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Promotion of transferrin folding by cyclic interactions with calnexin and calreticulin

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Calnexin, an abundant membrane protein, and its lumenal homolog calreticulin interact with nascent proteins in the endoplasmic reticulum. Because they have an affinity for monoglucosylated N-linked oligosaccharides which can be regenerated from the aglucosylated sugar, it has been speculated that this repeated oligosaccharide binding may play a role in nascent chain folding. To investigate the process, we have developed a novel assay system using microsomes freshly prepared from pulse labeled HepG2 cells. Unlike the previously described oxidative folding systems which required rabbit reticulocyte lysates, the oxidative folding of transferrin in isolated microsomes could be carried out in a defined solution. In this system, addition of a glucose donor, UDP-glucose, to the microsomes triggered glucosylation of transferrin and resulted in its cyclic interaction with calnexin and calreticulin. When the folding of transferrin in microsomes was analyzed, UDP-glucose enhanced the amount of folded transferrin and reduced the disulfide-linked aggregates. Analysis of transferrin folding in briefly heat-treated microsomes revealed that UDP-glucose was also effective in elimination of heat-induced misfolding. Incubation of the microsomes with an α-glucosidase inhibitor, castanospermine, prolonged the association of transferrin with the chaperones and prevented completion of folding and, importantly, aggregate formation, particularly in the calnexin complex. Accordingly, we demonstrate that repeated binding of the chaperones to the glucose of the transferrin sugar moiety prevents and corrects misfolding of the protein.

Keywords: calnexin/calreticulin/endoplasmic reticulum/folding/glucosylation

Introduction

In eukaryotes, the endoplasmic reticulum (ER) provides a unique oxidative environment (Hwang *et al.*, 1992) which favors formation of disulfide bonds (reviewed by Creighton *et al.*, 1995; Freedman, 1995). Most nascent polypeptides in the ER are *N*-glycosylated on asparagine residues and the completion of folding is coupled to egress to later compartments along the secretory pathway. Like the processes in the cytosol (Hartl, 1996), folding in the ER is also thought to be assisted by a group of molecular chaperones (Gething and Sambrook, 1992; Ruddon and Bedows, 1997). In addition to some ER chaperones such as BiP/GRP78 and GRP94 which have cytosolic counterparts, the ER contains several chaperones which apparently have no cytosolic counterparts. Hsp47 (Nagata, 1996), RAP (Bu and Rennke, 1996), calnexin and calreticulin are examples and it is thus likely that their chaperone activities represent unique aspects of folding occurring in the ER.

The chaperone functions of calnexin and its ER lumenal homolog calreticulin are tightly coupled to N-linked oligosaccharide processing. Transient interaction of nascent proteins with calnexin was originally described for class I molecules by Degen and Williams (1991). Later, a vast collection of proteins along the secretory pathway were reported to interact with calnexin and calreticulin (reviewed by Bergeron *et al.*, 1994; Williams and Watts, 1995). Since treatment with castanospermine, an inhibitor of α-glucosidases, abolished the calnexin–ligand interaction and since monoglucosylated oligosaccharides were previously proposed to play a role in the retention of misfolded proteins (Suh *et al.*, 1989), Hammond *et al.* (1994) postulated that such interactions may be caused by the recognition of nascent chain oligosaccharides bearing monoglucose. This intermediate in oligosaccharide processing has the unique property that it is produced by the successive actions of glucosidases I and II on the N-linked core oligosaccharides and is also regenerated from the non-glucosylated sugar by UDP-glucose:glycoprotein glucosyltransferase, which acts exclusively on unfolded glycoproteins (Sousa *et al.*, 1992; Sousa and Parodi, 1995). Indeed, it has been shown that calnexin and calreticulin have an affinity for monoglucosylated oligosaccharides (Ware *et al.*, 1995; Rodan *et al.*, 1996; Spiro *et al.*, 1996; Zapun *et al.*, 1997).

Involvement of calnexin in the folding of nascent membrane proteins has been suggested by pharmacological studies employing glucosidase inhibitors (Hammond *et al.*, 1994; Tector and Salter, 1995; Vassilakos *et al.*, 1996). It was also reported that in *Drosophila* cells expressing class I molecules, folding was enhanced by overexpression of calnexin (Vassilakos *et al.*, 1996). There are currently two views about the mechanism of the chaperone action of calnexin. It has been postulated that binding of calnexin causes protein–protein associations that persist until the substrate is folded and its surface is free of hydrophobic patches (Wada *et al.*, 1994; Ware *et al.*, 1995; Williams and Watts, 1995; Vassilakos *et al.*, 1996). Observations that removal of the sugar moiety from the substrates bound to calnexin did not affect the association support this hypothesis (Ware *et al.*, 1995; Zhang *et al.*, 1995). Also, binding of calnexin and calreticulin to a variety of non-glycoproteins has been reported (Arunachalam and Cresswell, 1995; Carreno *et al.*, 1995; Kim and Arvan,

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1995; Loo and Clarke, 1995; Wiest *et al.*, 1995; Cannon *et al.*, 1996). In contrast, Hebert *et al.* (1996) have suggested that 'on–off' cycles of calnexin/calreticulin may be essential for their chaperone function, as is the case for other heat shock proteins with ATPase activity. In any case, it is unclear whether the post-translational cyclic association, if it exists, plays any role in folding.

To analyze the role of the reglucosylation cycle on folding, we have focused on transferrin as a model substrate. This iron binding protein, which is secreted from hepatocytes, is known to interact with calnexin (Ou *et al.*, 1993) and calreticulin (Wada *et al.*, 1995) for exceptionally long periods in HepG2 cells. It has been shown that treatment of these cells with α-glucosidase inhibitors has little effect on the rate of transferrin secretion (Lodish and Kong, 1984; Yeo *et al.*, 1985), suggesting that the chaperones may not play significant roles in the folding of transferrin. However, interpretation of this data is difficult due to the fact that calnexin is a part of the ER retention machinery for misfolded molecules (Jackson *et al.*, 1994; Rajagopalan *et al.*, 1994). Furthermore, castanospermine, like other glycosylation inhibitors, is known to elicit induction of glucose-responsive proteins, including Grp78/Bip, Grp94 and ERp72, and alters the level of protein synthesis (Watowich and Morimoto, 1988; Pahl and Baeuerle, 1995). Accordingly, we have elected to study the process *in vitro*. The protein synthesizing system of dog pancreatic microsomes coupled to rabbit reticulocyte lysates (Pelham and Jackson, 1976; Walter and Blobel, 1983) has been used as a powerful method to study various ER events, including the processes of folding (Freedman, 1995, and references therein; Brunke *et al.*, 1996; Hebert *et al.*, 1996; Shtrom and Hall, 1996). However, although microsomes preferentially incorporate oxidized glutathione into the lumen, this system is known to require the presence of reticulocyte lysates for oxidative folding (Marquardt *et al.*, 1993), thus making the folding conditions ill defined.

Here we describe a simple alternative method to study post-translational folding. This system allows us to reinitiate oxidative folding in microsomes by incubating in a chemically defined buffer. We briefly pulse labeled HepG2 cells with [^{35}S]methionine, chilled them rapidly in ice/water, prepared microsomes using a discontinuous sucrose gradient and finally measured the folding states of transferrin. When the microsomes were isolated, nascent transferrin remained unfolded and contained some reduced cysteine residues. Incubation of the pulse labeled microsomes in the redox buffer resulted in conversion of the unfolded form to the folded form. However, efficient folding of transferrin in the microsomes required supplementation with UDP-glucose, which is the sugar donor for UDP-glucose:glycoprotein glucosyltransferase. Addition of UDP-glucose caused glucosylation of transferrin and triggered the cyclic interactions with calnexin and calreticulin. Furthermore, repeated interactions with the chaperones apparently redirected the misfolded transferrin to the correct folding process. Thus, we demonstrate here that under physiological conditions repeated cycles of association with and dissociation from calnexin and calreticulin play an important role in the maturation of transferrin.

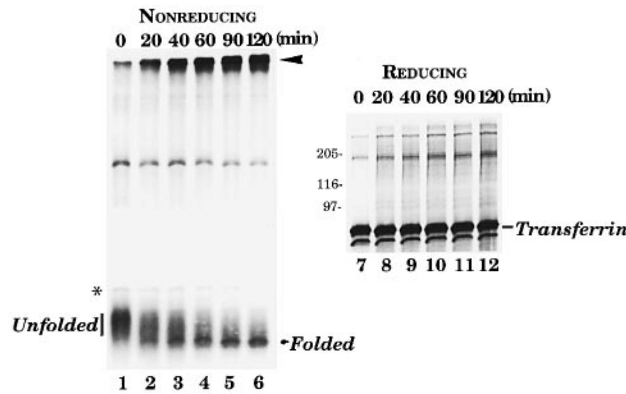
Results

Characterization of post-translational folding of transferrin in pulse labeled microsomes

Maturation of transferrin in the endoplasmic reticulum has been characterized by several groups using pulse–chase experiments in HepG2 cells (Fries *et al.*, 1984; Lodish and Kong, 1984, 1991). This iron binding protein consists of two homologous domains and contains 19 disulfide bonds with two asparagine-linked sugars (MacGillivray *et al.*, 1983). The folding process can be monitored by SDS–PAGE under non-reducing conditions (Lodish and Kong, 1991). Since UDP-glucose is known to be transported into the lumen of the ER (Perez and Hirschberg, 1986), it should be possible to examine the role of reglucosylation in folding by incubating the microsomes with exogenously added UDP-glucose. So far, an mRNA-programed protein synthesis system using rabbit reticulocyte lysates in the presence of microsomes has been used to characterize the process of folding in microsomes. However, it was necessary to re-isolate the microsomes to remove all components of the rabbit reticulocyte lysate and these re-isolated microsomes failed to oxidize nascent hemagglutinin even in the presence of 5 mM oxidized glutathione (Marquardt *et al.*, 1993). Post-translational folding was only observed when non-denatured reticulocyte lysate was remixed with the isolated microsomes, suggesting that protein factors may be required for uptake of oxidized glutathione into microsomes. Considering the complexity of reticulocyte lysates, we decided to develop an *in vitro* folding system where maturation could proceed in microsomes in a defined system. We first tested whether it was possible to isolate microsomes from pulse labeled HepG2 cells while maintaining the protein in its unfolded state and whether folding could then be re-initiated in these 'pulse labeled microsomes' in a chemically defined buffer.

To arrest folding, we chilled HepG2 cells that had been pulse labeled for 5 min at 37°C with [^{35}S]methionine with ice/water and prepared microsomes at 4°C. Since oxidative folding intermediates can be trapped by alkylating their free thiol groups (Creighton *et al.*, 1993, and references therein), we treated the microsomes with iodoacetamide and the stabilized folding intermediates of transferrin were then monitored by immunoprecipitation using transferrin antibodies followed by SDS–PAGE under non-reducing conditions. As shown in lane 1 of Figure 1A, the observed bands had the characteristic features of previously defined unfolded proteins, being very broad and diffuse and slow migrating (Lodish and Kong, 1991). These fuzzy bands remained for at least 2 h at 4°C (see Figure 2). Thus microsomes could be isolated while arresting the folding of nascent chains at 4°C. We often observed a faint band of fully reduced transferrin (* in Figure 1A). This reduced form should represent a fraction of the transferrin molecules exposed to the extravesicular buffer, where reduced glutathione was in excess. We then tested various conditions where transferrin folding could be allowed to proceed. We found that incubation of the isolated microsomes at 33°C in the cytosolic buffer supplemented with 1 mM reduced glutathione and 100 μM oxidized glutathione resulted in a decrease in the unfolded form and a corresponding increase in a single, faster migrating, sharply

A



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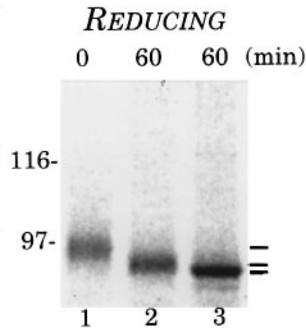
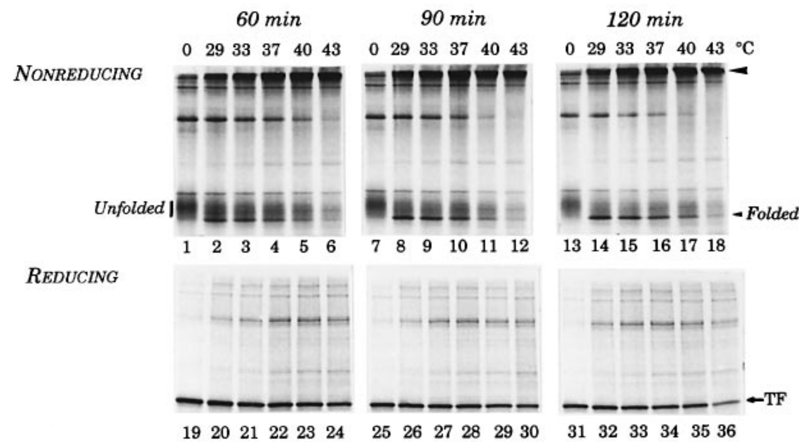


Fig. 1. Oxidative folding in pulse labeled microsomes in a defined redox buffer. (**A**) Microsomes were prepared from HepG2 cells pulse labeled with [^{35}S]methionine for 5 min as described in Materials and methods. The microsomes were incubated for the indicated periods of time at 33°C in the cytosolic buffer containing 1 mM reduced glutathione and 100 µM oxidized glutathione. Transferrin was immunoprecipitated from 50 µl microsomes using anti-transferrin antibody and the immunoprecipitates analyzed by SDS–PAGE under non-reducing (lanes 1–6) or reducing (lanes 7–12) conditions. The radioactive signal was visualized by a BAS2000 equipped with Pictrography. Note the aggregates (arrowhead) at the top of the non-reducing gel. The positions of folded transferrin and the region of unfolded transferrin are shown. *, the position of fully reduced transferrin. The positions of the molecular mass standards are shown on the left side of the reducing gel. (**B**) Pulse labeled microsomes, (50 µl) incubated for 0 (lane 1) or 60 min (lanes 2 and 3), were mixed with an equal volume of cholate lysis buffer in the presence of 1 mM Evans Blue–IACHS conjugate (lanes 1 and 2) or 5 mM iodoacetamide (lane 3). Evans Blue–IACHS conjugate was freshly synthesized by mixing Evans Blue and IACHS to a molar ratio of 10:1 in dimethylformamide for 24 h at 4°C, followed by lyophilization. The conjugate was dissolved in 100 mM triethanolamine acetic acid, pH 7.2, to yield 10 mM with respect to IACHS. Transferrin was then immunoprecipitated and analyzed under reducing conditions. The positions of the molecular mass standards are shown on the left.

demarcated band (Figure 1A, lanes 2–6), which has been previously defined as fully folded transferrin (Lodish and Kong, 1991). We found that folding was best achieved with a mixture of 100 µM oxidized and 1 mM reduced glutathione. Raising the oxidized glutathione concentration to 1 mM decreased the rate of folding to nearly 50% (data not shown). However, even in the optimized redox buffer we noticed that a significant amount of nascent transferrin failed to fold and formed disulfide-linked aggregates which were observed at the top of the non-reducing gels (lanes 2–6) but were not observed on reducing gels (lanes 8–12). Disulfide-linked aggregates are characteristic of misfolded products in the ER (Marquardt and Helenius, 1992).

Mature transferrin purified from serum contains 19 disulfide bonds. It was previously assumed that all the unfolded bands observed by SDS–PAGE under non-reducing conditions contained no free thiols, suggesting that conversion from the fuzzy, slow migrating bands to the faster, compact band reflected only disulfide exchange and not oxidation (Lodish and Kong, 1991). This assumption was based on a report which showed the absence of thiols in transferrin purified from the rough microsomal fraction (Morgan and Peters, 1985). In our experiments it was clear that the transferrin at the beginning of the incubation was not fully reduced, because the unfolded forms migrate more rapidly than the fully reduced form in non-reducing gels (Figure 1A, lane 1). However, since studies on *in vivo* folding of gonadotropin (Bedows *et al.*, 1993) as well as other proteins containing disulfide bonds have shown a stepwise oxidation of thiols (reviewed by Freedman, 1995), we tested whether nascent transferrin was indeed fully oxidized when the pulse labeled microsomes were isolated. We synthesized a high molecular weight thiol alkylating compound, Evans Blue–IACHS [6-(iodoacetamide)caproic acid *N*-hydroxysuccinimide ester] conjugate, which selectively modifies free thiol groups, and mixed this compound, instead of iodoacetamide, with the microsomal extracts. Modification of free thiols should result in a significant shift upon SDS–PAGE. When the transferrin immunoprecipitates were analyzed by reducing SDS–PAGE we found that the most nascent form migrated more slowly than iodoacetamide-treated transferrin (Figure 1B, lane 1). The size was estimated to be ~95 kDa. This form was converted to 86 kDa (lane 2) upon 60 min incubation in the redox buffer. Because alkylation with iodoacetamide gave an 85 kDa band (lane 3), the transferrin molecules in isolated microsomes should contain free thiols. Conversion of the unfolded to the folded form in our system should, therefore, involve disulfide bond formation as well as a rearrangement such as that observed for other known proteins.

It is known that oxidative folding in the pancreatic microsome/rabbit reticulocyte lysate system fails to proceed at physiological temperature (Hebert *et al.*, 1996). To characterize the folding process in our present system, we examined the effects of temperature on transferrin folding in pulse labeled microsomes. As shown in Figure 2A, conversion to the folded form was clearly detected at 37°C (lanes 4, 10 and 16). However, the amount of disulfide-linked aggregate at the top of the gel dramatically increased as the incubation temperature was raised (lanes 1–18). As a result, transferrin folding was severely hampered at higher temperatures (lanes 5 and 6, 11 and 12 and 17 and 18). In this case, the formation of disulfide-linked aggregates was more obvious than the decrease in folded transferrin, suggesting that the increase in aggregates was due to misfolded transferrin non-covalently trapping other interchain linked proteins, as reported



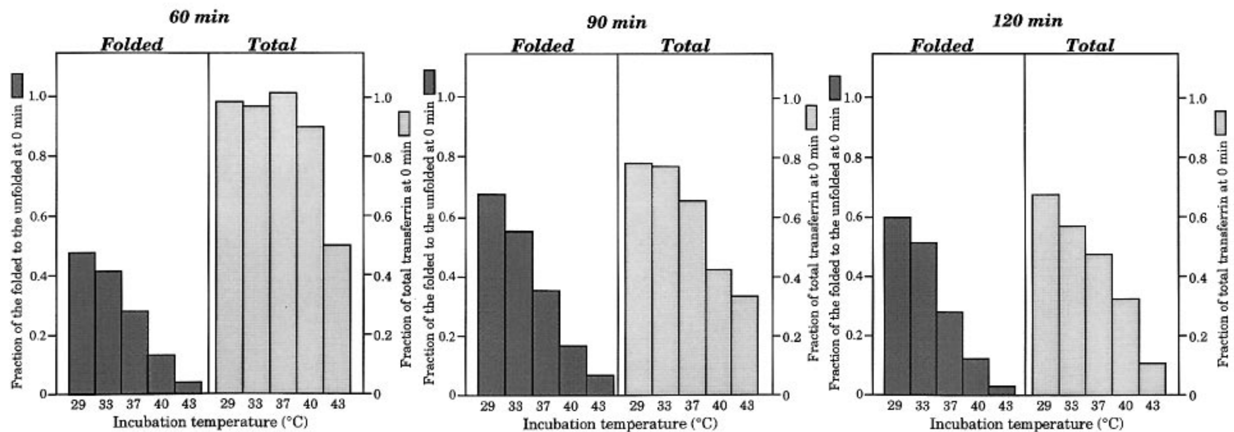


Fig. 2. Effect of various temperatures on transferrin folding in microsomes. Pulse labeled microsomes were incubated for 60, 90 or 120 min at various temperatures. Transferrin was then immunoprecipitated by anti-transferrin antibody as in Figure 1 and analyzed by reducing (bottom) or non-reducing (top) SDS–PAGE (**A**). TF, transferrin. Folding efficiencies were expressed as the fraction of folded transferrin (non-reducing gel) at any given point with respect to the unfolded form, which had received no incubation (**B**). Recoveries of total transferrin were expressed as the fraction of the total (reducing gel) at 0 min.

previously (Sawyer *et al.*, 1994). However, with the exception of the 43°C incubation (lane 24), the amount of total transferrin assessed under reducing conditions was not significantly decreased by incubation for 60 min (Figure 2A, lanes 19–23). Upon further incubation, the total amount of transferrin decreased, particularly at higher temperatures (lanes 30 and 36), quite likely due to degradation. Thus, although folding can, at least in part, be completed in the microsomes using the redox buffer, the extent of transferrin folding is highly dependent on temperature.

Addition of UDP-glucose induces glucosylation of transferrin and cyclic interactions with calnexin and calreticulin

In contrast to the results *in vitro* shown in Figure 2, transferrin folding occurring in cultured HepG2 cells was not significantly affected by heat treatment at up to 40°C and only small amounts of interchain linked aggregates were detected *in vivo* (data not shown). We presumed that the hypersensitivity of the *in vitro* maturation process to heat might be caused by the lack in microsomes of some intrinsic machinery, such as the reglucosylation cycle. Prior to testing this possibility, we did a series of experiments to examine whether the proposed cycle is operational in the current system. First, we examined whether transferrin was reglucosylated. Since the ER-derived microsomes should contain nascent proteins and are capable of transporting UDP-glucose into the lumen (Perez and Hirschberg, 1986), the addition of UDP-glucose should result in transfer of the glucose moiety to the aglucosylated oligosaccharides of transferrin if it is subject to the glucosylation cycle. We prepared microsomes from unlabeled HepG2 cells, incubated them with UDP-[^{14}C]glucose at 33°C, and analyzed total proteins by SDS–PAGE followed by phosphorimaging. As shown in lanes 2–5 of Figure 3, upon incubation of the microsomes in our assay buffer, a series of bands, including a major band of 85 kDa, appeared. Comparable patterns were obtained by incubations at 37, 40 and 43°C (data not shown). We observed that a signal was only detected in the presence of 5 mM ATP or 1 mM adenosine 5′-*O* (thio)triphosphate (ATPγS) (data not shown). Hence, unless otherwise specified, we included 5 mM ATP in our assay buffer. The signals were completely abolished by treatment with *N*-glycanase (lane 7), but not by treatment with jack bean α-mannosidase (lane 6). Since it has been previously shown that the glucose moiety of UDP-glucose is used

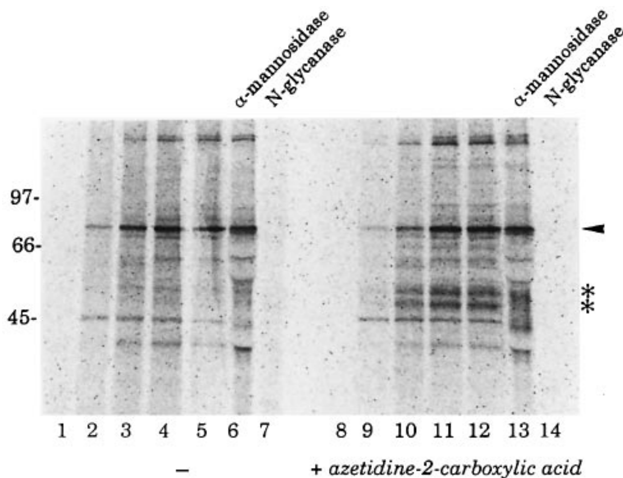


Fig. 3. Post-translational labeling of microsomes with UDP-[^{14}C]glucose. Microsomes isolated from unlabeled HepG2 cells treated with 5 mM azetidine-2-carboxylic acid for 3 h (lanes 8–14) or untreated (lanes 1–7) were incubated at 33°C in the cytosol buffer containing 5 mM ATP and 30 μM UDP-[^{14}C]glucose (sp. act. 15 Ci/mmol) for 0 (lanes 1 and 8), 5 (lanes 2 and 9), 10 (lanes 3 and 10), 20 (lanes 4 and 11) or 30 min (lanes 5–7 and 12–14). After incubation, 10 μl microsomes were directly subjected to SDS–PAGE under reducing conditions (lanes 1–5 and 8–12). For glycosidase digestion, the microsomes were lysed with an equal volume of 1% Sarkosyl, 10 mM triethanolamine–HCl, pH 7.5, and heated at 65°C for 10 min. The denatured samples were diluted 10-fold with either a buffer containing 1% Triton X-100, 50 mM sodium citrate, pH 4.3, and 0.1 U jack bean α-mannosidase or with a buffer containing 1% Triton X-100, 50 mM sodium phosphate, pH 7.0, and 10 mU *N*-glycanase. The mixture was incubated for 12 h and proteins were concentrated by chloroform/methanol precipitation (Wessel and Flugge, 1984) for analysis by SDS–PAGE (lanes 6, 7, 13 and 14). The two bands which appeared following treatment with azetidine-2-carboxylic acid are indicated with asterisks (*).

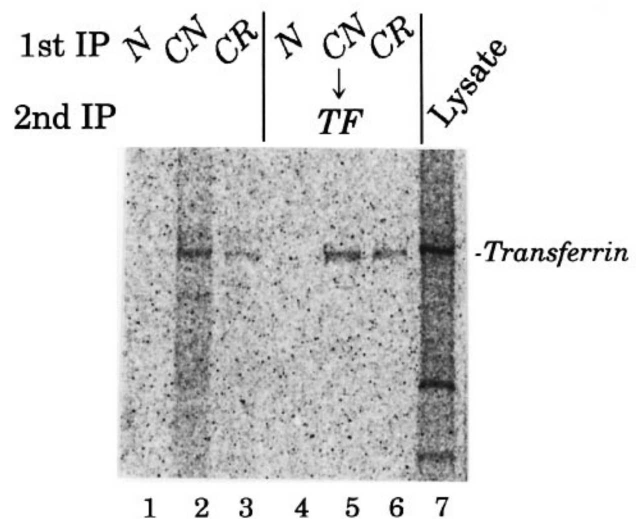


Fig. 4. Co-immunoprecipitation of [^{14}C]glucose-labeled transferrin with calnexin and calreticulin. [^{14}C]Glucose-labeled microsomes (100 μl) were prepared as described in Figure 3 by incubating with UDP-[^{14}C]glucose at 33°C for 30 min. An aliquot (5 μl) was directly used for SDS–PAGE under reducing conditions (lane 7). The rest (90 μl) was subjected to immunoprecipitation as described in Materials and methods using non-immune serum (lanes 1 and 4), anti-calnexin antibody (lanes 2 and 5) or anti-calreticulin antibody (lanes 3 and 6). One third of the immunoprecipitates was analyzed by SDS–PAGE (lanes 1–3). Transferrin was further isolated from the remaining two thirds of these immunoprecipitates using anti-transferrin antibody (lanes 4–6). N, non-immune serum; CN, calnexin; CR, calreticulin; TF, transferrin.

solely by UDP-glucose:glycoprotein glucosyltransferase in ER-derived microsomes (Parodi *et al.*, 1984), we concluded that the 85 kDa protein was reglucosylated by the transferase during folding.

A unique property of UDP-glucose:glycoprotein glucosyltransferase has been described by the group of Parodi (Sousa *et al.*, 1992; Sousa and Parodi, 1995). This enzyme only glucosylates unfolded proteins. If this enzyme is exclusively responsible for the [^{14}C]glucose labeling, misfolding should enhance the transfer of [^{14}C]glucose onto proteins. We thus prepared microsomes from HepG2 cells that had been preincubated with azetidine-2-carboxylic acid, a proline analog which causes irreversible misfolding (Beckmann *et al.*, 1990). As shown in lanes 9–12 of Figure 3, a new set of bands (e.g. 53 and 51 kDa) appeared as expected when the microsomes containing misfolded proteins were incubated with UDP-[^{14}C]glucose. In this case, the maximum intensity of the major 85 kDa band was not significantly enhanced by the azetidine-2-carboxylic acid pretreatment (lanes 4 and 11), suggesting that the transferrin molecules in microsomes isolated from untreated cells were largely unfolded.

Since calnexin and calreticulin have an affinity for monoglucosylated oligosaccharides, it can be expected that *in vitro* glucosylated transferrin should bind to the chaperones. Therefore, we examined whether the post-translationally glucosylated proteins in Figure 3 were associated with these chaperones. The unlabeled microsomes were incubated with UDP-[^{14}C]glucose and the proteins immunoprecipitated with anti-calnexin and anti-calreticulin antibodies were analyzed (Figure 4). The major 85 kDa band in the [^{14}C]glucose-labeled microsomes was immunoprecipitated with both of the anti-chaperone antibodies (lanes 2 and 3). The amount of the 85 kDa protein recovered with either antibody when compared with that observed in the lysate was 13% for calnexin and 6% for calreticulin. When the chaperone immunoprecipitates were dissociated by SDS treatment and subjected to a second immunoprecipitation with anti-transferrin antibody, we found that the major 85 kDa band observed in the lysates (lane 7) was indeed transferrin (lanes 5 and 6). Additionally, the data indicate that transferrin is the major chaperone substrate which is extensively reglucosylated.

The proposed function of the reglucosylation cycle on folding relies on rapid deglucosylation of the target molecules. Thus, we determined the turnover rate of post-translationally labeled glucose for transferrin in microsomes. We incubated the [^{14}C]glucose-labeled microsomes with an excess of unlabeled UDP-glucose at 37°C. This temperature was used to assess the rate under physiological conditions. We found that ~95% of the glucose was removed from transferrin within 15 min incubation (Figure 5, EXP. 1, lane 2). The half-time of removal was 5.8 min for the calnexin complex (EXP. 2, lanes 1–5), 4.7 min for the calreticulin complex (EXP. 2, lanes 6–10) and 4.0 min in the total lysate (EXP. 2, lanes 11–15). At 4°C deglucosylation of [^{14}C]glucose-labeled transferrin by the same UDP-glucose chase was not detected for at least 2 h (data not shown). When the post-labeling incubation was with unlabeled UDP-glucose in the presence of

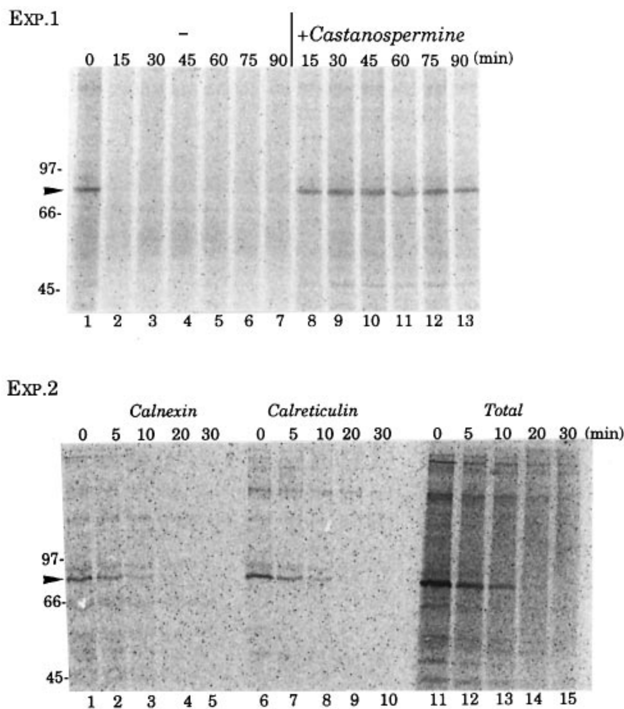


Fig. 5. Pulse–chase experiments with [^{14}C]glucose-labeled microsomes. [^{14}C]Glucose-labeled microsomes were prepared as described in Figure 3. The labeled microsomes were incubated at 37°C with 3 mM unlabeled UDP-glucose for the indicated periods of time (experiment 1, lanes 1–7 and experiment 2). In experiment 1 0.5 mM castanospermine was included during the incubations with unlabeled UDP-glucose (lanes 8–13). Ten microliters of total microsomes were resolved by SDS–PAGE under reducing conditions. In experiment 2 calnexin (lanes 1–5) or calreticulin (lanes 6–10) was immunoprecipitated from 30 μl [^{14}C]glucose-labeled microsomes and the immune complexes analyzed. Total microsomes (10 μl) were also resolved by SDS–PAGE (lanes 11–15).

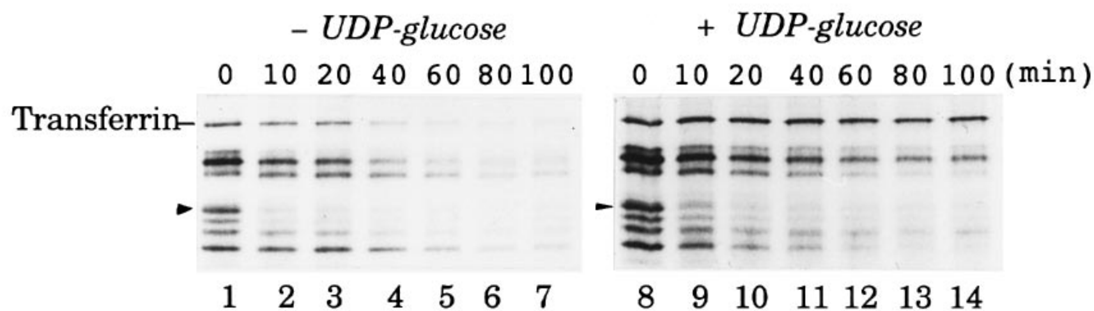
castanospermine (EXP. 1, lanes 8–13), glucose removal from transferrin was completely inhibited, indicating that α-glucosidase was responsible for deglucosylation of transferrin. Assuming that the calculated deglucosylation rates reflect the *in vivo* situation, the cycle may not occur on substrates such as α_1-antitrypsin that dissociate rapidly from calnexin (Ou *et al.*, 1993).

If the association of calnexin/calreticulin is exclusively determined by their binding to glucosylated oligosaccharides on transferrin, the transferrin–chaperone complex should dissociate with a half-time similar to the rate of deglucosylation, i.e. ~5 min in the absence of added UDP-glucose, and the addition of UDP-glucose should prolong the interaction. We prepared microsomes from HepG2 cells that had been pulse labeled with [^{35}S]methionine and measured the dissociation kinetics of transferrin from the chaperones. In HepG2 cells, secretory proteins associate with calnexin and calreticulin for markedly varied durations (Ou *et al.*, 1993; Wada *et al.*, 1995). When the pulse labeled microsomes were prepared, most of the *in vivo* ligands of calnexin and calreticulin were also found in association with the chaperones (Figure 6A and B, lane 1). The spectra of the chaperone substrates were almost indistinguishable from those obtained by pulse–chase experiments of intact HepG2 cells (data not shown). However, in chase incubations carried out in the absence of UDP-glucose, most of the ligands rapidly dissociated from calnexin and calreticulin, and sustained interaction with transferrin, as has been shown *in vivo* (Wada *et al.*, 1995), was not seen (Figure 6A and B, lanes 2–7). In contrast, supplementation of the incubation mixture with UDP-glucose strikingly prolonged the dissociation process of some ligands, particularly transferrin, without affecting the dissociation kinetics of other ligands, such as α_1-antitrypsin (Figure 6A, lanes 9–14). Similarly, association of calreticulin with transferrin could be sustained by inclusion of UDP-glucose (Figure 6B, lanes 9–14). In these experiments, we noticed that the dissociation kinetics of transferrin from calnexin in the absence of UDP-glucose were still slower than that of α_1-antitrypsin (Figure 6A, lanes 1–3). This may be due to a slow turnover ($T_{1/2} \mp 7$ min at 33°C) of endogenous UDP-glucose in the microsomes (data not shown). Considering the high deglucosylation rates of transferrin (Figure 5), we conclude that addition of UDP-glucose causes a rapid cyclic interaction of calnexin/calreticulin with the sugar moieties of transferrin.

Reglucosylation cycles in microsomes rescue transferrin from misfolding

To examine the effect of the chaperone cycle on folding of transferrin, we incubated pulse labeled microsomes with UDP-glucose in the presence of ATP at various temperatures and transferrin immunoprecipitates were separated by SDS–PAGE under non-reducing (Figure 7, lanes 1–8) and reducing (Figure 7, lanes 9–16) conditions. We found that at 33°C formation of disulfide-linked aggregates was clearly suppressed by supplementation with UDP-glucose (lane 2). At 37°C the effect of UDP-glucose supplementation was more prominent in that addition clearly increased recovery of the oxidized form as well as the folding intermediates (lane 4). The amount of folded transferrin was increased by nearly 2-fold when incubated with UDP-glucose at 40°C (lane 6), although considerable amounts of aggregates were formed even in the presence of UDP-glucose. Upon incubation at 43°C the proper folding intermediates and fully folded forms were only faintly detected in the absence of added UDP-glucose. However, the folded form was clearly visible with the addition of UDP-glucose (lane 8). Consistent with the results in Figure 1, the amounts of total transferrin were not significantly affected by a 60 min incubation in the presence or absence of UDP-glucose (Figure 7, lanes 9–16).

It is known that ATP, ATPγS, UDP-glucose, UDP-*N*-acetylglucosamine and UDP-*N*-acetylglucuronic acid can be incorporated into the microsomal lumen (Perez and Hirschberg, 1985, 1986; Clairmont *et al.*, 1992; Mayinger and Meyer, 1993; Bossuyt and Blanckaert, 1994; Radominska *et al.*, 1994). In Table I we summarize the results of experiments where the influence of various chemicals on the maturation process was examined. Among the various combinations of nucleotide analogs, the addition of UDP-glucose in the presence of ATP or ATPγS was markedly effective in increasing folding efficiency. The effect of UDP-glucose increased as the incubation temperature was raised, from a calculated folding efficiency of 134 ± 23% at 33°C to 215 ± 30% at 43°C (+UDP-glucose+ATP, Table I). Addition of UDP-glucose alone was ineffective, which is consistent with



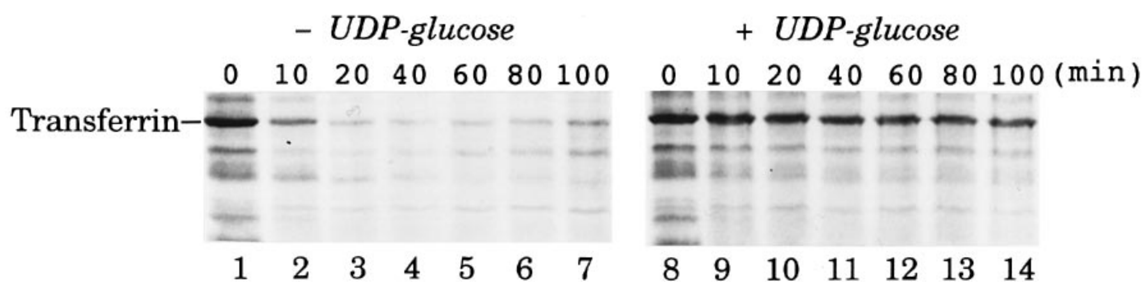


Fig. 6. Effect of UDP-glucose on the kinetics of interaction of calnexin and calreticulin with their substrates. Microsomes containing nascent proteins labeled with [^{35}S]methionine were incubated at 33°C in the cytosol buffer containing 5 mM ATP for the indicated periods of time in the absence (lanes 1–7) or presence (lanes 8–14) of 1 mM UDP-glucose. Fifty microliters of the microsomes were subjected to immunoprecipitation with anti-calnexin (**A**) or anti-calreticulin antibodies (**B**) as described under Materials and methods. The immunoprecipitates were separated by SDS–PAGE under reducing conditions. The position of transferrin is indicated. Arrowheads indicate the position of α_1-antitrypsin.

the observation that ATP or ATPγS was required for post-translational glucosylation of transferrin, as described above. So far, the only known reaction using UDP-glucose inside ER-derived microsomes is the transfer of glucose to substrates by UDP-glucose:glycoprotein glucosyl-transferase (Parodi *et al.*, 1984). Hence, we conclude that the occurrence of repeated associations of calnexin and calreticulin with the sugar moiety of transferrin enhances the folding efficiency of transferrin.

The results shown in Figure 7 and Table I imply two things; repeated interactions of calnexin and calreticulin with transferrin may: (i) prevent formation of the interchain linked aggregates; and/or (ii) correct the already misfolded structure. To evaluate the latter possibility, we designed an experiment in which correction of misfolding could be measured. We heat treated the pulse labeled microsomes at 45°C for 5 min in the minimum redox buffer, added ATP and then incubated the samples at 33°C for 90 min with or without UDP-glucose. Without heat pretreatment and UDP-glucose, formation of the folded form and the disulfide-linked aggregates, which may be non-covalently bound to the misfolded transferrin, were observed at the level previously described (Figure 8, lane 1). Upon heat pretreatment we observed that in the absence of UDP-glucose, the folded molecules, after 90 min at 33°C, were reduced to 58 ± 3% (Figure 8, lane 3) and the amount of interchain linked proteins, including transferrin and other non-specifically trapped misfolded proteins, was further increased (Figure 8, lane 7). Although the 5 min treatment at 45°C without further incubation at 33°C did not significantly reduce the amount of non-aggregates (not shown), the nascent transferrin at the end of the heat pretreatment must have been misfolded, thus being destined to form interchain linked aggregates by further incubation at 33°C. Supplementation with UDP-glucose without heat pretreatment reduced the amount of disulfide-linked aggregates (lane 2) and slightly increased folding efficiency, as described for 33°C incubations in Figure 7. Remarkably, incubation of the heat-treated microsomes with UDP-glucose completely restored folding efficiency (lane 4) back to the level observed without heat treatment (lane 2). Therefore, these results strongly indicate that the chaperone cycles also act on misfolded molecules and direct them to the normal folding pathway.

We next examined the state of folding in the chaperone complex. Post-translational treatment with castanospermine inhibits dissociation of substrates from calnexin and calreticulin by preventing cleavage of the innermost glucose (Hebert *et al.*, 1996). Indeed, as shown in Figure 9, incubation of pulse labeled microsomes with castanospermine inhibited normal dissociation of substrates from the chaperones (Figure 9, lanes 2–6 and 9–14). Transferrin was recovered from the immune complex and its folding status analyzed by non-reducing SDS–PAGE. Under these conditions we found that conversion of transferrin to the folded form was markedly repressed in both cases (lanes

22–26 and 29–34). Most of the slower migrating species representing unfolded transferrin remained even after 2 h incubation, although upon incubation the diffuse bands moved to the region of the distinct sharp band (representing folded transferrin) and the fastest edge of the fuzzy bands almost reached the position of the folded form. When the cyclic interactions were allowed to proceed by addition of UDP-glucose and total transferrin was recovered by double immunoprecipitation with anti-transferrin antibody (lanes 27 and 35), the progress of folding was observed as in Figure 7 with few misfolded products. Importantly, formation of disulfide-linked aggregates in the absence of added UDP-glucose (Figure 9, lanes 36–40) was evidently inhibited in the calnexin complexes (Figure 9, lanes 22–26 and 34) and less markedly, but significantly, in the calreticulin complex (Figure 9, lanes 29–33).

Finally, we determined the influence of the reglucosyl-

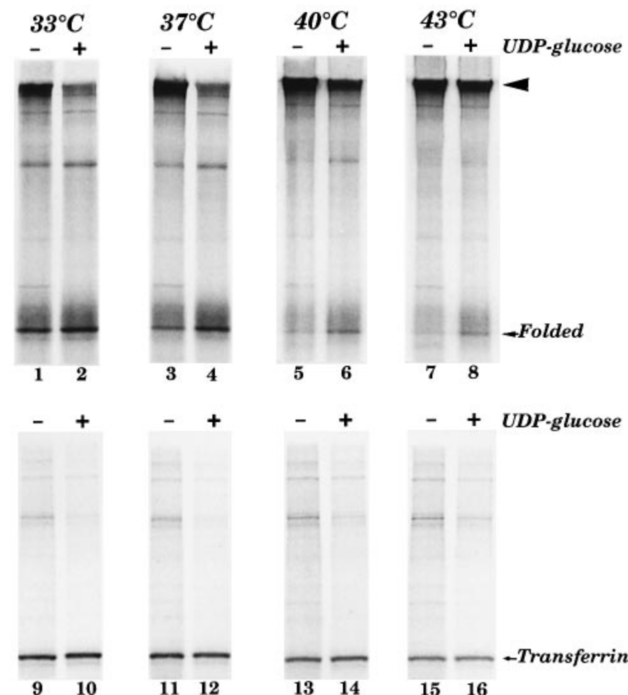


Fig. 7. Effect of UDP-glucose on transferrin folding. Fifty microliters of the pulse labeled microsomes from [^{35}S]methionine-labeled HepG2 cells were incubated with (even lanes) or without (odd lanes) 1 mM UDP-glucose at 33 (lanes 1 and 2), 37 (lanes 3 and 4), 40 (lanes 5 and 6) or 43°C (lanes 7 and 8) for 60 min in the presence of 5 mM ATP. At the end of the incubation the microsomes were treated with iodoacetamide and transferrin was immunoprecipitated as described under Materials and methods. The immunoprecipitates were subjected to SDS–PAGE under reducing (lanes 9–16) or non-reducing (lanes 1–8) conditions.

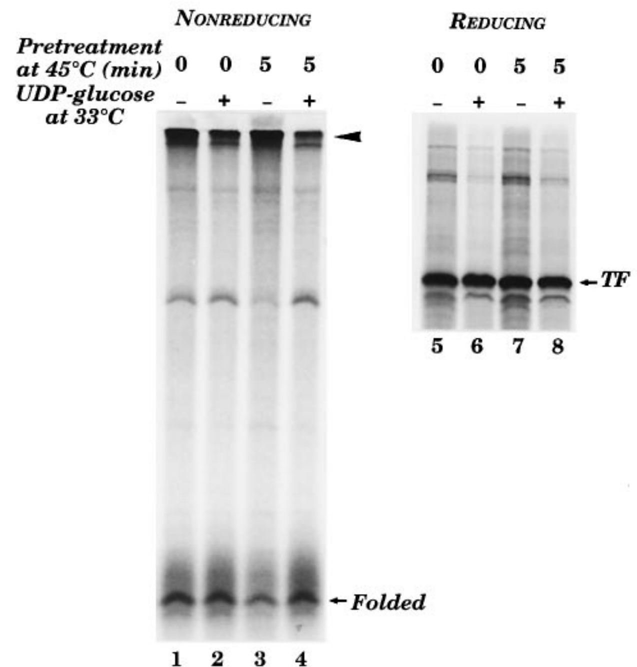


Fig. 8. Effect of UDP-glucose on transferrin folding in heat-treated microsomes. Microsomes (50 µl) pulse labeled with [^{35}S]methionine were preincubated at 45°C for 0 (lanes 1–2 and 5–6) or 5 min (lanes 3–4 and 7–8) in the minimum redox buffer as in Figure 1. After incubation ATP (5 mM) was added and the heat-treated microsomes were further incubated at 33°C for 90 min with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) 1 mM UDP-glucose. Transferrin was then immunoprecipitated and analyzed by SDS–PAGE under non-reducing (lanes 1–4) or reducing (lanes 5–8) conditions. Arrowhead, disulfide-linked aggregates.

Table I. Effects of various chemicals on transferrin folding

Compounds	Folding efficiency (% of +ATP)				Total transferrin (% of +ATP)			
(No. of experiments)	33°C	37°C	40°C	43°C	33°C	37°C	40°C	43°C
+ATP (5)	100	100	100	100	100	100	100	100
+ATPγS (3)	108	92	107	105	95	115	93	95
– (5)	92	82	78	71	95	88	86	74
+UDP-glc (3)	107	116	103	88	97	109	89	81
+UDP-glc+ATP (5)	133	149	179	214	114	108	110	118
+UDP-glc+ATPγS (3)	125	162	190	198	106	107	104	127
+UDP-GlcNAc+ATP (2)	97	93	99	95	109	102	104	88
+UDP-GRA+ATP (2)	103	99	105	91	117	103	97	106
+Glucose+ATP (2)	110	105	96	109	109	116	105	109

50 µl of the pulse-labeled microsomes were incubated for 60 min at various temperatures in the presence of various chemicals as indicated and nascent transferrin was immunoprecipitated as in Figure 7. ATP was added at 5 mM and other chemicals were added at 1 mM. The folding efficiency of transferrin estimated from non-reducing gels after the 60 min incubations was expressed as a percentage of the amount folded in the presence of ATP alone. In the presence of ATP, the recoveries of the folded transferrin from the unfolded which had received no incubation are 51 ± 8, 39 ± 7, 15 ± 3 and 9 ± 3% at 33, 37, 40 and 43°C, respectively. The amount of total transferrin after the incubations was expressed as a percentage of that in the presence of ATP alone. In the presence of ATP, the recoveries of the total transferrin upon 60 min incubation are 101 ± 11, 102 ± 13, 86 ± 6 and 53 ± 11% at 33, 37, 40 and 43°C, respectively. The data represent means of the experiments.

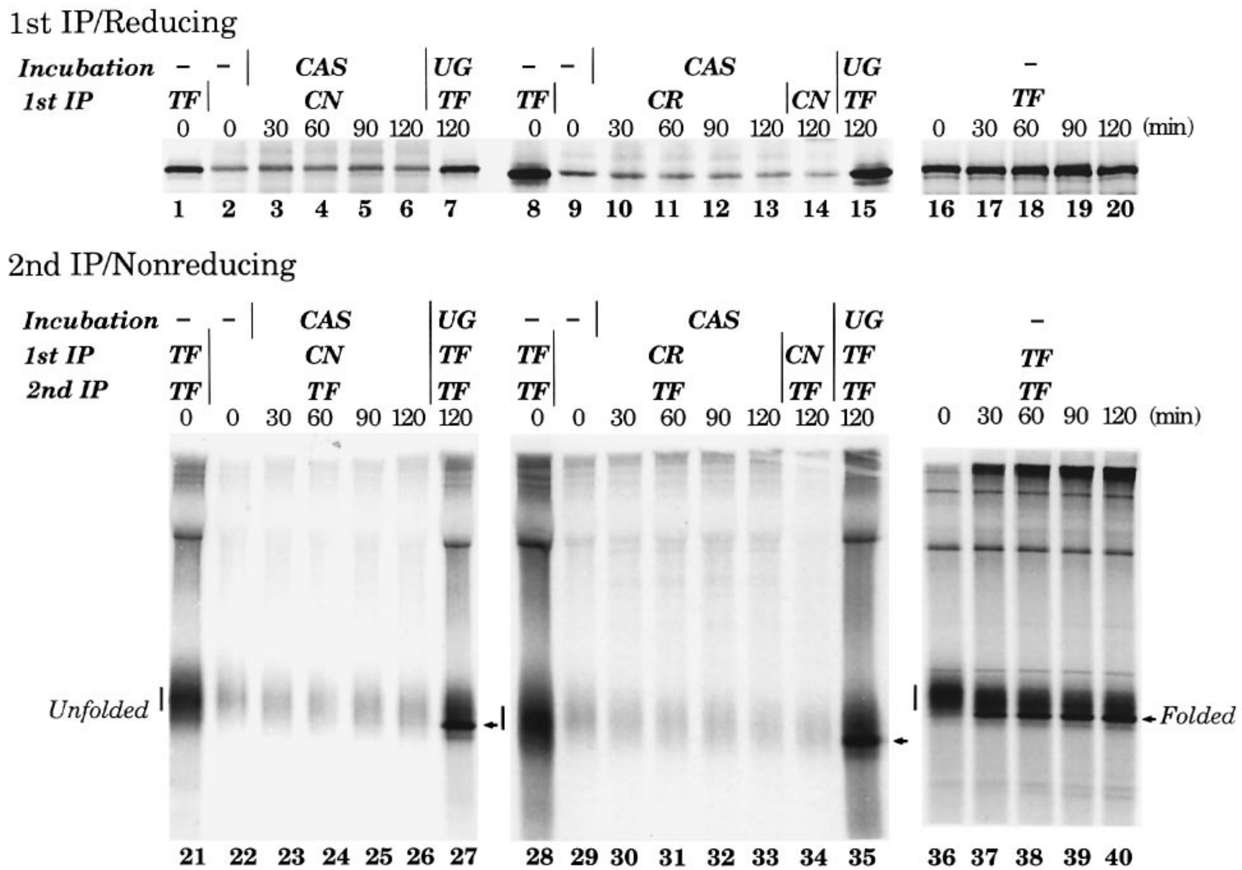


Fig. 9. Effect of castanospermine on transferrin folding in microsomes. One hundred microliters of microsomes pulse labeled with [^{35}S]methionine were incubated in the presence of 5 mM ATP at 33°C with 0.5 mM castanospermine (lanes 3–6, 10–14, 23–26, 30–34) or 1 mM UDP-glucose (lanes 7, 15, 27 and 35) or without additions (lanes 17–20) for the indicated periods of time. After incubation samples were alkylated and subjected to immunoprecipitation with anti-calnexin (lanes 2–6, 14, 22–26 and 34), anti-calreticulin (lanes 9–13 and 29–33) or anti-transferrin (lanes 1, 7–8, 15–20, 21, 27–28 and 35–40) antibodies. One quarter of each immunoprecipitate was separated by SDS–PAGE under reducing conditions (lanes 1–20). Transferrin was recovered from the other three quarters of each immunoprecipitate using anti-transferrin antibody and analyzed by SDS–PAGE under non-reducing conditions (lanes 21–40) as described in Materials and methods. The positions of unfolded and folded transferrin are indicated.

ation cycle on the rate of transferrin folding, because folding was almost completely impaired by the forced association of transferrin with calnexin or calreticulin following treatment with castanospermine (see Figure 9). Pulse labeled microsomes were incubated at 33°C with or without UDP-glucose in the presence or absence of ATP and the kinetics of transferrin folding were estimated by SDS–PAGE under non-reducing conditions. When we compared the kinetics of folding under the two conditions the rate of formation of the folded form was not significantly altered by adding UDP-glucose to the ATP-containing mixtures (Figure 10). These results suggest that the reglucosylation cycle may be a mechanism to optimize promotion of transferrin folding. On the other hand, we observed significant delay in folding in the absence of added ATP, particularly for the first 20 min (Figure 10, circles). The exact reason for the observed delay is currently unknown (see Discussion).

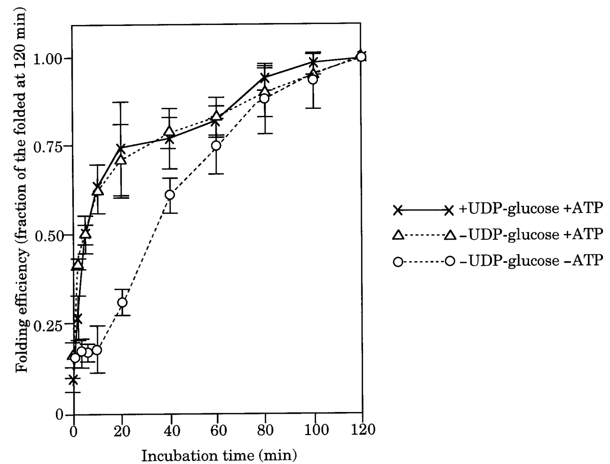


Fig. 10. Effects of UDP-glucose and ATP on the time course of transferrin folding. Fifty microliters of microsomes containing nascent protein labeled with [^{35}S]methionine were incubated at 33°C in the absence (○) or presence of 5 mM ATP with (×) or without (△) 1 mM UDP-glucose for the indicated periods of time. Transferrin was then immunoprecipitated as in Figure 8 and analyzed by SDS–PAGE under non-reducing conditions. The folding efficiency of transferrin estimated from non-reducing gels was expressed as a fraction of the folded observed at 120 min. The data represent mean ± SE (bars) of three different assays.

Discussion

Studies on nascent protein folding in the ER have thus far employed essentially three methodologies: (i) the use of membrane permeable inhibitors in pulse–chase experiments using cultured cells; (ii) overexpression in

cells of a certain chaperone(s) or its dominant negative form(s); (iii) cell-free folding in a dog pancreatic microsome/rabbit reticulocyte lysate system supplemented with oxidized glutathione. In the present study we have described a novel and simple assay by which the progress of folding can be easily analyzed. In our system, microsomes containing nascent unfolded proteins are isolated from pulse labeled cells and oxidative folding of a nascent protein proceeds in a defined buffer having a redox potential similar to that of cytosol (Hwang *et al.*, 1992).

Using the pulse labeled microsomes we have studied the role of reglucosylation cycles on the folding of transferrin. We have shown here that nascent transferrin bound to calnexin and calreticulin is rapidly dissociated from them by deglucosylation and re-associated by post-translational glucosylation. We have also found that the repeated association–dissociation cycles primarily promoted folding by preventing formation of disulfide-bonded aggregates, thus redirecting the misfolded molecules to the correct folding pathways. The mode of interaction with the chaperones in this system must be cyclic, because glucose residues on transferrin were rapidly turned over ($T_{1/2} \top 5$ min). This is basically in agreement with the results of Suh *et al.* (1989), in which they showed reglucosylation *in vivo* of high mannose-type oligosaccharides of misfolded vesicular stomatitis virus G protein. In the present investigation we have obtained clear evidence for the concept that repeated binding to the monoglucosylated sugar is responsible for the promotion of folding by the two chaperones. It is unlikely that direct protein–protein interactions are involved in causing association with the chaperones under the conditions described here, since binding/dissociation in the microsomes was dependent on addition of UDP-glucose or castanospermine, which is consistent with recent reports on the mode of association of the chaperones with RNase B (Rodan *et al.*, 1996; Zapun *et al.*, 1997). The following two possibilities may be conceptualized for the effects of UDP-glucose. One possibility is that unfolded transferrin may be stabilized by repeated binding of calnexin/calreticulin *per se*, consistent with a previous study (Hebert *et al.*, 1996). This hypothesis considers the glucose cycles as a mechanism analogous to the ATP-driven cycles of heat shock proteins such as BiP. The other possibility is that binding to calnexin/calreticulin may recruit the substrates to a microenvironment where other chaperones act on the substrates efficiently. In this scenario, folding of the substrates is arrested in the chaperone complexes, while aggregation is prevented, and accelerated by the action of other chaperones immediately after release from calnexin/calreticulin, thus compensating for the earlier reduced rate. The observation that the overall folding rate of transferrin was not significantly decreased by cycles triggered by addition of UDP-glucose (Figure 10) is consistent with this hypothesis. In this context, it is interesting to note that ER-60/ERp-57, a molecule having structural motifs similar to peptidyl disulfide isomerase, was reportedly found in the nascent chain complex with calnexin and calreticulin (Oliver *et al.*, 1997). Also, Michalak's group have reported that calreticulin is found in close contact with peptidyl disulfide isomerase in the yeast two-hybrid system, as well as other *in vitro* systems (Baksh *et al.*, 1995).

It has been reported that incubating the microsome/rabbit reticulocyte lysate system in the presence of castanospermine resulted in the sustained presence of folding intermediates of hemagglutinin and inhibition of trimer formation during post-translational incubation periods, although the majority of hemagglutinin was already folded at the end of the 2 h translation/folding period (Hebert *et al.*, 1996). Interestingly, they showed that involvement of calnexin and calreticulin enhanced the efficiency of post-translational folding but not that of co-translational folding. Post-translational folding was initiated by adding an excess of oxidized glutathione to the dithiothreitol (DTT)-containing translation mixture. They suggested that the differences observed during post- and co-translational folding may reflect the suboptimal conditions used for the post-translational folding process. However, considering that DTT is known to induce misfolding of several proteins (de Silva *et al.*, 1993; Sawyer *et al.*, 1994) and that the cyclic interactions of calnexin and calreticulin were effective in suppressing misfolding of transferrin, the difference under the two conditions used may also indicate the importance of the chaperone cycles, particularly for misfolded molecules.

The role of ATP in our system is unclear. Although we initially thought that addition of ATP to the assay mixture would result in cyclic interaction of BiP with substrates, we cannot rule out the possibility that ATP may be required for the proper function and structure of some other ER proteins. Indeed, it has been reported that, with the exception of a few chaperones, including BiP, several proteins in the ER bind to ATP without hydrolyzing it (Ou *et al.*, 1995; Dierks *et al.*, 1996). We also observed that glucosylation of transferrin in microsomes required ATP. This may explain our previous result in which depletion of ATP from MDCK cells reversed the effects of DTT (Wada *et al.*, 1994). While treatment of HepG2 cells with DTT caused rapid dissociation of calnexin from its ligands (our unpublished data), we have consistently observed that DTT treatment of MDCK cells resulted in sustained association of gp80 with calnexin. Currently we think that DTT causes misfolding of gp80 resulting in continuous reglucosylation of the molecule. Depletion of ATP from MDCK cells would inhibit reglucosylation and, as a result, gp80 would be released from calnexin by the action of glucosidase II, irrespective of whether the molecule was misfolded or not.

Several misfolded or incompletely folded proteins have been shown to be retained in the ER and calnexin has been shown to be responsible for this retention (Jackson *et al.*, 1994; Rajagopalan *et al.*, 1994). Recently, van Leewen and Kearse (1997) reported that the cellular content of unassembled T cell receptor α subunit, which is retained in the ER, decreased rapidly during the chase when N-linked glycan formation was impaired by treatment of BW cells with mannosamine, a chain terminator of core glycan elongation as well as an inhibitor of anchorage of membrane proteins by glycoinositol phospholipids (Lisanti *et al.*, 1991; Sevlever and Rosenberry, 1993). A similar observation was made in BWE cells, which are deficient in synthesis of dolichol-P-mannose. Reglucosylation was inhibited in both cases. While it is possible that sugar truncation itself may have affected the ability of the ER to determine the fate of the glycoproteins, the role of the reglucosylation cycle may also be important

in regulation of degradation in the ER. The system which we designed and describe in this paper may provide a powerful tool for the analysis of ER degradation, a process which is currently the target of wide and intensive studies.

Materials and methods

Materials

Antiserum against the C-terminal 19 amino acids of human calreticulin was a generous gift of StressGen (Victoria, Canada). UDP-[^{14}C]glucose (254 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). All other reagents were detailed previously (Wada *et al.*, 1995) or purchased from Sigma-Aldrich.

Preparation of microsomes

HepG2 cells, cultured to near confluency in 35 mm cell culture dishes, were labeled for 5 min with [^{35}S]methionine as described previously (Wada *et al.*, 1995) and chilled in ice/water at the end of labeling. All subsequent procedures were done at 4°C. The labeling medium was removed and cells were washed once with ice-cold homogenizing buffer (0.2 M sucrose, 10 μM leupeptin, 10 μM pepstatin, 25 mM triethanolamine acetic acid, pH 7.2). The cells were then scraped into 150 μl homogenization buffer and disrupted by repeated suction five times using a Hamilton 100 μl microsyringe. The homogenates were centrifuged for 5 min at 500 *g*. The pellets were rehomogenized in 50 μl homogenization buffer by three times repeated suction using the microsyringe and recentrifuged at 1500 *g* for 5 min. The combined post-mitochondrial supernatants were loaded on top of 300 μl 20% sucrose which had been overlaid onto 5 μl 80% sucrose in Beckman TL100.1 ultracentrifugation tubes. This was then centrifuged for 20 min at 90 000 r.p.m., after which the supernatants and the sucrose cushions were removed and the tube walls wiped with cotton swabs to minimize cross-contamination from the cytosol. The pellets were resuspended in 200 μl cytosolic buffer (120 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 25 mM triethanolamine acetic acid, pH 7.2) supplemented with 1 mM reduced glutathione and 100 μM oxidized glutathione. This suspension was used for the folding assay. When unlabeled microsomes were used, they were prepared from unlabeled HepG2 cells as above and resuspended to 0.1 eq./μl (Walter and Blobel, 1983) in the cytosolic buffer containing 1 mM reduced glutathione and 100 μM oxidized glutathione.

In vitro folding and immunoprecipitation

Pulse labeled microsomes were diluted to 0.1 eq./μl (Walter and Blobel, 1983) in the cytosolic buffer containing 1 mM reduced glutathione and 100 μM oxidized glutathione and incubated at the indicated temperatures. At the end of various incubation times the samples were chilled in ice/water and a one-tenth volume of 0.25 M iodoacetamide was added to alkylate the folding intermediates. All subsequent procedures were done at 4°C. An equal volume of 2% sodium cholate, 400 mM KCl, 10 μM leupeptin, 10 μM pepstatin, 50 mM triethanolamine acetic acid, pH 7.2, (lysis buffer) was added to the microsomes and further incubated with 10 μl 20% formalin-fixed *Staphylococcus aureus* and 2 μl 10% bovine serum albumin for 20 min on ice. The samples were centrifuged for 5 min at 12 000 *g* and the supernatants incubated for 60 min with antisera as indicated, followed by incubation with 10 μl *S.aureus* for 20 min. The immune complexes were isolated by centrifuging at 250 *g* for 3 min and washed once with 0.6 M KCl, 0.05% Triton X-100, 10 mM triethanolamine acetic acid, pH 7.2, for 10 min. The complexes were then rinsed with the wash buffer minus KCl. The pellets were resuspended in 30 μl 1% SDS, 2 mM EDTA, 5% sucrose, 10 mM triethanolamine acetic acid, pH 7.2, supplemented either with 5 mM iodoacetamide for analysis under non-reducing conditions or with 50 mM DTT for reducing conditions. The samples were heated for 20 min at 65°C and resolved by SDS–PAGE. Bands were visualized by phosphorimaging using a Fujix BAS2000 equipped with Pictrography. Quantification of radioactivity in the gels was done by software contained in the BAS2000.

Determination of UDP-glucose turnover in microsomes

Microsomes (10 eq.) isolated from unlabeled HepG2 cells were incubated for 30 min at 33°C in the ATP-containing cytosolic buffer supplemented with 1 mM UDP-glucose containing 20 μCi UDP-[^{14}C]glucose. After incubation microsomes were re-isolated as described above using the discontinuous sucrose density gradient. The recovered microsomes containing the translocated UDP-[^{14}C]glucose were resuspended in the cytosolic buffer containing 1 mM UDP-glucose and re-incubated at 33°C. After the incubation, castanospermine and iodoacetamide were added to yield 5 and 20 mM respectively and then the microsomes were re-isolated by the same sucrose density centrifugation. The membrane pellets were dissolved in 10 μl dimethylformamide, spotted directly onto polyethyleneimine–cellulose plates (Merck) and developed in 0.1 M KH_2PO_4. The radioactivity at the identified spots (R_f for UDP-glucose = 0.47) was quantitated with a BAS2000 phosphorimager.

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