Endoproteolytic Cleavage of Human Thyroperoxidase

ROLE OF THE PROPEPTIDE IN THE PROTEIN FOLDING PROCESS*

Valérie Le Fourn†, Mireille Ferrand, and Jean-Louis Franc§

From the Laboratoire de Recherche Interactions Cellulaires Neuroendocrinienennes, Unité Mixte de Recherche 6544, CNRS-Université de la Méditerranée, Faculté de Médecine, Institut Fédératif de Recherche Jean-Roche, 13916 Marseille, France and U555 INSERM, Faculté de Médecine, 13385 Marseille, France

Human thyroperoxidase (hTPO), the key enzyme involved in thyroid hormone synthesis, is synthesized in the form of a 933-amino acid polypeptide that subsequently undergoes posttranslational modifications such as N- and O-glycosylation and heme fixation. In the present study, it was established that the N-terminal part of hTPO is cleaved during the maturation of the enzyme. In the first set of experiments performed in this study, Chines hamster ovary (CHO) cells transfected with hTPO cDNA generated four different species after deglycosylation, namely a 98-kDa species, which corresponds to the full-length deglycosylated hTPO, and two 94-kDa and one 92-kDa species, which were truncated in the N-terminal parts. The three latter forms were detected only at the cell surface. A proprotein convertase inhibitor prevented these cleavages, and experiments using monensin and brefeldin A showed that they occurred in a post-endoplasmic reticulum compartment. Site-directed mutagenesis studies were performed in which Arg95 was identified as one of the cleavage sites. In the second part of the study, hTPO from human thyroid glands was purified using a monoclonal antibody recognizing the folded form of hTPO. Amino acid determination showed that the N-terminal part of this protein begins at Thr109. This cleavage process differs from that observed in CHO cells. The fact that this hTPO was endoglucosaminidase H-sensitive indicated that the cleavage of the propeptide occurs in the endoplasmic reticulum. To analyze the role of the hTPO prosequence, cDNAs with and without prosequence (Cys15–Lys108) were transfected into CHO cells. hTPO propeptide deletion drastically decreased the proportion of the folded hTPO form, and under these conditions the cell surface activity disappeared completely. These results strongly suggest that the prosequence plays a crucial role as an intramolecular chaperone, facilitating the folding of hTPO.
polymerase and buffer were obtained from Stratagene Europe, and dNTP mix came from Invitrogen. Pure plasmid DNA preparations were obtained with GenElute endotoxin-free plasmid midi prep kit from Sigma. Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (CMK) was obtained from Bachem (Weil am Rhein, Germany). hTPO cDNA was a gift from B. Rapoport (Cedars-Sinai Research Institute, Los Angeles, CA) (11). CHO RPE-40 cells were a gift from J. F. Suciu (University of Michigan, Flint, MI).

Cell Culture and Transfection Procedure—CHO cells and stably transfected hTPO-CHO cells were kept in Ham’s F12 medium supplemented with 10% foetal bovine serum, penicillin, and streptomycin in a humidified incubator (5% CO₂).

Transfection of hTPO cDNA was carried out by using the Lipofectamine reagent protocol (Invitrogen). CHO cells in 35-mm dishes were transfected with 1 μg of pcDNA3-hTPO construct for 3 h in serum-free culture medium. The transfection medium was replaced by complete medium, and cells were grown 48 h before use.

Metabolic Labeling and Extraction—For metabolic labeling, confluent cells incubated in culture cell medium supplemented with 10 mM sodium butyrate were incubated 24 h with 100 μCi/ml [³⁵S]Met/Cys. At the end of the labeling process, cells were washed four times with 2 ml of ice-cold PBS and subjected to either protein extraction or the bio-tinylation technique (see above).

For bio-tinylation experiments, cells were pre-incubated for 2 h in Met- and Cys-free Eagle’s minimum essential medium supplemented with 10% dialyzed fetal bovine serum and 10 mM sodium butyrate and then labeled for 30 min with 100 μCi/ml [³⁵S]Met/Cys. After the pulse step, cells were washed four times with 1 ml of PBS and chased by adding 1 ml of culture medium supplemented with 5 mM Met/Cys. At the chase times indicated, cells were washed twice with 2 ml of ice-cold PBS.

For N-terminal sequencing, cells were scraped into 600 μl of TCA extrac-tion buffer (50 mM Tris, pH 7.4, 0.15 μM NaCl, 1% Triton X-100, 0.3% DOC, protease inhibitor mixture, and 20 mM N-ethylmaleimide) and incubated on ice for 20 min. Samples were then centrifuged for 3 min at 10,000 × g, and the supernatants were kept for later use.

Immunoprecipitation and SDS-PAGE—The immunoprecipitation step was performed using two antibodies directed against TPO, mAb47, and mAb15, which were previously complexed with protein A-Sepharose by incubating them overnight at 4°C. As described above, mAb47 immunoprecipitated the unfolded hTPO forms, and mAb15 immunoprecipitated the partially and completely folded hTPO forms (5). The labeled supernatants obtained after the extraction process were incubated for 3 h with immune complexes. After being briefly centrifuged, the immune complexes were washed four times with TPO extraction buffer and then incubated with PBS. The immunoprecipitated complexes were then extracted from the immune complexes by boiling them for 5 min in 62.5 mM Tris, pH 6.8, containing 2% SDS, 1% β-mercaptoethanol, 5% glycerol, and 10% bromphenol blue. The samples were run on 7.5% acrylamide gels and examined by autoradiography with a phosphorimaging system (Fuji BAS 1000, Japan).

Cell Culture—hTPO-CHO cells metabolically labeled for 24 h with 100 μCi/ml [³⁵S]Met/Cys were incubated twice for 24 h, each time at 4°C in ice-cold PBS containing 0.5 mM captoethanol, respectively. Samples were then diluted with glycosidase and then centrifuged, the immune complexes were washed four times with TPO extraction buffer. The precipitated hTPO were denatured by boiling them for 5 min in 0.1% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol (β-ME) at 100°C, then transferred to ice-cold tubes, and the cell surface was washed once with 2% SDS and 1% N-ethylmaleimide. The cell surface hTPO was stored for further use, and the pellet (cell surface hTPO was separated from the intracellular pool by performing centrifugation at 4°C for 1 h, the supernatant was recovered and dialyzed against 10 mM Tris/HCl, pH 7.8, and 0.1 mM potassium iodide. Human TPO was then purified on a Mono S HR5/5 column (Pharmacia) and eluted with a linear gradient of potassium chloride (0-0.5 M KCl). The purified enzyme was then dialyzed against 10 mM Tris/HCl, pH 7.8, and 0.1 mM potassium iodide. The protein was concentrated and dialyzed against 10 mM Tris/HCl, pH 7.8, 0.1 mM potassium iodide, and 0.3% DOC. After 105,000 × g centrifugation at 4°C for 1 h, the supernatant was recovered and dialyzed against 10 mM Tris/HCl, pH 7.8, 0.1 mM potassium iodide. Human TPO was then purified on a Mono S HR5/5 column. After washing with 10 mM Tris/HCl, pH 7.8, 0.1 mM potassium iodide, 0.1% DOC, and 0.5 mM KCl, hTPO was eluted with 0.1 mM NaOH, 0.5% DOC, 1 mM KCl, and 0.1 mM potassium iodide. Each fraction was tested for peroxidase activity by guaiacol assay, and the most active fractions were pooled. This pool was extensively dialyzed against 0.1 mM sodium phosphate, pH 7.4, and 0.05% DOC buffer and stored at −80°C until use.

After deglycosylation with Endo H and reduction, 35 μM of native hTPO was resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. Western blotting was then performed using Problott polyclonal antibodies against human thyroid peroxidase in the presence of N-ethylmaleimide, which blocks endogenous peroxidase activity. Western blotting was performed using Problott polyclonal antibodies against human thyroid peroxidase in the presence of N-ethylmaleimide, which blocks endogenous peroxidase activity. Western blotting was then performed using Problott polyclonal antibodies against human thyroid peroxidase in the presence of N-ethylmaleimide, which blocks endogenous peroxidase activity. Western blotting was then performed using Problott polyclonal antibodies against human thyroid peroxidase in the presence of N-ethylmaleimide, which blocks endogenous peroxidase activity. Western blotting was then performed using Problott polyclonal antibodies against human thyroid peroxidase in the presence of N-ethylmaleimide, which blocks endogenous peroxidase activity.
0.5 ml PBS. Then 1 ml of ice-cold 20% (w/v) trichloroacetic acid, supplemented with 10^{-3} \text{M} potassium iodide, was added to each tube. After 20 min at 4°C, the suspension was centrifuged (2000 \times g for 6 min). The supernatant was discarded, and the acid insoluble irogenate obtained was washed three times with 2 ml of 10% trichloroacetic acid. The radioactivity remaining in the pellet was counted.

**RESULTS**

**Evidence for Endoproteolytic Cleavage of hTPO**—In a previous study using stably hTPO-expressing CHO cells metabolically labeled with \([35\text{S}]\text{Met/Cys}\), we reported that the \([35\text{S}]\text{-hTPO}\) gave a single band on SDS-PAGE and, after deglycosylation with PNGase F, yielded three distinct bands (7). We assumed that this was due to an incomplete action of glycanase. At the beginning of the present study, we reassessed this hypothesis by carrying out the following experiments.

After performing metabolic labeling for 24 h with \([35\text{S}]\text{Met/Cys}\), hTPO was immunoprecipitated using the pair of mAbs, mAb15 and mAb47. Under these conditions, as described previously (8), >90% of the hTPO was immunoprecipitated. The samples were then deglycosylated by Endo H or PNGase F (Fig. 1A). As reported previously by our group, \([35\text{S}]\text{-hTPO}\) run on SDS-PAGE gave a specific band of 110 kDa (Fig. 1A, lane 1). After Endo H digestion a supplementary 98-kDa band appeared (Fig. 1A, lane 2), and after PNGase F digestion the hTPO showed up on an SDS-polyacrylamide gel as 98-, 94-, and 92-kDa bands (Fig. 1A, lane 3). The 94-kDa band is actually composed of two distinct bands with very similar molecular masses.

To induce the expression of completely unglycosylated control hTPO samples, hTPO-CHO cells were treated with tunicamycin, an antibiotic that inhibits the formation of all \(N\)-linked oligosaccharides in proteins. Under these culture conditions, the 98-kDa hTPO band obtained (Fig. 1A, lane 4) was resistant to Endo H or PNGase F digestion (Fig. 1A, lanes 5 and 6) and therefore corresponded to the full-length unglycosylated hTPO. In comparison with this unglycosylated hTPO band, the shift in the 110- to 98-kDa hTPO band extracted from untreated control cells after PNGase F deglycosylation can therefore be attributed to the presence of \(N\)-linked glycans (Fig. 1A, lanes 3 and 4). By contrast, the migration of the two lower apparent molecular mass bands beyond the unglycosylated hTPO migration band suggests that the 94- and 92-kDa hTPO species cannot have been due to the glycosylation process but rather to the action of protease(s) on the polypeptide structure of the protein.

To determine whether these cleavages occur in the N- or the C-terminal part of the protein, a mutant form of hTPO lacking the cytoplasmic tail was used. The nucleotidic sequence encoding 61 amino acids from Trp^{873} to Leu^{933} was deleted from the hTPO cDNA. This mutant, WCT-hTPO, was expressed in CHO cells and, after immunoprecipitation, was subjected to a PNGase F deglycosylation step. Along lines similar to what occurs in the case of the electrophoretic migration of the full-length hTPO, after PNGase F digestion WCT-hTPO was found to contain the three different apparent molecular mass species (Fig. 1B, lanes 2 and 4). This result indicates that the endoproteolytic cleavages of hTPO occurred in the N-terminal part of the protein.

Because the hTPO cleaved species were resistant to Endo H and because this enzyme specifically cleaved the high-mannose type glycans, it is suggested that the endoproteolytic processing did not occur in the ER compartment but rather in the Golgi apparatus or at the cell surface. To confirm this assumption, after a metabolic labeling step the intracellular and cell surface \([35\text{S}]\text{-hTPOs}\) were separated using the cell surface biotinylation technique. Both intracellular and cell surface samples were then subjected to deglycosylation with Endo H or PNGase F.

**FIG.1. Evidence for the occurrence of an endoproteolytic cleavage of hTPO.** A, Endo H and PNGase F digestion of hTPO. After 24 h of \([35\text{S}]\text{Met/Cys}\) metabolic labeling of hTPO-CHO cells in the absence (lanes 1, 2, and 3) and presence (lanes 4, 5, and 6) of tunicamycin (Tu) (15 \(\mu\text{g/ml}\)), hTPO was extracted and immunoprecipitated with the pair of mAbs, mAb47 and mAb15 (lanes 1, 2, and 3) or with mAb47 alone (lanes 4, 5, and 6). Samples were then digested for 16 h at 37°C with 5 milliunits of Endo H (lanes 2 and 3) or 1 unit of PNGase F (lanes 3 and 6). Control assays were carried out under the same conditions, except that glycosidases were omitted (lanes 1 and 4). Samples were reduced and subjected to SDS-PAGE analysis. B, PNGase F digestion of hTPO lacking a cytoplasmic tail. CHO cells were transfected with the cDNA of hTPO lacking the cytoplasmic tail, WCT-hTPO. Forty-eight hours after transfection, full-length hTPO-transfected WT-hTPO CHO control cells (lanes 1 and 2) and WCT-hTPO-transfected CHO cells (lanes 3 and 4) were labeled for 24 h with \([35\text{S}]\text{Met/Cys}\). After extraction and hTPO immunoprecipitation with mAb47, samples were incubated or not with 1 unit of PNGase F and then reduced and subjected to SDS-PAGE analysis. C, Endo H and PNGase F digestion of intracellular and cell surface hTPO. After 24 h of \([35\text{S}]\text{Met/Cys}\) metabolic labeling of hTPO-CHO cells, a cell surface biotinylation step was performed with NHS-SS-biotin. After extraction and immunoprecipitation with mAb15, intracellular (lanes 1, 2, and 3) and cell surface hTPOs (lanes 4, 5, and 6) were separated with avidin-agarose beads. Samples were incubated for 16 h at 37°C with 5 milliunits of Endo H or 1 unit of PNGase F digestion and then reduced and subjected to SDS-PAGE analysis.

Intracellular and cell surface hTPO appeared on an SDS-polyacrylamide gel as a specific 110-kDa band (Fig. 1C, lanes 1 and 4). After Endo H and PNGase F digestion, the intracellular hTPO showed up on an SDS-polyacrylamide gel as a single 98-kDa band (Fig. 1C, lanes 2 and 3), whereas the cell surface hTPO yielded 98-, 94-, and 92-kDa bands (Fig. 1C, lane 6). The fact that the hTPO cleaved species bore complex-type \(N\)-glycans and were present only at the cell surface therefore suggests that the cleavage process occurred in a late compartment of the secretory pathway on mAb15 recognizing folded or partially folded hTPO forms. The subsequent hTPO immunopre-
Endoproteolytic Cleavage of hTPO

Effects of secretory pathway inhibitors on the hTPO endoproteolytic cleavage process. A, hTPO-CHO cells were metabolically labeled for 24 h with [35S]Met/Cys in the absence (A, lanes 1 and 2) or presence of 10 μg/ml BFA (A, lanes 3 and 4) or 10 μM monensin (Mon) (A, lanes 5 and 6). After incubation, hTPO-CHO cells were immediately subjected to protein extraction (A, lanes 1–6), or cell surface biotinylation was performed (B). hTPO was immunoprecipitated with mAb15. Samples were treated with PNGase F and then analyzed by SDS-PAGE.

To characterize the site of this processing more exactly, cell radiolabeling experiments were performed in the presence of brefeldin A (BFA) or monensin. These secretory pathway inhibitors induce the disassembly of the Golgi complex and block ER-Golgi transport and Golgi-cell surface vesicular transport of proteins, respectively. In the presence of BFA, which completely blocked the cell surface expression of hTPO (data not shown), only one of the lower bands (94 kDa) was retrieved after deglycosylation (Fig. 2A, lane 4). In the presence of monensin the cell surface expression of hTPO was not completely inhibited (Fig. 2B), but the deglycosylation of the hTPO remaining in the trans-Golgi network gave rise to three different apparent molecular mass populations (Fig. 2B). These results indicate that hTPO cleavages occurred after its transit through the Golgi apparatus but before it reached the cellular surface, and that one cleavage can occur when hTPO was retained in the Golgi apparatus. However, it is also worth noting that the hTPO cleavages were not affected by serine (aprotinin), as proreceptors or proproteases, we attempted to determine what role furin itself might possibly play in the processing of hTPO. Transfection of hTPO-pcDNA3 was then performed in the furin-deficient CHO cell strain RPE.40 (17) followed by the [35S]Met/Cys metabolic labeling experiments described above. The results showed that hTPO processing was not inhibited in this cell line (Fig. 4A, lanes 1 and 2) and was always sensitive to CMK treatment (Fig. 4A, lanes 5 and 6), based on comparisons with similar experiments using hTPO-transfected CHO cells (Fig. 4B, lanes 1–6). In addition, the co-expression of furin with hTPO in RPE.40 and in hTPO-CHO cells did not increase the rate of hTPO cleavage (Fig. 4A and B, lanes 3 and 4).

The effects of a proprotein convertase (PPC)-specific inhibitor, CMK, were also tested. Human TPO-CHO cells were metabolically labeled with [35S]Met/Cys for 24 h in the absence or presence of CMK. After extraction and immunoprecipitation, hTPO was digested with PNGase F before SDS-PAGE. Contrary to what occurred with the untreated hTPO-CHO cells in which the 94- and 92-kDa bands could be observed (Fig. 3, lane 2), CMK treatment prevented the formation of cleaved forms. In the presence of 50 or 100 μM CMK, only the 98-kDa band corresponding to the totally deglycosylated full-length hTPO was observed after digestion with PNGase F (Fig. 3, lanes 4 and 6). Moreover, under CMK treatment, a change in the pattern of mobility of glycosylated hTPO was observed. The large 110-kDa band obtained with control hTPO-CHO cells was observed with two distinct species (Fig. 3, lanes 3 and 5). The lower apparent molecular mass band (110 kDa) corresponded to the high mannose-type, N-glycan structure of the hTPO found in the intracellular hTPO pool under control culture conditions (Fig. 1C, lane 1), and the upper band (120 kDa) observed in CMK-treated hTPO-CHO cells can be attributed to the non-cleaved hTPO species containing complex-type N-glycan structures.

All these results show that the endoproteolytic cleavage of hTPO is specifically inhibited by CMK and strongly suggests that PPCs may be involved in the hTPO processing. This endoprotease family has been found to be implicated in the maturation of numerous precursors of biological active proteins and peptides such as hormones and neuropeptides (for review, see Ref. 16).

Because furin, a member of the PPC family, is known to be required in the processing of various surface glycoproteins such as proreceptors or proproteases, we attempted to determine what role furin itself might possibly play in the processing of hTPO. Transfection of hTPO-pcDNA3 was then performed in the furin-deficient CHO cell strain RPE.40 (17) followed by the [35S]Met/Cys metabolic labeling experiments described above. The results showed that hTPO processing was not inhibited in this cell line (Fig. 4A, lanes 1 and 2) and was always sensitive to CMK treatment (Fig. 4A, lanes 5 and 6), based on comparisons with similar experiments using hTPO-transfected CHO cells (Fig. 4B, lanes 1–6). In addition, the co-expression of furin with hTPO in RPE.40 and in hTPO-CHO cells did not increase the rate of hTPO cleavage (Fig. 4A and B, lanes 3 and 4).

These results suggest that PPCs other than furin are probably involved or may rescue the cleavage(s) of hTPO in these cell lines. However, it is also worth noting that the hTPO cleavage observed when hTPO-CHO cells were treated with BFA was insensitive to CMK digestion, which suggests that in this case an enzyme other than PPC may be involved.

Determination of the Amino Acid Sequences Involved in hTPO Cleavages in CHO Cells—Seven members of the subtilisin-like family of PCs, PC1/PC3, PC2, Furin/PACE, PC4, PACE4, PCS/Pc6, and PC7, have been found to cleave protein precursors at basic residues within the general motif (R/K)nX(R/K), where n = 0, 2, 4, or 6.

Analysis of the N-terminal part of hTPO showed the existence of three potential PPC cleavage sites composed of the KR...
paired basic amino acid residues at Arg^{48}, Arg^{65}, and Arg^{103} (Fig. 5A). Moreover, a method was recently improved for predicting PPC cleavage sites (18). Using the ProP1.0.b program (Fig. 5A), we scanned the N-terminal part of the hTPO. The results identified two of the three potential sites Arg^{48}. Moreover, a method was recently improved for predicting PPC cleavage sites (18). Using the ProP1.0.b program (Fig. 5A), we scanned the N-terminal part of the hTPO. The results identified two of the three potential sites Arg^{48}. Using the ProP1.0.b program dealing with this method, we scanned the N-terminal part of the hTPO. The results identified two of the three potential sites with relevant scores at the Arg^{48} and Arg^{65} positions.

To assess the involvement of these different potential cleavage sites in hTPO processing, site-directed mutagenesis experiments were performed to mutate any PPC recognition motifs. Because the basic residue was essential to recognition by PPCs, the Arg residues at position P1 in these potential sites (Lys-Arg^{65} and Lys-Arg^{103}) were replaced by Ala residues. CHO cells were then transfected with the hTPO-cDNA of each of which is composed of multiple other bands. The efficient deglycosylation of native hTPO with Endo H was found to sustain various species (Fig. 6, lane 2). Similar results were obtained with PNGase F (data not shown). The presence of various mRNA species encoding for different hTPO isoforms might explain the existence of different molecular mass species (3). However, the existence of various endoproteolytically processed forms of hTPO in CHO cells might also account for the great complexity of the electrophoretic bands observed after the deglycosylation of native hTPO.

To answer the above question, we carried out N-terminal sequencing on hTPO purified from human thyroid glands. After purification on a mAb15-Sepharose column, an aliquot of native hTPO was subjected to SDS-PAGE. Electrophoretic procedure was then performed onto ProBlott membrane. The two bands of interest were revealed by Amido Black staining (Fig. 6, lane 1, bands a and b), excised, and sequenced.

Surprisingly, the N-terminal sequencing data showed that the various native hTPO species all have the same N terminus sequence beginning at Thr^{109}. This result suggests that in the thyroid gland, as in CHO cells, the N-terminal part of the hTPO is subjected to proteolytic processing but that the potential propeptide sequence runs from the signal peptide cleavage site to the Thr^{109}. Based on the consensus PPC cleavage motif, we immediately identified a potential PPC cleavage site, KRKVNKL^{108} T^{109}, upstream of the N terminus of the native hTPO with a Lys residue in position P1 and an Arg residue at position P6, which suggests that a PPC may also be involved in hTPO processing in human thyroid gland.

If this cleavage also occurs in the CHO cell line, it might contribute to the formation of the 92-kDa band. However, the mutation of the Arg^{103} (Fig. 5B, lane 3) and Lys^{108} (data not shown) did not inhibit the cleavage in this cell line. Because it seems likely that the PPC involved in this cleavage process is not expressed in CHO cells, we performed experiments with two other cell lines, the erythroleukemic cell K562, in which the cleavage of the propeptide of another mammalian peroxi-
dase, myeloperoxidase, can occur (20), and the rat thyroid cell PCC3, which retains most of the typical markers of thyroid differentiation (21). The results obtained on these two cell lines were similar to those obtained with the CHO cells (data not shown).

Role of the Prosequence on the Folding of hTPO—Many pro-
teins are produced as precursors, and the peptidic sequence at the N terminus end of these proteins, which are called propeptides, has been found to be essential to the folding, stability, intracellular trafficking, and biological activation of several proteins such as proteases, hormones, and plasma glycoproteins (22, 23). Some of these propeptides act as intramolecular chaperones facilitating protein folding, and others are involved in the cell targeting and sorting processes or in the inhibition of the protein activity.

To test the role of the N-terminal hTPO propeptide in the folding of hTPO, we first generated an hTPO-cDNA construct called Δpro-hTPO and deleted the hTPO propeptide sequence, amino acids Cys^{15}–Lys^{108} (Fig. 7A). The full-length and Δpro-hTPO were synthesized by performing pulse-chase experiments after transfecting CHO cells. The hTPO folding process was monitored with the two antibodies mAb47, which recognized unfolded hTPO forms (Fig. 7, B and D), and mAb15, which immunoprecipitated the partially and completely folded hTPO forms (Fig. 7, E and F).

The results showed that the proportions of the unfolded and folded forms differed between Δpro-hTPO and the entire hTPO. During the chase step, the newly synthesized unfolded hTPO forms were retrieved in large proportions in the propeptide-deleted hTPO mutant (Fig. 7D), whereas the proportions of the three-dimensional hTPO structure recognized by mAb15 were
Determination of amino acid sequences involved in hTPO cleavages in CHO cells. A, scheme of site-directed mutagenesis of potential PPCs cleavage sites detected in the N-terminal part of hTPO. According to the consensus PPC cleavage site and the role of the P1-P2 dibasic motif present in the pattern of PPC substrate recognition, three potential PPC cleavage sites were observed at the Arg⁴⁸ (R⁴⁸), Arg⁶⁵ (R⁶⁵), and Arg¹⁰³ (R¹⁰³) positions. Mutagenesis of these sequences was performed using site-directed mutagenesis procedures in order to include point mutations and transform the Arg residue into an Ala residue (A⁴⁸, A⁶⁵, and A¹⁰³). The respective PPC cleavage site mutants are indicated under each sequence. The numbering of the amino acids includes the signal peptide sequence. (SP, signal propeptide; ED, extracellular domain; TM, transmembrane domain; CT, cytoplasmic tail). B, effects of potential PPC cleavage sites mutation on hTPO cleavage processes. CHO cells were transiently transfected with R48A– (lane 1), R65A– (lane 2), or R103A—pcDNA3 (lane 3). After 48 h of cell culture, hTPO mutant-transfected CHO cells and CHO cells expressing the wild type hTPO (lanes 4 and 5) were metabolically labeled for 24 h with [³⁵S]Met/Cys. After protein extraction and hTPO immunoprecipitation with mAb15, samples were digested or not with PNGase F and then analyzed by SDS-PAGE. C, effects of the mutation of Arg⁶⁵ on the cleavage observed in BFA-treated hTPO-CHO cells. R65A cdNA-transfected CHO cells and hTPO-CHO cells expressing the wild type protein were metabolically labeled for 24 h with [³⁵S]Met/Cys in the presence of 10 μg/ml BFA. hTPO was immunoprecipitated with mAb15 and digested with PNGase F. Samples were then reduced and subjected to SDS-PAGE analysis.

Fig. 5. N-terminal sequencing of hTPO extracted from human thyroid gland. hTPO extracted from human thyroid gland using a mAb15-Sepharose column was subjected to SDS-PAGE under reducing conditions before (lane 1) and after Endo H digestion (lane 2). The two major distinct bands (a and b) obtained without the Endo H deglycosylation step are indicated. Under the same conditions Western blotting was performed followed by Amido Black staining and, after their excision, these two bands were sequenced by the Protein Microsequencing Laboratory at the Pasteur Institute. The N-sequencing data showed that the N-terminal ends of these two different molecular mass hTPO species (a and b) have the same amino acid sequence.

Fig. 6. N-terminal sequencing of hTPO extracted from human thyroid gland. hTPO extracted from human thyroid gland using a mAb15-Sepharose column was subjected to SDS-PAGE under reducing conditions before (lane 1) and after Endo H digestion (lane 2). The two major distinct bands (a and b) obtained without the Endo H deglycosylation step are indicated. Under the same conditions Western blotting was performed followed by Amido Black staining and, after their excision, these two bands were sequenced by the Protein Microsequencing Laboratory at the Pasteur Institute. The N-sequencing data showed that the N-terminal ends of these two different molecular mass hTPO species (a and b) have the same amino acid sequence.

Assuming that the prosequence of hTPO plays this chaperone-like role, we investigated whether or not it can contribute to a folding assistance in trans, i.e., when the hTPO propeptide (pro-hTPO) and the pro-hTPO were co-expressed as independent units. Synthesis of pro-hTPO₁⁻¹⁰₈ in CHO cells was checked by introducing an influenza HA tag on the C terminus side of the prosequence sequence (Fig. 7A). Western blotting of the HA-tagged prosequence extracted from transfected CHO cells was performed using an anti-HA antibody. As we expected, an ≈11 kDa peptide was obtained that is consistent with the molecular mass of pro-hTPO₁⁻¹⁰₈-HA (data not shown).

CHO cells were then co-transfected with the two plasmids encoding Δpro-hTPO and pro-hTPO, and pulse-chase experiments similar to those described previously were carried out. The results showed that when the hTPO propeptide was co-expressed in trans with the propeptide-deleted hTPO mutant, the correct folding of hTPO protein was not restored (Fig. 7, B and E). These results demonstrate that in the human thyroid gland the hTPO prosequence must be attached to the protein to enable the correct folding of the thyroperoxidase to occur.

Effects of the Prosequence on the Cell Surface Expression and the Activity of hTPO—The fact that the hTPO propeptide deletion drastically decreased the proportion of the folded forms of hTPO suggested that a loss of cell surface expression and thyroperoxidase activity occurs in Δpro-hTPO transfected cells. To confirm this hypothesis, after performing metabolic labeling on Δpro-hTPO transfected cells, the intracellular and cell surface Δpro-hTPOs were separated using the cell surface biotinylation technique. As we expected, in comparison with the control hTPO-CHO cells, the cell surface expression of hTPO was completely blocked in the Δpro-hTPO transfected cells, whereas inhibition of the cleavage by CMK did not affect the rate of cell surface expression (Fig. 8A).

and identical results were obtained (data not shown).

The fact that no significant changes in the rate of hTPO molecule degradation were observed (Fig. 7C) suggests that the folding process rather than the degradation of unfolded forms was impaired. Both of these results suggest that the hTPO propeptide sequence might act as an intramolecular, chaperone-like protein.

It is worth noting that the signal peptide cleavage site is not known in the case of hTPO. Predictions made using diverse algorithms suggest that the cleavage may occur between residues 14 and 15, as well as between residues 18 and 19 or 26 and 27. To rule out the possibility that deleting part of the signal peptide may have prevented the Δpro-hTPO to fold correctly, we performed the same experiment after deleting the hTPO propeptide sequence consisting of amino acids Lys⁷⁷–Lys¹⁰₈,

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We then investigated the question as to whether inhibiting the endoproteolytic cleavage of hTPO might affect the enzymatic activity at the cell surface. Surprisingly, after 48 h of incubation in the presence of CMK, the activity of hTPO increased by 33% in comparison with that of the control cells (Fig. 8B). This finding suggests that in the human thyroid gland the efficient cleavage of hTPO may lead to a slight decrease in the activity of this enzyme at the cell surface. Both of the above results suggest that the presence of the propeptide is necessary for the correct folding and the intracellular trafficking of hTPO to occur but that the enzymatic activity does not depend on the occurrence of this cleavage.

**DISCUSSION**

After its synthesis, hTPO undergoes various posttranslational modifications such as N- and O-glycosylation and heme fixation. The folding of this enzyme seems to be a prerequisite for its maturation and for its ER sorting and cell surface expression (7, 8). In the present study, we obtained evidence that an endopeptidase cleaves the N-terminal part of the hTPO and that the proper folding of the protein depends on the presence of the propeptide.

In the first set of experiments we established that hTPO expressed in CHO cells is subjected to three different types of cleavage in a post-ER compartment. These cleavage processes are inhibited by CMK, a PPC inhibitor, but the fact that they still occurred in a furin-deficient CHO cell line indicates that PPCs other than furin cleave the prosequence.

It has by now been clearly established that many active forms of proteins are generated after a proteolytic processing step. PPCs are a family of subtilases involved in this cleavage process. Seven PPCs have been identified to date, namely PC1/3, PC2, furin, PC4, PACE4, PC5, and PC7. These enzymes cleave at basic residues (usually Arg) within the general sequence (K/R)Xn(K/R), where n = 2, 4, or 6 and X is any amino acid.
Acid except Cys and rarely Pro. However, in a number of cases precursors are cleaved at non-basic residues, and the proteinases involved in these proteolytic processes have not yet been identified (16).

In the present study, only one of the cleavage sites of hTPO in CHO cells was identified; it was found to be located at the Arg65 level, and the mutations of the other Arg residues located in positions 48 and 103, which might have been involved in cleavage by PPCs, were found to have no effects. It is also worth noting that, in cells treated with BFA, the hTPO is cleaved between the Arg65 and the Glu68 and that this cleavage is not inhibited by CMK. This finding indicates that in these cells another endoprotease was able to cleave the hTPO present in the ER when the transport between this compartment and the Golgi apparatus was blocked. On the other hand, the inhibition of the cleavages exerted by CMK did not prevent the intracellular trafficking of hTPO, and this inhibition led to an increase in the activity of this enzyme at the cell surface level, which indicates that the removal of the prosequence is not necessary to obtain an active enzyme.

Surprisingly, the N-terminal sequence of the hTPO molecules purified from the human thyroid gland using mAb15-Sepharose began at Thr109. Because this hTPO was sensitive to Endo H and also because it is known that hTPO is located mainly in the ER in thyroid cells (6), this cleavage presumably occurs in this compartment, and the propeptide will then probably be quickly removed after the folding of the protein.

Cleavage of the propeptide(s) therefore occurs in both CHO cells and thyroid cells, but the cleavage processes are different. This was confirmed by the fact that the mutation of Arg103 and Lys108, amino acid residues potentially involved in a cleavage mechanism between Lys108 and Thr109 in human thyroid, has no effect on the removal of the prosequence in CHO cells. It is therefore possible that, in CHO cells, PPCs other than the furin may be present in the Golgi apparatus where they participate in the cleavage of the prosequence, whereas in thyroid cells some other endoproteinases may cleave the prosequence at the ER level.

Five years ago, the first member of a new subfamily of kexin-like PPCs was characterized and found to be involved in the processing of various protein precursors at non-basic residues (24). This protease, called SKI-1, was found to process substrates along the secretory pathway, preferentially in the ER compartment, unlike the other PPCs that cleave protein precursors after their transit to the trans-Golgi network. The proposed consensus sequence for SKI-1 is (R/K)X-(hydrophobic)-Z1, where Z is any amino acid, preferably Leu, Thr, Lys, or Phe but excluding Val, Pro, Glu, Asp, or Cys (25–27). Because the sequence KRKVLK108 has an aliphatic residue in the P2 position, but not Arg or Lys in position P4, the cleavage in the human thyroid cell cannot be attributable to SKI-1 but must be due to an unknown endoprotease. By contrast, the sequence immediately following Arg65, which was previously identified as a potential furin-like cleavage site, also contains a potential SKI-1 cleavage site, R65GIL68, including the aliphatic residue Ile in P2 and an Arg in P4. Furthermore, SKI-1 is known to be expressed in CHO cells (28). Under these conditions, it seems likely that SKI-1 may be involved in the cleavage process observed in CHO cells exposed to BFA. This would explain why the ER retention-mediated cleavage of hTPO observed in hTPO-CHO cells treated with BFA was insensitive to CMK treatment but completely abolished when the Arg65 was replaced by an Ala residue (Fig. 5C).

As far as the role of this prosequence is concerned, many proteins are initially synthesized in the form of inactive precursors and need to be cleaved by PPCs in the secretory path-
Endoproteolytic Cleavage of hTPO

The conservation of amino acids in all the sequences and that the pair Lys102-Arg103 (Fig. 9), and the results show that the pair Lys64-Arg65 is

way before showing any biological activity; in other cases, the prosequence acts as an intramolecular chaperone, and the proper folding of the protein depends on the presence of this chaperone (for a review, see Refs. 22 and 23). In the present study, we observed that the presence of the prosequence is necessary for the correct folding of this glycoprotein to occur and that the cleavage of this prosequence is not a prerequisite for its transport to the cell surface or for its enzymatic activity; on the contrary, a slight decrease in the enzymatic activity was observed in CHO cells when hTPO was cleaved.

Another mammalian peroxidase, myeloperoxidase, undergoes a proteolytic trimming process resulting in the elimination of a propeptide consisting of 164 amino acids (29). Nothing is known so far about the exact nature of the enzyme involved in this cleavage process, but contrary to what occurs with hTPO, it seems to take place in the Golgi apparatus. On the other hand, no sequence homology was observed between the cleavage sites of hTPO and myeloperoxidase. As in the case of hTPO, the propeptide facilitates the folding of the protein, but it also facilitates both the export from the ER and the targeting for the storage granules (for a review, see Ref. 30). It is also worth noting that in CHO, BHK, and Sf9 cells (31–33) transfected with myeloperoxidase cDNA, no processing of myeloperoxidase occurs (31–33), whereas in human erythroleukemia K562 cells, still remain to be elucidated. What enzyme is involved in the cleavage of hTPO in the human thyroid gland, and does this process differ from one species to another?

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REFERENCES

1. Kimura, S., Kotani, T., McBride, O. W., Umeki, K., Hirai, K., Nakayama, T., and Ohtaki, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5555–5559
2. Elisei, R., Vassart, G., and Ludgate, M. (1991) J. Clin. Endocrinol. Metab. 72, 700–702
3. Ferrand, M., Le Fourn, V., and Franc, J. L. (2003) J. Biol. Chem. 278, 3793–3800
4. McLachlan, S. M., and Rapoport, B. (1992) Endocr. Rev. 13, 192–206
5. Taurog, A. (1999) Biochimie (Paris) 81, 557–562
6. Eckholm, R. (1981) Biochim. Biophys. Acta 660, 62–70
7. Faye, D., Niccoli, S., Piret, L., and Franc, J. L. (1998) Endocrinology 139, 4277–4285
8. Faye, D., Lisfranc-Fernandez, S., Janet, L., and Franc, J. L. (2000) J. Biol. Chem. 275, 15944–15954
9. Faye, D., Lisfranc-Fernandez, S., Janet, L., and Franc, J. L. (2000) Endocrinology 141, 859–866
10. Faye, D., Niccoli, S., Piret, L., and Franc, J. L. (1999) J. Biol. Chem. 274, 10533–10538
11. Magnusson, R. P., Chazenbalk, G. D., Gestautas, J., Seto, P., Filetti, S., and DeCroy, J. L. (1997) Mol. Endocrinol. 11, 856–861
12. Ru, J., Toubert, M. E., Czarnocka, B., Durand-Gorde, J. M., Ferrand, M., and Lissitzky, S. (1985) FEBS Lett. 190, 147–151
13. Makarova, O., Kamberev, E., and Margolis, B. (2000) BioTechniques 29, 970–972
14. Wang, J., and Wilkinson, M. F. (2000) BioTechniques 29, 976–978
15. Czarnocka, B., Ru, J., Ferrand, M., Carayon, P., and Franc, J. L. (1985) J. Clin. Endocrinol. Metab. 55, 1417–1423
16. Seidah, N. G., and Chretien, M. (1999) Endocrinology 140, 1211–1218
17. Makarova, O., Kamberev, E., and Margolis, B. (2000) BioTechniques 29, 970–972
18. Wang, J., and Wilkinson, M. F. (2000) BioTechniques 29, 976–978
19. Czarnocka, B., Ru, J., Ferrand, M., Carayon, P., and Lissitzky, S. (1985) J. Biol. Chem. 260, 147–151
20. Seidah, N. G., and Chretien, M. (1999) Endocrinology 140, 1211–1218
21. Fusco, A., Berlingieri, M. T., Di Fiore, P. P., Portella, G., Grieco, M., and Vecchio, G. (1978) Mol. Cell. Biol. 2, 1365–1377
22. Takagi, H., and Takahashi, M. (2005) Appl. Microbiol. Biotechnol. 63, 1–9
23. Shinde, U. G., and Inouye, M. (2000) Semin. Cell Dev. Biol. 11, 35–44
24. Seidah, N. G., Mowla, S. J., Hamelin, J., Mamarbachi, A. M., Benjannet, S., Toure, B. B., Basak, A., Munzer, J. S., Marcinkiewicz, J., Zhong, M., Baralle, J. C., Lazure, C., Murphy, R. A., Chretien, M., and Marcinkiewicz, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1321–1326
Endoproteolytic Cleavage of hTPO

25. Elagaz, A., Benjannet, S., Mammarbassi, A., Wickham, L., and Seidah, N. G. (2002) J. Biol. Chem. 277, 11265–11275
26. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000) Mol. Cell 6, 1355–1364
27. Lenz, O., ter Meulen, J., Feldmann, H., Klenk, H. D., and Garten, W. (2000) J. Virol. 74, 11418–11421
28. Mouchantaf, R., Watt, H. L., Sulea, T., Seidah, N. G., Alturaihi, H., Patel, Y. C., and Kumar, U. (2004) Regul. Pept. 120, 133–140
29. Hashinaka, K., Nishio, C., Hur, S. J., Sakiyama, F., Tsunawara, S., and Yamada, M. (1988) Biochemistry 27, 5906–5914
30. Gullberg, U., Bengtsson, N., Bulow, E., Garwicz, D., Lindmark, A., and Olsson, I. (1999) J. Immunol. Methods 232, 201–210
31. Moguilevsky, N., Garcia-Quintana, L., Jacquet, A., Tournay, C., Fabry, L., Pierard, L., and Bollen, A. (1991) Eur. J. Biochem. 197, 605–614
32. Cully, J., Harrach, B., Hauser, H., Harth, N., Robenek, H., Nagata, S., and Hasilik, A. (1989) Exp. Cell Res. 180, 440–450
33. Taylor, K. L., Uhlinger, D. J., and Kinkade, J. M., Jr. (1992) Biochem. Biophys. Res. Commun. 187, 1572–1578
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Valérie Le Fourn, Mireille Ferrand and Jean-Louis Franc

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