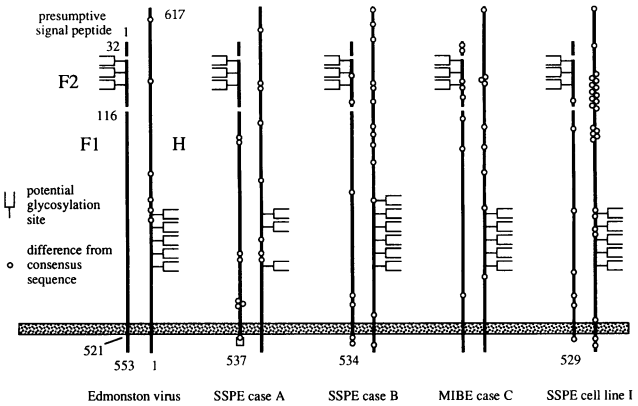

FIG. 1. Characteristics of the F and H proteins of MVs analyzed in this study deduced from the sequences of the corresponding genes. The predicted primary translation products of F and H protein genes, 553 and 617 amino acids long, respectively, are represented by vertical lines. The plasma membrane is shown as a stippled region. Amino acids differing from the F and H protein consensus sequences, defined on the basis of the sequences of six lytic and persistent viruses (12), are shown as circles. A frameshift in the intracellular domain of the F protein from patient A (case A), caused by a 1-nucleotide deletion, is indicated by a small open box. One potential glycosylation site situated in the presumptive F protein signal peptide has not been indicated. Note that the subcloning strategy used resulted in the substitution of the first 17 amino acids of the F protein signal peptide of each protein with the corresponding amino acids of strain E; in three F protein genes, the sequences were completely identical to the E strain sequence, but for the F protein from patient C two amino acids differed.

RESULTS

We have compared the F and H proteins expressed from cloned cDNAs produced from brain autopsy material of two SSPE patients (patients A and B) and a MIBE patient (patient C) and from a cell line derived from brain cells of another SSPE patient (cell line I) with the F and H proteins of the lytic strain E. The MV F protein (Fig. 1), a type I transmembrane protein with an extracellular amino terminus and an intracellular carboxyl terminus, is produced as a precursor, F_0. The signal peptide is first removed, and a subsequent cleavage yields a 20-kDa F_2 protein and a 40-kDa F_1 protein, covalently linked by one disulfide bridge (41). The H protein is a type II transmembrane protein with the opposite orientation.

Previous cDNA sequence analysis revealed several differences in the predicted sequences of the F and H proteins expressed in this study from the F and H protein consensus sequences (12) (these differences are shown by small circles in Fig. 1). Two point mutations and a 1-nucleotide deletion near the carboxyl-terminal coding region of the three SSPE F proteins result in the deletion or alteration of a majority of the 33-amino-acid F protein intracellular domain (12) (Fig. 1). Differences are also clustered around the first leucine (position 466) of the zipper structure essential for fusion (7), including a threonine-to-isoleucine change at position 464 in the F proteins from patients A and B and asparagine-to-

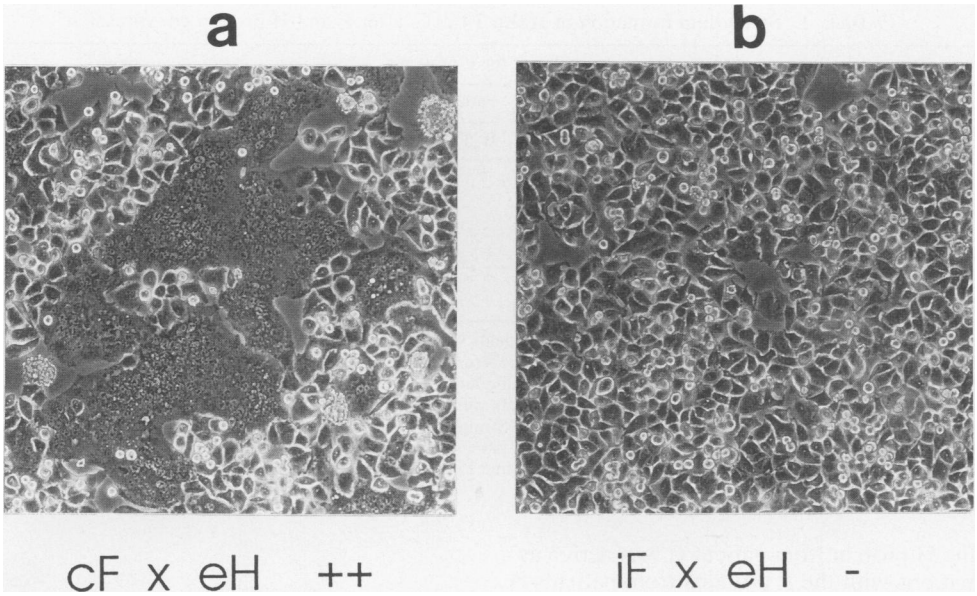

FIG. 2. Syncytium formation in HeLa T4 cells after F and H protein coexpression. HeLa T4 cells were infected with vTF7-3 and then cotransfected with the plasmids indicated. Cells were photographed 12 h after transfection. (a) Coexpression of the F protein from patient C (cF) with the H protein of strain E (eH). ++, extensive syncytium formation. (b) Coexpression of the F protein of cell line I (iF) with the same H protein (eH) as in panel a. −, no syncytium formation.

serine changes at position 465 in the F protein from patient A and at position 468 in the F proteins from patient A and cell line I. In addition, in the F protein from patient C, there is a glycine-to-valine change at position 127, within the fusogenic peptide located in the amino-terminal region of the F_1 subunit (60). As shown below, the F proteins of patients A, B, and C were functional, and thus, these differences did not have negative consequences.

Although mutations of the H protein genes of persistent MVs do not result in striking alterations of the reading frames, the H proteins accumulate more changes than the F proteins (Fig. 1) (46). The H protein of the vaccine strain E has six positions at variance with the consensus, whereas the H proteins from the four persistent strains have 8 to 25 differences from the consensus. Two of the five potential glycosylation sites are eliminated in the H protein from patient A. In the H protein from patient B, a potential glycosylation site is added. The H protein of cell line I, exhibiting strongly reduced hemadsorption activity (50, 59), shows a cluster of 16 amino acid changes due to a hypermutation event (8).

A functional test of F and H proteins: a fusion assay based on a vaccinia virus T7 RNA polymerase expression system. A vaccinia virus-based expression system has recently been used to study cell fusion induced by MV proteins, leading to the conclusion that fusion requires the expression of both MV F and MV H proteins (56, 64). We have used the vaccinia virus T7 RNA polymerase (vv-T7) expression system to study the interactions of the F and H proteins not only of the lytic strain E but also of the four persistent MVs described above.

Coexpression of certain combinations of F and H proteins in HeLa T4 cells resulted in more or less efficient cell fusion as visualized by syncytium formation (Fig. 2a), but other combinations of F and H proteins were not fusogenic (Fig. 2b). In our fusion assay, relative values were generally similar among different transfection series, but absolute values varied. For this reason, each experiment was done in quadruplicate, as presented in Table 1. The standards used to evaluate fusion efficiency are indicated in footnote *a* to Table 1; at least six fields were examined in each experiment.

The F proteins of the lytic strain E and of three of the four persistent MVs showed high fusion efficiency when tested in combination with the H protein of strain E (Table 1). The F protein of cell line I was only marginally functional in combination with the H protein of strain E (Table 1). In addition, the fusion efficiency of the five F proteins was tested in combinations with the H proteins of the four persistent MVs. Interestingly, the F protein from strain E was less fusogenic than the F proteins from patients A, B, and C when tested in combination with the H proteins from patients A, B, and C (Table 1; compare positions 2 to 4 in column 1 with the corresponding positions in columns 2 to 4).

The fusion assays presented in Table 1 can also be interpreted with respect to the H proteins. The H proteins from strain E and patient C cooperated efficiently with different F proteins to induce cell fusion. The H protein from patient B induced lower levels of cell fusion when coexpressed with three different F proteins. The H protein from patient A was weakly active when coexpressed with two F proteins, and the H protein from cell line I was completely inactive in fusion helper function. As demonstrated below, the fusion helper function of the different H proteins roughly correlated with the efficiency of their expression at the cell surface.

Homologous combinations of F and H proteins (underlined in Table 1), with the exception of that for patient A, showed higher fusion activity than most of the heterologous combinations. Unexpectedly, certain combinations of functional proteins were unable to induce efficient fusion. For example, the H protein from patient C could not cooperate with the F protein of strain E to produce cell fusion, in spite

TABLE 1. Syncytium formation in HeLa T4 cells after F and H protein coexpression[a]

H protein	Result for F protein					Fusion helper coefficient
	Strain E [0.6]	Patient			Cell line I [0.1]	
		A [1.1]	B [1.7]	C [1.2]		
Strain E	+, ++, +, ++	++, ++, ++, ++	++, ++, ++, ++	++, ++, ++, ++	−, −, (+), +	2.4
Patients						
A	−, +, (+), −	−, −, −, −	−, +, −, +	−, −, −, −	−, −, −, −	0.3
B	−, −, −, −	(+), (+), (+), (+)	+, +, +, +	−, −, (+), (+)	−, −, −, −	0.7
C	−, −, −, −	+, (+), +, +	+, +, ++, ++	+, +, ++, +	−, −, −, −	1.3
Cell line I[b]	−, −, −, −	−, −, −, −	−, −, −, −	−, −, −, −	−, −, −, −	0

[a] Data are from quadruplicate experiments. In each experiment, several fields were evaluated. Symbols for data for an average field (about 2,000 cells): ++, several syncytia with more than 10 nuclei (sometimes up to half of the nuclei were found in syncytia); +, one or two syncytia with more than 10 nuclei (and many more smaller syncytia); (+), several 4- or 5-nucleus syncytia and, occasionally, larger ones; −, two or fewer 4- or 5-nucleus syncytia (background). Homologous combinations are underlined. Fusion coefficients are given in brackets. Points were assigned as follows: −, 0; (+), 1; +, 2; ++, 3. Coefficients were calculated by adding the points and dividing the total by the number of fusion assays. Strain E is lytic; the other MVs are persistent. Patients A and B had SSPE, patient C had MIBE, and cell line I was derived from brain cells of another SSPE patient.

[b] Our failure to observe fusion helper activity with the H protein of cell line I is most likely due to the extremely reduced surface expression.

of the fact that the H protein from patient C was active as a helper in combinations with the F proteins from patients A, B, and C, and the F protein of strain E was fusogenic in combinations with the H protein of strain E and the H protein from patient A. This and other results, which cannot be explained by reduced cell surface expression of the corresponding proteins (see below), suggest that specific interactions between F and H proteins are required for efficient fusion.

The F protein of cell line I is processed aberrantly. In spite of the fact that preliminary experiments suggested that only a small fraction of the five F proteins examined reached the cell surface (data not shown), the functional analysis presented above indicated that four of the five F proteins can efficiently induce cell fusion. Only the F protein of cell line I is functionally impaired. To address the cause of this defect, we analyzed the expression of this protein, as well as the expression of the other four F proteins, by immunoprecipitation with two different antisera.

Immunoprecipitations with an antiserum recognizing preferentially the unfolded precursor, F_0, indicated that similar amounts of F_0 precursors are produced by all five plasmids (Fig. 3a, 0-h chase; the upper band corresponds to the completely glycosylated precursor, and the lower two bands correspond to forms not completely glycosylated). As expected, after a 3-h chase a large fraction of the precursor, F_0, disappeared (Fig. 3a).

An antibody recognizing preferentially the native, processed form of the F protein was used to detect the F_1 and F_2 subunits, which appeared as expected for proteins of strain E and from patients A, B, and C only after a 3-h chase (Fig. 3b). In all cases, only a fraction of the F_0 precursor was chased in the F_1 and F_2 subunits. Note also that the serum against total MV recognizes the native F protein from patient B poorly; this protein is precipitated at a level comparable to the level of the corresponding protein of strain E or that from patient C by the antiserum preferentially recognizing F_0 (Fig. 3a). The largest fraction of the F protein of cell line I, however, was detected as a broad 33- to 34-kDa band, which is 6 to 7 kDa shorter than the weak but sharp band corresponding to the F_1 protein (Fig. 3b and c, 3-h chase). Thus, we conclude that the poor functionality of the F protein of cell line I correlates with its aberrant processing.

Half of the samples immunoprecipitated with the antibody against native F protein were digested with endo H (Fig. 3c). This enzyme cleaves high-mannose oligosaccharides that are

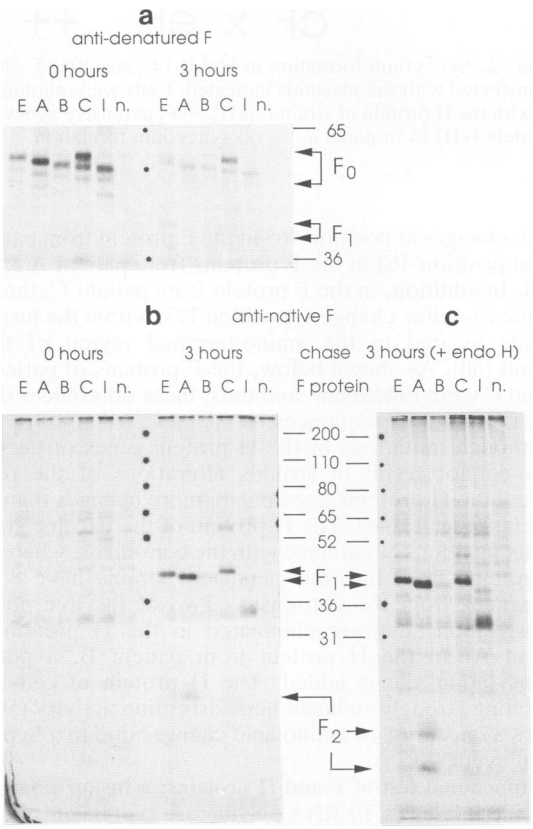

FIG. 3. Pulse-chase and endo H analyses of the expression of different F proteins in HeLa cells. Transfected HeLa cells expressing the F protein of strain E (lanes E), F proteins from SSPE patients A and B and MIBE patient C (lanes A, B, and C, respectively), and the F protein of the SSPE-derived cell line I (lane I) were labeled with [^{35}S]methionine for 1 h and then chased in medium containing unlabeled methionine for the indicated times. (a) Immunoprecipitation with antibodies against an F_1 protein fragment expressed in *E. coli* (anti-denatured F); (b and c) immunoprecipitation with antibodies against native E strain MV (anti-native F). The samples shown in panel c were treated with endo H. Molecular weights (in thousands) and the positions of the different forms of the F_0, F_1, and F_2 proteins are indicated. The positions of the molecular weight markers are indicated by dots. n., negative controls, in which no plasmid was used for transfection.

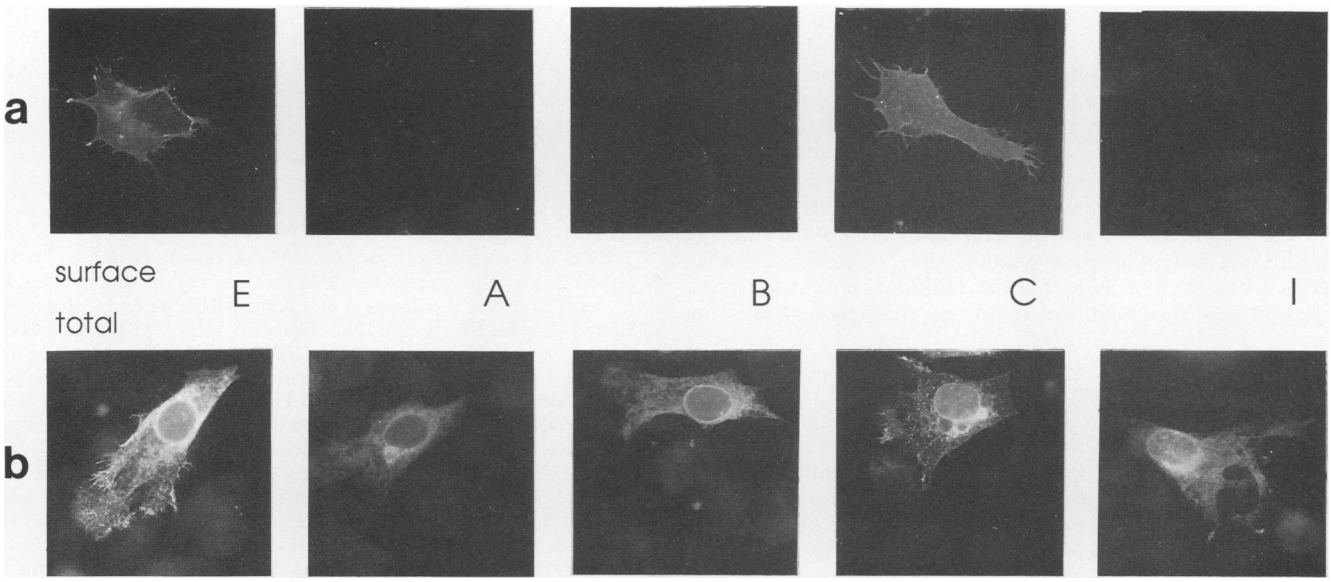

FIG. 4. Immunofluorescence analysis of H protein expression in HeLa cells. Transfected HeLa cells expressing the H protein of strain E (panels E), H proteins from SSPE patients A and B and MIBE patient C (panels A, B, and C, respectively), the H protein of the SSPE-derived cell line I (panels I), and a negative control (not shown) were stained with monoclonal antibody I29, recognizing an H protein epitope common to many different MV strains. Five hours after transfection, cells were fixed with 3% paraformaldehyde. (a) Analysis of surface H protein expression in intact cells; (b) analysis of total H protein expression in cells permeabilized with 1% Nonidet P-40.

added in the endoplasmic reticulum, but it does not cleave oligosaccharides which have been modified by the Golgi enzymes *N*-acetylglucosaminyltransferase and mannosidase II (25). Since *N*-acetylglucosaminyltransferase is located in the medial Golgi compartment, the acquisition of endo H resistance by glycoproteins marks their arrival at the medial Golgi compartment (14). As expected, when the F proteins were treated with endo H, a reduction in molecular weight was observed for the F_2 but not for the F_1 subunit, which is not glycosylated (51). In the three cases in which there was enough material to detect the F_2 subunit, equivalent amounts of two bands migrating 3 and 5 kDa faster than the untreated F_2 subunit were detected (Fig. 3c, lanes E, A, and C). This result indicates that after a 3-h chase about half of the F_2 molecules acquired processed glycans and, thus, had reached the medial Golgi or a subsequent compartment.

The three SSPE H proteins are defective in intracellular transport, oligosaccharide modification, and dimerization. To determine whether H proteins are expressed and transported to the cell surface, we analyzed their intracellular distribution in intact HeLa cells by immunofluorescence (Fig. 4a). Clear cell surface staining was detected for the H protein of strain E and the H protein from patient C, but for the H proteins from patients A and B and cell line I, surface staining was below the detection level (Fig. 4a).

To confirm that the H proteins from patients A and B and cell line I were expressed, immunofluorescence analysis was performed on permeabilized cells. Indeed, for the H proteins from patient A and cell line I, internal staining was clearly visible, but surface staining was again below the detection level (Fig. 4b). In contrast, for the H protein of strain E and the H protein from patient C, which showed strong internal staining, superimposed weak surface staining could be detected. The H protein from patient B was intermediate; whereas internal staining was also strong, the edges of the cells were less evident.

To further investigate the intracellular transport of the different H proteins, we studied their glycosylation and oligomerization. A pulse-chase labeling experiment was carried out with transfected HeLa cells as shown in Fig. 5. Half of the samples were analyzed directly after immunoprecipitation (Fig. 5a), whereas the other half of the samples were digested with endo H (Fig. 5b). Immediately after pulse labeling, a single, approximately 80-kDa form was detected for all five H proteins expressed (Fig. 5a, lanes 1 to 5). Strain E protein and the protein from patient C were always

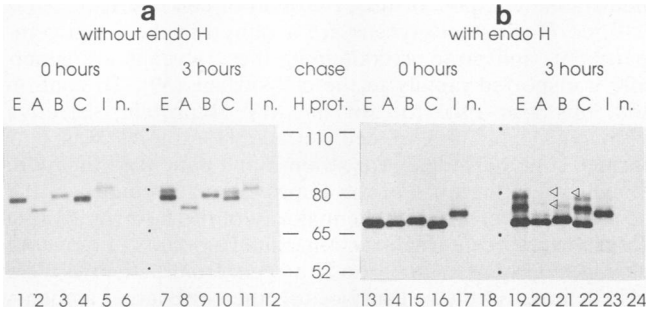

FIG. 5. Pulse-chase and endo H analysis of the expression of different H proteins in HeLa cells. Transfected HeLa cells expressing the H protein of strain E (lanes E), H proteins from SSPE patients A and B and MIBE patient C (lanes A, B, and C, respectively), and the H protein of the SSPE-derived cell line I (lanes I) were labeled with [^{35}S]methionine for 30 min and then chased in medium containing unlabeled methionine for the times indicated. Small amounts of endo H-resistant material from patient A are highlighted by arrowheads. The molecular weight markers are indicated by dots in the center lane of each panel, and their sizes (in thousands) are shown between the panels. n., negative controls, in which no plasmid was used for transfection. See Fig. 8 for a pulse-chase analysis in which a larger fraction of the H protein from patient A has received the processed glycans. Exposure times, 6 and 24 h for panels a and b, respectively.

expressed at the highest levels. When these proteins were treated with endo H, a 10-kDa reduction in size was observed for each H protein except that from patient A, for which a 5-kDa size reduction was observed (compare Fig. 5a, lanes 1 to 5, with Fig. 5b, lanes 13 to 17). This result indicates that at least one of the two potential glycosylation sites deleted in the H protein from patient A is used (in fact, both are used; see below). On the other hand, the reduction in the size of the H protein from patient B, which has an additional potential glycosylation site, was also about 10 kDa. Thus, we conclude that the additional potential glycosylation site present in the H protein from patient B is not used.

After a 3-h chase, two different forms of H protein each were detected for strain E and patient C (Fig. 5a, lanes 7 and 10). The lower band migrated slightly faster than the single band detected immediately after the pulse (Fig. 5a, lanes 1 and 4), whereas the upper band was more diffuse and its electrophoretic migration was slightly slower. A longer exposure of this gel indicated the presence of a weak upper band for the H proteins from patients A and B also. These new forms presumably resulted from oligosaccharide processing. To determine whether the proteins contained processed glycans, we digested the 3-h samples with endo H. For the H protein of strain E and the H protein from patient C, glycans were completely removed from about two-thirds of the proteins while the remainder contained endo H-resistant glycans (Fig. 5b, two upper bands in lanes 19 and 22). Small amounts of endo H-resistant material were detected also for the H protein from patient B (Fig. 5b, lane 21); even smaller amounts of endo H-resistant material detected for the H protein from patient A are highlighted by arrowheads (Fig. 5b, lane 20). The presence of 2 or 3 bands containing resistant glycans indicates heterogeneous processing of oligosaccharides. No processing of glycans on the H protein of cell line I was detected (Fig. 5b, lane 23). These results are consistent with what we observed by immunofluorescence microscopy and indicate relatively efficient transport of the H protein of strain E and the H protein from patient C, poor transport of the H proteins from patients A and B, and no detectable transport of the H protein of cell line I.

Endo H-resistant glycans are acquired in the Golgi compartment, and proteins containing these glycans are generally transported rapidly to the cell surface (39). To confirm that this was also true for the MV H protein, selective immunoprecipitation of cell surface H proteins was performed (Fig. 6). Indeed, for strain E and patient C, the more slowly migrating form of the H protein was detected in the surface fraction whereas the majority of the faster-migrating form was in the internal fraction. Small amounts of the more slowly migrating form of the H protein from patient B were also detected at the cell surface together with larger amounts of the faster-migrating form. We believe that the faster-migrating H protein is a contaminant of the surface fraction and that this contamination is caused by lysis of some cells before or during the first incubation or by antibody exchange. With a longer exposure of this gel, a weak cell surface signal was also detected for the H protein from patient A, whereas cell surface expression of the H protein of cell line I was always below the detection level. Note that low levels of surface H proteins detected with this assay (for example, H proteins from patients A and B) reflect not only inefficient cell surface expression but also limitations in the efficiency of our immunoprecipitation assay. In summary, the H protein of strain E and the H protein from patient C, which contain processed glycans, were readily detected at the cell surface. The H protein of cell line I, devoid of processed glycans, was not detected at the cell surface. The H proteins from patients A and B showed intermediate levels of processed glycans and reduced but detectable cell surface expression.

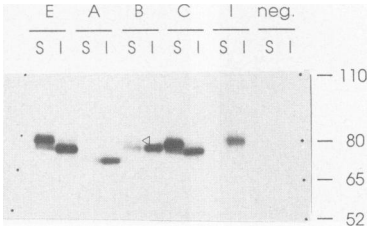

FIG. 6. Cell surface expression of different H proteins. Transfected HeLa cells expressing the H protein of strain E (lanes E), H proteins from SSPE patients A and B and MIBE patient C (lanes A, B, and C, respectively), and the H protein of the SSPE-derived cell line I (lanes I ["I" in top row]) were labeled with [^{35}S]methionine for 1 h and then chased in medium containing unlabeled methionine for 3 h. Cells were then detached from the plate with EDTA and incubated with specific antibodies at 0°C to limit endocytosis. After extensive washing to remove unbound antibodies, cells were lysed and the antibody-antigen complexes were collected as surface (S) material. The supernatant was then reprecipitated with antibodies, and the antibody-antigen complexes were collected as internal (I) material. The positions of the molecular weight markers are indicated by dots, and their sizes (in thousands) are shown on the right. neg., negative controls, in which no plasmid was used for transfection.

Oligomerization is an obligatory step in the intracellular maturation of the H proteins of MV and related viruses. Initially, disulfide-linked dimers and, then, tetramers and other noncovalently associated oligomers are formed (17, 34, 40). To compare the dimerization of the five H proteins, we analyzed a fraction of the samples used for the gel shown in Fig. 5a on another gel in the absence of a reducing agent in the sample buffer (Fig. 7). About one-third each of the H protein from strain E and the H protein from patient C was detected as a dimer immediately after the pulse (0 h). After a 3-h chase, the large majority of the H protein from the E strain and of the H protein from patient C was in the dimeric form. The same was true for about half of the H protein from patient B and for some of the H protein from patient A, but no material from cell line I was in the dimeric form. Thus, the levels of H-protein dimer formation correlated with the level of glycan processing and surface expression.

H protein expression: interference experiments. Defective MVs have been shown to interfere with lytic MVs in mixed infections (19, 23). To investigate whether the expression of defective MV H proteins can interfere with the expression of functional MV H proteins, we coexpressed the H protein from patient A and the H protein of strain E. The forms of these proteins without processed glycans have different electrophoretic mobilities and, thus, can be separated. Figure 8 shows an experiment in which the H protein of strain E and the H protein from patient A expressed alone or coexpressed at the amounts of plasmid DNAs indicated above the gel were labeled and then immunoprecipitated after either no chase (Fig. 8, lanes 1 to 6) or a 3-h chase (Fig. 8, lanes 7 to 12). No clear interference effect was observed; the H protein from patient A did not retain the H protein of strain E in the endoplasmic reticulum, as judged by the acquisition of processed glycans, and the H protein of strain E did not promote more-efficient transport of the H protein

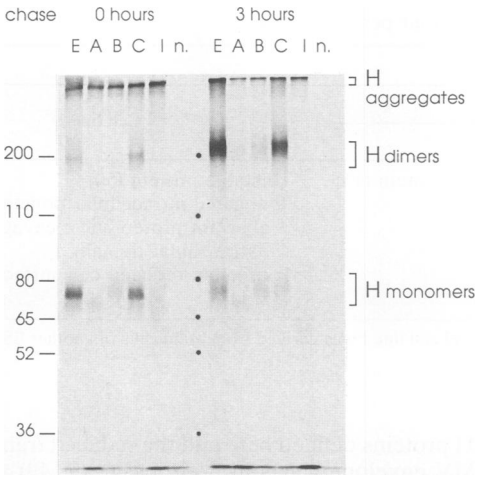

FIG. 7. Dimerization of different H proteins in HeLa cells. Transfected HeLa cells expressing the H protein of strain E (lanes E), H proteins from SSPE patients A and B and MIBE patient C (lanes A, B, and C, respectively), and the H protein of the SSPE-derived cell line I (lanes I) were labeled with [^{35}S]methionine for 30 min and then chased in medium containing unlabeled methionine for the times indicated. Samples were separated on a nonreducing gel. The positions of the H protein monomers, dimers, and aggregates are shown on the right. The molecular weight markers are indicated by dots in the center lane, and their sizes (in thousands) are indicated on the left. n., negative controls, in which no plasmid was used for transfection.

from patient A. The fact that the H protein from patient A failed to retain the H protein of strain E in the endoplasmic reticulum is probably related to its inability to dimerize efficiently (22, 45).

Four of the five potential glycosylation sites of the H protein are used. To determine how many of the five potential glycosylation sites on the H protein of strain E and of the three sites on the H protein from patient A are used, we performed a partial endo H digest. The time of digestion was varied so that the number of partially digested bands could be counted. Figure 9 shows the results of this analysis,

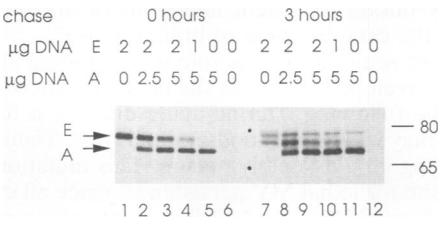

FIG. 8. The H protein from patient A does not retain the H protein of strain E in the endoplasmic reticulum. Proteins from transfected HeLa cells labeled with [^{35}S]methionine for 1 h were immunoprecipitated after either no chase (lanes 1 to 6) or a 3-h chase (lanes 7 to 12) in medium containing unlabeled methionine. The H protein of strain E and the H protein from patient A were expressed alone (lanes 1, 5, 7, and 11) or coexpressed at the amounts of plasmid DNAs indicated at the top (lanes 2 to 4 and 8 to 10). The H protein of strain E and the H protein from patient A without complex sugars migrate at different positions (lanes 1 and 5), as indicated on the left, but the proteins with processed glycans are almost exactly superimposed (lanes 7 and 11).

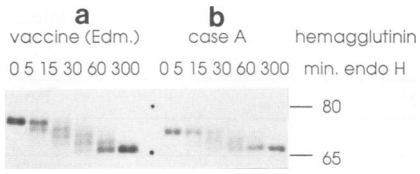

FIG. 9. Four of the five potential glycosylation sites of the H protein are used. Proteins from transfected HeLa cells labeled with [^{35}S]methionine for 1 h were immunoprecipitated and digested with endo H. Partial endo H digestions of the H protein of strain E (a) and of the H protein from patient A (case A) (b) are shown. The positions of the 80,000- and 65,000-molecular-weight markers are indicated by dots in the center lane. The length (in minutes) of the endo H treatment for each sample is indicated above each lane. Four bands in addition to the completely deglycosylated protein band can be detected for the H protein of strain E, and two bands can be detected for the H protein from patient A. Edm., Edmonston strain (strain E).

indicating that four bands in addition to the completely deglycosylated protein are detected for strain E (Fig. 9a) and that two bands are detected for the H protein from patient A (Fig. 9b). We conclude that four sites in the H protein of strain E and two sites in the H protein from patient A are used. Moreover, the analysis presented in Fig. 5 suggests that either one or two of the primary glycans are processed when the H proteins transit through the Golgi complex.

DISCUSSION

Intracellular transport of MV envelope proteins. To study the F and H proteins of persistent MVs, we used the vv-T7 system and HeLa cells (16). As a standard, we expressed the vesicular stomatitis virus G protein and found, as expected (63), that 80 to 90% of the G protein contained processed glycans after a 1-h chase (data not shown). The rate of acquisition of processed glycans by the MV E strain H protein was considerably slower. In different experiments, 20 to 60% of the H protein received processed glycans after a 3-h chase. In lytic MV E strain infections of HeLa cells, 40 to 80% of the H protein had acquired processed glycans after a 3-h chase (7a), a rate similar to the one reported for the same system by Kohama et al. (24) but slower than the rate recently measured by Ogura et al. (36). Thus, glycan processing in the vv-T7 system is slightly slower than in a lytic MV infection. In a finding similar to that for the H protein of strain E, 30 to 60% of the H protein from patient C acquired processed glycans after a 3-h chase. In contrast, after the same chase period, only 5 to 15% of the H protein from patient B, 2 to 10% of the H protein from patient A, and less than 1% of the H protein of cell line I acquired processed glycans.

It is noteworthy that the H protein from patient A, in which two of the four glycosylation sites used have been eliminated by mutation, is not dimerized efficiently and is poorly transported to the cell surface. One specific carbohydrate chain in the hemagglutinin neuraminidase of the other paramyxovirus, simian virus 5, plays a major role in promoting correct folding, and another chain, not necessary in the initial folding, plays a role in the aggregation of oligomers (33). It is likely that individual carbohydrate chains of the MV H protein have similar functions.

SSPE is typically characterized by alterations in the short F protein cytoplasmic domain of the persistent MV (48). It has been observed previously that alterations of the cyto-

TABLE 2. Defects in the envelope proteins of four persistent MVs

Protein	Protein defect[a]			
	Patient			Cell line I
	A	B	C	
M	None	Hypermutated protein (1)	Hypermutated protein (11)	Unstable protein (50)
F	Truncated intracellular domain (12)	Truncated intracellular domain (12)	None	Truncated intracellular domain (12) and aberrant proteolytic cleavage of the extracellular domain
H	Strongly reduced cell surface expression	Reduced cell surface expression	None	Extremely reduced cell surface expression

[a] References are given in parentheses. Patients A and B had SSPE, patient C had MIBE, and cell line I was derived from brain cells of another SSPE patient.

plasmic domain of another type I integral membrane protein capable of inducing fusion, the vesicular stomatitis virus G protein, bring about two effects: the transport rate and cell surface expression of G protein are reduced (43), and proteins are not incorporated efficiently into virus particles (63). On the other hand, human immunodeficiency virus types 1 and 2 carrying truncated transmembrane proteins retain infectivity and cytopathogenicity, at least in selected cell lines (32, 53, 65). MV F proteins with a truncated intracellular domain appear as efficient in fusion as proteins with a complete intracellular domain, suggesting that truncated F proteins may be transported to the cell surface as efficiently as F proteins with an intact intracellular domain, a situation more similar to the one observed with the human immunodeficiency virus type 1 and 2 envelope proteins than to that with the vesicular stomatitis virus envelope protein. We suggest that truncation of part of the F protein intracellular domain, which is highly conserved among all morbilliviruses (48), may reduce functional interactions with the M protein or nucleocapsid and, thus, impede efficient viral assembly and budding.

Specific interactions of F and H proteins are required for efficient fusion of HeLa cells. The roles of the F and H proteins of different paramyxoviruses in the cell fusion process have recently been studied intensively (20, 21, 30, 31). The F proteins of different viruses exhibit various degrees of fusion activity independently of H proteins in different expression systems, but the formation of syncytia can generally be enhanced by the coexpression of the homologous H proteins (20). In the vv-T7 system and HeLa or HeLa T4 cells, fusion by the MV F protein was completely dependent on the coexpression of H protein. Unexpectedly, we observed that not every combination of functional F and H proteins induced efficient fusion. Our data extend the observations of Hu et al. (21) that only homologous pairs of F and H proteins of different viruses are able to trigger cell fusion. In view of the significant homology of the F and H proteins of different MV strains (amino acid identity, 97 to 99%), our data suggest very specific cooperative interactions during fusion. It should be possible to define the individual amino acids influencing cell fusion efficiency by constructing hybrid proteins.

The fusion tests presented here, carried out with an efficient transient expression system in HeLa T4 cells, cannot be directly extrapolated to the fusion function of the same F and H proteins in resting brain cells. In neurons, certain viral surface glycoproteins are transported to specific cellular compartments (13), and it is conceivable that the MV envelope proteins are present locally at concentrations sufficient to induce cell fusion, even when the reduced transport rate of H proteins defined here and the reduced transcription of the MV envelope genes in brain tissue (9, 49) are taken into account. To obtain more information on the pathogenic significance of the functional defects defined in this study, it will be necessary to use a system approximating a persistent infection of human brain tissue.

Defective MV envelope proteins and lethal human diseases. Before the role of defective MV envelope proteins in disease is discussed, several facts should be recalled. First, we cannot be certain that the selected F and H cDNAs are representative of the mRNA population of a given brain or cell line, because MV genomes replicating in persistent infections are moderately heterogeneous (3, 48, 55, 66). For the rest of this discussion, however, we will assume that the randomly chosen single cDNAs reflect the properties of the majority of the viral mRNAs from the same source. Second, it should be stressed that SSPE (patients A and B) is distinct from MIBE (patient C) in two aspects: it has a longer incubation time and a stronger immune response, which has to be evaded by the spreading MVs (4, 37). Third, since MV mutations do occur during propagation of persistently infected cell lines (66), not all defects detected in the SSPE-derived cell line I are necessarily disease related.

Although it might not be statistically relevant, we noted a correlation between the length of the MV persistent infection and the number of detected defects in the envelope proteins. Table 2 shows that one defect was identified for the MIBE patient, two and three defects, respectively, were identified for SSPE patients A and B, and four defects were identified for the SSPE cell line I. Are these defects accumulating fortuitously during persistent infections or are they instrumental for the establishment of brain disease?

A plausible scenario for the role of mutational alterations of the MV envelope proteins in the development of SSPE or MIBE is the following. During acute disease, a fully functional MV may cross the blood-brain barrier. Then, a mutation impairing viral assembly occurs. This mutation may be a prerequisite for lethal MV persistence, since all characterized MVs from SSPE and MIBE patients show mutations throughout the entire M protein, the cytoplasmic domain of the F protein, or both. It appears likely that additional, more-subtle mutations, like those in the extracellular domains of the F and H proteins, are necessary to allow a defective virus not only to persist in one cell or in a localized brain area but to spread throughout the entire brain, overgrowing the wild-type virus (19, 67).

Support for the hypothesis that mutations are involved in disease development comes from the fact that MV genomes found in diseased brains are of clonal origin and contain mutations not compatible with lytic replication. For exam-

ple, a hypermutation event changing 132 of 266 U residues in the M protein gene was identified for all MV genomes from the brain of MIBE patient C analyzed (11). Moreover, recently, in the M protein gene of patient B, sequentially selected hypermutation events have been characterized (1). In summary, during persistent infection, many mutations which are neutral or which favor viral propagation only slightly may occur in all viral genes. However, one or a few mutations, causing defective viral assembly and possibly reduced cell surface expression of the envelope proteins, seem to be instrumental in the establishment of lethal MV persistence.

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