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Kinetics of Interactions of Sendai Virus Envelope Glycoproteins, F and HN, with Endoplasmic Reticulum-Resident Molecular Chaperones, BiP, Calnexin, and Calreticulin[1]

Yasuhiro Tomita,* Tetsuro Yamashita,* Hironori Sato,† and Hideharu Taira*,[2]

*Department of Bioscience and Technology, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550; and †Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640

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Sendai virus envelope glycoproteins, F and HN, mature during their transport through the endoplasmic reticulum (ER) and Golgi complex. To better understand their maturation processes in the ER, we investigated the time course of their interactions with three ER-resident molecular chaperones, BiP, calnexin (CNX), and calreticulin (CRT), in Sendai virus-infected HeLa cells. Pulse-chase and immunoprecipitation analyses using antibodies against each virus glycoprotein or ER chaperone revealed that F precursor interacted with CNX transiently ($t_{1/2}$=8 min), while HN protein displayed longer and sequential interactions with BiP ($t_{1/2}$=8 min), CNX ($t_{1/2}$=15 min), and CRT ($t_{1/2}$=20 min). HN interacted with the three ER chaperones not only as a monomer but also as a tetramer for several hours, suggesting mechanism(s) to undergo chaperone-mediated quality control of an assembled HN oligomer in the ER. The kinetics of dissociation of the HN-chaperone complexes exhibited a marked delay in the presence of proteasome inhibitors, suggesting that a part of HN associated with BiP, CNX, and CRT is destined to be degraded in the proteasome-dependent pathway. Further, the associations between virus glycoproteins and CNX or CRT were impaired by castanospermine, an inhibitor of ER glucosidase I and II, confirming that these interactions require monoglucosylated oligosaccharide on F_0 and HN peptides. These findings together suggest that newly synthesized F protein undergoes rapid maturation in the ER through a transient interaction with CNX, whereas HN protein requires more complex processes involving prolonged association with BiP, CNX, and CRT for its quality control in the ER.

Key words: BiP, calnexin, calreticulin, molecular chaperone, proteasome.

Like cellular glycoproteins, viral glycoproteins are generally synthesized *via* the host secretory pathway in the ER, which has a specialized environment for ensuring folding and assembly of newly synthesized secretory and integral membrane proteins, and for degradation of misfolded proteins (*1-3*). The ER of mammalian cells contains molecular chaperones and foldases, which are required for formation of the active structures of newly synthesized peptides and thus serve as components of the ER quality control system. The ER-resident chaperones include BiP, calnexin (CNX), and calreticulin (CRT). BiP, a member of the heat shock protein 70 (HSP70) family, interacts with hydrophobic surfaces of immature proteins in the ER (*4, 5*). CNX, a type I integral membrane protein (*6*), and its soluble homologue CRT (*7*) have lectin-like activity and preferentially bind to newly synthesized glycoproteins carrying monoglucosylated *N*-linked oligosaccharide(s). Many viral (*8-10*) and cellular (*11-15*) glycoproteins have been reported to interact with BiP, CNX, and/or CRT during their maturation processes in the ER.

Sendai virus is a negative-strand RNA virus which belongs to the paramyxoviridae. It has two envelope glycoproteins, fusion protein (F) and hemagglutinin-neuraminidase (HN), which mediate virus entry into host cells and cell-to-cell fusion between infected and uninfected cells. The two glycoproteins differ in their topology on the lipid-bilayer membrane, folding rate, proteolytic processing, and oligomerization pattern. The F glycoprotein is a type I integral membrane protein which consists of 565 amino acids, while the HN glycoprotein is a 575-amino-acid type II integral membrane protein. The F protein exhibits a folding half-time of 5 min, while the HN protein displays a longer half-time of 10-30 min for folding into an assembly-competent conformation (*16*). The F protein is synthesized as an inactive precursor (F_0), which is then cleaved by cellular proteases to form the biologically active protein consisting of disulfide-linked subunits F_1 and F_2 (*17-19*); but the HN protein does not undergo such proteolytic processing. Finally, the F protein forms a homo-oligomer

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

Abbreviations: ER, endoplasmic reticulum; CNX, calnexin; CRT, calreticulin; CST, castanospermine; endo H, endo-β-*N*-acetylglucosaminidase H.

shortly after its synthesis, while the HN protein forms a homo-tetramer consisting of two disulfide-linked dimers (*18, 19*). These differences raise the possibility that the F and HN proteins interact with different ER chaperones with different kinetics. However, the study of the interactions of Sendai virus glycoproteins and ER chaperones is limited to a single report, which suggests that HN protein interacts with BiP with a half-time of 30 min during its maturation process in BHK cells (*20*).

To understand the maturation processes of Sendai virus F and HN proteins in the ER, we investigated here the kinetics of interactions of the F and HN glycoproteins with three ER molecular chaperones, BiP, CNX, and CRT, in the Sendai virus-infected HeLa cells. For this purpose, we prepared antibodies against each of the Sendai glycoproteins and ER chaperones. Anti-chaperone antibodies were raised against synthetic peptides corresponding to the C-terminal region of each chaperone to obtain antibodies which sustain the chaperone-substrate interaction. We show here, on the basis of a series of pulse-chase and immunoprecipitation experiments using these antibodies, that F and HN proteins interact with different combinations of ER chaperones with different kinetics. In addition, we found that the ER chaperones interacted not only with an HN monomer but also with an HN tetramer and that some of them were selectively degraded by a proteasome-dependent pathway, providing the first evidence that the ER chaperones participate also in the quality control of an assembled oligomeric viral protein.

MATERIALS AND METHODS

Chemical Reagents—Antipain, leupeptin, Z-Leu-Leu-Leu-H (aldehyde) (MG-132), and Z-Leu-Leu-H (aldehyde) (ZLLal) were from Peptide Institute (Osaka). Castanospermine (CST) were from Wako Pure Chemical Industries (Osaka). EXPRE^{35}S^{35}S protein labeling mix was from Du Pont/NEN (Wilmington, DE). Hexokinase (type III: from bakers yeast) was from Sigma Chemical (St. Louis, MO). Protein A-Sepharose 4 Fast Flow was from Pharmacia Biotech (Uppsala, Sweden). Lactacystin was from Calbiochem (La Jolla, CA). Endo-β-*N*-acetylglucosaminidase H (endo H) was from Boehringer Mannheim Biochemica (Mannheim, Germany).

Cell Culture and Viruses—HeLa cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% newborn bovine serum (NBS) at 37°C in 5% CO_2. Sendai virus Z strain was prepared from the allantonic fluid of 9-day-old chick embryos after infection, purification by discontinuous sucrose gradient centrifugation, and determination of its plaque forming unit (PFU) in LLC-MK_2 cells (*21*).

Antibodies—Anti-Sendai virus antiserum was prepared by infecting rabbits with Sendai virus. Rabbit anti-F and anti-HN sera were raised against non-glycosylated F protein expressed in *Escherichia coli* and against HN protein purified from virions by SDS-PAGE, respectively, (*22*). Rabbit anti-human BiP, anti-human calnexin, and anti-human calreticulin antisera were raised against synthetic peptides corresponding to residues 632 to 653 (*23*), 579 to 592 (*24*), and 399 to 417 (*25*) for BiP, calnexin, and calreticulin, respectively. To obtain anti-chaperone antibodies which can sustain chaperone-substrate interaction, the C-terminal portion of each chaperone was chosen as immunogen because (i) the N-terminal portion of chaperone is the signal peptide and thus must be cleaved during maturation in the ER, and (ii) the internal portion of the chaperone is functionally important in substrate binding (*26*). The three synthetic antigenic oligopeptides were conjugated with keyhole limpet hemocyanin by using *m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester and injected subdermally into rabbits with Freund's complete adjuvant to raise polyclonal antisera. To increase specificity of antibodies, the sera were further purified using affinity columns (AF-Amino Toyopearl) coupled with the synthetic peptides (*27*).

Virus Infection and Metabolic Labeling—Confluent HeLa cells in 35-mm dishes were washed twice with MEM, then infected with Sendai virus at a multiplicity of infection of 10 PFU per cell for 1 h at 37°C, and the culture medium was replaced with fresh medium. At 6 h post infection, virus-infected cells were washed twice with methionine- and cysteine-free MEM and incubated in the same medium for 30 min at 37°C. The cells were pulse-labeled for 10 min with 200 μCi of [^{35}S]methionine/cysteine (EXPRE^{35}S^{35}S protein labeling mix) per ml of methionine- and cysteine-free MEM. The labeled cells were washed with prewarmed phosphate-buffered saline (PBS), then chased in MEM-10% NBS supplemented with 5 mM Met/Cys. At the end of the chase, the cells were washed with ice-cold PBS containing 10 mM iodoacetoamide (IAA) to alkylate free sulfhydryl groups, and lysed on ice for 10 min with 800 μl of RIPA buffer containing 1% (v/v) Nonidet P-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% (w/v) SDS supplemented with 10 mM IAA, 10 mM glucose, 2 U/ml hexokinase, and the protease inhibitors (1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml antipain, and 10 μg/ml leupeptin). The cell lysates were homogenated by sonicating for 30 s at 4°C, centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was subjected immediately to the immunoprecipitation.

For CST treatment of infected cells, CST (final concentration 1 mM) was added to growth medium for 2 h prior to pulse labeling. The equivalent concentration of CST was added to the medium during the starvation and the chase. In the case of CST-treated cells, PBS and RIPA buffer containing 20 mM IAA were used to prevent the formation of artificial intermolecular disulfide bonds between excess misfolded molecules. For proteasome inhibitor treatment, infected cells were incubated in growth medium containing 10 μM lactacystin, 5 μM MG-132, or 5 μM ZLLal for 2 h prior to pulse labeling. Equivalent concentration of inhibitor was added throughout the subsequent starvation, pulse labeling, and chase period. Dimethyl sulfoxide was added to medium of control cells at a final concentration of 0.1% during the starvation and the chase.

Immunoprecipitation and Electrophoresis—The anti-chaperone antibody preparations used for the immunoprecipitation were examined for their reactivity to the BiP, CNX, and CRT molecules derived from HeLa cells before experiments, and excess amounts of antibodies were used to ensure quantitative immunoprecipitation. Cell lysate was divided into four aliquots: one (200 μl) was incubated with 8 μl of a mixture of anti-F and anti-HN antisera, and the other three were incubated with an excess of anti-BiP, anti-CNX, or anti-CRT antibodies (3-10 μl), respectively,

at 4°C for 16 h. Twenty microliters of a 50% suspension protein A-Sepharose was added to each cell lysate/antibody mixture, which was then incubated at 4°C for 1 h. The immune complexes adsorbed on the beads were washed three times at 4°C with NET buffer containing 0.1% (v/v) NP-40, 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.02% (w/v) NaN_3.

For sequential immunoprecipitation, immune complexes obtained by use of anti-chaperone antibodies were dissolved in 20 μl of 1% SDS/TE buffer containing 1% (w/v) SDS, 10 mM Tris HCl (pH 7.5), and 1 mM EDTA by heating at 65°C, then diluted with 180 μl of dilution buffer containing 1% (w/v) NP-40, 10 mM Tris HCl (pH 7.5), and 100 mM NaCl. Twenty microliters of protein A-Sepharose was added to diluted samples to remove rabbit IgG used in the first round of immunoprecipitation, then cell lysates were incubated for 1 h at 4°C. After removing protein A-Sepharose, anti-F or anti-HN antiserum was added to the supernatant, and the mixture was incubated at 4°C for 16 h, then incubated with 20 μl of protein A-Sepharose for 1 h at 4°C.

The immune complexes were washed twice with NET buffer, denatured in SDS sample buffer containing 6.25 mM Tris HCl (pH 6.8), 2% (w/v) SDS, and 10% (v/v) glycerol in the presence or absence of 5% 2-mercaptoethanol (2-ME) by boiling for 5 min, then separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions or by 7.5% gel electrophoresis under non-reducing conditions (*22*). Viral protein size marker was prepared by labeling the Sendai virus-infected HeLa cells with 200 μCi/ml [^{35}S]Met/Cys for 1 h, followed by immunoprecipitation with anti-Sendai virus antiserum. Gels were fixed with a mixture of 10% acetic acid and 30% methanol, dried and scanned with a Molecular Imaging System GS-525 (Nippon Bio-Rad Laboratories). Radioactive bands were quantified with Multi-Analyst software and the imaging system.

Endoglycosidase H Digestion—The immune complexes adsorbed on protein A-Sepharose were resuspended in 50 μl of 50 mM sodium acetate buffer (pH 5.5) and incubated with 2 mU of endo H for 16 h at 37°C. After digestion, the immune complexes were denatured by boiling for 5 min with SDS-sample buffer containing 5% 2-ME, then separated by 10% SDS-PAGE.

RESULTS

Time Course of Processing of Sendai Virus F_0 and HN Proteins in HeLa Cells—To examine the maturation processes of F and HN in HeLa cells, Sendai virus-infected cells were pulse-labeled with [^{35}S]Met/Cys at 6 h post-infection and chased for up to 360 min. The cells were lysed, immunoprecipitated with a mixture of anti-F and anti-HN antisera, and analyzed by SDS-PAGE under the reducing or non-reducing conditions. As shown in the Fig. 1A, F protein precursor (F_0) was detected under the non-reducing conditions as two closely migrating bands: the faster one was detected only transiently and disappeared within 30 min of chase, while the slower one was detected after 10 min of chase and continuously thereafter. Proteolytic cleavage of F_0 protein was not observed during the 360-min chase period, which is consistent with the accepted notion that the cleavage does not occur in the tissue culture (*1*). The oligomeric form of F protein was not detected under the present experimental conditions, probably because the association between F monomers is weak and readily disrupted unless the cross-linker is present (*17*).

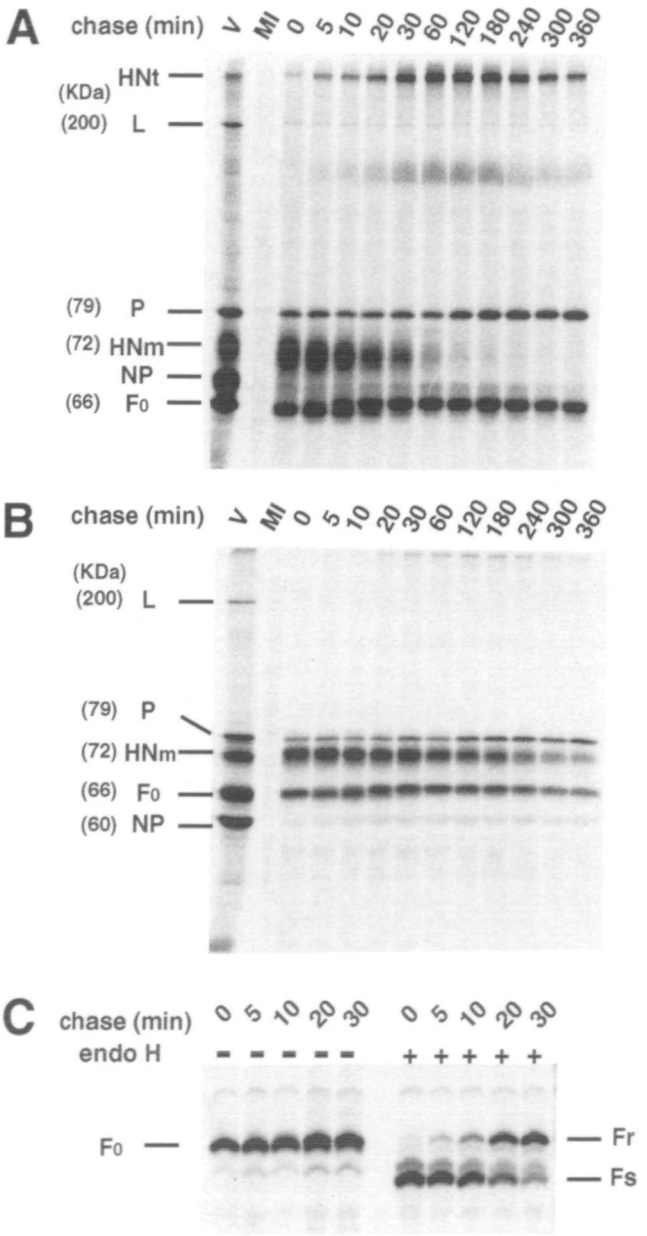

Fig. 1. **Time course of processing of Sendai virus F_0 and HN proteins in HeLa cells.** Sendai virus-infected HeLa cells were labeled for 10 min with [^{35}S]Met/Cys and chased between 0 and 360 min after the pulse labeling. The cells were lysed at the indicated times with RIPA buffer, alkylated with 10 mM IAA, and immunoprecipitated with a mixture of anti-HN and anti-F antisera. The immunoprecipitates were separated by 7.5% SDS-PAGE and visualized with a Molecular Imager. (A) SDS-PAGE under non-reducing conditions. (B) SDS-PAGE under reducing conditions. (C) F_0 proteins immunoadsorbed with anti-F antiserum on protein A-Sepharose were incubated in the presence (+) or absence (−) of 2 mU endoglycosidase H (endo H) at 37°C for 16 h, then separated by reducing 10% SDS-PAGE. V, viral marker; MI, mock infection; HNm, HN monomer; HNt, HN tetramer; Fr or Fs, endo H-resistant or -sensitive form of F_0 protein.

On the other hand, under the non-reducing conditions, a high molecular weight protein corresponding to the size of HN tetramer was detected together with a monomeric form of HN (Fig. 1A, HNm and HNt), as reported previously (*28*). The high molecular weight protein was converted to the size of the HN monomer on the reducing SDS-PAGE (Fig. 1B), indicating that it was indeed an assembled HN oligomer. In contrast, the HN dimer, an intermediate of the HN tetramer (*28*), was not detected under either non-reducing or reducing conditions, suggesting that its detection depends on the type of antibody used for the immunoprecipitation.

The two molecular forms of F_0 seen in the non-reducing SDS-PAGE were also detected in the reducing SDS-PAGE (Fig. 1B): the faster-migrating band was detected only transiently and replaced by a more slowly migrating band between 20 and 30 min of chase, suggesting that the difference in the electrophoretic mobility was not related to the disulfide bond formation of peptides. To examine the characteristics of glycosylation of the two F_0 proteins, they were subjected to endo-β-glycosidase H (endo H) digestion. As shown in Fig. 1C, the faster-migrating band was processed to the endo H-sensitive form (Fs), while the more slowly migrating one remained as the endo H-resistant form (Fr), indicating that the two closely migrating F_0 proteins differed in the processing of the *N*-linked oligosaccharides. Nearly all F_0 proteins had become resistant to the endo H at 30 min of chase, suggesting that newly synthesized F peptides were transported into the *medial* Golgi compartment within 30 min.

To examine the rate of transport of HN protein into the Golgi complex, susceptibility of HN to endo H was examined. Unexpectedly, however, all of the HN proteins remained sensitive to this enzyme even at 360 min of chase, suggesting that all of the oligosaccharides on the HN protein were processed to the high mannose type rather than the complex type during the maturation in the HeLa cells (data not shown). Indirect immunofluorescent staining of the formaldehyde-fixed cells using the anti-F or anti-HN serum showed that the intense fluorescent signals were localized on the cell surface of the Sendai virus-infected HeLa cells, and approximately 40% of the total labeled HN proteins were recovered by the cell-surface specific immunoprecipitation, suggesting that some, if not all, of the viral glycoproteins synthesized in the HeLa cells were transported to the cell surface (data not shown).

Quantification of radioactivity of the F_0 bands obtained with reducing SDS-PAGE in Fig. 1B showed that F protein reached the maximum level within 30 min, then decreased with a half-time of about 300 min (Fig. 2A). Similarly, the amount of HN protein was maximal within 30 min of chase, then decreased with a half-time of approximately 200 min (Fig. 2A). Formation of HN monomer and tetramer was also quantified on the basis of results of the non-reducing SDS-PAGE in Fig. 1A. The amount of HN monomer was maximal at 5 min of chase, then decreased with a half-time of approximately 25 min (Fig. 2B). In parallel with the decrease in HN monomer, the amount of HN tetramer increased and reached a maximum at 60 min of chase, maintained the maximum level between 60 min and 180 min of chase, then decreased with a half-time of approximately 240 to 300 min (Fig. 2B). The time courses of F_0 and HN monomer production and of HN oligomerization seen in

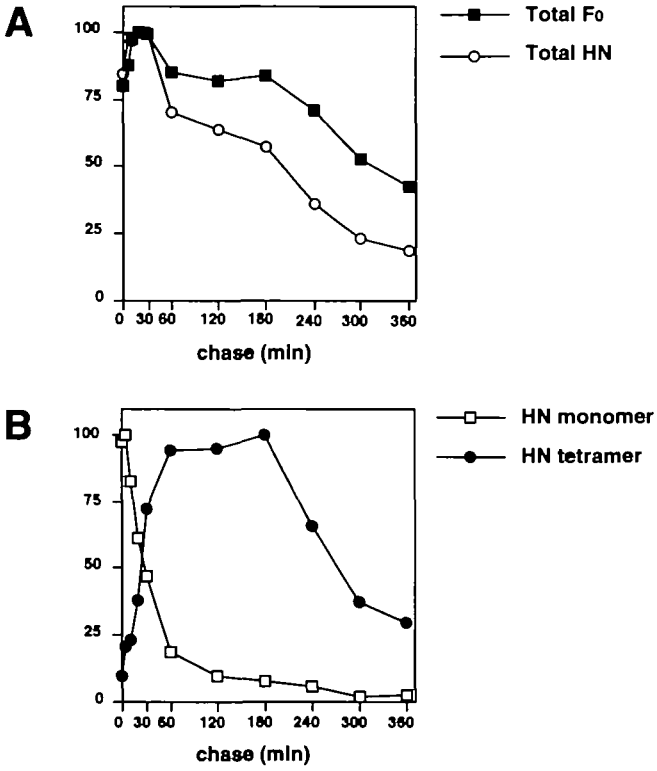

Fig. 2. **Quantitation of kinetics of F_0 and HN production in HeLa cells.** Radioactivity of the bands shown in Fig. 1, A and B, were quantified by use of a Molecular Imager and expressed as the percentage of the maximum amount of the immunoprecipitated F_0 or HN protein during the chase period. (A) Kinetics of production of the F_0 and HN proteins on the reducing gel in Fig. 1B. (B) Kinetics of production of HN monomer and HN tetramer on the non-reducing gel in Fig. 1A.

Fig. 2, A and B, were highly reproducible in the three independent experiments (data not shown) and were similar to those reported for the envelope glycoproteins of other paramyxoviruses (*1*).

Time Course of Interactions of F_0 and HN Proteins with ER Chaperones—To investigate the kinetics of interaction of F_0 and HN proteins with BiP, CNX, and/or CRT, Sendai virus-infected ^{35}S-labeled HeLa cells were chased between 0 and 360 min, lysed, and immunoprecipitated with antibodies to BiP, CNX, or CRT. A significant amount of HN monomer was co-precipitated with BiP immediately after the pulse labeling, while only a trace of F_0 monomer was detected at this time (Fig. 3A, HNm and F_0). Notably, we found that the BiP also associated with a high molecular weight protein corresponding to the size of HN tetramer during the 6-h chase period (Fig. 3A, HNt). This high molecular weight protein was converted to HN monomer on SDS-PAGE under the reducing conditions, indicating that it was indeed an assembled HN oligomer (data not shown). Furthermore, when an excess of ATP was added to the cell lysates, neither HN monomer nor tetramer were precipitated with the anti-BiP antibody, suggesting that the HN-BiP interaction seen in Fig. 3A was regulated by intrinsic ATPase activity (data not shown).

Both CNX and CRT are reported to bind preferentially to newly synthesized glycoproteins with monoglucosylated *N*-linked oligosaccharide(s) and thus are probably involved

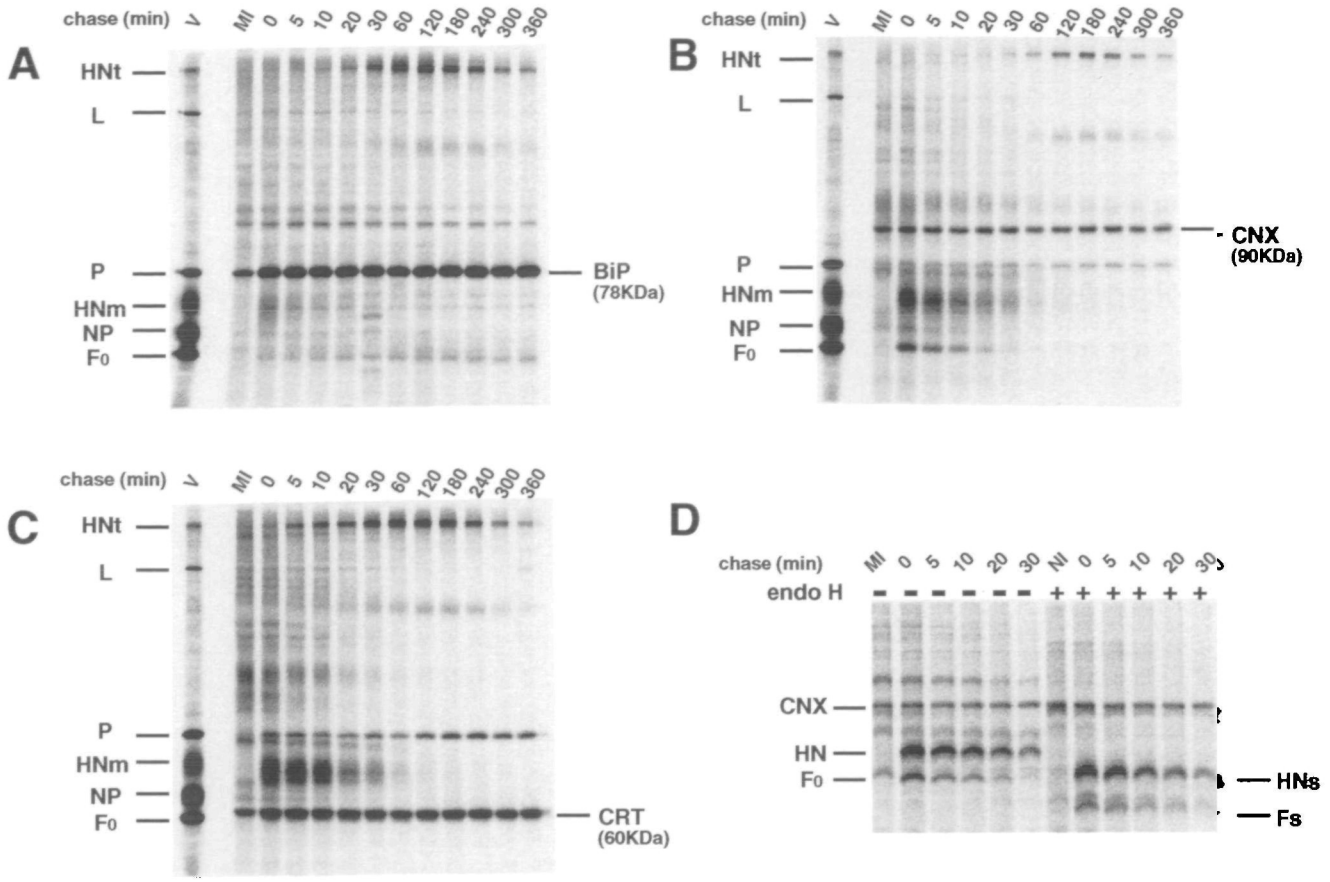

Fig. 3. **Time course of interactions of F_0 and HN proteins with ER chaperones in Sendai virus infected HeLa cells.** Aliquots of the samples used for the experiments in Fig. 1, A and B, were immunoprecipitated with anti-BiP (A), anti-CNX (B), or anti-CRT (C) antibody. The immunoprecipitates were separated by non-reducing 7.5% SDS-PAGE and visualized using a Molecular Imager. (D) Virus-infected cells were labeled for 10 min with [^{35}S]Met/Cys and chased for the indicated times. Proteins co-immunoprecipitated with anti-CNX antibody were incubated in the presence (+) or absence (−) of 2 mU endoglycosidase H at 37°C for 16 h, then analyzed by reducing 10% SDS-PAGE.

in maturation of F and HN. As shown in Fig. 3B, CNX was indeed able to complex with the F_0 and HN monomers. Interestingly, CNX also interacted with the HN tetramer, although the interaction was much slower and more prolonged than that with the monomer. Similarly, CRT interacted with both HN monomer and tetramer (Fig. 3C). Since the CRT migrated to a similar position to the F_0 protein, we could not determine from this experiment whether the CRT could bind to the F protein and assessed this issue later. Treatment of the immunoprecipitates with endo H at various chase periods showed that F_0 molecules co-precipitated with CNX were sensitive to endo H (Fig. 3D), confirming that the F_0-CNX interaction occurred in the ER and/or *cis* Golgi. The same type of analysis could not be applied to the HN-chaperone interactions because all of the HN was endo H sensitive during the chase period as descried above.

Quantification of the radioactivity of the bands corresponding to F_0 in Fig. 3B indicated that the amount of F_0-CNX complex became maximum during the 10 min of pulse labeling and decreased rapidly thereafter with a half-time of 8 min (Fig. 4A). The same kinetics was obtained with reducing SDS-PAGE (data not shown). Similarly, the amount of HN monomer bound to BiP, CNX, or CRT was maximum at 0 min of chase (Fig. 4B). The amount of HN monomer co-precipitated with BiP, CNX, and CRT decreased sequentially with a half-time of 8, 15, and 20 min, respectively (Fig. 4B). In contrast to the rapid and transient interactions between the viral protein monomers and chaperones, interactions between HN tetramer and ER chaperones proceeded much more slowly. The amount of HN tetramer co-precipitated with BiP or CRT started to increase from 20 min of chase, reached a maximum level between 60–120 min of chase, and decreased after 180 min of chase with a half-time of approximately 240 min (Fig. 4C). The amount of HN tetramer co-precipitated with CNX increased from 60 min of chase, reached a maximum level around 180 min of chase, then decreased with a half-time of 300 min. The ordered kinetics of interaction of HN proteins with the three ER chaperones seen in the Fig. 4, B and C, was highly reproducible in three independent experiments. The kinetics of the chaperone-HN tetramer interaction was not affected by the addition of cycloheximide in the chase media, indicating that these interactions were not caused by the overproduction of the viral proteins (data not shown).

To examine the potential association of CRT with F_0, and to confirm that the proteins co-precipitated with ER

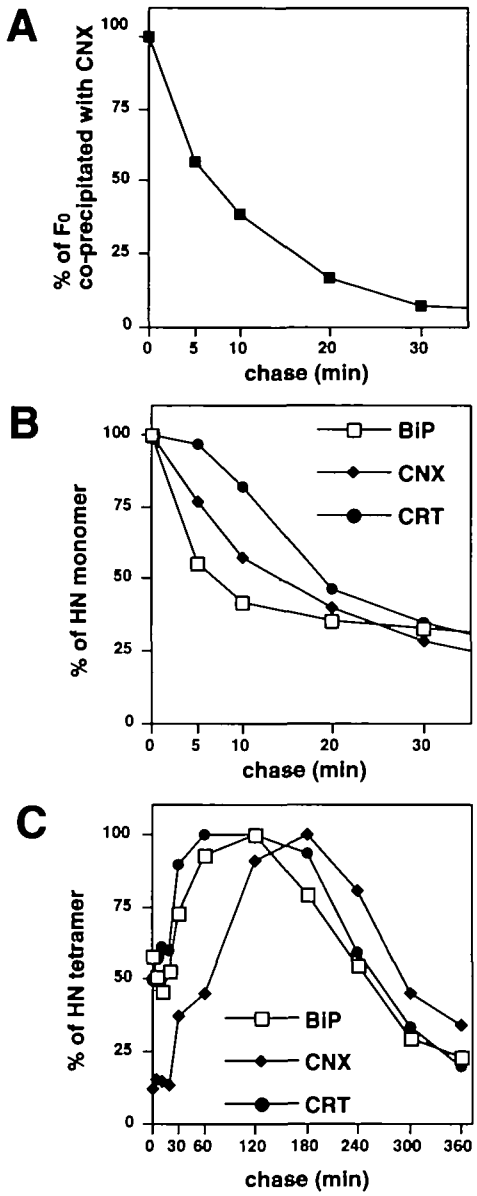

Fig. 4. **Quantification of kinetics of the F_0 and HN proteins co-precipitated with BiP, CNX, and CRT.** Radioactivity of the bands corresponding to F_0 monomer, HN monomer, and HN tetramer in Fig. 3, A, B, and C, were quantified using a Molecular Imager. The relative amounts of F_0 (A), HN monomer (B), and HN tetramer (C) co-precipitated with chaperones at each time point are expressed as a percentage of the maximum amount of F_0 or HN protein co-precipitated with the ER chaperones.

chaperones are the Sendai virus glycoproteins, the immunoprecipitates obtained with anti-chaperone antibodies were subjected to immunoprecipitation with anti-F or anti-HN antibody. As shown in Fig. 5A, a faint band was detected with the sequential immunoprecipitate obtained with anti-CRT and anti-F antibodies. However, the signal was much weaker than that obtained with anti-CNX and anti-F antibodies, suggesting that the association between F_0 and CRT, if there is any, is much more fragile or transient than that between F_0 and CNX. In contrast to the F_0 protein, HN monomers were readily recovered from all of the precipitates made with anti-BiP, anti-CNX, or anti-CRT antibody (Fig. 5B). Protein corresponding in size to the HN tetramer was again recovered by the sequential immunoprecipitation (Fig. 5B). This high molecular weight protein was converted to HN monomer on SDS-PAGE under reducing conditions, confirming that it was indeed an assembled HN oligomer (data not shown).

Effect of Proteasome Inhibitors on the Interactions between Viral Glycoproteins and ER Chaperones—As shown in Fig. 2A, amounts of viral glycoproteins in the infected cells stayed at a similar level from 60 to 180 min, then decreased from 180 to 360 min. This biphasic kinetics from 60 to 360 min of the chase period resembled the kinetics of HN tetramer production (Fig. 2B) or of HN tetramer-chaperone complexes formation (Fig. 4C) during the same chase period. These observations might reflect the incorporation of assembled viral glycoproteins into the budding virions after 180 min of chase. They might also reflect the intracellular degradation of misfolded or misassembled glycoproteins that were retained in the ER in association with chaperones. In this regard, a mutant of cystic fibrosis transmembrane conductance regulator protein, which lost the potential to be transported into the cell surface membrane and was thus retained in the ER, was rapidly degraded by the cytosolic proteasome (*29*).

To examine the possibility of the proteasome-dependent degradation of F_0 and HN proteins, Sendai virus-infected HeLa cells were treated with the following metabolic inhibitors before and after pulse labeling and chase, as studied for CD4 degradation mechanisms in the HeLa cells (*30*): lactacystin (LC), a proteasome-specific inhibitor (*31*); MG-132, an inhibitor of proteasomes and calpains; and ZLLal, a calpain-specific inhibitor (*32*). As shown in Fig. 6A, no significant effect was detected in the kinetics of processing of F_0 proteins in the presence of these three metabolic inhibitors, which was confirmed by the quantification of the radioactivity of the bands corresponding to the F_0 protein (Fig. 6B, left panel). In contrast, treatment of the LC resulted in a marked delay in the decrease of HN monomer and tetramer (Fig. 6A, Lactacystin). A similar effect was caused also by MG-132 but not by ZLLal (Fig. 6A, MG-132 and ZLLal). Quantification of the radioactivity of the bands corresponding to the HN monomer and tetramer in Fig. 6A indicated that HN monomer and tetramer decreased significantly more slowly in the presence of LC or MG-132 than they did in the presence of ZLLal or in the untreated control cells (Fig. 6B, center and right).

To ascertain the effects of these metabolic inhibitors on the interactions of F_0 and HN proteins with ER chaperones, the cell lysates used in the above experiments were immunoprecipitated with each of the three anti-chaperone antibodies and analyzed by SDS-PAGE. The radioactivity of bands corresponding to HN monomer or tetramer were quantified. LC, MG-132, and ZLLal did not affect the kinetics of the interaction of HN monomer with BiP or CNX. However, LC and MG-132 but not ZLLal caused significant delay in the kinetics of HN monomer-CRT dissociation, with a half-time of 90 min (Fig. 7, upper panels). Furthermore, the kinetics of dissociation of HN tetramers-chaperone complexes were equally delayed in the presence of LC and MG-132, with half-times from 4 h to over 6 h (Fig. 7, lower panels). These attenuated effects

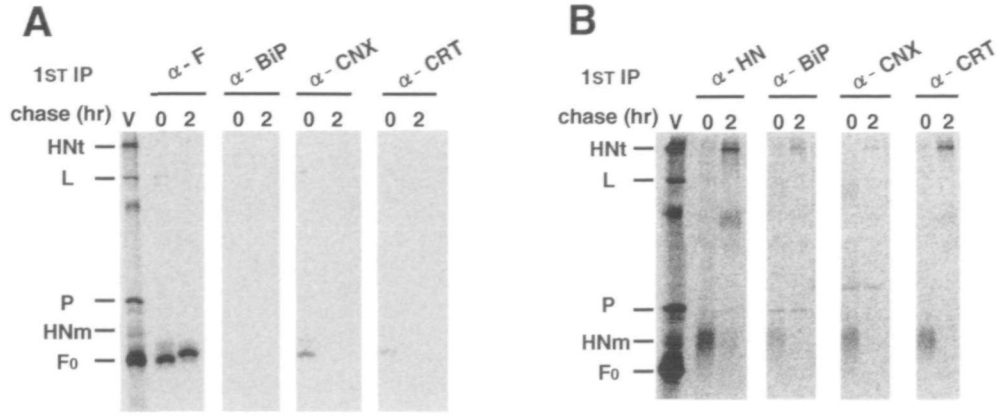

Fig. 5. **Sequential immunoprecipitation.** Sendai virus-infected cells were labeled for 10 min with [^{35}S]Met/Cys and chased for the indicated time, then immunoprecipitated with each anti-chaperone antibody. The immunoprecipitates were dissolved in buffer containing 1% (w/v) SDS and 1 mM EDTA at 65°C, diluted with excess amount of buffer containing 1% (w/v) NP-40, reacted with protein A-Sepharose to remove rabbit IgG, subjected to the immunoprecipitation with anti-F (A) or anti-HN (B) antiserum, and separated by non-reducing 7.5% SDS-PAGE.

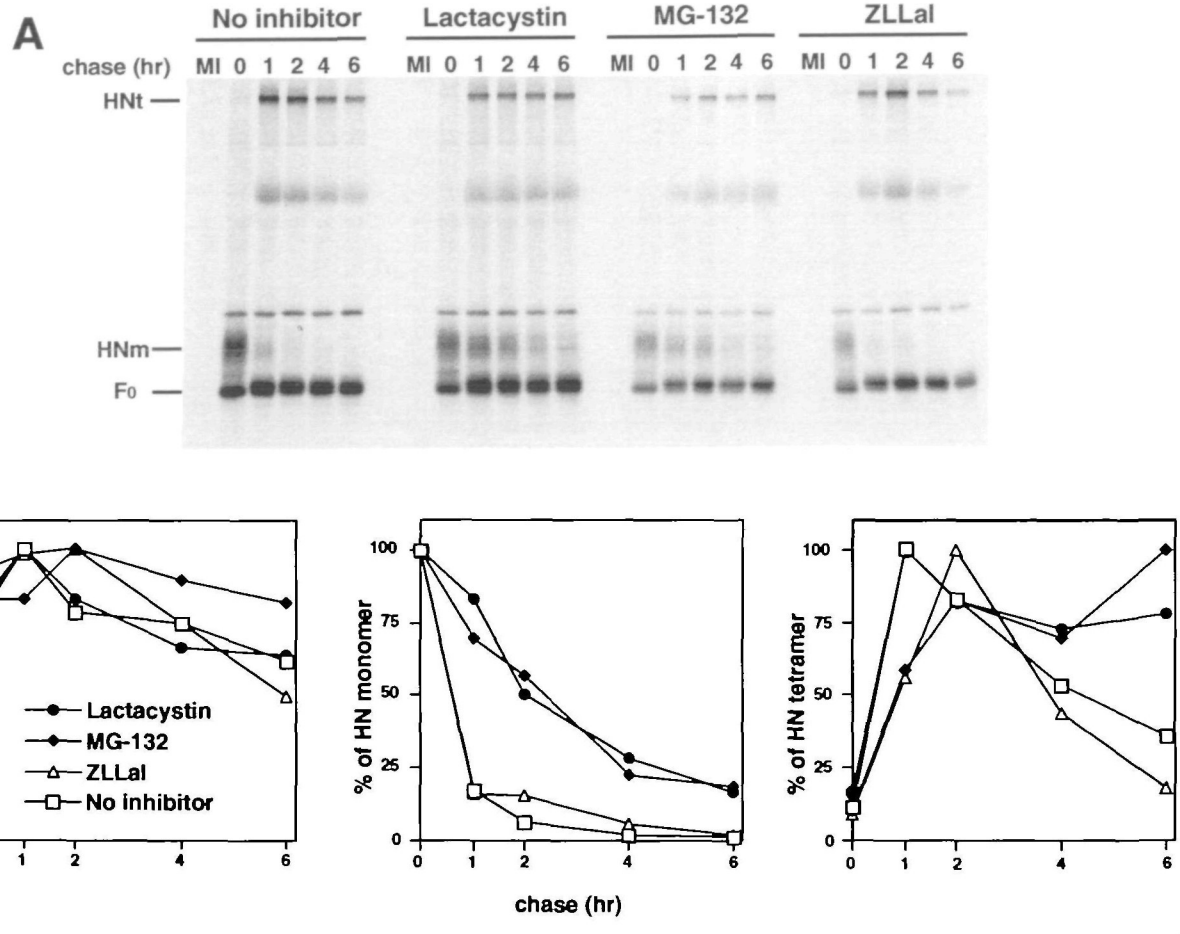

Fig. 6. **Effects of proteasome inhibitors on the processing of F_0 and HN proteins.** (A) Sendai virus-infected cells were incubated in the medium containing 10 μM lactacystin, 5 μM MG-132, 5 μM ZLLal, or 0.1% v/v DMSO for 2 h prior to pulse labeling, labeled for 10 min with [^{35}S]Met/Cys, and chased in the medium containing the same concentrations of metabolic inhibitors. Labeled cells were collected at the indicated time, immunoprecipitated with a mixture of anti-F and anti-HN antisera, separated by non-reducing 7.5% SDS-PAGE, and visualized with a Molecular Imager. (B) Quantitation of the radioactivity of the bands corresponding to F_0 (left panel), HN monomer (center panel), or HN tetramer (right panel) in the panel (A). The relative amounts of F_0 or HN proteins at each time point are expressed as the percentage of the maximum amount of the protein co-precipitated with respective antibody.

of LC and MG-132 on dissociation of HN-ER chaperone complexes were highly reproducible in repeated experiments.

Effect of Castanospermine on the Interactions between Viral Glycoproteins and ER Chaperones—Castanospermine(CST), an inhibitor of the ER glucosidase I and II, prevents the trimming of terminal glucose residues from

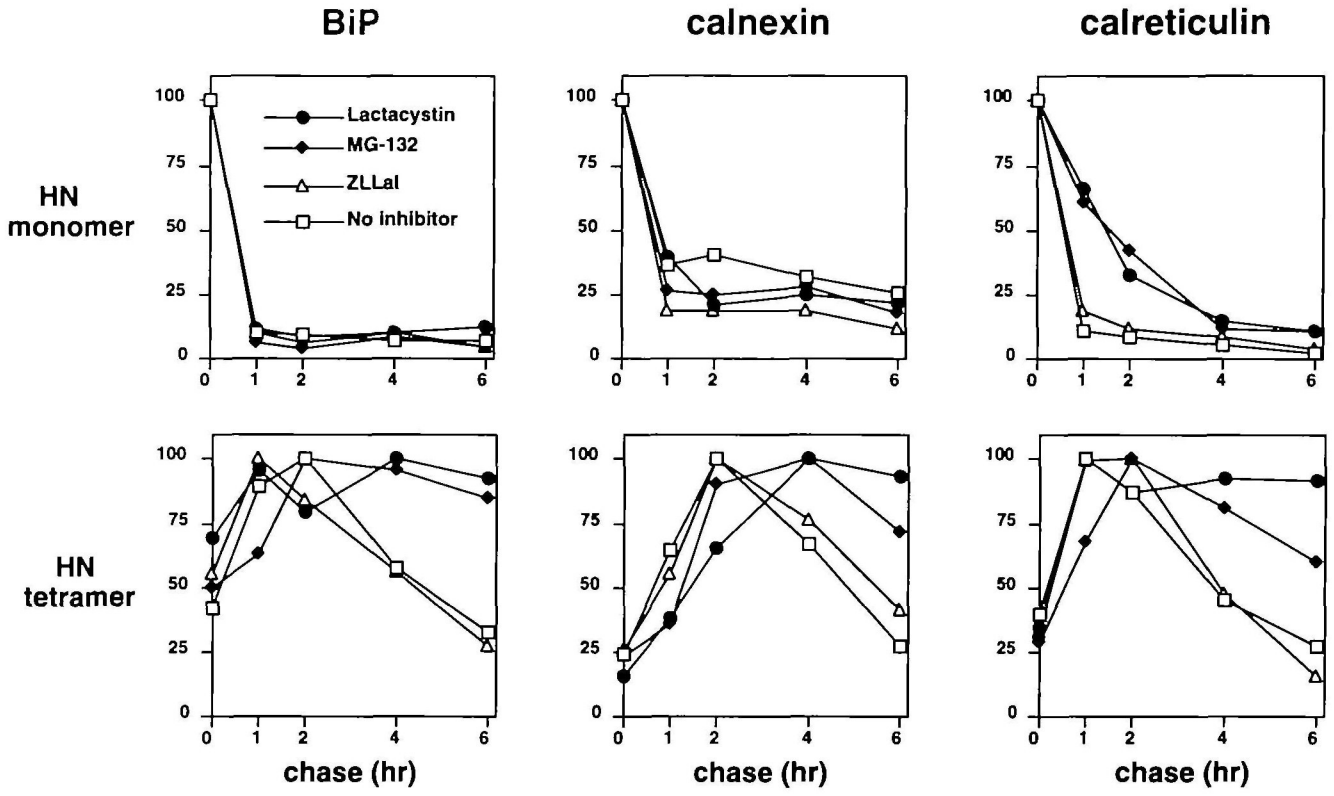

Fig. 7. **Effects of proteasome inhibitors on the interactions of F_0 and HN proteins with BiP, CNX, and CRT.** Aliquots of the samples used in the experiment in A were immunoprecipitated with anti-BiP, anti-CNX, or anti-CRT antibody. The immunoprecipitates were separated by non-reducing 7.5% SDS-PAGE, and visualized and quantified with a Molecular Imager. The relative amounts of HN monomer (upper panels) or HN tetramer (lower panels) co-precipitated with BiP (left panels), CNX (center panels), or CRT (right panels) at each time point are expressed as the percentage of the maximum amount of the protein co-precipitated with the respective antibody. Closed circles, lactacystin; closed diamonds, MG-132; open triangles, ZLLal; open squares, no inhibitor.

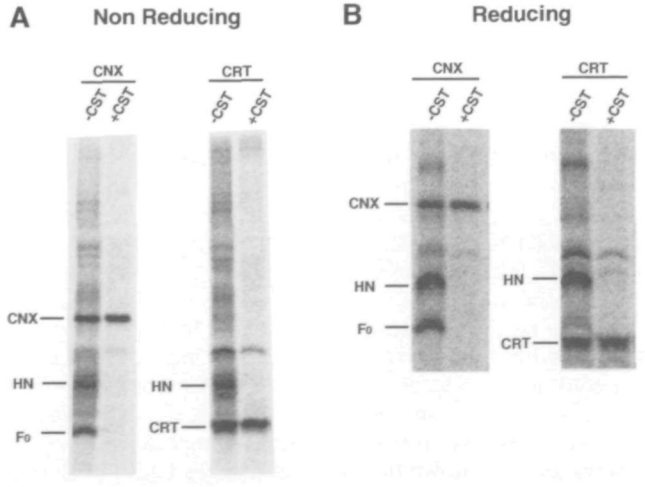

Fig. 8. **Effects of castanospermine (CST) on the interactions between viral glycoproteins and CNX or CRT.** Sendai virus-infected cells were incubated in the medium containing 1 mM CST for 2 h prior to pulse labeling, labeled for 10 min with [^{35}S]Met/Cys, and chased in the medium containing the same concentration of CST for the indicated time. Labeled cell lysates were immunoprecipitated with anti-chaperone antibodies, separated by SDS-PAGE, and visualized with a Molecular Imager. (A) SDS-PAGE under non-reducing conditions. (B) SDS-PAGE under reducing conditions.

core oligosaccharides on a glycoprotein, resulting in failure of the interaction of CNX and CRT with the glycoproteins (*33*). To determine the effects of CST on the interactions of F_0 and HN proteins with ER chaperones, Sendai virus-infected HeLa cells were incubated in medium containing 1 mM CST, pulse-labeled with [^{35}S]Met/Cys for 10 min, lysed immediately after the labeling, and immunoprecipitated with either anti-CNX or anti-CRT antibody. As shown in Fig. 8, CST effectively blocked the formation of F_0-CNX, HN-CNX, and HN-CRT complexes. Thus, binding of CNX or CRT to the viral glycoproteins observed in this study required the presence of monoglucosylated oligosaccharide on F_0 and HN peptides.

DISCUSSION

Although many newly synthesized viral glycoproteins are thought to interact with ER-resident molecular chaperones in the process of becoming functional, molecular aspects of the interactions, such as the types of chaperones, and interaction kinetics are often not well described. This is partly because of the difficulty of obtaining anti-chaperone antibodies, that do not interfere in the association between the ER chaperones and glycoproteins and thus can be used to monitor the protein complex formation and dissociation. In this study, by the choosing C-terminal portion of the chaperones for the immunogen, and by further purifying

antisera with affinity columns, we obtained a series of anti-ER chaperone antibodies that recognized the complexes composed of ER chaperones and Sendai virus glycoproteins. With these antibodies, we analyzed the detailed kinetics of interaction of the Sendai virus glycoproteins with three well-characterized ER-resident chaperones, BiP, CNX, and CRT.

The kinetics of formation of endo H-resistant protein, together with the kinetics of interaction with ER chaperones suggest that newly synthesized F_0 peptide undergoes rapid folding with a transient interaction with CNX in the ER of Sendai virus-infected cells. The majority of newly synthesized F_0 peptides became resistant to endo H digestion during 30 min of chase (Fig. 1C), indicating that the F_0 protein stays in the ER for no longer than 30 min and is transported efficiently into the Golgi complex. Of the three ER-resident chaperones, CNX was able to make a relatively stable complex with F_0 peptide (Figs. 3 and 5). The amount of F_0 associated with CNX was maximal within 10 min of pulse labeling, which is consistent with the accepted concept that the ER-resident chaperones bind to their newly synthesized substrates co-translationally and/or post-translationally. This F_0-CNX association was transient, as seen in many reported interactions chaperone-substrate. Dissociation of the F_0 peptide from the F_0-CNX complex occurred with a half time of 8 min during the subsequent chase period (Figs. 3B and 4A), accompanied with appearance of endo H-resistant F_0 (Fig. 1C), suggesting that CNX remains associated with F_0 until just before the F_0 is transported into Golgi complex.

In case of the maturation of influenza hemagglutinin (HA) protein, CNX promotes the intramolecular disulfide bond formation (*26, 33*). Because the F_0 peptide has 10 cysteine residues, all of which are required for maturation of F_0 precursor (*22*), it is conceivable that the multiple disulfide bond formation of newly synthesized F_0 peptide proceeds correctly and efficiently with the aid of molecular chaperone(s). CNX, which was confirmed in the present study to interact transiently with the F_0 peptide, probably helps in formation of the correct intramolecular bridging within a newly synthesized F_0 peptide.

In addition to binding to CNX, F_0 may also interact with BiP or CRT, because a faint band corresponding in size to the F_0 monomer was observed by immunoprecipitation with anti-BiP antibody (Fig. 3A) or sequential immunoprecipitation with anti-CRT and anti-F antibodies (Fig. 5A). However, the signals were much weaker than those of the F_0-CNX interaction and were not detected consistently in all types of analysis, suggesting that the associations between F_0 and BiP or CRT, if they exist, are much more fragile or transient than that between F_0 and CNX. Because BiP binds to hydrophobic surfaces of immature proteins (*4, 5*), the less efficient detection of F_0-BiP complex in the present study supports the possibility described above that the folding of a newly synthesized F_0 peptide in the ER proceeds rapidly and efficiently in the infected HeLa cells.

In contrast to the F_0 protein, the associations of HN monomer with chaperones were readily detected for all of the ER chaperones tested (Fig. 3), indicating that these interactions are more stable and continuous than those of F_0-ER chaperones. The HN monomer remained associated with CNX and CRT even after 30 min of chase (Fig. 3, B and C), by which time the vast majority of F_0 monomer had been transported into the Golgi complex (Fig. 1C). These observations are consistent with the previous suggestion that the HN monomer remains as immature forms longer than the F_0 monomer (*16*). Thus, folding of HN monomer in ER appears to be more complex than that of F_0 protein. Because the F_0 and HN proteins comprise similar numbers of amino acid residues and have similar numbers of potential *N*-glycosylation sites, the difference in complexity of the maturation processes of F_0 and HN proteins is seemingly paradoxical. Although more experimental data are needed to clarify this issue, a possible explanation is that a topological difference between the two proteins causes the difference in maturation complexity in the subsequent folding and processing processes. Consistent with this possibility, influenza neuraminidase (NA), a type II integral membrane protein, has a slower kinetics of folding and assembly than influenza HA, a type I integral membrane protein (*1*).

Newly synthesized HN monomers associated with BiP, CNX, and CRT were released from the complexes with the same time frame as that of the appearance of HN tetramers (Figs. 2B and 3), suggesting that the three ER chaperones are involved in formation of an assembly competent HN monomer. Of the three ER chaperones, BiP is less well characterized at the molecular level with regard to its function in mammalian cells. On the other hand, CNX and CRT are reported to bind to different domains of a newly synthesized peptide and promote local folding sequentially (*26, 34*). A similar scenario may be applied to the HN peptide folding. The extracellular portion of HN peptide consists of stalk and globular domains, both of which seem to have potential *N*-glycosylation sites and thus can be regions for CNX and CRT binding. These two domains may provide distinct binding sites for CNX and CRT. Thus, our reproducible observation that the HN monomers were released from HN-CNX complexes faster than from HN-CRT complexes (Fig. 4B) may imply that folding of HN peptide proceeds by sequential folding of distinct domains.

The three ER chaperones were found to interact not only with an HN monomer but also with an oligomeric form of HN protein (Figs. 3 and 5). This finding was totally unexpected, because ER chaperones are considered to interact with a newly synthesized monomer peptide. The present studies revealed the following characteristics of the HN oligomer-chaperone interactions, which contrast sharply with the HN monomer-chaperone interactions. First, the interactions began with much slower kinetics than those observed with monomer-chaperone interactions and reached the maximum level between 60 and 180 min after the pulse labeling (Fig. 4C), suggesting that they occur in parallel with or after dissociation of HN monomer-chaperone complexes. Second, they were much more stable than HN monomer-chaperone interactions and sustained for several hours (Fig. 4, B and C), suggesting that some of the assembled HN tetramers are retained for hours in the ER in association with chaperones. Third, the kinetics of dissociation of chaperones from the HN tetramers correlated well with the decrease in total amount of HN tetramer in the infected HeLa cells (Figs. 2B and 4C), suggesting that the HN oligomers are either incorporated into budding virions or degraded shortly after their dissociation from the chaperones.

Because it is unlikely that the correctly assembled HN

oligomers possesses hydrophobic surfaces or monoglucosylated oligosaccharide for the binding of ER chaperones, the present finding suggests that these chaperones interact with assembled HN peptides exhibiting incorrect conformation. The HN oligomers interacting with ER chaperones and thus remaining as immature forms would have at least two destinations. First, some of the HN oligomers interacting with ER chaperones would complete correct assembly and be subjected to the subsequent modifications in the Golgi complex in the normal maturation pathway. Second, the other part of HN oligomers might be unable to assemble correctly and thus be retained in the ER in association with chaperones, which would eventually be degraded. Because the local folding or assembly reaction itself proceeds rapidly, the unusually prolonged association of ER chaperones with HN tetramers of several hours (Fig. 4C) appears to reflect the HN oligomer-chaperone complexes involved in the latter processes. The difference in interaction kinetics between CNX and the other two chaperones indicated that CNX-binding site of HN tetramer might be exposed only after the HN tetramer was associated with BiP and/or CRT, or that CNX might indirectly bind with HN tetramer through the mediation of BiP or CRT.

Consistent with this idea, the kinetics of dissociation of HN tetramer-chaperone complexes displayed a marked delay in the presence of proteasome inhibitors (Fig. 7), suggesting that a part of HN associated with BiP, CNX, and CRT is indeed destined to be degraded in proteasome-dependent pathway during the maturation processes. However, the proteasome inhibitors may affect the oligomerization of the HN proteins by delaying the decrease of HN monomers (Fig. 6), and ER chaperones would be involved in the processes of correct assembly and degradation of oligomer.

In conclusion, the present study highlights marked differences in maturation processes between the two Sendai virus glycoproteins in the context of their interactions with ER-resident molecular chaperones. It also provides evidence that the oligomers are also subject to the ER chaperone-mediated quality control, as recently reported by Kearse (*35*). These findings are important in understanding the maturation processes of Sendai virus glycoproteins, as well as of other virus and cellular glycoproteins. Additional experiments, for example, using conformation-dependent antibodies against F and HN proteins or mutants deficient in folding, assembly or transport into the Golgi complex, would greatly improve the understanding of the chaperone-glycoprotein interactions identified in the present study.

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