Calnexin and BiP Act as Sequential Molecular Chaperones during Thyroglobulin Folding in the Endoplasmic Reticulum

Paul S. Kim* and Peter Arvan**

*Division of Endocrinology, Beth Israel Hospital, and †Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02215

Abstract. Before secretion, newly synthesized thyroglobulin (Tg) folds via a series of intermediates: disulfide-linked aggregates and unfolded monomers → folded monomers → dimers. Immediately after synthesis, very little Tg associated with calnexin (a membrane-bound molecular chaperone in the ER), while a larger fraction bound BiP (a lumenal ER chaperone); dissociation from these chaperones showed superficially similar kinetics. Calnexin might bind selectively to carbohydrates within glycoproteins, or to hydrophobic surfaces of secretory proteins while they form proper disulfide bonds (Wada, I., W.-J. Ou, M.-C. Liu, and G. Scheele. *J. Biol. Chem.* 1994. 269:7464–7472). Because Tg has multiple disulfides, as well as glycans, we tested a brief exposure of live thyrocytes to dithiothreitol, which resulted in quantitative aggregation of nascent Tg, as analyzed by SDS-PAGE of cells lysed without further reduction. Cells lysed in the presence of dithiothreitol under non-denaturing conditions caused Tg aggregates to run as reduced monomers. Cells lysed either way, after in vivo reduction, Tg coprecipitated with calnexin. After washout of dithiothreitol, nascent Tg aggregates dissolved intracellularly and were secreted ultimately. 1 h after washout, > 92% of labeled Tg was found to dissociate from calnexin, while the fraction of labeled Tg bound to BiP rose from 0 to ~40%, demonstrating a "precursor-product" relationship. Whereas intraluminal reduction was essential for efficient Tg coprecipitation with calnexin, Tg glycosylation was not required. These data are among the first to demonstrate sequential chaperone function involved in conformational maturation of nascent secretory proteins within the ER.

In recent years, molecular chaperones have been suggested to play important roles in the quality control of exportable protein biosynthesis, translocation, folding, and degradation (de Silva et al., 1990; Gething and Sambrook, 1992; Nicchitta and Blobel, 1993; Urade et al., 1993). With few exceptions (Flynn et al., 1991; Blond-Elguindi et al., 1993), molecular details about the peptide-binding specificity of individual chaperones are sparse. Nevertheless, there is a general consensus that a primary function of molecular chaperones is to enhance the rate and/or the efficiency of protein folding (Rothman, 1989). Such a conclusion is consistent with the one feature shared by all chaperones, namely, their enhanced recognition of unfolded proteins over those in the native state (Ellis et al., 1989).

At each cellular location in which protein folding takes place, chaperones can be found in groups, presumably working together (Hendrick and Hartl, 1993). In higher eukaryotes, particular attention has been paid to chaperones in the ER, not only because the ER is a specialized folding compartment for newly made exportable proteins (Helenius et al., 1992), but because of the high concentrations of chaperones that must work within this uniquely oxidizing environment (Freedman, 1989; Braakman et al., 1992a; Hwang et al., 1992). Since nascent secretory polypeptides are by definition structurally immature, it seems possible that exposure of these highly unfolded "substrates" might allow multiple groups of chaperones to bind at once. In theory, such concomitant interactions might be one way to account for chaperone cooperativity.

However, the possibility of distinct binding sites for different chaperones on nascent polypeptides leaves unclear whether in most proteins, such sites are sufficiently spatially segregated to allow these concomitant interactions. Indeed, depending on the particular substrate polypeptide, the relative abundance of different chaperones, and binding affinities for their respective sites, one may even conceive of a situation in which the interaction of certain chaperones might compete for the binding of other helper proteins, hindering the overall folding process. Specifically in CHO cells manufacturing von Willebrand factor, the selective overexpression of BiP, an ER lumenal chaperone, enhances and protracts BiP association with newly synthesized von Willebrand factor, suggesting that BiP might act as a "co-chaperone" in the folding process.
brand factor and interferes with its secretion, presumably as a consequence of retarded protein folding (Dorner et al., 1992). Indeed, differences in the balance of chaperone activities may be an important factor contributing to the folding environments in the ER of different cells, which vary in their ability to fold the same polypeptide (Braakman et al., 1991). One mechanism to minimize unregulated competition and enhance efficiency of chaperone function includes their sequential action, such as the ordered binding of certain bacterial proteins to DnaK, DnaJ, and GroEL/GroES (Langer et al., 1992). However, to our knowledge, reports of sequential chaperone function in the ER are limited to only few recent studies (Melnick et al., 1994).

We have been studying the conformational maturation of nascent thyroglobulin (Tg) in thyroid epithelial cells. After its synthesis, this secretory glycoprotein progresses through a series of discrete folding intermediates that ultimately achieve a dimeric state (Kim and Arvan, 1991) with an overall t1/2 of 25-55 min (Kim and Arvan, 1993). Either in culture, or physiologically within thyroid follicles, roughly half of nascent Tg can initially be detected as disulfide-linked protein aggregates (Kim et al., 1993). The fact that the Tg monomer approaches 3,000 amino acids in length and the observation of large Tg-containing complexes (Mr >2,000,000) certainly leaves open the possibility of simultaneous binding of multiple different chaperones. We recently reported that BiP is one chaperone that can bind both disulfide-linked aggregates and unfolded monomers of nascent Tg. However, at a moment in time in the steady state, on average, nearly 10 BiP molecules bind to each nascent Tg (Kim et al., 1992), and it is not obvious that this accumulated mass of BiP (totally 780,000 for each ~300,000 worth of Tg monomer) allows much room for binding of other chaperones. Nevertheless, the degree of BiP binding declines progressively during the folding of individual Tg molecules (Kim et al., 1992; Kim and Arvan, 1993); thus, it seems likely that the access and binding of other helper proteins to Tg may also change as a function of time. Consequently, it was of interest to examine the binding of calnexin, an integral membrane protein in the ER (Wada et al., 1991; David et al., 1993), which also functions as a chaperone for secretory proteins (Ou et al., 1993; Le et al., 1994), including those undergoing disulfide maturation (Wada et al., 1994). Using dithiothreitol to slow aggregate dissolution in vivo, we find that calnexin and BiP act as sequential molecular chaperones in the Tg folding pathway. (These findings were originally published in abstract form [Kim, P. S., and P. Arvan. 1993. Mol. Biol. Cell. 4:93a].)

Materials and Methods

Cell Culture

Porcine thyrocytes were isolated and seeded as previously described (Kim and Arvan, 1991) and then cultured in control medium (DME) or that containing hormones that stimulate Tg production (Kim and Arvan, 1993). Bovine thyroid-stimulating hormone (1 nM/ml), bovine insulin (1 µg/ml), hydrocortisone (1 nM), and human transferrin (5 µg/ml), when used, were obtained from Sigma Immunochemicals (St. Louis, MO). Experiments were carried out on day 7.

1. Abbreviations used in this paper: BFA, brefeldin A; IAA, iodoacetamide; Tg, thyroglobulin; VSV, vesicular stomatitis virus.

Cell Labeling and Lysis

For pulse-chase analysis, confluent monolayers were labeled with a mixture of [35S]methionine and [35S]cysteine (Exper, New England Nuclear, Boston, MA). In Figs. 7, 8, and 11 a, DTT was included in the labeling mixture. Otherwise (with the exception of Fig. 1), the labeled cells were washed three times in the presence of excess unlabeled methionine/cysteine, and were then exposed to PBS containing DTT for 10 min at 37°C. The cells were then washed three times to remove the DTT, and the chase was continued in control medium. Where indicated in some experiments, prolonged incubation was performed to collect chase media that contained secreted Tg. At the end of each chase, cells were quickly chilled to 4°C and treated with 50 mM iodoacetamide (IAA) in PBS for 10 min to alkylate intracellular sulfhydryls (Braakman et al., 1991).

Before lysis, the cells were washed three times in cold PBS, and then lysed in 0.5 ml of buffer containing 0.1 M NaCl, 25 mM Tris, pH 7.5, 10 mM IAA, 1% Triton X-100, 5 mM EDTA, and a cocktail of protease inhibitors as previously described (Kim et al., 1992). In our experiments, we observed little difference in the ability to coprecipitate Tg with calnexin, regardless whether EDTA, calcium, or neither were added to the lysis buffer. (When applied at 0-4°C, as in this report, the inclusion of EDTA does not interfere with the association of unfolded peptides with calnexin [Le et al., 1994].) In certain experiments, in preparation for calnexin immunoprecipitation, the cell lysis buffer omitted IAA and instead included DTT at either 1 mM in Fig. 8 (Wada et al., 1994) or 2.5 mM (See Figs. 4 and 9 b). When samples were to be used for immunoprecipitation of BiP, cells were alkylated in situ and neither IAA nor DTT were subsequently used, while 20 µg/ml apyrase was included in the lysis buffer (to enzymatically deplete ATP). After exposure to apyrase for 60 min, 10 mM IAA was added.

For steady-state analysis, thyrocytes were continuously labeled at constant 35S-specific radioactivity in normal growth media. The cells were fed as usual with these media over the course of 6 d. After this time, the cells were treated with DTT in vivo and then lysed as described above. Tunicamycin (Sigma) was used at a final concentration of 20 µg/ml for the last 3 h of the steady-state labeling period, or at a final concentration of 1 µg/ml for the last 18 h (Kim et al., 1992). Castanospermine (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used at the maximum soluble dose (1.32 mM). Brefeldin A (BFA) (Sigma) was used at 10 µg/ml.

Antibodies and Immunoprecipitations

Antisera to denatured Tg (Arvan and Lee, 1991) and a monoclonal antibody to BiP (Bole et al., 1986) were previously described. A polyclonal rabbit antiserum against a synthetic peptide corresponding to amino acids 487-505 of canine calnexin was the generous gift of Dr. J. Bergeron (McGill University, Montreal, Canada). Samples were preclerared in the presence of preimmune sera and protein A- or protein G-Sepharose beads or Zysorbin (Zymed Laboratories, South San Francisco, CA). The samples were then immunoprecipitated for 2 h at 4°C.

Gel Electrophoresis and Quantitation

Reducing and nonreducing SDS-PAGE, phosphorimaging, and band quantitations were carried out as previously described (Kim et al., 1992).

Results

Binding of Nascent Tg to Calnexin in the ER of Control Thyrocytes

When pulse-labeled thyrocytes are lysed in the absence of reducing agent, the nascent secretory protein Tg can be detected as disulfide-linked aggregates, which after several minutes of chase, progress to unfolded monomers (Kim et al., 1992). However, after a 5-min pulse labeling without chase, no more than about half of newly synthesized Tg is generally found in these aggregates (Kim and Arvan, 1991). These data suggest kinetic heterogeneity, such that substantial early folding and binding to molecular chaperones (including that occurring during the period of Tg translation-
translocation) may have already concluded at the 0 chase time.

We used a pulse-chase protocol to examine whether any newly synthesized Tg associated with calnexin. Immunoprecipitation with an antibody directed against a synthetic peptide from the sequence of canine calnexin recovered mostly previously synthesized calnexin (since no radioactive calnexin band was detected), although labeled thyroglobulin was specifically coprecipitated (Fig. 1). However, recovery of nascent Tg at the 0 chase time was <10% of that synthesized (as compared with direct immunoprecipitation of Tg); moreover, coprecipitation of labeled Tg fell to <2% within 10 min after synthesis (Fig. 1). Although technically and theoretically demonstrable chase kinetics representing dissociation from calnexin (i.e., including time points not earlier than 0 chase) bear superficial resemblance to dissociation kinetics for newly synthesized Tg from BiP (Kim et al., 1992), the low and rapidly declining binding to calnexin suggested that unlike BiP, most nascent Tg dissociated from calnexin cotranslationally.

To confirm adequate immunoreactivity of antibodies prepared against the canine calnexin synthetic peptide to porcine calnexin, we examined similar immunoprecipitates after labeling thyrocytes to steady state (see Materials and Methods). In this case, labeled calnexin was specifically recovered (Fig. 2, last three lanes) in comparison to the same cells immunoprecipitated for Tg (Fig. 2, first lane). In agreement with results from pulse-chase studies (Fig. 1), coprecipitation of labeled Tg with calnexin was nearly 0 in control samples (Fig. 2, second lane), indicating that relatively little labeled Tg is associated with calnexin at a moment in time in the steady state.

**Effect of DTT Exposure on Nascent Thyroglobulin Aggregation**

When added immediately postpulse, a brief exposure of live cells to DTT (a membrane-permeant reducing agent) can actually unfold newly synthesized proteins in the ER (Braakman et al., 1992a, b; Lodish et al., 1992; Kaji and Lodish, 1993; Lodish and Kong, 1993; Tatsumi et al., 1993). If thyrocytes treated with DTT in vivo are then lysed in the absence of additional reducing agent, newly synthesized Tg is recovered quantitatively as disulfide-linked aggregates, as measured by the complete absence of a labeled band in the Tg position upon nonreducing SDS-PAGE (Kim et al., 1993). Moreover, even after DTT removal, there was substantial prolongation of the chase time needed to convert disulfide-linked Tg aggregates into monomers (Fig. 3). However, when additional DTT was included in the nondenaturing cell lysis buffer, interchain disulfide linkages were broken and Tg aggregates ran as reduced monomers (Fig. 4, described further below).

While some variability in the rate of aggregate dissolution occurred between different preparations of primary thyrocytes, the most important factors in these kinetics were the dose and duration of DTT exposure. (The use of DTT solutions that were not freshly prepared immediately before use [as in Kim and Arvan, 1993] led to a significant loss of DTT potency.) Consequently to a delay in aggregate dissolution (Fig. 3), a DTT dose-dependent delay in labeled Tg export was observed. Nevertheless, a majority of the labeled Tg was secreted by 6 h of chase, even after brief exposure to 25 mM DTT (mM) 25 0 10 25

![Figure 1](#) Binding of nascent Tg to calnexin in control thyrocytes. Confluent thyrocytes were pulse labeled for 5 min and chased in complete medium for the chase times indicated. The cells were alkylated in situ, lysed in a buffer containing 10 mM IAA, and immunoprecipitated with an antibody to calnexin. Tg recovered upon calnexin immunoprecipitation was analyzed by reducing SDS-PAGE; the fraction of coprecipitated Tg was calculated by comparison to an aliquot of the same lysates immunoprecipitated directly for Tg, shown at bottom. No radiolabeled calnexin was recovered in this experiment (not shown).

![Figure 2](#) Binding of Tg to calnexin in steady-state labeled thyrocytes. Confluent thyroid epithelial monolayers were labeled continuously for 6 d. Before lysis, the live cells were either not treated or treated with DTT for 10 min at the doses indicated. The cells were then alkylated in situ, lysed as described in Materials and Methods, and immunoprecipitated with an antibody to calnexin. Coimmunoprecipitations were analyzed by reducing SDS-PAGE using an acrylamide gradient, followed by phosphorimaging. A control immunoprecipitation demonstrates the mobility of Tg (lane 1). The mobility of calnexin is indicated; the highly radiolabeled band recovered at the bottom of these gels has not been identified.

![Figure 3](#) Effect of DTT Exposure on Nascent Thyroglobulin Aggregation
Figure 3. Transient postpulse exposure of live thyrocytes to DTT causes quantitative aggregation of nascent Tg with prolonged aggregate dissolution. Confluent thyroid monolayers were pulse labeled for 15 min, exposed to 25 mM DTT for 10 min, and then washed and chased in complete medium lacking the reducing agent. At the chase times shown, thyrocytes were alkylated in situ, lysed as described in Materials and Methods, and analyzed by nonreducing SDS-PAGE. The asterisk indicates disulfide-linked Tg aggregates that are unable to penetrate into the resolving gel, as well as the delayed appearance of Tg monomers.

DTT (Fig. 5, see below). Thus, consistent with previous reports, effects of DTT exposure on thyrocytes were reversible.

Effect of DTT on the Binding of Tg to Calnexin in the ER

Using DTT exposure of live thyrocytes to prolong disulfide-linked aggregation of Tg, we reexamined Tg association with calnexin in the ER. In steady-state labeled thyrocytes, exposure to 10 mM DTT had no effect on the amount of immunoprecipitated calnexin, yet a significant increase in coprecipitated Tg was observed (Fig. 2, third lane); exposure to 25 mM DTT further increased the fraction of coprecipitated Tg to 25–40% of that recovered by direct Tg immunoprecipitation (Fig. 2, last lane). After DTT exposure, Tg coprecipitation with calnexin was specific, since it was not recovered in immunoprecipitates using control antibodies (not shown). After steady-state labeling, immunoprecipitation with anti-Tg recovers forms that are both sensitive and resistant to digestion with endoglycosidase H (ER and Golgi/post-Golgi, respectively) in a ~50/50 ratio (Arvan and Lee, 1991). Since roughly half of Tg is in the ER at a moment in time in the steady state and 25–40% of total cellular Tg is bound to the ER chaperone calnexin, the data indicate that DTT treatment causes a large fraction of Tg in the ER to associate with calnexin; furthermore, these data (Figs. 2 and 3) imply that calnexin recognizes disulfide-linked Tg aggregates as a predominant folding intermediate. However, while exposure to DTT in vivo was essential for the increased coprecipitation of Tg with calnexin (Fig. 2), interchain disulfide cross-linkages were not required because inclusion of DTT in the cell lysis buffer (Wada et al., 1994), which breaks interchain disulfide bonds, also yielded efficient coprecipitation of nascent secretory protein (Fig. 4).

Sequential Chaperoning of Nascent Tg by Calnexin and BiP in the ER

Since the effect of DTT on Tg aggregation is reversible, we surmised that nascent Tg and calnexin must disengage during the folding that follows DTT washout. We therefore compared the association of nascent Tg with calnexin and BiP during this recovery period. In pulse-labeled thyrocytes, binding of nascent Tg to calnexin started at its highest level and fell progressively during the chase (Fig. 6). By contrast, binding of newly synthesized Tg to BiP actually rose substantially after DTT washout (Fig. 6) and then declined subsequently. In later hours of chase, binding to both chaper-
After DTT washout, nascent Tg exhibits different kinetics of association with calnexin and BiP. Thyrocyte monolayers were pulse labeled, treated with DTT, and chased as in Fig. 3. The cells were then alkylated in situ, lysed without apyrase treatment, and immunoprecipitated at each chase time with antibodies to calnexin and BiP. The immunoprecipitates were analyzed by reducing SDS-PAGE followed by phosphorimaging. The fraction of coprecipitated Tg was calculated by comparison to an aliquot of the same lysates immunoprecipitated directly for Tg. On average, the data points from two independent experiments varied by ~20% of the mean values shown (i.e., 20% of 30% = 6%).

These reached comparably low levels (not shown), and a large fraction of labeled Tg was ultimately secreted (Fig. 5). Unfortunately, using this protocol (Fig. 6), the coprecipitation recoveries of Tg with either chaperone were not sufficiently quantitative to exclude the possibility that the two different chaperone-containing complexes represented nonoverlapping Tg subfractions. For this reason, we tested alternative protocols, exploiting the fact that DTT is more potent when it is included before initial disulfides have formed within the ER (i.e., UFT included before and during pulse labeling), rather than when it is added immediately after synthesis (Lodish and Kong, 1993).

Following published procedures for cotranslational DTT exposure (Braakman et al., 1992a), the interaction of nascent Tg with calnexin was reexamined. Even without DTT pretreatment, it was obvious that most Tg was bound to calnexin when synthesized during a 30-min labeling that included DTT at ~5 mM (Fig. 7). Next, we tested the kinetics of calnexin and BiP interactions with nascent Tg in pulse-labeled thyrocytes pretreated with 1 mM DTT for 5 min before a 30-min labeling period that included the reducing agent. Under these conditions, calnexin coprecipitation of labeled Tg at the 0 chase time yielded quantitative (i.e., 100%) recovery (Fig. 8, upper panel). Nascent Tg (~92%) dissociated from calnexin within 30 min of DTT washout, with complete dissociation achieved by 2 h. Similar to results obtained from postpulse exposure to DTT, washout of DTT led to a delayed yet marked (~40%) increase in Tg binding to BiP (Fig. 8, lower panel). While coprecipitation of newly synthesized Tg with BiP exhibited less than complete recovery, these data demonstrate unequivocally a precursor-product relationship in Tg association with the two chaperones. Thus, the data establish that at least some of the nascent Tg molecules are bound first to calnexin and then to BiP before folding and assembly is completed, indicating sequential chaperoning of Tg in the ER.

**Effect of DTT on the Association of Nonglycosylated Tg with Calnexin**

Based on studies of exportable proteins from which data is presently available, calnexin has been hypothesized to serve primarily as a lectin with a specificity for monoglucosylated proteins (Hammond and Helenius, 1993; Hammond et al., 1994). Alternatively, calnexin may bind directly to polypep-
tide chains through the recognition of a structural feature that correlates in some proteins with oligosaccharide processing. In addition to our studies of the effects of reducing agents on calnexin association with Tg (above), recent studies of hepatocyte transferrin and the gp80 secretory protein of MDCK cells suggest a strong kinetic and biochemical correlation of calnexin binding with disulfide bond rearrangement (Ou et al., 1993; Wada et al., 1994), a process that assists in burying hydrophobic peptide domains (Braakman et al., 1992a). Since Tg is richly endowed with N-linked glycans (>20), as well as intrachain disulfide bonds (>60), we attempted to distinguish between calnexin binding to sugars versus peptide by examining the effect of in vivo DTT exposure on the association of calnexin with nonglycosylated Tg, using thyrocytes pretreated with tunicamycin. In thyrocytes pulse labeled after 3 h of tunicamycin treatment, the binding of calnexin to nascent nonglycosylated Tg was negligible (Fig. 9, left panels, lane C), while binding to BiP was considerable (left panels, lane B). However after 10 mM DTT exposure, the nonglycosylated Tg that coprecipitated with anticalnexin rose substantially (right set of panels, lane C), while BiP coprecipitation of Tg appeared moderately reduced (left vs right sets of panels, lane B). Thus, as long as we perturbed intracellular redox potential, the absence of glycosylation did not interfere with the ability of Tg to associate with calnexin. Once again, these results were relatively insensitive to the presence of DTT in the lysis buffer itself (Fig. 9, a vs b).

Recent studies suggest that DTT may inhibit intracellular transport of nascent exportable proteins within the ER, as well as older molecules within the intermediate compartment and cis-Golgi network (Hammond and Helenius, 1994). To distinguish the effects of DTT on nascent nonglycosylated Tg from those on slightly older molecules, we reexamined the coprecipitation of nonglycosylated Tg in thyrocytes previously labeled to steady state. During the 3-h tunicamycin treatment, ER and Golgi pools of glycosylated Tg (upper portion of Tg band) decreased, while the pool of nonglycosylated Tg (lower portion of Tg band) rose (Fig. 10, first two lanes). Even in the absence of DTT exposure, this tunicamycin treatment causes Tg in the ER to be found in disulfide cross-linked aggregates, which bind fairly well to BiP (Kim et al., 1992). Nevertheless, formation of aggregates per se was not a basis upon which significant coprecipitation of Tg with calnexin could be promoted (Fig. 10, third lane). However, after a 10-min exposure to DTT in vivo, calnexin was at least as efficient in the coprecipitation of nonglycosylated Tg as that of residual glycosylated Tg (Fig. 10, last two lanes). Moreover, this increased coprecipitation of Tg using anticalnexin did not increase recovery of BiP protein (compare last three lanes); in similarly treated thyrocytes.

**Figure 9.** Coprecipitation of nascent nonglycosylated Tg with calnexin in thyrocytes treated with DTT. Confluent thyrocyte monolayers were treated with tunicamycin (20 µg/ml) for 3 h, to fully inhibit N-glycosylation of newly synthesized Tg (Kim et al., 1992). The cells were pulse labeled and then either treated or not treated with DTT for 10 min as shown. The cells were lysed in one of two ways. **(Lysis A)** Cells were prealkylated, washed, and then lysed in the absence of DTT or **(Lysis B)** the cells were not prealkylated but lysed in the presence of DTT, since this has been suggested to improve coprecipitation with calnexin (see text). The antibodies used were either anti-BiP (B) or anti-calnexin (C), with anti-Tg (T) included as a control. The immunoprecipitates were analyzed by reducing SDS-PAGE and phosphorimaging. Relatively little difference was obtained between the two lysis procedures.

**Figure 10.** Binding of nonglycosylated Tg to calnexin in steady-state labeled thyrocytes. Thyrocyte monolayers grown in the presence of hormones to enlarge the ER pool of Tg (Kim and Arvan, 1993) were labeled to steady state as in Fig. 3 before tunicamycin treatment (20 µg/ml) for 3 h to inhibit N-glycosylation. Before lysis, the cells were treated with DTT for 10 min at the doses shown above the figure. The cells were then lysed and immunoprecipitated with an antibody to calnexin (Calnexin). The immunoprecipitates were analyzed by reducing SDS-PAGE using an acrylamide gradient, followed by phosphorimaging. Two Tg immunoprecipitations (lanes 1 and 2) were included as controls, demonstrating the mobilities of glycosylated and nonglycosylated Tg (see text). The mobilities of calnexin and BiP are as indicated.
samples, Tg coprecipitation with anti-BiP diminished after DTT treatment (not shown). Thus, nonglycosylated Tg molecules <3 h old maintain their susceptibility to a DTT-induced shift from one kind of chaperone complex to another.

Finally, if thyrocytes were treated for 3 h with castanospermine (to inhibit deglycosylation of newly synthesized Tg) instead of tunicamycin, DTT-induced binding of nascent Tg to calnexin was not inhibited (Fig. 11 a). In addition, after extended treatment with brefeldin A (which blocks Tg export but allows retrograde traffic of Golgi sugar-processing enzymes into the ER compartment), SDS-PAGE mobility of labeled Tg after endoglycosidase H digestion indicated Golgi-type sugar processing of Tg glycans, yet the susceptibility of Tg to DTT-induced binding to calnexin was not impaired (Fig. 11 b). Lastly, there was no inhibition of calnexin coprecipitation of nonglycosylated Tg in thyrocytes treated more extensively with tunicamycin (for 18 h) to give glycosylated molecules that might form a putative bridge between Tg and calnexin, an ample opportunity for turnover (Fig. 11 c).

**Discussion**

In considering the interaction between folding polypeptides and molecular chaperones, it seems reasonable that, comparable to antigenic epitopes (Copeland et al., 1988; Yewdell et al., 1988), certain binding sites on nascent chains will be presented at relatively discrete points in time along the protein folding pathway. A second consideration is the extent to which different chaperones are available. In experimental systems, selective overexpression of one chaperone appears able to retard the process of secretory protein folding and transport (Dorner et al., 1992). In normal tissues, a proper balance of chaperone activities and ordered chaperone function are probably required for optimal folding, so that indiscriminate "piling on" (which could interfere with coordinated chaperone function) is avoided. We have recently reported that in concert with thyroid stimulated hormone-regulated flux of Tg through the ER, the steady-state levels of BiP, GRP94, and PDI appear to be regulated in thyrocytes to maintain or accelerate Tg folding rates (Kim and Arvan, 1993). The notion of an ordered, sequential binding to different classes of helper proteins represents another level of understanding of how teams of molecular chaperones work together (Hendrick and Hartl, 1993). Nevertheless, until recently (findings that were published in abstract form [Kim, P. S., and P. Arvan. 1993. Mol. Biol. Cell. 4:93a.]) data indicating sequential chaperone function in the ER has been limited (Melnick et al., 1994).

For this reason, we have examined a model of newly synthesized Tg folding after brief exposure of live thyrocytes to DTT, a membrane-permeant reducing agent that alters the conformation of disulfide-containing exportable proteins within the ER (Braakman et al., 1992a,b; Lodish et al., 1992; Kaji and Lodish, 1993; Lodish and Kong, 1993; Tu et al., 1993), potentially allowing exposure of chaperone-binding peptide regions that might otherwise have been buried. This reduction induces obvious structural rearrangement in nascent Tg. Presumably, the open conformation of monomers with available cysteine thiols leads to quantitative disulfide-linked Tg aggregation that is reversible but considerably prolonged (Figs. 3 and 5), as Tg is gradually converted to more oxidized monomers in a process that normally represents an early stage in Tg folding (Kim et al., 1992). This greatly slowed period of early folding has assisted exploration of the initial stages of Tg conformational maturation.

We have suggested that in general, BiP dissociation is most closely coupled to monomer folding rather than oligomerization of exportable proteins (Kim et al., 1992). Studies in yeast suggest that in relation to the process of translocation through the ER membrane (Deshaies and Schekman, 1991; Musch et al., 1992), BiP may act in close proximity (Vogel et al., 1990; Nguyen et al., 1991; Sanders et al., 1992). Nevertheless, since BiP is confined entirely to the ER lumen, it is possible that BiP does not have the first opportunity to interact with nascent chains as they emerge on the luminal side of the ER membrane. Furthermore, we have recently found that during thrombospondin synthesis in thyroid epithelial cells, the monomer folding to which BiP dissociation is coupled occurs after homooligomerization; thus, there is reason to believe that BiP dissociation may serve as a slightly downstream event in the protein folding pathway (Prabakaran, D., P. S. Kim, V. M. Dixit, and P. Arvan, manuscript submitted for publication). Calnexin, an ER membrane protein with a large luminal domain (Wada et al., 1991), known to be near the protein translocation site and in intimate prox-
imimity to a group of proteins formerly thought to be the signal sequence receptor (Gorlich et al., 1990; Hartmann et al., 1993), is ideally situated to have early access to emerging nascent chains. For this reason, it is not surprising that most calnexin binding and unbinding to Tg (a large secretory protein that requires >5 min to cross the ER membrane) may be largely finished before Tg is released into the ER lumen (Fig. 1), while BiP interactions are still ongoing (Kim et al., 1992). Such an explanation also helps to account for the greater degree of calnexin binding when thyrocytes are exposed to DTT during the Tg translational period rather than immediately after translation/translocation. Nevertheless, an initial reduction in the binding of nascent Tg to BiP when DTT is added posttranslationally (Figs. 6, 8, and 9) suggests that some of the effects of DTT may involve more than mere slowing of the normal Tg folding pathway.

A number of hypotheses might explain the dramatic effect of DTT on the protein folding environment to shift Tg association from BiP (Figs. 6, 8, and 9), to calnexin (Figs. 2, 7, 9, and 10). One hypothesis is that the change in redox potential alters the peptide binding or peptide release functions of BiP. This hypothesis seems highly unlikely, in that the effect on Tg binding was already quite marked after exposure to 1 mM DTT (Fig. 8), whereas equal or greater in vivo exposure to reducing agent has no adverse effect on BiP interactions with other exportable proteins (Braakman et al., 1992b; Melnick et al., 1994).

A second possibility is that calnexin and BiP recognize distinct structural features within Tg, and DTT-mediated reduction, while stabilizing interactions with calnexin, causes Tg to assume a conformation that is unable to bind BiP. This hypothesis also seems unlikely, not only based on the high number of potential BiP-binding sites on Tg (Kim et al., 1992) and the unfolding of exportable proteins usually achieved after treatment with reducing agents (Braakman et al., 1992a,b; Lodish et al., 1992; Kaji and Lodish, 1993; Lodish and Kong, 1993; Tatu et al., 1993), but by recent studies directly demonstrating BiP binding (and ATP-mediated release) using reduced denatured Tg (Nigmat et al., 1994). Nevertheless, the hypothesis that calnexin and BiP recognize distinct binding sites fits well with a recent proposal of calnexin as a lectin that binds selectively to monoglucosylated forms of exportable glycoproteins (Hammond and Helenius, 1993; Hammond et al., 1994). However, calnexin has been shown capable of binding to the CD3ε subunit of the T cell receptor complex (which lacks N-linked glycans [Rajagopalan et al., 1994]), and it has been proposed to interact with a nonglycosylated region near the membrane anchor of the heavy chain of the class I major histocompatibility complex molecule (Margolese et al., 1993). On the other hand, it has been argued that the latter study may have failed to identify primary calnexin binding to the oligosaccharide of the class I major histocompatibility complex heavy chain (Helenius, 1994). Thus, Tg is a valuable protein to clarify the role of calnexin in binding to monoglucosylated glycoproteins, since it has served as a prototype of exportable proteins subject to the deglucosylation-reglucosylation cycle (Parodi et al., 1983; Sousa et al., 1992).

Studies of Tg synthesis, consistent with another recent report (Wada et al., 1994), do not lend support to the concept of calnexin as a lectin because the data do not readily account for the dramatic increase in calnexin association with exportable proteins after intralumenal reduction. After DTT treatment, coprecipitation of nonglycosylated Tg with calnexin occurs with very high efficiency, regardless of whether the molecules are nascent (Fig. 9) or somewhat older (Fig. 10). Additional results with castanospermine (which inhibits deglucosylation of Tg, Fig. 11 a) or exposure for several hours to brefeldin A (which does not permit Tg export from the ER, but allows glycans on newly synthesized Tg to acquire Golgi-type sugar processing, Fig. 11 b) are consistent with this view. Of course, one can always consider the possibility that a high fraction of newly synthesized Tg, irrespective of glycans, could be mediated by a small fraction of glucosylated Tg that participates in Tg aggregates via interchain disulfide bonds, thereby bridging an association of the remaining labeled Tg with calnexin. This possibility can probably be excluded because (a) additional exposure to DTT in control cell lysates breaks most interchain disulfide bonds, yet the efficiency of coprecipitation is unimpaired (Figs. 4 and 9); (b) tunicamycin treatment by itself causes quantitative disulfide-linked aggregation of newly synthesized Tg (Kim et al., 1992), yet coprecipitation with calnexin requires that thyrocytes are exposed to DTT in vivo (Fig. 9); and (c) there is no inhibition of calnexin coprecipitation of nonglycosylated Tg in thyrocytes treated for 18 h with tunicamycin to give putative glucosylated bridging molecules time to fully turn over (Fig. 11 c). Thus, although not formally excluded, we think the concept that calnexin binds to Tg primarily as a lectin is implausible.

With this in mind, we hypothesize that reduction within the secretory pathway allows calnexin to compete better for substrate interaction, making BiP association less favorable. Intralumenal reduction could shift the competitive balance to favor calnexin binding by altering relative local availability of the two chaperones through sequestration (or complex formation) between chaperones and/or substrates, via simple steric hindrance, or via subcompartmentalization of different chaperone binding within the secretory pathway. In this regard, Hammond and Helenius (1994) have recently reported that DTT exposure is sufficient to induce a change in the immunofluorescence localization of a mutant vesicular stomatitis virus (VSV) G protein from the intermediate compartment and cis-Golgi network (where they suggest the exportable protein is bound to BiP) to an ER staining pattern (where the protein might be bound to calnexin, although this was not tested). Further, VSV G-calnexin complexes are largely devoid of BiP, and VSV G bound to BiP is largely devoid of calnexin (Hammond and Helenius, 1994). In thyrocytes, we propose that a DTT-induced reexposure of calnexin binding sites (that normally disappear during cotranslational Tg folding) leads to posttranslational association of Tg with calnexin, which in turn makes BiP association less favorable. The generality of this finding and the model of sequential chaperone function in the ER will only be established by studies of additional exportable proteins.

Our view at present is sufficiently broad to accommodate the notion that calnexin may bind to similar or unrelated structural features in Tg to those recognized by BiP (Flynn et al., 1991; Blond-Elguindi et al., 1993; Wada et al., 1994). Nevertheless, in other experiments, we observed that when
pulse-labeled thyrocytes exposed to DTT in vivo were subsequently lysed in the absence of reducing agents at different times after DTT washout (i.e., analyzed as in Fig. 3), only BiP but not calnexin was able to coprecipitate Tg monomers that dissociated in vivo from disulfide-linked aggregates (not shown). Further, quantitative analysis of the data in Fig. 8 indicates strongly that subsequent to DTT washout, binding to calnexin and BiP follows a precursor-product relationship. While these observations do not exclude sterile or other competition between the chaperones, they do support the notion that calnexin and BiP may recognize different structural elements sequentially exposed during the progression of nascent Tg folding.

We have hypothesized that in the physiological context, most ER chaperones and helper proteins function primarily to assist nascent protein folding rather than as quality controllers (Kim et al., 1992; Kim and Arvan, 1993), presumably by limiting certain kinds of counterproductive interactions or aggregation that can be essentially irreversible (Pelham, 1989; Rothman, 1989). The sequential action of chaperones figures prominently among the strategies that biological systems have evolved to optimize the kinetics and efficiency of proper protein folding (Langer et al., 1992). There is now reason to believe that this is also the case within the ER (Melnick et al., 1994; this report). While DTT treatment exaggerates kinetic differences in chaperone association (Figs. 6 and 8), our data nevertheless support the conclusion that even under routine conditions, calnexin and BiP are sequential Tg chaperones. Importantly, the sequential action of ER chaperones on Tg aggregates formed upon ER reduction did not involve significant protein degradation (Young et al., 1993), but rather redirected most Tg into the normal folding pathway (Fig. 5). Thus, the data suggest that an ordered sequence of chaperone binding assists proper secretory protein folding within the ER.

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