N-linked oligosaccharide chains of Sendai virus fusion protein determine the interaction with endoplasmic reticulum molecular chaperones

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Abstract The selectivity and individual roles of the *N*-linked oligosaccharide chains of Sendai virus fusion protein (F protein) in the interaction with endoplasmic reticulum molecular chaperones were investigated by analyses of transient expression of single *N*-glycosylation mutants and sequential immunoprecipitation. We demonstrated differential interactions depending on the location of the *N*-linked oligosaccharide chain, and showed that these interactions were correlated with the folding and transport of F proteins. Moreover, mutant F proteins that lacked the specific *N*-linked oligosaccharide chains required for disulfide bond formation showed increased association with ERp57.

Key words: Sendai virus fusion protein; Calnexin; BiP; ERp57; *N*-linked oligosaccharide chain

1. Introduction

The endoplasmic reticulum (ER) is the compartment in which most glycoproteins acquire their tertiary structure via processes such as disulfide bond formation and subunit assembly, with the assistance of molecular chaperones. It is widely accepted that calnexin (CNX), a type I membrane protein, and calreticulin (CRT), a soluble homolog of CNX, act as molecular chaperones via mechanisms related to their lectin activity; they recognize monoglucosylated *N*-linked oligosaccharide chains as the first step of their chaperone function, assist the folding of substrate glycoproteins and retain misfolded proteins in the ER [1]. Several reports have demonstrated that the chaperone function of CNX and CRT is achieved via mechanism involving ERp57, which is a homolog of protein disulfide isomerase and possesses thiol-reductase activity [2,3]. BiP also promotes the folding of nascent proteins by preventing their aggregation [4].

N-linked oligosaccharide chains are considered to be the important factors for the association of CNX with substrate glycoproteins. In most cases, the interaction of such proteins with CNX is prevented by *N*-glycosylation or glucosidase inhibitors [1,5]. Furthermore, it has been reported that the binding preferences of CNX and CRT are influenced in vivo and in vitro by the location and number of *N*-linked glycans [6–9].

Our previous results demonstrated that Sendai virus fusion protein (F protein), which is one of the envelope proteins of Sendai virus, interacts with ER molecular chaperones during its maturation: mainly with CNX and to lesser extents with CRT and BiP in Sendai virus-infected HeLa cells [10]. F protein contains three potential *N*-linked glycosylation sites (designated g1, g2 and g3 according to their order from the N-terminus) and they are linked to complex-form oligosaccharide chains. We demonstrated that the oligosaccharide chain linked to g2 is the most important oligosaccharide chain for the maturation and intracellular transport of F protein, and that the folding efficiency of F proteins single *N*-glycosylated at each of the three sites dependent on the site which the oligosaccharide chain added [11]. We were particularly interested in whether lectin-chaperone CNX, and also CRT, binds and utilizes specific oligosaccharide chains when they interact with F protein and in whether the differential folding efficiency of glycosylation-defective mutants is related to the interaction with ER molecular chaperones.

To address this issue, wild-type and singly *N*-glycosylation mutant F proteins were transiently expressed in HeLa cells, and their interactions with ER molecular chaperones were examined by sequential immunoprecipitation. The results presented here indicate that the specific *N*-linked oligosaccharide chains of F protein determine the interaction with ER chaperones in a manner dependent on the position of the glycosylation. Furthermore, these interactions are correlated with the maturation of various F proteins.

2. Materials and methods

2.1. Expression plasmids and antibodies

Plasmids encoding the genes of wild-type F protein (Fwt) and its single *N*-glycosylation mutants (Fg1, Fg2 and Fg3; see Fig. 1), based on the plasmid pSRD, were previously constructed in our laboratory [11]. Rabbit anti-CNX, and anti-BiP antisera were obtained as described previously [10]. Rabbit anti-ERp57 antiserum was generated against the 17-amino acid C-terminal peptide (VIQEEKPKKKKKAQEDL) of human ERp57. This peptide was synthesized in our laboratory, conjugated with keyhole limpet hemocyanin by using *m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester and injected into a rabbit. In order to avoid the non-specific interactions, anti-ER chaperone antisera were affinity-purified using AF–amino-toyopearl columns coupled with the corresponding synthetic peptides. Rabbit anti-F anti-

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Abbreviations: F protein, Sendai virus fusion protein; CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; CST, castanospermine; PBS, phosphate-buffered saline; TBS, Tris-buffered saline

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serum was prepared as follows: purified Sendai virus Z strain virions prepared from the allantoic fluid of 9-day-old chick embryos [10] were subjected to SDS–PAGE. The band corresponding to F protein was cut out from the gel and used as antigen.

2.2. Cell culture and transfection

HeLa cells were cultured in growth medium consisting of Eagle's minimal essential medium containing 10% new born calf serum at 37°C under 5% CO_2/95% air. Transient expression of wild-type and glycosylation mutant F proteins was carried out by the plasmid transfection method. 1 day prior to transfection, HeLa cells were plated onto 35-mm or 12-well tissue culture dishes. The next day, the cells (at 60–70% confluency) were transfected with 1 μg (35-mm dish) or 0.5 μg (12-well plate) of plasmid using LipofectAMINE reagent (Invitrogen™) in serum-free OPTI-MEM (Invitrogen™) for 4 h. Then the transfection medium was replaced with the growth medium described above.

2.3. Metabolic labeling and immunoprecipitation

At 16 h post transfection, the cells were starved for 30 min in methionine- and cysteine-free medium (Sigma, Japan) without serum, and then pulse radiolabeled with 200 μCi/ml of [^{35}S]methionine and cysteine (EXPRE^{35}S^{35}S protein labeling mix, NEN) for 3 min. In chase experiments, pulse-labeled cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and then incubated for the time indicated in each figure with 1 ml of the chase medium (growth medium containing 5 mM unlabeled cysteine and methionine). At the end of the chase period, the cells were washed with cold PBS and PBS containing 10 mM iodoacetamide for modification of free thiol groups. The cells were solubilized in lysis buffer (1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 50 mM HEPES (pH 7.5), 200 mM NaCl) containing 10 mM iodoacetamide and protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml antipain and 10 μg/ml leupeptin (Wako, Japan). To prevent the further activation of BiP by cellular ATP, 10 mM glucose and 2 U/ml type III hexokinase (Sigma, Japan) were added to the lysis buffer [10,12]. The solubilized cells were sonicated for 5 min. The nuclear pellet was removed and antibody was added to the supernatant. After incubation at 4°C for 1 h, the immunocomplex was recovered with Pansorbin Cell (Calbiochem). The immunocomplex was washed three times with washing buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, and 0.02% NaN_3 and sequential immunoprecipitation buffer A (10 mM Tris–HCl (pH 7.5) 1 mM EDTA and 2% SDS) was added to the immunopellet and boiled for 5 min to dissociate antigen from the antibody complex. Excess antibody was removed with Pansorbin Cell after dilution of SDS to 0.05% by addition of sequential immunoprecipitation buffer B (10 mM Tris–HCl (pH 7.5), 100 mM NaCl and 1% NP-40) and then a second immunoprecipitation using anti-F antiserum was carried out. After washing, SDS sample buffer without reducing agent was added to the immunocomplex and the mixture was boiled for 5 min. The samples were resolved by non-reducing SDS–PAGE on a 9% polyacrylamide gel, and then the gel was fixed with 30% methanol and 10% acetic acid. The fixed gel was enhanced with EN^{3}HANCE (NEN) and the protein bands were visualized by fluorography.

2.4. Immunoblotting

After transfected cells were cultured for 20 h in the presence or absence of 1 mM castanospermine (CST) (purchased from Wako), they were solubilized in lysis buffer, and samples for the SDS–PAGE analysis were prepared by removing of the nuclear pellet by centrifugation. Cleavage of cell-surface F protein with 0.1% trypsin (Difco) was previously described [11]. The samples were separated by SDS–PAGE on a 9% polyacrylamide gel under reducing condition. Proteins on the gel were electrophoretically transferred to a polyvinylidene difluoride membrane filter (Millipore) and blocked in Tris-buffered saline (TBS), pH 7.4, containing 1% polyvinylpyrrolidone and 0.05% Tween 20 at 4°C overnight. The filter was washed with TBS containing 0.05% Tween 20 and then incubated with anti-F antiserum diluted in TBS containing 1% gelatin. Immunoreactive bands were detected with protein-A conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) and visualized on X-ray film by ECL reagents (Amersham Pharmacia Biotech).

3. Results

3.1. Characterization of wild-type and N-glycosylation mutant F proteins

The F protein mutants used in this study are depicted schematically in Fig. 1b. Fwt (also illustrated in Fig. 1a) is a 66-kDa type I membrane protein containing a 24-amino acid transmembrane domain (amino acids 500 to 523) and a 25-amino acid signal sequence [13]. To investigate the specific role of each *N*-linked oligosaccharide chain in the maturation, intracellular transport, and interaction with ER molecular chaperones, each recombinant F protein expression plasmid was transfected into HeLa cells and the following experiments were carried out.

First, we examined the biosynthesis of the wild-type and the single *N*-glycosylation mutant F proteins. The HeLa cells were metabolically radiolabeled for 3 min and chased for between 0 and 60 min at 16 h after transfection. After solubilization of the cells and removal of the nuclear pellet, F protein was isolated by a sequential immunoprecipitation method using anti-F antiserum and subjected to SDS–PAGE under non-reducing conditions followed by autoradiography. It is thought that native F protein exists in an oligomeric state. However, the interaction among F protein monomers seems to be very weak, and thus oligomeric forms can not be detected by SDS–PAGE analysis without chemical cross-linking [10,14]. Therefore, in the present study, oligomeric forms of F protein were not detected.

At the beginning of the pulse-chase period, newly synthesized F proteins were detected as doublet bands and aggregated forms (Fig. 2a). The monomeric Fwt proteins were de-

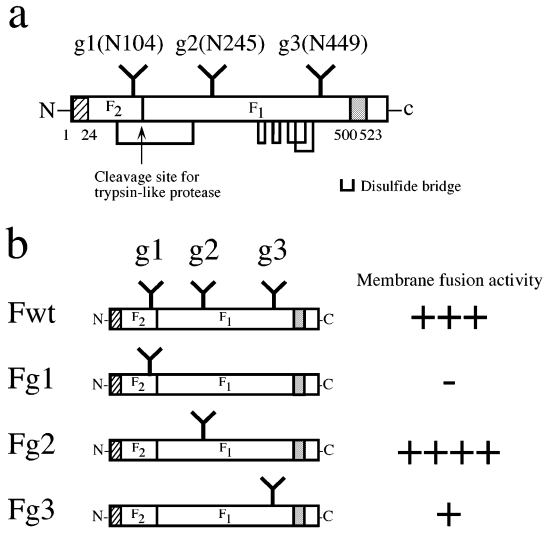

Fig. 1. Schematic representations of Fwt and its N-glycosylation mutants. (a) The transmembrane region at the C-terminus and the signal sequence of F protein are represented by shaded and hatched squares, respectively. Potential *N*-linked glycosylation sites, designated g1, g2 and g3, are indicated by Y symbols, and the amino acid positions of their asparagine residues are noted above. The asparagine residue of each *N*-linked glycosylation motif was replaced by glutamine via point-mutation PCR to prevent the addition of the oligosaccharide chain precursor [12]. (b) The wild-type and *N*-glycosylation mutant F proteins used in this study are illustrated. Biological activity of each F protein is indicated.

tected as 66-kDa and 68-kDa bands. Since Fwt that was pulse-labeled in the presence of 5 mM DTT was detected only as a 68-kDa band (data not shown), we considered that this dual mobility reflected the state of intramolecular disulfide bond formation: the slower and faster migrating forms of each F protein appeared to represent the forms which reduced and oxidized might reflect reduced and oxidized disulfide bonds, respectively. The fully oxidized F proteins were observed at 66 kDa (Fwt) and 62 kDa (Fg1, Fg2 and Fg3). These differences appear to reflect the loss of two

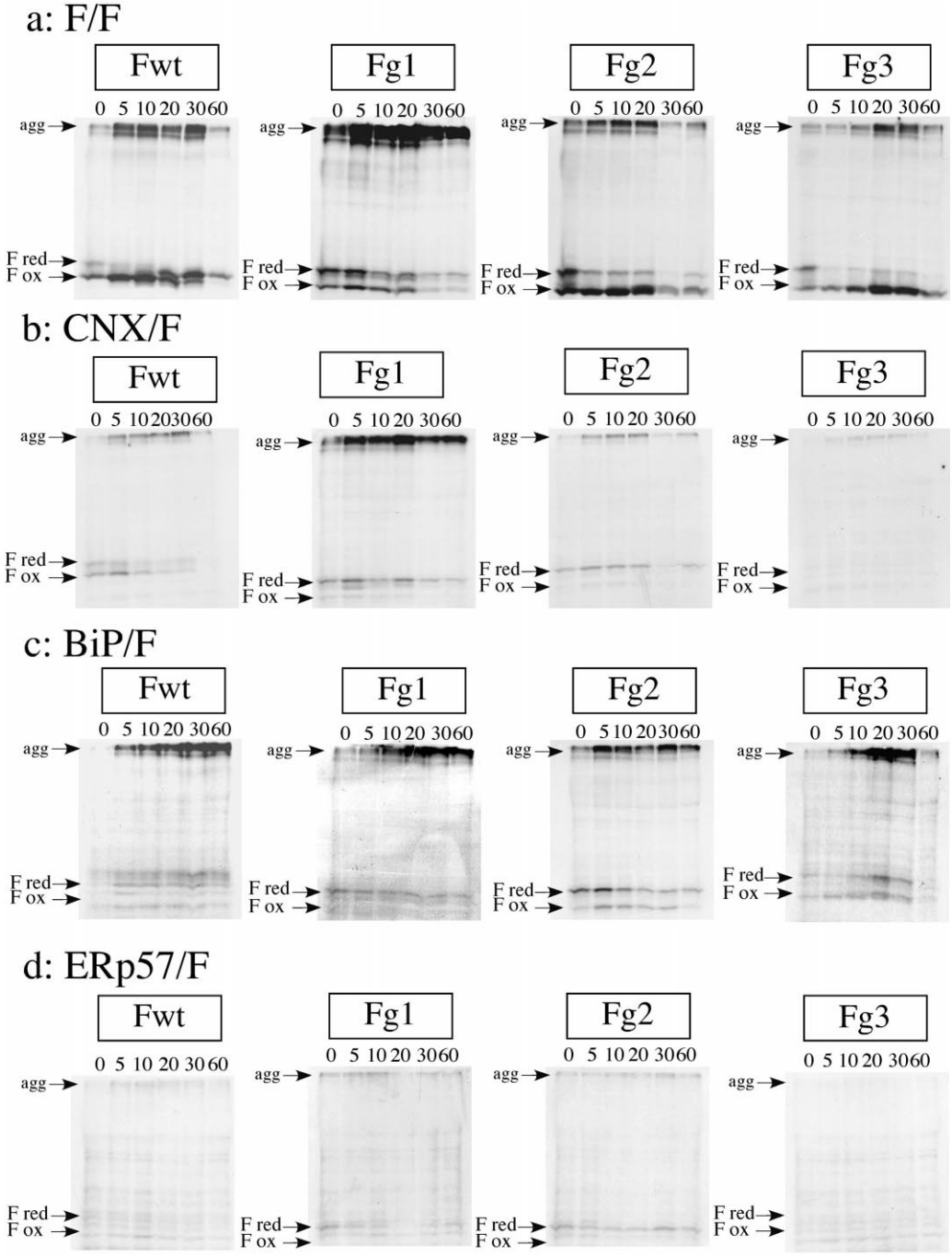

Fig. 2. Biosynthetic characterization of F proteins and interaction of CNX, BiP and ERp57 with F proteins. HeLa cells transfected with the indicated F protein expression plasmid were metabolically radiolabeled for 3 min and chased for the indicated periods. The cells were lysed in 1% CHAPS lysis buffer and subjected to sequential immunoprecipitation. First, anti-F antiserum or purified anti-CNX, BiP or ERp57 antibody was added to an equal aliquot of cell lysate (all aliquots contained equal cell equivalents) and immunocomplex were recovered using Pansorbin Cell. Sequential immunoprecipitation buffer A was added to the washed pellet and the bound proteins were dissociated by boiling for 5 min in the presence of SDS. The concentration of SDS was reduced by addition of sequential immunoprecipitation buffer B and then Pansorbin Cell to remove excess antibody. After the supernatant was transferred to a new tube, the F protein in the supernatant was immunoprecipitated. The immunocomplexes were resolved by 9% SDS–PAGE under non-reducing condition and visualized by autoradiography. F red and F ox represents reduced and oxidized forms of F protein, respectively. agg indicates the aggregated form of F protein. First immunoprecipitation was done using (a) anti-F antiserum, (b) anti-CNX antibody, (c) anti-BiP antibody and (d) anti-ERp57 antibody, respectively. Lane Fwt, Fg1, Fg2 and Fg3 are HeLa cells transfected with pSRD encoding Fwt, Fg1, Fg2 and Fg3 DNAs, respectively.

each *N*-glycans. The electrophoretic migration of Fwt was slightly decreased by 10–20 min of chasing. This seemed to be due to the modification of oligosaccharide chain in the medial Golgi, as confirmed by endoglycosidase H digestion experiments (data not shown).

To examine the role of *N*-linked oligosaccharide chains of F proteins in association with ER molecular chaperones, HeLa cells transfected with each F protein expression plasmid were metabolically pulse-labeled and chased. The cells were solubilized with lysis buffer containing mild detergent (1% CHAPS) to preserve protein–protein interactions, and equal cell equivalents of cell lysate were subjected to sequential immunoprecipitation. First, anti-CNX, -BiP or -ERp57 antibody was added to the cell lysate and immunoprecipitation was performed. The immunocomplexes obtained were dissociated by boiling in the presence of SDS, and subsequently F protein bound to the above ER chaperones in the supernatant was recovered by a second immunoprecipitation using anti-F antiserum.

3.2. CNX preferentially recognizes the g1 and g2 N-linked oligosaccharide chains of F protein

Fig. 2b shows that, in Fwt, Fg1 and Fg2, CNX preferentially interacted with the upper band of F protein rather than the lower band. These results demonstrate that CNX associates with immature F proteins, suggesting that this lectin-like chaperone plays some role in the folding of F proteins. These F proteins preferentially interacted with CNX rather than CRT during the chase period (data not shown). This tendency is consistent with our previously reported findings in Sendai virus-infected HeLa cells [10].

As shown in Fig. 2b, during the chase period, CNX hardly interacted with Fg3, which has an *N*-glycosylation site at the C-terminus of the F protein. In contrast, the other singly *N*-glycosylated F proteins, Fg1 and Fg2, were co-precipitated with CNX. These results suggest that when CNX interacts with F protein, it preferentially utilizes the g1 and g2 oligosaccharide chains, which are attached to amino acids farther (in terms of the amino acid sequence) from the transmembrane region of F protein than the g3 oligosaccharide chain. The selectivity of CRT for interaction with the *N*-linked oligosaccharide chains of F protein was similar to that of CNX (data not shown).

3.3. BiP interacts with both folding intermediates and aggregated forms of F proteins

Next, we performed sequential immunoprecipitation experiments to examine the contribution of another ER molecular chaperone, BiP, to the folding of F protein. BiP recognizes the hydrophobic region of the polypeptide that is exposed at the molecular surface, and prevents the non-specific aggregation of folding intermediates in a manner dependent on its ATPase activity [4]. BiP interacted more strongly with singly *N*-glycosylated F proteins than with Fwt (Fig. 2c). This result may be due to the increase of the hydrophobicity of the molecular surface of F proteins resulting from the absence of *N*-linked oligosaccharide chains. The fact that both aggregated and monomeric forms of each F protein were co-precipitated with anti-BiP antibody suggests that BiP facilitates the folding of each of the F proteins examined here, and prevents aggregated F proteins from exiting the secretory pathway.

3.4. Deletion of specific N-linked oligosaccharide chains induces the interaction of ERp57 with F proteins

ERp57 exhibits thiol-dependent reductase activity, and its association with substrate glycoproteins is inhibited by CST treatment [15]. As recent studies have suggested that the chaperone functions of CNX and CRT are exerted in collaboration with ERp57 [15,16], we used sequential immunoprecipitation to assess whether ERp57 interacts with F proteins. Fig. 2d shows that, in Fwt- or Fg3-expressing HeLa cells, bands indicative of such interaction were not detected or were detected only very faintly. However, Fg1 and Fg2 were co-precipitated with anti-ERp57 antibody and were detected as reduced forms. The fact that ERp57 showed almost no interaction with Fg3 is consistent with the results of the experiments examining the interactions of CNX and CRT with Fg3.

3.5. CST suppresses cell-surface expression of F protein

We have reported that CST, an ER glucosidase inhibitor, significantly reduces the interactions of CNX and CRT with F protein in Sendai virus-infected HeLa cells [10]. To investigate the effect of these interactions on the maturation of F protein, transfected HeLa cells were cultured in the presence of CST, and the cell-surface expression of F protein was examined by tryptic digestion. It should be noted that cleavage of Sendai virus F protein to the F_1 and F_2 subunits does not occur at the surface of cultured cells in the absence treatment with trypsin-like proteases [10,14]. Each cell lysate was subjected to SDS–PAGE under reducing conditions, followed by Western blotting analysis using anti-F antiserum.

As shown in Fig. 3, in the absence of CST, Fwt and Fg2 seemed to be transported to the cell-surface and to be in a cleavable form, while only a very small fraction of Fg1 was transported and processed by the tryptic digestion. Cell-surface expression of Fg3 was not detected. This tendency is consistent with the results of our previously published study

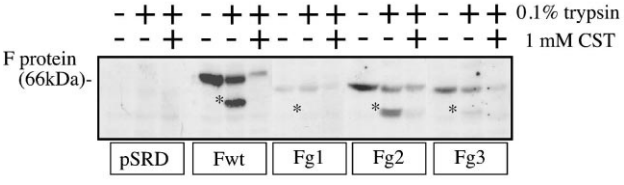

Fig. 3. Interaction with ER chaperones through the monoglucosylated *N*-linked oligosaccharide chain is required for cell-surface expression of F protein. HeLa cells in 12-well culture plate were transfected with plasmids encoding the indicated F protein. After transfection, the cells were incubated in the presence or absence of 1 mM CST for 20 h. In the case of examination of the cell-surface expression of F protein, the cells were washed with PBS containing 0.1% trypsin and then kept on ice for 5 min. After washing with growth medium twice and with cold PBS once, the cells were lysed and the nuclear pellet was removed. SDS sample buffer was added to the lysate and the mixture was boiled for 5 min in the presence of 2-mercaptoethanol. Samples were resolved by 9% SDS–PAGE under reducing condition and proteins in the gel were electrophoretically transferred to a polyvinylidene difluoride membrane filter. After blocking, the filter was incubated with anti-F antiserum (diluted 1:1000) at room temperature for 1 h, followed by detection with protein-A-conjugated horseradish peroxidase (diluted 1:4000) at room temperature for 1 h. Asterisks indicate the position of the F_1 subunit. Lane pSRD, pSRD transfected HeLa cells; lanes Fwt, Fg1, Fg2 and Fg3 are HeLa cells transfected with pSRD encoding Fwt, Fg1, Fg2 and Fg3 DNAs, respectively.

showing that the *N*-linked oligosaccharide chain of g2 plays important roles in the folding efficiency and fusion activity of F protein [11]. When transfected cells were incubated with 5 μg/ml brefeldin A, which inhibits the transport of glycoproteins from the ER to the Golgi, the F_1 subunit was not detected, which indicates that tryptic digestion occurred only at the cell-surface (data not shown).

In contrast, in the presence of 1 mM CST in the culture medium, the level of F_1 subunit, i.e. the expression of F protein at the cell-surface, was significantly decreased for all the *N*-glycosylated F proteins examined. Furthermore, the cell fusion activity of Fwt and Fg2, whose membrane fusion activity was higher than that of the other F proteins [11], in cell cultures the co-transfected with the hemagglutinin-neuraminidase gene, was also completely abolished by CST (data not shown). Therefore, for Fwt and Fg2, interaction with ER chaperones through the monoglucosylated oligosaccharide chain is required for the maturation and cell-surface expression of F protein.

4. Discussion

In the present study, we investigated the interactions of singly *N*-glycosylated F proteins with ER molecular chaperones. Differential interactions of F proteins with various ER chaperones were observed, and were closely related with the quality-control properties of Fg1 and Fg3, which rarely fold properly. The strongest interaction of CNX was observed with Fg1 among the F protein mutants examined here. The position of the oligosaccharide chain suggests that the potential folding capability of Fg1 is lower than that of the other F proteins. Therefore, immature Fg1 interacts strongly with ER molecular chaperones and is retarded in the ER. These results accord with our previous findings that intracellular Fg1 was strongly detected by indirect immunofluorescence staining, and that the oligosaccharide chain attached to g1 somehow suppresses the folding of F protein [11]. BiP interacted with the immature and mature forms of Fg3, while CNX, CRT and ERp57 showed almost no such interaction. Even though Fg3 passed through the check mechanism mediated by CNX and CRT and was transported through the post-ER secretory pathway, the Fg3 at the cell-surface was molecularly immature since the trypsin treatment did not cleave or completely digest the Fg3.

Radiolabeling and sequential immunoprecipitation experiments showed that CNX and also CRT interacted only with F protein *N*-glycosylated at g1 or g2, but not with F protein *N*-glycosylated at g3. This suggests that when CNX and CRT associate with Fwt protein, they particularly recognize the g1 and g2 *N*-linked oligosaccharide chains. Whether the binding selectivity of CNX or CRT is caused by steric hindrance during the synthesis of F protein per se or due to the involvement of other factors remains unclear at present. Because their binding preferences for *N*-linked oligosaccharide chains were similar, the function of CNX and CRT in the folding of F protein may involve the identical region in the tertiary structure. Numerous experimental findings support the idea that *N*-linked oligosaccharide chains on various proteins contribute to the stabilization of protein conformation and subunit assembly [17]. F protein contains five intramolecular disulfide bonds, and they are essential for the maturation and intracellular transport of the protein [18]. Four of them are located between the g2 and g3 *N*-glycosylation sites in the amino acid sequence, hence, these *N*-linked oligosaccharide chains are likely to contribute to correct disulfide bond formation, and their deletion may cause improper disulfide bond formation in the F protein [19]. Almost no interaction between ERp57 and Fwt was observed, whereas Fg1 and Fg2 were co-precipitated from cell lysates by anti-ERp57 antibody. These results suggest that ERp57 tends to interact with F proteins that have trouble forming intramolecular disulfide bonds. Allen et al. reported that misfolding of von Willebrand factor owing to a point-mutation causes prolonged interaction with ERp57 [20]. Collectively, these findings suggest that the interaction between ERp57 and CNX or CRT would be reinforced and serve to assist the folding of substrate glycoprotein when its intramolecular disulfide bond formation was impaired.

Several reports have shown that CST reduces the cell-surface expression and maturation of glycoproteins by preventing their interaction with CNX and CRT, and also ERp57 [15,21–23]. In the present study, analyses of tryptic digestion followed by immunoblotting clearly demonstrated that Fwt, Fg2 and Fg3 were transported to the cell-surface and cleaved, while the cleavage product of Fg1 was hardly detected. Furthermore, cell-surface expression of F proteins was prevented by CST treatment; hence, these results indicate that interaction with CNX and CRT is indispensable for expression of F protein at the cell-surface, and suggest that F protein folding largely depends on the chaperone function of CNX and CRT and also ERp57.

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