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Membrane IgG H chains turn over considerably more rapidly than secretory Ig H chains in the 18-81 A2 pre-B cell line. This rapid degradation occurs in proteasomes. N-Glycosylated membrane Ig H chains accumulate in the endoplasmic reticulum in the presence of proteasomal inhibitors, suggesting that retrotranslocation and proteasomal degradation of membrane Ig H chains may be closely coupled processes. Accelerated proteasomal degradation of membrane Ig H chains was also observed in transfected nonlymphoid cells. At steady state, the membrane form of the H chain associates more readily with Bip and calnexin than its secretory counterpart. The preferential recognition of membrane, as opposed to secretory, Ig H chains by some endoplasmic reticulum chaperones, may provide an explanation for the accelerated proteasomal degradation of the former. The Journal of Immunology, 2000, 164: 4713–4719.

Accelerated proteasomal degradation of membrane Ig H chains is mediated by a process that was originally described as ER degradation. This process is now believed to involve the retrograde translocation of proteins through the Sec61 protein channel into the cytosol, followed by their targeting to proteasomes (1–5). For a small subset of ER proteins that are degraded by this pathway, cytosolic intermediates have been identified (1, 6–9). For many proteins, however, cytosolic intermediates have not been detected, and it is presumed that some of these latter proteins might be translocated directly into proteasomes that are associated with the cytosolic face of the ER membrane (2). Proteins that are misfolded or improperly assembled must be identified before translocation across the Sec61 channel. This identification step might be mediated by ER resident chaperones. Chaperones that associate with misfolded proteins in the ER include Bip (10–12), calnexin (13–17), and calreticulin (18, 19). In yeast, Kar2, which is homologous to Bip, and Cne1, a homologue of calnexin, have been shown to be required for the proteasomal degradation of ER proteins (20, 21).

Bip is a homologue of heat-shock protein 70, which was originally described as a protein that binds to Ig H chains. It is known to associate with the CHI domains of Ig H chains (22) and binds preferentially to linear arrays of hydrophobic amino acids (23, 24). This chaperone participates both in the Sec61-dependent transport of proteins into the ER (25) as well as in the recognition and/or retrograde translocation of target proteins for proteasomal degradation.

Calnexin primarily binds to newly formed monoglucosylated proteins in the ER (26). N-Linked carbohydrates are maintained in the monoglucosylated state by a UDP-glucosyl:glycoprotein glucosyltransferase that specifically transfers a glucose moiety onto oligosaccharide chains of denatured deglucosylated proteins (27). Once a glycoprotein is fully folded, it is no longer recognized by this enzyme, fails to bind calnexin, and can thus leave the ER (28). Apart from its carbohydrate-dependent association with nascent glycoproteins (29, 30), calnexin also associates with protein aggregates in a carbohydrate-independent manner (31). Calnexin has been reported to bind unassembled B cell receptor complexes (14, 15, 32, 33). Although the proteasomal degradation of misfolded Ig L chains has recently been described (34), the mechanism by which unassembled Ig H chains are degraded is not known.

We have previously described a pre-B cell line in which the membrane form of the Ig H chain is rapidly degraded in a prelysosomal compartment, whereas the secretory form is relatively stable (35). We show in this study that the rapid degradation of membrane Ig H chains occurs in proteasomes. Membrane Ig H chains accumulate in the ER before retrotranslocation, suggesting that retrotranslocation and proteasomal degradation of this protein may represent closely coupled processes. The membrane form of the y H chain (γm) expressed alone in nonlymphoid cells is also subject to rapid proteasomal degradation. While both γm and the secretory form of the H chain (γs) associate with Bip, at steady state, Bip associates more readily with γm. In similar assays, calnexin was also found to associate with γm, but not with γs. Exposure to castanospermine does not alter the rate of degradation of γm, and also does not impair the association of γm with calnexin. Although polypeptides in the process of folding might be recognized transiently by calnexin via monoglucosyl moieties on the former, protein-protein interactions might be involved in the retention of some misfolded glycoproteins by this chaperone as a prelude to their degradation in proteasomes.

Materials and Methods

Cells and Abs

The 18-81 A2 pre-B cell line expresses γ2b H chains and has been described in our previous study (35). A20.25 and 2PK3 are murine B lymphoma lines that were obtained from the American Type Culture Collection (Manassas, VA). H564 hybridoma cells were kindly provided by Dr.
Endoglycosidase H treatment of affinity-isolated γm and γs

PAS pellets were washed as described above, and affinity-isolated Ig H chains were eluted by boiling in 50 μl of 0.4% w/v SDS in 50 mM sodium citrate, pH 5.5. The eluted proteins were incubated with or without 0.002 U endoglycosidase H (Oxford Glycoscience, Wakefield, MA) at 37°C for 3 h. Proteins were then taken up in sample buffer and separated on 10% polyacrylamide/SDS gels.

Trypsin treatment of isolated membranes

Metabolically labeled cells were allowed to swell on ice in 10 mM Tris, pH 7.5, 10 mM NaCl, and 3 mM MgCl2, (0.3% reticulocyte saline buffer or RSB). Cells were pelleted and dounced in a Potter-Elvehjem type homogenizer, and nuclei were removed by centrifugation for 10 min at 1,500 × g. Membranes were pelleted from the postnuclear supernatant by ultracentrifugation at 180,000 × g for 20 min in a Beckman TL.100 ultracentrifuge. Membranes were washed once in 0.3 × RSB, and one portion was treated with trypsin (200 μg/ml) for 30 min on ice. Proteolytic activity was blocked by using soybean trypsin inhibitor (500 μg/ml; Sigma) and 5 mM PMSF. Membrane pellets were lysed with 0.5% Triton X-100 in PBS, and IgG H chains were isolated using PAS before analysis on a 10% polyacrylamide/SDS gel.

Immunoblot assays of affinity-isolated and immunoprecipitated proteins

In experiments the association of calnexin with Ig H chains, lysosomes of cell lines and transfected COS cells were prepared in 0.6–2% CHAPS (Sigma) in PBS with 2 mM PMSF, 2 μg/ml of leupeptin, and 2.5 mM iodoacetamide. Ig H chains of the γ isotype were isolated by incubation with PAS. BSA was conjugated to Sepharose (4 mg protein/ml of packed gel) using vinyl sulfone (Sigma), and this was used as a control matrix. Lysates were also immunoprecipitated with control IgG (5 μg), or specific Abs (5 μg), and rocked for 2–16 h with 200 μl 3% PAS in PBS. In all cases, beads were collected by centrifugation and washed four times with 0.3% CHAPS in PBS. Ig molecules and associated proteins were eluted by boiling the samples in 50 μl SDS-PAGE sample buffer containing 5% (w/v) 2-ME. Proteins were separated on 10% polyacrylamide/SDS gels.

After electrophoresis, proteins were transferred onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA), blocked with 5% powdered milk in PBS containing 0.1% Tween 20, and probed with Abs, as described in the figure legends. The blots were washed in PBS containing 0.1% Tween 20 and were developed using a chemiluminescence detection kit from Pierce (Rockford, IL).

Results

In 18-81 A2 cells, the rapid degradation of γm occurs in a proteasomal compartment

We have previously demonstrated that γm is rapidly degraded in 18-81 A2 pre-B cells. This degradation was shown to be nonlysosomal and was presumed to occur in the ER. To determine whether the degradation of γm might occur in proteasomes, we sought to examine the degradation of this protein in the presence of proteasomal inhibitors. We pulse labeled 18-81 A2 cells for 20 min with [35S]methionine and examined affinity-isolated Ig H chains on SDS-PAGE both immediately after the pulse and after a 4-h chase period. As seen in Fig. 1, the membrane form of the γ chain was rapidly degraded. Degradation was markedly inhibited when cells were separately treated with proteasomal inhibitors such as ALLN (Fig. 1, a, left panel, and b), lactacystin (Fig. 1a, right panel), or ALLM (Fig. 1b). A similar inhibition of degradation was also observed with ZL VS (data not shown). Degradation was not inhibited by three inhibitors of lysosomal proteases, E64, leupeptin, and pepstatin A. These results strongly suggest that the rapid prelysosomal degradation of γm occurs in proteasomes.

As noted in our previous study, and shown here in Fig. 1, while most of the γm was degraded in the course of a 4-h chase, little, if any, γs was degraded during the chase period. Although in Fig. 1b ALLM appears to be more efficient than ALLN in inhibiting the degradation of γm, inhibition of degradation by ALLN was more efficient in other experiments. We believe that the variable extent
of protection seen with different proteasomal inhibitors may reflect experimental variability, rather than a greater sensitivity to ALLM.

Inhibition of proteasomal activity contributes to the accumulation of a glycosylated nonretrotranslocated form of γm

For a small subset of ER-derived proteins, deglycosylated degradation intermediates that accumulate in the presence of proteasomal inhibitors have been identified (1, 6–9). For many other proteins of ER origin that are also degraded in proteasomes, such intermediates have not been reported. We noted during our pulse-chase studies that the γm protein that accumulated in a proteasomal inhibitor-dependent manner was apparently unchanged in mobility during the chase. This suggested that γm might accumulate in the ER in a glycosylated form when proteasomal activity is inhibited. However, because γ2b proteins have a single N-glycan unit, and other posttranslational modifications (such as phosphorylation or ubiquitination) could theoretically mask a deglycosylation-induced size alteration, we considered it possible that our PAGE conditions might not have been able to readily discriminate between glycosylated and nonglycosylated γm forms. Ig H chains were isolated after the pulse, and after a 4-h chase performed in the presence of ALLN and lysed. One-half of the Ig H chain proteins isolated from each of the chase lysates was subjected to endoglycosidase H treatment.

Although the most obvious interpretation of the above data is that γm accumulates in the ER before retrotranslocation, it could be argued that the glycosylated γm that accumulates in the presence of ALLN may actually have undergone partial or complete retrotranslocation, but may not yet have been deglycosylated or released on the cytosolic side. Indeed, deglycosylated and ubiquitinated MHC class I H chains, which have been dislocated by US11 and are largely cytosolically disposed, remain associated with ER membranes (41). We isolated membranes from cells that had been incubated with ALLN for the entire 4-h chase period and treated these membranes with trypsin before detergent lysis and isolation of Ig H chains. If indeed this protein because trypsin would cleave the cytoplasmic tail (which contains a KVK sequence adjacent to the membrane followed by 25 aa), leaving the luminal and transmembrane domains intact. In the protected γm that accumulates in the presence of ALLN the presence of ALLN, the H chains that pretreated these membranes with trypsin before trypsin inhibitor before lysis with trypsin inhibitor (lane 3). Ig H chains were isolated on PAS and separated by SDS-PAGE.
untouched. As seen in Fig. 2b, a small, ~3-kDa decrease in size of \( \gamma_m \) was noted after trypsin treatment, suggesting that in the presence of proteasomal inhibitors, this protein accumulates in the ER before retrotranslocation. In all of our experiments, we noted a slight decrease in the intensity of the cleaved \( \gamma_m \) band (see Fig. 2b), as well as some decrease in the intensity of the \( \gamma_s \) band after trypsin treatment, when compared with the sample in the untreated lane. Some of the loss of both \( \gamma_m \) and \( \gamma_s \) probably reflects the existence of a small proportion of leaky or inside-out vesicles. The diffuse nature of the cleaved \( \gamma_m \) band may reflect the fact that there are a total of five potential tryptic sites (including the two lysines immediately adjacent to the membrane) in the cytoplasmic tail of \( \gamma_m \). In addition, there is a methionine residue in the tail, whose loss may contribute to the reduced intensity of the \( \gamma_m \) band in the trypsin-treated samples (middle lane in Fig. 2b) as compared with the untreated material. Although the existence of multiple tryptic cleavage sites and the loss of a radiolabeled residue in the tail may account for the less intense and more diffuse cleaved \( \gamma_m \) band after trypsin treatment of the membranes, it remains possible that some of the loss of intensity may have resulted from partial retrotranslocation of \( \gamma_m \). Overall, these data suggest that retrotranslocation and proteasomal degradation of \( \gamma_m \) are closely coupled processes.

Degradation of \( \gamma_m \) in COS cells also occurs in a proteasome-dependent manner

To examine the preferential degradation of \( \gamma_m \) in mechanistic detail, it was necessary to examine the fate of newly synthesized membrane and secretory Ig H chains. Expression constructs encoding \( \gamma_m \) or \( \gamma_s \) were separately transfected into COS cells, followed by pulse labeling and chase studies, as described for 18-81 A2 cells. As seen in Fig. 3a, \( \gamma_m \) was rapidly degraded in COS cells, as observed in the pre-B cell line, and this degradation process was attenuated in the presence of a proteasomal inhibitor (Fig. 3b). In contrast, however, degradation of \( \gamma_s \) occurred more slowly, and little loss of this form of the \( H \) chain was seen over a 4-h chase period. These experiments suggest that free \( \gamma_m \) H chains may be intrinsically more prone to proteasomal degradation than free \( \gamma_s \) H chains.

At steady state Bip associates more efficiently with \( \gamma_m \) than with \( \gamma_s \) in transfected COS cells

Bip associates with both membrane and secretory Ig H chains in the absence of L chains. This association is dependent in part on the presence of an exposed CH1 domain. Although the coprecipitation of labeled Bip with both \( \gamma_m \) and \( \gamma_s \) can be observed in metabolically labeled cells, as seen in Fig. 3a, the amount of Bip that is brought down will in part reflect the steady state accumulation of (primarily unlabeled) \( \gamma_m \) or \( \gamma_s \), as the case may be. A more quantitative approach requires the affinity isolation of Ig proteins, followed by an immunoblot assay for associated Bip. COS cells were transfected with expression constructs for either the membrane or secretory forms of \( \gamma H \) chains. When equivalent amounts of \( \gamma_m \) and \( \gamma_s \) were affinity isolated, considerably higher levels of coprecipitating Bip were seen with \( \gamma_m \) (Fig. 4). The development Ab used in the immunoblot assay recognizes an epitope on both Bip and Grp94. However, little, if any, Grp94 coprecipitated with either \( \gamma_m \) or \( \gamma_s \). This is perhaps not surprising, because the demonstration of the association of other proteins with Grp94 has required chemical cross-linking or the use of apyrase (42). In other affinity isolation experiments, we failed to show the association of calreticulin with either \( \gamma_m \) or \( \gamma_s \) (data not shown). These results also suggest some degree of specificity in the interaction of misfolded/unassembled Ig H chains with ER chaperones.

Calnexin associates preferentially with \( \gamma_m \) as compared with \( \gamma_s \) in B lineage cell lines and in transfected COS cells

As a first step toward examining whether the membrane and secretory forms of Ig H chains associate in an equivalent manner with calnexin, we chose to examine cell lines that express different proportions of \( \gamma_m \) and \( \gamma_s \) proteins. In 18-81 A2 pre-B cells, most of the \( H \) chain is of the secretory form at steady state (Fig. 5a), and this predominates, although some \( \gamma_m \) is also made. The size differences of \( \gamma_m \) in these cell lines primarily reflect a specific difference in size of the core polypeptides, although at steady state a proportion of the \( \gamma_m \) in A20.25 (but not in 18-81 A2 cells) has already transited.
Calnexin associates preferentially with \( \gamma_m \) a. Detergent lysates were prepared from equal numbers of murine 18-81 A2 pre-B cells and A20.25 B cells, and IgGs were affinity isolated using PAS. Samples were separated by SDS-PAGE, and immunoblot filters were probed for calnexin (upper panel) using SPA-860 and for IgG H chains (lower panel) using an affinity-purified anti-mouse IgG Ab. A. A schematic representation of the \( \gamma \) constructs used in COS cell transfections. Wild-type membrane-bound \( \gamma_2a \) is represented as \( \gamma_m \). The sequences of the entire spacer region and transmembrane domain as well as a portion of the cytoplasmic tail are presented here. The \( \gamma_m \Delta \) mutant contains a stop codon after the three proximal amino acids of the cytoplasmic tail. This –KVK cytoplasmic tail is identical to that of \( \mu_m \) and \( \delta_m \). Another mutant, denoted \( \gamma_m \Delta TM \Delta cyt \), lacks both the transmembrane region as well as the cytoplasmic tail, and is therefore not anchored to a membrane. It does contain the highly negative charged spacer region (encoded by membrane exon 1). Another soluble \( \gamma \) molecule, termed \( \gamma_s \), is a \( \gamma_2b \) protein. This was encoded by a construct including a rearranged murine \( \gamma_2b \) gene that was truncated up-stream of the membrane exons, and thus produces secretory \( \gamma_2b \) H chains that lack the spacer region, the transmembrane domain, and the cytoplasmic tail. c. Transfected membrane-anchored Ig H chains can bind calnexin. COS cells were mock transfected, transfected with \( \gamma_m \), \( \gamma_m \Delta cyt \), \( \gamma_m \Delta TM \Delta cyt \), or with \( \gamma_s \). Forty-eight hours posttransfection, cells were lysed and the lysate was incubated with control BSA-Sepharose (B-S) or PAS. Eluted proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and probed with an anti-calnexin Ab (SPA-860).

**Proteasomal degradation of \( \gamma_m \) is unaffected by castanospermine**

Castanospermine is an inhibitor of glucosidase I and II and thus prevents the trimming of the glucose residues on N-linked GlcNAc\(_2\)Man\(_2\)Glc\(_3\) side chains. As a result, monoglycosylated forms of glycoproteins are not generated. Although castanospermine could conceivably inhibit proteasomal degradation of ER proteins by preventing the association of calnexin with monoglycosylated targets, in general this inhibitor enhances the rate of proteasomal degradation of ER-derived proteins, suggesting a protective role for calnexin during the ER degradation of certain proteins (43–45). Although calnexin may participate in the identification of misfolded proteins in the ER for proteasomal degradation, by promoting the assembly of multisubunit proteins, calnexin may impede the rate of proteasomal degradation of certain polypeptides.

Exposure of A20.25 cells to castanospermine resulted in a predicted decrease in the migration of \( \gamma_m \) (Fig. 6b). This, however, did not inhibit the association of this protein with calnexin (Fig. 6a). These results suggest that the binding of membrane Ig H chains to calnexin at steady state may not depend on the recognition of N-linked oligosaccharides. In studies performed in parallel using 18-81 A2 cells, the association of calnexin with Ig H chains was not influenced by castanospermine (data not shown).
18-81 A2 cells were pulse labeled with [35S]methionine for 20 min, as rated on SDS-PAGE, and transferred proteins were revealed with an anti-calnexin Ab (SPA-860). Lysis from 90% of the cells was set aside for detection of calnexin association. Cell lysates were incubated with a control matrix (BSA-Sepharose, B-S) or with PAS. Proteins were separated for detection of calnexin association. Cell lysates were incubated with 1 mM castanospermine (CST) for 6 h. Lysate from 90% of the cells was set aside by lysis and isolation of H chains using PAS. Glucose residues on the γ-chain were not trimmed in the cells treated with castanospermine.

FIGURE 6. Castanospermine does not affect the steady state association of calnexin with γm, and does not influence the rate of proteasomal degradation of γm. a, A20.25 B cells were mock treated or incubated with 1 mM castanospermine (CST) for 6 h. Lysate from 90% of the cells was set aside for detection of calnexin association. Cell lysates were incubated with a control matrix (BSA-Sepharose, B-S) or with PAS. Proteins were separated on SDS-PAGE, and transferred proteins were revealed with an anti-calnexin Ab (SPA-860). b, 10% of the mock-treated or castanospermine-treated A20.25 cells were metabolically labeled with [35S]methionine, followed by lysis and isolation of H chains using PAS. Glucose residues on the γ-chain were not trimmed in the cells treated with castanospermine. c, 18-81 A2 cells were pulse labeled with [35S]methionine for 20 min, as described in legend to Fig. 1, and chased for 4 h with or without ALLN or CST, or both. Lysates were obtained from the pulse-labeled cells and from cells chased with or without proteasomal and/or glucosidase inhibitors. Ig H chain proteins were isolated using PAS, and proteins were separated using SDS-PAGE. d, 18-81 A2 cells were pulse labeled with [35S]methionine for 20 min and chased either without (left panel) or with (right panel) castanospermine for 1, 2, and 4 h. Ig H chains were isolated using PAS, and proteins were analyzed by SDS-PAGE.

Presence and absence of castanospermine revealed that degradation of γm does not occur in an accelerated manner in the presence of castanospermine (Fig. 6d). This result is consistent with an earlier study indicating that the association of calnexin with membrane IgM H chains is dependent on the transmembrane domain of the Ig chain (32), and suggests that if calnexin influences the proteasomal degra-

Discussion

We have demonstrated that the rapid degradation of γm in a pre-B cell line and in transfected COS cells occurs in proteasomes. This establishes that free Ig H chains, like misfolded Ig L chains (34), are degraded in proteasomes. It is likely that γs is also degraded in proteasomes, but that the process occurs more slowly for this form of the H chain protein. The accumulation in the presence of proteasomal inhibitors of N-glycosylated γm in the ER before retrotranslocation suggests that retrotranslocation and proteasomal degradation of γm may be closely coupled processes. Why exactly a deglycosylated form of the protein does not apparently build up in the presence of proteasomal inhibitors is unclear.

The quality control process for misfolded or incompletely assembled proteins in the ER dictates that these molecules be first identified as candidates for degradation. Once marked, these proteins are then transported across the ER membrane and targeted to proteasomes. We consider it likely that γm is kinetically more prone to misfolding than γs. As a result, γm may associate more stably with ER chaperones such as Bip and calnexin. It is likely that the interaction of these chaperones with γm identifies this protein as a target for retrograde translocation and eventual proteasomal degradation. The preferential association of these chaperones with γm may correlate with the far more rapid proteasomal degradation of γm as opposed to γs originally observed in the 18-81 A2 cell line.

Carbohydrate-independent interactions with calnexin have been demonstrated for MHC class I molecules (46), MHC class II molecules (47), thyroglobulin (48), and P glycoprotein (49, 50). It is possible that in all of these interactions, the initial contact between calnexin and its targets depends on the recognition of monoglycosyl oligosaccharide chains on the latter; subsequently, after the polypeptide has attained its native conformation, protein-protein interactions might lead to the continued retention of certain polypeptides that await their partners. In the case of ribonuclease A, it is clear that the binding of this protein to calnexin is exclusively a carbohydrate-lectin interaction (29, 30). A potential criticism of studies on calnexin-substrate interactions that have employed either pharmacological agents such as tunicamycin or point mutations to abrogate N-linked sugar addition is that these manipulations may lead to the misfolding and aggregation of proteins, thus causing them to associate tightly with calnexin.

In the case of the Ig H chain protein, preferential binding of calnexin to the membrane form is observed, although the secretory form is equivalently modified by the cotranslational addition of oligosaccharides. It has been shown previously that apart from interacting with monoglycosylated oligosaccharide side chains, calnexin also associates with protein aggregates (51). The preferential association of calnexin with γm as opposed to γs may actually reflect the propensity of γm to misfold and form aggregates more readily than γs. Aggregated γm may therefore preferentially associate with calnexin, and, perhaps as a result, be more rapidly degraded.

ER chaperones recognize misfolded proteins and may remain associated with them till they fold properly and exit the ER; alternatively, they may deliver these misfolded proteins to the retrotranslocation machinery. Chaperones go through cycles of substrate release and rebinding, and if a retained protein substrate cannot properly refold, it is apparently targeted for degradation. How exactly ER proteins are marked for degradation and transferred from chaperones to the retrotranslocation machinery remain intriguing issues that are presently not well understood.
Barely detectable amounts of membrane Ig H chains exit the ER and are transported to the cell surface in pre-B cells (52). The synthesis and assembly of components of the pre-B receptor and of the B cell receptor occur with equivalent efficiency; however, pre-B cells are unable to transport complexes of Ig H chains assembled with either endogenous surrogate L chains, or transfected conventional L chains, to the surface (53). Pre-B cells appear to have an intriguing but poorly understood ability to retain membrane Ig-containing complexes and to target them for proteasomal degradation.

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**References**


