Pivotal Role of Calnexin and Mannose Trimming in Regulating the Endoplasmic Reticulum-associated Degradation of Major Histocompatibility Complex Class I Heavy Chain*

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We have established a mammalian semipermeabilized cell system that faithfully reconstitutes the proteasome-mediated degradation of major histocompatibility complex Class I heavy chain. We show that degradation required unfolding of the protein and was cytosol- and ATP-dependent and that dislocation and degradation required proteasome activity. When the interaction of heavy chain with calnexin was prevented, the rate of degradation was accelerated, suggesting that an interaction with calnexin stabilized heavy chain. Stabilization of heavy chain to degradation was also achieved either by preventing mannose trimming or by removal of the N-linked glycosylation site. This demonstrates that glycosylation and mannose trimming are required to ensure degradation of heavy chain. When degradation or mannose trimming was inhibited, heavy chain formed a prolonged interaction with immunoglobulin heavy chain binding protein, ERP57, and protein disulfide isomerase. Taken together, these results indicate that calnexin association and mannose trimming provide a mechanism to regulate the folding, assembly, and degradation of glycoproteins entering the secretory pathway.

The ability of the endoplasmic reticulum (ER)† to recognize and retain unassembled and malfolded proteins has been identified as an important regulatory mechanism to ensure that only correctly folded and assembled proteins are transported through the secretory pathway (1). One consequence of this “quality control” is that retained proteins are selectively degraded by a mechanism that is distinct from lysosomal degradation and has previously been suggested to take place within the ER (2). It is now clear from a number of studies that this degradation takes place within the cytosol, is mediated by the proteasome, and requires dislocation of the polypeptide targeted for degradation from the ER to the cytosol (reviewed in Refs. 3 and 4). However, it is not clear how the cell regulates the ER-associated degradation (ERAD) process or how proteins destined for degradation are transported from the ER to the proteasome.

Several workers have tried to address these questions, and a consensus is emerging as to the sequence of events that results in the degradation of proteins originally located within the ER. Once translocated into the ER, newly synthesized polypeptides will normally fold and assemble to form native protein molecules that can be further transported to the Golgi apparatus. However, during conditions of stress (5), in the absence of interacting subunits (6), or after viral infection (7, 8), newly synthesized proteins are unable to adopt the correct native structure and either form prolonged interactions with ER molecular chaperones or are rapidly degraded. A similar process occurs when mutant proteins that are unable to fold correctly are expressed, either in yeast (9, 10) or in mammalian cells (11, 12). Degradation is coupled to dislocation, probably via the Sec61 complex, in a process believed to be the reverse of translocation into the ER. Evidence for the role of Sec61 complex in dislocation comes from both genetic (13, 14), and biochemical approaches (8, 15, 16) that have shown a reduction in degradation of model substrates in Sec61 mutant yeast strains and an interaction of proteins targeted for degradation with components of the Sec61 complex. Once dislocated to the cytosol, the polypeptide chains can then be degraded directly by the proteasome (6) or can be deglycosylated (7) or ubiquitinated (16) prior to degradation.

From the sequence of events outlined above, it is clear that a close relationship must exist between folding and assembly on the one hand and targeting for degradation on the other. It has been suggested that one mechanism whereby proteins can be targeted for degradation is by regulating the trimming of N-linked oligosaccharide chains (17, 18). Prior to folding, most glycoproteins interact with either calnexin, calreticulin, or both; the interaction with these proteins is regulated by the glucosylation state of the oligosaccharide side chain (19). If the glycoprotein is released from calnexin prior to correct folding, the Manα,GlcNAc2 side chain can be deglucosylated by UDP-glucose-glycoprotein glucosyltransferase, thereby initiating another round of binding to calnexin. This binding to calnexin retains glycoproteins within the ER until they fold correctly or assemble with other subunits to form multi-subunit complexes. The oligosaccharide side chain can also be modified within the ER by the action of mannosidase I that removes terminal mannose residues. Interestingly, inhibition of the mannose trimming either by expression in ER mannosidase-deficient yeast strains (17) or by incubation of mammalian cells in the presence of deoxymannojirimycin (12, 18, 20, 21) results in stabilization of polypeptide chains that would normally be degraded. These results have lead to the hypothesis that mannose...
Mannose Trimming Regulates ERAD

trimming provides a timing mechanism allowing the lifetime of a protein within the ER to be regulated.

To investigate the molecular interactions occurring during the process of ERAD, we have developed a cell-free system to study the degradation of MHC Class I heavy chain. Cell-free systems have been used in the past to demonstrate that the degradation process is proteasome-mediated, requires ATP, can involve ubiquitination of the substrate, and occurs after dislocation through the Sec61 complex (10, 14, 22). Our results highlight the pivotal role played by calnexin in regulating assembly and degradation.

MATERIALS AND METHODS

Monoclonal antibody HC10 was a gift from Dr. Hidde Ploegh (Harvard Medical School, Boston, MA); rabbit polyclonal antibodies against TAP1 and TAP2 were a gift from Dr. Jacques Neefjes (Netherlands Cancer Institute, Amsterdam, Netherlands); W6/32 antibody was purchased from Serotec Ltd. (Oxford, United Kingdom); rabbit polyclonal antibody to β2 microglobulin was purchased from AMS Biotechnology (Whitney, United Kingdom); rabbit polyclonal antibody to BiP was purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada); rabbit polyclonal antibody to e-Myc was purchased from Santa Cruz Biotechnology (Santa Cruz, California, United States); rabbit polyclonal to calreticulin was from Chemicon (Temecula, California, United States); rabbit polyclonal to BiP was purchased from Stressgen (Victoria, British Columbia, Canada); rabbit polyclonal to calreticulin, and BiP were purchased from Promega (Southampton, United Kingdom). Rabbit reticulocyte lysate (Flexi-lysate®, Promega) for 60 min at 30 °C. Flexi-lysate was slowly layered onto a 9.5-ml 5–25% sucrose gradient. Proteins in the gradient were collected and analyzed by SDS-PAGE.

RESULTS

MHC Class I Molecules Are Assembled and Can Be Targeted for Degradation in Semipermeabilized Cells—We have previously demonstrated that when MHC Class I heavy chain was translated in the presence of SP cells derived from the human cell line HT1080, a heavy chain/β2 microglobulin heterodimer could be formed, as judged by immunoprecipitation with the conformational specific antibody W6/32 (26). This assembly was inefficient, probably due to a limited amount of β2-microglobulin in the cell line. To improve efficiency of assembly, we used here the human embryonic CEM, which is lymphoblastoid in origin and has been used previously to study MHC Class I assembly (27). For these studies we have used the B27 allele of human MHC Class I heavy chain. When B27 RNA was translated in the presence of CEM cells, the polypeptide chain was N-glycosylated, as judged by decay in electrophoresis.

In Vivo Translation—Translations were carried out in rabbit reticulocyte lysate (Flexi-lysate®, Promega) for 60 min at 30 °C. Flexi-lysate does not contain added DTT and, therefore, supports the formation of disulfide bonds in newly synthesized polypeptides (26). A standard translation mixture consisted of 16.5 µl of reticulocyte lysate, 0.5 µl of rabbit reticulocyte lysate, and 1 µl of transcribed RNA, 1 µl of [35S]methionine (10 µCi/µl), and 5.5 µl of KH buffer (100 mM KOAc, 2 mM MgOAc, 20 mM HEPES, pH 7.2) or semipermeabilized (SP) cells (—5 × 10^6) prepared as described previously (26). Where appropriate, SP cells were pretreated prior to translation with either 1 mM castanospermine or 1 mM 1-deoxymannorijimycin for 10 min at 30 °C.

Proteinase K Digest—Translation products were incubated with proteinase K (200 µg/ml) on ice for 25 min. Proteinase K was then inhibited with 0.5 mM phenylmethylsulfonyl fluoride on ice for 5 min. Samples were prepared for SDS-PAGE by the addition of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, bromphenol blue) containing DTT (50 mM).

In Vitro SP Cell Degradation Assay—Translation of heavy chain mRNA was carried out in the presence of SP cells for 60 min as described above. For these experiments, the total volume of translation mixture was 250 µl, i.e. 10 times the standard mixture. Further protein synthesis was then inhibited by the addition of cycloheximide to 5 mM. The SP cells were isolated by centrifugation for 2 min at 12,000 × g and resuspended in 1 ml of KH buffer. The resuspended SP cells were divided into two aliquots of 140 µl and resolated by centrifugation for 2 min at 12,000 × g. Isolated SP cells were resuspended in either 10 µl of KH buffer or 5 µl of untreated reticulocyte lysate and 5 µl of energy regeneration system (0.5 mM ATP, 10 mM phosphocreatine, 1.6 mg/ml creatine phosphokinase, 40 mM Tris-HCl, 5 mM MgCl2, 2 mM DTT). Degradation of heavy chain was carried out at 37 °C for various times up to 4 h. For proteasome inhibition studies, untreated reticulocyte lysate or SP cells were pretreated for 15 min with either MG132 (25 µM), ALLN (25 µM), clasto-lactacystin β-lactone (100 µM), or lactacystin (1 µM).

ATP Depletion—SP cells and untreated reticulocyte lysate were depleted of endogenous source of ATP by pretreatment with 0.1 units/ml hexokinase (1.5 units/ml stock), 0.22 mM glucose, and 6.5 mM MgCl2 for 15 min at room temperature. For the stabilization of BiP-heavy chain complexes, SP cells were incubated as above with hexokinase, glucose and MgCl2, and dissociated with the addition of excess ATP at 1 mM for 15 min at room temperature. SP cells were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) and subjected to immunoprecipitation.

Immunoprecipitation—SP cells isolated from translation reactions were solubilized in 500 µl of lysis buffer. Protein A-Sepharose (50 µl) (10% w/v) was added, and samples were incubated for 30 min on ice. Samples were centrifuged for 15 s, and supernatants transferred to a fresh 1.5-ml tube. Antibodies were then added (1 µl of PDI, ERp57, calreticulin, and β2 microglobulin; 2 µl of calnexin, TAP1, TAP2, W6/32, and BiP; and 10 µl of HC10) along with protein A-Sepharose (50 µl), and samples were incubated for 16 h. Samples were centrifuged for 15 s to pellet protein A-Sepharose, and supernatant was discarded. Pellets were washed three times with lysis buffer, and pellets were resuspended in 30 µl of SDS-PAGE sample buffer containing DTT (50 mM) prior to analysis by electrophoresis.

SP Cell Fractionation following Degradation Assay—Individual aliquots (10 µl) of SP cells in untreated reticulocyte lysate from the degradation assay were layered on top of 300 µl of sucrose cushion (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, and 0.5 mM sucrose) and centrifuged at 150,000 × g at 4 °C for 10 min in Beckman ultracentrifuge (TLA100.1 rotor) to yield a supernatant and pellet. The proteins were precipitated from the supernatants by the addition of 10% (w/v) trichloroacetic acid and 10% (v/v) acetone and placed on ice for 10 min. Insoluble proteins were isolated by centrifugation at 14,000 × g at room temperature for 10 min, washed with 0.5 ml of ice-cold 100% acetone for 5 min, air-dried, and resuspended in SDS sample buffer containing DTT (50 mM). The pellets containing SP cells were resuspended in SDS-PAGE sample buffer containing DTT (50 mM).

Sucrase Gradient Analysis—All sucrose solutions were prepared in gradient buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (v/v) Triton, 1 mM phenylmethylsulfonyl fluoride). Equal volumes of 25 and 5% (w/v) sucrose were differentially mixed using a gradient maker to create a 10–50% (w/v) sucrose gradient. This was gradually layered onto 0.5 ml of 50% (w/v) sucrose in a Beckman SW-41 rotor at 4 °C. Following translation, SP cells were isolated and lysed for 30 min in 0.5 ml of gradient buffer. The lysate was centrifuged at 12000 × g for 15 min, and the supernatant was slowly layered onto a 9.5-ml 5–25% sucrose gradient. Proteins were recovered in the supernatant and sedimented through the gradient by centrifugation at 40,000 × g (Beckmann SW-41 rotor) for 18 h at 4 °C.

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translation prior to protease digestion is due to the removal of the cytoplasmic tail. The product of translation was completely degraded when the cells were solubilized with Triton X-100 prior to digestion, demonstrating that the translation product was not intrinsically resistant to proteolysis (Fig. 1A, lane 4). It is worth noting that the reticulocyte lysate used during translation does not contain any added DTT and therefore supports the formation of disulfide bonds as demonstrated previously (26). That the heavy chain had folded correctly and formed a dimer with β2-microglobulin was demonstrated by co-immunoprecipitating the translation product with antibodies to this protein (Fig. 1A, lane 8). This precipitation was specific, as no translation product was immunoprecipitated with preimmune serum (Fig. 1A, lane 6). Assembly into a complex could also be demonstrated by immunoprecipitation with the conformational specific antibody W6/32 (28) (Fig. 1A, lane 7). Immunoprecipitation of heavy chain with the monoclonal antibody HC10 (which recognizes unassembled chains) (29) shows that the newly synthesized heavy chain is in excess (Fig. 1A, lane 5), the yield of complex thereby being limited by the availability of free β2 microglobulin.

The rabbit reticulocyte lysate used for the translation of heavy chain contains added hemin, a reagent that has been shown previously to inhibit proteasomal activity (30). Therefore, to be able to study ERAD, we first isolated cells from the translation mixture after inhibiting further protein synthesis by the addition of cycloheximide. The SP cells were resuspended either in buffer alone or in the presence of an untreated reticulocyte lysate that did not contain added hemin (25). When the SP cells were resuspended in the presence of this lysate, an ATP regeneration system, and DTT, a time-dependent decrease in the translated heavy chain was observed (Fig. 1B, lanes 1–6). When the SP cells were resuspended in buffer alone, no such decrease occurred (Fig. 1B, lane 7). It is important to note that no immunoprecipitation of translation products was carried out, so this observed decrease is due to degradation of the translation product. The degradation of heavy chain in the presence of untreated reticulocyte lysate was shown to be ATP-dependent (Fig. 1B). However, endogenous ATP bound to ER chaperones such as BiP could be released during the degradation time course. This would account for the small reduction in heavy chain (approximately 20%) observed during the time course. Degradation of heavy chain in the presence of untreated reticulocyte lysate was accelerated by the addition of DTT. DTT is a reducing agent that will break the intrachain disulfide bonds within the translated heavy chain, thereby accelerating unfolding. Hence, the effect of DTT on the degradation process is likely to be due to the unfolding of unassembled heavy chain and disassembly of MHC Class I molecules formed during translation. When heavy chain is translated in the absence of cells, it will not fold correctly, and when this translation product was added to the degradation assay, a more rapid process of degradation was observed (Fig. 1B, bottom panel). This more rapid degradation could be due to either the heavy chain being unfolded or the fact that there was no requirement for transport from the ER. These results demonstrate that the translated glycosylated heavy chain can be degraded posttranslationally from SP cells by a process that is dependent on an added cytosolic fraction (in this case, an untreated reticulocyte lysate) and ATP and can be accelerated by agents that induce the unfolding of this protein. In all subsequent degradation assays, DTT was included to ensure unfolding of the heavy chain.

To evaluate whether the degradation process that we were observing in our SP cells was mediated by the proteasome, we determined the ability of ALLN, MG132 and clasto-lactacystin

![Fig. 1](http://www.jbc.org/content/258/14/21226/F1.large.jpg)


**Fig. 2.** Inhibition of ERAD and location of proteasome activity. A, mRNA coding for heavy chain was translated for 60 min in the presence SP cells. Further protein synthesis was inhibited with cycloheximide (5 mM), and the SP cells were isolated by centrifugation and washed in KHM buffer. Aliquots of SP cells were resuspended either in reticulocyte lysate (without hemin) treated with ALLN, MG132, or clasta-lactacystin β-lactone and an energy regeneration system containing DTT (lanes 1–3) or in KHM buffer and an energy regeneration system containing DTT (lane 4). Degradation was terminated by the addition of SDS-PAGE sample buffer containing DTT (50 mM). Products were separated by SDS-PAGE and analyzed by autoradiography. B, mRNA coding for heavy chain was translated for 60 min in the presence SP cells. Following translation, isolated cells were pretreated with clasta-lactacystin β-lactone for 10 min at 37 °C. Cells were reisolated and resuspended in untreated reticulocyte lysate (without hemin) (lanes 4–6) and an energy regeneration system containing DTT. Alternatively, heavy chain mRNA was translated for 60 min in the presence of SP cells and degradation analyzed as described above in the presence of reticulocyte lysate (without hemin) that had been treated with clasta-lactacystin β-lactone (lanes 1–3). Degradation was terminated by the addition of SDS-PAGE sample buffer containing DTT (50 mM). Products were separated by SDS-PAGE and analyzed by autoradiography. Band intensity at each time point was quantified by phosphorimager analysis and plotted against time of assay. Each point represents an average value from three experiments, with the error bars representing S.E.

**Fig. 3.** Heavy chain is not dislocated from the ER to the cytosol and remains inserted in the ER membrane. A, mRNA coding for heavy chain was translated for 60 min in the presence SP cells. Further protein synthesis was inhibited with cycloheximide (5 mM); the SP cells were isolated by centrifugation and washed in KHM buffer. Aliquots of SP cells were resuspended either in reticulocyte lysate (without hemin) treated with ALLN and an energy regeneration system containing DTT (lanes 1–5) or in KHM buffer and an energy regeneration system containing DTT (lane 6). Degradation was terminated by the addition of 20 mM NEM, and samples were separated through a sucrose cushion. Supernatants were removed and proteins precipitated with 10% (w/v) trichloroacetic acid and resuspended in SDS-PAGE sample buffer containing DTT (50 mM) (supernatants, lanes 1–6). Pellets were resuspended in SDS-PAGE sample buffer containing DTT (50 mM) (pellets, lanes 1–6). Products were separated by SDS-PAGE and analyzed by autoradiography. B, mRNA coding for heavy chain was translated for 60 min in the presence SP cells. Further protein synthesis was inhibited with cycloheximide (5 mM), and the SP cells were isolated by centrifugation and washed in KHM buffer. Aliquots of SP cells were resuspended either in reticulocyte lysate (without hemin) treated with ALLN and an energy regeneration system containing DTT (lanes 1–4) or in KHM buffer and an energy regeneration system containing DTT (lane 5). Samples were treated with proteinase K (lanes 2–5) and resuspended in SDS-PAGE sample buffer containing DTT (50 mM). Products were resuspended in SDS-PAGE and analyzed by autoradiography.

β-lactone (lactacystin) to inhibit this process. ALLN and MG132 are nonspecific inhibitors of the proteasome, whereas lactacystin is specific, modifying an essential threonine residue within the catalytic core (31). The three inhibitors blocked the degradation of heavy chain from SP cells when they were incubated in the presence of untreated reticulocyte lysate, ATP, and DTT (Fig. 2A, lanes 1–4). Lactacystin was found to inhibit degradation by approximately 90% when included at a concentration of 30 μM (results not shown). No degradation was observed when SP cells were incubated in the presence of a reticulocyte lysate containing added hemin (results not shown), further indicating that the degradation was proteasome-mediated. To determine whether the proteasome activity was present in the SP cells or the added reticulocyte lysate, we preincubated either the SP cells or reticulocyte lysate with lactacystin, which irreversibly inhibits the proteasome (31). Pretreatment of SP cells did not prevent degradation, whereas pretreatment of reticulocyte lysate inhibited degradation (Fig. 2B). This further verifies that the proteolytic activity is present in the added reticulocyte lysate and is mediated by the proteasome.

The Degradation and Dislocation of Heavy Chain Are Coupled Processes—Previous studies into the mechanism of degradation of heavy chain in the presence of the CMV protein US2 or US11 has demonstrated that in the presence of proteasome inhibitors, heavy chain can be dislocated into the cytosol, where it appears as a soluble, deglycosylated protein (7, 8). We investigated whether heavy chain could be dislocated in the presence of proteasome inhibitors within our SP cell system. We isolated SP cells after a 4-h incubation in the presence of the untreated reticulocyte lysate and determined whether the heavy chain remained within the membranes of the SP cells or was released. Under these conditions, we detected no soluble heavy chain released from the SP cells (Fig. 3A). This does not exclude the possibility that heavy chain was dislocated but remained associated with the outside of the ER membrane of the SP cells during the course of this experiment. To test this, we carried out a proteinase K digest to determine whether the heavy chain was still sequestered in the ER membrane. The results (Fig. 3B) clearly demonstrate that the heavy chain present within the membranes is protected from proteolysis. Furthermore, the
cytosolic tail remains sensitive to degradation by proteasome K, demonstrating that the protein is still integrated in the membrane and that the cytosolic tail is still intact. Hence, in the absence of proteasome activity, the degradation of heavy chain is inhibited, and the dislocation from the ER membrane is also prevented. This suggests that at least in non-virally infected cells, the dislocation of MHC Class I heavy chain is coupled to degradation.

The Degradation of Heavy Chain Is Accelerated in the Absence of an Interaction with Calnexin—When MHC Class I heavy chain is expressed in cell lines deficient in calnexin, assembly can still occur, and a fully assembled complex can be expressed at the cell surface (27). Similarly, when heavy chain is expressed in insect cells in the presence of β2 microglobulin, assembly occurs; however, co-expression of calnexin prevents heavy chain degradation and thereby increases the yield of MHC Class I molecules (32). Calnexin, therefore, is not an absolute requirement for assembly but may be required to prevent the premature degradation of heavy chain. To investigate the role of calnexin in ERAD, we translated heavy chain in SP cells derived from the calnexin-deficient cell line CEM-NKR. Heavy chain translated under these conditions assembled to form a stable MHC Class I molecule that could be immunoprecipitated with antibodies to β2 microglobulin and with the conformational specific antibody W6/32 (results not shown). When the SP cells were isolated from the translation mix and added to the untreated reticulocyte lysate, very rapid degradation was observed (Fig. 4A, lanes 1–6). This degradation was dependent on the presence of the lysate (Fig. 4A, lane 7) and was inhibited both by ALLN and lactacystin, suggesting that it was mediated by the proteasome (results not shown).

As an alternative approach, we prevented the interaction of calnexin and calreticulin with heavy chain by carrying out translations in the presence of castanospermine. Calnexin and calreticulin associate specifically with monoglucosylated glycopeptides, so inhibition of glucose trimming with reagents such as castanospermine prevents interactions with these proteins (19). We also demonstrated that castanospermine prevented an interaction with calnexin and calreticulin during translation of heavy chain in SP cells (results not shown). When the SP cells were isolated from these translations and added to untreated reticulocyte lysate in the presence of castanospermine, a more rapid degradation of heavy chain was observed than that occurring with translation products synthesized in the absence of castanospermine (Fig. 4B). This degradation of heavy chain in the presence of castanospermine was inhibited with ALLN (results not shown). These results demonstrate that preventing an interaction with calnexin or calreticulin accelerated the rate of degradation. Given that this accelerated degradation occurs in CEM-NKR cells that contain calreticulin, this accelerated degradation is probably due to a lack of an interaction with calnexin.

It has been shown previously that calnexin associates with heavy chain at an early stage of MHC Class I assembly prior to association with β2 microglobulin (33). In our system, we are translating and assembling heavy chain prior to studying degradation, and therefore, after translation, most of the heavy chain will have dissociated from calnexin. To determine whether this is the case and whether calnexin interacts with heavy chain during the time course of degradation, we immunoprecipitated translation products at various times after translation. At the beginning of the time course, calnexin did not associate significantly with heavy chain; however, after 2 h, a clear increase in calnexin association was observed (Fig. 5A, lanes 1 and 2). After 4 h, the amount of material associating with calnexin diminished, coinciding with heavy chain degradation (Fig. 5A, lanes 2 and 3). A similar interaction of heavy chain with calnexin was observed when degradation was inhibited both with ALLN (Fig. 5B) and lactacystin (Fig. 5C). The opposite effect was observed with calreticulin. At the beginning of degradation...
of the time course of degradation carried out in the presence of ALLN, calreticulin was associated with heavy chain, consistent with its presence in a peptide-loading complex (34) (Fig. 5D, lane 1). After 4 h, most (>90%) of the heavy chain dissociated from calreticulin (Fig. 5D, lane 3). This dissociation of calreticulin from heavy chain mirrors the disassembly of the heavy chain/β2 microglobulin dimer (>80%) and also dissociation of heavy chain from the TAP complex (>80%) (Fig. 5, E and F). Thus, at the beginning of the time course, heavy chain is present in a loading complex with TAP and calreticulin but not calnexin. During the time course of degradation that takes place in the presence of DTT, the heavy chain unfolds, dissociating the complex and associates with calnexin. These results and the previous experiments demonstrating that preventing calnexin association accelerates degradation suggest that calnexin rather than calreticulin association delays the degradation of heavy chain by partitioning unfolded heavy chain away from the dislocation and degradation pathway. Preventing degradation could lead to an accumulation of unfolded heavy chain within the ER that remains glucosylated and associated with calnexin.

By preventing the degradation of heavy chain, we have shown that dislocation can be blocked, which should lead to a build-up of unfolded heavy chain in the ER. We looked at the possibility that under conditions in which degradation is inhibited, heavy chain could interact with other ER proteins. In this experiment, we carried out immunoprecipitations of translation products during the time course in the presence of ALLN with antibodies to PDI, ERP57, and BiP. To stabilize the interaction of heavy chain with BiP, immunoprecipitations were carried out after ATP depletion (35). A steady increase in the amount of heavy chain immunoprecipitated with PDI and ERP57 was observed over the time course (Fig. 6, A and B, lanes 1–3). MHC Class I does not interact with nonspecific antibodies, such as α-Myc, a polyclonal antibody to the c-Myc epitope (Fig. 6A, lane 4). A more marked increase in the heavy chain co-immunoprecipitated with antibodies to BiP was observed (Fig. 6C, lanes 1–3). This interaction was shown to be ATP-dependent, as incubation of the immunoprecipitate with ATP lead to dissociation of the complex (Fig. 6C, lane 4). These results show that other ER molecular chaperones, as well as calnexin, interact with heavy chain when degradation is inhibited.

**Requirement for Glycosylation and Mannose Trimming for ERAD of Heavy Chain**—To further study the role of glycosylation during the ERAD of heavy chain, we constructed a mutant form of heavy chain that lacked the N-linked glycosylation site. Such a mutated protein has been constructed previously and has been shown to be capable of assembly into transport competent MHC Class I molecules (36). Mutating the glycosylation site also results in a lack of binding to calnexin, again suggesting that calnexin association is not an absolute requirement for assembly. When the SP cells were isolated from the translation mixture and incubated in the presence of untreated reticulocyte lysate, no degradation of the S112A mutant was observed (Fig. 7A). This is in marked contrast to glycosylated wild type and suggests that either the oligosaccharide side chain is required to target the protein for ERAD or the unassembled heavy chain associates with other ER proteins. Alternatively,
the S112A mutant could form an aggregate, thereby preventing its dislocation and subsequent degradation. However, fractionation of the translation products on a sucrose gradient revealed that the S112A protein remains soluble and does not form high molecular weight aggregates (Fig. 7B).

If heavy chain requires an oligosaccharide side chain to ensure its ERAD and binding to calnexin delays the process, what regulates binding to calnexin? It is well established that deglucosylation of oligosaccharide side chains prevents binding to calnexin. Subsequent removal of mannose residues has also been hypothesized to either prevent rebinding to calnexin (17) or, conversely, stabilize calnexin/glycoprotein interactions due to a reduced deglucosylation of Glu1Man8GlcNAc2 oligosaccharides (18). To test the role of mannose trimming in ERAD, we translated heavy chain in the presence of an inhibitor of ER mannosidases DMJ. SP cells were isolated and incubated in the presence of cytosol and DMJ. The presence of DMJ resulted in the stabilization of the heavy chain to degradation (Fig. 8A).

This confirms previous studies on the ERAD of proteins in both yeast (17) and mammalian cells (20, 12, 18) and highlights the crucial role of mannose trimming in targeting proteins for degradation. Interestingly, if DMJ is included in the degradation time course, heavy chain not only is stabilized in the presence of the inhibitor of ER mannosidases DMJ. SP cells were isolated and incubated in the presence of cytosol and DMJ. The presence of DMJ resulted in the stabilization of the heavy chain to degradation (Fig. 8A). This confirms previous studies on the ERAD of proteins in both yeast (17) and mammalian cells (20, 12, 18) and highlights the crucial role of mannose trimming in targeting proteins for degradation. Interestingly, if DMJ is included in the degradation time course, heavy chain not only is stabilized but also forms a prolonged interaction with calnexin (Fig. 8B). Thus, when either mannose trimming or degradation is inhibited, the untrimmed or unfolded heavy chain remains associated with calnexin.

We have demonstrated that when translations were carried out in the presence of castanospermine, the rate of degradation was accelerated (Fig. 4B). As castanospermine inhibits deglucosylation, this may affect mannose trimming and therefore degradation. We determined whether preventing mannose trimming could inhibit the accelerated degradation of heavy chain after synthesis in the presence of castanospermine. When DMJ, in addition to castanospermine, was included during the degradation time course, no degradation occurred (Fig. 8C). This clearly shows that preventing deglucosylation itself does not target the protein for rapid degradation but that trimming of the core mannose oligosaccharide is also required. These results suggest that the acquisition of a mannose-trimmed oligosaccharide is an absolute requirement for degradation to occur.

**DISCUSSION**

It has now become well established that at least one of the mechanisms for degradation of proteins from the ER involves dislocation of the substrate from the luminal side of this organelle to the proteasome within the cytosol (3, 4). Although the general principle is now accepted, the underlying molecular mechanisms involved in regulating the process are not understood, nor have the proteins potentially involved in facilitating the process been identified or characterized. The general process can be outlined as follows: (i) either misfolded proteins or unassembled subunits are recognized as such and targeted for transport to the site of degradation; (ii) polypeptides are dislocated from the ER to the cytosol through the Sec61 transloca-
polypeptide could then assemble with the removal of a single mannose residue. The resulting
body or calnexin, the action of ER mannosidase I could result in
interacting with calnexin. This interaction with calnexin would
acting on heavy chain (HC) synthesis and translocation
into the ER lumen, the polypeptide can fold and assemble with β2 microglobulin (β2m) or can be reglucosylated and interact with calnexin (C). If β2 microglobulin is limiting, then the oligosaccharide side chain on heavy chain can become modified by ER mannosidase I (ERMI), which removes a single mannose residue. This step can be blocked by DMJ, thereby inhibiting degradation. The man8 form of heavy chain can be reglucosylated and associate with calnexin, can assemble with β2 microglobulin, or can be degraded by a process inhibited by ALLN or lactacycin (LC). Interactions with calnexin can be blocked by preventing the initial glucose trimming with CST or by synthesis in a calnexin-deficient cell line (CEM-NKR). This blocking of an interaction with calnexin leads to an acceleration of degradation.

degradation of glycoproteins.

The tenet on the right side of this hypothesized process is summarized in a modification of a scheme described previously (18) (Fig. 9). During and after translocation, the newly synthesized heavy chain interacts with calnexin, an interaction that is regulated by the glucosylation state of the oligosaccharide side chain. Once released from calnexin, either the heavy chain is recognized by UDP-glucose glycoprotein glycosyltransferase as unfolded and reglucosylated, thereby initiating rebinding to calnexin, or it assembles with β2 microglobulin to form a heterodimer. Rather than interacting directly with β2 microglobulin or calnexin, the action of ER mannosidase I could result in the removal of a single mannose residue. The resulting polypeptide could then assemble with β2 microglobulin, could be reglucosylated and reassociate with calnexin, or could be degraded by the proteasome. It has been suggested (18) that the association with calnexin itself targets the protein for degradation. In this case, the formation of the Man8NAc2Glc1 oligosaccharide side chain would prevent dissociation from calnexin, leading to a partitioning away from the folding pathway. However, we have shown that prevention of an interaction with calnexin (either by synthesis of the polypeptide in the presence of castanospermine, or by prevention of the formation of the monoglucosylated oligosaccharide side chain, or by synthesis in calnexin-deficient cells) leads to accelerated degradation. Hence, degradation can occur in the absence of an interaction with calnexin or calreticulin, arguing against either of these molecular chaperones targeting the protein for degradation.

In our model, the newly synthesized heavy chain can escape from degradation by assembling with β2 microglobulin or by interacting with calnexin. This interaction with calnexin would provide more time for the polypeptide to fold correctly or assemble with β2 microglobulin. If this interaction is blocked, then degradation is accelerated. We and others (12, 20, 21) have also shown that inhibition of mannos trimming significantly blocks degradation. Our findings also show that when calnexin association and mannos trimming are blocked, degradation is still inhibited. This demonstrates that the formation of the Man8NAc2 oligosaccharide side chain is essential to ensure degradation of glycoproteins. This raises a further question as to how ER resident glycoproteins are protected from similarly trimmed oligosaccharides. It could be that a combination of mannos trimming and unfolding is required to ensure degradation occurs, with the unfolded protein interacting with ER chaperones, such as BiP (5, 13). The Man8NAc2 oligosaccharide could then interact specifically with a lectin that would target the protein to the translocon, as has been suggested previously (17, 21). Thus, the combined actions of calnexin binding and mannos trimming would provide a timer mechanism initially allowing folding to occur and then, if this does not proceed, targeting the protein for degradation.

In our experimental system, we synthesize a mixed population of molecules during the initial translation, with heavy chain either being assembled with β2 microglobulin or remaining unassembled but partially folded. We show that at the end of this incubation, there is little binding of either form to calnexin, an observation that is consistent with previous results indicating that calnexin only associates during the initial stages of MHC Class I assembly (33). When we add a reducing agent and start the degradation assay, we initially see a reassociation with calnexin prior to degradation. This suggests that reglucosylation of heavy chain occurs and that interaction with calnexin competes with degradation. Interestingly, when we inhibit degradation we see an interaction with other ER chaperones, such as BiP, ERp57, and PDI. Under these conditions, the mannos residues on the oligosaccharide side chain would be trimmed. Hence, even though calnexin is not essential for correct folding and assembly of the MHC Class I molecule, it does provide a mechanism whereby unassembled heavy chain can remain competent to fold and associate with β2 microglobulin.

Such a preference for unfolded heavy chain to bind to calnexin rather than be targeted for degradation could also explain the lack of dislocation of heavy chain under conditions in which degradation has been inhibited. Other workers have shown the intimate relationship between degradation and dislocation and suggested that the proteasome itself could facilitate the dislocation process (37). One would predict that the dislocation of polypeptides from the ER to the cytosol would rely upon release of the polypeptide chain from any interactions occurring within the ER. This release, combined with either proteasomal activity or modifications to the polypeptide chain within the cytosol, such as ubiquitination, could ensure dislocation. It has also been shown recently that the cytosolic tail of heavy chain is required for dislocation, indicating that a physical association between the cytosolic tail and cytosolic factors is required to facilitate this process (38). Heavy chain does not appear to be ubiquitinated prior to dislocation (38, 39), so the dislocation would be reliant upon release from calnexin, cytosolic factors, and proteasomal activity. Thus, in our system, a lack of proteasomal activity coupled to a preferential interaction of unfolded heavy chain with calnexin leads to a block in dislocation and an accumulation of polypeptide chains interacting with calnexin and other ER chaperones. This is not the case in CMV-infected cells, in which the US2 and US11 gene products themselves facilitate rapid dislocation.
of heavy chain into the cytosol, even in the absence of proteasomal activity (7, 8). Also, when heavy chains are expressed in cells deficient in β2 microglobulin or TAP, the amount of unfolded heavy chain synthesized could saturate the ability of ER chaperones to prevent dislocation, leading to an accumulation of dislocated polypeptides in the cytosol (40).

The results presented in this paper clearly demonstrate that ERAD of MHC Class I heavy chain requires both glycosylation and modification of the oligosaccharide side chain, at least in our SP cell system. Others have shown similar requirements for ERAD of model proteins within both yeast (9, 17) and mammalian cells (11, 12, 18), indicating that we are truly reconstituting this process in our SP cell system. Our current hypothesis would suggest the role of additional factors that both target the modified protein for dislocation and facilitate the extraction of the polypeptide to the cytosol. This experimental approach will greatly facilitate the identification of these proteins and will allow a dissection of the dislocation process, in which proteins are targeted for ERAD from the ER to the cytosol.

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Pivotal Role of Calnexin and Mannose Trimming in Regulating the Endoplasmic Reticulum-associated Degradation of Major Histocompatibility Complex Class I Heavy Chain

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