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Functional Analysis of N-Linked Glycosylation Mutants of the Measles Virus Fusion Protein Synthesized by Recombinant Vaccinia Virus Vectors

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The role of N-linked glycosylation in the biological activity of the measles virus (MV) fusion (F) protein was analyzed by expressing glycosylation mutants with recombinant vaccinia virus vectors. There are three potential N-linked glycosylation sites located on the F_2 subunit polypeptide of MV F, at asparagine residues 29, 61, and 67. Each of the three potential glycosylation sites was mutated separately as well as in combination with the other sites. Expression of mutant proteins in mammalian cells showed that all three sites are used for the addition of N-linked oligosaccharides. Cell surface expression of mutant proteins was reduced by 50% relative to the wild-type level when glycosylation at either Asn-29 or Asn-61 was abolished. Despite the similar levels of cell surface expression, the Asn-29 and Asn-61 mutant proteins had different biological activities. While the Asn-61 mutant was capable of inducing syncytium formation, the Asn-29 mutant protein did not exhibit any significant cell fusion activity. Inactivation of the Asn-67 glycosylation site also reduced cell surface transport of mutant protein but had little effect on its ability to cause cell fusion. However, when the Asn-67 mutation was combined with mutations at either of the other two sites, cleavage-dependent activation, cell surface expression, and cell fusion activity were completely abolished. Our data show that the loss of N-linked oligosaccharides markedly impaired the proteolytic cleavage, stability, and biological activity of the MV F protein. The oligosaccharide side chains in MV F are thus essential for optimum conformation of the extracellular F_2 subunit that is presumed to bind cellular membranes.

Measles virus (MV) is an enveloped RNA virus which contains a linear single-stranded RNA genome of negative polarity. The MV particle contains two integral surface glycoproteins, the hemagglutinin (HA) and fusion (F) proteins, in addition to a nonglycosylated matrix (M) protein that forms the inner layer of the envelope. The MV ribonucleoprotein core contains the genomic RNA and three associated proteins: the nucleocapsid (NP), a phosphoprotein (P), and a presumed RNA polymerase large protein (L) (25).

The predicted primary structure of the MV F protein is composed of 550 amino acids with a calculated mass of 59,510 Da (32). The MV F protein is required for virus penetration into the host cell (6). This is accomplished through a process of membrane fusion between the virus envelope and the host cell membrane. When the full-length F protein cDNA was inserted into the DNA genome of human adenovirus type 5, high levels of biologically active MV F protein expression were reported (3). Other virus systems have also been used to direct the synthesis of biologically active F protein in insect and mammalian cells (40, 43).

In MV-infected cells, the F protein is synthesized as an inactive precursor, F_0, which is subsequently cleaved by host proteolytic enzymes to generate a nonglycosylated F_1 and a glycosylated F_2 polypeptide subunit linked together by a disulfide bond (12, 35). Cleavage of the F_0 precursor generates a new hydrophobic amino terminus on the F_1 polypeptide. F_1 contains the carboxy-terminal region of the precursor and is anchored in the viral membrane, while the F_2 subunit contains the original amino terminus minus the signal peptide (35). The amino-terminal hydrophobic region of F_1 is highly conserved among paramyxoviruses and has considerable homology with the human immunodeficiency virus type 1 (HIV-1) gp41 amino terminus (5). This region of the F_1 polypeptide is believed to mediate the membrane fusion activity of paramyxoviruses (36). Previous work with the paramyxovirus simian virus 5 F protein has indicated that the hydrophobic amino terminus of F_1 is capable of interacting directly with cellular membranes (29).

Glycosylation of viral membrane proteins has been shown to influence stability and intracellular transport (reviewed in reference 9). Inhibition of N-linked glycosylation by using tunicamycin has been shown to retard the transport of many but not all glycoproteins (26). There have been many indications that carbohydrate side chains may function to protect against proteolytic degradation, promote correct folding, and improve the solubility of glycoproteins (26). Evidence for a role for glycosylation in proteolytic cleavage of viral precursor glycoproteins has been reported. Inhibition of N-linked glycosylation inhibited the processing of Sindbis virus envelope protein E2 (20) and the Newcastle Disease virus F glycoprotein (23), suggesting a carbohydrate requirement for specific proteolytic cleavage. Others have found that site-specific glycosylation can influence cleavage of the CK/Penn strain of avian influenza virus hemagglutinin (7). Studies on the HIV-1 enve-

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TABLE 1. Oligonucleotides used to generate MV F N-linked glycosylation mutants

Oligonucleotide[a]	Sequence[b] (5′→3′)	Resulting mutation(s)
G1	CAT TGG GGC **CAG** CTG TCT AAG ATA	Asn-29 to Gln
G2	TTA ATG CCC **CAG** CTG ACT CTC CTC	Asn-61 to Gln
G3		Asn-67 to Gln
RW441	ATGGGTCTCAAGGTGAAC	
RW442	ACCCTCGTGCACTGATTGAGGAGAGTTATATTGGGC	
RW443	TCTCCTCAAT**CAG**TGCACGAGGGTAGAGATTGC	
RW444	GCTCGCTCTCAGATTGTC	
G23	TTA ATG CCC **CAG** CTG ACT CTC CTC AAT **CAG** TGC ACG AGG	Asn-61 to Gln, Asn-67 to Gln

[a] The G3 mutant was generated by using the four PCR primers indicated.
[b] Mutated bases are underlined.

lope glycoprotein gp160 have shown that its cleavage into a biologically active protein is dependent on the addition of N-linked oligosaccharides (8).

Although much is known about the biological activities of paramyxovirus F proteins, the structural features involved in cleavage activation and/or membrane fusion activity are still not well understood. The importance of N-linked glycosylation in the fusion activity of the MV F protein has been suggested previously (3). Proteolytic cleavage of the F_0 precursor protein has been shown to be completely abolished in the presence of tunicamycin. Recent studies on the HIV-1 envelope protein indicated that the switch from a non-syncytium-inducing to a syncytium-inducing phenotype was associated with the length and number of potential N-linked glycosylation sites in the V2 hypervariable loop (13).

We have now examined the role of glycosylation in the processing, intracellular transport, and fusion activity of the MV F protein. Our results show that all three potential glycosylation sites are utilized. Elimination of all three sites resulted in an unstable, biologically inactive protein which was not transported to the cell surface. Inactivation of individual sites reduced cleavage and cell surface expression and altered fusion activity. The severity of the defect depended on both the number and the positions of the mutated glycosylation sites.

In addition, we provide evidence that MV F protein expressed by vaccinia virus recombinants was capable of inducing syncytium formation and that the HA glycoprotein enhanced this biological activity.

MATERIALS AND METHODS

Cell cultures and viruses. The NCI-H460 (human lung large cell carcinoma), CV1, and Vero cell lines were obtained from the American Type Culture Collection. NCI-H460 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Hyclone Laboratories) at 37°C in the presence of 5% CO_2. MV (Edmonston strain) was obtained from the laboratory of Erling Norrby (Karolinska Institute, Stockholm, Sweden). MV stocks were prepared by infecting confluent monolayers of Vero cells (grown in Dulbecco's modified Eagle's medium [DMEM] with 10% FBS) at a multiplicity of infection (MOI) of 0.001 PFU/cell. Virus-containing supernatants were harvested after 72 to 96 h of incubation at 37°C, when more than 90% of the cells were involved in cell fusion.

Oligonucleotide-directed mutagenesis and construction of recombinant vaccinia viruses. Recombinant plasmids containing various modifications of the MV F protein were constructed by standard cloning techniques as reported previously (2, 3). Plasmid pJV-NheI, which contains the full-length coding region of the wild-type MV F protein, has been described previously (43). The *Nhe*I-*Bam*HI DNA fragment of pJV-NheI represents most of the MV F coding region, including the ATG initiation codon and the F_2 subunit sequences, which contain all the potential N-linked glycosylation sites. The *Nhe*I-*Bam*HI cDNA insert was purified and subcloned into the *Bam*HI site of the double-stranded replicative form of pBluescript SK(+/−) phagemid (20, 38). The resulting plasmid was designated SK/MVF.

Oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. (17). Single-stranded DNA prepared from SK/MVF was used as the template for in vitro mutagenesis. Four oligonucleotides were synthesized by the Biotechnology Research Institute Facility, Montreal, with a DNA synthesizer. The oligonucleotides (Table 1) were designed so that the Asn-encoding codons were mutated to Gln-encoding codons. Mutants were verified by DNA sequencing by the dideoxynucleotide chain termination method (33). Elimination of all three glycosylation sites was achieved by using FG1 and FG23 oligonucleotides as primers on the single-stranded SK/MVF template. The G3 mutation was constructed with a PCR fragment. The fragment was generated by priming pRW908 with RW441 and RW442. A second PCR with pRW908 as the template was primed with RW443 and RW444. The two PCR products were pooled, primed for a final PCR with RW441 and RW444, and digested with *Hpa*I and *Nae*I. The resulting 352-bp *Hpa*I-*Nae*I DNA fragments of each mutant were purified and used to replace the equivalent fragment in pRW908. Plasmid pRW908, which contains the vaccinia virus KIL gene and directs insertion to the ATI locus, also contains the wild-type MV F gene linked to the vaccinia virus H6 promoter. Plasmids generated from pRW908 containing glycosylation mutations were used for in vivo recombination and selected by using a host range selection system similar to that described by Perkus et al. (30). In this case, the highly attenuated host range-restricted vaccinia virus strain NYVAC (38) was the rescuing virus. Recombinants were selected by plating on RK-13 cells, which are nonpermissive for growth of NYVAC. Inclusion of the KIL host range gene in the insertion plasmid with the mutated MV F genes allowed recombinants to be selected on this cell line.

Antibodies. Rabbit antisera were prepared against gel-purified proteins or synthetic oligopeptides. Peptide antisera were raised against the carboxy terminus of MV F protein (F_{COOH}) as described previously (31). Rabbit polyclonal antiserum (anti-F_{Ele}) was raised against MV F electroeluted from gel slices containing the uncleaved precursor F_0 synthesized in insect cells (43). Gel-purified F_0 was used to immunize rabbits for antibody production as described previously (31). Anti-F_{pure} antibodies were kindly provided by Tamas Varsanyi (Karolinska Institute, Stockholm, Sweden) and generated against MV F protein purified from MV-infected Vero cells (42). All rabbit antisera were affinity purified on Affi-Gel protein A gel columns (Bio-Rad). Eluted immunoglobulin G

(IgG) fractions were concentrated and resuspended at a final stock concentration of 1 mg/ml.

Analysis of mutant proteins, metabolic labelling of cells, and immunoprecipitation. Confluent monolayers of CV1 cells grown in 60-mm dishes were infected with the recombinant vaccinia viruses at an MOI of 10 PFU/cell. At 6 h postinfection (p.i.), cells were starved in phosphate-buffered saline (PBS) for 30 min and then labelled for 1 h with [^{35}S]methionine (Amersham) at 50 μCi/ml in methionine-free DMEM (Flow Laboratories) containing 10% FBS (Hyclone). Cell lysates were prepared and analyzed as previously described (1, 2). Briefly, cellular proteins were extracted in RIPA buffer (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.5 mg of aprotinin per ml, 10 mM Tris-HCl [pH 7.4]). Lysed cells were microfuged for 30 s, and the supernatants were used for immunoprecipitation with the anti-MV F polyclonal antibodies described in the previous section. Antigen-antibody complexes were precipitated with protein A-Sepharose beads (Pharmacia) and washed three times in RIPA buffer. Precipitated proteins were removed from protein A-Sepharose beads by boiling in lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions.

Tunicamycin treatment and endo H digestion. Infected CV1 cells labelled with [^{35}S]methionine were treated with tunicamycin by previously described methods (2). The drug (1-mg/ml stock solution in dimethyl sulfoxide) was added 2 h before metabolic labelling and was maintained at a final concentration of 20 μg/ml throughout the labelling period. Endoglycosidase H (endo H) digestion was performed on immunoprecipitated glycosylated MV F mutant proteins. Confluent monolayers of CV1 cells were grown in 60-mm dishes and infected, in duplicate, at an MOI of 10 PFU/cell. At 4 h p.i., infected cells were washed with PBS and pulse labelled for 30 min with 200 μCi of [^{35}S]methionine (Amersham) per ml. One set of infected cells was washed with PBS, lysed in RIPA buffer, and immunoprecipitated as described in the previous section. This was considered the time zero of the chase period. The other set of infected cells was washed with PBS before addition of fresh medium containing nonradioactive methionine, and incubation was continued for 4 h. After the chase period, cells were lysed and immunoprecipitated. Antigen-antibody complexes were eluted from protein A-Sepharose beads by boiling for 5 min in 50 mM Tris-HCl (pH 7.0) containing 0.5% SDS. Supernatants were removed, and an equal volume of 0.1 M sodium acetate (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride and 5 mU of endo H (Boehringer Mannheim GmbH) was added. Protein samples were digested with endo H for 20 h at 37°C. After digestion, gel sample buffer was added, and samples were analyzed on SDS–12% polyacrylamide gels by the method of Laemmli (18).

Fluorescence-activated flow cytometric analysis. CV1 cells or NCI-H460 cells (grown in 60-mm dishes at a density of 2×10^6 per dish) were infected with the recombinant vaccinia viruses at an MOI of 1 PFU/cell (CV1 cells) or 10 PFU/cell (NCI-H460 cells). At 20 h p.i., cells were washed and resuspended in PBS containing 0.1% bovine serum albumin. Each sample (10^6 cells) was incubated with anti-F_{pure} at a 1:100 dilution at 4°C for 1 h. Subsequently, cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rabbit IgG (diluted 1:3,000 in washing buffer) (Tago Immunologicals) for 1 h at 4°C. Cells were washed and resuspended at 10^6 cells per ml and analyzed immediately with a flow cytometer (Epics Profile II; Coulter Electronics Inc., Hialeah, Fla.) equipped with a logarithmic amplifier. Background staining of cells infected with the control vaccinia virus vector was subtracted from all test values for mutant- and wild-type-infected cells. The values shown in Fig. 5 were calculated by the cumulative subtraction method described by Overton (27).

Cell fusion activity and hemolysis assays. To inhibit the observed low levels of cell fusion activity caused by vaccinia virus, subconfluent monolayers of NCI-H460 cells were treated with 100 μg of rifampin per ml (16) (Sigma) and then infected with the recombinant vaccinia viruses. Cells were either infected at an MOI of 20 PFU/cell with F recombinants alone or coinfected with both HA and F recombinants (each at an MOI of 10 PFU/cell) for 20 h. Syncytium formation was monitored under a phase-contrast microscope (Leitz), and cells were photographed with Kodak Technical Pan Film at a magnification of ×320.

For the hemolysis assays, African green monkey erythrocytes (Connaught Laboratories, Toronto, Canada) were washed three times with PBS and resuspended in PBS to give a 10% suspension. Aliquots of 1.5 ml of the erythrocyte suspension were overlaid on infected NCI-H460 cells and incubated at 37°C for 24 h. The erythrocyte suspension was removed from each infected-cell monolayer, and erythrocytes were sedimented by low-speed centrifugation. Hemolysis was quantitated by using a spectrophotometer at a wavelength of 540 nm.

RESULTS

Design of N-linked glycosylation mutants and construction of recombinant vaccinia viruses. There are four potential N-linked glycosylation sites (Asn-X-Ser/Thr) in the predicted amino acid sequence of the MV fusion protein (32). Since the first site at asparagine 6 (Asn-Val-Ser) lies within the signal peptide sequence which is cleaved during protein processing, it does not contribute directly to glycosylation of the final F protein product. The second site is at asparagine 29 (Asn-Leu-Ser), the third is at asparagine 61 (Asn-Ile-Thr), and the fourth is at asparagine 67 (Asn-Cys-Thr). These potential carbohydrate attachment sites are all located on the F_2 subunit. The larger F_1 subunit is a nonglycosylated polypeptide. This is a unique characteristic for MV F since other members of the paramyxovirus family are known to have potential glycosylation sites on both the F_1 and F_2 subunits (4, 10, 28).

The addition of N-linked oligosaccharides was prevented by changing the first codon in the consensus sequence (Asn-X-Thr/Ser) from asparagine to glutamine in all glycosylation mutants that were constructed. This change is fairly conservative, since it probably induces the least conformational change in the protein molecule. The oligonucleotides used to mutate the codons for asparagine residues 29, 61, and 67 are shown in Table 1. The mutants in which consensus sequences were altered singly were designated G1, G2, and G3. The positions of these N-linked sites are numbered sequentially from the amino terminus of MV F (Fig. 1). The double mutant is referred to as G23, and the triple mutant is referred to as G123 (Fig. 1).

The oligonucleotides were used as primers to synthesize double-stranded DNA on the single-stranded SK/MVF DNA template. Plasmid SK/MVF DNA contains the complete coding sequences for wild-type MV F. Mutations were confirmed by DNA sequence analysis with an oligonucleotide primer representing nucleotides 19 to 37 downstream of the initiator ATG codon of MV F. The elimination of all glycosylation sites was accomplished by using both oligonucleotides FG1 and FG23 as primers on the single-stranded SK/MVF template. DNA fragments containing mutant F genes were purified and inserted into the vaccinia virus genome. This resulted in the development of recombinant vaccinia viruses that contained the mutated F genes (Fig. 1). The recombinant viruses were

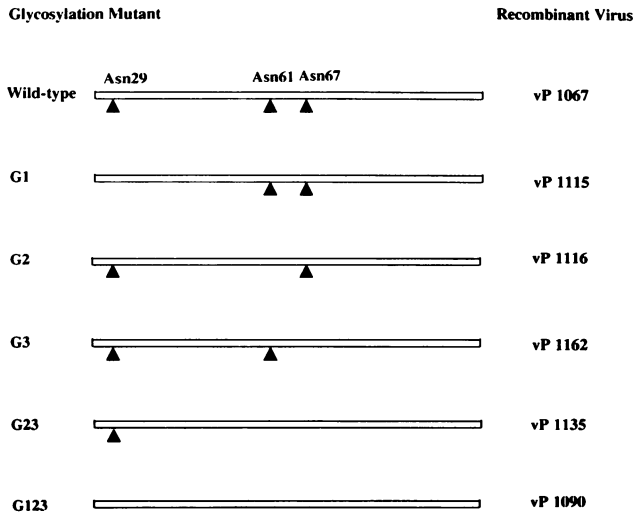

FIG. 1. Schematic representation of MV F glycosylation mutants generated by site-directed mutagenesis. Wild-type MV F protein contains three N-linked glycosylation sites at asparagine residues 29, 61, and 67. All three sites are located on the F_2 subunit polypeptide. Only the F_2 subunit is represented in this diagram. The designations of N-linked carbohydrate chains (G1, G2, G3, etc.) are given on the left. For example, G1 represents a mutant protein in which Asn-29 has been inactivated. Functional glycosylation sites are represented by solid triangles. The designations of recombinant vaccinia viruses containing mutant F genes are shown on the right.

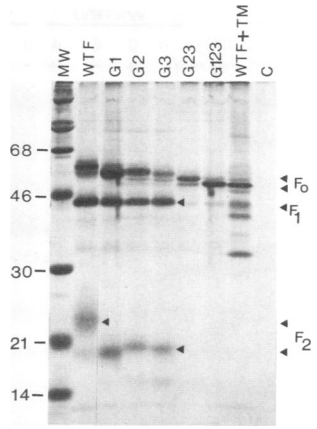

FIG. 2. Expression of glycosylation mutants in infected CV1 cells. Cells were infected (MOI, 10 PFU/cell) with recombinant vaccinia viruses expressing wild-type (WTF) and mutant MV F proteins. Infected cells were labeled with [^{35}S]methionine for 1 h, and MV F-specific proteins were immunoprecipitated with F_{COOH} antibodies. MV F-specific polypeptides were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel. Lane MW, size markers (in kilodaltons). Lane C, polypeptides immunoprecipitated from control-infected cells; lane WTF + TM, polypeptides immunoprecipitated from cells infected with the wild-type F vaccinia virus recombinant in the presence of 20 μg of tunicamycin (TM) per ml.

used to analyze the expression, intracellular stability, cell surface transport, and fusion activity of the altered F proteins.

Analysis of proteins expressed by MV F glycosylation mutants. The expression of F glycosylation mutants was examined by metabolic labelling of recombinant vaccinia virus-infected CV1 cells with [^{35}S]methionine. The wild-type F recombinant virus expressed the expected three protein bands: the uncleaved F_0 precursor, the unglycosylated F_1 polypeptide, and the glycosylated F_2 subunit. The gel mobilities of mutant F_0 proteins increased proportionally with the number of inactivated glycosylation sites (Fig. 2, F_0).

The difference in electrophoretic mobility between wild-type and mutant polypeptides was more obvious in the F_2 band. As shown in Fig. 2, the F_2 polypeptides encoded by G1, G2, and G3 migrated faster than the wild-type F_2 subunit. These differences are consistent with the absence of one of the three N-linked glycosylation sites, each of which contributes approximately 2,000 to 3,000 Da to the relative mass of the F protein. Moreover, when wild-type-infected cells were treated with tunicamycin, the unglycosylated F protein (lane WTF + TM, Fig. 2) comigrated with the G123-encoded deglycosylated mutant protein from untreated cells. These results provide evidence that all three sites (G1, G2, and G3) are utilized for N-linked glycosylation. Figure 2 also shows that G23- and G123-encoded mutant proteins are defective in cleavage, since the F_1 and F_2 subunits are absent from their cell lysates. Only one major band was present in the immunoprecipitates of cells infected with either G23- or G123-encoding viruses.

Quantitation of the relative amount of F polypeptides made in recombinant vaccinia virus-infected CV1 cells compared with those of F polypeptides made in MV-infected cells is shown in Fig. 3. Densitometric analysis of five X-ray film exposures of the same autoradiogram indicated that the amount of F protein made in wild-type F-encoding vaccinia virus-infected cells was at least 10 times higher than that made in MV-infected cells. By a similar approach, the levels of all mutant F proteins were found to be similar to those observed for the wild-type F in the vaccinia virus system (data not shown). The different antibodies used to immunoprecipitate F polypeptides determined that F_{Ele} antiserum showed less immunoreactivity towards F proteins whether native or unglycosylated. Scanning of four different exposures of the autora-

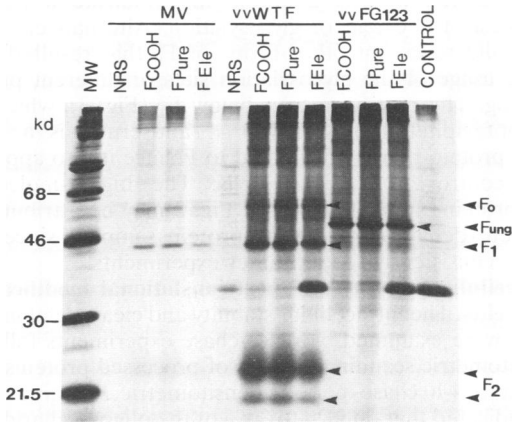

FIG. 3. Immunoreactivity of MV F-specific rabbit antisera and quantitation of relative amounts of F protein made by vaccinia virus recombinants compared with those made by MV. CV1 cells were infected at an MOI of 10 PFU/cell with the indicated virus (MV, vaccinia virus-MV wild-type F [vvWTF], or vaccinia virus-MV F G123 [vvFG123]) for 6 h and then labelled with [^{35}S]methionine. Cell lysates were prepared and immunoprecipitated with the three different MV F-specific antibodies described in Materials and Methods. Equivalent numbers of cells were loaded in each lane. Note that F_{Ele} antibodies were not as efficient as F_{COOH} or F_{pure} antibodies in immunoprecipitation of MV F polypeptides. Lane MW, size markers. F_{ung}, unglycosylated F. NRS, normal rabbit serum; control, vaccinia virus vector which does not contain MV F-specific DNA sequences.

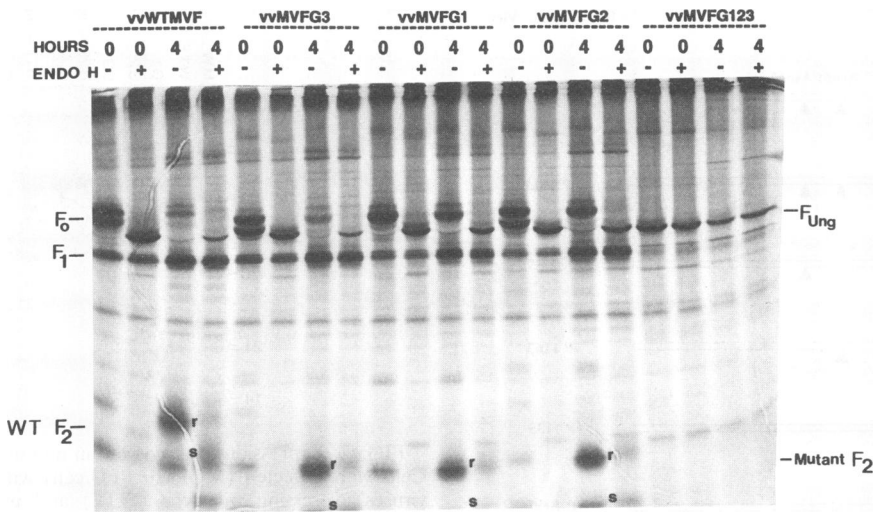

FIG. 4. Pulse-chase experiment for glycan processing and cleavage of wild-type and mutant F proteins. CV1 cells were infected with vaccinia virus (W) recombinants and then pulse labeled with [^{35}S]methionine for 30 min (time zero) and chased for 4 h as indicated. Cell lysates were prepared at each time point and immunoprecipitated with F_{COOH} antibodies. Each immunoprecipitated sample was divided into two equal portions. One portion was digested with endo H (lanes +). The other portion was incubated without endo H treatment (lanes −). Endo H-resistant (r) and -sensitive (s) carbohydrate-containing proteins are indicated to the left of the corresponding bands. F_{ung}, unglycosylated F protein. The WT F_2 marks the F_2 band in the third lane which appears to be more intensely labelled. The corresponding F_2 band at 0 h is in the first lane, above the marked band. Longer exposures of this autoradiogram were also analyzed by densitometry.

diogram shown in Fig. 3 revealed that the relative amounts of F precipitated with F_{Ele} were 40 to 50% of those precipitated with the F_{COOH} and F_{pure} polyclonal antisera.

It was assumed that the fainter band below F_2 observed in the immunoprecipitates shown in Fig. 2 and 3 is another form of F_2 that probably resulted from differences in carbohydrate trimming and modification. We have previously observed F_0 protein as a doublet band in 293 cells infected with either MV or an MV F-adenovirus recombinant (3). The multiple F_0 bands observed in Fig. 2 and 3 may indicate a possible difference in the extent of glycosylation. Alternatively, these bands could represent other forms of F_0 that resulted from selective usage of the glycosylation sites or different protein processing. The protein species below F_1 (Fig. 3), which was immunoprecipitated only with F_{Ele} antiserum, is a cross-reactive protein that is not related to F since it also appeared in the control-virus-infected cells. The higher-molecular-weight protein bands observed in Fig. 3 may be attributed to incomplete denaturation of the protein samples, since they were not consistently seen in other experiments.

Intracellular stability and posttranslational modifications of F proteins. The intracellular stability and cleavage of mutant proteins were examined by pulse-chase experiments followed by densitometric scanning analysis of processed proteins (Fig. 4). After a 4-h chase period, densitometric scanning of F_2 bands indicated that only 20 to 30% of the oligosaccharides on any of the proteins were resistant to endo H digestion (Fig. 4). These results were further confirmed by scanning the F_0 bands before and after endo H treatment. No endo H resistance was observed for either the double mutant G23 (data not shown) or the triple mutant G123 (Fig. 4). Acquisition of endo H resistance indicates that the glycoprotein has reached the medial Golgi (14, 26).

It is evident that elimination of all N-linked oligosaccharide addition sites impaired intracellular transport of the G23 and G123 mutant proteins to the Golgi. Based on the rate at which the G1, G2, and G3 mutant proteins acquired endo H resistance, it was evident that removal of any single N-linked glycosylation site did not seem to block intracellular transport to the Golgi. All single-site mutant proteins were relatively stable after a 4-h chase period. The relative amounts of wild-type and single-site mutant F polypeptides remaining after the chase period were determined to be between 75 and 80%. In contrast, double or triple mutant proteins showed a decrease in their rate of accumulation over time, with only 40 to 50% of G123 remaining at the end of the chase (Fig. 4).

The results in Fig. 4 were also used to determine the efficiency of cleavage of precursor (F_0) proteins. The amount of F_0 at the end of the pulse (determined by a scanning densitometer) was taken as 100%. Therefore, 100% cleavage represented no detectable F_0 in cell lysates. Wild-type F_0 showed 90% cleavage after a 4-h chase. Cleavage of mutant proteins showed different values under the same experimental conditions with equivalent amounts of cell lysates. Mutant G3 was efficiently cleaved into F_1 and F_2 subunits, with 20% of its F_0 precursor remaining after the chase. Elimination of the G1 or G2 site resulted in reduced cleavage efficiency and increased accumulation of uncleaved F_0. Densitometric scanning of precursor F_0 bands indicated that the intensities of G1 and G2 bands were 50% of those found at 30 min postlabelling (Fig. 4). No cleavage products were observed for either the G23 or G123 mutant polypeptide (Fig. 2, 3, and 4).

Cell surface expression of glycosylation mutants. Among the anti-F antibodies used in this study, only anti-F_{pure} antibodies were able to detect expression of F at the cell surface (Fig. 5A). As expected, anti-F_{COOH} antibodies were negative in cell surface staining of living cells, since the region of the F molecule against which these antibodies were raised is located inside the cell. Surface expression of mutant proteins was analyzed by flow cytometry. Forward and right-angle light scatter techniques were used to establish appropriate gates for the cells, excluding nonviable cells. The fluorescence distribution of 10,000 cells was accumulated for analysis. The results of cell surface staining shown in Fig. 5B are expressed as percent positively stained cells, as determined by cytofluorographic analysis. Cells infected with recombinant viruses encoding

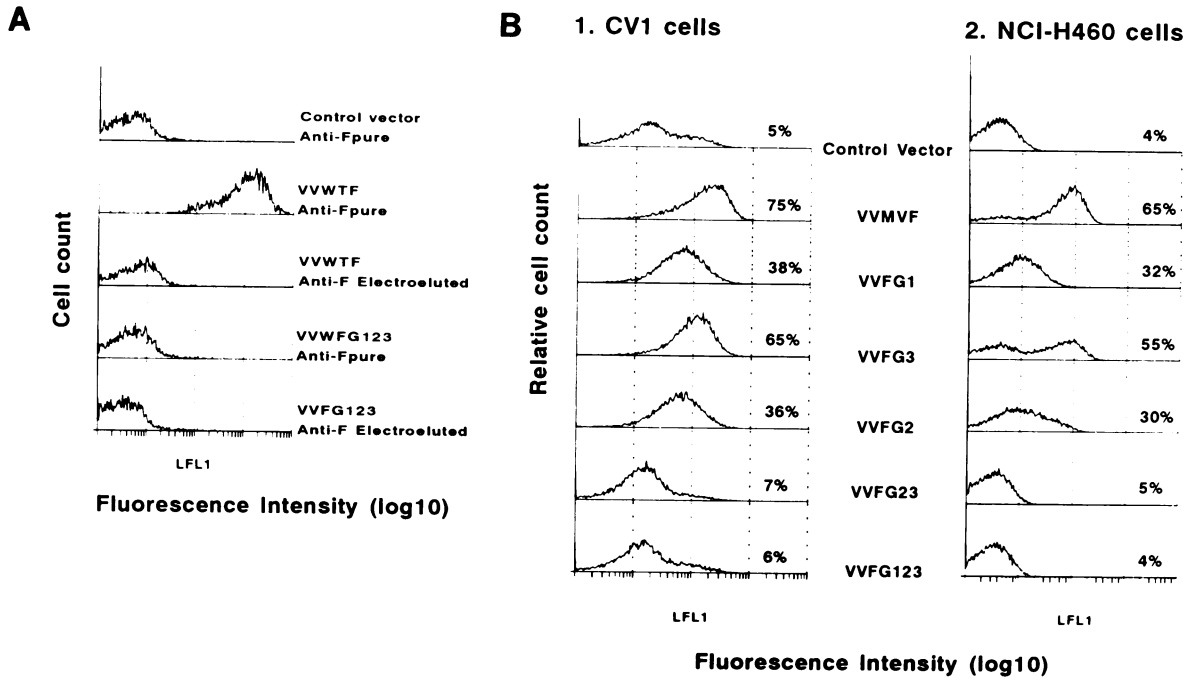

FIG. 5. Immunofluorescence staining of cells infected by wild-type (WTF) and mutant F-vaccinia virus (VV) recombinants. Cells were infected with the indicated recombinant virus at an MOI of 1 PFU/cell (CV1 cells) or 10 PFU/cell (NCI-H460 cells) for 20 h. After infection, cells were washed three times with PBS and reacted with anti-F_{pure} antiserum (primary antibody). After 1 h of incubation on ice, unbound antibodies were washed off with PBS. FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG (secondary antibody) was used for indirect staining of 10^6 cells. MV F-specific surface expression was analyzed with a flow cytometer. Background immunofluorescence represents staining of cells infected with the control vector virus. Each histogram represents the analysis of 10,000 cells. (A) Cell surface immunoreactivity of two different MV F-specific rabbit antisera (F_{pure} and F_{Elc}). Note that only anti-F_{pure} antibodies could detect the F protein expressed at the cell surface. (B) Analysis of the relative levels of cell surface F proteins made by vaccinia virus recombinants encoding MV F glycosylation mutants in two different cell lines. The values are the result of calculating the percentage of positively stained cells within the gated region of viable cells.

single-site mutants expressed lower levels (compared with wild-type F) of mutant F proteins at the cell surface. The levels of the G3 mutant protein detected at the cell surface were only 10% lower than wild-type levels, while the G1 and G2 surface levels were reduced to 50% of the wild-type levels (Fig. 5B). Significant cell surface expression could not be detected with either G23- or G123-encoded proteins (Fig. 5B). These experiments were repeated four times for each cell line, and the results were reproducible without significant variation.

Analysis of membrane fusion activity of expressed F proteins. The cell line NCI-H460 was used to monitor the cell fusion activity of wild-type and mutant F proteins. Infection of this cell line with recombinant vaccinia viruses did not result in the severe cytopathic effect typical of vaccinia virus infections. This made it possible to observe the fusogenic activities of wild-type and mutant F proteins. Subconfluent monolayers of NCI-H460 cells were treated with rifampin before infection with the recombinant viruses. Rifampin was used to inhibit the low cell fusion activity caused by vaccinia virus (16). Very few foci of cell fusion were observed in control-virus-infected NCI-H460 cells without rifampin treatment. In the presence of rifampin, control-infected cells did not show any significant cell fusion activity. Cells expressing wild-type F or the G2 or G3 mutant formed syncytia that were enhanced by the presence of the MV HA glycoprotein (Fig. 6). Coexpression of the F and HA proteins resulted in a two- to threefold increase in the number and size of syncytia compared with those observed with F alone. In contrast, cells expressing the other F mutants (G1, G23, or G123) did not show significant syncytium formation. The inability of G1 to cause cell fusion could not be reversed by addition of the HA protein. Syncytium formation was not observed in either uninfected cells, cells infected with the control virus, or cells infected with the HA-vaccinia virus recombinant. MV-infected NCI-H460 cells showed typical MV-induced fusion.

To confirm these results and obtain a more accurate measure of fusion activity, a hemolysis assay was used (3, 43). Since MV HA was found to enhance syncytium formation, HA-vaccinia virus and F recombinants were used to coinfect NCI-H460 cells. The results (Table 2) indicate that cells expressing wild-type, G2, and G3 F proteins were capable of inducing hemolysis. No significant hemolysis was observed after incubation of G1-, G23-, or G123-expressing cells with monkey erythrocytes. Similarly, significant hemolysis was not seen in uninfected cells or in cells infected with either the control virus or the vaccinia virus-MV HA recombinant. It is evident that the hemolysis results reported in Table 2 support the microscopic observations of syncytium formation.

DISCUSSION

In this study, the contribution of carbohydrate groups to the structure and biological activity of the MV F protein was investigated by using a panel of site-specific mutant proteins with mutations involving the asparagine residue in the consensus glycosylation sequence (Asn-X-Ser/Thr). The results demonstrate that all three potential N-linked glycosylation sites are utilized for oligosaccharide addition in vivo. It was interesting to find that the third site (G3) within the sequence (Asn-Cys-Thr) was used for N-linked glycosylation. This site was not

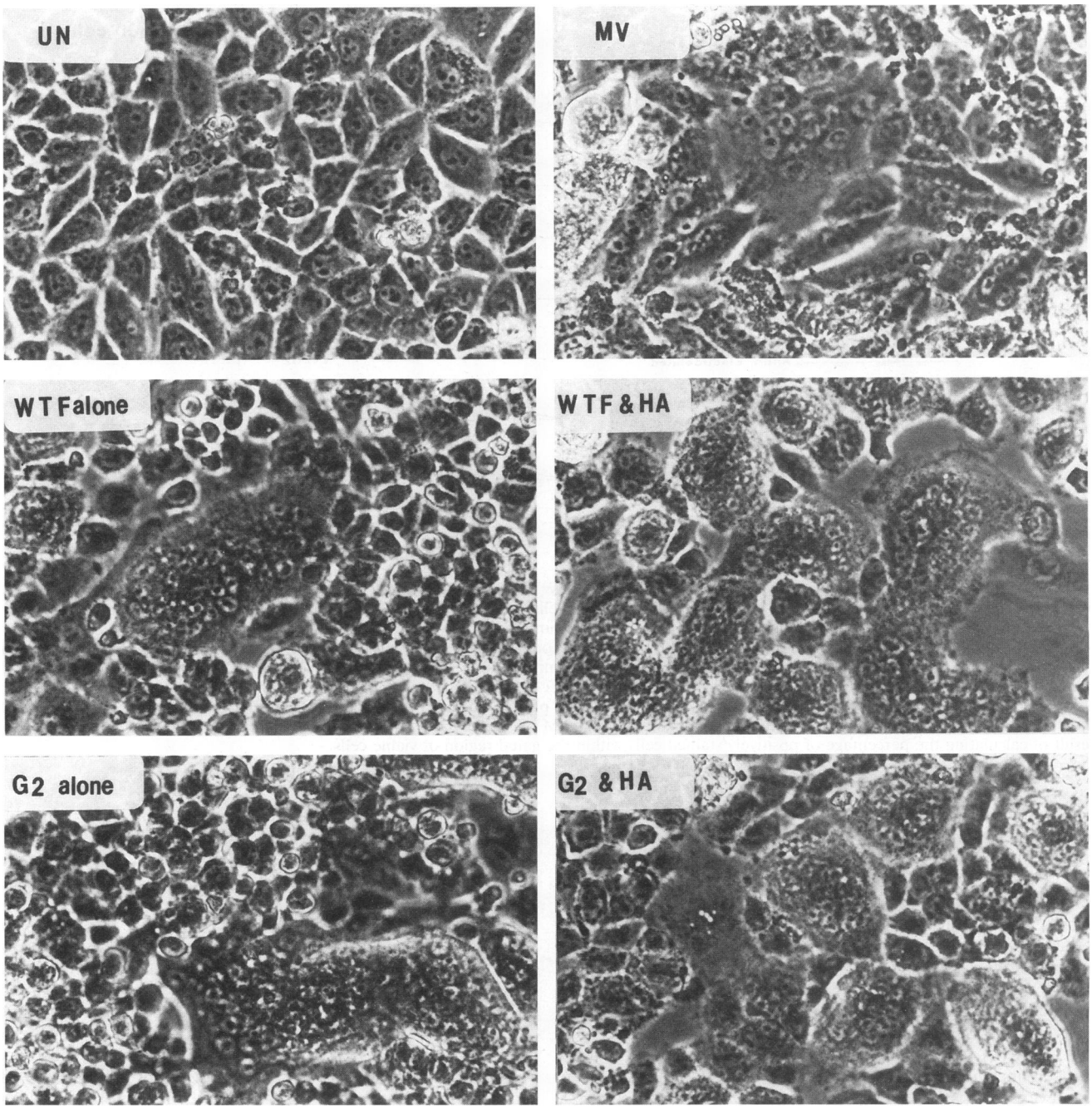

FIG. 6. Syncytium formation induced by wild-type (WTF) and mutant F proteins. NCI-H460 cells were treated with rifampin (100 μg/ml) and then infected with F recombinants or coinfected (F and HA) at an MOI of 20 PFU/cell for 18 h. After incubation at 37°C, cell monolayers were washed with PBS and photographed at a magnification of ×320.

expected to be used, since it contains the only cysteine residue found in the F_2 subunit (32). This cysteine residue must be involved in the formation of the disulfide bond that holds the two subunits together. It seems that two adjacent amino acids are involved in two different interactions. Asn-67 in glycosylation and Cys-68 in disulfide bond formation. This is another unique structural feature of the MV F protein that has not been described for other paramyxovirus F proteins.

It was demonstrated that single-site mutants showed different biological characteristics. These differences were dependent on the position of the inactivated glycosylation site. Similar findings have been reported previously for the simian virus 5 HN glycoprotein (24). The glycosylation site most proximal to the amino terminus (G1) seemed to be more important than the other two sites (G2 and G3). Although the G1 and G2 mutant proteins were similarly affected in cleavage efficiency and transport to cell surface, the G1 protein was incapable of inducing cell-cell fusion and hemolysis (Fig. 6 and Table 2), whereas G2 did exhibit such biological activity. The predicted secondary structure of the MV F protein (32) revealed that the F_2 polypeptide is probably involved in the formation of a globular head that serves in attachment of the

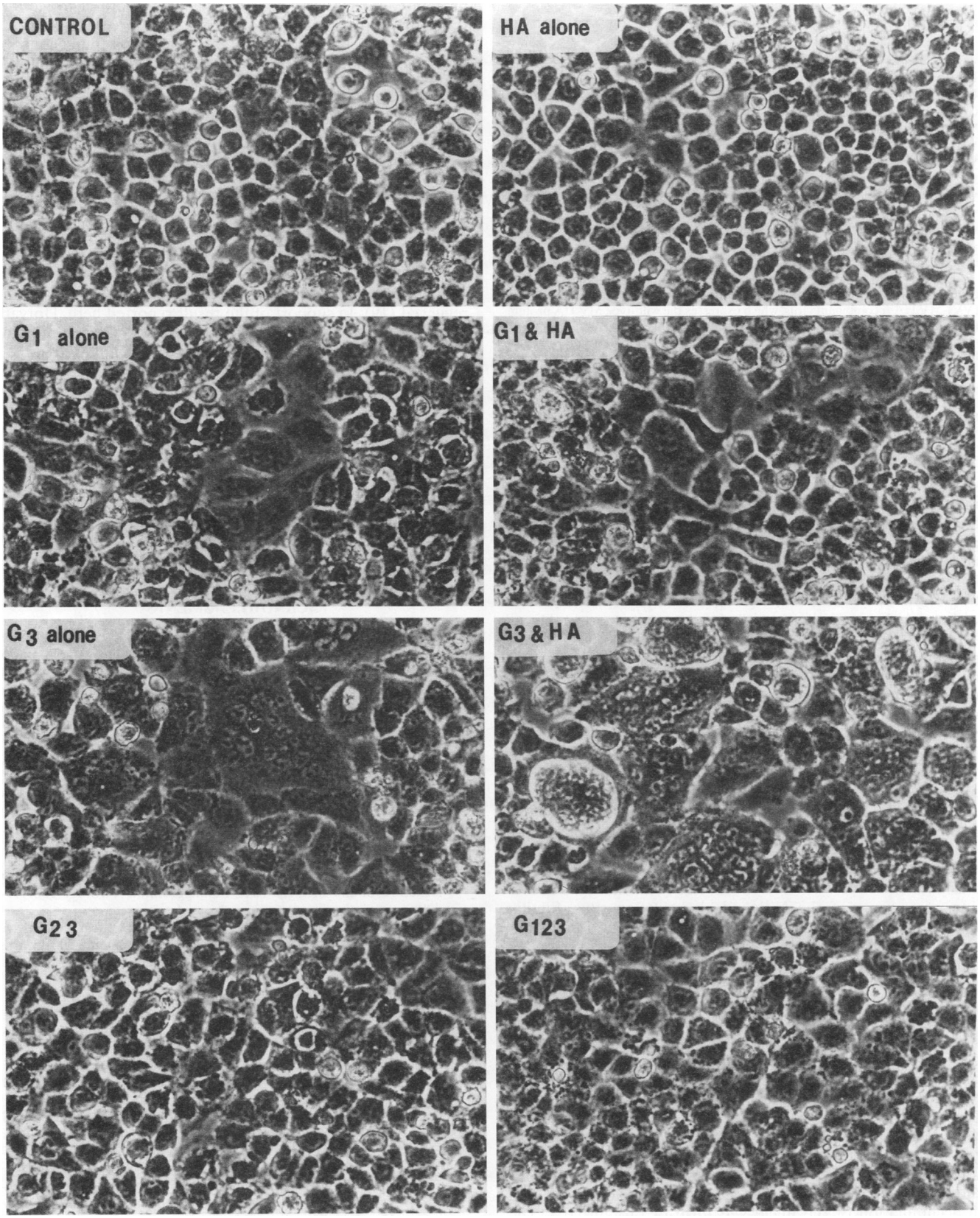

FIG. 6—*Continued.*

TABLE 2. Hemolysis of monkey erythrocytes induced by wild-type and mutant F proteins

Assay components[a]	Hemolysis (OD_{540})
Erythrocytes alone	0.020
Uninfected cells	0.028
Control virus (no F gene)	0.038
VV-MV F (wild-type F)	0.475
VV-MV F G1	0.035
VV-MV F G2	0.265
VV-MV F G3	0.370
VV-MV F G23	0.030
VV-MV F G123	0.045
VV-MV HA only	0.048
MV[b]	0.820

[a] Cells (2×10^6 per dish) were treated with rifampin (100 μg/ml) and then coinfected with the indicated F recombinant virus plus the vaccinia virus (VV)-MV HA recombinant virus, which expresses the wild-type MV HA protein, (at an MOI of 10 PFU/cell for each virus. Infected cells were overlaid with 10% monkey erythrocyte suspension as described in Materials and Methods. Hemolysis was quantitated by measuring the optical density (OD) of supernatants at 540 nm. Values represent the mean of three different samples processed at the same time. Deviations from the mean value were not significant ($P < 0.005$).

[b] NCI-H460 cells (2×10^6) were infected with MV at an MOI of 5 PFU/cell for 24 h without rifampin treatment.

virus to the host cell. It is possible that N-linked oligosaccharide chains present only on the F_2 subunit constitute a major factor in determining the proposed globular structure of this polypeptide. It is also possible that the carbohydrate group added to the Asn-29 site (G1) plays a critical role in maintaining the correct conformation of the F_2 subunit, since inactivation of this site abolishes the fusogenic function of the protein without abolishing either its cleavage or its transport to the cell surface. The data presented here, however, do not exclude the possibility that the observed biological inactivity of the G1 mutant can be attributed to the mutated asparagine residue rather than to the absence of the glycan. Previous studies have indicated that the asparagine residue itself is critical for the biologically active conformation of human major histocompatibility class 1 antigen (34). Although similar findings have not been reported for other viral glycoproteins, it remains a possibility that the Asn-29 residue is itself critical for F protein conformation. Site-directed mutagenesis of the serine residue at position 31 (the third amino acid in the same glycosylation site [Asn-29–Leu-30–Ser-31]) would likely resolve this issue.

It was also demonstrated that transport to the Golgi and the plasma membrane was affected but not abolished by inactivation of any one of the N-linked glycosylation sites. Similar studies on influenza virus HA indicated that the loss of any single N-linked site is tolerated but elimination of three or more sites partially or completely blocked transport to the cell surface (11). Other studies with N-linked glycosylation mutants of the vesicular stomatitis virus G protein have demonstrated that only one of the two normal sites is sufficient for cell surface expression (21). It seems that oligosaccharides at the G1 and/or G2 sites may contribute more significantly to cell surface transport than those at the G3 site. A direct role for N-linked glycosylation in transport of vesicular stomatitis virus G protein to the cell surface has been suggested (14). However, the results presented here imply that the loss of carbohydrate groups alters the conformation of MV F, which may then affect transport to the cell surface. The pulse-chase experiments show that elimination of either the G1 or the G2 site resulted in accumulation of the uncleaved form of protein (F_0). The reduced cleavage efficiency was probably due to a change in conformation which decreased the association of the cleavage enzyme(s) with its F_0 substrate. Therefore, a change in F protein conformation might have affected both cleavage and transport to plasma membranes.

This study demonstrated that the HA glycoprotein enhanced syncytium formation caused by the MV F protein but that MV F alone was capable of causing cell fusion. This result does not support that obtained by Wild et al. (44) and Taylor et al. (40), who did not observe cell fusion in Vero cells infected with the MV F-vaccinia virus recombinant. These different results may be attributed to the cell lines used for infections. The cell line (NCI-H460) used in this study was resistant to vaccinia virus infection. This made it possible to observe syncytium formation caused by the MV F protein in the absence of the severe cytopathic effect caused by vaccinia virus in Vero cells, which are permissive for vaccinia virus infection. The factors influencing syncytium formation caused by paramyxovirus fusion proteins have recently been re-evaluated (15) and recently reviewed (19). In support of the result reported here, Horvath et al. (15) have demonstrated that paramyxovirus F proteins capable of inducing syncytium formation are enhanced in their fusion activity by coexpression of the homotypic HN protein.

In summary, we have used site-directed mutagenesis to study the contribution of N-linked glycosylation to the biological activity of the MV F protein. Our data indicate that the N-linked carbohydrates are important for maintaining the proper conformation and stability of the MV F protein. It seemed that some of the carbohydrate residues are close to regions in the fusion molecule that are important for interaction with target membranes. Therefore, any alteration in glycosylation may have induced a change in protein conformation, resulting in modulation or loss of fusion activity.

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