Transient Aggregation of Nascent Thyroglobulin in the Endoplasmic Reticulum: Relationship to the Molecular Chaperone, BiP

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Abstract. Because of its unusual length, nascent thyroglobulin (Tg) requires a long time after translocation into the endoplasmic reticulum (ER) to assume its mature tertiary structure. Thus, Tg is an ideal molecule for the study of protein folding and export from the ER, and is an excellent potential substrate for molecular chaperones. During the first 15 min after biosynthesis, Tg is found in transient aggregates with and without interchain disulfide bonds, which precede the formation of free monomers (and ultimately dimers) within the ER. By immunoprecipitation, newly synthesized Tg was associated with the binding protein (BiP); association was maximal at the earliest chase times. Much of the Tg released from BiP by the addition of Mg–ATP was found in aggregates containing interchain disulfide bonds; other BiP-associated Tg represented non-covalent aggregates and unfolded free monomers. Importantly, the immediate precursor to Tg dimer was a compact monomer which did not associate with BiP. The average stoichiometry of BiP/Tg interaction involved nearly 10 BiP molecules per Tg molecule. Cycloheximide was used to reduce the ER concentration of Tg relative to chaperones, with subsequent removal of the drug in order to rapidly restore Tg synthesis. After this treatment, nascent Tg aggregates were no longer detectable. The data suggest a model of folding of exportable proteins in which nascent polypeptides immediately upon translocation into the ER interact with BiP. Early interaction with BiP may help in presenting nascent polypeptides to other helper molecules that catalyze folding, thereby preventing aggregation or driving aggregate dissolution in the ER.

E XIT from the ER is a rate-limiting step in the secretion of many nascent secretory polypeptides (Lodish et al., 1983). The prevailing model of transport envisions that diffusible contents in the lumen of the ER travel to the Golgi complex via non-selective, vesicular bulk flow, thus obviating the need for any positive transport signal on exportable proteins (Pelham, 1989). The observation that most exportable proteins leave the ER substantially more slowly than the rate of bulk flow suggests two possibilities which are not mutually exclusive: interactions with relatively long-lived residents of the ER slow the transport of soluble polypeptides, or the intrinsic solubility of exportable proteins prior to folding limits their diffusion into Golgi-bound transport vesicles. Indeed, the possible existence of protein aggregates as intermediates in the folding pathway for exportable proteins has been previously hypothesized (Pfeffer and Rothman, 1987).

The most frequently identified ER protein in the family of molecular chaperones is the binding protein (BiP)[1] (Geth-

ing and Sambrook, 1991). Besides the induction of its synthesis during cellular stress (Watowich and Morimoto, 1988), BiP plays a role in preparing normal proteins for export from the ER (Hendershot, 1990). Two alternate models of BiP function have emerged in recent years: (a) a catalyst of proper folding before protein export (Rothman, 1989), and (b) a quality control monitor which binds and retains unassembled or misfolded proteins in the ER (Hurtley et al., 1989). These varying views are based upon observations that BiP transiently associates with some exportable proteins, yet stably associates with a variety of misfolded proteins and unassembled subunits. The difference between views may be more apparent than real, since the molecular basis for BiP binding to misfolded proteins (i.e., quality control function) is likely to be equivalent to its recognition of unfolded proteins (i.e., chaperone function) as they are first translocated into the ER lumen (Flynn et al., 1991). Thus, involvement of BiP and other chaperones would be expected at the time of polypeptide translocation in either model, with a high degree of BiP association remaining until either proper folding takes place or the protein is degraded. However, this prediction has been difficult to test for many proteins, especially for smaller molecules with simpler tertiary structure, in which the steps of nascent peptide folding can be excep-

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tionally rapid. Indeed, certain newly synthesized exportable proteins exhibit no detectable association with chaperones at any time, although technical difficulties in detecting transient association (e.g., rapid kinetics of dissociation) cannot be excluded in such cases. Thus the precise stages of exportable protein folding and assembly in which molecular chaperones play a role, and the structural requirements for their association, both are areas of intense interest.

One useful approach to address these issues has been to study the interactions between purified oligopeptides and chaperones in vitro (Flynn et al., 1991). However, since additional factors within the ER environment as well as structural complexity of real proteins may influence their folding and transport, a physiological understanding also requires in vivo studies. N-linked glycosylation is one factor which may influence the structure of exportable proteins as well as BiP binding (Dorner et al., 1987). More recent studies of exportable proteins, either integral membrane (Machamer and Rose, 1988; Hurtley et al., 1989) or luminal (Kassenbrock et al., 1988; Singh et al., 1990), have concentrated on the idea that BiP binding can be related to the presence of improper disulfide bonds in the nascent polypeptide, presumably by coincident exposure of aliphatic side chains (Flynn et al., 1991). Indeed, mutational analysis of vesicular stomatitis virus G protein has clearly demonstrated that BiP binds most strongly to those forms which are incompletely or aberrantly disulfide bonded (Machamer et al., 1990).

We have been studying the folding and assembly of nascent thyroglobulin (Tg), the major secretory protein product of thyroid epithelial cells and the precursor protein in the manufacture of thyroid hormones. This enormous molecule, whose monomeric subunits contain 2,750 amino acids (Malthiery et al., 1989), is ideally suited for studies of protein folding and export from the ER because of (a) high expression level (13% of total thyroid protein synthesis [Wagar, 1984]), and (b) exceptional slowness to assume mature quaternary (dimeric) structure (Kim and Arvan, 1991). Consequently, studies of Tg have provided a unique opportunity to resolve kinetically discrete stages of normal polypeptide folding in the ER lumen of non-transformed, non-infected cells. Using a system of cultured porcine thyrocytes we have shown that for up to 15 min after biosynthesis, a fraction of nascent Tg contains aberrant interchain disulfide bonds and is found in protein aggregates (Kim and Arvan, 1991). Remarkably, this aggregation is transient, and is followed by spontaneous dissolution and the formation of free monomers, which go on to assemble into dimers that are transport competent. The formation and dissolution of nascent Tg aggregates occur entirely within the ER. We have now investigated the association of BiP with nascent Tg as it proceeds through its folding pathway in the ER, and we have attempted to examine the hypothetical role of BiP as catalyst of aggregate dissolution versus other proposed models of BiP function.

Materials and Methods

Cell Culture

Porcine thyrocytes were isolated and cultured as described previously (Kim and Arvan, 1991). Some cells were cultured in the presence of bovine TSH (1-10 mU/ml, Sigma Chemical Co., St. Louis, MO) alone or with bovine insulin (1 μg/ml, Sigma Chemical Co.), human transferrin (5 μg/ml, Sigma Chemical Co.) and hydrocortisone (1 nM, Sigma Chemical Co.), before experiments conducted on day 7 of culture.

Cell Labeling and Lysis

Except where otherwise indicated, confluent monolayers were labeled as described previously (Kim and Arvan, 1991) for 5 min with ^{35}S Express (New England Nuclear, Bedford, MA) and chased for the indicated periods in the presence of excess unlabeled methionine and cysteine. At the conclusion of the chase period cells were chilled to 4°C and treated with 50 mM iodoacetamide or N-ethyl maleimide for 10 min to alkylate intracellular sulfhydryls. The cells were then washed three times in ice-cold phosphate-buffered saline and lysed in 0.5 ml of a solution containing 0.1 M NaCl, 25 mM Tris, pH 6.8, 5 mM EDTA, 1% Triton X-100, 10 U/ml apyrase (Sigma Chemical Co.), and a cocktail of protease inhibitors including 0.1 mM leupeptin, 10 μM pepstatin, 1 mM diisopropylfluorophosphate, and 1 mU/ml aprotinin. Alkylating agents were not added during the lysis step in order to maintain apyrase activity, but controls confirmed that Tg had already been fully alkylated. After exposure to apyrase for 1 h, 10 mM iodoacetamide was added before immunoprecipitation or storage.

For experiments to measure BiP/Tg stoichiometry, thyrocytes in 24-well plates were labeled continuously, twice for 2 d (4 d total), in complete medium containing 0.25 mCi per well of [^{35}S]methionine (New England Nuclear); this protocol ensured labeling at a constant specific radioactivity. Preliminary studies showed that amino acids were not limiting during cell culture labeling. After 4 d, the cells were briefly washed and lysed as described above.

Gel Electrophoresis and Quantitation of Tg Recovery

Two-dimensional (2-D) PAGE was carried out as described previously (Kim and Arvan, 1991) with two modifications used to improve resolution: the amount of cell lysate loaded in these gels was reduced to 15 μl, and the electrophoresis time in the first, non-denaturing dimension was lengthened so that the dye front ran off the gel. As in previous studies (Kim and Arvan, 1991), Tg dimer and monomer positions were located using Tg standards. Scanning densitometry of gel fluorographs was used to quantitate different forms of the Tg band in all experiments except those shown in Fig. 6, in which quantitation was performed by phosphorimaging (400B; Molecular Dynamics, Benton, NJ). For both analyses, data was coupled to the Image-Quant software package (Molecular Dynamics).

Immunoprecipitations

Antisera to denatured porcine Tg, or a mAb to BiP (Bole et al., 1986) were used in all immunoprecipitations. Notably, the buffer used, identical to the cell lysis buffer, was free of SDS. Samples were pre-cleared in the presence of protein A- or protein G-Sepharose beads only. Subsequent non-immune or immune precipitation was carried out for 2 h at 4°C. Since the BiP antibody subtype is rat IgG$_2$ we used protein G-Sepharose beads (or those coated with anti-rat IgG), whereas the secondary reagent for precipitation of Tg was protein A-Sepharose. Repeat precipitations were performed on several occasions to check the efficiency of the immunoprecipitation protocol; for both proteins, the efficiency of our method ranged from 60-75%. In some experiments, co-precipitated Tg was eluted from BiP by incubation with 1 mM Mg-ATP for 20 min at 37°C.

Inhibition of N-glycosylation

Tunicamycin, generously provided by Dr. A. Elbein (University of Texas, San Antonio, TX), was added to confluent monolayers at a final concentration of 20 or 0.5 μg/ml. At high dose, cells were exposed for 2 h, then pulse labeled for 5 min, and finally chased for 15-60 min in the presence of the drug. At low dose, cells were pre-exposed to tunicamycin for 16 h, and the cells then labeled identically. Preliminary studies established that the doses used in these protocols were optimal to achieve complete inhibition of glycosylation with the minimum effects on thyrocyte protein synthesis. Nevertheless, high-dose tunicamycin treatment inhibited overall protein synthesis ~70% while low-dose treatment inhibited ~60%, as measured by TCA-precipitable counts relative to control cells in the standard labeling protocol.

Cycloheximide Pretreatment to Deplete Tg from the ER

Cells were exposed to cycloheximide (Sigma Chemical Co.) at 10 μg/ml in

DME for a time equivalent to three half-times for exit of Tg from the ER (6 h, which included 45 min in met-free, cys-free medium). Before pulse radiolabeling, cells were rinsed three times with PBS to wash out cycloheximide and allowed to recover in the absence of the inhibitor for 10 min. Cycloheximide treatment reduced protein synthesis to ~5% of control values, while cycloheximide washout plus recovery allowed restoration of protein synthesis to ≥60%. Pulse-labeled cells were lysed as described above and samples were then analyzed by SDS-PAGE under non-reducing and reducing conditions.

Results

Resolution of Tg Folding Intermediates

We showed previously that a large fraction of nascent Tg involves protein aggregates containing interchain disulfide bonds; no other pulse-labeled proteins have been found associated with Tg in these precipitates (Kim and Arvan, 1991). The aggregates serve as precursor to monomeric Tg seen by 2-D PAGE. After 60 min of chase, monomeric Tg has dimerized within the ER and is transported to the Golgi complex (Kim and Arvan, 1991).

Through improvements of our 2-D PAGE analysis (see Materials and Methods), we have now been able to resolve discrete monomer Tg folding intermediates. As shown by 2-D PAGE at 10 min of chase (Fig. 1 *a* shows the non-denaturing dimension running from left to right), the predominant Tg form (labeled *U*) was a slow, broadly migrating band suggesting expanded and complex monomeric conformations. Shortly thereafter, a discrete, faster-migrating band was resolved in the first dimension (to the right, labeled *F*). The migration of this spot suggests it is a more compact monomer; thus the U- and F-forms are taken to represent unfolded and folded monomers, respectively. It must be noted that both forms exhibited identical mobility in the second, denaturing dimension (SDS-PAGE, from top to bottom), co-migrating with Tg standard; thus, the F-form is not a proteolytic fragment of the U-polypeptide. In control thyrocytes, the F-form at no time represented a major intermediate, maximally accounting for ~15% of newly synthesized Tg at 30 min of chase (Fig. 1 *b*). Instead, labeled Tg dimer (D, Fig. 1 *a*) appeared nearly in parallel with the appearance of label in the F-form. Such an observation suggests that Tg dimerization derives from the relatively rapid combination of F-monomers. Together, our data support precursor-product relationships between U-, F-, and D-forms, and suggest that the F-form is an obligatory intermediate in Tg dimerization (see Discussion). By visual inspection at 60 min of chase (Fig. 1 *a*), dimerization was virtually complete with only a negligible amount of F-monomer remaining.
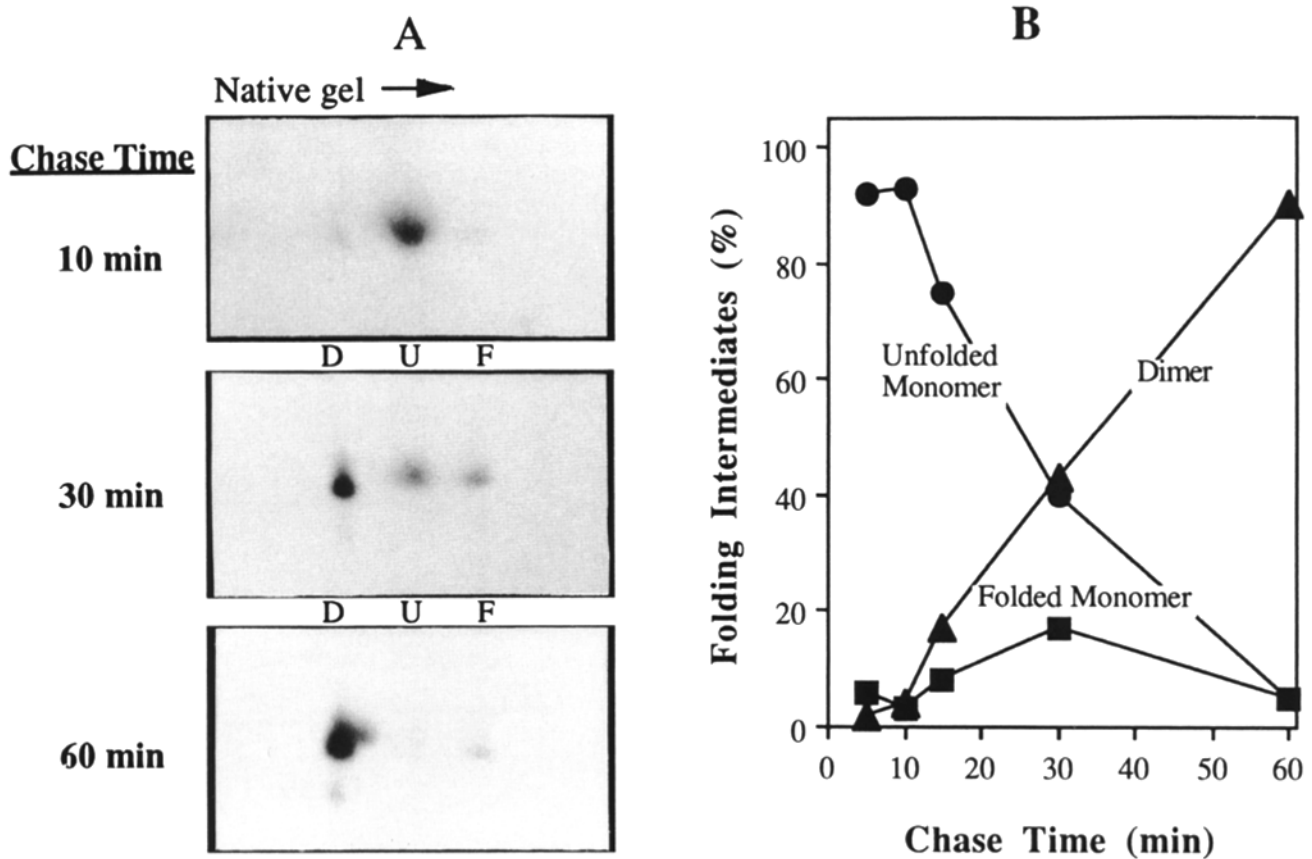

Figure 1. Identification of folded monomer by 2-D PAGE. (*A*) Confluent monolayers of porcine thyrocytes were pulse labeled for 10 min and chased for up to 1 h at 37°C. At the times indicated, the cells were lysed under non-denaturing conditions and analyzed by 2-D PAGE as described in Materials and Methods. The 2-D PAGE patterns appeared identical whether or not apyrase was included in the lysis buffer; thus, BiP association is unlikely to be preserved during the gel analysis. (*B*) Tg bands obtained by 2-D PAGE were quantitated by scanning densitometry. The data is expressed as a percent of total labeled Tg on the gel at each chase time.

Kinetics of BiP Interaction with Tg Folding Intermediates

Our previous inability to detect other newly synthesized proteins associated with Tg folding intermediates (Kim and Arvan, 1991) might be explained by the observation that resident proteins of the ER lumen have a long half-life and a low rate of synthesis compared to secretory proteins; thus they are only weakly labeled in short labeling protocols. To reexamine this question, we used a long-term labeling protocol to continuously radiolabel potential polypeptide chain binding proteins. By immunoprecipitation (see Materials and Methods), we consistently were able to detect small amounts of BiP co-precipitating with anti-Tg (e.g., Fig. 4, see below). Mock precipitations with non-immune serum failed to recover either polypeptide (not shown).

We wished to understand the relationship between the Tg folding pathway and BiP interaction. Using a mAb to BiP, immunoprecipitation at 1–60 min of chase recovered similar amounts of pulse-labeled BiP (not shown) but dramatically different amounts of newly synthesized Tg (Fig. 2 a). At the earliest time points tested (1–10 min of chase after a 10-min pulse) the recovery of nascent Tg with anti-BiP was observed to be maximal, representing ≥90% of that obtained by precipitation with a polyclonal serum directed against Tg (Fig. 2 b). In the interval 15–20 min, ≤20% of the Tg molecules could be co-precipitated, indicating a significant decrease in BiP association. By 30 min (and thereafter, Fig. 2 b), a time when >50% of Tg is still monomeric (Fig. 1 b), BiP association had declined further so that <5% of Tg was co-precipitated. All of the BiP-associated Tg was sensitive to digestion with endoglycosidase H (not shown). These results suggested a specific association of BiP with one or more early Tg folding intermediates in the ER. The kinetics of BiP dissociation in Fig. 2 b seems to correlate reasonably well with the dissolution of nascent Tg aggregates into monomers,

given our previous observation that such aggregation was prominent at 5–10 min of chase yet was fully resolved by 20 min of chase (Kim and Arvan, 1991).

Identification of Specific Tg Folding Intermediates Recognized by BiP

To examine directly the association of BiP with nascent Tg aggregates, we exploited the fact that BiP binding is maximal to exportable polypeptides with improperly coupled cysteine residues (Machamer et al., 1990); Tg aggregates clearly contain aberrant disulfide bonds (Kim and Arvan, 1991). Interchain disulfide links (which are not present in Tg that exits from the ER) result in an inability of Tg aggregates to enter an SDS–polyacrylamide gel without prior reduction. Tg specifically bound to BiP was co-precipitated with anti-BiP after pulse radiolabeling, and eluted from BiP by incubation with 1 mM Mg-ATP, a method which dissociates bound proteins without denaturation. Equal aliquots of the eluted Tg were then analyzed by SDS-PAGE under non-reducing and reducing conditions (Fig. 3, *left* and *middle* panels, respectively). At the earliest chase times, ≥50% of Tg released from BiP could not enter a non-reducing gel (*left*) as determined by comparison to a reducing gel (*middle*); instead, material was routinely accumulated at the top of the stacking gel (not shown). As the chase progressed, considerably less labeled protein was eluted because of reduced BiP association at later stages of Tg maturation. These data provide evidence that early BiP association occurs directly with Tg aggregates containing interchain disulfide bonds.

We noted (Kim and Arvan, 1991; and this report) that disulfide-linked aggregates never account for all of the labeled Tg. To characterize the remaining fraction of BiP-associated Tg molecules, an equal aliquot of the ATP eluate from each BiP immunoprecipitate was analyzed by native PAGE (we emphasize that interchain disulfide-linked aggre-
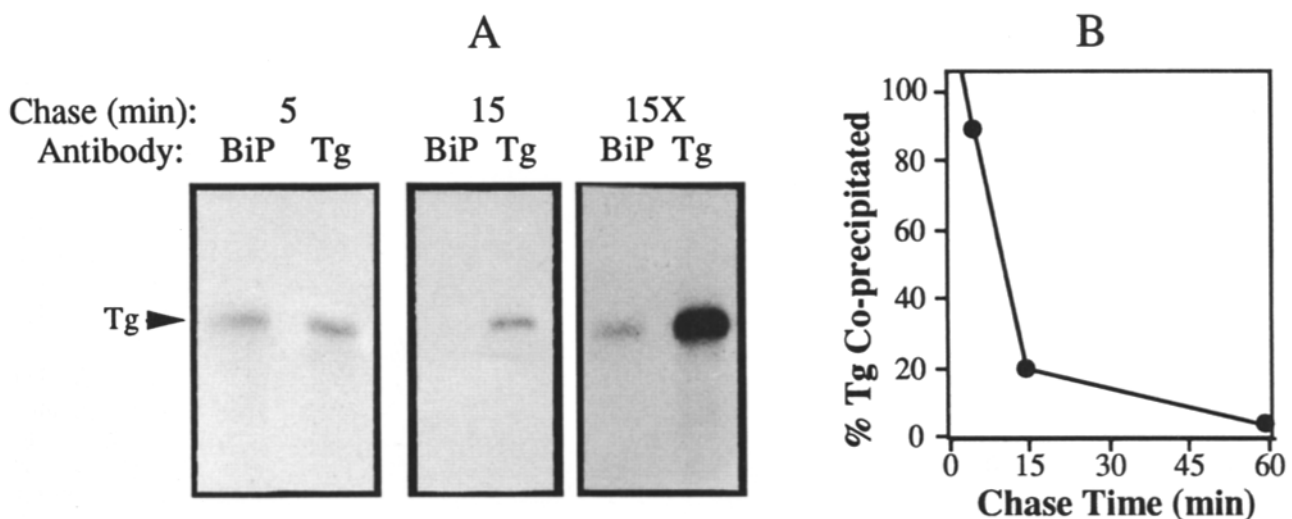

Figure 2. Co-precipitation of nascent Tg with BiP as a function of chase time. (A) Cells were pulse-labeled as in Materials and Methods and chased for the times indicated. The cells were lysed under non-denaturing conditions as described in Materials and Methods. Cell lysates were subjected to parallel immunoprecipitations with antibody either to BiP or to Tg, and analyzed by 4–8% SDS-PAGE under reducing conditions. The gel at 15 min of chase was intentionally overexposed (*15X*) to reveal Tg co-precipitated with BiP. (B) Scanning densitometry of fluorographs like those shown in A were used to calculate the fraction of Tg co-precipitated with anti-BiP. Not shown in the figure, recovery at 1 min of chase was measured at 118%.
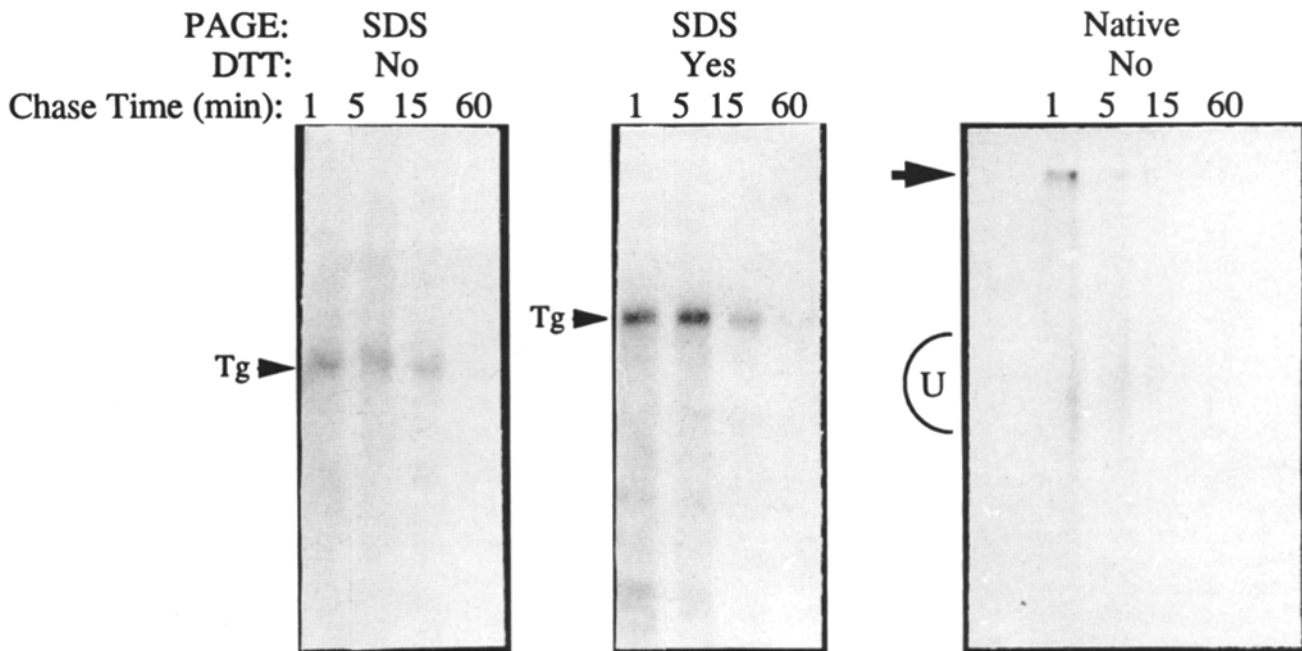
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Figure 3. Direct demonstration of BiP association with Tg aggregates. Thyrocyte monolayers were pulse labeled for 10 min and then lysed under non-denaturing conditions as described in Materials and Methods. The cell lysates were immunoprecipitated with a mAb to BiP. Co-precipitated Tg was specifically eluted from BiP with Mg–ATP as described in Materials and Methods. Equal aliquots of the eluate were then analyzed by 4% PAGE under non-reducing and denaturing (*left*), reducing and denaturing (*middle*), or native (*right*), gel conditions. Diminished recovery of nascent Tg is evident by non-reducing (*left*) versus reducing (*middle*) SDS-PAGE (∼50% after accounting for band width). Further analysis of non-reduced samples indicates that Tg which is resolved by SDS-PAGE (*left*) can be further fractionated by native PAGE (*right*) into an aggregate accumulated at the top of the resolving gel (*arrow*), or in the unfolded monomer region (*U*). Within the limits of error in the measurement, scanning densitometry of the native gel at 1 min of chase fully accounts for the Tg recovered by non-reducing SDS-PAGE.

gates are excluded from this gel). In the cells at 1 min of chase, a small fraction of the Tg released from BiP (perhaps 10%) migrated with a mobility of unfolded monomers (*U*, Fig. 3, *right*). In addition, a larger fraction was unable to enter the resolving gel under non-denaturing conditions and accumulated at the top (*arrow*). As the chase progressed, these bands decreased in signal, but no F-monomer or dimer ever appeared, even though these forms were clearly present by direct 2-D PAGE (Fig. 1). The recovery of unfolded monomers was less than that obtained by non-reducing SDS-PAGE (Fig. 3, *left*), but adding to this the material recovered at the top of the native gel could fully account for the Tg obtained in the left panel. Although broad migration in the native gels made precise quantitation difficult, it appears that in addition to interchain disulfide-linked Tg aggregates, a considerable fraction (up to 40%) of BiP-associated Tg transiently exists in forms that are resolved in a polyacrylamide gel only after the addition of SDS. We refer to these forms as noncovalently linked aggregates (see Discussion).

Analysis of Tg Aggregates Formed after Tunicamycin Treatment

N-glycosylation of Tg can be completely inhibited by exposure of thyrocytes to a low dose of tunicamycin (0.5 μg/ml) for 16 h. In this case, Tg aggregates are formed which accumulate in the ER; this differs from normal Tg which aggregates only in a transient fashion (Kim and Arvan, 1991).

In cells labeled for 2 d with [^{35}S] amino acids, tunicamycin treatment resulted in a considerable increase in the amount of BiP that was co-precipitated with anti-Tg (Fig. 4 *a*). Analysis of the cell lysates after pulse labeling showed that a 16 h pre-exposure to tunicamycin increased the synthesis of molecular chaperones (e.g., BiP and GRP94, Fig. 5 *b*) resulting in dramatically elevated intracellular levels (data not shown). Lysates of pulse-labeled, tunicamycin-treated cells failed to reveal a labeled BiP band after precipitation with anti-Tg (Fig. 5 *a*), not because of failure of BiP association, but because most of the co-precipitated BiP was previously rather than newly synthesized (Fig. 4 *a*). The data indicate that like the Tg aggregates which occur normally, aggregates of non-glycosylated Tg also serve as excellent substrates for BiP binding.

N-glycosylation of Tg can also be completely inhibited within 2 h using a higher dose of tunicamycin (20 μg/ml), also resulting in the formation of nonglycosylated Tg aggregates which cannot exit the ER. In this case, however, there was no significant difference in the synthesis or levels of molecular chaperones compared to control cells (Fig. 5 *b*). Even without increased levels of BiP, >90% of pulse-labeled Tg was recovered by coprecipitation with anti-BiP (Fig. 5 *a*). Compared to control cells in which Tg aggregates exist only transiently, the fraction of cellular BiP bound to nonglycosylated Tg aggregates was increased by >10-fold (P. Kim and P. Arvan, unpublished data). The data imply that
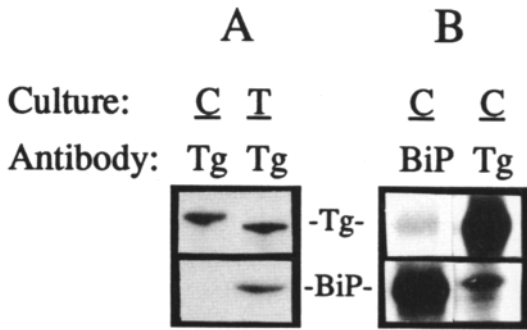
Figure 4. Co-precipitation of Tg-BiP in thyrocytes after long-term radiolabeling. (A) Thyrocytes were labeled for 2 d during which cells were untreated (C) or treated with 0.5 µg/ml tunicamycin for 16 h (T) before lysis. Equal aliquots (normalized to TCA-precipitable counts) were immunoprecipitated by anti-Tg antibody and analyzed by 4–8% reducing SDS-PAGE. Non-glycosylated Tg migrates faster than normally glycosylated Tg. (B) Confluent monolayers were steady-state labeled for 4 d with [³⁵S]methionine, lysed, and subjected to parallel immunoprecipitation. Both precipitations were analyzed on the same 4–8% reducing SDS-PAGE. Exposure of fluorographs was optimized for densitometric scanning of the co-precipitate bands. For the figure, Tg and BiP bands were juxtaposed for a direct comparison of their relative intensities.

as glycosylated Tg aggregates with bound BiP begin to fold, they liberate BiP, making it once again available in the ER lumen. By contrast, non-glycosylated Tg aggregates with bound BiP release little or none of it. Progressive accumulation of these aggregates recruits more of the available BiP into complexes, which presumably induces thyrocytes to increase their synthesis of BiP.

Stoichiometry of BiP/Tg Binding

The observed aggregation of nascent Tg in normal thyrocytes, and the finding that Tg aggregates bind significant

amounts of BiP, raise two related questions: (a) how much BiP is required for a Tg molecule to fold; and (b) might the level of available BiP and other chaperones in the thyrocyte ER limit the rate of initial Tg folding? To determine the average BiP/Tg stoichiometry in normal thyrocytes, cells were labeled to steady-state with [³⁵S]methionine at a constant specific radioactivity (for 4 d, see Materials and Methods) and then lysed under non-denaturing conditions. Equal aliquots were subjected to parallel immunoprecipitation either for Tg or BiP. Co-precipitated Tg and BiP (seen in Fig. 4 b) were quantitated by scanning densitometry and corrected for the relative methionine content of the polypeptides. From these data, it was calculated that on average, nearly 10 BiP molecules are associated with each Tg molecule (Table I).

As noted previously, pulse-labeled Tg aggregates from tunicamycin-treated cells are completely unrecovered by PAGE in the absence of reducing agents (Kim and Arvan, 1991) even under conditions in which molecular chaperones are markedly elevated (Fig. 5 b). To test whether the compensatory increase in molecular chaperones is sufficient to handle the magnitude of misfolded, non-glycosylated Tg that accumulates in the ER lumen, we used identical methodology to that described above for examination of the BiP/Tg stoichiometry. Interestingly, after 16 h of tunicamycin treatment, the average BiP/Tg ratio was only ∼4 (Table I).

Availability of Molecular Chaperones

To establish the importance of the availability of BiP and other helper proteins in the Tg folding pathway, we exploited the difference between the extremely long lifetime of BiP and other resident chaperones in the ER lumen ($t_{1/2} \geqslant 24$ h; Suzuki et al., 1991), versus the relatively short residence time for exportable proteins (for Tg export: $t_{1/2} \sim 2$ h; Kim and Arvan, 1991). Consequently, thyrocytes were treated with 10 µg/ml cycloheximide to inhibit protein synthesis for 6 h, sufficient to deplete ∼90% of the Tg from the ER; under these conditions, the concentrations of the ER chaperones BiP, GRP94, and PDI were unaffected (unpublished data).
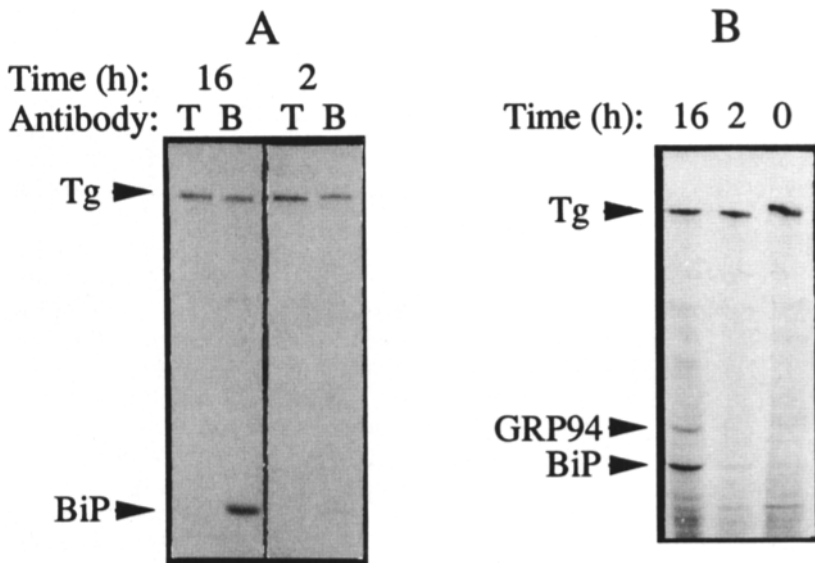

Figure 5. Misfolding of non-glycosylated Tg by tunicamycin treatments in cells with elevated or normal BiP levels. (A) Thyrocytes were treated with tunicamycin at 0.5 µg/ml for 16 h or at 20 µg/ml for 2 h, to produce non-glycosylated Tg that aggregated irreversibly in the ER (Kim and Arvan, 1991). Each set of cells was then pulse-labeled for 15 min, chased for 15 min, and lysed as in Materials and Methods. Equal aliquots were subjected to parallel immunoprecipitation and analyzed as in Fig. 2 a. After either treatment, precipitation with anti-BiP (lanes marked B) recovered ⩾90% of the non-glycosylated Tg that was recovered by anti-Tg (lanes T). (B) Cells treated as in A and control cells were labeled and then lysed in boiling SDS in the presence of reducing agent, and analyzed without immunoprecipitation by 4–12% SDS-PAGE. Non-glycosylated Tg from cells treated with tunicamycin for 16 or 2 h migrates faster than normally glycosylated Tg (0 h). Note the induction of BiP and GRP94 in cells treated for 16 h, in marked contrast to cells exposed for only 2 h.

Control	Tunicamycin
9.5	4.4

Finally, cycloheximide was removed (see Materials and Methods) and protein synthesis was permitted to resume for 10 min at which time the cells were pulse labeled. Fig. 6 shows that in control cells at 5 min of chase, ≤50% of the pulse-labeled Tg could be recovered by SDS-PAGE under non-reducing conditions compared to reducing conditions, due to the formation of interchain disulfide-linked Tg aggregates. By contrast, in cells drained of previously-synthesized Tg with the intention of increasing the chaperone/Tg ratio, pulse-labeled Tg was now fully recovered even under non-reducing conditions (Fig. 6). In this case, it appears that the relative rates of aggregate formation and dissolution were altered so that aggregates were no longer observed.

Discussion

We have been interested in understanding whether BiP and other molecular chaperones in the ER are primarily involved in the more proximal period of folding of exportable proteins, or in more distal steps such as protein oligomerization. Recent studies in yeast have indicated that alterations in BiP function lead, within minutes, to a phenotype exhibiting arrest of translocation into the ER lumen (Vogel et al., 1990; Nguyen et al., 1991). Based on the conservation of many protein export functions between yeast and higher species (Novick et al., 1981), it seems probable that BiP action oc-
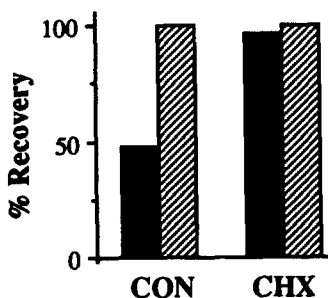

Figure 6. Effects of increasing "available" BiP level on nascent Tg aggregation, by depleting pre-existing Tg from the ER. Confluent thyrocytes were either untreated, or treated with cycloheximide (10 μg/ml) for 6 h to deplete ~90% of pre-existing Tg from the ER (without detectable change in the levels of resident chaperones, see text). After washout of cycloheximide to restore protein synthesis, the cells were labeled, chased for 5 min, and lysed as described in Materials and Methods. The lysates were then examined by SDS-PAGE under non-reducing and reducing conditions. The recovery of Tg bands was quantitated by phosphorimaging. Tg recovered under reducing conditions was set as ~100% (based on Kim and Arvan, 1991).

curs in very close proximity to protein translocation. By contrast, actual measurements of BiP association using multiple test proteins in different mammalian cell lines (Gething and Sambrook, 1990; Hurtley et al., 1989; Dorner et al., 1987; Hendershot, 1990; Singh et al., 1990) have reported a range of results. We wish to reconcile the two extremes of this range; first, transient BiP interaction (shortly after synthesis) will be considered. Later in this discussion, we consider the stable, "long-term" association that has been observed in specific instances.

Considerable controversy regarding transient BiP binding has focused on a co- and posttranslational period (<5 min) during which folding kinetics in vivo are sufficiently fast that multiple early steps in the structural maturation of typical model proteins (i.e., those conveniently expressed in cell lines) have been difficult to resolve without temperature blocks or pharmacological intervention. It is therefore of particular interest to examine the relationship between BiP association and the assembly of proteins in higher eukaryotes that exhibit physiologically normal expression levels of endogenous proteins and chaperones. To this intent, we have exploited the exceptionally well resolved conformational maturation of nascent Tg. Shortly after synthesis, Tg is found in protein aggregates that are converted into monomeric, and ultimately, dimeric Tg that is transported from the ER. Immunoprecipitation does not reveal other newly synthesized proteins contained in nascent Tg aggregates (Kim and Arvan, 1991), but a similar precipitation after steady-state labeling (which enables long-lived ER resident proteins to become labeled) co-precipitates BiP (Fig. 4 *b*).

The degree of BiP-protein interaction in this system is consistent with the report that BiP binding is highest for exportable proteins containing incompletely or improperly formed disulfide bonds (Machamer et al., 1990). Since (*a*) the formation of disulfides in the ER is initiated co-translationally (Freedman et al., 1989; Hurtley et al., 1989; Machamer et al., 1990); (*b*) nascent Tg aggregates clearly contain aberrant disulfides (Kim and Arvan, 1991); and (*c*) nascent proteins before folding are most likely to expose peptide stretches suitable for BiP binding (Flynn et al., 1991), it is expected that BiP association should be maximal at the earliest possible times after emergence of Tg in the ER lumen. In tunicamycin-treated cells, non-glycosylated Tg is found in mispaired disulfide-linked aggregates (Kim and Arvan, 1991); virtually all is associated with BiP (Fig. 5 *a*). Furthermore, when thyrocytes are cooled to 15°C (to slow the steps of early Tg folding as well as blockage of vesicular exit, (Kim and Arvan, 1991) not only is the dissolution of Tg aggregates substantially retarded, but the association of nascent Tg with BiP is prolonged in parallel (Kim and Arvan, unpublished data). Thus, Tg aggregates with mispaired, interchain disulfide-bonds appear to be a favored target for BiP association. The decline in BiP association is most dramatic during the first 15 min of chase (Fig. 2 *b*), correlating best with the dissolution of Tg aggregates (Kim and Arvan, 1991); these data indicate that quantitatively, BiP plays its most important role in early Tg folding.

The finding that some Tg released from BiP with Mg-ATP can be detected by non-reducing SDS-PAGE clearly demonstrates that folding intermediates without interchain disulfide links are also associated with BiP. We have characterized these BiP-associated intermediates by native gel electropho-

resis and found that most of the Tg that enters such a gel is found in a large complex at the top of the resolving gel (Fig. 3 *a, right*). Thus, in addition to Tg aggregates held together by interchain disulfide bonds, BiP is also associated with aggregates that do not contain such bonds. Although we have not yet obtained direct evidence, these "non-covalent aggregates" may represent Tg molecules that still possess aberrant intrachain disulfide bonds, given that each Tg monomer has >100 cysteine residues, most of which eventually form proper intrachain disulfides (Malthiery et al., 1989). We do not know if non-covalent aggregates exist in vivo or if they are obtained by aggregation of U-monomers after release from BiP in vitro. In either case, these molecules serve as outstanding substrates for BiP binding (Fig. 3). We must emphasize that pulse–chase studies highlight newly synthesized Tg molecules, thus we have not excluded the alternative that unlabeled chaperones other than BiP could be bound to non-covalent aggregates, contributing to their inability to enter a native gel. We can only state that the estimated relative molecular weight cut-off for entry into our native and SDS-PAGE gels is ≥1 million kD.

By improving our native gel electrophoresis technique over previous studies, we have now resolved monomeric intermediates into unfolded (U) and folded (F) forms. After chronic hormonal stimulation of thyrocytes, we have been able to demonstrate an unequivocal precursor–product relationship between U- and F-forms, as well as between the F-form and Tg dimer (Arvan and Kim, manuscript in preparation); under the culture conditions described in this paper, the association of folded subunits to form the Tg dimer is sufficiently fast to prevent significant accumulation of the F-form (Fig. 1). The improved resolution of our gel system has permitted two independent advances: first, kinetic criteria (comparing Figs. 1 *b* and 2 *b*) reveal that BiP is quantitatively less important in the later stages of Tg folding. Second, we have found by direct release from BiP with Mg-ATP that the monomeric species interacting with BiP (albeit seemingly weakly) is the U-form; within the limits of sensitivity of our assay, the F-form does not bind BiP. Taken together, these data strongly support our conclusion that BiP is not important in the dimerization of folded monomers. To reconcile this conclusion with that concerning proteins such as immunoglobulins (in which the role of BiP is thought to involve stable, long-term associations with monomeric subunits pending oligomerization; Hendershot, 1990), we believe it is necessary to reconsider the stoichiometry of the BiP/substrate interaction.

Using parallel co-precipitation assays from steady-state labeled cells, we have been able to calculate an average molar ratio of the BiP which interacts with Tg (Table I). We must emphasize that this is not a ratio of the total expression levels of the two proteins, but merely the fraction of molecules that happen to be interacting at a single moment in time in the steady state. The antibody to BiP is a monoclonal that does not block the ability of BiP to bind a substrate molecule, while the parallel antibody is a polyclonal against denatured Tg which potentially may displace BiP from some of its binding sites. If for this or any other reason, BiP molecules were to be dissociated from Tg during or after cell lysis, the recovery of BiP by anti-Tg precipitation would be underestimated. Given these facts, we find the BiP:Tg molar ratio of 9.5 remarkable. Since this value represents an average of all

BiP:Tg combinations, including many which are considerably lower, it follows that some Tg molecules have even higher levels of association with BiP. Although the high number of BiP binding sites are undoubtedly related to the large size of Tg, it should be pointed out that while the monomer molecular weight of Tg is only ∼300 kD, 10 BiP molecules have an accumulated mass of ∼780 kD. Thus, it is likely that nascent Tg molecules are extensively involved with BiP, and BiP molecules may even be close packed along the nascent chain.

It appears that the induction of BiP (and other chaperone) synthesis to markedly elevated levels after tunicamycin treatment (Fig. 5 *b*), as a cellular compensation for the aggregated Tg that has accumulated in the ER (Kim and Arvan, 1991), still is insufficient to bring the average BiP/Tg ratio up to control values (Table I). Since it has been shown that some proteins which are described as "irreversibly misfolded" can actually fold properly in vivo under specialized conditions such as altered temperature (Gibson et al., 1979; Machamer and Rose, 1988), a relative deficiency of molecular chaperones (i.e., the concentration of available chaperones relative to the concentration of exportable proteins) may be an important factor in protein aggregation, especially for complex proteins like Tg. Altering the relative availability of chaperones in the thyrocyte ER is made possible by cycloheximide pretreatment, which allows preexisting Tg to be depleted by export from the ER; such conditions do not disturb the levels of BiP and other resident proteins which are known to have much slower turnover. In this case, interchain disulfide-linked Tg aggregates were not observed (Fig. 6); either aggregate formation was inhibited or aggregate dissolution was accelerated. Once again, these data support the hypothesis that available chaperones are important to minimize nascent protein aggregation.

Although a large fraction of the BiP molecules dissociate during the proximal period of Tg folding, at later chase times (when very few Tg aggregates can be found) (Kim and Arvan, 1991; and this report, Fig. 3), a declining range of 20–5% of newly synthesized Tg still can be co-precipitated with anti-BiP (Fig. 2 *b*). These data suggest that at prolonged chase times when most Tg molecules have undergone considerable folding, a measurable fraction of Tg molecules still have one or two attached BiP molecules. In the cycles of BiP unbinding and rebinding as proposed by Flynn et al. (1989), these last few BiP molecules would be unable to rebind only when conformational changes in Tg, i.e., the F-form, eliminate or hide potential BiP binding sites. Of course in the case of some proteins, these last binding sites may finally be hidden only upon oligomerization. Myeloma cells display "stable" association of BiP molecules with immunoglobulin heavy chains (Bole et al., 1986). However, since these heavy chains undergo long-term maturation arrest due to the absence of a partner for heterodimerization (Hendershot, 1990), the observation of a stable association with BiP tends to weight one's impression toward a major role of BiP in protein oligomerization per se. Although exceptions are always possible, it may be preferable to view this association as simply the end of a spectrum of progressively declining stages of relative BiP binding during maturation of the nascent chain.

We have shown that BiP binds to Tg with aberrant interchain (and presumably intrachain) disulfides, during a transient period when most nascent Tg exists in an aggregated

state. BiP interaction has been hypothesized to cause aggregate solubilization (Pelham, 1986) in a catalytic manner (Rothman, 1989). However, no evidence has been obtained for BiP as a direct catalyst of protein folding, and Dorner and colleagues (1987 and 1988) have suggested an inverse correlation between the degree of BiP association and the efficiency of ER export. These data obtained in cell lines have implied that BiP binding could retard the folding of exportable proteins, unlike resident proteins such as protein disulfide isomerase (PDI; Freedman et al., 1989) which are likely to interact with nascent Tg to catalyze its folding in the ER lumen. Nevertheless, we believe that BiP helps rather than hinders the process of protein folding in vivo; to clarify this view, we suggest the following hypothesis. We propose that BiP recognition occurs co-translationally (Vogel et al., 1990); aberrant disulfide bonds indirectly facilitate the association (Machamer et al., 1990) to peptide side chains (Flynn et al., 1991) by serving to stabilize highly unfolded conformations. While slowing (mis)folding of nascent proteins by retarding the random motion of peptide stretches that have yet to acquire secondary structure, BiP "presents" exportable polypeptides to PDI and other potential catalysts of polypeptide folding. During periods of BiP unbinding driven by ATP, this stretch of nascent polypeptide has an opportunity to fold. A normal protein uses this opportunity, and buries or eliminates the BiP recognition site, thereby reducing progressively the degree of BiP association. In the folding of normal proteins, a balance exists between the action of BiP as a catalyst (Rothman, 1989) versus quality controller (Hurtley et al., 1989). To the extent that BiP can present proteins to PDI (for the rectification of mispaired disulfides) or to other catalysts, folding will be accelerated. To the extent that BiP sterically hinders the access of other catalysts, or that other catalysts are unavailable, folding will be slowed and quality control more evident. Thus we propose that BiP works in tandem with other chaperones to facilitate protein folding in the ER.

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