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Association of ERp57 with Mouse MHC Class I Molecules Is Tapasin Dependent and Mimics That of Calreticulin and not Calnexin

Michael R. Harris,* Lonnie Lybarger,† Yik Y. L. Yu,† Nancy B. Myers,† and Ted H. Hansen2†

Before peptide binding in the endoplasmic reticulum, the class I heavy (H) chain-β2-microglobulin complexes are detected in association with TAP and two chaperones, TPN and CRT. Recent studies have shown that the thiol-dependent reductase, ERp57, is also present in this peptide-loading complex. However, it remains controversial whether the association of ERp57 with MHC class I molecules precedes their combined association with the peptide-loading complex or whether ERp57 only associates with class I molecules in the presence of TPN. Resolution of this controversy could help determine the role of ERp57 in class I folding and/or assembly. To define the mouse class I H chain structures involved in interaction with ERp57, we tested chaperone association of Ld mutations at residues 134 and 227/229 (previously implicated in TAP association), residues 86/88 (which ablate an N-linked glycan), and residue 101 (which disrupts a disulfide bond). The association of ERp57 with each of these mutant H chains showed a complete concordance with CRT, TAP, and TPN but not with calnexin. Furthermore, ERp57 failed to associate with H chain in TPN-deficient .220 cells. These combined data demonstrate that, during the assembly of the peptide-loading complex, the association of ERp57 with mouse class I is TPN dependent and parallels that of CRT and not calnexin. The Journal of Immunology, 2001, 166: 6686–6692.

The biosynthesis of oligomeric proteins entering the secretory pathway is facilitated by chaperone proteins in the endoplasmic reticulum (ER)† that are thought to provide quality control to insure correct folding. For example, MHC class I molecules require assembly in the ER of three subunits, a heavy (H) chain, β2-microglobulin (β2-m), and peptide ligand (1). Recent studies demonstrate that assembly intermediates of class I are associated with several ER proteins including calnexin (CXN), calreticulin (CRT), TAP, tapasin (TPN), and ERp57 (2). The dynamics of ER chaperone interactions with class I assembly intermediates and the selective roles of each ER chaperone in class I biogenesis remain topics of considerable interest and controversy.

Although several details remain unclear or controversial, the temporal order of events involved in class I assembly is starting to emerge. As the MHC class I H chain is translocated into the ER, intrachain disulfide bonds are formed within the α2 and α3 domains (3). Interestingly, the formation of these two H chain disulfide bonds may be temporally distinct (4, 5). This observation raises the possibility that the α3 and α2 disulfide bond formation may correspond with H chain interaction with β2-m and peptide, respectively, according to a kinetic model (6, 7). Both non- and disulfide-bonded H chains can be detected in association with CXN, an ER chaperone implicated in either stabilizing free H chains and/or promoting H chain assembly with β2-m (3, 8, 9). After β2-m assembly, class I-β2-m heterodimers are detected in association with CRT-TAP-TPN, a complex referred to here as the peptide-loading complex (2, 10–16). The order of assembly of this peptide-loading complex is unclear (2). However, once a peptide binds to form the fully assembled class I molecule, it is no longer associated with the peptide-loading complex, thus releasing class I molecules from the ER for transit to the cell surface (10–12).

Several H chain mutations have been described that disrupt its interaction with members of the peptide-loading complex. For example, α1 residue N86 (17), α2 residues 116 (18) and 128–136 (19–21), and α3 residues 223–229 (12, 22, 23) have all been implicated in H chain interaction with the peptide-loading complex. However, it remains unclear which, if any, of these are direct sites of interaction with TAP, TPN, or CRT. Part of the problem in defining direct interaction sites is the fact that cooperative binding among members of the peptide-loading complex may be required for their stable association with class I (21, 24). Furthermore, these mutagenesis approaches have been technically limited because of the lack of reagents capable of precipitating ER proteins while associated with class I molecules and the lack of reagents capable of identifying specific class I alleles while associated with ER molecules. Despite these limitations, a model has been proposed whereby CRT specifically interacts with the N-linked glycan at H chain residue 86, whereas TPN interacts with residue 128–136 and 223–229 in the α2 and α3 domains, respectively (25). However, this model must be considered speculative because of the aforementioned cooperative binding issue as well as the fact that the model does not consider the contribution of the most recently identified participant in class I assembly, ERp57 (26–28).

ERp57 has thiol-dependent reductase activity (29), cysteine-dependent protease activity (30, 31), and is known to interact with glycoproteins in a manner that can involve forming complexes.

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‡ Abbreviations used in this paper: ER, endoplasmic reticulum; β2-m, β2-microglobulin; CXN, calnexin; CRT, calreticulin; TPN, tapasin.
with either CXN and CRT (32–34). Three recent reports demonstrated that ERp57 is detected in association with class I molecules before peptide binding (26–28). However, important details regarding this association remain controversial, such as whether ERp57 association with class I is dependent upon TPN or whether ERp57 can associate with class I before its association with other members of the peptide-loading complex. Resolution of this controversy could help define the primary function of ERp57, which, reportedly, may include H chain disulfide bond formation, peptide trimming, or ER degradation of misfolded class I proteins (26).

In this report, we study the association of a series of H chain mutations with ERp57, TPN, and other ER molecules associated with class I during its assembly. This analysis includes mutations in all three extracellular domains that have been reported to disrupt H chain association with CRT-TPN-TPAP and have not been previously tested for association with ERp57. The advantage of a mutagenesis approach is that a particular mutant may reduce or disrupt class I interaction with a particular ER protein and may thus prolong associations with other ER proteins that may otherwise be difficult to detect. In this regard, perhaps the most interesting mutation tested is C101S, because it prevents the disulfide bridge in the a2 domain that is critical for the formation of the peptide binding groove. The simplest model consistent with these findings suggests that, during the assembly of the peptide-loading complex, ERp57 associates with CRT, and ERp57-CRT complexes only bind class I-β2m in the presence of TPN.

**Materials and Methods**

**Cell lines**

All the cell lines used in this report have been previously described. Mutant forms of Ld expressed in various cell lines are referred to by a single letter designation of native amino acid followed by the position number, which is then followed by a single letter designation of the newly introduced amino acid. The mouse L cell transfectants used in this study were: L-Ld (34), L-Ld D227K and L-Ld D227K/E229K (12), L-Ld K131D and L-Ld T134K (21), L-Ld N86Q, L-Ld N176Q, L-Ld N86K, and L-Ld S88W (17), and L-Ld C101S (16). Two human lymphoblastoid cell lines transfected with the Ld cDNA were also used, LCL721.220-Ld and LCL721.221-Ld (13, 35, 36).

**Antibodies**

The Abs used in this study are listed in Table I. Briefly, mAb 30-5-7 (37) was used to precipitate fully assembled forms of Ld, and mAb 64-3-7 (38) was used to precipitate open forms of Ld indiscriminate of their BBM1 (43) to human mAb 148.3 (42) to human TAP was a kind gift of Dr. R. Tampe, and mAb Table I.

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<th>Specificity</th>
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<td>64-3-7</td>
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<td>Residues 11–34 m TPN</td>
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*a* Ra, Rabbit; Ch, chicken; Ha, Armenian hamster; m, mouse; hu, human.

* New reagent.

**Results and Discussion**

Using Ld to study class I interactions with ER proteins is advantageous because mAb 64-3-7 can be used to specifically detect this class I allele while associated with CXN, CRT, TAP, TPN, and/or ERp57 (12, 21). In other words, the detection of Ld by mAb 64-3-7 is not sterically blocked when Ld binds any of these ER proteins, either directly or in complexes. Thus, several Ld mutants were constructed to probe how class I assembly intermediates interact with ER proteins during class I biogenesis. To further strengthen the Journal of Immunology | 6687

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Table I. Abs used in this study

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the mutagenesis approach reported here, new reagents were developed including a rabbit serum to mouse ERp57 and mouse TPN that can precipitate these ER proteins while associated with class I (Table I). Furthermore, a new hamster mAb that specifically detects mouse TPN by Western blotting was developed (Fig. 1). With these combined reagents, the association of mutant H chains with salient ER proteins could be rigorously tested.

Mutations in H chain residues 227 and 229 disrupt association with ERp57, TPN, CRT, but not CXN

Several studies have now demonstrated, using mouse Ld (12), Db (22), or Dv (23) molecules, that mutations in the α3 domain can disrupt association with TPN-TAP. To determine whether α3 mutation also affects ERp57 association, lysates of cells expressing Ld H chains with a single mutation at residue D227K or a double mutation at residues D227K and E229K were tested. Results obtained by precipitating Ld, Ld D227K, or Ld D227K/E229K and then blotting with anti-CXN, -CRT, -ERp57, -TAP, -TPN, or -Ld H chain are shown in Fig. 2. As shown, comparable amounts of Ld, Ld D227K, and Ld D227K/E229K H chains were detected in all three lysates, and all three of these H chains displayed strong association with CXN. By contrast with these findings, the α3 mutant H chains displayed weaker (Ld D227K) or no (Ld D227K/E229K) association with ERp57, TAP, and TPN, as also previously reported (12, 21). To extend these findings, precipitations were also performed using anti-ERp57 or anti-TPN followed by blotting for Ld H chain association. In data not shown, comparable amounts of Ld mutant H chains were detected in association with either TPN or ERp57 compared with wild-type Ld H chains. It is noteworthy that, in all of our comparisons, the double mutation was considerably more efficacious at ablating associations with ER proteins relative to the single mutation. This finding is consistent with an earlier report by Kulig et al. (22). In any case, TAP association with these α3 mutants clearly parallels that of TAP-TPN-CRT. Furthermore, based on studies of α3 mutations of Dv that were associated with CRT and not TPN-TAP, it was predicted that the α3 domain may be a site on the class I H chain that directly interacts with TPN. In such a model, it is assumed that only TPN, and not TAP, directly contacts class I (14).

FIGURE 1. Specificity of the rabbit polyclonal Ab (number 2668) and hamster mAb 5D3 for mouse TPN. Cell lysates from .221, .220, and .220 transfected with mouse TPN were tested by Western blotting with the indicated Ab. These Abs were raised against a region of mouse TPN that shows a divergence from human TPN. Furthermore, this region is absent in the truncated human TPN produced by .220 cells (51). Thus, the finding that no unique bands are present in .221 cells (human TPN-positive) relative to .220 cells demonstrates that these Abs are mouse TPN specific and that the common bands seen with rabbit antiserum are nonspecific.

Mutation in the α2 loop residue 134 of the H chain ablates ERp57 association

Residue 134 was originally shown to affect class I association with the peptide-loading complex in studies of HLA-A2.T134K (19, 20). More recent studies in our laboratory have defined the extent of the region surrounding residue 134 involved in TAP-TPN-CRT association by mutagenesis of Ld (21). In this previous report, amino acid substitutions of Ld residues 128–136 significantly reduced association with TAP-TPN-CRT with the exception of Ld K131D. Interestingly, the K131D mutation appeared as a hyper-morph in terms of loading complex association, because it had enhanced association with TAP-TPN in comparison with wild-type Ld (21). Furthermore, of the mutations that reduced association, T134K was among the most efficacious. To determine whether the K131D and T134K mutations affected ERp57 association, lysates of L cells expressing wild-type Ld, Ld K131D, or Ld T134K were precipitated with mAb 64-3-7 and blotted with anti-CXN, anti-CRT, anti-ERp57, anti-TAP, and anti-TPN. As shown in Fig. 3A, Ld and Ld K131D molecules were found to be associated with all ER proteins tested. By contrast, Ld T134K showed no detectable association with ERp57, TAP, TPN, and CRT. Importantly, each of these cell lines synthesized readily detectable Ld H chains and displayed high levels of CXN association (Fig. 2A). However, the level of Ld H chains expressed by each of these cell lines was clearly disparate, potentially influencing the detection of associated ER proteins. Thus, this experiment was repeated using samples in which the Ld H chains were normalized based on expression differences determined by densitometry (see Fig. 3B legend for details). Normalizing for Ld H chain expression resulted in similar qualitative differences in ER protein associations as obtained using cell equivalents (compare Fig. 3, A and B). Furthermore, normalizing for Ld H chain provided clear evidence that Ld K131D had increased association with CRT-ERp57-TAP-TPN, whereas Ld T134K lacked association with these same ER proteins (Fig. 3B). Interestingly, CXN associated comparably with Ld, Ld K131D, or Ld T134K. Thus, ERp57 association with the K131D and T134K mutants correlates precisely with that of the peptide-loading complex (TAP-TPN-CRT) and not CXN. Based on studies
cells expressing wild-type L^d or L^d N86K and L^d S88W mutant molecules were precipitated with Ab to the various ER proteins and blotted with mAb 64-3-7 to detect associated L^d H chains (Fig. 4C). Using each of these approaches, neither the L^d N86K or L^d S88W mutants were found to be associated with ERp57, CRT, TPN, or TAP. It should also be noted that both mutants were associated with CXN. Thus, these combined studies clearly show that ERp57, like other members of the loading complex, is dependent upon the N86 glycan for association with the class I H chain.

Based on the lectin activity of CRT (33), it was proposed that N86 is specifically required for CRT to interact with the class I H chain (17). Given the validity of this conclusion, the findings in Fig. 3 indicated that TPN-TAP and ERp57 are dependent upon CRT to form stable, steady-state associations with L^d. Interestingly, CXN binds H chains lacking the N86 glycan (Fig. 3A and Ref. 17). However, CXN association with L^d is clearly increased by N-linked glycosylation (M. R. Harris and T. H. Hansen, unpublished observation) as predicted from studies using glucosidase inhibitor castanospermine in studies of other class I molecules (45, 46). The advantage of the mutagenesis approach taken here is that L^d has three N-linked glycans, allowing us to clearly demonstrate that the N86 glycan is uniquely required for H chain association with CRT and the loading complex. By contrast, preventing complete glycosylation can result in aggregation and nonspecific association of aglycosylated proteins with CXN (47). The fact the CRT requires the glycan in the α2 domain of the H chain may be influenced by the fact that CRT is a soluble protein, whereas CXN is membrane anchored. Furthermore, the location of the H chain α1 domain glycan positions CRT near the site of peptide loading, possibly rendering CRT better able to interact with other members of the peptide-loading complex.

**Mutation at cysteine residue 101 of the H chain disrupts association ERp57**

All MHC class I molecules have two disulfide bonds, one between residues C101 and C164 in the α2 domain and the other in the α3 domain between C203 and C259. Because ERp57 has thiol-dependent reductase activity and has been implicated in the peptide-loading complex, it is attractive to speculate that ERp57 may facilitate disulfide bond formation in the α2 domain. If this were the case, then the C101S mutant might be expected to display high levels of ERp57 association. To test this hypothesis, lysates of L-L^d C101S cells and control L-L^d cells were precipitated with mAb.

FIGURE 3. Mutation in the α2 domain at residue 134 disrupts H chain association with ERp57 as well as TAP, CRT, and TPN. A, Aliquots of lysates representing cell equivalents of L-L^d, L-L^d K131D, and L-L^d T134K were precipitated with 64-3-7 and blotted with the various Abs to ER proteins (specific Abs listed in Fig. 1 legend). B, Same as A except the quantity of sample loaded was normalized according to the relative amount of L^d H chain that was determined by densitometry. Relative levels of L^d H chain were determined to be 8:1:1 for L-L^d, L-L^d K131D, and L-L^d T134K, respectively.

The N-linked glycan at residue 86 is specifically required for H chain association with ERp57 and CRT but not CXN

We previously showed that association of L^d with the complex of TAP-TPN-CRT requires the N-linked oligosaccharide at residue 86 (17). This conclusion was based on mutagenesis studies of L^d, a class I molecule with three N-linked glycans, one in each of the three external domains. In this previous study, L^d mutation at N86 was reported to ablate association with TPN-TAP-CRT. To determine whether ERp57 association with L^d shows this same glycan dependency, two mutations that ablate the N86-linked glycan were tested, N86K and S88W. Thus, lysates of cells expressing wild-type L^d or L^d N86K and L^d S88W mutant molecules were precipitated with mAb 64-3-7 to detect L^d H chains and were blotted to detect associated proteins (Fig. 4A, cell equivalents, and Fig. 4B, normalized L^d H chains). In a reciprocal experiment, lysates of HLA-B27 mutants (M. R. Harris, L. Lybarger, N. B. Myers, C. Hilbert, J. Solheim, T. H. Hansen, and Y. Y. L. Yu, manuscript in preparation), it was predicted that residues around 134 of the H chain may directly interact with TPN.

FIGURE 4. The N-linked glycan at residue 86 is required for H chain association with ERp57, CRT, TPN, and TAP but not with CXN. A, Aliquots of lysates representing cell equivalents of L-L^d, L-L^d N86K, and L-L^d S88W were precipitated with mAb 64-3-7, and then each precipitate was blotted with the Ab listed along the left (see Fig. 1 legend for specific Ab used for blotting). B, Same as A except quantity of sample loaded was normalized according to the relative amount of L^d H chain that was determined by densitometry to be 16:2:1 for L-L^d, L-L^d N86K, and L-L^d S88W, respectively. C, Normalizing for the amount of L^d H chains, aliquots of lysates of L-L^d, L-L^d N86K, and L-L^d S88W were precipitated with anti-TAP (502), anti-TPN (2668), anti-ERp57 (2890), or anti-open L^d (64-3-7). Each precipitate was then blotted with mAb 64-3-7.

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64-3-7 and then blotted with Abs to various ER proteins. As shown in Fig. 5A, L\textsubscript{d} C101S was found to be highly associated with CXN. By contrast, ERp57, CRT, TPN, and TAP displayed substantially reduced association with L\textsubscript{d} C101S relative to wild-type L\textsubscript{d}. Furthermore, when these respective lysates were precipitated with anti-TAP, anti-TPN, or anti-ERp57 (Fig. 5B), only wild-type L\textsubscript{d}, and not L\textsubscript{d} C101S, molecules were detected by blotting for L\textsubscript{d} H chains. Thus, C101S mutation of L\textsubscript{d} severely reduces its ability to associate with ERp57 as well as TAP, TPN, and CRT but not CXN. It should be noted that failure of L\textsubscript{d} C101S to associate with TAP-TPN-CRT proteins confirms an earlier report (16) and is consistent with studies that used DTT to identify H chain disulfide bond intermediates (5, 48, 49). These combined studies indicate that the \(\alpha\)2 disulfide bond, like the \(\alpha\)3 disulfide bond, likely formed before steady-state association of H chain with TAP-TPN-CRT. The original observation reported here is that formation of the H chain \(\alpha\)2 disulfide bond is also required for ERp57 to display steady-state association. The fact that the C101S mutation does not increase H chain association with ERp57 raises questions regarding the role of ERp57 in formation of the disulfide bond in the \(\alpha\)2 domain of the class I H chain.

**ERp57 association with L\textsubscript{d} is not detected in human TPN-deficient .220 cells**

There are conflicting reports in the literature as to whether human class I H chains are detected in association with ERp57 in TPN-deficient .220 cells. Furthermore, to the best of our knowledge, there are no reports of the TPN dependency of ERp57 association with mouse class I. To address this question, ERp57-L\textsubscript{d} association was compared in TPN-deficient .220-L\textsubscript{d} and TPN-positive .221-L\textsubscript{d} cells. From lysates of each of these cell lines, L\textsubscript{d} molecules were precipitated with either mAb 30-5-7, which detects fully assembled L\textsubscript{d}-\(\beta\)\textsubscript{2}m-peptide heterotrimers, or mAb 64-3-7, which detects open forms of L\textsubscript{d} H chains in a \(\beta\)m-independent manner. In this experiment, .220-L\textsubscript{d} and .221-L\textsubscript{d} lysates were also precipitated with a mAb to human TAP (148.3) or a mAb to human \(\beta\)\textsubscript{2}m (BBM1). As indicated in Fig. 6, each of these precipitates was blotted with anti-human TAP or anti-human ERp57. The experiment shown in Fig. 6 makes several important points. As previously noted, only 64-3-7-positive forms of L\textsubscript{d} are detected in association with TAP (12), and this association is only seen in .221-L\textsubscript{d} cells and not .220-L\textsubscript{d} cells (36). Interestingly, ERp57-L\textsubscript{d} association showed the identical result. Thus, L\textsubscript{d} association with ERp57, like TAP, is TPN dependent. Furthermore, \(\beta\)\textsubscript{2}m association with ERp57 was only observed in .221-L\textsubscript{d}, suggesting that L\textsubscript{d}-\(\beta\)\textsubscript{2}m heterodimers require TPN to maintain stable association with ERp57. Furthermore these data show that ERp57 only interacts with L\textsubscript{d}-\(\beta\)\textsubscript{2}m heterodimers and not free H chains.

In summary, multiple lines of evidence are presented showing that ERp57 association with mouse L\textsubscript{d} correlates precisely with TAP-TPN-CRT and not CXN. This finding clearly validates the conclusion that ERp57 is a bona fide member of the peptide-loading complex (26–28) and, furthermore, suggests a model consistent with the data reported here as well as most of the published literature (2). In this model, ERp57-H chain-\(\beta\)\textsubscript{2}m complexes are only associated with CRT and not CXN. Given our findings as well as certain other published reports using .220 cells (26), we propose that ERp57-CRT complexes are TPN dependent in their association with class I-\(\beta\)\textsubscript{2}m complexes. This latter dependency is consistent with published findings that CRT is also TPN dependent in its association with class I (14, 16). Although ERp57 could bind directly to TPN and not CRT, we consider this unlikely. ERp57 is known to interact in combination with CRT or CXN with several different proteins besides class I (32–34), whereas CXN is thought to be a class I-specific chaperone (2). Thus, the simplest model consistent with these conclusions is that, during the assembly of the peptide-loading complex, ERp57 binds CRT, and ERp57-CRT only binds to class I-\(\beta\)\textsubscript{2}m in the presence of TPN.

Interestingly, a recent report by Diedrich et al. (50) used human cells to define an ER complex of ERp57-CXN-TAP-TPN. These authors proposed that, coincident with class I binding, this intermediate complex replaces CXN with CRT to form the peptide-loading complex. However, regardless of the fate of this intermediate, the study by Diedrich et al. (50) is in complete agreement with our report concerning two underlying conclusions: first, that the steady-state association of ERp57 with class I is dependent upon TPN and, second, that when class I is bound, the loading complex includes steady-state levels of CRT and not CXN. This concordance between these two studies indicates that the rules governing the association of ERp57 with the peptide-loading complex are similar for mice and humans. It should be noted that ERp57 can clearly also be detected in association with CXN (32–34).
In another report, ERp57 association with class I was reported to coincide association with class I of ERp57 and TPN-TAP may be consistent with our finding that ERp57 may have class I-re-
sisting the failure to detect steady-state association of ERp57 with the C101S H chain mutant, suggests that ERp57 may have class I-re-

Acknowledgments

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References


