A Plasminogen-Like Protease in Thyroid Rough Microsomes Degrades Thyroperoxidase and Thyroglobulin

Annie Giraud, Pierre-Jean Lejeune, Jocelyne Barbaria, and Bernard Mallet

Institut National de la Santé et de la Recherche Médicale Unité Mixte de Recherche 476 (A.G.) and Institut National de la Santé et de la Recherche Médicale Unité 555 (P.-J.L., J.B., B.M.), Faculté de Médecine, 13385 Marseille, France

Proteasome activity takes place in the cytosolic compartment and acts to degrade several proteins translated and unfolded. In transfected CHO cells expressing thyroid peroxidase (TPO), just-translated TPO undergoes proteasome activity, and then a second proteolytic system degrades more mature forms of TPO. A plasminogen-like (Pl-like) protease is found in microsomal liver membranes and in the thyroid. In the thyroid, this Pl-like protease is localized in the follicular lumen and efficiently degrades thyroglobulin (Tg) in vitro. Here we checked for the presence, in purified endoplasmic reticulum (ER) membranes of transfected CHO and in rough microsomes purified from thyroid tissue, of a second proteolytic system, different from the proteasome, and active against the two major proteins of the thyroid gland, TPO and Tg. We first confirmed that this proteolytic system was able to degrade folded endogenous TPO. We showed also that externally added TPO (folded form) was degraded by opened vesicles of ER in the same system. For thyroid tissue, we showed that added TPO, as well as purified Tg, was degraded by some unknown membrane-associated protease(s) in human and porcine thyroid rough microsomes, whereas BSA and IgG were not. These results indicated that major thyroid glycoproteins are preferential substrates of such protease(s). Immunoblot and zymography experiments identified the unknown membrane-associated protease in rough microsomes from thyroid tissues as being a Pl-like protease. These results highly suggest that this system acts as a nonproteasomal degradation enzyme at the ER level, and we hypothesize that it contributes in regulating the level of major thyroid glycoproteins. (Endocrinology 148: 2886–2893, 2007)
In the present study, we first confirmed by a direct experiment on CHO expressing human TPO that a proteolytic system in the ER was able to degrade the intrinsic TPO recognized by mAb15 but no obvious degradation of the TPO recognized by mAb47. Furthermore, we showed that external TPO (purified by affinity chromatography on mAb15-Sepharose) was degraded by opened vesicles of ER in the same system. We then turned to thyroid tissue and first showed that purified TPO and Tg were degraded by a proteolytic system in human and porcine thyroid rough microsomes. Parallel experiments performed with BSA and IgG showed no degradation of these molecules. These results indicated that major thyroid glycoproteins were preferential substrates. In addition, the immunological and enzymatic properties of this thyroid proteolytic system associated with rough microsomes proved to be those of a PI-like protease.

Materials and Methods

Materials

CHO cells expressing TPO (referred to as CHO-TPO cells) were described previously (7). Na125I was from Amersham Pharmacia Biotech (Piscataway, NJ). Expre35S35S was from NEN Life Science Products Life Science Products (Boston, MA). Fetal bovine serum (FBS) and Complete (protease inhibitor cocktail tablets) were from Roche Molecular Biochemicals (Meylan, France). Penicillin and streptomycin were from Life Technologies (Grand Island, NY). Anti-TPO mAb, anti-Tg mAb, and purified TPO were kindly given by Dr. Ruf. Antihuman Pl rabbit serum was from Dako, (Carpinteria, CA). Lactoperoxidase, casein, human Pl, and antimouse and antirabbit IgG peroxidase conjugate were from Sigma (Saint Quentin France). Protein A-Sepharose-4B was from Zymed Laboratories Inc. (San Francisco, CA). Lactoperoxidase, casein, human Pl, and antimouse and antirabbit IgG peroxidase conjugate were from Sigma (Saint Quentin Fallavier, France).

CHO-TPO cell culture and metabolic labeling

The cells were cultured in Ham’s F12 medium supplemented with 10% FBS, penicillin (100 UI/ml), and streptomycin (100 μg/ml) and 10% (Petri dish of 10 cm diameter). After reaching confluence, proteins TPO were kindly given by Dr. Ruf. Antihuman Pl rabbit serum was from Dako, (Carpinteria, CA). Lactoperoxidase, casein, human Pl, and antimouse and antirabbit IgG peroxidase conjugate were from Sigma (Saint Quentin Fallavier, France).

Preparation of ER membranes from CHO-TPO cells

The membranes were prepared from unlabeled cells or from cells labeled with [35S]Met+Cys. All the subsequent steps were performed at 4 C. After being washed with PBS, the cells were scraped in PBS (1 ml/dish) and centrifuged (200 × g for 5 min). ER membranes were prepared according to Urbani and Simonì (11). In brief, the pellet containing the cells from four dishes was washed twice with 2 ml PBS and then suspended in 2 ml 10 mM Tris-HCl (pH 7.5) (hypothenic medium) and incubated for 20 min on ice. After another centrifugation (1900 × g for 5 min), the supernatant (containing the cytoplasmic soluble materials) was discarded, the membrane pellet was resuspended in 1 ml 10 mM Tris-HCl (pH 7.5), transferred into a Dounce homogenizer, and incubated on ice for 5 min. After one stroke of tight-fitting pestle, 1 ml 0.5 m sucrose solution in 10 mM Tris-HCl (pH 7.5) was added, and more strokes were given. The homogenate was made 55% in sucrose by addition of 6.5 ml 63.5% sucrose (wt/wt) and was transferred to an ultracentrifuge tube. Three layers, 0.5 ml each, of 28, 31, and 25% sucrose in 1 ml Tris-EDTA were added. The membranes were then separated by ultracentrifugation (3 h in a Beckman Swinging 27 rotor, at 24,000 rpm). The membranes at the interface between 55 and 38% sucrose were collected manually and washed by three volumes of 1 mM Tris EDTA (pH 8.0). They were mainly ER membranes with possibly minor contamination by plasma membranes (11). After centrifugation in a 60 Ti rotor (100,000 × g for 1 h), the supernatant was discarded. The pellet was resuspended in 400 μl 1 mM Tris-EDTA (pH 8.0), and the ER fractions were stored at ~70 C.

Degradation of endogenous TPO molecules

The method was adapted from the cell extraction and immunoprecipitation procedures described by Fayadat et al. (7). [35S]Met+Cys-labeled ER membranes from CHO-TPO cells were thawed on ice and suspended on 1 ml Tris-EDTA (pH 8.0) (100 μl/ER membranes from one standard dish, i.e., about 70 μg protein). They were then incubated in four tubes each containing 50 μl suspended ER membranes. Two of these samples were immediately extracted for 30 min on ice in extraction buffer (EB) [50 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Triton X-100, 0.3% sodium deoxycholate (DOC), and protease inhibitor mixture], with occasional gentle manual agitation. After 3 min of centrifugation at 10,000 × g, the supernatant was stored at −20 C until the next day. The two other samples were incubated for 24 h at 37 C. They were then treated like the two nonincubated samples. The four supernatants were immunoprecipitated for 2 h at room temperature with anti-TPO mAb15 or mAb47 previously complexed with protein A-Sepharose (overnight incubation at 4 C of mAb with protein A-Sepharose in EB followed by four washes in EB). Immune complexes were then retrieved by brief centrifugation of the pellets with DOC (10 μl × g for 10 sec) and washed four times with 1 ml EB and once with 1 ml PBS. The precipitated proteins were separated from the antibody-Sepharose complex by a 5-min treatment in boiling water with the Laemmli sample buffer supplemented with 2-mercaptoethanol. The samples were then analyzed by SDS-PAGE using 7.5% acrylamide gels. The radioactivity was visualized and quantified by a phosphorimager (Fuji BAS 1000; Fuji, Tokyo, Japan).

Preparation of human and porcine thyroid microsomes

Fragments of normal human thyroid tissue adjacent to benign nodules were obtained after extemporaneous examination of thyroid samples during surgical removal. Porcine thyroids were obtained from the local slaughterhouse. Normal human and porcine rough microsomes were prepared as described in (12, 13). All procedures were done at 4 C. After being sliced, the tissue was homogenized by fractions of 6 g in 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec. The homogenates were centrifuged for 10 min at 10,000 × g. The filtered postmitochondrial supernatants were layered over 1.35 ml 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec. The homogenates were centrifuged for 10 min at 10,000 × g. The filtered postmitochondrial supernatants were layered over 1.35 ml 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec. The homogenates were centrifuged for 10 min at 10,000 × g. The filtered postmitochondrial supernatants were layered over 1.35 ml 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec. The homogenates were centrifuged for 10 min at 10,000 × g. The filtered postmitochondrial supernatants were layered over 1.35 ml 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec. The homogenates were centrifuged for 10 min at 10,000 × g. The filtered postmitochondrial supernatants were layered over 1.35 ml 18 ml 1% dextran 500/0.5 mM sucrose, using an Ultra-Turax mixer at half-maximal speed, twice for 12 sec.

Sequential extraction of microsomal membrane proteins

Depending on the experiments, ER membranes or RMH were solubilized by addition of detergents: Triton X-100 (final concentration 1%), Nonidet P-40 (NP-40) (final concentration 1%), or SDS (final concentration 0.1%). In addition, in some experiments, ER membranes from CHO-TPO cells or porcine RMH were sonicated (three times for 10 sec). After the first experiments, membrane proteins of ER from CHO-TPO cells, of RMH from porcine thyroids, and of RMH from normal human thyroids were sequentially extracted with NP-40 and with SDS (about 2 mg protein/ml of detergent solution). Soluble fractions and pellets were obtained by ultracentrifugation (100,000 × g for 1 h) of NP-40-treated membranes (4 mg/2 ml). The proteolytic activity was distributed be-
between supernatants and pellets, but it was mainly in the pellets. The pellets were further extracted with 0.1% SDS, and soluble and insoluble materials were obtained by a second ultracentrifugation (100,000 × g for 1 h). The proteolytic activity of each extract was assayed by studying the degradation of exogenous molecules (see below).

**125I** protein labeling

The molecules to be labeled were iodinated with 125I by a peroxidase-catalyzed reaction. In the case of TPO labeling, the TPO catalyzed its own iodination. When other molecules had to be iodinated, lactoperoxidase was added to the reaction mixture. In brief, 2 μl (0.2 μg) of NaO125I was added to 10 μg (in 5 μl) of the protein to be labeled. When the protein was not TPO, 0.1 μg in 1.1 μl of lactoperoxidase was added to the mixture. The iodination reaction was started by addition of 20 μl 0.3 mM H2O2, followed by sequential addition at 1-min intervals and at room temperature of 20 μl 1.0 mM H2O2, 20 μl 3 mM H2O2, and 20 μl 9.0 mM H2O2. The reaction mixture was increased to 1 ml by adding PBS containing 0.5% Triton X-100. Excess of reagents was removed by passage over a PD 10 (Sephadex G-25) column. Two fractions of 1 ml, containing the 125I-labeled proteins, were pooled and stored at −20 C. This pooled solution was referred to as 125I-labeled proteins solution. About 70–90% of the radioactivity engaged was found associated with the labeled protein (TPO, IgG, or BSA), with the exception of Tg, which fixed only about 50% of the radioactivity added.

**Degradation of exogenous [125I]TPO by RMH**

Depending on the experiments, 125I-labeled proteins (about 2 μl of a 1/100 dilution of the 125I-labeled protein solution) or unlabeled proteins, used as putative substrates of the membrane protease(s), were added to ER membrane homogenates from CHO-TPO cells or to RMH from porcine or human thyroid (from 1–100 μg of membranes, although generally, 50 μg was used; see Results). The protease activity was also checked on fractions obtained by sequential extraction of RMH with detergents. The presence of protease activity was checked by following the disappearance of these added proteins at 37 C under various conditions of incubation time, pH, membrane concentration, and solubilization. After incubation, the samples were placed in the Laemmli buffer under reducing conditions and were analyzed by SDS-PAGE (15). To study the disappearance of 125I-labeled proteins, the gels were fixed, dried, and exposed to an imaging plate radioactive energy sensor (BAS-IP.MP 2040S; Fuji). The exposed plate was analyzed using a phosphorimager and quantified with the Tina 2.09 image software program. The percentage of degradation was evaluated as the radioactivity (arbitrary units) associated with the control TPO minus the radioactivity remaining associated with TPO after the degradation assay, divided by radioactivity associated with the control TPO. ([labeled TPO before degradation] − [labeled TPO remaining after degradation])/([labeled TPO before degradation]).

The disappearance of unlabeled proteins was followed by immunoblot analysis or sometimes by Coomassie blue staining after SDS-PAGE. In these latter cases, the results were not quantified.

**Immunoblot analysis**

After SDS-PAGE separation, the proteins were electrophoretically transferred from the acrylamide gels onto polyvinylidene difluoride membranes. The membranes were blocked for 1 h at room temperature in 3% nonfat dried milk in Tris-buffered saline (TBS) (20 mM Tris, 0.5 mM NaCl, pH 7.4) supplemented with 0.1% Tween 20 and incubated overnight at 4 C with anti-TPO mAb47 (generally a 1/2000 dilution of the ascites), with a mixture of several mAb raised against human Tg (same dilution), or with a rabbit polyclonal antibody raised against human PI, depending on the protein used as protease substrate. The membranes were washed four times in 0.3% fat-dried milk in TBS supplemented with 0.1% Tween 20 and incubated for 2 h at room temperature with antimouse or antirabbit IgG peroxidase conjugate (Sigma) (1/10,000 to 1/20,000 dilution). After four washes in 0.3% fat-dried milk in TBS/0.1% Tween 20 followed by one wash in TBS/0.1% Tween 20 without fat-dried milk, bound antibodies were detected with an ECL detection reagent (Super Signal West Fento Maximum Sensitivity Substrate from Pierce, Rockford, IL).

**Zymography**

Zymography was performed as described previously (4, 16) using 0.2% protein copolymerized in a 10% SDS-polyacrylamide gel. Electrophoresis was done at 4 C under nonreducing conditions. Depending on the experiments, the protein used was casein, human Tg, or BSA. After electrophoresis, the gels were washed for 1 h at room temperature in 50 mM Tris/HCl (pH 8.0) containing 2.5% Triton X-100 and then incubated overnight in 50 mM Tris/HCl (pH 8.0). After incubation, the gels were washed for 5 min with H2O and fixed for 30 min in 50% ethanol/7.5% acetic acid in water. They were stained with Coomassie blue and recorded with a Kodak Image Station 440.

**Protein determination**

Proteins were quantified by the micro-BCA method, using the kit commercialized by Pierce. In particular cases, the protein analysis was done by AccQ-Taq system (Waters Associated, Milford, MA).

**Results**

An ER-associated degradation system specifically degrades endogenous folded TPO

It is known that mAb15 recognizes a conformational epitope localized in one of the two major antigenic domains of the TPO molecule only after TPO has acquired a folded form (10). After fractionation of 35S-labeled CHO-TPO cells, ER purification, and incubation of purified ER membranes at 37 C, we observed that the folded TPO forms recognized by mAb15 were degraded in vitro (Fig. 1A, lane 2). In contrast, the TPO forms recognized by mAb47, mainly unfolded forms of TPO, normally degraded by the proteasome (7), were not degraded after 24 h of incubation (Fig. 1A, lane 4), which indicated that the purified ER displayed no proteasome activity in our experimental conditions. These results confirmed that ER membranes contained an active proteolytic system, different from the proteasome.

![Image](https://example.com/image.png)

**Fig. 1.** Proteolytic activity of ER membranes from CHO-TPO cells. A, Endogenous proteolytic activity. CHO-TPO cells were labeled for 2 h with [35S]Met Cys, and the ER membranes of these cells were purified. Immunoprecipitation with anti-TPO mAb was performed on membranes treated with 1% Triton X-100 and 0.3% DOC and incubated or not at 37 C. Lanes 1 and 3, No incubation; lanes 2 and 4, incubation for 24 h. Immunoprecipitation was performed either with mAb15 (lanes 1 and 2) or with mAb47 (lanes 3 and 4). B, Exogenous proteolytic activity. ER membranes from CHO-TPO cells degrade exogenous TPO. [125I]TPO was added to 50 μg ER membranes obtained from CHO-TPO cells and treated or not by sonication or by 1% Triton X-100, this volume of sample was used for SDS-PAGE. For 1% Triton X-100, this volume of sample contained about 30% of the protein concentration compared with sample treated by sonication, for the sample with untreated membrane or for the sample without membrane. This difference was due to the dilution in this case (see Materials and Methods). The acrylamide gel was dried, and the radioactivity was visualized and quantified with a phosphorimager. Lane 1, Control TPO (no membrane); lane 2, TPO after incubation with untreated membranes; lane 3, TPO after incubation with a membrane extract obtained by sonication; lane 4, TPO after incubation with a membrane extract obtained with Triton X-100; right lane, molecular weight markers.
Degradation of exogenous $^{125}$I-TPO by purified ER from CHO-TPO cells

To study this ER-associated proteolytic activity, we tried to degrade a labeled exogenous protein with purified ER from unlabeled CHO-TPO cells. For these experiments, we used human TPO extracted from Basedow tissue and purified by affinity chromatography on mAb15 and labeled with $^{125}$I ($^{125}$I-TPO). When $^{125}$I-TPO was incubated for 5 h at 37 C either without membrane or with untreated membranes (Fig. 1B, lanes 1 and 2, respectively), no protease activity was detected. On the other hand, when $^{125}$I-TPO was incubated with a membrane extract obtained by sonication (lane 3) or treated by Triton X-100 (lane 4), exogenous TPO was partially degraded. Quantification of the TPO-associated radioactivity, taking into account the amount of $^{125}$I-TPO used for lane 4 (see Fig. 1 legend), allowed us to estimate that about 34% and 20% of the total TPO was degraded after treatment of the purified ER membrane by sonication or by Triton X-100, respectively. These results confirmed that a proteolytic activity occurred in the ER membranes of CHO-TPO cells. But more interesting was that intact ER membranes displayed no activity and that to detect TPO degradation, the purified ER vesicles had to be opened and/or extracted, either by sonication or by detergent treatment. We presumed that the proteolytic agent was inside the vesicles and/or was membrane associated. The kinetics of TPO degradation, with sonicated membranes, was rapid during the first 60 min of incubation and slowed down afterward, with maximum degradation after 5 h of incubation. Moreover, the proteolytic activity was not modified at pH 8 (data not shown).

Degradation of exogenous $^{125}$I-TPO by thyroid microsomes

Although ER membranes purified from CHO-TPO cells showed the existence of ER membrane-associated protease(s), different from the proteasome, we sought to confirm these preliminary results by using RMH preparations from thyroid tissue. $^{125}$I-TPO was added on porcine thyroid RMH and on ER membranes obtained from CHO-TPO cells (60 μg sonicated membrane protein per sample), and the samples were incubated or not (controls) for 5 h at 37 C. At the end of the incubation, all the samples were analyzed by SDS-PAGE, and the radioactivity associated with TPO was quantified. Porcine thyroid RMH were able to degrade 42 ±10% of $^{125}$I-TPO, whereas ER membranes from CHO-TPO cells degraded only 20 ±10% (n = 3 experiments). Accordingly, to better evaluate the proteolytic activity for the following experiments, we used only thyroid RMH. With thyroid RMH, we observed that detergent extraction allowed the recovery of proteolytic activity, especially when ionic (acidic) detergents were used. Figure 2A shows that, after incubation for 5 h at 37 C, the TPO-associated radioactivity decreased by 23, 37, and 57% after treatment of the membranes with 1% Triton X-100, 1% NP-40, or 0.25% DOC, respectively. Thus, extraction with 0.25% DOC led to a better recovery of the proteolytic activity than extraction with 1% Triton X-100 or 1% NP-40. On the other hand, when proteolytic activity was evaluated with thyroid RMH treated with 0.1% SDS, about 90% of the $^{125}$I-TPO was degraded (Fig. 2B, lane 2). In contrast, if the concentration of SDS was increased to 0.5%, no proteolytic activity was observed (lane 4). Furthermore, the association of 1% Triton X-100 with 0.1% SDS partially limited the efficiency of the 0.1% SDS, because about 80% of the initial radioactivity (Fig. 2B, lane 5) disappeared after incubation (lane 6). It was logical to assume that 0.1% SDS increased the efficiency of the Triton X-100 to activate the degradation process of $^{125}$I-TPO by the porcine thyroid RMH. Thus, a low concentration (0.1%) of SDS was the most effective way to recover the proteolytic activity; all additional proteolytic degradation experiments were done in the presence of 0.1% SDS, either alone or added to nonionic detergent. Moreover, to rule out the possibility the proteasome participates in this proteolytic activity, we performed the same experiments in the presence or not of β-lactone (a specific inhibitor of the proteasome). Figure 2C reveals that the proteolytic activity of the porcine RMH treated with 0.1% SDS was 88 and 89% in absence or presence of β-lactone, respectively (Fig. 2C, lanes 2 and 3). Accordingly, the efficiency of TPO degradation by porcine thyroid microsomes was not related to the proteolytic activity of the proteasome. Finally, in another set of experiments, we observed that $^{125}$I-TPO was only weakly degraded at pH 6.2 but that degradation was highly effective at pH 7.2 and 8.2 (data not shown).
shown). The latter results thus also indicated that the acidic lysosomal enzymes were not involved in the degradation.

**The protease activity of thyroid microsomes is strongly membrane anchored**

The proteolytic activity recovered after 0.1% SDS extraction was very efficient. Almost all the activity was associated with the soluble fraction obtained by ultracentrifugation for 1 h at 100,000 \( \times \) g (Fig. 3A, lane 2). About 78% (77.6 ± 15.2%; \( n = 3 \)) of the labeled TPO was degraded by the soluble fraction. However, reported as the protein concentration present in each fraction (soluble and pellet), the relative proteolytic activity was of the same order, because at least 80% of the proteins from thyroid RMH were in the soluble fraction. When extraction was performed with 1% NP-40, we noted that this compound was less efficient in solubilizing proteins anchored on thyroid RMH. The soluble fraction contained 63% (63.5 ± 8.2%; \( n = 3 \)) of the initial protein concentration in the thyroid RMH, whereas about 37% (36.8 ± 9.8%) was still anchored to the membrane and was found in the pellet. By SDS-PAGE (Fig. 3B), the TPO degradation by the soluble fraction was evaluated to be 88% (lane 2), compared with the 94% of the activity found in the pellet (lane 4). The activity associated with the ultracentrifugation pellet obtained after 1% NP-40 extraction could, in turn, be solubilized by treating this pellet with 0.1% SDS (data not shown), a process that solubilized the proteins associated with the 1% NP-40 pellet. This finding was also observed with porcine and human thyroid RMH, which indicated strong membrane anchorage for both species.

**Relative specificity of thyroid RMH extracts activity toward thyroid proteins**

Because thyroid RMH were effective in degrading exogenous and endogenous TPO, we evaluated their ability to degrade either nonthyroid proteins, like BSA and bovine Ig, or the major protein of the thyroid, Tg. Figure 4 shows that the proteolytic activity of sequential RMH extracts was effective toward TPO and Tg (Fig. 4, A and D, respectively), whereas neither bovine Ig nor BSA were hydrolyzed (Fig. 4, B and C, respectively).

**An active Pl-like protease is present in thyroid RMH**

A PI-like protease selectively degraded stearoyl-CoA desaturase in liver microsomes (3, 17). This protease has properties reminiscent of those we observed with the thyroid microsomal protease (nonproteasomeric proteolytic activity at neutral and slightly basic pH, strong membrane association, and selective degradation of a tissue-specific, microsomal-anchored protein). We also observed that a PI-like protease was apically secreted in the follicular lumen of the thyroid in a sulfated form (4, 5). Therefore, we checked whether the proteolytic activity in microsomes was linked with a PI-like protease. For this, immunoblots and zymography were performed on whole microsomes to check thyroid RMH for the presence of an active PI-like-protease. Again, the specificity of the protease toward thyroid proteins, observed on sequential detergent extracts, was observed when whole microsomal fractions were used.

The immunoblot performed on microsomes, using a commercial polyclonal antibody against human PI, displayed a pattern highly similar to the one obtained with purified serum PI, although the relative intensity of the fragments slightly differed (Fig. 5A). The pattern obtained by zymography indicated also that a PI-like protease was present in microsomes as the main, if not the only, protease associated with this subcellular fraction. This protease was able to degrade casein (Fig. 5B), a typical substrate of PI, as well as Tg (Fig. 5C). In both cases, a protein with an apparent molecular weight of about 80 kDa was found. This protein corresponded to the molecular mass of PI. This major band was also associated with fragments of lower molecular weight, which were also found in human control PI, with the possible exception of some low-molecular weight active fragments found only in the microsomal enzyme after overnight incubation at 37 C. Note that in contrast with what was observed with human serum PI, the enhancement of the degradation activity of microsomal PI-like protease was maximum without addition of tissue PI activator (t-PA), even after overnight incubation at 37 C (data not shown). This finding suggested that the PI-like molecule in RMH was in an activated state and/or that a PI activator was also present in this fraction. Moreover, because BSA was not hydrolyzed by human PI or microsome-associated protease, we confirmed the specificity of the proteolytic activity of this PI-like protease (Fig. 5D).

**Discussion**

In the present work, we show that a PI-like protease is strongly associated with the membranes of rough thyroid microsomes and is able to degrade specifically the main thyroid proteins, a matured form of TPO and Tg. This proteolytic enzyme possesses the properties of the second ER-associated proteolytic system, different from the proteasome, described previously (7) and studied here in more detail by using exogenous substrates in addition to endogenous TPO.
The first observations on this proteolytic activity were done using ER membrane preparations from CHO-TPO cells, and the same results were confirmed using endogenously labeled TPO, purified with a discriminating mAb. This ER-associated protease was able to degrade matured TPO and not just synthesized unfolded TPO. In addition, exogenous $^{125}$I-labeled TPO was also degraded by the same ER membranes provided by sonication or detergent extraction. This finding suggests that the enzyme is located in the membrane and/or that ER vesicles must be opened to allow contact between the added TPO and the protease. This protease activity was then identified in thyroid RMH, demonstrating that it was present in both CHO and thyroid cells, and this proteolytic activity was later recovered in sequential detergent extracts of the same RMH. This protease differed both from the proteasome and from acidic lysosomal proteases, because it was still active in the presence of β-lactone and inactive at acidic pH, whereas slightly basic pH (up to pH 8.4) did not impair its activity. Moreover, it displayed specificity toward major thyroid proteins, namely the matured form of TPO and Tg, but it did not degrade other molecules like BSA or Ig, reflecting specificity toward thyroid proteins. We previously found that thyroid cells synthesize a Pl-like protease able to degrade Tg, but it was a secreted sulfated protease, and its location was mainly luminal (4, 5), although a weak intracellular location was also detected (4). Moreover, Heinemann et al. (3) showed the presence of a Pl-like protease in liver microsomes. Thus, in addition to the Pl secreted and exported in the plasma, liver cells were able to produce a membrane-associated Pl-like protease able to specifically degrade stearyl-CoA desaturase, an integral membrane protein of the ER. This result prompted us to check whether the protease in thyroid RMH was also a Pl-like protease. We revealed by SDS-PAGE that the thyroid microsomal protease has the same electrophoretic mobility as control human Pl and that it was recognized by a specific anti-Pl antibody. Moreover, like Pl, the thyroid microsomal protease was active against casein and Tg. When Tg was used as substrate, the zymographic patterns revealed several bands both for control human Pl and for thyroid RMH protease. As for SDS-PAGE, the major band with an apparent molecular weight of 80 kDa was associated with several active cleaved bands whose activity increased upon incubation and particularly with the smallest peptide of about 40 kDa. This latter peptide was also identified by immunoblot with a control human Pl. So, we had here a Pl-like protease, bound to microsomal membranes tightly enough to necessitate sequential detergent extraction to extract the enzymatic activity. Because TPO is located mainly in the ER of thyroid cells, ER-associated proteases are probably involved in the regulation of the intracellular pool and/or the traffic of TPO. The Pl-like protease found here is associated with purified ER membranes in the case of CHO-TPO cells and with RMH in the case of thyroids. Although no subcellular fractionation is able to give 100% purified ER membranes, and even if a plasma membrane location cannot be totally excluded, it is more than likely that this membrane...
Pl-like protease is associated with ER membranes (14). Although there is no intracellular pool of Tg, the fact that Tg is a substrate of the rough microsome-associated Pl-like protease suggests it may play a role in the regulation of Tg secretion in addition to its previously described role in luminal Tg degradation (4). In a recent paper, endoproteolytic cleavage of human TPO was described; it occurred between Lys 108 and Thr 109, probably in the ER compartment of thyroid cells (18). The protease responsible for this cleavage was not identified; it could be Pl (preferential cleavage Lys-/Arg-/Xaa>Arg-/Xaa). However, in our in vitro experiments, TPO was completely degraded, although a transitory band was sometimes visible. Why a normally secreted protein is so strongly linked to membranes is unclear. Several possibilities exist, involving either posttranslational modifications or binding to lysine on the ER membrane through Kringle domains (3,19,20). In view of the results presented here, this Pl-like protease participates in the nonproteasomal system that degrades the TPO recognized by mAb15. Our results suggest that a Pl-like protease is probably part of some basic intracellular enzymatic equipment, and we hypothesize that its proteolytic properties contribute to regulate the pool and/or the trafficking of molecules involved in specialized functions like TPO and Tg in the thyroid or stearoyl-CoA desaturase in the liver (3). Zymography experiments with Tg evidenced only the presence of a Pl-like protease, suggesting that it is the main enzyme associated with the rough microsomal fraction visible in our experimental conditions. It might not be the only one, because it is a serine protease, and in addition to serine protease inhibitors, some threonine protease inhibitors are able to lower TPO degradation by the second proteolytic system of the ER (7). Depending on tissues or cell lines, other proteases acting on other substrates were reported to be part of the enzymatic equipment of the ER, e.g. ER-60, an ER-localized protein that has both proteolytic and chaperone activities and that is involved in the nonproteasomal degradation of apolipoprotein B 100 in HepG2 cells, although it is not clear whether it acts as a protease or as a chaperone (21); cathepsin W in NK cells (22); or caspase 12, which is activated during ER stress-induced apoptosis in various tissues and cell lines (23,24) and could contribute to regulatory secretion of specialized proteins. Other specialized proteases in the ER are ubiquitously needed, e.g. the signal peptidase, a membrane-bound endopeptidase that cleaves the signal peptides from secretory proteins (25,26) and the signal peptide peptidase that cleaves the signal peptide remaining in the membrane of the ER (27–30). Indeed, up to recently, Pl was considered to be synthesized in the apical extracellular environment of thyroid epithelial cells, degrades thyroglobulin in vitro. Biochem Biophys Res Commun 338:1000–1004.

In the present work, we found that microsomal Pl is activated when incubated overnight at 37 °C, without the addition of exogenous t-PA. In fact, in some experiments, we added t-PA and obtained the same pattern in zymography as when no activator was added (data not shown). This finding could indicate the presence of t-PA in thyroid microsomes. In contrast, exogenous t-PA had to be added to the luminal Pl to reach full activity. Several studies showed the presence of t-PA (2,36–39) and of t-PA inhibitors (40) in the thyroid. But t-PA were not necessarily involved here; when associated with membranes, generally through lysine residues on membrane proteins, Pl acquires a conformation that is more active than that of soluble Pl (41–43). For the thyroid, it had never been suggested that Pl had an intracellular function, and although cultured thyroid cells were able to synthesize and to secrete Pl and Pl activators (2,36,37,39), this action was associated with extracellular degradation. We suggest here that depending on its location, Pl has different substrates and fulfills different functions.

Acknowledgments
We thank Dr. Catherine De Micco for providing human thyroid tissues.

Received January 10, 2007. Accepted February 16, 2007.

Address all correspondence and requests for reprints to: Bernard Mallet, Laboratoire de Biochimie, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France. E-mail: bernard.mallet@medecine.univ-mrs.fr.

This study was supported by Institut National de la Santé et de la Recherche Médicale Unité 555, Institut Fédératif de Recherche 125, and Université de la Méditerranée.

Disclosure Statement: All the authors have nothing to disclose.

References
12. Ronin C, Bouchilloux S 1978 Cell-free labeling in thyroid rough microsomes
of lipid-linked and protein-linked oligosaccharides. I. Mannosylated units. Biochim Biophys Acta 539:470–480