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Endoplasmic Reticulum-Associated Degradation-Induced Dissociation of Class II Invariant Chain Complexes Containing a Glycosylation-Deficient Form of p41

Lisa M. Sevilla, Sarah S. Comstock, Kevin Swier, and Jim Miller

The quality control system in the secretory pathway can identify and eliminate misfolded proteins through endoplasmic reticulum-associated degradation (ERAD). ERAD is thought to occur by retrotranslocation through the Sec61 complex into the cytosol and degradation by the proteasome. However, the extent of disassembly of oligomeric proteins and unfolding of polypeptide chains that is required for retrotranslocation is not fully understood. In this report we used a glycosylation mutant of the p41 isoform of invariant chain (Ii) to evaluate the ability of ERAD to discriminate between correctly folded and misfolded subunits in an oligomeric complex. We show that loss of glycosylation at position 239 of p41 does not detectably affect Ii trimerization or association with class II but does result in a defect in endoplasmic reticulum export of Ii that ultimately leads to its degradation via the ERAD pathway. Although class II associated with the mutated form of p41 is initially retained in the endoplasmic reticulum, it is subsequently released and traffics through the Golgi to the plasma membrane. ERAD-mediated degradation of the mutant p41 is dependent on mannos trimming and inhibition of mannosidase I stabilizes Ii. Interestingly, inhibition of mannosidase I also results in prolonged association between the mutant Ii and class II, indicating that complex disassembly and release of class II is linked to mannosidase-dependent ERAD targeting of the misfolded Ii. These results suggest that the ERAD machinery can induce subunit disassembly, specifically targeting misfolded subunits to degradation and sparing properly folded subunits for reassembly and/or export. The Journal of Immunology, 2004, 173: 2586–2593.

Abbreviations used in this paper: ER, endoplasmic reticulum; ERAD, ER-associated degradation; Ii, invariant chain; Man, mannose; EDEM, endoplasmic reticulum degradation-enhancing α-mannosidase-like protein (EDEM) (13, 15, 16). Inhibition of mannosidase I can protect misfolded proteins from ERAD and overexpression of EDEM can increase degradation of folding-incompetent proteins (12–14). How EDEM-protein complexes are targeted back to the translocon and the extent of disassembly of oligomeric proteins and unfolding of polypeptide chains that is required for retrotranslocation of the misfolded subunits to the cytosol are not understood.

We have used a glycosylation mutant of the p41 isoform of invariant chain (Ii) to evaluate the ability of ERAD to discriminate between correctly folded and misfolded subunits in an oligomeric complex. Ii is a type II transmembrane glycoprotein the independently functioning domains of which impact several stages of MHC class II biosynthesis (17, 18). Shortly after synthesis, Ii rapidly forms trimers, a process facilitated by its transmembrane domain (19) and a C-terminal α-helical domain (20, 21). Newly synthesized class II αβ heterodimers associate with the Ii trimer by transmembrane domain interactions (22) and the binding of the CLIP segment of Ii to the class II peptide-binding groove (23, 24). Once fully assembled, the class II-Ii nonamer translocated from the ER to the cytosol via the Sec61 complex and are ubiquitinated and degraded by the 26S proteasome (11). However, the specific signals that target proteins to this process are not well understood. Recently, mannose trimming from N-glycans has been implicated as one of the targeting signals (12–16). High mannose sugars added to newly synthesized proteins in the ER initially contain 9 mannose (Man) residues. Proteins containing Man9 glycans are substrates for UDP-glucose:glycoprotein glucosyltransferase and can remain in the calnexin cycle until completely folded. Misfolded glycoproteins that are retained for extended times can be cleaved by the slow-acting mannosidase I, generating Man8 glycans. Glycoproteins containing Man8 glycans are poor substrates for glucosyltransferase and are released from the calnexin cycle and recognized by the lectin ER degradation-enhancing α-mannosidase-like protein (EDEM) (13, 15, 16). Inhibition of mannosidase I can protect misfolded proteins from ERAD and overexpression of EDEM can increase degradation of folding-incompetent proteins (12–14). How EDEM-protein complexes are targeted back to the translocon and the extent of disassembly of oligomeric proteins and unfolding of polypeptide chains that is required for retrotranslocation of the misfolded subunits to the cytosol are not understood.

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Departments of *Biochemistry and Molecular Biology and †Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637; and ‡Center for Vaccine Biology and Immunology, Aab Institute, and 3Department of Microbiology and Immunology, University of Rochester, Rochester, NY 14642.

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2 Current address: Keratinocyte Laboratory, Cancer Research U.K., 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom.

3 Current address: Department of Biological Sciences, Chicago State University, 9501 South King Drive, Chicago, IL 60628.

4 Address correspondence and reprint requests to Dr. Jim Miller, Center for Vaccine Biology and Immunology, University of Rochester, Box 609, 601 Elmwood Avenue, Rochester, NY 14642-8609. E-mail address: jim_miller@urmc.rochester.edu.

5 Abbreviations used in this paper: ER, endoplasmic reticulum; ERAD, ER-associated degradation; Ii, invariant chain; Man, mannose; EDEM, endoplasmic reticulum degradation-enhancing α-mannosidase-like protein; dMNJ, 1-deoxymannojirimycin; LfL, L-fucosyllectosamine; Hp, H-fucosyllectosamine; GRP78.
exits the ER and transits the Golgi, and two acidic dileucine-like motifs in the Ii cytoplasmic tail direct the complex to late endosomal-lysosomal compartments (25–28). In these compartments, Ii is degraded and released from class II molecules that are then free to bind antigenic peptides for presentation to CD4+ T cells. Ii mRNA is alternatively spliced, resulting in two isoforms (p31 and p41) that differ in the inclusion of a 64-aa luminal domain that is one of the most highly conserved regions of Ii. The p41-specific fragment is a competitive inhibitor of the lysosomal cysteine protease cathepsin L and can facilitate presentation of certain antigenic epitopes (29–31).

In this report, we show that mannosidase I-dependent targeting of an ER-retained mutant p41 to ERAD results in release of associated class II molecules. These results suggest that the ERAD machinery can induce subunit disassembly, specifically targeting misfolded subunits to degradation and sparing properly folded subunits for reassembly and/or export.

Materials and Methods

**DNA constructs**

The pcEXV-3 expression vector (32) and the p41 cDNA clone (33) have been previously described. Mutations in N-glycosylation sites within the exon 6b-encoded p41 fragment, T241A and S255A, were generated by overlapping PCR.

**Biochemicals and Abs**

Unless otherwise mentioned chemicals were purchased from Sigma-Aldrich (St. Louis, MO), cell culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA), and radiochemicals were obtained from Amersham Biosciences (Piscataway, NJ), Valeant Pharmaceuticals (Costa Mesa, CA), and PerkinElmer (Wellesley, MA). mAbs used were P4H5, specific for the luminal domain of murine Ii (34); In-1, specific for the cytosolic domain of Ii (35); 10-2-16, specific for I-Aβ (36); and 1D4B, specific for LAMP-1 (Developmental Studies Hybridoma Bank, Iowa City, IA). ER chaperone GRP78 (BiP) antiserum was purchased from Stressgen (Victoria, British Columbia, Canada). Cell surface expression of class II was determined by flow cytometry using the mAb MKD6. PE-conjugated secondary Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell culture, cell lines, and transfections**

All cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, 40 μg/ml gentamicin, and 50 μM 2-ME (D10 medium). To maintain selection of transfected genes, G418 (200 μg/ml) and/or 6 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine were added as appropriate. Ltk− fibroblast cells stably expressing I-Aα either alone or with the p31 or p41 isoforms of Ii have been described (37). Stable transfectants expressing I-Aα and T241A or S255A were generated in the same manner. For transient transfections, 0.5 × 10^6 Ltk− cells were plated per 60-mm tissue culture dish with D10 medium 12–24 h before transfection. Cells were washed twice in DMEM-HEPES (DMEM, 2 mM glutamine, 10 mM HEPES) before incubation in 2 ml of DMEM-HEPES containing 500 μg of DEAE-dextran (Amersham Biosciences), 50 μM chloroquine, and 2–5 μg of appropriate DNA. After 3 h at 37°C, cells were treated with 10% DMSO in DMEM-HEPES for 1 min at room temperature and then washed once with D10 medium before incubation in D10 medium for 48 h before experimentation.

**Radiolabeling, immunoprecipitation, and Western blots**

Cells (1 × 10^6/sample) were incubated with cell culture medium lacking either leucine or methionine-cysteine for at least 1 h before labeling with medium containing either 300 μCi/ml [3H]leucine or 150 μCi/ml [35S]methionine and cysteine, respectively. After radiolabeling, cells were either lysed or incubated in nonradioactive medium for various chase periods. In some experiments, either 1-deoxynojirimycin (dMNJ; 2 mM), N-acetylcysteine, norleucine (LNNL; 100 μM), NH4Cl (20 mM), or brefeldin A (BFA; 10 μg/ml) was included in the chase medium. For chemical cross-linking experiments, transfecteds were lysed in buffer containing 0.5% Nonidet P-40 (Valeant Pharmaceuticals), 20 mM bicine (pH 8.2), and 0.13 M NaCl in the presence or absence of 200 μg/ml dithiothreitol (suc-cinimidyl propionate) (Pierce, Rockford, IL). After 30 min, the reaction was quenched by addition of glycine to a final concentration of 100 mM, followed by incubation in the presence of 5 mM DTT during pulse labeling and then washed three times with PBS before incubation with medium lacking DTT for various chase periods. Before lysis, cells were placed on ice and treated with 20 mM N-ethylmaleimide in PBS to block free thiols by alkylation. Immunoprecipitation and gel electrophoresis were conducted as previously described (38). In some cases, immunoprecipitates were incubated with endoglycosidase H or N-glycosidase (Roche Applied Science, Indianapolis, IN) as described (39). Western blotting was performed as previously described (40). Blots were incubated with appropriate dilutions of primary Abs, followed by incubation with HRP-conjugated secondary Abs (Amersham Biosciences). Blots were washed extensively and developed with ECL (Amersham Biosciences).

**Immunofluorescence microscopy**

Cells were plated on microscope slides 24 h before staining. Immunofluorescent labeling of specimens was performed as previously described (41). Secondary Abs with either FITC or Texas Red fluorochrome were obtained from Jackson ImmunoResearch Laboratories. Samples were analyzed using a Zeiss Axiosvert microscope equipped with a Micromax cooled CCD camera and LUDI excitation filter wheel and shutter. Image capture and deconvolution analysis was performed using Slidebook software (Intelligent Imaging Innovations, Denver, CO).
Results
Elimination of N-glycosylation at Asn 239 results in ER retention of p41 Ii

In addition to N-glycosylation sites at Asn 113 and Asn 119, the p41 isoform of Ii contains two N-glycosylation sites at Asn 239 and Asn 253 located within the alternatively spliced fragment (Fig. 1). In the ER, core oligosaccharides are added to asparagine residues of nascent proteins with the consensus sequence Asn-X-Ser/Thr. To determine the function of the N-linked glycans at positions 239 and 253 within the p41 fragment, point mutations were introduced in the N-glycosylation consensus sequences substituting Thr 241 with alanine or Ser 255 with alanine (Fig. 1A). Deletion of the carbohydrate addition sites was confirmed by increased mobility in SDS-PAGE (Fig. 1B). Elimination of glycosylation at Asn 253 (S255A) had no significant effect on Ii transport and function. In contrast, elimination of glycosylation at Asn 239 (T241A) resulted in retention of p41 Ii in the ER (Fig. 2). After pulse labeling, wild-type p41, T241A, and S255A were sensitive to endoglycosidase H; and after a 2-h chase period, significant levels of wild-type p41 and S255A acquired endoglycosidase H resistance, indicative of ER to Golgi transport (Fig. 2, A and C). However, T241A remained sensitive to endoglycosidase H after the chase, suggesting that it was retained in the ER (Fig. 2B). This result was confirmed at steady state by immunofluorescence microscopy (Fig. 3). Wild-type p41 and S255A have similar post-Golgi staining patterns with little colocalization with the resident ER protein, BiP. However, T241A exhibits a reticular ER-like distribution that extensively overlaps with that of BiP. Furthermore, wild-type p41, but not T241A, is colocalized with the late endosomal-lysosomal marker, LAMP-1 (Fig. 3B). Taken together, these data indicate that glycosylation at Asn 239 is required for efficient ER export of p41 Ii.

The absence of glycosylation at Asn 239 does not result in global misfolding

Because N-glycans can directly facilitate protein folding by increasing protein solubility and preventing aggregation, it is not uncommon for partially glycosylated proteins to be retained in the ER due to misfolding (3). We did not anticipate an important role for N-glycosylation in p41 folding, because when the p41 fragment was bacterially expressed, it folded extremely efficiently as demonstrated by HPLC and inhibition of cathepsin L enzymatic activity (29). However, N-glycans may be required for the proper folding of the p41 fragment when it is expressed in the context of the full length Ii molecule within the ER of eukaryotic cells. To determine whether T241A misfolds, the ability of Ii to self-associate into trimers was examined (Fig. 4). Both N-glycosylation mutants trimerize equally well as wild-type p41, resulting in a band that migrates around 120 kDa (Fig. 4B). Importantly, when the cross-linked samples were incubated with the reducing agent 2-ME (Fig. 4C), the levels of monomeric Ii were equivalent to those observed in the absence of cross-linking (Fig. 4A). This result indicates that negligible quantities of T241A are lost in insoluble aggregates, as has been reported to occur with misfolded Ii (19). In other experiments, we found equivalent levels of T241A and wild-type p41.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** The majority of T241A molecules do not acquire endoglycosidase H resistance. Ltk<sup>-</sup> cells stably expressing I-A<sup>k</sup> and either wild-type (WT) p41, T241A, or S255A were metabolically labeled for 30 min with [35S]methionine and cysteine (pulse) and then chased in the absence of radiolabel for 2 h (chase). At each time point, cells were lysed, and proteins were immunoprecipitated from lysates with anti-Ii mAb PH5. Immunoprecipitates were either mock treated (M) or incubated with either endoglycosidase H (H) or N-glycosidase (N) before resolution on 10% SDS-PAGE under reducing conditions. Positions of molecular mass markers (kilodaltons) and endoglycosidase H-resistant (Ii<sub>R</sub>) and -sensitive (Ii<sub>S</sub>) Ii are indicated on the ordinates.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** T241A localizes to the ER. The intracellular localization of Ii was determined by immunofluorescent staining of Ltk<sup>-</sup> cells stably co-expressing I-A<sup>k</sup> and wild-type (WT) p41, T241A, or S255A. Cells were stained with rat anti-Ii mAb (In-1) and rabbit anti-BiP (A) or with rat anti-LAMP-1 mAb and rabbit anti-Ii (B) followed by anti-rat FITC and anti-rabbit Texas Red-conjugated secondary Abs. Isolated channels and composite images are shown.
association with the ER chaperones BiP and calnexin, indicating that T241A had neither a defect in chaperone association that could result in misfolding nor sustained chaperone association that might indicate local misfolding (data not shown).

In several experiments, we observed high molecular mass disulfide-linked protein complexes in T241A, but not wild-type p41, when immunoprecipitates were analyzed on unreduced gels (data not shown). Although these protein complexes represented only a small fraction of total T241A, their presence raised the possibility of a defect in disulfide bond formation between the six conserved cysteine residues in the p41 fragment. To examine this possibility, the kinetics of disulfide bond formation was analyzed after DTT washout (42). In the presence of DTT, Ii migrates with decreased mobility as predicted for the absence of disulfide bonds (Fig. 5A; T = 0, +DTT). Once disulfide bonds form within the p41 fragment, Ii migrates with an increased mobility due to its more compact structure (Fig. 5A; T = 1, 3, +DTT). After DTT washout, there was no detectable difference in the kinetics of disulfide bond formation between wild-type p41, T241A, and S255A, even within the short time points analyzed. Furthermore, when the DTT washout experiments were chased for longer time periods, wild-type p41, but not T241A, generated higher molecular mass forms indicative of Golgi transport (Fig. 5C; T = 30, +DTT). These results indicate that loss of glycosylation at Asn 239 does not interfere with the kinetics of disulfide bond formation. Furthermore, delaying the formation of disulfide bonds by adding DTT to the pulse label did not result in release of T241A from ER retention.

Interestingly, in the absence of disulfide bond formation, the efficiency of glycosylation within the p41 fragment is enhanced (Fig. 5). The two potential glycosylation sites in the p41 segment are not always used, resulting in electrophoretic mobility variants (33). The doublets seen in the pulse-labeled mobility variants (T = 0, −DTT, +), which result from differential glycosylation, are lost in the presence of DTT (T = 0, +DTT) where only the fully glycosylated forms are present. This suggests that disulfide bond formation can interfere with N-glycosylation and implies that these processes are occurring coincidently.

Finally, T241A efficiently associates with MHC class II (Fig. 6). Stable Ltk− cell transfectants coexpressing I-Aβ class II with wild-type or mutant p41 were pulse labeled with [3H]leucine, and class II and coassociated Ii were immunoprecipitated from cell lysates with the anti-I-Aβ mAb 10–2-16. Similar levels of wild-type p41 and T241A were found to coimmunoprecipitate with class II (Fig. 6). In six independent experiments, the relative association of T241A with class II compared with wild-type p41 was 1.03 ± 0.4. Taken together, these results indicate that the elimination of glycosylation at Asn 239 does not result in substantial misfolding, and this protein appears to be normally assembled into Ii trimers and class II-Ii complexes. This does not preclude the possibility that some local misfolding does occur and is recognized by quality control mechanisms resulting in ER retention.

**Class II that associates with T241A is not irreversibly retained in ER**

Because T241A associates with class II, yet does not exit the ER, it was surprising to find that expression of T241A did not reduce surface class II expression in stably transfected cell lines (data not shown). To evaluate the effect of higher levels of T241A expression on the steady state localization of class II, we analyzed the impact of transiently transfected wild-type and mutated p41 in class II-positive cells (Table I). There was no decrease in surface

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**FIGURE 4.** Ii glycosylation mutants do not have a defect in homotrimerization. Ltk− cells were transiently transfected with 2 μg of plasmid DNA of empty vector (Vect), wild-type p41 (WT), T241A, or S255A. Forty-eight hours posttransfection, cells were lysed in the absence (A) or presence (B and C) of the reducible cross-linker dithiobis(succinimidyl propionate) (DSP, 200 μg/ml). After immunoprecipitation with anti-Ii mAb In-1, Positions of molecular mass markers, monomeric and trimeric Ii, and mature Ii (★) are indicated.

**FIGURE 5.** Disulfide bonds form with equivalent kinetics in wild-type p41, T241A, and S255A after DTT washout. Ltk− cells stably expressing I-Aβ and wild-type p41, T241A, or S255A were pulse labeled for 15 min with [35S]methionine and cysteine in the presence of 5 mM DTT. After washout of the DTT, cells were chased in medium lacking radiolabel and DTT for 0, 1, 3, or 5 min (A and B) or for 0, 5, 15, and 30 min (C). Proteins were immunoprecipitated from cell lysates with anti-Ii mAb In-1 and samples were resolved by 10% SDS-PAGE in the absence (A and C) or presence (B) of 2-ME and analyzed by Western blotting with anti-Ii mAb In-1. Positions of molecular mass markers, monomeric and trimeric Ii, and mature Ii (★) are indicated. Incompletely glycosylated Ii (★) is present at the pulse time point only in the absence of DTT treatment; see text for comment.
class II expression in cells coexpressing T241A, suggesting that class II must dissociate from T241A to allow for transport of class II to the plasma membrane.

Early after biosynthesis, class II efficiently associates with wild-type p41 and with T241A, and both complexes remain endoglycosidase H sensitive, indicative of ER localization (Fig. 7A). After 2 h of chase, the majority of class II-wild-type p41 complexes have increased in apparent molecular mass and gained endoglycosidase H resistance, indicative of transport through the medial Golgi (Fig. 7B). In contrast, class II-T241A complexes remain largely endoglycosidase H sensitive, indicating that at this time point, class II associated with T241A is retained in the ER.

To examine class II-Ii dissociation, longer chase times were examined (Fig. 8). In cells expressing wild-type p41, class II-Ii complexes have largely matured into higher molecular mass, Golgi-modified forms by 1 h of chase. By 2 h of chase, class II-Ii dissociation has begun, and the p12 N-terminal endosomal degradation intermediate of Ii appears, indicating normal transit to post-Golgi endosomal compartments. By 8 h, p12 is largely lost, and class II, now free of Ii, is presumably released to the cell surface. The fate of class II-T241A complexes is quite different. Class II-T241A dissociation also appears to begin around 2 h and continues through 8 h of chase. However, T241A does not undergo an increase in molecular mass indicating transit through the Golgi and the p12 proteolytic fragment is not generated, suggesting that class II-T241A complexes are not transported to endosomes. Class II maturation does occur, but it is significantly delayed, appearing only after T241A has dissociated (at 4 h of chase). In addition, class II that was associated with T241A has an altered migration pattern that suggests a higher level of N-glycan modification, a phenotype associated with class II that matures in the Golgi in the absence of Ii (39). This supports the idea that class II only transits the Golgi after dissociation from T241A. The half-life of class II coexpressed with T241A is similar to that coexpressed with wild-type p41, an indication that class II is not degraded together with T241A. Overall, these data suggest that class II that associates with T241A is initially retained in the ER, but after T241A dissociation in the ER, class II, now free of T241, is able to exit the ER, transit the Golgi, and traffic to the plasma membrane.

**Targeting of T241A to ERAD induces class II-T241A dissociation**

The pulse-chase data discussed above suggest that T241A is degraded in a pre-Golgi compartment, possibly through the ERAD pathway. To address the site of T241A degradation, a pulse-chase analysis was performed in the presence or absence of either an inhibitor of pH-sensitive endosomal-lysosomal protease activity, NH_4Cl, or the proteasome inhibitor, LLnL (Fig. 9, A and B). Although incubation with NH_4Cl protects mature wild-type p41 from endosomal degradation, it does not stabilize T241A. This confirms that wild-type p41, but not T241A, is degraded in endosomal-lysosomal compartments. Degradation of T241A is inhibited by the proteasome inhibitor, LLnL, suggesting that T241A is degraded only after retrotranslocation into the cytosol, a feature of the ERAD pathway.

It has been shown that one of the signals that target proteins to ERAD is excessive trimming by mannosidase I, that can occur when glycoproteins have been retained in the ER for an extended time (12). The resulting Man8 structure functions as a signal that directs ER retained misfolded or improperly assembled proteins for ERAD. Because T241A does not exit the ER, its N-glycans may be cleaved by ER mannosidase I, resulting in subsequent targeting to ERAD. To examine this possibility, a pulse-chase analysis with cells stably expressing I-A^a and either wild-type p41 or T241A was conducted in the presence or absence of the mannosidase inhibitor dMNJ (Fig. 9C). In untreated cells after 4 h of chase, the majority of wild-type p41 and T241A has been degraded. Although inhibition of mannosidase with dMNJ does not stabilize wild-type p41, it protects T241A from degradation (Fig. 9C). Treatment with BFA, which blocks Golgi transport that in some cases is necessary for ERAD (43–45), stabilizes both wild-type and T241A.

Interestingly, stabilization of T241A with dMNJ results in extended class II association (Fig. 9D). After 4 h of chase, both wild-type and T241A p41 are degraded, and most of the class II has matured through the Golgi (see Fig. 8). Addition of dMNJ to the chase inhibits T241A degradation, and class II remains associated with the stabilized T241A. It is not possible to follow carbohydrate maturation through the Golgi in dMNJ-treated cells, because initial cleavage of high mannose sugars by mannosidase I is required for subsequent modification by Golgi glycosyltransferases. To determine whether the class II-T241A complexes exit the ER, NH_4Cl was added with dMNJ to the chase. In cells expressing wild-type p41, NH_4Cl addition protects class II-associated Ii from endosomal degradation. In contrast, addition of NH_4Cl did not increase the pool of class II-associated T241A, indicating that these complexes did not translocate the Golgi to endosomes.

Proteasome inhibition also lead to the stabilization of class II-T241 association (Fig. 9B). This supports the idea that at least for some proteins, the proteasome plays an active role in retrotranslocation of ERAD substrates from the ER (46, 47). Furthermore, the continued association of T241 with class II, when T241A degradation is blocked with either dMNJ or LLnL, suggests that mannosidase I-dependent and proteasome-dependent targeting of T241A to ERAD induces class II-Ii dissociation.

**Table 1. Expression of T241A does not inhibit cell surface expression of class II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA</th>
<th>MFI</th>
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<tbody>
<tr>
<td>Vector</td>
<td>1383</td>
<td></td>
</tr>
<tr>
<td>WT p41</td>
<td>1318</td>
<td></td>
</tr>
<tr>
<td>T241A</td>
<td>1391</td>
<td></td>
</tr>
</tbody>
</table>

a Ltk transfectants stably expressing class II, I-A^a, were transiently cotransfected with GFP and with wild-type (WT) p41 or T241A. Forty-eight hours posttransfection, the cells were stained with anti-class II mAb, MKD6, and PE-labeled secondary Ab and analyzed by two-color flow cytometry. Cells were gated into green-fluorescent protein-positive (transfected) pools and analyzed for the mean fluorescence intensity (MFI) of class II staining.
Asn 239 is located in a glycosylation site that the presence of an N-glycan can make the formation of compact conformations such as β turns more favorable. In fact, one-third of N-glycans examined are located in β turns (5). Although Asn 239 is located in a β turn, the polypeptide backbone structure around Asn 239 is very similar in the crystal structure of naturally generated and glycosylated p41 fragment in association with cathepsin L and the NMR structure of a chemically synthesized and unglycosylated polypeptide corresponding to the p41 fragment (50, 51). This finding suggests that glycosylation at this site is not required for the formation of this turn. However, it is possible that glycosylation at Asn 239 is required to stabilize the turn in the context of the full length Ii molecule or in the environment of the ER. Overexpression of wild-type p31 Ii with T241A did not result in increased ER export, suggesting that the folding defect is not caused by a high local concentration of p41 fragments in the Ii trimer, relative to when it is expressed as a soluble fragment (data not shown). Interestingly, two isoforms were generated upon in vivo folding of the synthetic p41 fragment that differed in the pattern of disulfide bond formation, Cys 226-Cys 235 and Cys 233-Cys 254, instead of the normal pattern Cys 226-Cys 233 and Cys 235-Cys 254 (51). It is possible that this isoform is favored in the T241A mutant in vivo, resulting in localized misfolding and ER retention, without interfering with Ii trimerization and class II association.

Our data suggest that ER-retained T241A is eventually targeted by mannosome trimming for cytosolic degradation by the 26S proteasome. It has been well established that inactivation of ER mannosidase I results in the stabilization of many misfolded or incompletely assembled proteins by blocking targeting to ERAD (12-14). However, the precise mechanism by which mannose trimming results in degradation of glycoproteins remains controversial. Initial studies with a C-terminal truncation mutant of α1-antitrypsin indicated that the Man8 oligosaccharide structure generated by ER mannosidase I results in a prolonged association with calnexin that leads to subsequent ERAD (52). Other studies with apolipoprotein A and mutant forms of thyroglobulin and protein C demonstrate that when posttranslational glucose trimming is inhibited, enhanced interactions with calnexin and calreticulin do not result in an increase in ERAD, but rather protection from degradation (12, 53). Furthermore, when glucose trimming is inhibited so that monoglycosylated glycans cannot be generated, ER mannosidase-dependent targeting to ERAD is unaffected (12, 53, 54). These data suggest that glycoproteins with the Man8 oligosaccharide structure can be effectively targeted to ERAD in the absence of calnexin association. A newly identified protein, EDEM, is a good candidate for the recognition of misfolded proteins carrying Man8 oligosaccharides (13, 15, 16). EDEM preferentially binds to a misfolded mutant form of α1-antitrypsin with the Man8GlcNAc2 structure. Furthermore, when overexpressed, this protein accelerates the degradation of α1-antitrypsin in an ER mannosidase I-dependent fashion.

Our data demonstrate that class II molecules that assemble with T241A are initially retained in the ER. Later, the complex is dissociated, T241A is degraded, and class II is released to the Golgi. Inhibition of mannosidase-dependent targeting of Ii to ERAD or inhibition of proteasome-dependent retrotranslocation and degradation results in sustained association of class II and T241A and apparent retention of the complex in the ER. It is possible that T241A may be targeted to ERAD only after spontaneous dissociation. In this case, the apparent sustained association of class II and T241A in the presence of dMNJ would result from continual re-association of class II with T241A that is now protected from degradation. However, this would imply that class II-Ii complexes
can undergo subunit exchange in the ER, and current data on the kinetics of class II-II assembly and transport do not support this possibility.

Instead, we favor a model in which mannosidase-dependent ERAD targeting itself induces disassembly of class II-T241A complexes. Proteins targeted to ERAD are retrotranslocated through the Sec61 complex which has a flexible pore (ranging in size from 15 to 50 Å) that can accommodate folded domains and glycosylated proteins (55). However, the class II-II nonameric complex is likely to be too large to remain intact during retrotranslocation. It is possible that once EDEM associates with T241A, other chaperones such as BiP induce dissociation from class II. To our knowledge, this is the first study indicating that the ERAD machinery can discriminate between correctly folded and misfolded subunits in an ER-retained multimer. Both the α and β subunits of class II are N-glycosylated, and because class II is retained by T241A in the ER, it would be predicted to have Man8 glycans. However, as class II is not degraded along with T241A by ERAD, two possibilities exist. In the first, the glycans of class II are not mannose trimmed, perhaps because they are inaccessible to the mannosidase I enzyme. In the second, the glycans of class II are mannose trimmed, but this signal alone is insufficient for targeting to ERAD. This possibility fits well with the observation that many proteins are mannose trimmed before ER exit. In addition, recent data demonstrating that EDEM remains associated with a misfolded protein even after its N-glycans have been enzymatically removed give further support to this idea (15). Molinari et al. hypothesize that EDEM binds to protein determinants of misfolding in addition to the Man8 structure. This model fits well with our observation that T214A, but not class II, is selectively recognized and targeted to ERAD. Furthermore, our data raise the possibility that the dissociation of oligomeric complexes before commitment to degradation may allow for the rescue of correctly folded subunits for reassembly and/or export.

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**References**
