Degradation of Human Thyroperoxidase in the Endoplasmic Reticulum Involves Two Different Pathways Depending on the Folding State of the Protein

Laurence Fayadat, Sandrine Siffroi-Fernandez, Jeanne Lanet, and Jean-Louis Franc

From the U38 INSERM, Faculté de Médecine, Université de la Méditerranée, 13385 Marseille cedex 5, France

Human thyroperoxidase (hTPO), a type I transmembrane glycoprotein, plays a key role in thyroid hormone synthesis. In a previous paper (Fayadat, L., Niccoli, P., Lanet, J., and Franc, J. L. (1998) Endocrinology 139, 4277–4285) we established that after the synthesis, only 15–20% of the hTPO molecules were recognized by a monoclonal antibody (mAb15) directed against a conformational structure and that only 2% were able to reach the cell surface. In the present study using pulse-chase experiments in the presence or absence of protease inhibitors followed by immunoprecipitation procedures with monoclonal antibodies recognizing unfolded or partially folded hTPO forms we show that: (i) unfolded hTPO forms are degraded by the proteasome and (ii) partially folded hTPO forms are degraded by other proteases. It was also established upon incubating endoplasmic reticulum (ER) membranes in vitro that the degradation of the partially folded hTPO was carried out by serine and cysteine integral ER membrane proteases. These data provide valuable insights into the quality control mechanisms whereby the cells get rid of misfolded or unfolded proteins. Moreover, this is the first study describing a protein degradation process involving two distinct degradation pathways (proteasome and ER cysteine/serine proteases) at the ER level, depending on the folding state of the protein.

Thyroperoxidase (TPO) is a type I transmembrane heme-containing glycoprotein that plays a key role in thyroid hormone synthesis. Although TPO catalyzes both thyroglobulin iodination and hormone synthesis at the apical cell surface of thyroid cells, this enzyme is located mainly in the endoplasmic reticulum (ER) and the perinuclear membrane, and only a small proportion is present at the apical surface (reviewed in Ref. 1). Kulawat et al. (2) and Penel et al. (3) report that no more than 30% of the immunoprecipitated TPO was detected at the cell surface of porcine thyrocytes cultured on porous filters. In a previous study (4), we proposed a model for the folding, degradation, and intracellular trafficking of human TPO (hTPO) expressed in CHO cells. hTPO was stably expressed in the CHO cell line, and its folding was studied with two monoclonal antibodies: mAb47, which recognizes a linear epitope, and mAb15, which recognizes a conformational epitope present in the mature protein. The results showed that only 15–20% of the hTPO molecules were able to acquire a suitable conformation to be recognized by mAb15 (4). On the other hand, only a fairly small proportion (~15%) of the latter were able to reach the plasma membrane. These data indicated that after being synthesized, only 2% of the hTPO molecules were able to exit from the ER and reach the cell surface. In the steady state, the hTPO recovery rate at the cell surface ranged between 10 and 15%. This firmly suggests that the process involved in the folding and intracellular trafficking of this glycoprotein is the same in both thyroid and CHO cells and that the inefficient maturation of the wild type hTPO is an intrinsic characteristic of this molecule. The speed at which the remaining molecules were degraded varied depending on their folding state. The incorrectly folded hTPO was degraded faster than that recognized by mAb15 (half-time 2 h versus 7 h) (4). “Quality control” is a term that refers to the process whereby conformational maturation is continuously controlled in the ER (5). The conformational maturation of nascent polypeptides in the ER is facilitated by transient physical interactions with folding catalysts (protein disulfide isomerase, peptidyl isomerase, etc.) and molecular chaperones (BiP, GRP 94, calnexin, etc.). The ER is the site of entry of proteins into the secretory pathway. As a rule, unfolded proteins, folding intermediates, unassembled subunits, and incompletely assembled oligomers remain in the ER and are ultimately targeted by the quality control apparatus responsible for intracellular degradation. In previous studies, we reported that (i) interactions between hTPO and calnexin and calreticulin are of crucial importance to the folding of hTPO (6) and (ii) the insertion of the heme is essential for the hTPO to be able to exit from the ER (7). It is generally agreed that any misfolded proteins that fail to fulfill the requirements for being transported out of the ER are delivered to cytoplasmic proteasomes to be degraded (8), although exactly how the decision to degrade misfolded or unassembled membrane proteins is made has not yet been completely elucidated. There are now some evidence, however, that ER proteases may also be involved in the degradation of proteins (9–11).

In the present study, we focused on the degradative processes underlying the degradation of hTPO, since the speed at which hTPO molecules were degraded varied depending on their folding state. The incorrectly folded hTPO was degraded faster than that recognized by mAb15 (half-time 2 h versus 7 h) (4). “Quality control” is a term that refers to the process whereby conformational maturation is continuously controlled in the ER (5). The conformational maturation of nascent polypeptides in the ER is facilitated by transient physical interactions with folding catalysts (protein disulfide isomerase, peptidyl isomerase, etc.) and molecular chaperones (BiP, GRP 94, calnexin, etc.). The ER is the site of entry of proteins into the secretory pathway. As a rule, unfolded proteins, folding intermediates, unassembled subunits, and incompletely assembled oligomers remain in the ER and are ultimately targeted by the quality control apparatus responsible for intracellular degradation. In previous studies, we reported that (i) interactions between hTPO and calnexin and calreticulin are of crucial importance to the folding of hTPO (6) and (ii) the insertion of the heme is essential for the hTPO to be able to exit from the ER (7). It is generally agreed that any misfolded proteins that fail to fulfill the requirements for being transported out of the ER are delivered to cytoplasmic proteasomes to be degraded (8), although exactly how the decision to degrade misfolded or unassembled membrane proteins is made has not yet been completely elucidated. There are now some evidence, however, that ER proteases may also be involved in the degradation of proteins (9–11).

In the present study, we focused on the degradative processes underlying the degradation of hTPO, since the speed at
which hTPO molecules are degraded varies depending on their folding state. This is the first time the degradation of a wild type protein has been found to involve two distinct pathways at the ER level, depending on its folding state: the cytosolic proteasome pathway and a pathway involving some ER membrane-bound cysteine/serine proteases.

EXPERIMENTAL PROCEDURES

Materials—The following materials were supplied by Sigma: MG132, N-acetyl-Leu-Leu-norleucinal (ALLN), cycloheximide, emetine, leupeptin, aprotinin, pepstatin A, phosphoramidon, 3,4-dichloroisocoumarin, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), leupeptin, aprotinin, pepstatin A, phosphoramidon, 3,4-dichloroisocoumarin, N-2-mercaptoethanol, brefeldin A, and dithiothreitol. Clastolactacystin (100 μg/ml) and streptomycin were from Life Technologies, Inc.; Geneticin was from Life Technologies, Inc. Experprotease inhibitors mixture, and 100 μM iodoacetamide. The cells were then thawed on ice and diluted 1:1 with ice-cold 150 mM sucrose, 10 mM Tris-HCl, pH 7.5, and then vigorously sonicated for 3 × 10 s on ice. Cells were then centrifuged for 1 h at 100,000 × g and separated into supernatant and pellet subfractions. Pellets were resuspended in 150 mM sucrose, 10 mM Tris-HCl, pH 7.5, and then incubated at 37 °C for various periods of time with or without protease inhibitors. After performing extraction and immunoprecipitation, samples were prepared as described previously.

RESULTS

Determination of Two Different Populations of hTPO Molecules Differing in Their Three-dimensional Structures—To discriminate between different populations of hTPO molecules differing in their three-dimensional structures, two mAbs were used, namely mAb15 and mAb47. These two mAbs were selected among a panel of 12 mAbs directed against hTPO (13). The mAb47 recognizes a linear epitope (14), and it has been previously established that this mAb does not recognize hTPO when this molecule acquires a partially or a totally folded form (4, 15). The mAb15 recognizes a three-dimensional epitope in the hTPO, which is localized in one of the two major antigenic domains of the molecule and is unable to recognize unfolded hTPO molecules (13). Using the mAb15 + mAb47 couple in immunoprecipitation experiments, more than 90% of the hTPO expressed in CHO cells was recovered (data not shown). When these mAbs were used separately in a pulse-chase experiment using 35S(Met+Cys) (Fig. 1), the amount of hTPO recognized by mAb47 reached a maximum after the pulse, and no hTPO recognized by mAb15 was immunoprecipitated at this time. During the chase period, the amount of hTPO recognized by the mAb47 decreased, whereas that recognized by the mAb15 increased. The total quantity of hTPO (hTPO immunoprecipitate by mAb15 and by mAb47) decreased during the chase. However, it is not clear from the results of this experiment whether or not any overlapping occurred between the populations of hTPO molecules recognized by mAbs 15 and 47. We then performed sequential immunoprecipitations: hTPO-CHO cells were labeled for 0 h with 35S(Met+Cys), and after the extraction step, the first immunoprecipitation was performed with either mAb15 or mAb47, followed by a second one using the other mAb (Fig. 2). Regardless of the order in which the two mAbs were used, the hTPO immunoprecipitation rate was practically the same in the case of both mAbs. This demonstrates that using these two mAbs, it is possible to define two distinct populations of hTPO molecules: those having the unfolded forms recognized by mAb47 and those with the partially and/or completely folded forms recognized by mAb15. In a previous study (4), we hypothesized that if a hTPO molecule recognized by mAb15 was not able to exit from the ER, it was certainly because this molecule had not reached the “completely folded form” and was therefore not able to satisfy the quality control requirements of the ER. The possibility cannot be ruled out, however, that the “hTPO15+ form” present at the cell
are degraded and determining the underlying degradation mechanisms, we performed pulse-chase analyses on hTPO-CHO cells. Since the proteasome pathway has recently been found to mediate the degradation of many ER proteins, we focused on the possible involvement of the proteasome in the degradation of hTPO. Cells were treated with or without lactacytin, a highly specific irreversible inhibitor of the multicatalytic proteasome, with and without cycloheximide, the well known protein synthesis inhibitor, which is also known to inhibit protein degradation. CHO cells were preincubated for 2 h with or without lactacytin and then pulse-labeled with $^{35}$S(Met+Cys), chased with or without lactacytin or cycloheximide. hTPO was then immunoprecipitated using mAb47 (Fig. 3a). It was observed that the unfolded forms recognized by mAb47 (the hTPO47+ forms) were rapidly degraded (half-life: $\sim 3$ h) and that treating the cells with lactacytin strongly inhibited this degradation. Similar results were obtained using MG132 (data not shown), whereas cycloheximide treatment had no noticeable effects on the degradation of the hTPO47+ forms.

In Vivo, the Degradation of Partially Folded hTPO Forms Requires Ongoing Protein Synthesis—Similar pulse-chase experiments to those described in Fig. 3a were carried out, except that hTPO was immunoprecipitated at the end of the chase period using mAb15 (Fig. 3b). Here it was observed that the degradation of the partially folded forms (hTPO15+ forms) was slower than that of the hTPO47+ forms (half-life: $\sim 20$ h as compared with 3 h) and was insensitive to lactacytin, whereas cycloheximide treatment strongly inhibited the degradation of the hTPO15+ forms. The degradation of the hTPO15+ forms therefore did not involve the proteasome pathway but a pathway inhibited by cycloheximide. To determine whether the degradation of the partially folded hTPO forms requires ongoing protein synthesis or whether the hTPO15+ forms were degraded via a process that is specifically inhibited by cycloheximide, we performed additional pulse-chase experiments with another protein synthesis inhibitor, emetine, which blocks protein synthesis via a different mechanism. The results indicated that the degradation of the hTPO15+ forms was inhibited by emetine (Fig. 4), which suggests that the degradation of the hTPO15+ forms requires ongoing protein synthesis.

In Vivo, the Degradation of the Partially Folded hTPO Forms Does Not Require Transport through the Golgi Apparatus and Is Nonlysosomal—Further studies were then carried out with a view to determining the degradation system involved in hTPO15+ form degradation and its cellular location. Eukaryotic cells contain two main systems responsible for the intracellular degradation of proteins: the ubiquitin-proteasome pathway and the lysosomal pathway. Since we knew that partially folded hTPO forms did not undergo proteasome-mediated degradation, we investigated the question as to whether these molecules are degraded in the lysosomes. Pulse-chase experiments were therefore performed in which lysosomotropic agents (chloroquine or NH$_4$Cl), which increase the intravesicular pH level, were added to the chase media, thereby inactivating low pH-dependent lysosomal proteases. After the chase, hTPO was immunoprecipitated with mAb15 (Fig. 5). The finding that the lysosomotropic agents used did not inhibit the degradation rate of the hTPO15+ forms argues against a lysosomal mechanism being responsible for the degradation of these forms. On the contrary, for unknown reasons these drugs increased the rate of degradation. To determine whether a passage through the Golgi apparatus was required for the intracellular degradation of the hTPO15+ forms to be possible, we performed additional pulse-chase experiments using brefeldin A, an inhibitor of protein transport through the Golgi

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**FIG. 1.** Pulse-chase analysis of the folding of hTPO expressed in CHO cells. Cells were preincubated for 2 h in Met- and Cys-free medium supplemented with 10% dialyzed FBS, 10 mM sodium butyrate, and 10 $\mu$Ci of $^{35}$S(Met+Cys)/ml. After the pulse, cells were chased for various times in Ham’s F-12 medium, 10% FBS supplemented with 5 mM Met and 5 mM Cys. Immunoprecipitation was performed with mAb15 (A) or mAb47 (B). Samples were separated by SDS-PAGE analysis, and the radioactivity was detected and quantified using a phosphorimaging device. This figure gives the results of an experiment that is representative of four identical experiments performed.

**FIG. 2.** Sequential immunoprecipitation of $^{35}$S-hTPO with mAb15 and mAb47. Cells were incubated for 3 h in Met- and Cys-free medium supplemented with 10% FBS, 10 mM sodium butyrate, and 10 $\mu$Ci of $^{35}$S(Met+Cys)/ml. At the end of the incubation period, hTPO was immunoprecipitated with either mAb47 (A) or mAb15 (B), and the supernatant was then immunoprecipitated again using mAb15 (A) or mAb47 (B). Samples were separated by SDS-PAGE analysis, and the radioactivity was detected and quantified using a phosphorimaging device. This figure gives the mean values from three different experiments.

surface may be similar to that present in the ER.

In Vivo, Unfolded hTPO Undergoes Proteasome-mediated Degradation—With a view to defining the kinetics and the extent to which unfolded (those recognized by mAb47) and partially folded hTPO molecules (those recognized by mAb15)
complex that induces a redistribution of the Golgi-apparatus proteins into the ER. The fact that degradation of the hTPO15 forms still occurred in the presence of brefeldin A shows that the degradation occurred in a pre-Golgi location, at the ER level (data not shown).

**In Vivo, Cysteine/Serine Proteases Degrade the Partially Folded hTPO Forms in the ER—**To obtain further insights into the degradation mechanisms involved in the degradation of the hTPO15+ forms and to determine the specificity of this degradation pathway, pulse-chase experiments were carried out in which various protease inhibitors were added to the chase medium at concentrations not affecting the cell viability. The following protease inhibitors were used for this purpose: leupeptin, aprotinin, pepstatin A, phosphoramidon, ALLN, TPCK, EDTA, E64d, and 3,4-dichloroisocoumarin and were added after the pulse with 35S(Met+Cys) to the chase medium for 2 h or 48 h. hTPO was then immunoprecipitated using mAb15 (Fig. 3). Among the protease inhibitors tested, (i) leupeptin, aprotinin, and alln were found to substantially reduce the degradation rate of hTPO immunoprecipitated by mAb15, (ii) pepstatin A weakly reduced this degradation rate, (iii) for...
unknown reasons the metalloprotease inhibitors (phosphoramidon, EDTA) and the chymotrypsin inhibitor (TPCK) accelerate this degradation at 48 h (Fig. 6), (iv) other inhibitors of cysteine proteases (E64d) and serine proteases (4-dichloroisocoumarin) had no effect on the degradation rate (data not shown). To examine this point more closely, we performed additional pulse-chase experiments using the three protease inhibitors, which significantly reduced the degradation of the hTPO forms: leupeptin (a serine/cysteine protease inhibitor), aprotinin (a serine protease inhibitor), and ALLN (which inhibits cysteine proteases and calpain and which is also known to inhibit proteasomal degradation) (Fig. 7). The results confirmed our previous finding that the degradation of the hTPO forms was noticeably inhibited by leupeptin, aprotinin, and ALLN, which suggests that the hTPO forms were degraded by cysteine and serine proteases at the ER level (cytosol, ER membrane, or ER lumen level). Note that the effects of leupeptin, aprotinin, and ALLN were not additive, since they did not lead to any additional enhancement of the stability of the hTPO forms (data not shown).

In Vitro, the Degradation of the Partially Folded hTPO Forms Is Sensitive to Cysteine/Serine Protease Inhibitors—To further define the location of the pathway responsible for the degradation of the partially folded hTPO forms, we performed cell fractionation and purified the ER membrane. After incubating these isolated ER membranes in vitro (Fig. 8a), we observed that the degradation of the hTPO forms persisted, which indicates that the proteases involved were located at the ER membrane level and not in the cytosol. Furthermore, this degradation process was always inhibited by ALLN (Fig. 8b) and to a weaker extent by leupeptin and aprotinin (Fig. 8a) but not by lactacystin (data not shown). These data rule out the possibility that proteasomes bound to the ER (18) may have been responsible for the degradation of the hTPO forms in vitro. It is worth noting, however, that the inhibitory effects of leupeptin and aprotinin were less noticeable in vitro than in CHO cells (Figs. 8a and 7a).

To determine whether the “lumen-free” membrane fragments still contained the proteases necessary for the degradation of the partially folded hTPO forms, ER membranes were sonicated, and the resulting disrupted ER membranes were isolated by ultracentrifugation. With these membranes, the in vitro degradation of the hTPO forms still occurred,
and its rate was even greatly enhanced in comparison with that recorded in the case of the control ER membranes (Fig. 8, c and d). The degradation of the hTPO15+ forms therefore does not require the presence of soluble luminal proteases. Membrane fragments conserve the proteases necessary for the degradation of the partially folded hTPO forms. The results of our experiments therefore indicate that some ER membrane-bound cysteine/serine proteases are involved in the degradation of the hTPO15+ forms both in vivo and in vitro. It is worth mentioning that a great increase in the hTPO15+ forms was observed in the presence of ALLN during the incubation periods (Fig. 8b), which may possibly be due to the hTPO47+ forms being transformed into hTPO15+ forms.

**DISCUSSION**

In a previous study (4), we described the occurrence of two crucial steps in the folding of the hTPO molecules expressed in CHO cells. Only 15–20% of the hTPO molecules were able to acquire a suitable three-dimensional structure to be recognized by mAb15, and only 2% were able to reach the cell surface. In the present study, we observed that the rate at which the remaining molecules were degraded varied depending on their folding state (Fig. 3). The disappearance of the hTPO molecules recognized by mAbs may have been due to the formation of complex or insoluble forms of the molecule, as previously found to occur in the case of the CFTR and T cell receptor α molecules (17, 18). Using the techniques described in the latter two studies, we were unable to detect the presence of insoluble hTPO molecules (data not shown). But we did observe that the degradation of the unfolded forms was significantly inhibited by lactacystin or MG132, which indicates that these molecules underwent proteasome-mediated degradation (Fig. 3). However, under these conditions, we were unable to detect either ubiquitinated or deglycosylated hTPO in the soluble or the non-soluble fractions (data not shown). In another study (6), we reported that when the association of hTPO with calnexin or calreticulin was impaired, hTPO was dramatically degraded, and that this degradation involved the proteasome pathway. The latter data are consistent with the present data, since they indicate that treating the cells with castanospermine led to a dramatic decrease in the proportion of the hTPO molecules that were able to reach the partially folded state and be recognized by mAb15. In the present study, the degradation of the partially folded forms of hTPO was not inhibited by lactacystin (Fig. 3). Since the hTPO15+ forms were not degraded by the proteasome, we investigated the nature of the proteases involved in their degradation. Among the panel of protease inhibitors tested, leupeptin, aprotinin, and ALLN significantly reduced the degradation rates of these forms. The degradation of the hTPO15+ forms therefore involved cysteine/serine proteases. On the other hand, this degradation process was not affected by treating the cells with lysosomotropic agents or brefeldin A, which indicates that the degradation occurs in a pre-Golgi compartment. Note that in vivo, the inhibitory effects of aprotinin and leupeptin may have been underestimated, since aprotinin is a 58-residue peptide, and the cells might not be completely permeable to this component, whereas leupeptin is a tripeptide that is likely to be membrane-permeant. The results of further in vitro studies carried out with purified ER membranes indicated that the hTPO15+ molecules were still degraded and that the degradation was sensitive to leupeptin.

**FIG. 8.** Effects of leupeptin, aprotinin, and ALLN on the in vitro degradation of the partially folded form of hTPO. hTPO-CHO cells were labeled for 2 h with 35S(Met + Cys), the cells were then harvested, and ER fractions were purified. To determine whether or not the lumen-free membrane fragments still contained the proteases necessary for the degradation process, ER membranes were sonicated, and the resulting disrupted ER membranes were isolated by ultracentrifugation. a and b, ER membrane control; c and d, ER membranes sonicated. ER fractions were incubated at 37 °C for various periods of time without (C) or with 10 μM leupeptin (a and c, ○), 10 μM aprotinin (a and c, □), or ALLN (b and d, ○). Control experiments were performed in which the same quantity of corresponding solvent was added to the media (PBS for a and c and Me2SO for b and d). hTPO was then extracted using extraction buffer, and immunoprecipitation was performed using mAb15. Samples were separated by SDS-PAGE. The radioactivity was detected and quantified using a phosphorimaging device. This figure gives the results of an experiment that is representative of four identical experiments performed.
Degradation of hTPO in the ER Involves Two Different Pathways

Aprotinin, and ALLN. These data suggest that the process of \textit{in vitro} degradation of hTPO15+ forms resembles the \textit{in vivo} process, whereas, in the case of HMG-CoA reductase, the \textit{in vivo} and \textit{in vitro} degradation processes are different (19). \textit{In vivo}, this protein is degraded by the proteasome, whereas the \textit{in vitro} degradation is not inhibited by lactacystin but is inhibited by cysteine protease inhibitors. Further experiments performed with luminal-free ER membrane fragments showed that a soluble luminal content was not required for the degradation of the partially folded hTPO forms to occur. These results therefore showed that these forms were degraded by some ER membrane-bound cysteine/serine proteases. \textit{In vivo}, the finding that the degradation of the partially folded forms is inhibited by cycloheximide and emetine, two protein synthesis inhibitors, suggests that ongoing protein synthesis is required for this degradation process to occur. As was to be expected, cycloheximide had no noticeable effects on isolated ER membranes, in which the mechanism of protein synthesis is impaired (data not shown). Our hypothesis is that \textit{in vivo} cycloheximide might either inhibit the synthesis of the protease(s) involved in the degradation of these forms or inhibit that of a component, contributing to the activation of this protease(s).

A number of proteins known to undergo ER-associated degradation have been found to be degraded via the proteasome pathway (8), although there exists new evidence in favor of the idea that a cytosolic giant protease (TPP II) might take over some of the functions of the proteasome (20, 21). On the other hand it has been suggested that (i) asialoglycoprotein receptor subunit is degraded by two different pathways in a pre-Golgi compartment (22), (ii) the degradation pathway of mutants of \(\alpha\)1 antitrysin (Z and S) is at least partly different from that of asialoglycoprotein subunits (23), (iii) cystic fibrosis transmembrane conductance regulator (24), COOH-truncated human \(\alpha\)-1 antitrysin (25), and mutated carboxypeptidase Y (26) might be degraded via the proteosomal and via a nonproteosomal pathway. However, relatively little is known so far about the ER proteases possibly involved in protein degradation in the ER. ER proteases are involved in the degradation of resident ER proteins, stearyl-Coenzyme A desaturase (9), and in that of P-glycoprotein (10), whereas proteasome and a cysteine protease are selectively involved in the differential processing of antigen presentation by major histocompatibility complex class I (11). In the present study, the inhibition of the degradation of the hTPO15+ forms by leupeptin, aprotinin, and ALLN suggests that multiple proteases may be involved in the degradation process or that a single protease with multiple enzymatic activities may take part in the process. One of the main aims of our ongoing studies is to identify and eventually clone the relevant ER proteases. The term “ER degradation” refers here to a degradation process that takes place in the ER or in the close vicinity. In this study, it was definitely established that depending on its folding state, hTPO is degraded either by the cytosolic proteasome pathway or by a novel degradative system involving some still unknown membrane-bound cysteine/serine proteases present in the ER itself. This second degradation system might be used by the cell to regulate the continuous turnover of proteins and to eliminate resident ER proteins.

All in all, our data provide valuable insights into the quality control mechanisms whereby the cells get rid of misfolded or unfolded proteins, and to our knowledge, this is the first time a wild type protein has been described that is degraded via two distinct degradation pathways (the proteasome and ER cysteine/serine proteases), depending on the folding state of the protein.

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