# Competition between Calnexin and BiP in the Endoplasmic Reticulum Can Lead to the Folding or Degradation of Human Thyroperoxidase†

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ABSTRACT: Previous studies on the fate of human thyroperoxidase (hTPO) molecules have shown that, after being synthesized, these glycoproteins interact with calnexin and calreticulin and that only some of them are able to acquire a partially folded structure. The aim of the present study was to further investigate the potential role of BiP, another major protein chaperon. Co-immunoprecipitation experiments showed the occurrence of interactions between hTPO and BiP. Pulse-chase studies showed that, when hTPO was expressed in a Chinese hamster ovary cell line overexpressing 5 times more BiP than the parent cells, the rate of hTPO recognized by a monoclonal antibody directed against a conformational structure decreased by 50% after 5 h of chase. Overexpression of the BiP-ATPase mutant G37T also led to a decrease in the correct folding rate of hTPO. When this protein was pulsed in the presence of 35S-(Met + Cys) and the reducing agent dithiotreitol and then chased in a culture medium without dithiothreitol, a 2.5-fold decrease in the correct folding rate was observed in cells overexpressing BiP, whereas co-overexpression of calnexin and Erp57 led to an increase in both the unfolded and partially folded form of hTPO after the pulse step. All of these findings show that BiP and calnexin have opposite effects on the folding behavior of hTPO and that the action of specific molecular chaperones may therefore crucially determine the fate of glycoproteins.

Human thyroperoxidase (hTPO)[1] is a type-I transmembrane heme-containing glycoprotein that plays a key role in the process of thyroid hormone synthesis. hTPO, which consists of 933 amino acids, has a large extracellular domain, a transmembrane domain consisting of 60 residues, and a short intracytoplasmic tail. Four potential asparagine-linked glycosylation sites (Asn129, Asn307, Asn342, and Asn569) are present in the extracellular region of this protein. There is some evidence suggesting the presence of disulfide bonds in this part of the molecule (*1*, *2*). Although hTPO catalyzes both thyroglobulin iodination and iodotyrosine coupling at the apical cell surface of thyroid cells, this enzyme is located mainly in the endoplasmic reticulum (ER) and only a small proportion is present at the apical surface (*3*).

In previous studies using stably transfected Chinese hamster ovary (CHO) cells, we established that only 15−20% of the hTPO synthesized is able to acquire a partially folded form and that only 15−20% of this partially folded hTPO is able to acquire a completely folded form and reach the cell surface. This means that only 2% of the hTPO molecules synthesized are able to exit from the ER and reach the cell surface (*4*). Moreover, hTPO is degraded at the ER level via two different pathways, depending upon its folding state (*5*). The completely nonfolded form is degraded by the proteasome, while the partially folded form is degraded by an unknown system involving some ER membrane-bound cysteine/serine proteases.

After their translocation, proteins synthesized at the ER level interact with several molecular chaperones. We previously established that hTPO interacts with calnexin (CNX) and calreticulin (CRT) and that these interactions are required for the correct folding of this glycoprotein to occur (*6*). BiP, one of the other main molecular chaperones present in the ER, which is a member of the Hsp70 family, has been reported to interact with numerous glycoproteins. However, the correct folding of the protein is known to require several association/dissociation cycles with different chaperone proteins (*7*). Moreover, Molinari and Helenius have established that, during the process of synthesis, glycoproteins can first interact with either CNX, CRT, or BiP and that the

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[1] Abbreviations: CHO, Chinese hamster ovary; CNX, calnexin; CRT, calreticulin; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ER, endoplasmic reticulum; FBS, fetal bovine serum; hTPO, human thyroperoxidase; mAb, monoclonal antibody; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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chaperone selected depends upon the position of the first *N*-glycan, because CNX and CRT interact with this *N*-glycan (*8*). If the first *N*-glycan is located among the 50 first amino acids, the protein will be associated with CNX or CRT, but if the glycan is located farther away from the N-terminal end, the protein will be associated with BiP.

In the present study, we studied the role of BiP, one of the main molecular chaperones, and that of ERp57, a partner of CNX and CRT, which rearranges disulfide bonds.

## EXPERIMENTAL PROCEDURES

*Materials.* Nutrient mixture F-12 (HAM) was from Invitrogen (Cergy Pontoise, France). Fetal bovine serum (FBS) was supplied by HyClone/Perbio (Brebiéres, France). Dulbecco's modified Eagle medium (DMEM), glutamine, protease inhibitor cocktail, Triton X-100, sodium deoxycholate, *N*-ethylmaleimide, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). LipofectAMINE PLUS reagent was obtained from Invitrogen and protein A-Sepharose 4B conjugate from Zymed Laboratories, Inc. (San Francisco, CA). EZ-Link sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), ImmunoPure Immobilized Avidin Gel, and Super Signal West Dura were purchased from Pierce/Perbio (Brebiéres, France). NEG-772 Easytag express protein labeling mix, 35S-(Met + Cys), was obtained from Perkin−Elmer Life Sciences, Inc. (Boston, MA). Pure plasmid DNA preparations were obtained with GenElute Endotoxin-Free plasmid Midi-prep kit from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-calnexin polyclonal antibody (SPA-860), rabbit anti-ERp57 polyclonal antibody (SPA-585), mouse anti-Hsp70 monoclonal antibody (SPA-822), and rabbit anti-Grp78 (BiP) polyclonal antibody (SPA-826) were obtained from Stressgen (Victoria, Canada). The full-length cDNA coding for the CNX was cloned into the Ap$^r$M8 vector (*9*). The human Erp57-pBK-CMV vector was a gift from B. Bourdi (*10*). The BiP wild-type pMT and the BiP-T37G pMT expression vectors were a gift from Dr. L. Hendershot (*11*). Monoclonal antobody directed against thyroglobulin was a gift from J. Ruf (*12*).

*Cell Culture and Transfection Procedure.* The parent CHO cell line (DUKX-B11 cells) and cells overexpressing BiP (BiP−CHO cells) (a generous gift from A. Dorner) have been previously described (*13*). DUKX cells were kept in α medium containing 10% fetal bovine serum, penicillin (100 IU/mL), streptomycin (0.1 mg/mL), and 10 μg/mL of adenosine, deoxyadenosine, and thymidine. BiP−CHO cells were maintained in the same medium with FBS, without nucleotides but supplemented with $10^{-7}$ M methotrexate before being incubated in a saturated atmosphere (5% $CO_2$/95% air) at 37 °C. They were then transfected with pcDNA3-hTPO using lipofectAMINE PLUS reagent and selected with G418 (400 μg/mL) to obtain a pool of cells overexpressing hTPO.

CHO cells and stably transfected hTPO−CHO cells were grown in Ham-F12 medium supplemented with 10% FBS and protease inhibitor cocktail (Sigma) in a saturated atmosphere (5% $CO_2$/95% air) at 37 °C. They were transiently transfected with ERp57, CNX, BiP, or BiP-T37G plasmid constructs using lipofectAMINE PLUS reagent according to the instructions of the manufacturer.

*Specificity of the Monoclonal Antibodies (mAb) Directed against hTPO.* To discriminate between different populations of hTPO molecules differing in their three-dimensional structures, two mAbs were used, namely, mAb15 and mAb47 (*14*). mAb47 recognizes a linear epitope, and it has been previously established that this mAb does not recognize hTPO when this molecule has acquired a partially or completely folded form (*5*). mAb15 recognizes a three-dimensional epitope in hTPO and is therefore unable to recognize unfolded hTPO molecules. Immunoprecipitation experiments were performed using these antibodies complexed with protein A-Sepharose beads (Zymed Laboratories, Inc., San Francisco, CA) for 3 h at room temperature in hTPO-extraction buffer containing 1% Triton X-100, 0.3% sodium deoxycholate, 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 20 mM *N*-ethylmaleimide, and protease inhibitor cocktail.

*Pulse-Chase Experiments and Extraction of hTPO.* The cells (in 9.6 $cm^2$ dishes) were preincubated for 2 h in Met- and Cys-free DMEM supplemented with 10% dialyzed FBS and 10 mM sodium butyrate and pulsed for 30 min in the same medium supplemented with 35S-(Met + Cys) (66 μCi/mL). After the pulse step, the medium was removed and the cells were washed twice in phosphate-buffered saline (PBS) and then chased for various times in culture medium supplemented with 5 mM Met and 5 mM Cys. When the chase was completed, cells were washed twice with 2 mL of ice-cold PBS and scraped off into 600 μL of hTPO extraction buffer. Cells were then tumbled for 20 min at 4 °C and centrifuged for 3 min at 10000*g*. The supernatants obtained were incubated for 2 h at room temperature with previously prepared mAb15− or mAb47−protein A-Sepharose complexes. Immune complexes were retrieved by briefly centrifuging the preparation and washing it 4 times with 1 mL of hTPO extraction buffer and once with 1 mL of PBS. They were then reduced and denatured, and after being centrifuged, the samples were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (7.5%). The radioactivity associated with the hTPO bands was detected and quantified using a phosphorimager (Fudjix BAS 1000, Japan, or GS-363 Molecular Imager System, BioRad).

*Sequential Immunoprecipitation Procedure.* Metabolic labeling and immunoprecipitation were performed as described above, except that 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was used instead of 1% Triton X-100 and 0.3% sodium deoxycholate in the extraction buffer and in the binding step between protein A-Sepharose and anti-BiP or anti-Hsp70 antibodies. CHO−hTPO cells were incubated for 2 h with 10 mM sodium butyrate and radiolabeled for 30 min with 35S-(Met + Cys). The immunoprecipitation step with the first antibody (anti-BiP or anti-Hsp70) was performed as described above. After the first immunoprecipitation step, the proteins were separated from the protein A-Sepharose pellets by heating the pellets for 5 min at 100 °C after adding 10 μL of 10% SDS diluted with 500 μL of CHAPS buffer. After a 3 min centrifugation at 10000*g*, the protein A-Sepharose pellet was discarded, and the supernatant was incubated overnight at 4 °C with the two associated anti-hTPO mAbs (mAb15 and mAb47) preabsorbed on protein A-Sepharose beads. After immunoprecipitation and a washing step, the second precipitates were resuspended in Laemmli buffer, boiled for 5 min, and loaded onto SDS−PAGE.

Alternatively hTPO−CHO cells were extracted with hTPO extraction buffer supplemented or not with 1% SDS, and after diluting SDS to 0.05%, immunoprecipitation was performed using anti-CNX or anti-BiP antibodies. Western blotting was then performed using mAb47 and HRP-coupled second antibody.

*Cell-Surface Biotinylation.* hTPO−CHO and BiP−CHO confluent monolayers were metabolically labeled for 5, 16, or 48 h with 66 $\mu$Ci/mL 35S-(Met + Cys) in the presence of 10 mM sodium butyrate, and cell-surface biotinylation was performed as previously described (*4*). Cells were washed twice with PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and exposed to a 0.5 mg/mL Immunopure NHS-SS-biotin (Pierce, Oud Beijerlank, The Netherlands) for 20 min at 4 °C. The cross-linker was removed, and the same procedure was repeated once. The biotin reagent was quenched by incubating the preparation with 50 mM $NH_4Cl$ in PBS for 10 min at 4 °C. Cells were washed with PBS and harvested. To recover the immunoprecipitated antigens, the complexes were supplemented with 10 $\mu$L of 10% SDS, and the mixture was boiled for 5 min, diluted with 600 $\mu$L of hTPO-extraction buffer, and centrifuged (10000*g* for 3 min). The supernatant containing total hTPO was incubated for 2 h with avidin-agarose (Pierce, Oud Beijerlank, The Netherlands). Biotinylated surface TPO and intracellular hTPO were separated by centrifugation (10000*g*, 10 s). The beads were washed 4 times with hTPO extraction buffer and once with PBS, resuspended in Laemmli buffer supplemented with 2-mercaptoethanol, and boiled for 5 min. The supernatants were analyzed by SDS−PAGE.

## RESULTS

*Interaction between hTPO and BiP.* To determine whether BiP participates in the folding and/or retention of hTPO in the ER, hTPO−CHO cells were screened. Cells were incubated for 30 min with 35S-(Met + Cys), and cell extracts were then immunoprecipitated with antibodies directed against BiP or with Hsp70. Numerous proteins were found to be associated with both of these molecular chaperones (lanes 2 and 5 in Figure 1A). The results obtained after performing a second immunoprecipitation step using mAbs directed against hTPO showed that hTPO interacted with BiP and Hsp70 (lanes 3 and 6 in Figure 1A). Hsp70 is located in the cytoplasm, but it has been established that the transmembrane protein can interact with Hsp70 and Hsp90 via its cytoplasmic domain (*15*). The specificity of the interaction between BiP and hTPO was confirmed by performing immunoprecipitation tests under native (lane 3 in Figure 1B) and denaturing conditions (lane 1 in Figure 1B). Under denaturing conditions, no interactions were detected.

To investigate whether BiP contributes to the folding of hTPO, we used a CHO cell line overexpressing 5 times more BiP than a parent cell line (*13*) and we transiently transfected the cDNA of the hTPO into these two cell lines. The folding of hTPO was monitored by performing pulse-chase experiments. After a 30 min pulse with 35S-(Met + Cys), hTPO was immunoprecipitated by either mAb15 or mAb47 (parts A and B of Figure 2). Quantification of these bands showed the existence of no significant differences in the rate of disappearance of the unfolded form of hTPO recognized by

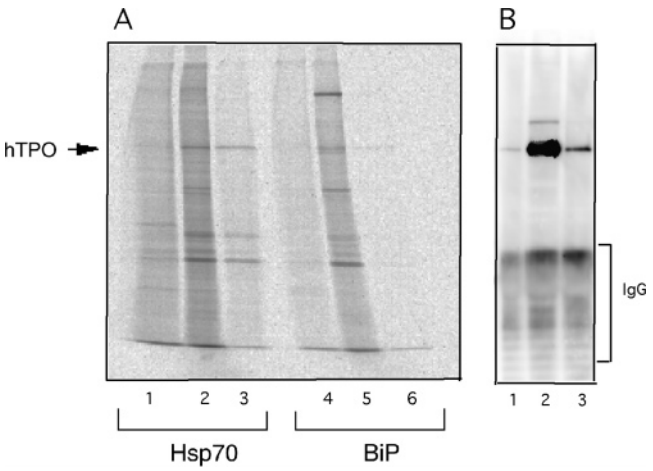


FIGURE 1: Co-immunoprecipitation of hTPO with BiP and Hsp70. (A) hTPO−CHO cells were incubated for 30 min with 35S-(Met + Cys) in DMEM without Met and Cys and supplemented with 10% FBS and 10 mM sodium butyrate. Antibodies against Hsp70 (lanes 1−3) or BiP (lane 4−6) were used to immunoprecipitate cell extracts. After the supernatant was eluted from protein A-Sepharose, it was run on 7.5% SDS−PAGE (lanes 2 and 5) or immunoprecipitated using mAb15 and mAb47 (lanes 3 and 6) or a mAb directed against thyroglobulin (lanes 1 and 4). Samples were run on 7.5% SDS−PAGE, and the bands were detected with a phosphorimager. (B) hTPO−CHO cells were extracted with buffer supplemented (lane 1) or not (lanes 2 and 3) with 1% SDS, and after diluting SDS to 0.05%, immunoprecipitation was performed using anti-CNX (lane 2) or anti-BiP antibodies (lanes 1 and 3). Western blotting was then performed using mAb47 and HRP-coupled second antibody.

the mAb47 (mAb47+ forms) (Figure 2C). In the parent cells, after 5 h of chase, 40% of the hTPO synthesized acquired a structure that was recognized by mAb15 (mAb15+ forms) (Figure 2D). In cells overexpressing BiP, this population decreased and was found to be approximately 50% smaller than in the parent cells at all of the chase times used. These results were not due to any changes in the levels of expression of the other molecular chaperones (CNX, CRT, Erp57, and grp94) present in these two cell lines (lane 1 versus lane 3 and lane 2 versus lane 4 in Figure 3). The expression of hTPO in these cell lines did not have any effect on the levels of expression of these chaperones.

To check whether the decrease in the formation of mAb15+ forms in the hTPO−BiP−CHO cells may have affected the hTPO level present at the cell surface, these cells and the parent cells were incubated in the presence of 35S-(Met + Cys) for 48 h and the hTPO present at the cell surface was separated from the intracellular hTPO by performing cell-surface biotinylation. Because the expression time of hTPO at the cell surface is between 3 and 5 h (*4*), a larger amount of hTPO was expected to be present at the cell surface after 48 h of labeling. Surprisingly, no changes were detected in the hTPO levels present at the cell surface. The fact that approximately 30% of the total hTPO was consistently recovered at the cell surface (Figure 4) indicated that the overexpression of BiP did not affect the relative rate of hTPO cell-surface expression.

The cotranslational interactions between proteins and molecular chaperones, which occur in the ER, are a prerequisite for the folding and proper formation of disulfide bonds via PDI family proteins associated with the chaperones. In the case of several proteins, cotranslational disulfide

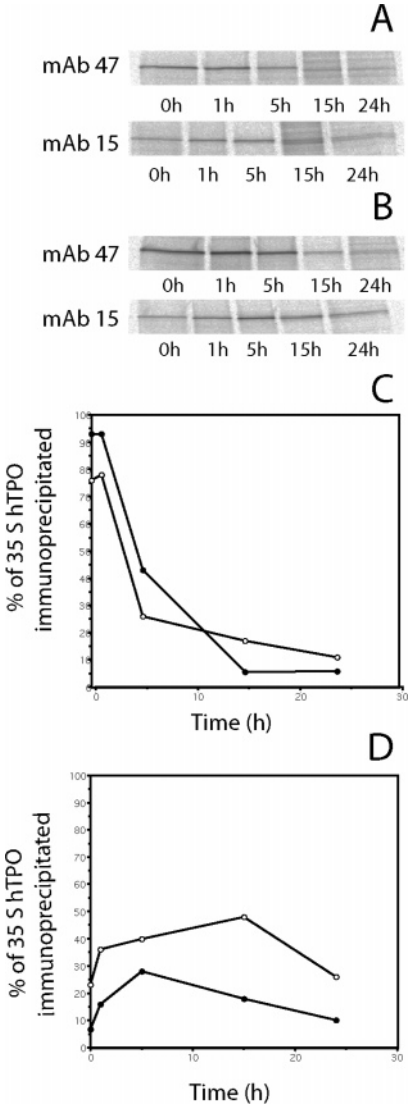


FIGURE 2: Effects of BiP overexpression on the folding of hTPO in CHO cells. A cell line overexpresssing BiP (BiP−CHO cells; B; ●) and the parent cell line (DUKX cells; A; ○) were transfected with hTPO-pcDNA3. After 48 h, pulse-chase analysis was performed. Cells were preincubated for 2 h in Met- and Cys-free medium supplemented with 10% dialyzed FBS and 10 mM sodium butyrate and then pulsed for 30 min in the same culture medium supplemented with 66 $\mu$Ci of 35S-(Met + Cys)/mL. After the pulse, cells were chased for various times in Ham-F12 medium and 10% FBS supplemented with 5 mM Met and 5 mM Cys. Immunoprecipitation was performed using mAb47 (A, B, and C) or mAb15 (A, B, and D). Samples were separated by SDS−PAGE, and the radioactivity was detected and quantified using a phosphorimager device. The 100% value was that obtained with the newly synthesized hTPO immediately after the pulse. This figure gives the results of an experiment that is representative of three identical experiments performed.

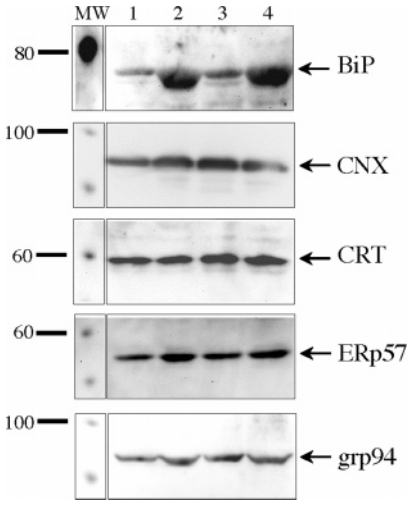


FIGURE 3: Expression of various molecular chaperones in BiP−CHO cells and DUKK cells expressing hTPO or not. Transfection of cells were performed as described in Figure 2. After 48 h, cells were scraped off into hTPO extraction buffer. After samples were denatured in Laemli buffer and reduced, they were analyzed using SDS−PAGE and Western blotting procedures with anti-BiP, anti-CNX, anti-CRT, anti-Erp57, and anti-grp94 antibodies. DUKK cells (lanes 1 and 3) BiP cells (lanes 2 and 4), and cells expressing hTPO (lanes 3 and 4).

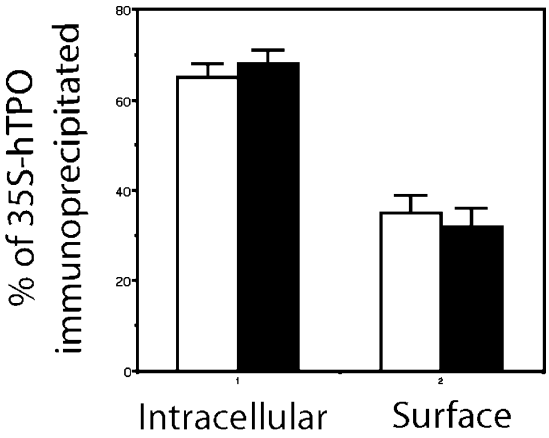


FIGURE 4: Cell-surface expression of hTPO in cells overexpressing BiP. Cells were incubated in DMEM without Met and Cys, supplemented with 10% FBS and 10 mM sodium butyrate, and then radiolabeled for 48 h with 35S-(Met + Cys). Cell monolayers were then incubated with NHS-SS-biotin as described in the Experimental Procedures, and after an extraction step, the hTPO was immunoprecipitated with mAb15 + mAb47. Intracellular and cell-surface hTPO were separated with avidin agarose. Samples were analyzed by performing SDS−PAGE, and the bands corresponding to hTPO were quantified with a phosphorimager device. Parent cell line (white bars), and cells overexpressing BiP (black bars). Values are mean ± standard error of the mean (SEM) of three different experiments.

bond formation, folding, and oligomerization of proteins within the ER are known to be reversibly inhibited when a reducing agent is added (*16*−*18*). To determine whether the formation of a completely unfolded form of hTPO might be reversed differently between parent cells and cells overexpressing BiP, hTPO transiently transfected parent cells and BiP cells were pulsed in the presence of 35S-(Met + Cys) and DTT and chased for various times in a culture medium containing no radioactivity or DTT. The hTPO was then immunoprecipitated with either mAb47 or mAb15. As was to be expected, DTT decreased the percentage of the partially folded mAb15+ forms recovered at the end of the pulse step and practically all of the hTPO was unfolded and immunoprecipitated with mAb47 (parts A and B of Figure 5). In the parental cell line, 40% of the 35S-hTPO was in the mAb15+ form after 5 h of chase, whereas the cells overexpressing BiP showed levels of mAb15+ forms that were at least 2.5 times lower throughout the chase than those recorded in the parent cells. These results indicate that cotranslational formation of disulfide bridges is not necessary for the hTPO

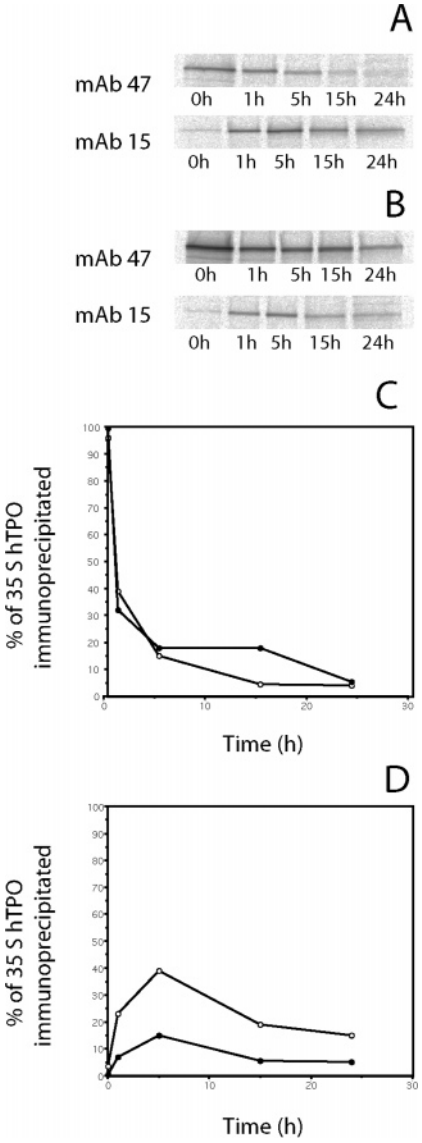


FIGURE 5: Processing of hTPO in the presence of DTT in cells overexpressing BiP or parent cells. A cell line overexpresssing BiP (BiP−CHO cells; B, ●) and the parent cell line (DUKX cells; A, ○) were transfected with hTPO-pcDNA3. After 48 h, pulse-chase analysis was performed. Cells were preincubated for 2 h in Met- and Cys-free medium supplemented with 10% dialyzed FBS, 10 mM sodium butyrate, and 10 mM DTT and then pulsed for 30 min in the same culture medium supplemented with 66 $\mu$Ci of 35S-(Met + Cys)/mL. After the pulse, cells were chased for various times in Ham-F12 medium and 10% FBS supplemented with 5 mM Met and 5 mM Cys. Immunoprecipitation was performed using mAb47 (A, B, and C) or mAb15 (A, B, and D). Samples were separated by performing SDS−PAGE analysis, and the radioactivity was detected and quantified using a phosphorimager device. The 100% value was that obtained with the newly synthesized hTPO directly after the pulse. This figure gives the results of an experiment that is representative of three identical experiments performed.

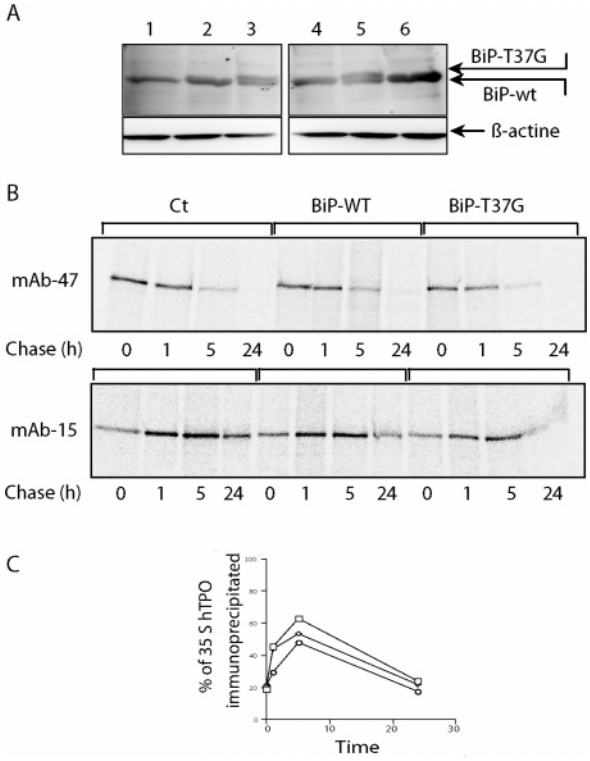


FIGURE 6: Effects of wild-type BiP and T37G-BiP mutant overexpression on hTPO folding. Stable CHO−TPO cells were transiently transfected with the BiP-WT-pMT plasmid (A, lanes 2 and 6; B; C, ◇), the BiP-T37G-pMT plasmid (A, lanes 3 and 5; B; C, ○), or the empty vector (A, lanes 1 and 4; B; C, □). Western blot analysis was performed on cell lysates with a specific anti-BiP antibody (A). This analysis was performed 48 h (lanes 1−3) or 72 h (lanes 4−6) after transfection. As expected, this antibody recognized the endogenous (lanes 1 and 4) and transfected BiP wild type (lanes 2 and 6), giving a specific band under the 80 kDa marker band. The BiP-T37G mutant was taken to have a slightly higher molecular weight, on the basis of the difference in N-glycosylation (*21*) (lanes 3 and 5). A total of 48 h after the transfection step, pulse-chase experiments were performed (B). Cells were preincubated for 2 h in Met- and Cys-free medium supplemented with 10% dialyzed FBS, 10 mM sodium butyrate, and 10 mM DTT and then pulsed for 30 min in the same culture medium supplemented with 66 $\mu$Ci of 35S-(Met + Cys)/mL. After the pulse and PBS washing, cells were chased in Ham-F12 medium supplemented with Met and Cys for various times. Immunoprecipitation of cell extracts was performed using mAb47 (upper panel of B) or mAb15 (lower panel of B; C). Samples were then separated by SDS−PAGE, and the radioactivity was detected (B) and quantified (C) with a phosphorimager device. The 100% value was that obtained with the newly synthesized hTPO immediately after the pulse. This figure gives the results of an experiment that is representative of three identical experiments performed.

to fold correctly and also confirm that BiP interacts negatively in the folding of hTPO.

The results obtained with the CHO cells overexpressing BiP suggest that BiP may drive hTPO along the degradation pathways rather than contributing to its maturation processes. To study the role of the BiP in the folding and/or degradation of hTPO more closely, pulse-chase experiments were performed using wild-type BiP and T37G-BiP, a conformational change mutant. The latter mutant was able to bind to protein but was unable to release its substrates after binding to ATP, and the folding of the substrate protein was therefore incomplete (*19*, *20*). Transfection of wild-type BiP cDNA into CHO cells led to a 1.7-fold expression of BiP (lanes 2 and 6 versus lanes 1 and 4 in Figure 6A).

Transfection of BiP-T37G cDNA resulted in the detection of two immunoreactive bands because the mutant migrates slightly more slowly than endogenous BiP, as a result of a difference in the glycosylation rates (*19*, *21*). The expression of the mutant led to a decrease in the endogenous BiP, as can be seen from the lower and upper bands, respectively, in Figure 6A (lanes 3 and 5 versus lanes 1 and 4), along with a 1.5-fold increase in the total BiP. This small increase

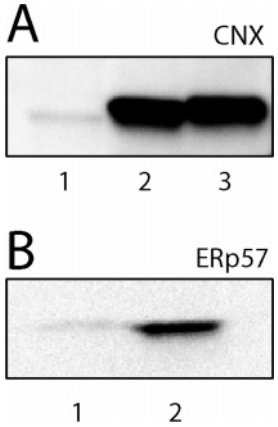


FIGURE 7: Overexpression of CNX and Erp57 in hTPO−CHO cells. hTPO−CHO cells were transiently transfected with CNX-Ap$^r$M8 (A, lanes 2 and 3) and ERp57-pBK-CMV (B, lane 2) plasmids, or empty plasmids (A, lane 1; B, lane 1) using lipofectAMINE PLUS reagent according to the instructions of the manufacturer. After 48 h (A and B, lanes 2) or 72 h (A, lane 3), cells were scraped off, and after an extraction step, samples were run on SDS−PAGE. After we performed Western blotting, the PVDF membranes were incubated with a CNX- (A) or an ERp57-rabbit polyclonal antibody (B). After the membranes were washed, they were incubated with a monoclonal anti-rabbit peroxidase-conjugated immunoglobulin. The signal was developed using Super Signal West Dura.

in total BiP was due to a feedback regulation exerted by the protein translation process (*22*). Under these conditions, the mAb15+ forms retrieved after 5 h of chase from CHO−TPO cells overexpressing BiP wild type decreased by 15% (parts B and C of Figure 6). In the presence of BiP-T37G, the maturation process was more severely impaired and decreased by 25% (parts B and C of Figure 6). However, this decrease was less pronounced than that obtained with the Dorner's BiP−CHO cells, probably because the latter cells overexpress BiP more efficiently (5-fold versus 1.5-fold). These results strongly suggest that, by promoting the degradation of the newly synthesized forms, the stable binding of BiP to hTPO reduces the folding ability of hTPO.

*Effects of Calnexin and ERp57 on the Folding of hTPO.* In a previous study, we reported that the binding of CNX and CRT to hTPO is a prerequisite for the initial molecular folding step to be possible (*6*). It has by now been established that CNX and CRT interact with ERp57, a thiol oxidoreductase belonging to the PDI family (*23*). One possible explanation for the incomplete folding of hTPO occurring in cells overexpressing CNX might be that they contain a low level of ERp57 in comparison with the CNX levels present in CNX−hTPO−CHO cells. To test this hypothesis, we transiently coexpressed CNX and ERp57 in hTPO−CHO cells. Under these conditions, the expression of CNX and ERp57 increased 25- and 7-fold, respectively (Figure 7) and the level of expression of CNX remained unchanged between the end of the pulse and after 24 h of chase (lanes 2 and 3 in Figure 7A). Pulse-chase experiments were then performed in the presence of 35S-(Met + Cys), and hTPO was immunoprecipitated with either mAb47 or mAb15 (parts A and B of Figure 8). In our previous study, we observed that overexpression of CNX increased the overall quantity of hTPO after the pulse. In the present study, the unfolded and partially folded forms were also recovered in larger amounts at the end of the pulse step (parts C and D of Figure 8).

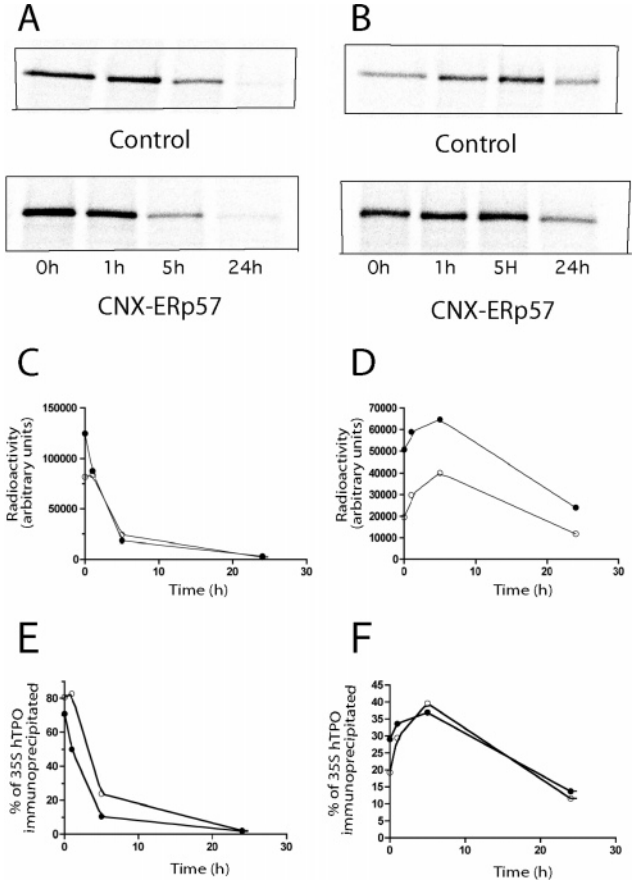


FIGURE 8: Effects of CNX and Erp57 overexpression on hTPO folding and stability. hTPO−CHO cells were transiently transfected with ERp57-pBK-CMV and CNX-Ap$^r$M8 plasmids (●) or empty plasmids (○) using lipofectAMINE PLUS reagent according to the instructions of the manufacturer. After 48 h, pulse-chase analysis was performed. Cells were preincubated for 2 h in Met- and Cys-free medium supplemented with 10% dialyzed FBS and 10 mM sodium butyrate and then pulsed for 30 min in the same culture medium supplemented with 66 $\mu$Ci/mL of 35S-(Met + Cys). After the pulse, cells were chased for various times in Ham-F12 medium and 10% FBS supplemented with 5 mM Met and 5 mM Cys. Immunoprecipitation was performed using mAb47 (A, C, and E) or mAb15 (B, D, and F). Samples were separated by performing SDS−PAGE analysis, and the radioactivity was detected and quantified using a phosphorimager device. The 100% value was that obtained with the newly synthesized hTPO immediately after the pulse. This figure gives the results of an experiment that is representative of three identical experiments performed.

However, the overexpression did not affect the relative proportions of the two forms (parts E and F of Figure 8).

The data obtained here, along with those obtained in our previous study (*6*), show that hTPO interacts with these three molecular chaperones and that these interactions lead to the folding of the glycoprotein in the case of CNX and to its degradation in the case of BiP. It has been previously reported (*8*) that competitive processes can occur between CNX and BiP, depending upon the location of their respective first binding sites. The first hTPO *N*-glycan is located on the Asn129, and the strongly predictive BiP-binding site algorithm applied using the Blond−Elguindi method (*24*) showed that the first three binding sites were located at positions 80, 113, and 133 in the hTPO sequence. To promote interactions between hTPO and CNX or CRT, we performed mutagenesis to add an N-glycosylation site in position 35 or 61. These two cDNAs were transfected into a CHO cell

line. After metabolic labeling in the presence of 35S-(Met + Cys), no change was observed in the proportions of mAb47+ and mAb15+ forms or in the relative levels of hTPO present at the cell-surface level (data not shown). It therefore does not seem at all likely that adding a *N*-glycan near the N-terminal end may have affected the folding or cell-trafficking properties of this glycoprotein.

## DISCUSSION

In eukaryotic cells, the efficiency of protein production processes is known to be monitored by quality-control machinery (*25*). This process is an extremely complex one and seems to differ from one (glyco)protein to another and from one cell line to another. In this paper, we provide new insights on the role played by BiP in the folding of hTPO.

In the first part of the present study, hTPO was found to interact with Hsp70 and BiP. We then focused on the interaction with BiP. The latter protein is a member of the Hsp70 family and is one of the main ER molecular chaperones. It binds to newly synthesized protein, facilitates its translocation (*26*), and contributes to protein folding and assembly processes in cooperation with the protein disulfide isomerase (*27*). BiP seems to be also essential to the transport of the unfolded polypeptides back across the membrane (*28*).

To ascertain whether BiP contributes importantly to the hTPO-folding process, we performed studies using a CHO line overexpressing this molecular chaperone (*13*). Contrary to what has been found to occur with CNX (Figure 7 and ref *6*), BiP was found to have negative effects on the folding of this glycoprotein (Figure 2). In the case of other glycoproteins, BiP can have either positive (*29*, *30*) or negative effects (*31*−*33*) on the folding process. It has been established that BiP protects some proteins, such as immunoglobulin light chains, from degradation, and that BiP must be released from the protein for complete protein folding to occur (*34*). Positive effects of BiP on the protein-folding process can be assessed using BiP ATPase mutants, because these mutants are not released and the protein is therefore unable to undergo complete disulfide bond formation (*11*). In the present study, the presence of BiP ATPase mutant T37G also led to a decrease in the folding of the hTPO. The fact that this negative effect was not much greater than that observed with BiP (Figure 6) confirms the negative effects of the latter. To examine whether the inhibition of disulfide bridge formation may have affected the folding of hTPO and its interactions with molecular chaperones, experiments were performed in the presence of DTT. The oxidative environment maintained in the ER is necessary for proteins containing disulfide bridges to fold properly. The presence of a reducing agent can lead to the formation of a fully reduced form and then to an unfolding form of these proteins. The effects of reducing agents have been described in the case of numerous proteins (*16*−*18*). Adding DTT or 2-mercaptohetanol results in the accumulation of unfolded forms of proteins in the ER, but if the reducing agents are removed, the proteins can acquire a proper three-dimensional structure (*18*, *35*, *36*). When hTPO was synthesized in the constant presence of DTT, this glycoprotein did not fold properly and was quickly degraded (data not shown). However, when hTPO was pulse-labeled in the presence of 35S-(Met + Cys) and DTT before being chased in the absence of the reducing agent, hTPO was able to form apparently normal post-translational disulfide bonds (parts A and B of Figure 5). However, it is worth noting that the proportion of the hTPO able to acquire a conformation recognized by mAb15 decreased in comparison with that obtained without any DTT (Figure 5B versus Figure 2B). This difference may have been due to the fact that, during the pulse, the degradation of the mAb47+ form is a faster process (Figure 5A versus Figure 2A). When similar experiments were performed on the CHO cells overexpressing BiP, the formation of correctly folded hTPO decreased 2.5-fold after 5 h of chase. These data indicate that the increased binding rate of BiP did not enhance the folding of the protein but rather led to its degradation and thus confirm that BiP had negative effects on the folding of hTPO.

In a previous study (*6*), we reported that the binding step with CNX and/or CRT is crucial to the folding of hTPO, as well as protecting hTPO from being degraded by the proteasome. The results of the present study confirm this finding and show that the overexpression of CNX and Erp57 increases the quantity of hTPO obtained at the end of the pulse step by stabilizing the protein immediately after its synthesis. The role of CNX, like that of BiP, can vary depending upon the protein involved. CNX has been found to increase the folding rate (*37*, *38*), to stabilize unfolded glycoproteins (*39*, *40*), and to cause glycoproteins to be degraded by the proteasome (*41*). It is therefore obvious that the presence of a specific molecular chaperone can be crucial to the correct folding of a glycoprotein.

A few years ago, Molinari and Helenius described the competitive process occurring between the CNX and BiP pathways during the translocation of a newly synthesized glycoprotein (*8*), and it has been established that, in the case of some glycoproteins, CNX and BiP can act sequentially (*42*, *43*). Our own results have shown that CNX and CRT are necessary for hTPO to fold properly and that the binding of hTPO to BiP impairs the folding process. Because the position of the first *N*-glycan might determine the choice of the first chaperone to be bound (*8*) and because the interactions with CNX or CRT increase when this *N*-glycan is present within the first 50 residues of the $NH_2$-terminal sequence, we performed mutagenesis to add an N-glycosylation site in position 35 or 61. These sites were glycosylated, but the folding and intracellular trafficking of these forms were not greatly improved in comparison with the wild-type hTPO. Unfortunately, because low levels of interaction were not detectable in our system, it was not possible to assess whether adding an *N*-glycan to the N-terminal part of the protein led to an increase in the binding rates of CNX and CRT to hTPO. However, the results of the present study show that adding an *N*-glycan to the first 50 *N*-glycans did not suffice to improve the folding of all of the glycoproteins in which binding to CNX or CRT is required for complete folding of hTPO to occur.

In other systems, adding an *N*-glycan or its presence in the N-terminal part of a glycoprotein can enhance the secretion process, but the exact role of these *N*-glycans has not yet been elucidated (*44*). On the other hand, it has by now been clearly established that CNX can interact with both glycosylated and nonglycosylated proteins (*45*, *46*). In the latter case, the competition between CNX and BiP will not be glycan-dependent. As far as hTPO is concerned, we

previously reported that the inhibition of the binding to CNX induced by castanospermine led to the rapid degradation of hTPO (*6*). This finding strongly suggests that the interactions between hTPO and CNX are mediated by the *N*-glycans.

From the results of all of these experiments, it is not really clear whether the first chaperone that binds to hTPO during its translocation into the ER is a crucial requirement for the folding of hTPO to occur or whether the presence of other BiP- and CNX-binding sites may be a more decisive factor. However, the fact that introducing an N-glycosylation site near the N-terminal end of hTPO did not increase the percentage of correctly folded protein seems to indicate that the first binding site does not play a critical role in this folding process. All of these results therefore show that the balance between the levels of expression of BiP and CNX (or CRT) determines the stability and folding of hTPO at the ER level; these two chaperones compete for the possibility of binding to neosynthesized hTPO and drive it to either the maturation or degradation pathway.

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