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A Role for the Transmembrane Domain in the Trimerization of the MHC Class II-Associated Invariant Chain

Jonathan B. Ashman* and Jim Miller2*†

MHC class II and invariant chain (Ii) associate early in biosynthesis to form a nonameric complex. Ii first assembles into a trimer and then associates with three class II αβ heterodimers. Although the membrane-proximal region of the Ii luminal domain is structurally disordered, the C-terminal segment of the luminal domain is largely α-helical and contains a major interaction site for the Ii trimer. In this study, we show that the Ii transmembrane domain plays an important role in the formation of Ii trimers. The Ii transmembrane domain contains an unusual patch of hydrophilic residues near the luminal interface. Substitution of these polar residues with nonpolar amino acids resulted in a decrease in the efficiency of Ii trimerization and subsequent class II association. Moreover, N-terminal fragments of Ii were found to trimerize independently of the luminal α-helical domain. Progressive C-terminal truncations mapped a homotypic association site to the first 80 aa of Ii. Together, these results implicate the Ii transmembrane domain as a site of trimer interaction that can play an important role in the initiation of trimer formation. The Journal of Immunology, 1999, 163: 2704–2712.

The MHC class II-associated invariant chain (Ii) is a type II transmembrane glycoprotein that self-associates rapidly after biosynthesis to form a trimer (1, 2). Deletion mutagenesis has identified the major trimer interaction site within the luminal domain in a segment encoded by exon 6 (3–5). However, in vivo proteolytic fragments of Ii lacking this domain remain associated, suggesting that there are additional N-terminal interaction sites within Ii other than the luminal trimerization domain (6, 7). This apparent discrepancy led to the interpretation that the luminal domain interactions played an essential role in the initiation of trimerization and, once formed, the N-terminal interactions could remain assembled after C-terminal cleavage in vivo. Consistent with the importance of this luminal domain in trimerization, it has been found that a recombinant fragment of Ii from aa 118 to 194 can trimerize independently in solution with a highly folded, largely α-helical structure (8).

Ii trimerization is thought to precede class II assembly. First, Ii trimerization is rapid and monomeric intermediates have not been detected (2, 9). Second, Ii trimerization appears to precede class II association kinetically, and intermediates in assembly that contain one or two class II heterodimers associated with an Ii trimer have been detected (2). Thus, the final assembled class II-Ii complex is a nonamer, consisting of a single Ii trimer associated with three class II heterodimers (2, 10). The major class II-Ii interaction site maps to the CLIP region (aa 82–110) that binds to the class II peptide binding site (3, 11–18). Occupancy of the class II peptide binding site by Ii plays an important role in class II assembly (19), preventing the aggregation of class II chains (20), and promoting the transport of class II out of the endoplasmic reticulum (ER) and through the Golgi (21, 22). Consistent with the importance of Ii binding within the class II antigenic peptide groove, addition of an Ii peptide or CLIP peptide alone suffices to enhance the folding and transport of class II heterodimers (23). In addition to the core class II-binding sequence, the flanking segments 81–89 and 101–110 of CLIP appear to interact with class II and may regulate Ii association to different class II alleles (16, 17, 24, 25). Finally, class II association sites outside of the CLIP region also might contribute to the Ii-class II complex (13, 26, 27).

After transit through the Golgi, the class II-Ii complex is diverted from the constitutive transport route from the trans-Golgi network (TGN) to the plasma membrane. Instead, most of the class II-Ii complexes are sorted directly from the TGN to the endosomal pathway (28). TGN sorting and endosomal targeting require two dileucine-like signals within the cytosolic tail of Ii (29–32). Interestingly, trimerization of Ii plays an important role in TGN sorting, because class II-Ii complexes containing only a single intact Ii cytosolic tail are not sorted in the TGN and appear at the plasma membrane (33).

In addition to the dileucine signals, the Ii transmembrane domain (TMD) has been implicated in lysosomal targeting following internalization from the plasma membrane (34, 35). This raised the possibility that the Ii TMD functions in concert with the dileucine signals as an endosomal targeting signal at the TGN. However, studies of Ii localization in polarized cells suggest that sorting within the endosomal pathway may be distinct from the sorting at the TGN (36, 37). In this study, we have addressed the role of the Ii TMD in class II-Ii complex assembly and transport within the secretory pathway. Examination of the Ii TMD sequence and hydrophobicity profile reveals a patch of embedded polar amino acids. Surprisingly, mutation of these residues interferes with efficient Ii trimerization and Ii-class II assembly. These and other results implicate a critical role for the TMD in the initiation of Ii trimerization.
Materials and Methods

DNA constructs

The pcEXV-3 expression vector (38), the p41 Ii cDNA clone (39), and the D31 Ii construct (32) have been described previously. Two rounds of overlapping PCR were used to generate TMmut Ii. First, the T49I, T50A mutations were engineered into the TMD of wild-type p41 Ii, and the Q47A substitution then was added to T49I, T50A p41 Ii. For in vitro transcription/translation reactions, p41 Ii and TMmut p41 Ii were subcloned into the pBluescript expression vector (Stratagene, La Jolla, CA). D31-TM mut Ii was generated by replacing the SacI/PvuII fragment of D31 Ii with this fragment from TM mut p41 Ii. For pcEXV-3 1–108 Ii, a stop codon and an EcoRI site were introduced after position L108 using PCR. The PCR product was digested with EcoRI and cloned into pcEXV-3. After subcloning p41 Ii into pCF1E, 1–80 Ii was generated by making an internal HindIII/SpeI deletion. pcEXV-3 1–131 Ii (40) was provided by Dr. R. Germain (National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD). All constructs generated through PCR techniques were confirmed by automated sequencing.

Cell lines and transfections

LtK transfectants expressing wild-type p41 Ii have been described previously, and stable transfectants expressing TMmut p41 Ii or D31 Ii were generated in the same manner (32, 39). Cells were maintained in DMEM supplemented with 10% FCS, 10 mM HEPES, 0.1 mM nonessential amino acids, and 10 μg/ml gentamicin. To maintain selection of the transfected genes, G418 (200 μg/ml), MXH (6 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine), or blasticidin (10 μg/ml) was added as appropriate.

For transient transfections, 1 × 10⁶ Ltk− cells were plated per 60-mm tissue culture dish with DMEM and 10% FCS 24 h before transfection. Cells were washed twice in DMEM, 2 mM glutamine, 10 mM HEPES (DMEM-HEPES) before incubation in 2 ml DMEM-HEPES containing 500 μg DEAE-dextran, 50 μM chloroquine, and 2–10 μg Ii DNA. After 3 h at 37°C, the cells were treated with 10% DMSO in DMEM-HEPES for 1–2 min at room temperature, then incubated at 37°C for 48 h in DMEM and 10% FCS before immunoprecipitation experiments.

Radiolabeling and immunoprecipitation

Cells were incubated with cell culture media lacking leucine or methionine for at least 1 h before pulsing with media containing either 300 μCi/ml [3H]leucine or 200 μCi/ml [35S]methionine for the appropriate time periods. Immunoprecipitation and gel electrophoresis were conducted as previously described (33). Abs used were 10.2.16, specific for I-Ak (41); P4H5, specific for the luminal domain of murine Ii (42); and In-1, specific for the cytosolic domain of Ii (43).

FIGURE 1. Full-length and truncated Ii molecules used in this study. A, Hydrophobicity plots of wild-type (WT) and TMmut Ii were generated using the Kyte-Doolittle scale with a window size of 7 aa. Positive and negative values represent relatively hydrophilic and hydrophobic regions, respectively. The Ii amino acid sequence is displayed beneath each plot. Note the expansion of the core hydrophobic segment of the WT Ii TMD (underlined) after the amino acid substitutions (arrows) in TMmut Ii. B, N- and C-terminal truncations of Ii are depicted schematically. Domains within p41 Ii are indicated by boxes (with the amino acid positions shown below) and include the TMD (lightly stippled), the core CLIP-class II binding site (wavy lines), the luminal α-helical trimerization domain (darkly stippled), and the p41-specific exon 6b (hatched). Sites of N-linked glycosylation (+) and TMD substitutions (arrow) are indicated. The locations of the In-1 and P4H5 mAb epitopes are indicated by lines below Ii.
Chemical cross-linking

Transfectants were labeled as above, but lysed in buffer containing 0.5% Nonidet P-40, 20 mM bicine, pH 8.2, and 0.13 M NaCl with or without 200 µg/ml dithiothreitol/succinimidyl)propionate (DSP; Pierce, Rockford, IL). After 30 min, the reaction was quenched by the addition of glycine to 100 mM. Immunoprecipitates were eluted in 2% SDS buffer without 2-ME. One half of each sample was boiled for 2 min and separated by 7% SDS-PAGE, while the second half was adjusted to 2% 2-ME before being boiled and separated by 10% SDS-PAGE.

In vitro transcription and translation

Transcription reactions were driven off the T7 promoter using the Ribomax Large Scale mRNA kit (Promega, Madison, WI). Translation reactions were performed using rabbit reticulocyte lysates and canine microsomal membranes (Promega) in the presence of 1 mCi/ml [35S]methionine for 1 h at 30°C. Microsomal membranes were isolated by centrifugation for 5 min at 14,000 rpm at room temperature and resuspended in bicine lysis buffer in the presence or absence of 25 µg/ml DSP. Immunoprecipitation and electrophoresis were performed as above.

Results

Mutations within the Ii transmembrane domain

In previous studies, the Ii TMD has been predicted to extend from a cytosolic anchor at Arg20 to a luminal termination near Gln56–58 (Fig. 1A) (44, 45). Assignment of Tyr55 to the luminal interface would be consistent with the finding that tyrosine residues are preferentially located at the lipid/aqueous interface (46). However, examination of the amino acid sequence in conjunction with the hydrophobicity profile suggested an unusual feature at the luminal end of this segment. The major region of hydrophobicity within the TMD begins at Leu52 and ends only 15 aa away. This stretch is followed by a hydrophilic spike (attributed to the amino acids Gln57, Thr59, and Thr58) and a second hydrophobic patch (Fig. 1A). The presence of Gln57 is especially striking within the TMD. In a study of single pass type I transmembrane proteins, glutamine is resolved from the class II context of the alternatively spliced gene product, p41, because p41 results suggest that mutations within the Ii TM exert an effect very early in the Ii/class II biosynthetic pathway before passage through the Golgi.

We reasoned that this unusual stretch of polar amino acids within the putative Ii TMD could be functionally important for the intracellular sorting of the MHC class II-Ii complex at the TGN. Golgi-resident proteins rely in part upon the length of their TMD for proper localization (47). Indeed, the TMD of Golgi proteins average only 15 aa compared with an average of 20 aa for plasma membrane-resident proteins (48). This is consistent with a mechanism of integral membrane protein sorting based upon the physical interactions of the TMD with the lipid bilayer. Because the structure of the Ii TMD is similar to that of Golgi-retained proteins, we reasoned that this feature might delay the transport of class II-Ii complexes within the TGN, facilitating the recruitment of the transport machinery by the Ii cytosolic tail dileucine motifs.

To examine the role of this hydrophilic spike within the Ii TMD, we generated an Ii molecule, termed TMmut_Ii, with Glu47 substituted by Ala, Thr49 substituted by Ile, and Thr50 substituted by Ala. Substitution with nonpolar amino acids effectively eliminates the patch of hydrophilicity and results in a strongly hydrophobic stretch of 24 aa (Fig. 1A). We chose to express TMmut_Ii in the context of the alternatively spliced gene product, p41, because p41 is resolved from the class II a-chain in SDS-PAGE, whereas p31 and the a-chain comigrate. p31 and p41 Ii are indistinguishable in trimerization, class II association, and intracellular trafficking, so any effect on these events could be generalized to both isoforms.

Ii containing TM mutations fails to assemble with MHC class II

We first evaluated the effect of the TMD mutations on Ii assembly with MHC class II. Stable Ltk− cell transfectants coexpressing class II with either wild-type p41 Ii or TMmut p41 Ii were contin-

FIGURE 2. TMmut Ii fails to associate with MHC class II. Stable transfectants expressing I-AΔ and either wild-type (WT) p41 Ii or TMmut p41 Ii were radiolabeled for 2 h with [35S]methionine and immunoprecipitated with the mAbs 10.2.16 (anti-I-AΔ; A) or P4H5 (anti-Ii; B). Positions of mature p41 Ii (p41 IiΔ), immature p41 Ii (p41 Ii), and class II α- and β-chains are indicated between panels, and positions of molecular mass markers are shown on the right side. Mature p41 Ii is only observed in the context of WT Ii. Coassociation of the α- and β-chains of I-AΔ with WT Ii, but not with TMmut Ii, is evident from both 10.2.16 and P4H5 precipitates. Similar results were found with transfectants coexpressing WT Ii or TMmut Ii with I-AΔ.

lously labeled for 2 h in the presence of [35S]methionine. After lysis in Nonidet P-40, immunoprecipitations were conducted with mAbs against either Ii or MHC class II. Wild-type Ii runs as a triplet on SDS-PAGE (Fig. 2B). The two faster migrating bands are the result of alternative glycosylation of the four possible carbohydrate addition sites in p41 Ii. The broader, upper band represents maturation of these sugars as they transit the Golgi. Immunoprecipitation with anti-class II Ab demonstrates the association of both mature and immature forms of wild-type Ii with MHC class II (Fig. 2A). Although the addition of the core N-linked glycans indicates ER insertion, TMmut Ii fails to undergo significant maturation, suggesting a defect in ER to Golgi transport (Fig. 2B). Furthermore, very little TMmut Ii can be detected in association with MHC class II (Fig. 2A). Pulse/chase analysis confirmed that TMmut Ii largely is retained within the ER (data not shown). These results suggest that mutations within the Ii TM exert an effect very early in the Ii/class II biosynthetic pathway before passage through the Golgi.

TMmut Ii fails to associate in trimers

Ii trimerization is thought to precede assembly with MHC class II (2). The failure of TMmut Ii to associate with MHC class II raised the possibility that the primary defect in Ii-class II association could be a failure of Ii trimerization. To assess the oligomeric state of TMmut Ii, stable transfectants were radiolabeled and then lysed in the absence or presence of the reducible cross-linker DSP (2, 10). After immunoprecipitation with anti-Ii mAb, samples were eluted and separated by SDS-PAGE under either reducing or nonreducing conditions. In the absence of cross-linker, wild-type Ii migrates at m.w. corresponding to monomeric and dimeric forms of Ii with MHC class II (Fig. 2A). Pulse/chase analysis confirmed that TMmut Ii largely is retained within the ER (data not shown). These results suggest that mutations within the Ii TM exert an effect very early in the Ii/class II biosynthetic pathway before passage through the Golgi.

TMmut Ii fails to associate with MHC class II. Stable transfectants expressing I-AΔ and either wild-type (WT) p41 Ii or TMmut p41 Ii were radiolabeled for 2 h with [35S]methionine and immunoprecipitated with the mAbs 10.2.16 (anti-I-AΔ; A) or P4H5 (anti-Ii; B). Positions of mature p41 Ii (p41 IiΔ), immature p41 Ii (p41 Ii), and class II α- and β-chains are indicated between panels, and positions of molecular mass markers are shown on the right side. Mature p41 Ii is only observed in the context of WT Ii. Coassociation of the α- and β-chains of I-AΔ with WT Ii, but not with TMmut Ii, is evident from both 10.2.16 and P4H5 precipitates. Similar results were found with transfectants coexpressing WT Ii or TMmut Ii with I-AΔ.
One concern with these studies was that a significant portion of the TMmut Ii was lost upon cross-linking. This is most evident in comparison of the level of TMmut Ii in the samples with and without DSP in the reduced gel in Fig. 3A. This loss of material raised the possibility that the TMD substitutions could be inducing non-specific aggregation and then large aggregates could have been lost during preclearing steps before immunoprecipitation. Aggregation of TMmut Ii seems unlikely because TMmut Ii sediments as a monomer in sucrose gradients (data not shown) and because overexpression in a transient transfection system drives TMmut Ii trimerization (Fig. 4). In addition, significant loss of TMmut Ii is not detected when we expressed either wild-type Ii or TMmut Ii using an in vitro transcription and translation assay (Fig. 3B). In this assay, wild-type Ii readily forms dimers and trimers, as previously noted (3). Consistent with the in vivo results, TMmut Ii does not form dimers efficiently (Fig. 3B). Moreover, upon cross-linking, TMmut Ii remains largely as a monomer with little detectable trimer. Aggregation is not a concern in this assay because loss of material in the presence of cross-linker was equivalent between wild-type Ii and TMmut Ii. Together, these results suggest that TMmut Ii is defective in its ability to efficiently self-assemble into homotypic trimers.

To independently confirm this defect in self-assembly of TMmut Ii without depending on chemical cross-linking, we took advantage of the alternative splicing of Ii to perform coprecipitation experiments. Full-length p41 Ii was coexpressed with a p31 Ii molecule lacking the first 17 aa of the cytosolic tail, termed Δ31 Ii (Fig. 1B). Δ31 Ii shares with p41 Ii the luminal epitope for the mAb P4H5, but lacks the cytosolic tail In-1 mAb epitope. Because p31 and p41 Ii randomly associate into mixed trimers (33, 51), any Δ31 Ii appearing in an In-1 immunoprecipitate must be assembling with homotypic trimers.
Cells expressing wild-type p41 Ii were supertransfected either with Δ31 Ii or Δ31-TMmut Ii. Stable subclones were matched for equivalent levels of Δ31 Ii expression. To assess Ii oligomerization, cells were continuously labeled for 1 h with [35S]methionine, postnuclear lysates were divided in half, and immunoprecipitations were performed with either P4H5 or In-1. P4H5 reacts with all Ii species equivalently and illustrates the equivalent levels of expression of each of the Ii molecules (Fig. 5A). The efficient coprecipitation of Δ31 Ii with p41 Ii in the In-1 precipitate demonstrates the formation of mixed complexes containing wild-type TMD. In contrast, In-1 does not coprecipitate Δ31-TMmut Ii with p41 Ii, consistent with an inability of TMmut Ii to trimerize. To further support these findings, reciprocal experiments were performed by coexpressing TMmut p41 Ii with Δ31 Ii or Δ31-TMmut Ii. In this case, neither Δ31 Ii nor Δ31-TMmut Ii can be coprecipitated with TMmut p41 Ii (Fig. 5B). Therefore, in agreement with the cross-linking data, the coprecipitation experiments confirm that the mutations within the TMD inhibit Ii trimerization. Moreover, the lack of detectable trimers upon cross-linking is not the result of a conformational change within an intact trimer that prohibits the cross-linking reaction. Together, these data show that substitution of polar residues within the Ii TMD with small hydrophobic residues results in monomeric Ii molecules that do not efficiently assemble into trimers.

Trimerization of N-terminal fragments of Ii

Carboxyl-terminal Ii truncations expressed in vivo or in vitro have not been observed to form trimers (3–5), yet analogous Ii molecules generated through in vivo proteolytic processing remain trimeric in MHC class II-associated nonamers (6, 7). Our observation that sequences within the TMD play an important role in the trimerization of full-length Ii led us to reexamine the ability of N-terminal fragments of Ii to trimerize. To test whether the TMD could mediate oligomerization independently of the luminal trimerization domain, we generated a C-terminal truncation mutant of Ii, 1–108 Ii, which corresponds approximatively to the intracellular (p12) proteolytic fragment that remains trimerized (Fig. 1B). Transient transfection of 1–108 Ii into Ltk− cells was conducted in the absence of class II, and, 2 days posttransfection, radiolabeling and cross-linking were performed as above. Both disulfide-linked dimers and cross-linked trimers of 1–108 Ii could be detected (Fig. 6B). Nonreducing/reducing two-dimensional gel electrophoresis confirmed that the bands identified as dimeric and trimeric Ii were in fact derived from 1–108 Ii (data not shown). This observation is consistent with the ability of truncated Ii molecules to trimerize independently of the luminal α-helical domain.

To confirm association of N-terminal truncations in the absence of cross-linking the In-1 epitope was deleted from 1–108 Ii to create 18–108 Ii (Fig. 1B). These two constructs were transiently transfected alone and in combination into class II-negative L cells, and equivalent expression of both forms of Ii was confirmed by immunoprecipitation with P4H5 (Fig. 7). 18–108 Ii was detected in In-1 precipitates only when it was coexpressed with 1–108 Ii, confirming that N-terminal fragments of Ii can associate in the absence of the luminal trimerization domain. Coprecipitation of these N-terminal fragments was less efficient than previously detected with Ii constructs containing the luminal trimerization domain (see Fig. 5), possibly because interactions mediated through the TMD are destabilized in the presence of detergent.

To further map this N-terminal association site, 1–80 Ii was generated by the deletion of the CLIP region (Fig. 1B). Upon cross-linking, 1–80 Ii also can be demonstrated to migrate at a position of cross-linking the In-1 epitope was deleted from 1–108 Ii to create 18–108 Ii (Fig. 1B). These two constructs were transiently transfected alone and in combination into class II-negative L cells, and equivalent expression of both forms of Ii was confirmed by immunoprecipitation with P4H5 (Fig. 7). 18–108 Ii was detected in In-1 precipitates only when it was coexpressed with 1–108 Ii, confirming that N-terminal fragments of Ii can associate in the absence of the luminal trimerization domain. Coprecipitation of these N-terminal fragments was less efficient than previously detected with Ii constructs containing the luminal trimerization domain (see Fig. 5), possibly because interactions mediated through the TMD are destabilized in the presence of detergent.

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To further map this N-terminal association site, 1–80 Ii was generated by the deletion of the CLIP region (Fig. 1B). Upon cross-linking, 1–80 Ii also can be demonstrated to migrate at a position corresponding to trimers (Fig. 6C). Therefore, these results identify a site of homotypic interaction within the first 80 aa of Ii. Because mutation of TMD polar residues can inhibit trimerization in the context of either full-length (Figs. 3–5) or truncated Ii (data not shown), this N-terminal interaction site most likely maps to the Ii TMD.

To attempt to reconcile our data with previous studies that did not detect trimerization of N-terminal fragments of Ii (3–5), we have explored several technical differences between these studies. First, in earlier studies, the shortest molecule reported not to trimerize was 1–127 Ii (4, 5), which contains the luminal glycosylation sites and the initial portion of the α-helical domain not present in either 1–80 Ii or 1–108 Ii. To test whether the length of...
These constructs might account for differences in trimerization seen in ours and previous studies, we assayed the oligomeric state of 1–131 Ii, a construct analogous to 1–127 Ii that was used in the previous studies (Fig. 1B). Consistent with our results for 1–80 Ii and 1–108 Ii, 1–131 Ii was found to form trimers upon cross-linking (Fig. 6A). Thus, the presence of the amino acid segment between residues 108 and 131 and the N-linked carbohydrate sites at residues 113 and 119 does not interfere with trimerization of N-terminal fragments of Ii. Second, these truncated trimers may be particularly sensitive to detergent-induced dissociation if their assembly is mediated by the TMD. However, we have found that trimers of 1–80 Ii, 1–108 Ii, and 1–131 Ii can be detected in several different detergents, including Nonidet P-40 and C12 E 9 (data not shown). Third, we have found equivalent trimerization of 1–131 Ii in Ltk– cells and in COS cells (data not shown), which were used in previous studies (4, 5). Finally, although the N-terminal trimers spontaneously dissociate more rapidly than intact trimers, trimers of both 1–108 Ii and 1–131 Ii remain intact 60 min after cell lysis in either Nonidet P-40 or C12 E 9 (data not shown). Therefore, while it is not clear why oligomerization of C-terminal truncation mutants of Ii has not been previously observed, the data presented in this study clearly demonstrate that the ability of soluble recombinant Ii to trimerize could reflect differences between the in vitro conditions and the ER environment. Sample concentrations of purified, soluble Ii used in vitro ranged from 0.3 to 2 mM (8, 52, 53).

These concentrations are likely to be 100-1000-fold greater than the concentration within the ER of moderately expressed proteins such as Ii (57). In vivo, trimerization of Ii may depend on the initial TMD-mediated associations to increase the relative concentration of the luminal domain trimerization site. Consistent with this idea, we found that overexpression of Ii TMmut Ii in cells can drive trimerization, possibly by increasing the relative concentration of Ii and allowing luminal domain interactions in the absence of efficient TMD-mediated association.

Aberrant binding to TMmut Ii by proteins such as ER chaperones may physically block luminal domain interactions. The observation that TMmut Ii is retained within the ER suggests associations with ER chaperones. Calnexin is an ER chaperone that can interact both with the N-linked carbohydrate side chains and with the TMD (58–60). Both of these interactions have been implicated in calnexin-Ii association (9, 40, 50). Interestingly, three times as much TMmut Ii than wild-type Ii has been found associated with calnexin (data not shown), raising the possibility that mutations in the Ii TMD created an exceptionally strong calnexin binding site. The stoichiometry of calnexin association to wild-type Ii trimers has been reported to be one calnexin molecule for each Ii trimer (57). However, treatment with either tunicamycin or castanospermine reduces the concentration of efficiently TMD-mediated association.

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This 3-fold increase then could be reflective of a 1:1 ratio of calnexin to monomeric TMmut Ii. This raised the possibility that increased calnexin binding to TMmut Ii could prohibit trimerization, as has been shown for influenza hemagglutinin (61). However, treatment with either tunicamycin or castanospermine reduces the amount of calnexin-associated TMmut Ii to a proportion equivalent to that for wild-type Ii, but does not allow TMmut Ii to trimerize (data not shown). Thus, the carbohydrates appear to be the major site of calnexin association for both wild-type and mutant Ii, and the specific mutations within the Ii TMD do not appear to create a
high affinity calnexin binding site. These results support a conclusion that increased association of calnexin is not actively inhibiting trimerization, but is instead a consequence of the retention of monomeric Ii within the ER. This result does not rule out the possibility that another protein(s) may be associating with TMmut Ii to block trimerization.

One possible scenario that could account for a role of the TMD in Ii trimerization is that newly synthesized Ii monomers form transient intramolecular interactions between the luminal domain and a membrane-proximal segment. Initial interactions mediated by the polar residues within the TMD of Ii monomers would increase the dissociation of these cis interactions, freeing the α-helical domain for intermolecular trimerization. Under normal conditions, Ii assembly is a rapid process and Ii monomers are typically not detected even with short pulse times (2, 9). In the TMmut Ii, loss of the polar residues within the TMD would decrease the efficiency of TMD interaction and would effectively stabilize this monomeric intermediate.

The importance of the polar residues in the TMD and the ability of short N-terminal fragments of Ii to trimerize support a structure of the Ii trimer that is maintained by interactions at both the luminal trimerization domain and the TMD (62). The intervening sequences between these two trimerization domains contain the major class II interaction sites, yet display little secondary structure (52, 53). Ii trimerization mediated through both N-terminal and C-terminal interactions may impose some structural order onto the CLIP region that could facilitate the assembly of class II with Ii. In our studies, mutations of the TMD that interfere with trimerization also interfere with class II binding. These results suggest that trimerization of Ii, either by disrupting intramolecular interactions or by adding structural order to the CLIP region, may play an important role in class II-Ii assembly. Consistent with this idea, when trimerization of TMmut Ii is driven by overexpression (Fig. 4), the resulting trimers do assemble with class II (data not shown).

If the initiation of Ii trimerization is dependent upon the interactions among TMD, this TMD-mediated association should function independently of the trimerization domain. We have mapped such an N-terminal interaction site to the amino acid segment 18–80, containing only part of the cytosolic tail, the TMD, and the membrane-proximal luminal segment. TMD have been demonstrated to play an important role in the assembly of a number of oligomeric complexes, including glycophorin A (63, 64), FcγRIII (65), TCR (66, 67), MHC class II (68), and influenza hemagglutinin (69–71). Considering this diverse array of complexes, a mechanism must exist to generate specificity of interactions among TMD. TMD are thought to fold into high affinity coiled coil trimers (72). Specificity of helix-helix interactions should then arise from the preference for close packing of the side chains at the protein-protein interface compared with packing at the lipid-protein interfaces (73). Interestingly, single buried polar residues such as asparagine and glutamine have been shown to confer specificity upon the oligomeric state and the orientation of soluble coiled coils (74, 75). Indeed, there is a strong preference for glutamine residues within coiled coil trimers (76, 77). Similarly, oligomerization and Golgi localization of the transmembrane M protein of the avian coronavirus depend upon a single glutamine...
within its TMD (78, 79). In addition, a recent study has implicated a TMD-specific glutamine in the dimerization of the bovine papilloma virus E5 protein and in its association with its target platelet-derived growth factor β receptor (80). Finally, serine and threonine residues can affect the structure of α-helices by forming both inter- and intrahelical hydrogen bonds (81). Seen in this context, the embedded polar residues within the II TMD may play a significant role in promoting the specific assembly of II trimers. In the absence of these residues, II trimerization is inhibited and the II TMD may lose specificity and interact with the TMD of other proteins within ER. This may account for the loss of TMmut II observed after cross-linking. In this context, the biochemical data presented in this study support an important role for the TMD in the initiation of homotypic interactions between II monomers that lead to their rapid assembly into trimers.

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