J Biochem. 128, 65–72 (2000)

Functional Analysis of the Individual Oligosaccharide Chains of Sendai Virus Fusion Protein[1]

Hiroaki Segawa,*[2] Tetsuro Yamashita,* Masao Kawakita,† and Hideharu Taira*[3]

*Department of Bioscience and Technology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020-8550; and †Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613

Received March 1, 2000; accepted April 24, 2000

The roles of *N*-linked glycosylation in the intracellular transport and fusion activity of the Sendai virus fusion (F) protein were studied. Each of three potential glycosylation motifs (designated g1, g2, and g3) in the F protein was mutated separately or in combination with the other sites. When the mutant F proteins were transiently expressed in COS cells, they showed significant changes in electrophoretic mobility, indicating that all three motifs in the F protein are glycosylated. Glycosylation-defective mutants which lacked the g2-oligosaccharide chain showed decreased immunoreactivity with a monoclonal antibody specific for the native conformation and were inefficiently transported to the cell surface. Such mutants, with the exception of a double mutant lacking g1 and g2-oligosaccharide chains, were also not able to induce syncytia formation when cells expressing them plus the hemagglutinin-neuraminidase protein were treated with trypsin. Mutations at the other glycosylation sites did not significantly affect the immunoreactivity with the monoclonal antibody or the efficiency of intracellular transport of the F protein. These results indicate that the *N*-linked oligosaccharide chain attached at g2 is important for efficient intracellular transport and for the fusion activity of the F protein.

Key words: cell fusion, fusion protein, intracellular transport, *N*-linked oligosaccharide chain, Sendai virus.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

Glycoproteins of paramyxoviruses, the fusion (F) and hemagglutinin-neuraminidase (HN) proteins, are integral membrane proteins which form spike-like projections on the outer surface of the viral envelope. The HN protein exhibits both hemagglutinating and neuraminidase activities, while the F protein has been shown to be involved in virus penetration, hemolysis and cell fusion (*1*). The F protein is inserted co-translationally into the endoplasmic reticulum, where the nascent polypeptide forms disulfide bonds and receives oligosaccharide chains. The correctly folded F protein molecule is then transported to the Golgi apparatus where further modification of oligosaccharide chains occurs, and then to the cell surface, where virus particles are assembled. Unlike other paramyxoviral F proteins, which are cleaved during intracellular transport to acquire fusion-competence (*2*), Sendai virus F protein is cleaved later on the cell surface by trypsin-like proteases such as tryptase clara in mouse lung (*3*), or blood clotting factor X in chicken egg (*4*).

The amino acid sequences of F proteins are well conserved among paramyxoviruses in the fusion-inducing domain, in which the cleavage site is located, and in the transmembrane domain. Cysteine residues at several positions are also conserved, and we showed previously that they are important for maintaining the structure of Sendai virus F protein (*5*). In contrast, *N*-glycosylation sites of paramyxoviral F proteins are not conserved, but vary among species. Sendai virus F_0 protein has three potential *N*-glycosylation sites designated g1, g2, and g3, as shown in Fig. 1, but the functional significance of each site remains unknown.

We are interested in how the oligosaccharide chains attached to the F protein of Sendai virus affect the function of the protein, since *N*-linked oligosaccharide chains may affect protein functions in many ways. These include promotion of proper folding, maintenance of protein conformation and stability, protection of a protein from proteolysis, and modulation of biological activities of proteins (*6*). Biological roles of *N*-linked oligosaccharide chains are often assessed by using tunicamycin, a potent inhibitor of *N*-glycosylation (*7, 8*). However, tunicamycin treatment, which inhibits the synthesis of dolichol intermediates, causes a total loss of *N*-linked oligosaccharide chains, and is prone to artifacts, including nonspecific aggregate formation of misfolded unglycosylated proteins (*9–11*). Site-directed mutagenesis at specific *N*-glycosylation sites would be a useful alternative for analyzing the roles of specific oligosaccharide chains.

In this report, we describe the biological roles of individual oligosaccharide chains of Sendai virus F protein analyzed by site-directed mutagenesis, and show that only one

[1] This work was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

[2] Present address: Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613

[3] To whom correspondence should be addressed. Tel +81-19-621-6154, Fax. +81-19-621-6177, E-mail: taira@iwate-u.ac.jp

© 2000 by The Japanese Biochemical Society.

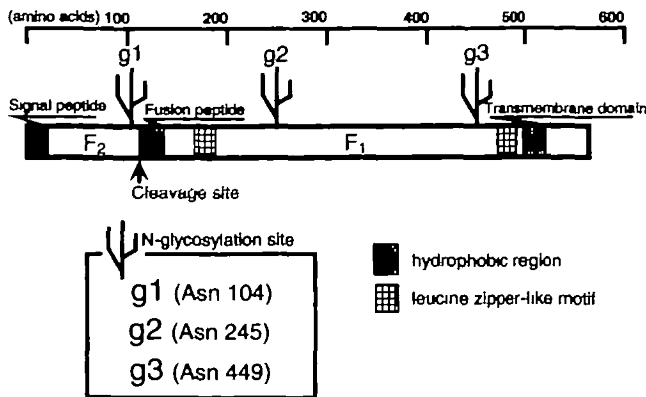

Fig 1 **Schematic representation of the locations of the potential *N*-glycosylation sites in the Sendai virus F protein sequence.**

TABLE I **Synthetic oligonucleotides used in this study.**

Mutagenesis site	Primer sequence (5'→3')
g1	Mutagenic primers 5'-mutagenic primer 5'-Fg1 GTC ACC **CAA** GAT ACG ACA CA 3'-mutagenic primer 3'-Fg1 TGT CGT ATC **TTG** GGT GAC AGT T Outer primers 5'-outer primer N· G GGC TTG GGA AAC ATG ACA GC 3'-outer primer Fcla2: G CGT C AT CGA TTC TTT GAT GAG CGC
g2	Mutagenic primers 5'-mutagenic primer 5'-Fg2: AC TCT GCT **CAG** ATT ACT GAG 3'-mutagenic primer 3'-Fg2· CTC AGT AAT **CTG** AGC AGA GT Outer primers 5'-outer primer N· G GGC TTG GGA AAC ATG ACA GC 3'-outer primer 3'-F1CS4 TC CCC CAG GAT AGA CTT TTG C
g3	Mutagenic primers 5'-mutagenic primer 5'-Fg3 GTC CAG **CAG** TTG ACA GTC G 3'-mutagenic primer 3'-Fg3. C GAC TGT CAA **CTG** CTG GAC Outer primers 5'-outer primer Fmut-3· GGT CGT CGC CAG AAG AGA TTC TTC GG 3'-outer primer C GAC ATC TGA TAA TGG TCG TGA TC

Bold letters show mutated bases.

oligosaccharide chain at the g2 site is important for folding and biological activity of the F protein. At the same time, we show that even nonglycosylated F protein could be folded into the mature form and transported to the cell surface.

MATERIALS AND METHODS

Virus Strain and Plasmids—Sendai virus Z strain was grown in the allantoic fluid of 9-day-old chick embryos, and purified by discontinuous sucrose gradient centrifugation. Titration of the virus preparation in terms of plaque forming units (PFU) was carried out as described (*12*) using LLC-MK_2 cells. The expression plasmid pc-DL-SRD (pSRD) (*13*) was a kind gift from Dr. S. Ohno (Yokohama City University). pUC-A35, which contains the N-terminal portion of the F gene, was kindly provided by the late Dr. H. Shibuta (Medical Science, The University of Tokyo). It was previously reported that the F protein of Sendai virus Z strain possesses four potential *N*-glycosylation motifs (*14*), but we found a point mutation at the cytosine 782 to adenine, which resulted in loss of an *N*-glycosylation motif due to a serine to tyrosine alternation Since this tyrosine residue is conserved among the F proteins of other Sendai virus strains (*15–17*), we decided to analyze the other three *N*-glycosylation motifs.

Replacement of Asparagine by Glutamine Residues and Construction of Expression Plasmids—To substitute each asparagine residue of the three glycosylation motifs, we employed site-directed mutagenesis by PCR (*18*) as described previously (*5*), using the oligonucleotides listed in Table I as primers and pUC-A35 as a template for the g1-site and pUC-F (*19*) as a templates for the g2- and g3-site mutagenesis.

The expression plasmid pSRD-Fg1⁻ was constructed as follows. a PCR fragment lacking the g1-glycosylation motif generated by mutagenic PCR was digested with *Cla*I and ligated with a 1.2-kb fragment (Fc) obtained by *Cla*I and *Pvu*II digestion of pUC-F. The resulting DNA fragment was inserted into pSRD, which had been digested with *Eco*RI and treated with Klenow fragment of DNA polymerase I.

The expression plasmids pSRD-Fg2⁻ and pSRD-Fg3⁻ were constructed as follows: appropriate PCR products generated by mutagenic PCR were digested with *Bam*HI or *Nde*I for the g2- or g3-mutated fragment, respectively, and the mutated DNA fragments were inserted into the *Bam*HI or *Nde*I site, respectively, of pBS-Fc (*5*). The resulting plasmids named pBS-Fcg2⁻ and pBS-Fcg3⁻, respectively, were digested with *Pst*I, and 1.0 kbp fragments were used to replace the corresponding stretch in pSRD-F (*2*) to yield pSRD-Fg2⁻ and pSRD-Fg3⁻.

The expression plasmid pSRD-Fg23⁻ was constructed as follows: the 900-bp fragment obtained by *Bam*HI digestion of the g2-mutated DNA fragment was inserted into the *Bam*HI site of pBS-Fc3⁻ to give a plasmid named pBS-Fcg23⁻, of which the 1.0-kbp-*Pst*I fragment was in turn used to replace the corresponding stretch in pSRD-F to yield pSRD-Fg23⁻.

The expression plasmids pSRD-Fg12⁻, pSRD-Fg13⁻, and pSRD-Fg123⁻ were constructed as follows: pSRD-Fg1⁻ was digested with *Cla*I, and the resulting fragment which contained the SRα promoter and the N-terminal portion of F cDNA was purified and used to replace the equivalent fragment in pSRD-Fg2⁻, pSRD-Fg3⁻, and pSRD-Fg23⁻, respectively.

Tandem expression plasmids coding for HN and glycosylation-defective mutant F proteins were constructed as follows: The mutant F genes were excised from each expression plasmid by digesting with *Hin*dIII and *Sal*I, and treated with Klenow fragment of DNA polymerase I, and then the fragments were inserted into pSRD-HN (*2*) which had been digested with *Hin*dIII and treated with Klenow fragment of DNA polymerase I.

The mutations at the desired sites of all the mutant DNAs were confirmed by DNA sequencing (*20*).

Cell Culture and DNA Transfection—Monkey COS-1 cells (*21*) were grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (FCS) at 37°C, in 5% CO_2. HeLa cells were grown in minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% FCS. Transient expression of the F protein using the calcium phosphate precipitation method (*22*) was carried out as described previously (*5*).

Antibodies—Antibodies described previously (*5*) were used in the present study. Monoclonal antibody (MAb) f-49 reacts with the mature form of F protein and MAb f-236 reacts with both mature and relatively immature forms of

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

F protein (*5*). Both were generous gifts from Dr. H. Tozawa (Kitasato University).

Indirect Immunofluorescence Staining—Indirect immunofluorescence staining was performed as described previously (*5*).

Pulse-Chase Experiment and Immunoprecipitation—Transfected cells were starved for 20 min in methionine- and cysteine-free MEM, and then labeled for 10 min in the same medium supplemented with [^{35}S]methionine and [^{35}S]cysteine (200 μCi/ml, 1,000 Ci/mmol; Du Pont/NEN Life Science Products, Boston, MA). The cells were chased with DMEM supplemented with 5% FCS, 5 mM methionine, and 5 mM cysteine for 1 h, and then immunoprecipitation was carried out as previously described (*5*). Tunicamycin (10 μg/ml, Wako Pure Chemical Industries, Osaka) was added to the medium during the starvation, pulse-labeling, and chase where indicated.

Western Blot Analysis—The procedures for Western blot analysis was described previously (*5*).

Trypsin Treatment—COS cells transfected with various plasmids were incubated in PBS containing 0.1% trypsin 250 (DIFCO Laboratories, Detroit, MI) on ice for 5 min at 48 h after transfection. The cells were washed twice with DMEM containing 10% FCS and once with ice-cold PBS, and then lysed in 100 μl of radio immunoprecipitation assay (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 subjected to Western blot analysis.

Fusion Assay—HeLa cells were washed once with PBS and then with PBS containing 0.1% trypsin at 12 h after transfection The cells were further incubated in growth medium for 6 h, washed once with PBS, and fixed with methanol at room temperature for 10 min. They were then stained with Giemsa's stain solution (Nacalai tesque, Kyoto) at room temperature for 10 min, washed three times with water, air dried, and examined under an optical microscope at the magnification of 200×.

RESULTS

Expression of the Glycosylation-Defective Mutant F Proteins—To examine whether individual *N*-glycosylation motifs are utilized and whether the oligosaccharide chains contribute to the F protein structure and function, we constructed mutant F proteins defective in glycosylation at specific sites by site-directed mutagenesis. In order to prevent addition of oligosaccharide chains, an asparagine residue in each *N*-glycosylation motif was altered to a glutamine residue by PCR-mutagenesis. Each of the three potential glycosylation sites was mutated separately as well as in combination with other sites. Seven expression plasmids for these glycosylation-defective mutants named Fg1$^-$, Fg2$^-$, Fg3$^-$, Fg12$^-$, Fg23$^-$, Fg13$^-$, and Fg123$^-$ were constructed.

These plasmids were introduced transiently into COS-1 cell by means of the calcium-phosphate method. The cells

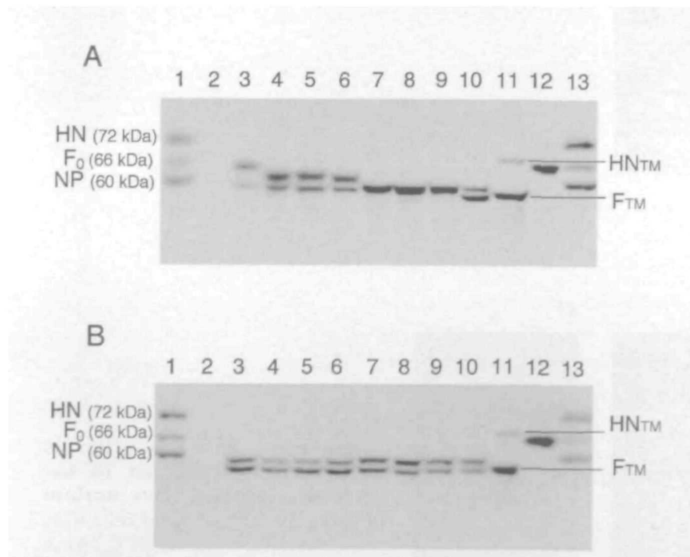

Fig. 2. **Expression of the glycosylation-defective F proteins.** COS cells transfected with the following plasmids were labeled for 10 min and chased for 1 h in the absence (A) or presence (B) of tunicamycin at 48 h after transfection. The cell lysates were precipitated with anti-F antibody and then analyzed by SDS-PAGE. Lanes 1 and 13, Sendai virus-infected COS-1 cell extract immunoprecipitated with anti-Sendai virus antiserum to serve as size marker; lane 2, pSRD; lane 3, pSRD-F, lane 4, pSRD-Fg1$^-$; lane 5, pSRD-Fg2$^-$; lane 6, pSRD-Fg3$^-$, lane 7, pSRD-Fg12$^-$; lane 8, pSRD-Fg23$^-$; lane 9, pSRD-Fg13$^-$; lane 10, pSRD-Fg123$^-$; lane 11, tunicamycin-treated Sendai virus–infected cell extract immunoprecipitated with an anti-F antiserum; lane 12, Sendai virus–infected cell extract immunoprecipitated with anti-F antiserum.

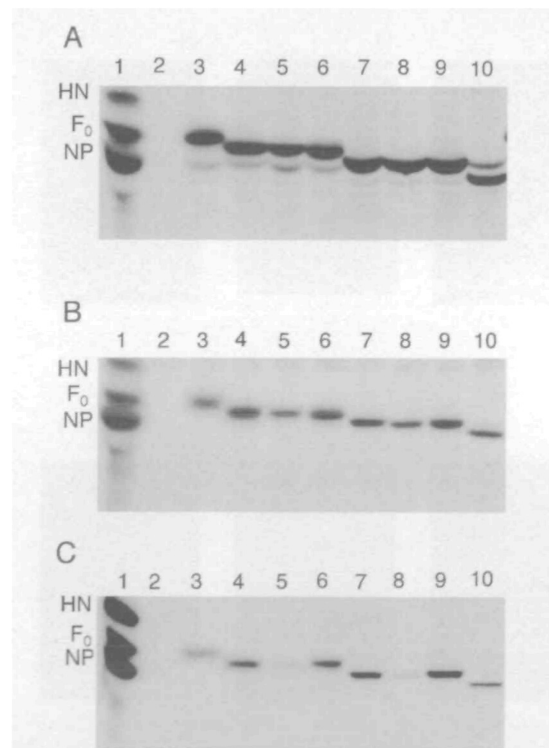

Fig. 3 **Immunoreactivity of the glycosylation-defective mutant F proteins with MAbs.** COS cells transfected with the following plasmids were labeled for 10 min and chased for 1 h at 48 h post transfection. The cell lysates were immunoprecipitated with anti-F antiserum (panel A), MAb f-236 (panel B), or MAb f-49 (panel C) Lane 1, Sendai virus–infected COS-1 cell extract immunoprecipitated with anti-Sendai virus antiserum; lane 2, pSRD; lane 3, pSRD-F; lane 4, pSRD-Fg1$^-$; lane 5, pSRD-Fg2$^-$; lane 6, pSRD-Fg3$^-$; lane 7, pSRD-Fg12$^-$; lane 8, pSRD-Fg23$^-$; lane 9, pSRD-Fg13$^-$, lane 10, pSRD-Fg123$^-$.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

were metabolically labeled with EXPRE^{35}S^{35}S for 10 min and then chased for 1 h in the presence or absence of the glycosylation inhibitor tunicamycin. F proteins were immunoprecipitated with anti-F antiserum and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (*23*). As shown in Fig. 2A, the wild-type F protein had mobility identical to that of the uncleaved F_0 polypeptide (about 66 kDa) expressed in Sendai virus–infected cells (lane 3), while the three single-site mutant polypeptides, Fg1$^-$, Fg2$^-$, and Fg3$^-$, showed increased electrophoretic mobility (corresponding to about 64 kDa) compared to that of the wild-type F_0 (lanes 4–6). Two-site mutants, Fg12$^-$, Fg23$^-$, and Fg13$^-$, migrated faster than the single-site mutants and showed an apparent molecular mass of 60 kDa (lanes 7–9). These differences are consistent with the loss of one or two of the three *N*-linked oligosaccharide chains, each contributing approximately 2,000 to 3,000 Da to the molecular mass of the F protein. The Fg123$^-$ mutant protein migrated even faster, and with the same mobility as the unglycosylated F protein (F_{TM}, Fig. 2A lane 10) synthesized in tunicamycin-treated Sendai virus–infected cells. An unidentified polypeptide, whose mobility is indistinguishable from that of two-site mutant F proteins, was found precipitated by the anti-F antibody. We have not attempted to characterize this polypeptide in detail because it did not occur in Sendai virus–infected cells, but only in F-gene transfected cells. It

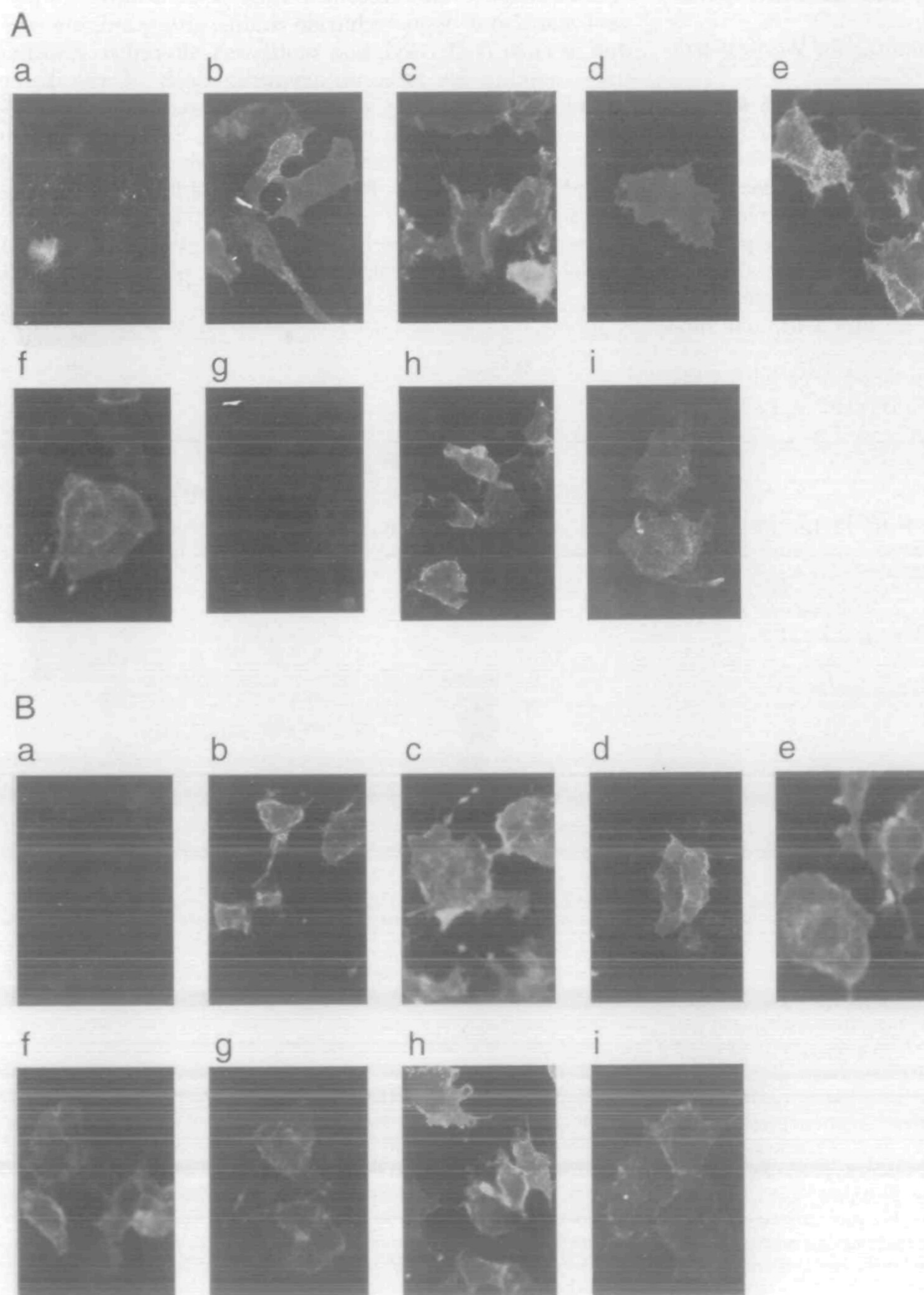

Fig. 4 **Effect of temperature on cell surface transport of the glycosylation-defective mutant F proteins.** Transfected cells were incubated for 48 h at 37°C (A) or 31.5°C (B) as described under "MATERIALS AND METHODS." The cells were then fixed with paraformaldehyde and incubated with anti-F antiserum. Bound antibodies were visualized with FITC-conjugated anti–rabbit IgG. Cells were photographed at a magnification of ×200 a, pSRD, b, pSRD-F; c, pSRD-Fg1$^-$; d, pSRD-Fg2$^-$; e, pSRD-Fg3$^-$; f, pSRD-Fg12$^-$; g, pSRD-Fg23$^-$, h, pSRD-Fg13$^-$, i, pSRD-Fg123$^-$

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

may be pertinent to note that the polypeptide does not seem to be glycosylated, since its mobility is unaffected by tunicamycin treatment as can be seen in Fig 2B below. Figure 2B shows that polypeptides showing the same mobility as F_{TM} were produced in every case where wild-type and glycosylation-defective mutant F proteins were synthesized in the presence of tunicamycin (lanes 3–11). These results led us to conclude that all three glycosylation motifs of F protein are utilized for *N*-linked glycosylation. Unglycosylated HN protein co-precipitated with unglycosylated F protein in lane 11 probably reflected the aggregation of F and HN proteins in tunicamycin-treated cells, since such co-precipitation was not observed in untreated cells (lane 12).

Immunoreactivity of the Glycosylation-Defective Mutant F Proteins with Monoclonal Antibodies—To examine whether the glycosylation-defective mutant proteins can acquire a mature structure or not, we analyzed the reactivity of mutant proteins with a polyclonal antiserum and conformation-specific MAbs f-236 and f-49. MAb f-236 recognized both mature and immature forms of F protein, while MAb f-49 recognized only the mature form of F protein. As shown in Fig. 3A, almost equal amounts of wild-type and glycosylation-defective mutant F proteins were precipitated with the polyclonal antiserum. When the same cell lysates were immunoprecipitated with MAb f-236, wild-type, and glycosylation-defective mutant F proteins were also recovered using this MAb (Fig. 3B), but mutants missing oligosaccharide chain at g2, except for Fg12⁻, were less reactive with this MAb (Fig. 3B, lanes 5, 8, and 10). Differential reactivity with MAbs among the mutant proteins was more pronounced with MAb f-49. Mutant F proteins Fg2⁻, Fg23⁻, and Fg123⁻ showed definitely reduced reactivity toward the MAb (Fig. 3C, lanes 5, 8, and 10). These results indicated that the oligosaccharide chain attached at the g2 site is important for the proper folding of the F protein.

Interestingly, mutants lacking an oligosaccharide chain at g1 showed increased immunoreactivity with MAb f-49 (Fig. 3C, lanes 4, 7, and 9). Thus, Fg2⁻ was less reactive with MAb f-49 than Fg12⁻ (Fig. 3C, lanes 5 and 7) and mutant Fg23⁻ was less reactive with the antibody than mutant Fg123⁻ (Fig. 3C, lanes 8 and 10). This implies that the oligosaccharide chain at g1 may somehow interfere with the folding of the F protein.

Cell Surface Expression of Glycosylation Mutants—Loss of oligosaccharide chains often leads to misfolding of the proteins, and incorrect folding leads to impairment of their cell surface expression. Therefore we tested the transport of the glycosylation-defective mutant F proteins to the cell surface by indirect immunofluorescence staining. As shown in Fig. 4A, cells expressing wild-type F and mutants Fg1⁻, Fg3⁻, and Fg13⁻ (panels b, c, e, and h, respectively) were brightly stained, while those expressing Fg2⁻, Fg12⁻, and Fg123⁻ were faintly stained. Cells expressing Fg23⁻ showed no fluorescence. These results indicated that the oligosaccharide chain attached to the glycosylation site g2 is important for cell surface transport of the F protein. It is noteworthy that the unglycosylated form of F, Fg123⁻ protein was transported to the cell surface, while Fg23⁻ protein was not. The Fg23⁻ protein has only one oligosaccharide chain, at the glycosylation site g1. The oligosaccharide chain attached at g1 may somehow suppress the intracellular transport of the F protein.

Reduced temperature has been reported to alleviate impaired cell surface expression of mutant proteins in some cases (*24–26*). Thus, we tested the effect of temperature reduction on cell surface transport of glycosylation-defective mutant F proteins. Transfected cells were incubated at 37°C for 12 h, and then, after a temperature shift to 31.5°C, were further incubated for 36 h. Indirect immunofluorescence staining was carried out as described under "MATERIALS AND METHODS." Although Fg23⁻-expressing cells were not stained at all after incubation at 37°C, the cells were faintly but positively stained and fluoresced after incubation at 31.5°C (Fig. 4, A and B, panel g). These results showed that the oligosaccharide chains attached to the F protein affected the efficiency of intracellular transport of the F protein, but they were not absolutely required for the transport to the cell surface.

Cell Fusion Activity of Glycosylation-Defective Mutant F Proteins—Cell fusion induction by Sendai virus glycoproteins requires the cleavage of inactive precursor F_0 to the active form complex, F_1-F_2, and co-expression of the HN protein on the surface of the same cells (*2*). Therefore, we first analyzed whether the F_1 peptide is formed from glycosylation-defective mutant F proteins upon treatment with trypsin. COS-1 cells transfected with wild-type or glycosylation-defective mutant F cDNA were washed once with PBS containing 0.1% trypsin at 48 h after transfection The cells were lysed with RIPA buffer, and then the lysate was subjected to Western blot analysis for the occurrence of the F_1 peptide. As shown in Fig. 5, wild-type and all glycosylation-defective mutant F proteins were detected. The mutant proteins showed altered mobilities reflecting the loss of

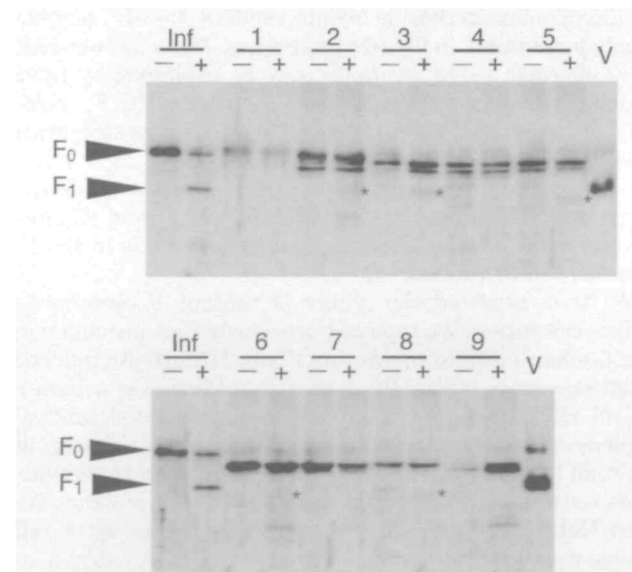

Fig. 5 **Detection of F_1 peptide derived from the glycosylation-defective mutant F_0 protein.** Transfected COS-1 cells were treated with (+) or without (−) 0 1% trypsin at 48 h after transfection Then the cells were lysed in RIPA buffer and cell lysates were analyzed by Western blotting using 1:1,000 dilution of anti-F antiserum. Bound antibodies were detected by 1 10,000 dilution of HRP-conjugated anti–rabbit IgG and ECL system Lane 1, pSRD; lane 2, pSRD-F; lane 3, pSRD-Fg1⁻; lane 4, pSRD-Fg2⁻; lane 5, pSRD-Fg3⁻; lane 6, pSRD-Fg12⁻; lane 7, pSRD-Fg23⁻; lane 8, pSRD-Fg13⁻; lane 9, pSRD-Fg123⁻; lanes Inf, Sendai virus–infected COS-1; lanes V, Sendai virus grown in egg. Asterisks indicate F_1 peptides cleaved from F_0 proteins

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

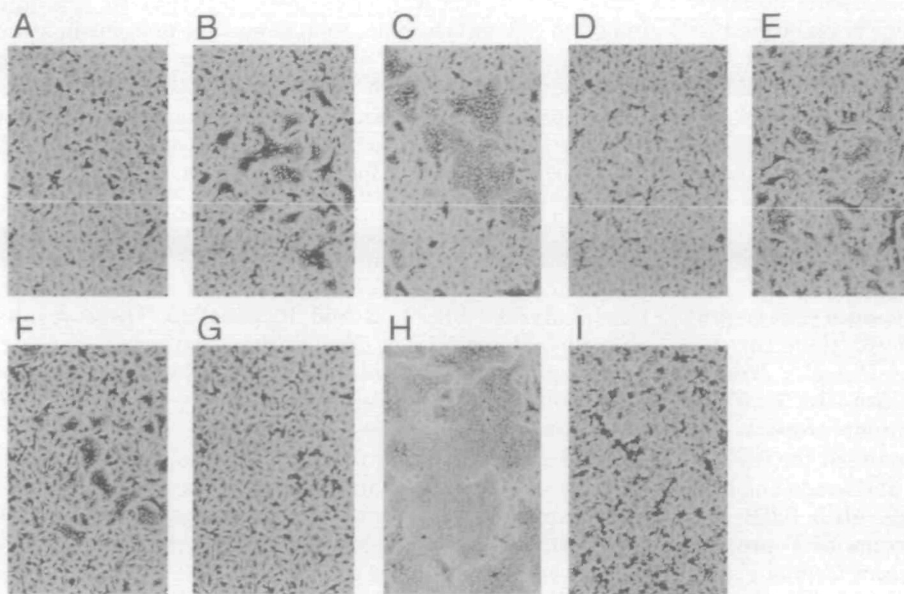

Fig. 6 **Cell fusion induced by co-expressed HN and glycosylation-defective mutant F proteins.** HeLa cells were treated with 0.1% trypsin at 12 h after transfection and further incubated at 37°C for 6 h. The cells were stained with Giemsa's stain and photographed at a magnification of ×200 A, pSRD; B, pSRD-HN-F; C, pSRD-HN-Fg1⁻, D, pSRD-HN-Fg2⁻, E, pSRD-HN-Fg3⁻; F, pSRD-HN-Fg12⁻, G, pSRD-HN-Fg23⁻, H, pSRD-HN-Fg13⁻, I, pSRD-HN-Fg123⁻.

oligosaccharide chains (lanes 3, 4, 5, 6, 7, 8, and 9). F_1, the cleavage product of F_0, was clearly detected with wild-type F and the $Fg1^-$, $Fg3^-$, $Fg12^-$, and $Fg13^-$ mutants [lanes 2(+), 3(+), 5(+), 6(+), and 8(+), respectively], while the F_1 forms of the mutants lacking an oligosaccharide chain attached at g2, except for $Fg12^-$, were not detected [lanes 4(+), 7(+), and 9(+)]. The F_1 fragment derived from the $Fg12^-$ mutant protein seemed to be further degraded into smaller peptides [lane 6(+)]. This implies that the absence of the g2-oligosaccharide chain renders the F_1 peptide highly susceptible to further proteolysis. The apparent lack of F_0 cleavage in $g2^-$ mutants may be explained by their inefficient transportation to the cell surface. The F_1 molecule detected in $Fg1^-$ expressing cells co-migrated with wild-type F_1, whereas those detected in $Fg3^-$, $Fg12^-$, and $Fg13^-$-expressing cells migrated faster than the wild-type F_1 molecule. This reflects the fact that the g2 and g3 sites are located in the F_1 portion, while the g1 site is in the F_2 portion of the F protein.

We then assessed the ability of mutant F proteins to induce cell fusion. We reported previously that introduction of a tandem plasmid containing F and HN cDNAs induced cell fusion more efficiently than co-transfection of separate F and HN cDNAs (*2*). Therefore, we constructed tandem expression plasmids which carried each mutant F gene in addition to the HN gene. Cells transfected with these plasmids were treated with trypsin to cleave the F proteins. We used HeLa cells in the experiments that follow, since cell fusion was readily detectable in these cells. As shown in Fig. 6, wild-type F and glycosylation-defective mutant proteins $Fg1^-$, $Fg3^-$, $Fg12^-$, and $Fg13^-$ co-expressed with HN protein induced cell fusion (panels B, C, E, F, and H, respectively). In contrast, the cells expressing $Fg2^-$, $Fg23^-$, and $Fg123^-$ proteins in addition to HN did not undergo cell fusion. Extensive cell fusion was noted in cells expressing $Fg1^-$ or $Fg13^-$ protein together with the HN protein. We also examined whether cell fusion could be observed at 31.5°C, since cell surface expression of g2 mutants at 37°C was very low, as shown in Fig. 4A. However, we could not observe significant syncytia formation at this temperature either. These results showed that the oligosaccharide chain attached at g2 is important for correct folding of the F protein and induction of cell fusion. On the other hand, lack of the oligosaccharide chain at g1 enhances the cell fusion activity of the F protein.

DISCUSSION

Oligosaccharide chains attached to glycoproteins play important roles in formation and maintenance of their functional structures and their biological functions (*6*). Previous studies on the roles of oligosaccharide chains attached to paramyxovirus glycoproteins F and HN showed that the loss of oligosaccharide chains leads to a deficiency of the intracellular transport, reduction or loss of function and decrease of stability of the proteins (*10*, *27–30*). However, the functional significance of individual sugar chains has not been clearly defined yet. Here we studied the usage of three potential *N*-glycosylation sites and the significance of individual oligosaccharide chains to the biological activities of Sendai virus F protein. Our data clearly showed that all of these sites are occupied by oligosaccharide chains (Fig. 2). Deletion of oligosaccharide chains attached to these sites variously affected the structure and function of the F protein.

In previous reports in which folding of Sendai virus glycoproteins was analyzed in virus-infected cells (*8*) and *in vitro* translation systems (*31*), glycosylation of the F protein was shown to facilitate correct disulfide bond formation and proper folding. Our analysis using mutant F proteins defective in specific glycosylation sites showed that the oligosaccharide chain attached to the g2-site was largely responsible for this folding-promoting effect. At the same time, we showed that the oligosaccharide chain at the g1 site has an opposite effect, namely, its deletion promoted the folding of the F protein (Fig. 3C). Many studies have shown that oligosaccharide chains facilitate the folding of proteins by interacting with molecular chaperones such as calnexin and calreticulin (*32–35*). Tomita *et al.* showed that Sendai virus F protein interact with calnexin during its

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

folding process (*35*). We are now investigating the interactions of various glycosylation-defective mutants with various molecular chaperons to elucidate the roles of individual oligosaccharide chains folding of F protein.

Loss of oligosaccharide-attachment site g1 or g3 did not severely affect the transport of the F protein to the cell surface, while loss of g2 did (Fig. 4). The efficiency of transport of glycosylation-defective mutant F proteins to the cell surface largely paralleled their reactivity with MAb f-49. This is consistent with the notion that loss of oligosaccharide chains causes incorrect folding, and leads to impairment of intracellular transport of proteins (*24, 26, 36, 37*). In fact, our preliminary experiments indicated that more endo H–sensitive products were accumulated in cells expressing mutant F proteins devoid of g2-oligosaccharide chain after 1 h chase following 10 min pulse labeling than those expressing wild-type F protein (Segawa, unpublished observation). It should be noted, however, that this is not always the case, since loss of the g1 site partially alleviated the effect of g2-site deficiency. As a result, nonglycosylated F, namely Fg123⁻ protein, was transported to the cell surface. The deficiency of the intracellular transport of glycosylation-defective mutants was improved at reduced temperature (*24, 26*), so that all the defective proteins, including Fg23⁻, were detected on the cell surface at 31.5°C (Fig. 4B). This implies that glycosylation of F protein is not an absolute requirement for their transport to the cell surface.

Glycosylation-defective mutants lacking an oligosaccharide chain at g2 were very susceptible to trypsin treatment, and glycosylation at g1 again oppositely affected the susceptibility (Fig. 5). Thus, when wild-type and mutant F proteins were co-expressed with HN and the cells were treated with trypsin, the F_1 peptide was detected on the surface of cells expressing wild-type F, Fg1⁻, Fg3⁻, Fg12⁻, and Fg13⁻, but not in those expressing Fg2⁻, Fg23⁻, and Fg123⁻ Syncytia formation roughly paralleled the presence of the F_1 peptide after trypsin treatment (Fig. 6). Loss of the oligosaccharide chain at g1 slightly increased the amount of F_1 protein detected, and markedly enhanced the syncytia formation. The g1-oligosaccharide may negatively regulate the cleavage efficiency of the F protein and the fusion-promoting activity of the F_1-F_2 complex. This consistent with the observation concerning avian influenza virus hemagglutinin (HA) protein, that loss of the oligosaccharide chain near the cleavage site increased cleavage efficiency, and that the virus containing the mutant HA showed strong virulence (*38*). Absence of the g1-oligosaccharide may partially compensate for the destabilizing effect of loss of the g2-oligosaccharide, and may confer fusion-inducing activity on the Fg12⁻ mutant protein.

In conclusion, systematic analyses of Sendai virus F protein mutants defective in glycosylation at well-defined sites led us to conclude that the sugar chain at g2 is indispensable for correct folding and biological activity of the F protein. Our analyses also revealed that the oligosaccharide chain at the g1 site appears to suppress protein folding, and exerts negative control over the efficiency of syncytia formation. Biological significance of this unusual effect of glycosylation is intriguing but unclear. The glycosylation site g3 is definitely glycosylated, but the oligosaccharide chain at this site does not appear to affect any of the biological activities of F protein tested so far.

REFERENCES

1. Morrison, T and Portner, A. (1991) *The Paramyxoviruses* (Kingsbury, D.W, ed.) pp 347-382, Plenum Press, New York
2. Taira, H., Sato, T, Segawa, H., Chiba, M, Katsumata, T, and Iwasaki, K. (1995) Transfection of Sendai virus F gene cDNA with mutations at its cleavage site and HN gene cDNA into COS cells induces cell fusion. *Arch Virol* **140**, 187–194
3. Tashiro, M, Yokogoshi, Y, Tobita, K., Seto, J T, Rott, R., and Kido, H. (1992) Tryptase Clara, an activating protease for Sendai virus in rat lungs, is involved in pneumopathogenicity *J Virol.* **66**, 7211–7216
4. Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N M, Hamaguchi, M, and Nagai, Y. (1990) An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo *EMBO J* **9**, 4189–4195
5. Segawa, H, Kato, M, Yamashita, T., and Taira, H (1998) The roles of individual cysteine residues of Sendai virus fusion protein in intracellular transport *J Biochem* **123**, 1064–1072
6. Rademacher, T.W, Parekh, R.B., and Dwek, R.A. (1988) Glycobiology *Annu Rev Biochem.* **57**, 785–838
7. Schwarz, R T, Rohrschneider, J M., and Schmidt, M F (1976) Suppression of glycoprotein formation of Semliki Forest, influenza, and avian sarcoma virus by tunicamycin *J Virol* **19**, 782–791
8. Mottet, G, Portner, A., and Roux, L (1986) Drastic immunoreactivity changes between the immature and mature forms of the Sendai virus HN and F_0 glycoproteins. *J Virol* **59**, 132–141
9. Machamer, C E, and Rose, J.K. (1988) Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J Biol. Chem* **263**, 5948–5954
10. Ng, D.T, Hiebert, S W., and Lamb, R.A. (1989) Different roles of individual N-linked oligosaccharide chains in folding, assembly, and transport of the simian virus 5 hemagglutinin-neuraminidase. *Mol Cell Biol.* **10**, 1989–2001
11. Malvoisin, E and Wild, F (1994) The role of *N*-glycosylation in cell fusion induced by a vaccinia recombinant virus expressing both measles virus glycoproteins. *Virology* **200**, 11–20
12. Ono, A. and Kawakita, M (1994) Transport of envelope proteins of Sendai virus, HN and F_0, is blocked at different steps by thapsigargin and other perturbants to intracellular Ca_2^+ *J. Biochem.* **116**, 649–656
13. Takebe, Y., Seiki, M., Fujisawa, J, Hoy, P, Yokota, T, Arai, K., Yoshida, M, and Arai, N (1988) SRα promotor: an efficient and verstile mammalian cDNA expression system composed of the simian virus 40 early promotor and the R-U5 segment of the human T-cell leukemia virus type 1 long terminal repeat *Mol Cell. Biol.* **8**, 466–472
14. Shioda, T, Iwasaki, K., and Shibuta, H (1986) Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L protein. *Nucleic Acids Res.* **14**, 1545–1563
15. Blumberg, B M., Giorgi, C, Rose, K., and Kolakofsky, D. (1985) Sequence determination of the Sendi virus fusion protein gene. *J Gen. Virol* **66**, 317–331
16. Itoh, M, Shibuta, H, and Homma, M. (1987) Single amino acid substitution of Sendai virus at the cleavage site of the fusion protein confers trypsin resistance. *J. Gen. Virol* **68**, 2939–2944
17. Miura, N, Ohtsuka, E., Yamaberi, N, Ikehara, M, Uchida, T., and Okada, Y (1985) Use of the deoxyinosine-containing probe to isolate and sequence cDNA encoding the fusion (F) glycoprotein of Sendai virus (HVJ). *Gene* **38**, 271–274
18. Landt, O., Grunert, H.-P, and Halm, U. (1990) A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene* **96**, 125–128
19. Taira, H, Ranin, L., and Iwasaki, K. (1990) Construction of expression plasmids for the fusion protein of Sendai virus, and their expression in *E. coli* and eucaryotic cells. *FEBS Lett.* **262**, 39–41
20. Sanger, F., Nicklen, S., and Coulson, A.R (1977) DNA sequenc-

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

ing with chain-terminating inhibitors. *Proc. Natl. Acad Sci USA* **74**, 5463–5467
21. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175–182
22. Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature* **227**, 680-685
24. Machamer, C.E. and Rose, J.K. (1988) Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding *J Biol Chem.* **263**, 5955–5960
25. Long, D, Cohen, G.H., Muggeridge, M.I., and Eisenberg, R.J. (1990) Cysteine mutants of herpes simplex virus type 1 glycoprotein D exhibit temperature-sensitive properties in structure and function *J. Virol.* **64**, 5542–5552
26. Gallagher, P.J., Henneberry, J.M, Sambrook, J.F, and Gething, M.J. (1992) Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus *J. Virol.* **66**, 7136–7145
27. Alkhatib, G, Shen, S.H., Briedis, D., Richardson, C, Massie, B, Weinberg, R., Smith, D., Taylor, J, Paoletti, E, and Roder, J (1994) Functional analysis of *N*-linked glycosylation mutants of the measles virus fusion protein synthesized by recombinant vaccinia virus vectors. *J. Virol.* **68**, 1522–1531
28. McGinnes, L.W. and Morrison, T.G (1995) The role of individual oligosaccharide chains in the activities of the HN glycoprotein of Newcastle disease virus. *Virology* **212**, 398–410
29. Bagai, S. and Lamb, R.A. (1995) Individual roles of *N*-linked oligosaccharide chains in intracellular transport of the paramyxovirus SV5 fusion protein *Virology* **209**, 250–256
30. Pastey, M.K. and Samal, S.K. (1997) Role of individual *N*-linked oligosaccharide chains and different regions of bovine respiratory syncytial virus fusion protein in cell surface transport. *Arch. Virol* **142**, 2309–2320
31. Vidal, S., Mottet, G, Kolakofsky, D., and Roux, L. (1989) Addition of high-mannose sugars must preced disulfide bond formation for proper folding of Sendai virus glycoprotein. *J Virol.* **63**, 892–900
32. Ou, W.J, Cameron, P.H, Thomas, D.Y., and Bergeron, J.J.M. (1993) Association of folding intermediates of glycoproteins with calnexin during protein maturation *Nature* **364**, 771–776
33. Wada, I, Imai, S, Kai, M, Sakane, F, and Kanoh, H. (1995) Chaperone function of calreticulin when expressed in the endoplasmic reticulum as the membrane-anchored and soluble forms. *J. Biol Chem.* **270**, 20298–20304
34. Trombetta, E.S and Helenius, A. (1998) Lectins as chaperones in glycoprotein folding. *Curr. Opin. Struct. Biol.* **8**, 587–592
35. Tomita, Y., Yamashita, T., Sato, H, and Taira, H. (1999) Kinetics of interactions of Sendai virus envelope glycoproteins, F and HN, with endoplasmic reticulum-resident molecular chaperones, BiP, calnexin, and calreticulin *J Biochem* **126**, 1090–1100
36. Matzuk, M.M. and Boime, I (1988) The role of the asparagine-linked oligosaccharides of the alpha subunit in the secretion and assembly of human chorionic gonadotrophin *J. Cell Biol* **106**, 1049–1059
37. Feng, W., Matzuk, M.M, Mountjoy, K., Bedows, E, Ruddon, R.W., and Boime, I (1995) The asparagine-linked oligosaccharides of the human chorionic gonadotropin beta subunit facilitate correct disulfide bond pairing. *J. Biol Chem.* **270**, 11851–11859
38. Deshpande, K.L, Fried, V.A., Ando, M, and Webster, R.G. (1987) Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc. Natl. Acad. Sci. USA* **84**, 36–40

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012