ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly

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The assembly of newly synthesized MHC class I molecules within the endoplasmic reticulum and their association with the transporter associated with antigen processing (TAP) is a process involving the chaperones calnexin and calreticulin. Using peptide mapping by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to identify a new component, we now introduce a third molecular chaperone, the thiol-dependent reductase ER-60 (ERp57/GRP58/ERp61/HIP-70/Q2), into this process. ER-60 is found in MHC class I heavy chain complexes with calnexin that are generated early during the MHC class I assembly pathway. The thiol reductase activity of ER-60 raises the possibility that ER-60 is involved in the disulfide bond formation within heavy chains. In addition, ER-60 is part of the late assembly complexes consisting of MHC class I, tapasin, TAP, calreticulin and calnexin. In a β_2 -microglobulin (β_2m)-negative mouse cell line, S3, ER-60–calnexin–heavy chain complexes are shown to bind to TAP, suggesting that β_2m is not required for the association of MHC class I heavy chains with TAP.

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Introduction

The function of a major histocompatibility complex (MHC) class I molecule is to present peptides, of intracellular origin, on the cell surface to cytotoxic T lymphocytes (CD8⁺). To fulfill this function, MHC class I molecules must first be synthesized and properly assembled within the endoplasmic reticulum (ER). In this organelle, there exists a network of molecular chaperones evolved to assist membrane and secretory proteins in the folding process (Helenius *et al.*, 1997; Tatu and Helenius, 1997). The first of these proteins to be identified in association with the MHC class I heavy chain (HC) was calnexin, an 88 kDa ER-resident chaperone (Degen and Williams, 1991). Calnexin binding to the newly synthesized MHC class I HC requires trimming of the oligosaccharide moiety

(Glc₃Man₉GlcNAc₂) to its monoglucosylated form (Kearse *et al.*, 1994; Zhang *et al.*, 1995). Another equally important early step in class I folding is the formation of the highly conserved disulfide bonds, which has been shown to occur within the first few minutes after synthesis (Neefjes *et al.*, 1993). Following these initial early folding steps is the association of β_2 -microglobulin (β_2m) with the calnexin–HC complex. At this point, the trimeric calnexin–HC– β_2m complex requires only a suitable peptide before it can be completed. The peptides bound by the MHC class I HC are supplied to the ER by the transporter associated with antigen processing (TAP) from the cytosol, where they were generated by the proteasome (Rock *et al.*, 1994). Inhibition of the proteasome results in a decrease in the expression of MHC class I molecules on the cell surface and an increase in TAP-associated complexes within the ER, supporting the role of the proteasome as the major protease involved in the generation of peptides for class I (Suh *et al.*, 1996). The association of ‘empty’ MHC class I molecules with TAP is well established (Ortmann *et al.*, 1994; Suh *et al.*, 1994, 1996); however, the purpose of the MHC class I–TAP association remains unknown. Despite examples of peptides binding to MHC class I molecules in a TAP-independent manner (Hammond *et al.*, 1993; Zweerink *et al.*, 1993; Bachmann *et al.*, 1995), TAP-deficient cell lines and mice have demonstrated that TAP is the primary source of peptides within the ER (van Kaer *et al.*, 1992; Koopman *et al.*, 1997). The current belief is that peptide binding by the class I HC induces a conformational change within the molecule that results in the dissociation of the assembly complex, leaving the folded MHC class I molecule free to exit the ER and transit to the cell surface (Elliott, 1997).

The use of cell lines deficient in surface MHC class I expression has been of great assistance in defining the assembly pathway of class I molecules. Recently, two additional factors, tapasin and calreticulin, were added to this pathway (Grande *et al.*, 1995; Sadasivan *et al.*, 1996; Li *et al.*, 1997; Solheim *et al.*, 1997b) utilizing the mutant cell line 721.220 (tapasin-negative). Tapasin is a transmembrane protein and its proposed role is to act as a bridge between empty MHC class I molecules and TAP, as it is an essential factor for class I association with TAP. Calreticulin is an ER-resident chaperone, and a model for class I assembly in human cell lines has been proposed in which calreticulin replaces calnexin upon binding of β_2m to the calnexin–HC complex (Solheim *et al.*, 1997a) as calreticulin appears to be the predominant chaperone bound to TAP; however, complexes of calnexin–HC– β_2m have also been shown in human cells (Carreno *et al.*, 1995). A somewhat different profile is seen in mouse cells, where calnexin is the predominant chaperone associated with TAP (Williams, 1995). Despite mouse HC’s preference for calnexin (Nöbner and Parham, 1995),

complexes of calreticulin-HC- β_2m can also be found associated with TAP (van Leeuwen and Kears, 1996).

With the recent addition of two new factors to the pathway of MHC class I assembly, this study was undertaken to see if other, as yet unidentified, factors exist which participate in the assembly of MHC class I molecules. Matrix-assisted laser desorption/ionization (MALDI) peptide mass mapping and sequence database searching was used to identify TAP-associated proteins isolated by immunoprecipitation and two-dimensional gel electrophoresis. Here we show that a previously unidentified component, ER-60/the thiol-dependent reductase ERp57, exists in a complex with TAP and MHC class I molecules within the ER and that ER-60 is involved in the early steps of complex formation, specifically in the assembly of MHC class I molecules, fulfilling the role of a molecular chaperone.

Results

Immunoprecipitation of TAP molecules from digitonin lysates of metabolically labeled EL4 cells (Figure 1A) revealed multiple co-precipitating spots which were not seen in the control immunoprecipitates using normal rabbit serum (Figure 1B). Whereas many of these spots corresponded to known proteins, such as β_2m , calnexin or the various alleles of HC, with determinations being made utilizing known molecular weights and pI values and/or control immunoprecipitations with the relevant antibodies, several co-precipitating spots could not be readily identified (Figure 1A, arrows). As the TAP1, TAP2 and tapasin proteins possess isoelectric points (pIs) of 8.8, 7.1 and 7.0 respectively, these proteins do not enter into the isoelectric focusing gel. These observations were repeated using a second mouse cell line, CMT 64.5, stimulated with interferon- γ (IFN- γ ; 100 U/ml) (data not shown) to ensure that the co-precipitating material was not simply an artifact or anomaly peculiar to one cell line. The cell line CMT 64.5 possesses a defect in antigen presentation that is overcome by treating the cells with IFN- γ (Klar and Hammerling, 1989; Sibille *et al.*, 1992).

To identify the unknown proteins, the immunoprecipitations were repeated using 20×10^6 EL4 cells, the immunoprecipitates resolved utilizing two-dimensional gel electrophoresis and the co-precipitating proteins visualized using colloidal Coomassie stain (Figure 2A). The bulk of the Coomassie-stainable material corresponded to the IgG heavy chains (55 kDa) and light chains (25 kDa). Many of the expected co-precipitating proteins (HC, tapasin, TAP) are obscured beneath the Ig heavy chain (IgH) spots. Each protein spot appearing in the anti-TAP immunoprecipitate (Figure 2A) which did not appear in the control immunoprecipitate (not shown) was excised. Gel slices containing a single 'unidentified' protein spot were washed, reduced, S-alkylated and subjected to overnight in-gel tryptic digestion. The resulting peptide mixtures were then analyzed using MALDI mass spectrometry. The high mass accuracy peptide mass maps generated by MALDI mass spectrometry have proven to be highly specific probes for database searches (Jensen *et al.*, 1996a; Shevchenko *et al.*, 1996b). In this method, a set of measured tryptic peptide masses derived from a protein is compared with the predicted tryptic peptide masses of

Fig. 1. TAP-associated proteins. Two-dimensional autofluorographs of an anti-TAP immunoprecipitate (A) from metabolically labeled EL4 cells using the rabbit anti-ham1 antibody showing an abundance of co-precipitating proteins. Known proteins have been identified utilizing known molecular weights and isoelectric points, and by immunoprecipitation (see Materials and methods for the list of antibodies). β_2m , β_2 -microglobulin; CNX, calnexin; HC, heavy chain. Spots corresponding to background observed in the control immunoprecipitate (B) are identified with an asterisk. Unidentified proteins are indicated with open, large and small arrows. The orientation of the isoelectric focusing gel is indicated as H^+ and OH^- .

every sequence entry in a database. A protein is identified as the sequence entry with the most peptide mass matches. The mass spectrum obtained from the spot indicated by the large arrows (Figures 1A and 2A respectively) is shown in Figure 2B. The set of tryptic peptide masses, i.e. the peptide mass maps, was used to query a comprehensive sequence database and retrieved mouse ER-60 [EC 5.3.4.1] (Hempel and DeFranco, 1991) (Swissprot P27773) as the only top candidate with seven peptide mass matches. The next protein sequences in the list only matched five peptide masses, including rat and bovine ER-60 and several unrelated proteins. Detailed evaluation of the 57 kDa mouse ER-60 sequence and the peptide mass map assigned additional peptide masses to the sequence for a total of 11 peptide matches with an error of <50 p.p.m. (indicated with circles in Figure 2B). The assigned peptides covered

Fig. 2. (A) Coomassie-stainable proteins present in an anti-TAP immunoprecipitate similar to that described in Figure 1A. Proteins not observed in the control immunoprecipitation are indicated with large and small arrows and were excised from the gel and subjected to further analysis. Bands corresponding to the immunoglobulin light and heavy chains are indicated as IgL and IgH, respectively. (B) Peptide mass map obtained by MALDI mass analysis of the peptide mixture generated by in-gel digestion of the 57 kDa protein spot indicated in (A) (large arrow). The isotopes of all peptide signals were resolved (inset) and monoisotopic masses were determined. Searching a comprehensive sequence database with the list of tryptic peptide masses unambiguously identified this protein as mouse ER-60 (Swissprot P27773). A total of 11 peptide masses matched the ER-60 amino acid sequence with a mass error of <50 p.p.m. (labeled with circles). Two terminal peptide sequence tags generated due to adjacent tryptic cleavage sites in the ER-60 sequence (indicated by arrows) were detected.

24% of the ER-60 amino acid sequence (Table I). Two terminal peptide sequence tags produced due to adjacent tryptic cleavage sites in the protein sequence (Jensen *et al.*, 1996b) were detected in the mass spectrum (Figure 2B). The mass differences between these peptides corresponded to one lysine and one arginine residue, and are specific signatures of a protein. Additionally, the retrieved protein sequence originated from a mouse, as expected, and the calculated molecular weight and pI fit the position on the gel (57 kDa, pI 6.17), leading to an unambiguous identification of the protein, ER-60. The broad distribution

of spots (Figure 1A, open arrow and large solid arrow), each identified as ER-60, is probably due to various isoforms of the protein, as the more acidic isoform is known to be phosphorylated (Merrick *et al.*, 1993). Another protein spot on the gel (Figures 1A and 2A, small arrows) was also unambiguously identified using MALDI mass spectrometry as the molecular chaperone calreticulin (Swissprot P14211), with 15 peptide mass matches yielding 40% sequence coverage. Whereas the identification of calreticulin co-precipitating with TAP in either human or mouse cell lines has been described previously

Table I. Peptide sequences identified by MALDI mass spectrometry

1	MRFSCLALLP	GVALLLASAR	LAAASDVLEL	TDENFESRVS	DTGSAGMLLV	EFFAPWCGHC	60
61	KRLAPEYEAA	ATRLKIVPLA	KVDCTANTNT	CNKYGVSGYP	TLKIFRAGEE	AGAYDGPRTA	120
121	DGIVSHLKQ	AGPASVPLRT	EEEFKFI	KDASVVGFFR	DLFSDGHSEF	LKAASNLRDN	180
181	YRFAHTNIES	LVKEYDDNGE	GITIFRPLHL	ANKFEDKTVA	YTEKKMTSAK	IKKFIQDSIF	240
241	GLCPHMTEDN	KDLIQGKDLL	TAYYDVDEYK	NAKGSNYWRN	RVMVAKKFL	DAGHKLNFV	300
301	ASRKTFSEL	SDFSLESTTG	EVVVAIRTA	KGEKFMQEE	FSRDGKALEQ	FLQEYFDGNL	360
361	KRYLKSEPIP	ESNEGPVKV	VAENFDDIVN	EEDKDVLEF	YAPWCGHCKN	LEPKYKELGE	420
421	KLSKDPNIVI	AKMDATANDV	PSPYEVKGFP	TIYFSPANKK	LTPKKYEGGR	ELNDFISYLO	480
481	KEATNPPIIQ	EEKPKKKKKA	QEDL				504

The amino acid sequence of ER-60 with the peptide sequences identified by MALDI mass spectrometry indicated with boxes (~24% sequence coverage; 120 of 504 amino acids). ER-60 contains a 24 amino acid signal peptide that is not present in the full-length protein, making the N-terminal amino acid Ser25. ER-60 also possesses a C-terminal QDEL ER-retention signal.

(Sadasivan *et al.*, 1996; van Leeuwen and Kearse, 1996), the identification of the protein, ER-60 (ERp57/GRP58/ERp61/HIP-70/Q2) (Mobbs *et al.*, 1990; Srivastava *et al.*, 1991; Hirano *et al.*, 1994, 1995; Mazzarella *et al.*, 1994), co-precipitating in such a complex has not.

ER-60 is an ER-resident protein (504 amino acids, mol. wt 56 627 Da, pI 6.17) that shares considerable sequence homology with protein disulfide isomerase (PDI) (Freedman *et al.*, 1994). Initially thought to be an isozyme of phosphoinositide-specific phospholipase C (Bennett *et al.*, 1988), ER-60 has since had several activities attributed to it, including that of a thiol-dependent reductase (Srivastava *et al.*, 1991), a cysteine protease (Urade and Kito, 1992; Urade *et al.*, 1992; Otsu *et al.*, 1995) and a carnitine palmitoyl transferase (Murthy and Pande, 1994). Recent reports have added an additional activity to this list, a proposed chaperone activity with a specificity for *N*-glycosylated proteins within the ER (Elliott *et al.*, 1997; Oliver *et al.*, 1997).

To characterize the role of this newly identified protein, we studied its association with TAP using pulse-chase experiments. A primary TAP immunoprecipitate from metabolically labeled murine EL4 cells is presented in Figure 3A. Reprecipitation of ER-60 from the supernatant of SDS-disrupted anti-TAP immunoprecipitates from metabolically labeled murine EL4 cell lysates (Suh *et al.*, 1994) (Figure 3B) showed a relatively stable level of ER-60 within these complexes. Densitometric analysis revealed the half-life of ER-60 in these complexes to be ~120 min, whereas the MHC class I allele D^b had an apparent half-life of 65 min, comparable with values previously reported (Suh *et al.*, 1994). Interestingly, bands corresponding to the TAP subunits and MHC class I HC (70 and 46 kDa respectively) were also found to co-precipitate with ER-60. The co-precipitation of these molecules with ER-60 may be due in part to poor dissociation of the immunoprecipitated complexes, as the dissociation procedure was performed with mild heating (37°C) and contains no reducing agent. Another possibility is that these proteins are tightly associated within these complexes, as the reprecipitation buffer contains 1% NP-40, a detergent known to disrupt weak protein-protein interactions. The relative intensity of these proteins with respect to one another is most likely due to the bias induced by the primary immunoprecipitate as well as the difference in labeling efficiency between these proteins as seen in the primary immunoprecipitate (Figure 3A).

Additionally, it has been demonstrated that up to four MHC class I HCs may be found in association with a single TAP dimer, so that reprecipitation of such a complex would certainly make proteins present in higher stoichiometries appear more abundant.

To see if these were the only proteins associating with ER-60, primary immunoprecipitations of ER-60 were performed. Immunoprecipitation of ER-60 from digitonin lysates of metabolically labeled EL4 cells using a rabbit anti-rat ER-60 serum (Otsu *et al.*, 1995) revealed co-precipitation of several proteins (Figure 4), among which HC was the predominant species. However, small amounts of tapasin, calreticulin, calnexin, β_2m and an unidentified 65 kDa protein were also observed to co-precipitate with ER-60. As the 65 kDa species was not observed in the anti-TAP immunoprecipitates, it has not been pursued further at this time. The absence of the TAP proteins from the anti-ER-60 immunoprecipitate is most likely to be due to differences in the protein pool sizes. Whereas each TAP molecule possesses 1–4 associated ER-60–HC complexes, the pool of free ER-60–HC complexes within the ER may be substantially larger. An additional possibility is that association of the ER-60–HC complex with the TAP–tapasin complex may obscure epitopes within the ER-60 protein, making the TAP-associated pool of ER-60 less efficiently immunoprecipitated.

Since TAP-associated HCs are known to be sensitive to Endo H digestion, we investigated whether the ER-60-associated form of HC was also sensitive to Endo H (Figure 5). Using metabolically labeled EL4 cells, ER-60 and its associated proteins were immunoprecipitated. Complexes were disrupted with SDS and the associated HCs reprecipitated using the monoclonal antibody 28.14.8s, specific for the α_3 domain of D^b, and treated with Endo H. As expected, the ER-60-associated form of HC was indeed sensitive to Endo H digestion. The presence of Endo H-sensitive oligosaccharides is consistent with these molecules being a part of an ER-resident protein complex.

To further define the role of ER-60 in the assembly of MHC class I molecules, immunoprecipitations of ER-60 and TAP were performed using several cell lines with well-characterized defects in class I expression (Figure 6). Analysis of the co-precipitating material should allow us to determine ER-60's place in the assembly pathway with respect to these known molecules. As expected, anti-TAP immunoprecipitates from the tapasin-negative cell line

Fig. 3. (A) Primary anti-TAP immunoprecipitate from mouse EL4 cells. Cells were starved in Cys⁻/Met⁻ medium for 30 min and then metabolically labeled for 15 min. Immediately following the labeling process, the cells were lysed in a 1% digitonin buffer and the TAP molecules immunoprecipitated using the anti-ham1 sera. Bands corresponding to the TAP subunits, ER-60, calreticulin (CRT), tapasin, MHC class I HC and β_2m are identified. Bands corresponding to background are marked with an asterisk. (B) Pulse-chase analysis of TAP-associated ER-60. Mouse EL4 cells were starved in Cys⁻/Met⁻ medium for 30 min and then metabolically labeled for 5 min. Labeling medium was removed and replaced with chase medium containing a 1000-fold excess of cold Met. Aliquots corresponding to 4.0×10^6 cells were removed, pelleted, lysed with 1 ml of a 1% digitonin lysis buffer (ice cold) and immunoprecipitations were performed as described. Primary immunoprecipitated protein complexes were disrupted in 1% SDS at 37°C for 60 min. The supernatant was removed, diluted to a final concentration of 0.2% SDS with a 5% milk/PBS/1% NP-40 solution, and the ER-60 reprecipitated using the rabbit anti-rat ER-60 sera. Bands corresponding to TAP, ER-60 and MHC class I HC are indicated.

721.220 (Figure 6A) showed no co-precipitating material that was not also seen in control immunoprecipitates (data not shown). This observation is consistent with previously published reports showing tapasin to be an essential factor for association of MHC class I molecules with TAP. The absence of the TAP proteins from this immunoprecipitate is due to the relatively poor metabolic labeling efficiency

Fig. 4. Primary immunoprecipitates of ER-60 from metabolically labeled mouse EL4 cells using the rabbit anti-rat ER-60 protease serum. Known proteins are indicated using the abbreviated names as in Figure 1. The unidentified protein with an apparent molecular weight of 65 kDa is designated p65. As this protein was not observed in the anti-TAP immunoprecipitates, it has not been pursued further at this time. Spots observed in the control immunoprecipitate (Figure 1B), therefore corresponding to background, are marked with an asterisk.

Fig. 5. ER-60-associated MHC class I heavy chains are sensitive to Endo H digestion. Anti-TAP immunoprecipitates from metabolically labeled mouse EL4 cells were SDS disrupted (as described in Figure 3B) and the associated HCs reprecipitated using the monoclonal antibody 28.14.8s. This material was then either mock treated or digested with 2000 U of Endo H (New England BioLabs, Beverly, MA) for 20 h at 37°C. Samples were resolved on a 12.5% polyacrylamide gel and visualized by autofluorography as described.

of the human TAP molecules (Ortmann *et al.*, 1994, 1997). Analysis of ER-60 immunoprecipitates from 721.220 cells (Figure 6B) showed co-precipitation of both HC and the unknown 65 kDa species observed earlier (Figure 4). These data show that ER-60 association with HC occurs prior to association of the complex with TAP and that this association is tapasin independent. Anti-TAP immunoprecipitates from the cell line 721.221 (HLA-A,B,C-negative) (Figure 6C) show association of multiple proteins with TAP. The observation that the small amounts of HLA-E, F and/or G that exist within these cells co-precipitate with the TAP-tapasin complex is sufficient to explain the observed co-precipitation of β_2m and calreticulin. This observation is confirmed by the data of Gobin *et al.* (1997) showing that non-classical class I molecules associate with TAP. Anti-ER-60 immunoprecipitates from these cells (Figure 6D) also show co-precipitation of HLA-E, F and/or G heavy chains, β_2m , calnexin, calreticulin and the unknown 65 kDa species previously observed. To assess the role of β_2m , we utilized the β_2m -negative mouse cell

Fig. 6. Analysis of known antigen presentation-defective cell lines. Anti-TAP (A, C, E and G) and anti-ER-60 (B, D, F and H) immunoprecipitates from the antigen presentation-defective cell lines: 721.220 (A and B), 721.221 (C and D), EL4-S3 (E and F) and T2 (G and H). All cells were metabolically labeled for 15 min and lysed in a 1% digitonin solution (see Materials and methods). For convenience, the lower region of each gel, below 30 kDa and containing β_2m , has been excised. These gels are similar to that in Figure 4, with the exception of the mouse β_2m -negative cell line S3, which lacks β_2m . The spots corresponding to TAP and tapasin are marked as 'T' and 't' respectively. Spots corresponding to background are marked with an asterisk.

line S3 which is a derivative of EL4. Immunoprecipitation of TAP from these cells (Figure 6E) revealed association of both HC and calnexin with the TAP–tapasin complex despite the absence of β_2m , suggesting that β_2m is not required for association of the calnexin–HC complex with tapasin–TAP. Longer exposures of this gel (data not shown) also show association of calreticulin in this complex. The presence of ER-60 in this complex could not be verified due to the increased background associated with longer exposures. However, immunoprecipitation of ER-60 (Figure 6F) did show co-precipitation of HC, showing that β_2m is not required for the association of ER-60 and HC. The decreased levels of ER-60 present in the anti-TAP immunoprecipitates from these cells are most likely due to the decreased association of HCs, in comparison with the β_2m -positive parent cell line EL4 (Figure 1A), and not a dependence on β_2m for ER-60 association, as analysis by Western blotting confirmed the presence of ER-60 within the TAP-associated complex (data not shown). To see how many components of the final TAP-associated complex can be found in association with ER-60, we used the TAP-negative human cell line T2. As expected, anti-TAP immunoprecipitates are devoid of co-precipitating proteins that are not also observed in control immunoprecipitates (Figure 6G). Anti-ER-60 immunoprecipitates from T2 cells (Figure 6H) included β_2m , calreticulin and calnexin among the co-precipitating proteins.

Likewise, immunoprecipitation with an anti-calreticulin serum brought down ER-60 and MHC class I (data not shown). Together, these data show that complete pre-TAP-associated complexes, which include ER-60, are capable of forming in human cells and that these complexes contain at least one other chaperone, calnexin or calreticulin. This observation was confirmed by previous reports showing that ER-60 exists in complexes with other chaperones (Otsu *et al.*, 1995; Elliott *et al.*, 1997; Oliver *et al.*, 1997).

As ER-60 binding to HC is independent of β_2m (Figure 6F), we postulated that the binding of ER-60 to HC could be an early step in the assembly of the TAP-associated complex. It is known that calnexin is among the earliest proteins to bind to newly synthesized HCs in the ER and that this binding requires the trimming of the oligosaccharide moiety to its monoglucosylated form (Williams, 1995). Calreticulin and ER-60 also require the presence of monoglucosylated oligosaccharides to bind to their substrates within the ER (Peterson *et al.*, 1995; Oliver *et al.*, 1997). To show that this was also the case here, the inhibitor castanospermine was used. Castanospermine is known to inhibit glucosidase I, the first enzyme involved in oligosaccharide trimming within the ER. EL4 cells were either treated with 4 mM castanospermine or left untreated during the metabolic labeling (Figure 7). In the anti-ER-60 immunoprecipitates there is an obvious decrease in the amount of HC associated with ER-60 in the treated sample

Fig. 7. (A) Glucose trimming is important for the binding of ER-60 to MHC class I heavy chains. EL4 cells were either treated with 4 mM castanospermine or left untreated during metabolic labeling (see Materials and methods). Cells were lysed in 1% digitonin buffer and immunoprecipitated with the rabbit anti-rat ER-60 serum or with the monoclonal antibody 28.14.8s (anti-HC) as described. (B) ER-60 co-precipitates with MHC class I heavy chains. Digitonin extracts of human tapasin-deficient 721.220 cells were probed with a variety of monoclonal antibodies known to recognize MHC class I HCs. Immunoprecipitates from 2.5×10^6 cells were resolved on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes. Western blots were probed with anti-ER-60 sera (1:800) and peroxidase-conjugated goat anti-rabbit IgG, Fc fragment (Dianova) (1:5000) and visualized by ECL using Super Signal Substrate (Pierce). Whole cell lysate equivalent to 5.0×10^5 cells was included as a positive control for the ER-60 protein. Immunoprecipitates containing antibody and phosphate-buffered saline were included to demonstrate cross-reactivity between the Ig heavy chain (IgH) and the secondary antibody.

when compared with the untreated control. This indicates that the presence of a trimmed glucose moiety is required for ER-60 to bind to HC or that ER-60 binding is dependent upon the binding of at least one other chaperone, either calnexin or calreticulin, which requires the proper oligosaccharide. Immunoprecipitation of HC from the castanospermine-treated cells shows that glucosidase I is indeed inhibited, as observed by the upward shift in the electrophoretic mobility due to the presence of the larger oligosaccharides on the HCs of the treated sample which is not seen in the untreated control. A possible explanation for the absence of ER-60 in the anti-HC immunoprecipitates is that these HCs represent the entire cellular pool of MHC class I molecules, while ER-60 interacts with only a portion of the ER-resident HCs. Thus, the ER60-

HC complexes would comprise only a small percentage of the total cellular pool of MHC class I.

To demonstrate that ER-60 could indeed be found associated with MHC class I HCs, anti-HC immunoprecipitations were performed from digitonin extracts of the human tapasin-negative cell line 721.220 (Figure 7B). Since tapasin-negative cells do not express MHC class I on the cell surface (Grande *et al.*, 1995), the entire pool of MHC molecules within these cells must reside within the ER. As expected, ER-60 could be found in association with immunoprecipitated MHC class I HCs (Figure 7B, antibodies B9.12, W6/32 and HC10 respectively) and could be found to a lesser extent when the HCs were precipitated via β_2m (Figure 7B, BBM.1). This is consistent with an earlier observation that showed low levels of β_2m in the anti ER-60 immunoprecipitates (Figures 4 and 6D). The lower band in the blot corresponds to IgH and results from cross-reactivity with the secondary antibody used in the detection method.

Discussion

Molecular chaperones mediate the correct assembly of other proteins, but are not themselves a part of the final structure (Hendrick and Hartl, 1993). The role of ER-60 as a molecular chaperone was first proposed by Hirano *et al.* (1995), and is consistent with the observations made in this report. ER-60 clearly participates in the assembly of MHC class I molecules, associating with HCs shortly after their synthesis, independently of tapasin and β_2m , and can be found in complexes with the TAP transporter, i.e. leading to the formation of the final product, HC- β_2m -peptide. MHC class I HCs do not appear to be degraded during their association with ER-60, as judged by the relative stability of the HCs seen in Figures 3 and 5, and these HCs have a normal turnover consistent with that of HCs exiting the ER. Therefore, one is left to conclude that the role of ER-60 in these complexes is that of a molecular chaperone.

The co-precipitation of multiple chaperones simultaneously associated with TAP/tapasin and HC (Figures 1 and 4, respectively) is another observation of interest. In TAP-expressing cell lines, one might expect to see multiple chaperones associated with TAP given the recent results of Ortmann *et al.* (1997) showing the stoichiometry of the TAP-associated proteins to be four MHC class I-tapasin complexes per TAP heterodimer. However, in the TAP-negative cell line, T2, we also observe the chaperones calnexin and calreticulin co-precipitating with ER-60 and HC (Figure 6H). This observation demonstrates that at least one of these chaperones is complexed simultaneously with the same substrate as ER-60, since ER-60 was the precipitating species. This may also represent trimeric chaperone complexes, ER-60-calnexin-calreticulin-HC, although a mixture of paired chaperone-HC complexes is the simplest explanation. Similar results were obtained in the anti-ER-60 immunoprecipitate from 721.220 cells (Figure 6B) showing ER-60-calnexin-HC complexes. From these data, one cannot rule out the possibility that ER-60 binds calnexin and calreticulin as substrates. However, this appears unlikely as recent reports have demonstrated paired chaperone-substrate complexes, calnexin-calreticulin (Otteken and Moss, 1996; Tatu and

Helenius, 1997), as well as ER-60-calnexin and ER-60-calreticulin (Elliot *et al.*, 1997; Oliver *et al.*, 1997). Additionally, the relative abundance of MHC class I HC co-precipitating with ER-60 with respect to calnexin or calreticulin favors ER-60-HC complexes as the predominant species observed in the anti-ER-60 immunoprecipitates. In the latter studies, the existence of chaperone complexes was shown using cross-linking reagents. The same technique demonstrated the association of ER-60 with yeast α factor and IFN- γ using an *in vitro* translation system (Oliver *et al.*, 1997). An additional interesting aspect of the present work is that we did not require cross-linking to observe the association of ER-60 with a natural substrate *in vivo*, namely MHC class I. The observation of multiple chaperones interacting with a single substrate is in agreement with a developing trend in protein folding, that chaperones work together in a concerted effort to fold proteins (Helenius *et al.*, 1997; Tatu and Helenius, 1997).

Previous studies have shown that calnexin associates with MHC class I HC early after biosynthesis. Following association with β_2m , calnexin is replaced by calreticulin, explaining the predominance of calreticulin in MHC class I-tapasin-TAP complexes (Sadasivan *et al.*, 1996; Figure 6C). In contrast, ER-60 is found, to comparable extents, in both early and late MHC class I assembly complexes. The present data also show that the replacement of calnexin by calreticulin is not quantitative, as some calnexin is still found in complexes precipitated via TAP. The presence of TAP-associated calnexin may represent complexes of calnexin-HC (without β_2m).

Complexes of calnexin-MHC class I, calreticulin-MHC class I and now ER-60-MHC class I have clearly been shown in association with TAP in both mouse (Figure 1) and human (Figure 6C). Additionally, we have also observed paired chaperone-HC complexes and possibly trimeric chaperone-HC complexes. The abundance of these complexes relative to one another has yet to be determined, but the existence of all forms of chaperone-MHC class I complexes in both species is consistent with the reported 'shared' specificity of these chaperones. The subtle differences observed between species is more likely to be due to differences in either the affinities or relative concentrations of the chaperones rather than to distinctly different MHC class I assembly pathways.

While demonstrating the association of ER-60 with HC was independent of β_2m in the mouse β_2m -negative cell line, S3, we observed the association of calnexin-HC complexes with TAP (Figure 6E). These complexes were not observed in the human β_2m -negative cell line, Daudi (Sadasivan *et al.*, 1996), possibly due to the difference in affinities between human and mouse MHC class I HCs for TAP and calnexin (Nöbner and Parham, 1995; Neisig *et al.*, 1996). The observation of calnexin-HC complexes associating with TAP independently of β_2m suggests that β_2m is not required for TAP association as previously proposed (Carreno *et al.*, 1995; Solheim *et al.*, 1997a,b). In contrast to these published reports, anti-TAP immunoprecipitates from 721.221 cells (HLA-A,B,C-negative) (Figure 6C) show sufficient amounts of non-classical MHC class I HCs associated with TAP to explain the observed co-precipitation of β_2m and calreticulin. The TAP-associated pool of non-classical MHC HCs would not have been removed by pre-clearing of the 721.221

cells with W6/32 (Solheim *et al.*, 1997b) as W6/32 does not recognize MHC class I molecules when they are associated with TAP (Carreno *et al.*, 1995; Neisig *et al.*, 1996), thus explaining the observation of TAP-associated β_2m .

ER-60 appears to be a chaperone that possesses two distinct enzymatic activities. The thiol-dependent reductase activity of ER-60 is well established (Srivastava *et al.*, 1991; Kozaki *et al.*, 1994; Hirano *et al.*, 1995). The association of MHC class I HCs with ER-60 before the association of HC with β_2m coincides with the formation of disulfide bonds within the HC (Ribaudo and Margulies, 1992; Neefjes *et al.*, 1993; Tector *et al.*, 1997), introducing the possibility that the thiol-dependent reductase activity is involved in this step. However, attempts to identify such a folding intermediate have so far been unsuccessful. ER-60 has also been suggested to possess proteolytic activity (Urade and Kito, 1992; Urade *et al.*, 1992; Otsu *et al.*, 1995). While a clearly defined substrate specificity is lacking, recent work by Urade *et al.* (1997) convincingly demonstrates a cysteine protease activity in the C-terminal cysteines in the CGHC motifs that is converted to a serine protease activity upon substitution of the active site cysteine with a serine residue. The observation that ER-60 associates via 'empty' MHC class I molecules with the TAP transporter and that ER-60 is able to bind TAP-transported peptides within the ER (J.O.Koopman, G.J.Hammerling and F.Momburg, unpublished observations) raises the possibility that ER-60 is involved in trimming of peptides binding to MHC class I.

Materials and methods

Cell lines

Cells were maintained in RPMI 1640 (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), 15 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, 2 mM glutamine, non-essential amino acids and pyruvate at 37°C in a humid, 7% CO₂ atmosphere.

The cell lines used in this study were the murine T-cell lymphoma EL4 (H-2^b) and its β_2m -negative derivative S3 (Stürmhöfel and Hammerling, 1990), and the human cell lines T2 (TAP-negative), 721.220 (tapasin-negative) and 721.221 (HLA-A,B,C-negative).

Antibodies

The antibodies used in this study were: the monoclonal antibodies anti-TAP1.28, anti-TAP2.17 (Nijenhuis *et al.*, 1996), BBM.1 (Brodsky *et al.*, 1979), B9.12 (Rebai and Malisen, 1983), HC10 (Stam *et al.*, 1986), W6/32 (Parham *et al.*, 1979), APH 266 (IgG2a), HD20 (IgG1), 28.14.8s (anti-D^b α_3), anti-calnexin (MA3-027 Affinity Bioreagents Inc.), and rabbit anti-calreticulin serum (PA3-900 Affinity Bioreagents Inc.), rabbit anti-bovine PDI serum (BioMol), rabbit anti-rat ER-60 serum (M. Kito), ham1 and ham2 rabbit sera (K.Fruh) and rabbit anti-peptide (tapasin 2-20) serum (Rgp48N) (P.Cresswell).

Metabolic labeling

For two-dimensional gels, 10×10^6 cells were starved for 30 min at 37°C in RPMI 1640 Cys⁻/Met⁻ (Gibco-BRL) supplemented with dialyzed 5% FCS, 15 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, 2 mM glutamine and pyruvate. Cells were labeled for 15 min at 37°C with 1 mCi [³⁵S]ProMix (Amersham) and lysed in 1 ml of ice-cold digitonin lysis buffer [1% digitonin (Sigma) 20 mM HEPES pH 7.4/5 mM MgOAc] for 30 min at 4°C with rotation.

Immunoprecipitation

Nuclei were removed from the cell lysates by centrifugation at 15 000 g for 15 min at 4°C and the resulting supernatants pre-cleared with *Staphylococcus aureus* and normal rabbit serum (DAKO). After pre-clearing, the proteins of interest were immunoprecipitated with the

corresponding antibody and protein A–Sepharose at 4°C; in the case of mouse monoclonal antibodies, a rabbit anti-mouse antibody was also included. After immunoprecipitation, the pellets were washed three times with a low digitonin wash buffer (0.05% digitonin/150 mM NaCl/50 mM Tris pH 7.4/5 mM EDTA), resuspended in 50 µl of sample buffer, and heated at 95°C for 5 min, briefly centrifuged and stored at –20°C until needed.

Isoelectric focusing

Isoelectric focusing gels were run as described in Celis *et al.* (1993). Ampholines pH 3.5–10 and pH 5–7 were purchased from Sigma.

SDS–PAGE

SDS–PAGE was performed as described (Laemmli, 1970).

Autofluorography

Polyacrylamide gels were washed twice in dimethylsulfoxide (DMSO) for 45 min with constant agitation and then incubated in DMSO/PPO (Rotifluorosint D, Roth) for 60 min with constant agitation. Following PPO treatment, the gels were rehydrated in deionized H₂O for 60–90 min and then dried at 80°C for 150 min. The dried gels were exposed to film (BioMax MR, Kodak) and stored at –80°C.

Densitometry

Autoradiographic films were scanned on a 'StudioScan IIsi' scanner (Agfa) using the software 'FotoLook SA 2.05' (Agfa). Films were scanned as 'grey scale' images with a density of 300 p.p.i. The scanned images were then imported into the software 'NIH Image' (v1.56 Wayne Rasband, NIH). After determining that the film had not received a saturating exposure, the densities of each band were measured as pixels per unit area (constant for each measurement) and the background subtracted. The average of five independent measurements was plotted versus time to determine the half-life of each protein band.

Coomassie staining

After electrophoresis, the polyacrylamide gels were fixed in a 10% trichloroacetic acid (TCA) solution for 90 min and then stained overnight using colloidal Coomassie stain (Neuhoff *et al.*, 1990). Gels were destained briefly in 25% methanol and stored in deionized H₂O. Visualized protein spots were excised using a scalpel and transferred to a 1.7 ml Eppendorf tube containing 0.5 ml of deionized H₂O. Samples were stored at –20°C until analyzed.

Protein analysis

Electrophoretically separated proteins were excised from the gel and analyzed as described (Shevchenko *et al.*, 1996a; Jensen *et al.*, 1997b). Briefly, proteins within the gel slice were washed, reduced, S-alkylated and subjected to overnight tryptic digestion at 37°C. A 0.3 µl aliquot of the tryptic digestion was removed and analyzed by MALDI mass spectrometry. Samples were analyzed using a modified Bruker-Daltonics (Bremen, Germany) REFLEX time-of-flight (TOF) mass spectrometer (Jensen *et al.*, 1996a). The measured sets of peptide masses were then screened against a comprehensive sequence database utilizing the PeptideSearch software as previously described (Mann *et al.*, 1993; Jensen *et al.*, 1997a).

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