

# The Roles of Individual Cysteine Residues of Sendai Virus Fusion Protein in Intracellular Transport<sup>1</sup>

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**The role of intramolecular disulfide bonds in the fusion (F) protein of Sendai virus was studied. The 10 cysteine residues were changed to serine residues using site-directed mutagenesis. None of the cysteine mutant F proteins reacted with a monoclonal antibody specific for the mature conformation of the F protein, but eight of ten mutants reacted with an immature conformation-specific monoclonal antibody. The transport of these mutant proteins to the cell surface was drastically reduced. All of the cysteine mutant F proteins remained sensitive to endoglycosidase H (endo H) for 3 h after their synthesis. Moreover, cell surface transport of the hemagglutinin-neuraminidase (HN) protein co-expressed with each of these cysteine mutant F proteins was also reduced. These results suggest that all cysteine residues participate in the formation of intramolecular disulfide bonds, that co-translational disulfide bond formation is crucial to the correct folding and intracellular transport of the F protein, and that interaction of the F and HN proteins takes place intracellularly.**

**Key words:** disulfide bond, F protein, HN protein, intracellular transport, protein folding.

The two glycoproteins of paramyxoviruses, the fusion (F) and hemagglutinin-neuraminidase (HN) proteins, are present as integral proteins which form spike-like projections on the outer surface of the viral envelope. The HN protein exhibits both the hemagglutinating and the neuraminidase activities, while the F protein has been shown to be involved in virus penetration, hemolysis and cell fusion (for reviews, see Refs. 1 and 2). The F protein is synthesized as an inactive precursor, F<sub>0</sub>, which is cleaved by proteases to form the biologically active protein consisting of the disulfide-linked subunits F<sub>1</sub> and F<sub>2</sub>. The N-terminal portion of the F<sub>1</sub> subunit is very hydrophobic, a feature which is highly conserved among paramyxovirus F proteins, and is suggested to mediate fusion of the virus envelope with the target membrane as well as cell fusion resulting in the syncytium formation.

The sequences of paramyxovirus F proteins reveal the conservation not only of amino acid residues of the fusion-inducing domain, cleavage site, and transmembrane domain, but also of cysteine residues at specific positions. As shown in Fig. 1, mature Sendai virus F<sub>0</sub> protein has 10 cysteine residues, designated C1 to C10, and the relative position of cysteine residues is highly conserved among paramyxovirus F proteins (2, 3). This suggests that these cysteine residues play important structural and functional roles. Two cysteine residues are present in the signal

peptide and are not likely to participate in intramolecular disulfide bonds of the mature protein. The C1 cysteine is in the F<sub>2</sub> subunit, and the remaining 9 cysteine residues are in the F<sub>1</sub> subunit. Eight of the 9 cysteine residues are clustered in a narrow region near the transmembrane domain.

The formation of disulfide bonds takes place in the ER and is co-translationally catalyzed by protein disulfide isomerase. It is suggested that only native disulfide bonds are found in the principal folding intermediates and that disulfide bond formation plays an integral role in the folding. The mechanisms of folding, oligomeric assembly, and sorting of viral membrane proteins have been characterized in recent years (for review, see Ref. 4). To date, the roles of individual cysteine residues in the folding of Newcastle disease virus (NDV) (5) and measles virus (6) have been reported. On the other hand, the roles of individual cysteine residues of paramyxovirus F proteins have not been characterized and remain largely unknown. Intramolecular and intermolecular disulfide bonds are essential components of the structure of the majority of proteins, and it is of great interest to understand their functions. Roles of disulfide bonds that have been suggested to include (i) aiding in protein folding and maturation and (ii) maintenance of stability and solubility. Two general approaches, *in vivo* reduction using reducing reagents (7-10) and site-directed mutagenesis to substitute each of the cysteine residues with other amino acids (5, 6, 11-14), have been used to study the functional role of disulfide bond formation within cells.

Recently, the sites of the disulfide bonds of Sendai virus F protein were determined using protein sequencing analysis, and all 10 cysteine residues were shown to participate in disulfide bond formation (15). Thus, mutagenesis to substitute each cysteine residue should provide additional

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Abbreviations: endo H, endoglycosidase H; MAAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride.

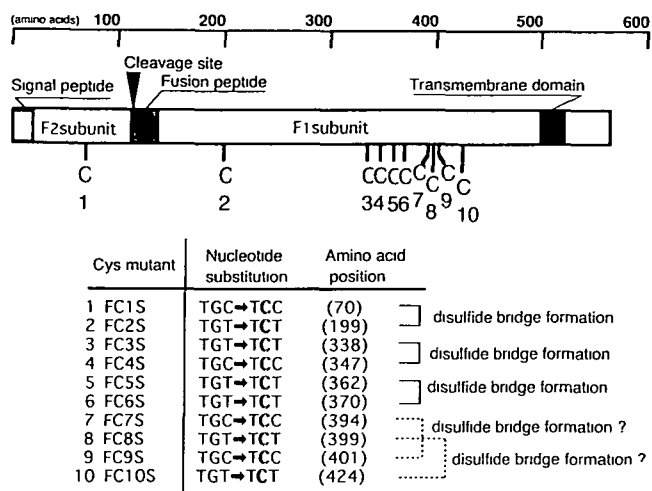


Fig. 1. Schematic representation of the locations of the cysteine residues in the Sendai virus F protein sequence. Numbers correspond to the cysteine mutant F proteins listed below.

findings regarding the functional role of each disulfide bond in the folding, intracellular transport and antigenicity. Previously, we described the efficient expression of Sendai virus F gene cDNA with mutations at its cleavage site and HN gene cDNA to induce cell fusion (16). In this study, we employed site-directed mutagenesis to prepare mutant F protein genes at each of the 10 cysteines and analyzed the role of each cysteine residue in F protein intracellular processing, cell surface expression, and immunoreactivity with different monoclonal antibodies (MAbs) after transient expression of the cysteine mutants in COS cells.

#### MATERIALS AND METHODS

**Replacement of Individual Cysteine Residues and Construction of Expression Plasmids**—Mutagenesis of PCR fragments by use of mismatched primers was performed in a three-step PCR (17). To replace each cysteine, except for C4, we used the sets of four primers listed in Table I. In the first PCR, two separate PCR reactions were run using the 5'-mutagenic and 3'-outer primers and the 3'-mutagenic and 5'-outer primers to amplify separate, overlapping sequences of the template plasmid pUC-F (18). The 5'-mutagenic and 3'-mutagenic primers have overlapping homologous regions containing a mutation of interest. Following amplification, the PCR products were purified, mixed, then denatured and re-annealed. An overlapping duplex was formed and extended by the second PCR reaction without primer to give the full-length target sequence containing the mutation. This mutated PCR fragment was amplified by the third PCR using the 5'-outer primer and 3'-outer primer used in the first PCR.

Plasmid pSRD-FC1S was constructed as follows: the PCR product containing a mutation at its C1 site was digested with *Cla*I and ligated to the Fc fragment, which was excised from pUC-F by digestion with *Cla*I and *Hind*III. The ligated whole F gene was purified by gel extraction, treated with Klenow fragment of DNA polymerase I (Klenow fragment) to create blunt ends, then treated with T4 polynucleotide kinase and ligated into the expression vector pcDL-SRD (pSRD) (19), which had been

TABLE I. Synthetic oligonucleotides used in this study.

Mutagenesis sites	Primer sequence (5'→3')	
C1	Mutagenic primers 5'-F2S G AAT GGG T CC GGA ACA GCC CA 3'-F2S: GC TGT TCC G GA CCC ATT CTC Outer primers 5'-outer primer N G GGC TTG GGA AAC ATG ACA GC 3'-outer primer Fclal2 G CGT <u>CAT CGA</u> TTC TTT GAT GAG CGC	
	C2	Mutagenic primers 5'-F1S GAA TTA GGC T CT GAG ACT GCT GC 3'-F1S A GGC AGC AGT CTC A GA GCC TAA TTC Outer primers 5'-outer primer 5'-F2S G AAT GGG T CC GGA ACA GCC CA 3'-outer primer Fxbal CT <u>CIC TAG</u> ATC CAC ATC TAT CAC
		C3
C4		
	C5	
C6		Mutagenic primers 5'-F1CS5 CA AGG T CT CCT GTC ACA AAA GT 3'-F1CS5 C AGG A GA CCT TGT TGT GTC Outer primers were the same as for C3
		C7
	C8	
C9		
		C10

Nucleotides in larger letters indicate introduced mutations. Underlines of Fclal2, Fxbal and 3'-F1CS3 indicate restriction enzyme sites for *Cla*I, *Xba*I and *Bam*HI, respectively

linearized by digestion with *Eco*RI and treated with Klenow fragment.

Plasmid pSRD-FC2S was constructed as follows: the PCR product containing a mutation at its C2 site was digested with *Bgl*III and *Pst*I and used to replace the corresponding fragment of pUC-F. Subsequently, the FC2S gene was excised by digestion with *Hind*III, blunt-ended by treatment with Klenow fragment, and inserted into pSRD, which had been linearized by digesting with *Eco*RI and treated with Klenow fragment.

To construct the expression plasmids pSRD-FC3S, pSRD-FC5S, pSRD-FC6S, pSRD-FC7S, pSRD-FC8S, pSRD-FC9S, and pSRD-FC10S, we used the following procedure: a cDNA fragment containing the carboxyl terminal portion of F protein from 235 to 565 amino acid residues was subcloned into pBluescript II SK+ (Stratagene Cloning Systems, La Jolla, CA) at the *Pst*I site to yield pBS-Fc. Mutagenesis was performed as described above, and the resulting PCR products containing a mutation at each cysteine site were digested with *Xmn*I and *Nde*I, then inserted into pBS-Fc, which had been digested with *Sma*I and *Nde*I. The resulting plasmids were digested with *Pst*I, and the mutated Fc fragment was ligated with 4.1 kb *Pst*I-digested pSRD-F.

Plasmid pSRD-FC4S was constructed as follows: PCR was performed using N primer and mutagenic primer 3'-F1CS3. The PCR product was digested with *Bam*HI and inserted into pBS-Fc, which had been digested with the same restriction enzyme. The resulting plasmid was digested with *Pst*I, and the mutated Fc fragment was ligated with 4.1 kb *Pst*I-digested pSRD-F as described above. The mutations at the desired sites of all the mutant DNAs were confirmed by DNA sequencing (20). As shown in Fig. 1, a total of 10 cysteine mutants were prepared and designated FC1S to FC10S from the amino terminus of the F<sub>0</sub> protein to the carboxyl terminus, corresponding to the cysteine residues at amino acid positions 70, 199, 338, 347, 362, 370, 394, 399, 401, and 424, respectively. The expression plasmid pSRD-HN was constructed by inserting the whole HN gene into pSRD as described previously (16).

**Cell Culture and DNA Transfection**—Monkey COS-1 cells (21) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO<sub>2</sub>. For transient expression of the F protein, COS cells were grown to 80% confluency in 35-mm tissue culture dishes, then transfected with 5 µg of expression plasmid per dish by calcium phosphate precipitation (22). Cells incubated for 48 h after transfection were analyzed by immunoprecipitation (18).

**Antibodies**—Anti-Sendai virus antiserum was prepared from Sendai virus-infected rabbits. The antiserum used for immunoprecipitation was obtained from rabbits immunized with F proteins, which were expressed in *Escherichia coli* as maltose-binding fusion proteins and purified by amylose resin as described by the manufacturer (New England Biolabs, Beverly, Massachusetts). Anti-F serum used to detect cell surface or intracellular expression of the F protein was obtained from rabbits immunized with the F<sub>1</sub> protein, which was purified from virions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (23). Anti-HN serum used to detect HN protein by Western blotting was obtained from rabbits immunized with the HN protein, which was purified from virions by SDS-PAGE. Monoclonal antibodies (MAbs) f-49, f-236, f-921, and γ1-111 were the generous gift of Dr. H. Tozawa (Kitasato University). MAb f-49 reacts with the mature form of F protein (24), and f-236 recognizes both F<sub>0</sub> and F<sub>1</sub> in immunoprecipitation but reacts specifically with F<sub>0</sub> in Western blot analysis. MAb f-921 reacts with the immature form of F protein. In a competitive binding assay, the MAbs were divided into two groups, F-I and F-II. MAbs f-236 and f-921 belonged to the F-I group and f-49 belonged to the F-II group (25). MAb γ1-111 recognizes the HN protein.

**Indirect Immunofluorescence Staining**—Indirect immunofluorescence staining was performed as described previously (26). In general, COS cells were grown on glass coverslips and transfected with plasmid DNA as described above. Forty-eight hours after transfection, cells were washed with ice-cold phosphate buffered saline (PBS) and fixed with acetone for 10 min at -20°C or with 3.7% paraformaldehyde in 100 mM sodium phosphate buffer (pH 6.5) for 30 min at room temperature. The cells were washed twice with PBS for 10 min, then incubated for 1 h at 37°C in PBS containing 3% BSA, 0.02% NaN<sub>3</sub>, and anti-F rabbit serum or γ1-111 (diluted 1:100). After incubation with the antiserum, cells were washed with PBS twice, then incubated for 1 h at 37°C in PBS containing NaN<sub>3</sub>, BSA, and

fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-mouse IgG (Wako Pure Chemical Industries), each diluted 1:100, as secondary antibody to anti-F rabbit serum or γ1-111, respectively. Immunofluorescence was examined by fluorescence microscopy.

**Western Blot Analysis**—COS-1 cells (in 12-well dishes) were transfected with plasmid DNA as described above. Forty-eight hours after transfection, cells were washed with ice-cold PBS containing 20 mM iodoacetamide (IAA), lysed in 100 µl of RIPA buffer [0.01 M Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] containing 2 mM PMSF and 20 mM IAA, and sonicated for 30 s. Twenty-five microliters of 5×SDS sample buffer [312.5 mM Tris-HCl (pH 6.8), 25% 2-mercaptoethanol (2-ME), 50% glycerol, 10% SDS, 0.05% Bromophenol blue] was added to the cell lysates and boiled for 3 min. Fifteen microliters of each sample was electrophoresed on a 9% polyacrylamide gel. Proteins were transferred to Immobilon-P membrane (Millipore) by semi-dry electroblotting. The membrane was preincubated in Tris-buffered saline (TBS) [0.02 M Tris-HCl (pH 7.6), 0.137 M NaCl] containing 0.05% Tween20, 5% low fat milk for 1 h at room temperature. The membrane was then washed in TBS containing 0.05% Tween20, incubated for 1 h at room temperature in TBS-Tween buffer containing a 1:1,000 each dilution of the anti-F and anti-HN antisera, washed twice for 30 min in TBS-Tween, and incubated for 1 h at room temperature in TBS-Tween containing a 1:4,000 dilution of horseradish peroxidase (HRP)-conjugated protein A (E. Y Laboratories). After extensive washing of the membrane, bound antibodies were detected using the ECL Western blotting detection reagent system (Amersham).

**Immunoprecipitation**—COS-1 cells (in 35-mm dishes) were transfected with plasmid DNA as described above. Forty-eight hours after transfection, the medium was replaced with methionine- and cysteine-free minimum essential medium (MEM). Twenty minutes later, cells were labeled with 100 µl of methionine- and cysteine-free MEM supplemented with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (200 µCi/ml, 1,000 Ci/mmol; Du Pont/NEN), and labeling was continued for 10 min. The cells were chased with DMEM supplemented with 5% FBS, 5 mM methionine, and 5 mM cysteine for the time indicated in each figure legend. Then the cells were washed twice with ice-cold PBS containing 20 mM IAA, lysed in 300 µl of RIPA buffer containing 20 mM IAA, sonicated for 30 s, and centrifuged for 10 min at 15,000 rpm. Fifty microliters of the supernatant (cellular extract) was incubated with 5 µl of antibody raised against recombinant F protein expressed in *E. coli* or MAbs reacting with various epitopes (25) on ice for 1 h. Twenty microliters of a suspension of protein-A Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) was added to the lysates. The mixture was incubated at 4°C for 1 h with gentle mixing, and immune complexes adsorbed on the beads were washed three times in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.25% (w/v) gelatin, 0.1% (v/v) NP-40, 0.02% (w/v) NaN<sub>3</sub>, then denatured in SDS sample buffer [6.25 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol] in the presence or absence of 5% 2-ME by boiling for 3 min. The samples were analyzed by 9% SDS-PAGE followed by fluorography.

**Glycosidase Treatment**—The immune complexes adsorbed on protein-A Sepharose were resuspended in 100 µl

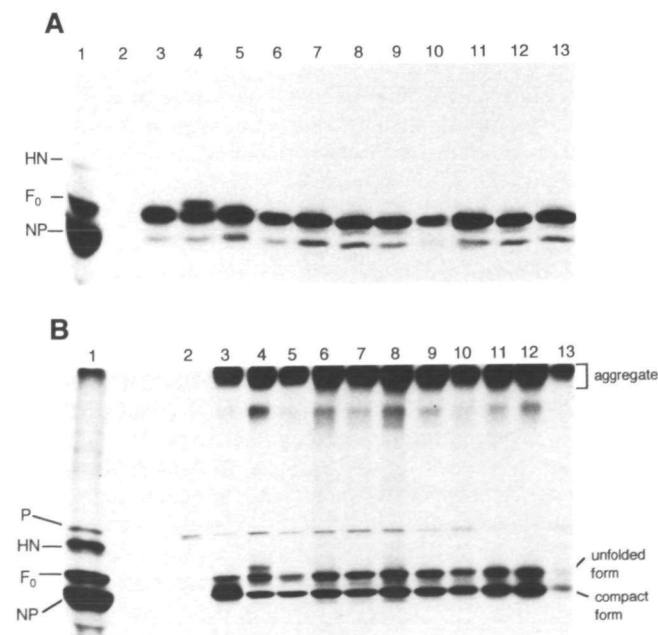
of 50 mM sodium acetate buffer (pH 5.5), then samples were incubated with 2 mU of endo- $\beta$ -*N*-acetylglucosaminidase H (endo H; Boehringer Mannheim Biochemical) for 16 h at 37°C. After digestion, the immune complexes on the beads were recovered by centrifugation and denatured in SDS sample buffer containing 5% 2-ME by boiling for 3 min. The immune complexes were analyzed by SDS-PAGE followed by fluorography.

## RESULTS

**Expression of the Cysteine Mutant F Proteins**—The F protein of the Z strain of Sendai virus possesses 12 cysteine residues. The first and second cysteine residues are present in the signal peptide while the rest are distributed across the entire ectodomain of the protein. The signal peptide is cleaved in the ER, and thus the remaining 10 cysteine residues are present in the mature F protein. The relative locations of these cysteine residues are highly conserved among paramyxovirus F proteins, and these cysteines are likely to participate in intramolecular disulfide bonds in the mature protein. To examine the processing of the intramolecular disulfide bonds and their contribution to the structure of the F protein, site-directed mutagenesis using PCR was used to substitute each cysteine residue by a serine residue to prevent the formation of disulfide bonds. Ten expression plasmids of the cysteine mutant F proteins, named FC1S to FC10S, were prepared as shown in Fig. 1. Recombinant plasmids were introduced into COS cells by the calcium phosphate precipitation method (22), and the cells were incubated for 48 h. Transfected cells were labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 10 min, chased for 1 h, then lysed in RIPA buffer. The lysates were immunoprecipitated with anti-F antiserum and analyzed by 9% SDS-PAGE under reducing (Fig. 2A) or nonreducing (Fig. 2B) conditions. In Fig. 2A, pSRD-F transfected cells gave one main band (lane 3) with mobility corresponding to the uncleaved form of F, or F<sub>0</sub>, synthesized in Sendai virus-infected COS cells as reported previously (16). The 10 cysteine mutant-transfected cells also gave one main band corresponding to the uncleaved F<sub>0</sub> protein. A minor band of about 60 kDa was also detected in all cells transfected with recombinant plasmids (lanes 3–13), and this seems to be nonspecifically degraded F proteins or unglycosylated F proteins, because the unglycosylated F proteins and immature F proteins could be detected by the antiserum used here, which was raised against the unglycosylated F proteins expressed in *E. coli*. The cysteine mutant pSRD-FC1S (Fig. 2A, lane 4) transfected cells gave three bands, one main band corresponding to the F<sub>0</sub> protein, the common minor band (60 kDa), and another higher molecular weight species of F protein. The alteration of the cysteine residue of the F<sub>2</sub> subunit to a serine residue introduced a new glycosylation site in this protein sequence. Thus, the higher molecular weight species seems to be the F protein utilizing the additional new glycosylation site. Approximately equal levels of most of the F proteins in the cells transfected with the above plasmids were detected by Western blot analysis (data not shown). This showed that these 10 cysteine mutant F proteins were efficiently expressed at almost the same level as wild-type F protein in pSRD-F transfected cells and were relatively stable.

Under nonreducing conditions, formation of disulfide

bonds in a protein should allow it to assume a more compact form and to migrate faster on SDS-PAGE than unfolded forms without disulfide bonds, as reported by Machamer *et al.* (27). As shown in Fig. 2B, the compact and unfolded forms of monomer F proteins and aggregates were detected with the mutants as well as the wild-type F protein. The compact form migrated as a 60 kDa band and the unfolded form migrated as a 66 kDa band, which is similar to the molecular mass of the reduced form of F protein as shown in Fig. 2A. Most of the wild-type F protein migrated as the compact form (lane 3). On the other hand, the relative amount of the unfolded form of the each 10 cysteine mutant F proteins was increased, and the ratio of the compact form to unfolded form was roughly 1 as determined by densitometry (lanes 4–13). This indicated that all cysteine residues contributed to make the compact form. No obvious oligomeric form of F proteins was detected in the mutant or wild-type F protein-expressing cells. This implied that F protein does not form disulfide-linked oligomers. Although the aggregates disappeared from the top of the gel when the radiolabeled cell lysates were reduced by boiling in 5% 2-mercaptoethanol before SDS-PAGE, treatment with thiol alkylation reagents such as iodoacetamide (IAA) or *N*-ethylmaleimide (NEM) had little effect on the formation of the aggregates. In preliminary experiments, we did not detect such aggregates in Sendai virus-infected cells analyzed under nonreducing conditions. These observations indicated that these aggregates were formed before sample preparation and that other viral factors might be required



**Fig. 2. Expression of the cysteine mutant F proteins.** COS cells transfected with the following plasmids were labeled for 10 min and chased for 1 h at 48 h post-transfection. The cell lysates were precipitated with anti-F antibody, then analyzed by SDS-PAGE. A: reducing conditions, B: non-reducing conditions; lane 1, Sendai virus-infected COS-1 immunoprecipitated with anti-Sendai virus antiserum and dissociated with SDS sample buffer containing 2-ME as a size marker; lane 2, pSRD; lane 3, pSRD-F; lane 4, pSRD-FC1S; lane 5, pSRD-FC2S; lane 6, pSRD-FC3S; lane 7, pSRD-FC4S; lane 8, pSRD-FC5S; lane 9, pSRD-FC6S; lane 10, pSRD-FC7S; lane 11, pSRD-FC8S; lane 12, pSRD-FC9S; lane 13, pSRD-FC10S.

for efficient folding of F protein.

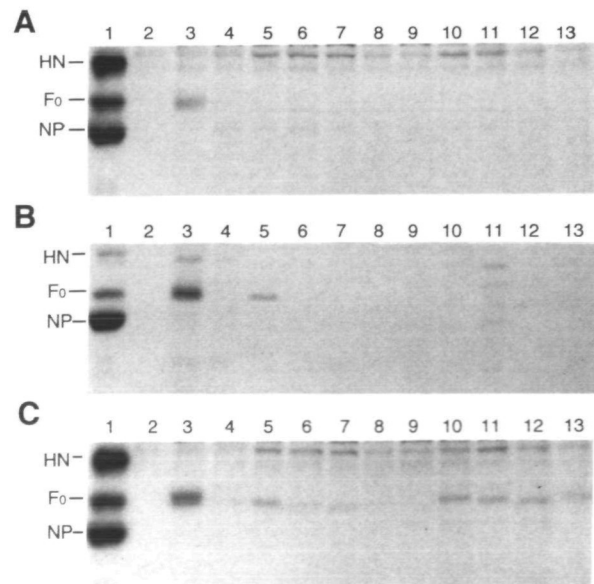
**Immunoreactivity of the Cysteine Mutant F Proteins to Monoclonal Antibodies**—To examine the antigenicity of the cysteine mutant proteins, the cell lysates were immunoprecipitated with MAbs f-49, f-236, and f-921 and analyzed by SDS-PAGE under reducing conditions. In preliminary experiments, f-921 detected intracellular F proteins but not cell surface-transported F proteins. MAb f-236 recognized both F<sub>0</sub> and F<sub>1</sub> in the immunoprecipitation, but specifically recognized F<sub>0</sub> in Western blot analysis (unpublished data). MAb f-49 recognized the mature form of F protein (24). Thus, these MAbs bind independent epitopes. As shown in Fig. 3, only the wild-type F protein was precipitated with anti-mature conformation MAb f-49 (panel A, lane 3), the wild-type F protein and a small amount of FC2S protein were precipitated with MAb f-236 (panel B, lanes 3 and 5), and the wild-type F protein and most of the cysteine mutant F proteins, except for the FC5S and FC6S proteins, were precipitated with MAb f-921 (panel C, lanes 3–7 and 10–13). These data indicated that loss of any one of the cysteine residues of the F protein had dramatic effects on the immunoreactivity to anti mature MAbs and that all of the cysteine mutant F proteins were blocked at some stage in their maturation.

**Cell Surface Transport of Cysteine Mutant F Proteins**—To examine the expression of cysteine mutant F proteins in the cells and their transport to the cell surface, the cells were fixed with acetone or paraformaldehyde and examined by indirect immunofluorescence staining using anti-F antiserum. To detect subcellular localization, the cells expressing wild-type and cysteine mutant F proteins were fixed with acetone, then examined by indirect immunofluorescence staining (Fig. 4, panels A, C, E, G, I, K, and M). We expected that a pair of cysteine mutants which had altered cysteine residues involving the same disulfide bond might show the same expression phenotype. Recent results by Iwata *et al.* (15) identified specific disulfide bonds between C1 and C2, C3 and C4, and C5 and C6, while C10 appears to be linked to C7, C8, or C9. Thus, only the 5 cysteine mutants FC1S, FC3S, FC5S, FC7S, and FC8S were shown in Fig. 4. The COS cells transfected with vector plasmid pSRD showed no fluorescence (panel A), whereas the cells transfected with pSRD-F (panel C), pSRD-FC1S (panel E), pSRD-FC3S (panel G), pSRD-FC5S (panel I), pSRD-FC7S (panel K), and pSRD-FC8S (panel M) displayed a bright staining pattern, and the frequency of positive cells was estimated to be 5–10% of total cells in each case. As shown in panel C, the cells expressing wild-type F protein displayed internal staining throughout the cytoplasmic reticulum as well as in the juxtannuclear region. On the other hand, the cysteine mutant-expressing cells showed an intracellular staining pattern limited to a reticular perinuclear structure (panels E, G, I, K, and M). This indicates that cysteine mutant F proteins were efficiently expressed in these cells. To detect whether the expressed proteins were transported to the cell surface, the cells were fixed with paraformaldehyde instead of acetone, then examined by indirect immunofluorescence staining. The cells transfected with pSRD-F displayed a staining pattern on the cell surface (panel B), whereas the surface fluorescence of the cells transfected with the cysteine mutant F plasmids was clearly much less intense than that of cells expressing wild-type F protein (panels D, F, H, J,

and L). This indicates that the wild-type F protein was properly transported to the cell surface, but these cysteine mutant F proteins were not transported to the cell surface.

In Sendai virus-infected cells, both F protein and HN protein are transported to the cell surface. To examine the effect of HN on cell surface expression of wild-type and cysteine mutant F proteins, cells co-transfected with the HN gene and wild-type F or one of the cysteine mutant F genes were fixed with paraformaldehyde, then cell surface expression of F or HN proteins was tested with anti-F antiserum or anti-HN MAb  $\gamma$ 1-111. The cell surface expression of cysteine mutant F proteins was not detected (data not shown). Furthermore, as shown in Fig. 5A, the cell surface expression of the HN protein was drastically reduced in the cells co-expressing HN and cysteine mutant F proteins. In panels (b) and (c), HN protein was efficiently detected at the cell surface of pSRD-HN- or pSRD-F and pSRD-HN-transfected cells. On the other hand, the cell surface expression of the HN protein was drastically reduced in the cells co-transfected with pSRD-HN and one of the cysteine mutant F genes (examples shown in Fig. 5A panels d, e, and f). As shown in Fig. 5B, in Western blot analysis, both HN and wild-type or the cysteine mutant F proteins were detected in the cells co-transfected with HN and wild-type or cysteine mutant F genes (lanes 4–14). The expression levels of HN proteins in these cells and in the cells transfected with HN gene alone were similar (lanes 3–14).

These results indicated that association of F and HN proteins takes place intracellularly and that the transport



**Fig. 3. Immunoreactivity of the cysteine mutant F proteins with MAbs.** COS cells transfected with the following plasmids were labeled for 10 min and chased for 1 h at 48 h post-transfection. The cell lysates were immunoprecipitated with f-49 (panel A), f-236 (panel B), and f-921 (panel C). Lane 1, Sendai virus infected COS-1 immunoprecipitated with anti-Sendai virus antiserum; lane 2, pSRD; lane 3, pSRD-F; lane 4, pSRD-FC1S; lane 5, pSRD-FC2S; lane 6, pSRD-FC3S; lane 7, pSRD-FC4S; lane 8, pSRD-FC5S; lane 9, pSRD-FC6S; lane 10, pSRD-FC7S; lane 11, pSRD-FC8S; lane 12, pSRD-FC9S; lane 13, pSRD-FC10S.

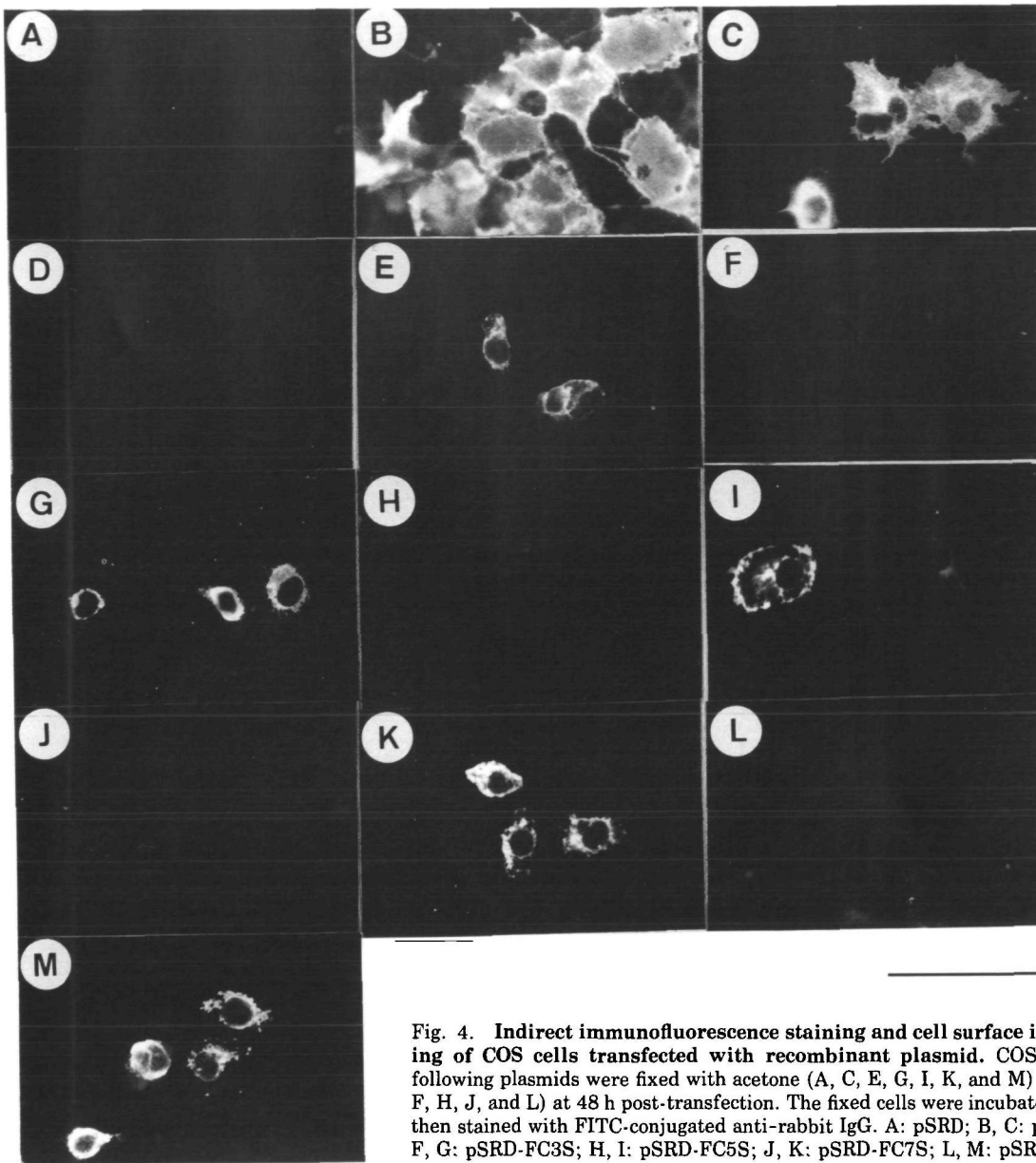


Fig. 4. Indirect immunofluorescence staining and cell surface immunofluorescence staining of COS cells transfected with recombinant plasmid. COS cells transfected with the following plasmids were fixed with acetone (A, C, E, G, I, K, and M) or paraformaldehyde (B, D, F, H, J, and L) at 48 h post-transfection. The fixed cells were incubated with anti-F rabbit serum, then stained with FITC-conjugated anti-rabbit IgG. A: pSRD; B, C: pSRD-F; D, E: pSRD-FC1S; F, G: pSRD-FC3S; H, I: pSRD-FC5S; J, K: pSRD-FC7S; L, M: pSRD-FC8S. Bar: 100  $\mu$ m.

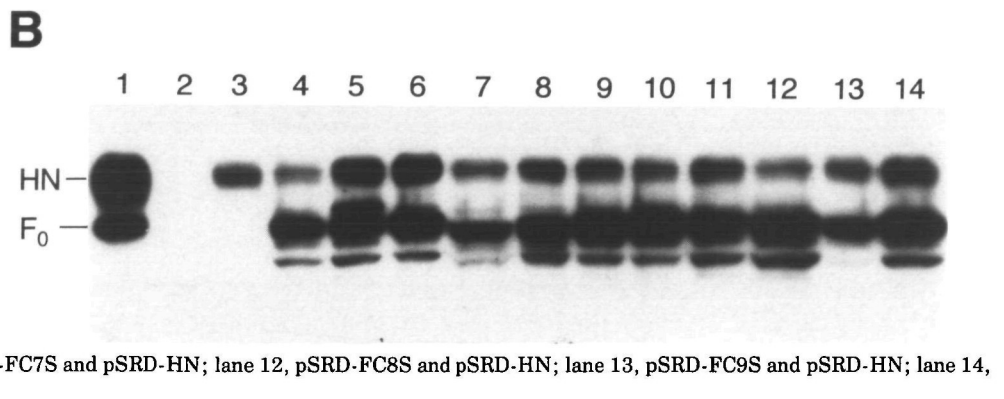
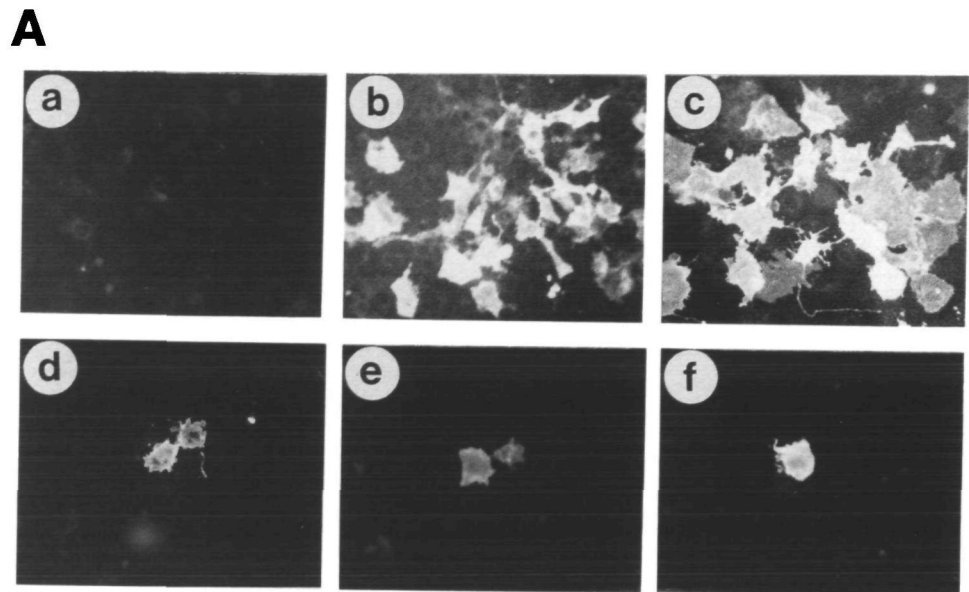
deficiency of the cysteine mutant F proteins affected the intracellular transport of the HN protein.

**Intracellular Transport of F Proteins**—The F protein has three *N*-glycosylation motifs and contains complex-type sugar chain(s) (28). The F protein is synthesized on the rough ER, then glycosylated in a well-defined sequence in the ER and Golgi apparatus in the process of transport to the cell surface. High mannose-type sugar chains of the glycoprotein in the ER and the *cis*-Golgi compartments are sensitive to digestion with endoglycosidase H (endo H), while sugar chains once trimmed and further processed in the medial and *trans*-Golgi compartments are not. We therefore determined the critical state in the transport process of the cysteine mutant F proteins by testing endo H sensitivity of the sugar chains. To follow the process of maturation of glycoproteins, the transfected cells were labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 10 min at 48 h after transfection. After being chased for 3 h, cells

were lysed and the lysates were analyzed after immunoprecipitation with anti-F followed by endo H digestion, as described in "MATERIALS AND METHODS." As shown in Fig. 6, endo H-resistant molecule (Fr) was only detected in the cells transfected with pSRD-F [Fig. 6, lane 1(+)], indicating that a part of the wild-type F protein was processed to the endo H-resistant form during the 3 h chase, with a large amount of the sensitive form remaining. This may in part be due to the efficient immunoprecipitative recovery of the immature F protein from the cell lysates. On the other hand, all cysteine mutant F proteins remained sensitive to digestion with endo H even after a chase of 3 h [Fig. 6, lanes 2(+), 3(+), 4(+), 5(+), 6(+), 7(+), 8(+), 9(+), 10(+), and 11(+)]. Although two species of F proteins were found in the cells transfected with pSRD-FC1S, the migration patterns were similar to other cysteine mutants after endo H digestion [Fig. 6, lane 2(+)]. This indicated that the higher molecular weight species of F protein detected in

Fig. 5. Co-expression of HN and wild-type or cysteine mutant F proteins. (A) Detection of cell surface expression of HN protein in COS cells co-transfected with HN and wild-type or the cysteine mutant F genes. COS cells transfected with the following plasmids were fixed with 3.7% paraformaldehyde at 48 h post-transfection. Then cells were incubated with anti-HN MAb  $\gamma$ 1-111 and stained with FITC-labeled anti-mouse IgG as described in "MATERIALS AND METHODS."

(A) a, pSRD; b, pSRD-HN; c, pSRD-F and pSRD-HN; d, pSRD-FC1S and pSRD-HN; e, pSRD-FC3S and pSRD-HN; f, pSRD-FC8S and pSRD-HN. Bar: 100  $\mu$ m. (B) Western blot analysis of COS cells co-transfected with HN and wild-type or the cysteine mutant F genes. Cell lysates were prepared from the COS cells transfected with the following plasmids at 48 h post-transfection and proteins were detected by Western blot analysis as described in "MATERIALS AND METHODS." Lane 1, Sendai virus infected COS-1; lane 2, pSRD; lane 3, pSRD-HN; lane 4, pSRD-F and pSRD-HN; lane 5, pSRD-FC1S and pSRD-HN; lane 6, pSRD-FC2S and pSRD-HN; lane 7, pSRD-FC3S and pSRD-HN; lane 8, pSRD-FC4S and pSRD-HN; lane 9, pSRD-FC5S and pSRD-HN; lane 10, pSRD-FC6S and pSRD-HN; lane 11, pSRD-FC7S and pSRD-HN; lane 12, pSRD-FC8S and pSRD-HN; lane 13, pSRD-FC9S and pSRD-HN; lane 14, pSRD-FC10S and pSRD-HN.



pSRD-FC1S-transfected cells was partially glycosylated F protein modified at a novel glycosylation motif generated by the mutagenesis.

Because the antiserum used in this study reacts efficiently with immature F protein, relatively large amounts of endo H-sensitive species of F proteins were detected even in the cells expressing wild-type F protein. These data showed that cysteine mutant F proteins were trapped in the ER or the *cis*-Golgi region, which prevented their transport to the cell surface.

#### DISCUSSION

We used mutagenesis of the cysteine residue of the F protein to examine the effect of the disruption and rearrangement of the disulfide bonds on the folding of the F protein and its transport to the cell surface. We found that all cysteine mutant F proteins were efficiently expressed in COS cells (Fig. 2A), but failed to fold into the compact form and to achieve proper conformation (Fig. 3). They were retained in the ER to *cis*-Golgi compartments (Fig. 6), formed aggregates (Fig. 2B), and failed to be transported to the cell surface (Fig. 4). This indicated that disulfide bond formation involving all cysteine residues is required for the F protein to exit the ER. Although our cysteine mutant F

proteins were glycosylated, none of them acquired native structure.

Assignment of disulfide bonds of the F protein has been reported (15); thus, we expected that we would be able to detect some specific folding intermediates corresponding to the lack of individual disulfide bonds by analyzing individual cysteine mutant F proteins under nonreducing conditions. When mutations are made in either one of a pair of cysteines that are normally linked in a mature structure, the phenotype of the mutant proteins is often similar. In our study, two species of F proteins were detected in the cysteine mutant F proteins under nonreducing conditions: folded F protein and unfolded F protein. This indicated that formation of incorrect disulfide bonds led to rapid unfolding or rearrangement of disulfide bonds. As shown in Fig. 3, almost all cysteine mutants exhibited a drastic decrease in reactivity to the mature conformation specific MAb f-49. This result showed that cysteine residues contribute to the whole protein structure through disulfide bridging, even though they are fairly distant from each other in the primary amino acid sequence. MAb f-921 could not immunoprecipitate FC5S and FC6S proteins but could immunoprecipitate the rest of the mutants. This implied that the epitope of f-921 is present in the loop formed by the C5 and C6 disulfide bond, and the antigenic site is exposed

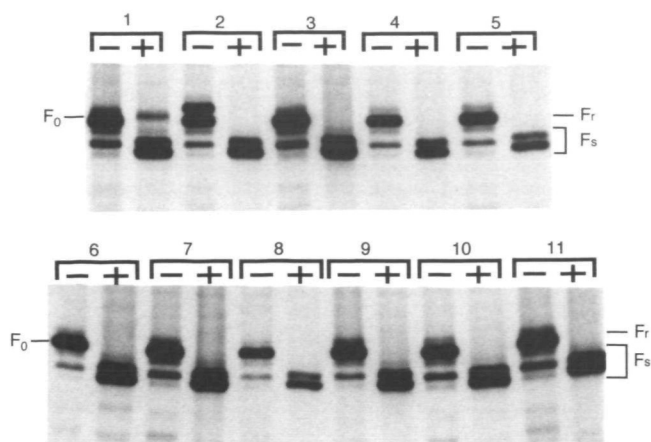


Fig. 6. Endo H digestion of F proteins synthesized in transfected COS cells. COS cells transfected with individual plasmids were labeled for 10 min at 48 h post-transfection, then chased for 3 h. The cells were lysed in RIPA buffer and the cell lysates were immunoprecipitated with anti-F antiserum. Precipitates were then incubated at 37°C for 16 h with (+) or without (-) 2 mU of endo H. The positions of the intact  $F_0$  (Fr) and the digested ( $F_s$ ) forms of the F proteins are indicated. Lane 1, pSRD-F; lane 2, pSRD-FC1S; lane 3, pSRD-FC2S; lane 4, pSRD-FC3S; lane 5, pSRD-FC4S; lane 6, pSRD-FC5S; lane 7, pSRD-FC6S; lane 8, pSRD-FC7S; lane 9, pSRD-FC8S; lane 10, pSRD-FC9S; lane 11, pSRD-FC10S.

when the F protein is inside the cells, but buried when the F protein is expressed on the cell surface. FC2S showed only slight reactivity to MAb f-236. These data indicated that f-236 and f-921 may recognize cysteine-rich domains and supported the idea that the cysteine-rich domain in the bunched structure of paramyxovirus F proteins is recognized by MAbs (29).

Since the formation of the correct intramolecular disulfide bonds seems to be a complex process involving disruption and rearrangement of disulfide bonds during intracellular transport, the three-dimensional structure of these cysteine mutants was changed, and the immunoreactivity of MAbs with these mutant proteins decreased dramatically. These data suggested that co-translational disulfide bond formation is an absolute requirement for subsequent folding.

Because of the incorrect folding of cysteine mutant F proteins, their transport to the cell surface was reduced drastically and they contained only high mannose-type oligosaccharides. These results indicated that the mutant proteins were retained in the ER. Viral glycoprotein mutants that had a temperature-sensitive phenotype and folded correctly at 32°C but misfolded at 37°C have been reported (4). In HSV-1, the cysteine mutant gD proteins showed temperature sensitive cell surface expression (11). We examined whether cysteine mutant Sendai virus F proteins show temperature sensitivity, but we could not detect cell surface expression of the cysteine mutant F proteins by incubating mutant gene-expressing cells at 33°C or at 31.5°C (data not shown).

These results suggest that the inhibition of the expression of the cysteine mutant F proteins on the cell surface occurred at a stage involved in the intracellular transport.

Though many reports have shown that cysteine mutant proteins are improperly folded and found as disulfide-

linked aggregates in the ER, there are some reports that cysteine mutant proteins could be transported to the cell surface, e.g., Newcastle disease virus (NDV) HN, measles virus H, and asialoglycoprotein receptor H2b subunit, all of which have type II topology (5, 6, 14). It seems that type I transmembrane proteins more strictly require disulfide bond formation than do type II proteins in order to exit from the ER.

We previously found that cleavage of an intrinsically protease-sensitive mutant F, Fmut, was enhanced by co-expression with HN protein (16), indicating that F protein associates with HN protein intracellularly. Thus, we examined the effect of co-expression of HN on cell surface expression of cysteine mutant F proteins. Although the expression of both proteins was detected at roughly equal levels by Western blot analysis (Fig. 5B), cell surface expression of cysteine mutant F proteins was not detected, and a decrease of cell surface expression of HN protein was observed (Fig. 5A). These observations are in accord with the down-regulation of the human parainfluenza type 3 (HPIV3) HN protein cell surface expression by the mutant F protein containing an intracellular retention signal (30). Recently, many reports have shown a direct interaction of paramyxovirus F and HN proteins (31-34). However, the cell surface expression of F and HN proteins is reported to occur at different rates because of the different degrees of association with BiP (35). Thus, decrease of cell surface expression of HN protein in the cysteine mutants co-expressing cells may be caused not only by direct interactions of the F and HN proteins but also by indirect interaction affected by binding of molecular chaperons such as BiP or calnexin.

Morrison *et al.* reported that NDV F protein undergoes conformational change with disruption and rearrangement of disulfide bonds during intracellular transport, and that the conformational change occurs before the cleavage reaction (36). Because of the intracellular transport deficiency of the cysteine mutant F proteins, we could not identify the cysteine residues involved in such disulfide bond rearrangements. To study sequential disulfide bond formation and bond rearrangements, *in vivo* reduction experiments using well-characterized MAbs are also now under way.

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