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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. Hansen†‡

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 (β2m), and peptide ligand (1). Recent studies demonstrate that assembly intermediates of class I are associated with several ER proteins including calnexin (CNX), 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 to H chain interaction with β2m and peptide, respectively, according to a kinetic model (6, 7). Both non- and disulfide-bonded H chains can be detected in association with CNX, an ER chaperone implicated in either stabilizing free H chains and/or promoting H chain assembly with β2m (3, 8, 9). After β2m assembly, class I-β2m 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; β2m, β2-microglobulin; CNX, calnexin; CRT, calreticulin; TPN, tapasin.

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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-TAP 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 α2 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: Ld-Ld (34), Ld-Ld D227K and Ld-Ld D227K/E229K (12), Ld-Ld K131D and Ld-Ld T134K (21), Ld-Ld N86Q, Ld-Ld N176Q, Ld-Ld N86K, and Ld-Ld S88W (17), and Ld-Ld C101S (16). Two human lymphoblastoid cell lines transfected with the Ld cDNA were also used, LCL.721.220-Ld and LCL.721.221-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 (35, 39–41) 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

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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

Immunoprecipitation

Cells were washed three times in cold PBS and were lysed in Tris-buffered saline that contained 1% digitonin (Wako, Richmond, VA) with 20 mM iodoacetamide (Sigma, St. Louis, MO) and 0.5 mM freshly added PMSF. The lysates were supplemented with a saturating amount of mAb or rabbit Ab before addition to pelleted cells. After incubation for 45 min on ice, nuclei were removed by centrifugation, and lysates were incubated with protein A-Sepharose beads (Pharmacia, Piscataway, NJ). The beads were washed four times with 0.1% digitonin in 10 mM Tris-buffered saline (pH 7.4) with 20 mM iodoacetamide, and the samples were eluted by boiling in 0.125 M Tris (pH 6.8)-2% SDS-12% glycerol-2% bromophenol blue. For Western blots, SDS-PAGE gels were transferred to Immobilon P membranes (Millipore, Bedford, MA). After overnight blocking in 10% milk-0.05% Tween 20, membranes were incubated in a dilution of Ab for 2 h, washed three times with PBS-0.05% Tween 20, and incubated for 1 h with biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag Laboratories, San Francisco, CA), goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or rabbit anti-chicken/turkey IgG (Zymed, San Francisco, CA). Following three washes with PBS-0.05% Tween 20, membranes were incubated for 1 h with streptavidin-conjugated HRP (Zymed), washed three times with PBS-0.3% Tween 20, and incubated with ECL chemiluminescent reagents (Amer sham Pharmacia Biotech, Piscataway, NJ).

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 D (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, -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 (Fig. 2) or with CRT, TAP, or 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, significantly less α3 mutant Ld H chain was 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, ERp57 association with these α3 mutants clearly parallels that of TAP-TPN-CRT. Furthermore, based on studies of α3 mutations of D 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.

**FIGURE 2.** Mutation at residues in the α3 domain disrupt association with ERp57, CRT, TAP and, TPN but not with CXN. Lysates of Ld, Ld D227K, and Ld D227K/E229K cells were precipitated with mAb 64-3-7 to specifically detect open forms of Ld. Aliquots representing cell equivalents of each of these precipitates were then blotted with the Ab listed along the left side. Specific blotting reagents used for this experiment were anti-CXN (SPA-860), anti-CRT (PA1-903), anti-ERp57 (2890), anti-TAP (502), anti-TPN (mAb 5D3), and anti-Ld (64-3-7).

**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). 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 Ld 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 Figs. 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...
ERp57 association with L^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^d association was compared in TPN-deficient .220-L^d and TPN-positive .221-L^d cells. From lysates of each of these cell lines, L^d molecules were precipitated with either mAb 30-5-7, which detects fully assembled L^d-B_m-peptide heterotrimers, or mAb 64-3-7, which detects open forms of L^d H chains in a B_m-independent manner. In this experiment, .220-L^d and .221-L^d lysates were also precipitated by a mAb to human TAP (148.3) and a mAb to human B_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^d are detected in association with TAP (12), and this association is only seen in .221-L^d cells and not .220-L^d cells (36). Interestingly, ERp57-L^d association showed the identical result. Thus, L^d association with ERp57, like TAP, is TPN dependent. Furthermore, B_m association with ERp57 was only observed in .221-L^d, suggesting that L^d-B_m heterodimers require TPN to maintain stable association with ERp57. Furthermore these data show that ERp57 only interacts with L^d-B_m heterodimers and not free H chains.

In summary, multiple lines of evidence are presented showing that ERp57 association with mouse L^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-B_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-B_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 TPN 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-B_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-CNX-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–

FIGURE 5. Mutation C101S disrupts H chain association with ERp57, CRT, TPN, and TAP but not with CXN. A, Samples representing cell equivalents of lysates from L-L^d C101S and L-L^d were precipitated with mAb 64-3-7, and aliquots of each of these precipitates were blotted with the reagent listed along the left (specific identity of reagents used listed in legend of Fig. 1). B, Aliquots of lysates of L-L^d C101S and L-L^d were immunoprecipitated with anti-ERp57 (2890), anti-TAP (502), anti-TPN (2668), or anti-L^d (mAb 64-3-7). All precipitates were then blotted with mAb 64-3-7.

FIGURE 6. L^d is only detectable in association with ERp57 in the presence of functional TPN. Aliquots of lysates of .220-L^d and .221-L^d were precipitated with 30-5-7 (assembled L^d), 64-3-7 (open L^d), 148.3 (anti-human TAP), or BBM1 (anti-human B_m). Each of these precipitates was then blotted with anti-human TAP (148.3) or anti-ERp57 (2890).


