# Chaperone Selection During Glycoprotein Translocation into the Endoplasmic Reticulum

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**A variety of molecular chaperones and folding enzymes assist the folding of newly synthesized proteins in the endoplasmic reticulum. Here we investigated why some glycoproteins interact with the molecular chaperone BiP, and others with the calnexin/calreticulin pathway. The folding of Semliki forest virus glycoproteins and influenza hemagglutinin was studied in living cells. The initial choice of chaperone depended on the location of N-linked glycans in the growing nascent chain. Direct interaction with calnexin and calreticulin without prior interaction with BiP occurred if glycans were present within about 50 residues of the protein's $NH_2$-terminus.**

Proteins synthesized in the endoplasmic reticulum (ER) rely during their folding and assembly extensively on the assistance of molecular chaperones and other factors present in the ER lumen. Interactions with such ER-resident folding factors begin, typically, as growing nascent chains enter the lumen through the translocon complex and continue until the newly synthesized proteins are folded, assembled, and ready to leave the ER. Proteins interact differently with the molecular chaperones. Some interact first with BiP and then with calnexin (Cnx) (*1, 2*), some bind Cnx and calreticulin (Crt) but do not associate with BiP (*3–5*), some are sequentially assisted by BiP and GRP94 (*6*). Formation of intramolecular disulfide bonds can be catalyzed by protein disulfide isomerase (PDI), ERp57, or by both oxidoreductases acting together (*7*). The rules that govern such differential chaperone selection are only poorly understood.

To learn more about selection of folding factors, the maturation of two glycoproteins, p62 and E1 of the Semliki forest virus (SFV), was analyzed in infected Chinese hamster ovary (CHO) cells. SFV suppresses host protein translation without affecting protein folding and maturation. In contrast to noninfected cells, infected cells only express viral proteins (Fig. 1A). Among these, E1 and p62 are translocated into the ER. They are type I membrane glycoproteins with four and one N-linked glycans, respectively, and they have numerous intrachain disulfide bonds in their ectodomain.

Infected cells were pulse-labeled for 1 min with [$^{35}$S]methionine and cysteine and chased for 1 to 72 min (Fig. 1B). Cellular extracts were prepared and analyzed by nonreducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as described (*7*). After a 1-min chase, most of the label was in nascent chains. Because of their heterogeneous size, the nascent chains run as diffuse smears. The labeled, full-length E1 and p62 differed in the extent of intrachain disulfide bonding and were thus distributed in multiple bands. The folding of E1 involved three intermediates (E1red, E1ox1, and E1ox2). E1red had the same electrophoretic mobility as the E1 protein run under reducing conditions. E1red was rapidly converted to E1ox1, a partially oxidized intermediate that persisted for 6 to 10 min before being converted into E1ox2, which had the same mobility as mature E1. In contrast to E1, the oxidation of p62 took place during the first 20 min without distinct intermediates. That the spike glycoproteins followed a productive maturation pathway was shown by the cleavage of p62 into E2, by the formation of noncovalent E1:p62 heterodimers ($t_{1/2}$ = 15 min), by the transition of E1 and p62 from a dithiothreitol (DTT)-sensitive to a DTT-resistant conformation ($t_{1/2}$ = 15 to 20 min), and by the release of mature viral particles in the culture medium.

We established by coimmunoprecipitation that BiP associated with early intermediates of E1 (nascent chains, E1red, and E1ox1, but not E1ox2; Fig. 1B). In contrast, Cnx and Crt associated with nascent chains, folding intermediates of p62, and some late forms of E1 (E1ox2), indicating that Cnx and Crt associated with E1 only after it had been released from BiP. Thus E1 and p62 expressed different chaperone preferences, and the differences were already manifested when they were growing nascent chains.

To provide detailed information about cotranslational events, we investigated the interactions occurring between chaperones and growing nascent chains, using a two-dimensional (2-D) SDS-PAGE technique in which the first dimension is nonreducing and the second reducing (*7, 8*). After a 1-min pulse and a 1-min chase (Fig. 2A), the full-length viral proteins appeared as spots, whereas the nascent chains formed streaks starting on the diagonal in the bottom left corner. The presence of radioactive streaks below the diagonal indicated that intramolecular disulfide bonds were forming cotranslationally. Oxidation started when nascent chains were about 35 kD in size (see arrowhead 1, Fig. 2A).

When the extracts were precipitated with antibody to BiP, the 2-D gel pattern showed coprecipitation of E1 nascent chains with molecular masses of 30 kD and larger (Fig. 2B, arrow a). Since these nascent chains migrated on or close to the diagonal, we concluded that BiP associated with E1 nascent chains that had not been extensively oxidized. Although it has been speculated that BiP binds to growing nascent chains, these data suggest that such binding can occur in cells.

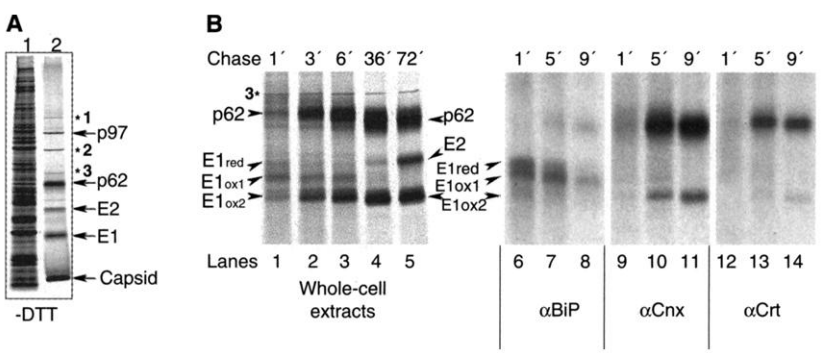


**Fig. 1.** Folding and interaction with ER-resident chaperones of SFV glycoproteins. (**A**) Lane 1, CHO cells were pulsed for 2 min with 500 μCi of [$^{35}$S]methionine and cysteine and chased for 30 min without radioactivity. Cell extract was prepared as described in (*7*) and analyzed by nonreducing gel electrophoresis. Lane 2, CHO cells were infected with 30 plaque-forming units per cell of SFV 4 hours before pulse-chase. Infected cells mainly express viral structural proteins (indicated). Cellular or nonstructural viral proteins are indicated with asterisks 1, 2, and 3. (**B**) Whole-cell extract (lanes 1 to 5), oxidative folding of SFV glycoproteins E1 and p62 was analyzed by nonreducing SDS-PAGE. Viral proteins in different oxidation states are labeled. E2 derives from p62 processing in the Golgi compartment (lanes 4 and 5). Cell extracts were precipitated with antibody to BiP (lanes 6 to 8), Cnx (9 to 11) and Crt (12 to 14). The E1 form coprecipitating with the lectins after 5- and 9-min chase has faster electrophoretic mobility than the E1 forms coprecipitating with BiP.

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The chaperone molecule BiP also coprecipitated with early folding intermediates of E1 (E1red and E1ox1 in Figs. 1B and 2B) and interchain disulfide-bonded complexes visible above the diagonal (Fig. 2, A and B). The complexes corresponded to mixed disulfides between E1 and PDI (*7*). Evidently they contained BiP as a third component that was not covalently bound. The fully oxidized form of E1 (E1ox2) did not coprecipitate with BiP (Figs. 1B and 2B), consistent with preferential BiP-binding to less folded forms of E1.

Nascent chains of p62 coprecipitated with Cnx (in Fig. 2C, arrow b indicates p62 nascent chains without intrachain disulfides and arrow c, p62 nascent chains that are partially oxidized). Unlike E1 oxidation, p62 oxidation started cotranslationally when 30 to 50% of the polypeptide chain had entered the ER lumen. Previous work has shown that oxidation of p62 is catalyzed by the oxidoreductase ERp57 (*7*).

When access to the Cnx/Crt chaperone system was blocked by adding the reducing agent DTT to the medium (*9*), both p62 and E1 were seen to associate with BiP (Fig. 3A). When the DTT was washed out, the glycoproteins were rapidly transferred to the Cnx/Crt chaperone system, and proper folding was reestablished. The same was observed when access to Cnx and Crt was blocked by addition of castanospermine, an inhibitor of glucose trimming (Fig. 3B): both E1 and p62 showed prolonged association with BiP. Thus, the reason BiP failed to bind to p62 normally was not because p62 lacked BiP binding sites but rather that the bound Cnx and Crt prevented the interaction.

In trying to explain these results, we noted that one difference between E1 and p62 was that p62 possessed glycosylation sites close to the $NH_2$-terminus that might provide a site for early lectin binding to nascent chains. To test the notion that the position of N-linked glycans in the sequence of a glycoprotein correlates with differential chaperone binding, we made use of mutants of influenza hemagglutinin (HA) in which consensus sites for N-linked glycosylation were altered either close to the $NH_2$-terminus or more toward the COOH-terminus [legend of Fig. 4 and (*10*)]. The results with wild-type HA confirmed that BiP does not bind despite the presence of multiple, potential BiP binding sequences [Fig. 4 and (*3, 11–13*)]. When maturation of the mutants was analyzed, it was found that removal of glycosylation sites in positions 8 and 22, or—even better—in positions 8, 22, and 38, resulted in binding of BiP. As already observed for SFV E1 (Figs. 1B and 2B), and for vesicular stomatitis virus (VSV) glycoprotein (*1*), it was the less oxidized forms of M1 and M2, that is, those migrating slower in the nonreducing gel (labeled IT1 and IT2 in Fig. 4), that preferentially associated with BiP. The fully oxidized HA form (NT) was mostly associated with Cnx (*10*).

Taken together, the results indicated that, during translocation of a glycoprotein in the ER, a choice is made between chaperone

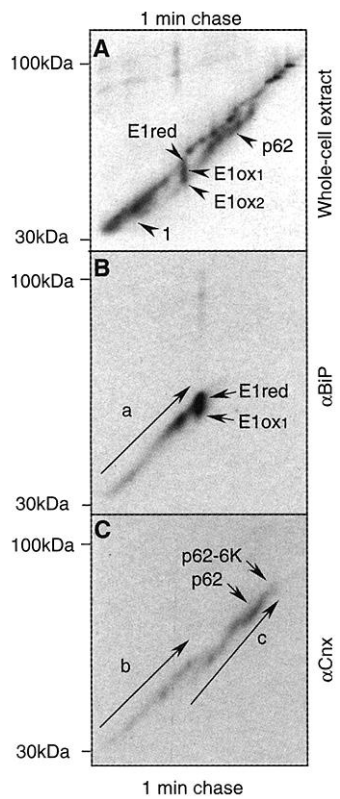


**Fig. 2.** Cotranslational folding and interaction with ER-chaperones of SFV glycoproteins analyzed by 2-D SDS-PAGE. Whole-cell extract (**A**), αBiP (**B**), and αCnx (**C**) precipitates were subjected to 2-D SDS-PAGE in which the first dimension was nonreducing and the second, reducing (*7*). The full-length labeled viral glycoproteins, as well as E1 (arrow a) and p62 growing nascent chains (arrows b and c) are indicated.

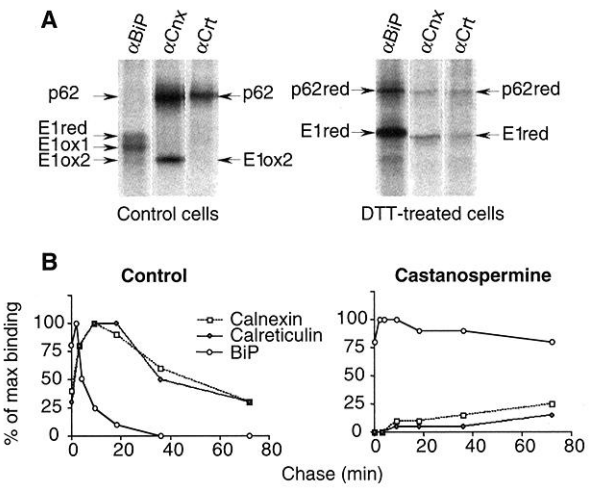


**Fig. 3.** SFV glycoproteins expression in the presence of DTT or castanospermine. (**A**) The binding of E1 and p62 to BiP, Cnx, and Crt was tested in control cells (left) and in cells treated with 5 mM DTT during pulse and 5-min chase (right). (**B**) BiP rapidly released E1 folding intermediates before complete oxidation ($t_{1/2}$ = 5 min, refer also to Fig. 1B). The viral-derived radioactivity associated with Cnx and Crt increased during the first 15 min of chase as the E1 released by BiP completed its folding, in association with p62, upon binding to the ER lectins. In the presence of castanospermine (right), association of p62 with Cnx and Crt was blocked. As observed in the presence of DTT, p62 bound to BiP.

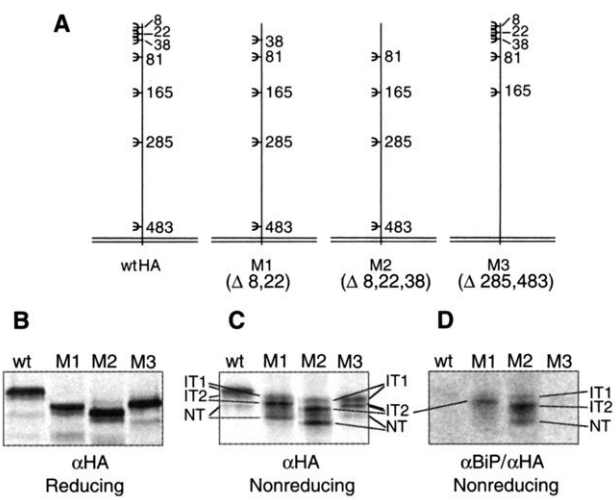


**Fig. 4.** Deletion of N-linked glycans and glycoprotein association with BiP. (**A**) The position of N-linked glycans in the four constructs used is shown. Proteins were transiently expressed in CHO cells using a recombinant vaccinia virus with T7 polymerase and cowpox *hr* gene as described in (*10*). (**B**) The different mobility in the reducing gel (left panel) corresponds to the different number of N-linked glycans each of them contributing approximately 2 kD to the protein molecular mass. (**C**) In unchased samples, folding of the HA molecules was still incomplete, and in the nonreducing gel (central panel) the different oxidation states (IT1, IT2, and NT) of wild-type and mutant HAs are indicated. Note that oxidative folding was somewhat faster for the mutants than for the wild-type HA (*10*). (**D**) Association with BiP was determined by double immunoprecipitation (αBiP, then αHA). Only M1 and M2, in which the glycans located at the $NH_2$-terminus were deleted, associated with BiP during the early step of oxidative folding.

systems. One of the systems comprises BiP and possibly PDI, and the other consists of Cnx, Crt, and ERp57. The chaperones compete for binding sites on the incoming nascent chain. Whichever binds first apparently dominates during early stages of co- and posttranslational folding. Its association delays or prevents binding of the other chaperones. Later in the folding process a transfer from one pathway to the other can, however, take place as observed for E1 here and VSV glycoprotein in a previous study (*1*).

The results indicate, moreover, that glycoproteins such as p62 and HA that possess glycans close to the $NH_2$-terminus enter the Cnx/Crt pathway directly without prior binding to BiP. Glycoproteins like E1 and VSV glycoprotein that have their glycans more COOH-terminally in the sequence associate first with BiP. Inhibitors of the Cnx/Crt pathway, such as castanospermine, direct proteins such as p62 and HA that normally do not bind BiP to the BiP pathway. This shows that the presence of N-linked glycans, as such, does not prevent BiP binding but rather promotes the association of the alternative chaperones. A literature survey of glycoproteins for which chaperone requirements are known is consistent with the notion that proteins with N-linked glycans within the first 50 residues do not undergo transient interaction with BiP (*14*).

Thus, in summary, our findings demonstrated that to understand the molecular basis for chaperone selection in the ER it is important to analyze growing nascent chains and to consider the precise timing of cotranslational modifications such as N-linked glycosylation and oligosaccharide trimming. Attention must be paid particularly to the location of glycosylation sites in the linear sequence.

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14. A number of cellular and viral proteins with N-linked glycans close to the $NH_2$-terminus have been shown to fold independently of BiP; examples follow: influenza HA [first glycan at position 8, I. Braakman *et al.*, *J. Cell Biol.* **114**, 401 (1991)], SFV p62 (13, this work), factor V [23, D. D. Pittman *et al.*, *J. Biol. Chem.* **269**, 17329 (1994)], prepro-α-factor [22, K. Su *et al.*, *J. Biol. Chem.* **268**, 14301 (1993)], Sindbis virus (SIN) PE2 [11 (*4*)], rotavirus NS28 [8, A. Xu *et al.*, *J. Virol.* **72**, 9865 (1998)], CD-mannose 6-P receptor [31, J.-X. Zhang *et al.*, *Biochem. J.* **295**, 841 (1993)]. BiP requirement for folding has been shown for proteins with glycans late in the sequence. As examples VSV glycoprotein [178 (*1*)], von Willebrandt factor [93, A. J. Dorner *et al.*, *J. Cell Biol.* **105**, 2665 (1987)], SFV E1 (141, this work), SIN E1 [139 (*4*)], HIV gp160 [88, P. L. Earl *et al.*, *J. Virol.* **65**, 2047 (1991)], amyloid precursor protein [542, Y. Yang *et al.*, *J. Biol. Chem.* **273**, 25552 (1998)], Ig heavy chain [99–160, I. G. Haas and M. Wabl, *Nature* **306**, 387 (1983)], and simian virus HA [110, D. T. Ng *et al.*, *J. Cell Biol.* **109**, 3273 (1989)].


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# Convergence of the Secretory Pathways for Cholera Toxin and the Filamentous Phage, CTXϕ


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**Virulence of *Vibrio cholerae* depends on secretion of cholera toxin (CT), which is encoded within the genome of a filamentous phage, CTXϕ. Release of CT is mediated by the extracellular protein secretion (eps) type II secretion system. Here, the outer membrane component of this system, EpsD, was shown to be required for secretion of the phage as well. Thus, EpsD plays a role both in pathogenicity and in horizontal transfer of a key virulence gene. Genomic analysis suggests that additional filamentous phages also exploit chromosome-encoded outer membrane channels.**


Secretory systems are essential mediators of bacterial pathogenesis, for they enable release of a diverse array of bacterial virulence factors. Export of these extracellular effectors usually depends on one of four distinct multiprotein secretion systems (type I to type IV) to transport macromolecules through the inner and outer bacterial membranes (*1*). Some components of these systems probably evolved from common ancestral genes, and additional related proteins mediate pilus assembly and contribute to viral and conjugal transfer of DNA (*2, 3*). However, very few proteins are known to be integral components of more than one secretion apparatus (*4, 5*). Instead, for example, similar but distinct outer membrane pores have been shown to be essential for type II and type III secretion, type IV pilus assembly, and release of filamentous coliphages (*2*).

The genome of CTXϕ, the filamentous phage of *Vibrio cholerae* that encodes cholera toxin (CT), resembles those of the paradigmatic filamentous phages of *Escherichia coli* (e.g., f1) in many respects (*6*). However, the CTXϕ genome lacks a homolog of f1's gene IV, which encodes the outer membrane pore ("secretin"), pIV, through which f1 exits from its host (*7*). Interactions between pIV, pI (an f1-encoded inner membrane protein thought to regulate channel opening), and phage coat proteins mediate the simultaneous assembly and secretion of f1 virions (*8*). Very specific physical constraints underlie the pIV/pI interaction; thus, the secretin from the closely related coliphage Ike cannot substitute for f1 pIV (*9*). Because CTXϕ resembles f1 in that it does not lyse its host to gain release and because CTXϕ encodes proteins related to f1 phage coat proteins and assembly proteins (*6*), secretion of CTXϕ from *V. cholerae* was expected to require an outer membrane channel similar to pIV. Consequently, we investigated whether *V. cholerae* produces a pIV-like protein from a chromosomal locus that serves as a CTXϕ secretin.

A comparison of f1 pIV with the database of the *V. cholerae* genome (*10*) revealed that *V. cholerae* encodes a similar (51%) protein: EpsD, the putative outer membrane pore for the *eps* (extracellular protein secretion) type II secretion system. The *eps* apparatus is essential for secretion of CT, protease, and chitinase by *V. cholerae* (*11*). Because the CT released by infecting bacteria induces most of the symptoms of cholera, it is clear that the *eps* system plays a critical role in cholera pathogenesis (*12*). However, like other type II systems, it is only known to contribute to protein secretion.

To assess the role of *epsD* in secretion of CTXϕ, we generated Kn-insertion mutations within *epsD* in classical (O395) and El Tor (Bah-2) biotype strains of *V. cholerae* (*13*). The wild-type and mutant strains were then transformed with pCTX-Ap, the replicative form of


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