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A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules

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An important mammalian defence strategy against intracellular pathogens is the presentation of cytoplasmically derived short peptides by major histocompatibility complex (MHC) class I molecules to cytotoxic T lymphocytes. MHC class I molecules assemble in the endoplasmic reticulum (ER) with chaperones, including calnexin and calreticulin, before binding to the transporter associated with antigen processing (TAP). We show here that the thiol-dependent reductase ERp57 (also known as ER60 protease) is involved in MHC class I assembly. ERp57 co-purified with the rat TAP complex (comprising TAP1 and TAP2), and associated with MHC class I molecules at an early stage in their biosynthesis. This association was sensitive to castanospermine, which inhibits the processing of glycoproteins. Human MHC class I molecules were also found to associate with ERp57. We conclude that ERp57 is a newly identified component of the MHC class I pathway, and that it appears to interact with MHC class I molecules before they associate with TAP.

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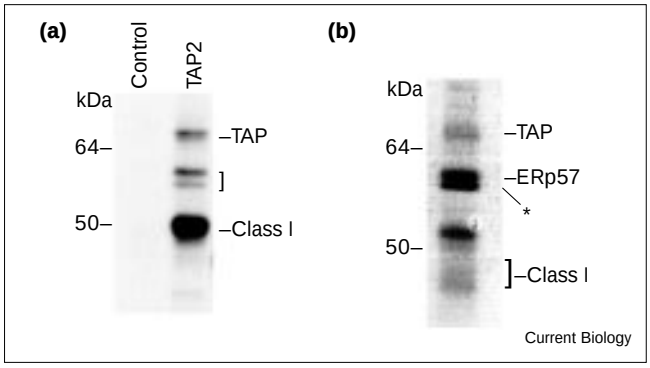
Results and discussion

Co-immunoprecipitation of proteins associated with TAP from metabolically labelled rat C58 thymoma cells lysed in digitonin gives two proteins of apparent molecular weights of around 59 kDa when separated by SDS–PAGE — a region of polyacrylamide gels previously reported to contain calreticulin [1]. In order to identify the components of this doublet, TAP was immunoaffinity purified from digitonin-solubilised C58 cells, and Coomassie-stained gel bands (Figure 1b) were subjected to mass spectrometry after 'in-gel' tryptic digestion. Database searching with the mass of the obtained fragments allowed us to confirm the presence of rat TAP and rat MHC class I, and to determine that the upper band of the 59 kDa doublet contained ERp57 thiol-dependent reductase (Table 1 and Figure 1).

Figure 1



Analysis of TAP-associated polypeptides. (a) C58 cells were labelled for 15 min with [^{35}S]methionine, lysed in digitonin-containing buffer, and immunoprecipitated with antisera to TAP2, followed by SDS–PAGE and autoradiography. The bracket indicates the doublet of bands under investigation; bands known to contain the TAP heterodimer and MHC class I molecules are also indicated. Sepharose beads alone were used as a control. (b) Coomassie-stained polyacrylamide gel of the TAP complex immunopurified from C58 cells, with product identities ascribed by subsequent mass spectrometry (derived from this and other purifications). MHC class I polypeptides run as a diffuse band, possibly due to the use of non-reducing sample buffer. No signal in the mass spectrometry analysis of proteins from rat cells was obtained for the band marked with an asterisk (*), though the equivalent doublet in this region from a human cell line contained mixtures of Erp57 and calreticulin. Also, in immunoprecipitates from a human cell line, we have identified the band immediately above MHC class I as containing tapasin (data not shown). Rat tapasin was not identified, possibly due to its absence from the sequence database.

ERp57 is an ER-resident polypeptide which has been ascribed functions as a thiol-dependent reductase [2] and a cysteine protease [3], and which is involved in glycoprotein-specific interactions with nascent polypeptides in the ER [4]. We therefore investigated what role ERp57 might have in the complex containing TAP and MHC class I molecules. Immunoprecipitations of MHC class I molecules with the pan-MHC class I monoclonal antibody OX18, or of TAP with antisera to rat TAP1 or TAP2, were probed by western blot analysis for the presence of ERp57. We found that ERp57 associated with the TAP complex in digitonin but not in NP40 (Figure 2a), nor with the bulk of OX18-reactive MHC class I molecules, most of which will be at the cell surface. TAP complexes in digitonin-lysed C58 cells also contained calreticulin (Figure 2b). Multiple rounds of

Table 1

Peptide matches for ERp57 obtained by mass spectrometry.

Start	End	MH+	Considered modification	Sequence
62	73	1347.70		RLAPEYEAAATR
105	119	1652.77	+ PO_4	IFRDGEEAGAYDGPR
162	179	2086.96	+ PO_4	DLFSDGHSEFLKAASNLR
195	218	2821.38		EYDDNGEGITIFRPLHLANKFEDK
289	304	1746.93		TFLDAGHKLNFAVASR
305	329	2733.41		KTFSHELSDFGLESTTGEIPVVAIR
336	344	1172.54		FVMQEEFSR
352	363	1529.78		FLQEYFDGNLKR
472	482	1397.71		ELNDFISYLQR

For each ERp57 peptide (given in single-letter amino-acid code), the amino-acid number of the first and last residue and the molecular weight by mass spectrometry (MH+) is indicated, as are any modifications predicted from the molecular weight. Considered modifications included oxidation of methionine and phosphorylation of serine, threonine and tyrosine residues. Matched peptides covered 28% (142 out of 505 amino acids) of the protein.

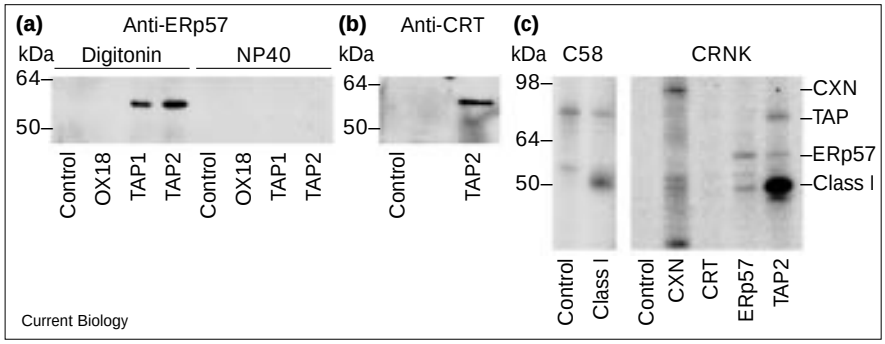
depletion of a digitonin cell lysate using an anti-ERp57 antibody reduced but did not completely ablate the presence of TAP, suggesting that not all TAP complexes contain ERp57 (data not shown).

We next determined if ERp57 could be found in association with MHC class I molecules that were not associated with TAP, as has been found for calreticulin [5]. For these experiments we studied both C58 cells and the rat natural killer cell line CRNK16, which produced essentially identical results. Metabolically labelled cells, lysed in digitonin, were immunoprecipitated with reagents specific for MHC class I, calnexin, calreticulin, ERp57 and TAP. A band of protein of similar molecular mass to that of class I heavy chains was seen in association with calnexin, ERp57 and TAP (Figure 2c). Longer exposure of the gel revealed that class I heavy chains also associated with calreticulin (data not shown). When proteins were separated on gels containing a higher proportion of polyacrylamide, heterodimers of β2-microglobulin and the class I heavy chain were found to associate with calnexin, ERp57 and TAP (data not shown). Two-dimensional gel analysis confirmed that MHC class I molecules associated with TAP and ERp57 (data not shown).

We also determined the putative order of interaction of MHC class I molecules with calnexin, ERp57 and TAP. CRNK16 cells were metabolically labelled for 5 minutes and chased for various time periods, followed by lysis in digitonin and immunoprecipitation. MHC class I molecules assembled rapidly with calnexin, but this interaction decreased after 10 minutes of chase (Figure 3a). There was a delay in the interaction of ERp57 with MHC class I molecules in comparison with calnexin. The ERp57 interaction occurred with similar kinetics to that seen with TAP, increasing during the 30 minute chase period. The kinetics of the association between ERp57 and MHC class I closely resemble those reported for calreticulin and mouse H-2K^b molecules [6].

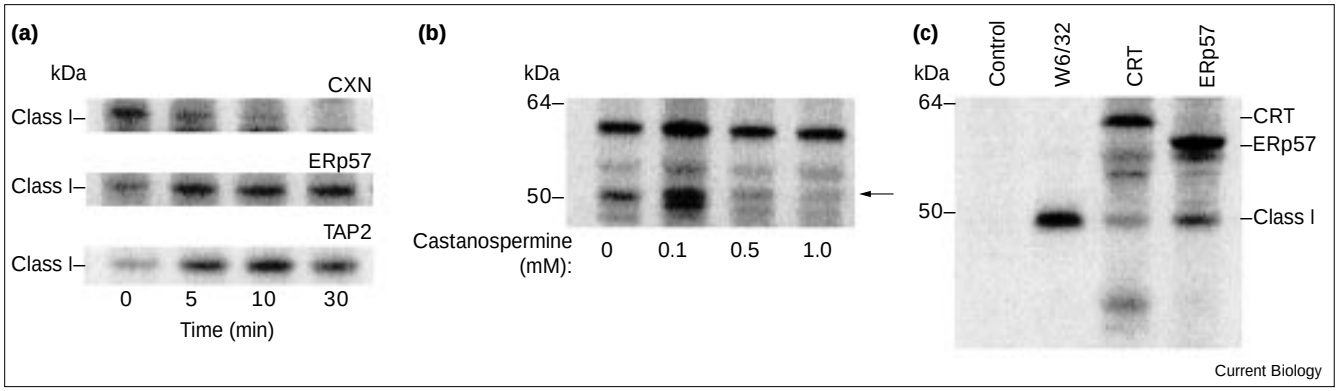
The interaction of calreticulin with glycoproteins in general [4], and MHC class I molecules specifically [5,6] is disrupted by castanospermine, an inhibitor of glucosidases I and II. To test if the interaction of ERp57 was also carbohydrate dependent, we immunoprecipitated complexes of ERp57 and MHC class I molecules from cells incubated and labelled in the presence of increasing amounts of castanospermine. As shown in Figure 3b, castanospermine treatment decreased the association of MHC class I molecules with ERp57 (and also TAP; data not shown). Finally, we tested the ability of human ERp57 and MHC class I molecules to interact. The human lymphoblastoid cell line T1 was metabolically

Figure 2



Erp57 is part of the TAP complex and associates with MHC class I molecules. (a) C58 cells were lysed in either digitonin-containing or NP40-containing buffers and immunoprecipitated with antibodies to MHC class I (OX18), TAP1 or TAP2. Sepharose beads alone were used as a control. Samples were separated by SDS–PAGE, blotted onto nitrocellulose, and probed with antisera to ERp57. (b) In a similar experiment, TAP2 immunoprecipitates from digitonin-lysed cells were probed for calreticulin (CRT). (c) C58 and CRNK16 cells were labelled for 15 min with [^{35}S]methionine, lysed in digitonin, and immunoprecipitated with antibodies specific for MHC class I, calnexin (CXN), calreticulin (CRT), ERp57 and TAP2, followed by SDS–PAGE analysis. The main immunoprecipitated products are indicated.

Figure 3



ERp57 associates with MHC class I early in biosynthesis, in an interaction dependent on carbohydrate side chains, and also with human MHC class I. (a) CRNK16 cells were labelled for 5 min with [^{35}S]methionine, chased for the indicated times, lysed in digitonin and sequentially immunoprecipitated with antibodies to calnexin (CXN), ERp57 and TAP2, followed by analysis by SDS–PAGE. For clarity, only the region of the gel containing MHC class I is shown. (b) CRNK16 cells were preincubated with the indicated concentrations of castanospermine, labelled for 15 min with [^{35}S]methionine, lysed in digitonin and immunoprecipitated with ERp57 antibodies, followed by SDS–PAGE analysis. Co-precipitating MHC class I molecules are indicated by an arrow. Concomitant with the loss of association between ERp57 and MHC class I molecules at higher concentrations of castanospermine is the appearance of slightly smaller species, possibly representing *N*-linked glycan processing intermediates. (c) Human T1 cells were labelled for 15 min with [^{35}S]methionine, lysed in digitonin, and immunoprecipitated with either the anti-MHC class I monoclonal antibody W6/32, anti-calreticulin (CRT) antibody, or anti-ERp57 antibody. The main immunoprecipitated products are indicated.

labelled, solubilised in digitonin and immunoprecipitated for MHC class I (using the antibody W6/32), calreticulin or ERp57. Both the calreticulin and ERp57 reagents co-immunoprecipitated a protein of the same apparent size as that brought down by W6/32, strongly suggesting that it was MHC class I (Figure 3c), and its identity was confirmed by two-dimensional gel analysis (data not shown). Thus, like the rat MHC class I assembly pathway, the human pathway also appears to include ERp57.

The results described here indicate that ERp57 can act as an integral part of the assembly of MHC class I molecules within the ER. The data imply that ERp57 and calreticulin may be interchangeable in function, though this remains to be determined in full. Due to a lack of defined peptide epitopes, we have not been able to test whether the complex between ERp57 and MHC class I in rat cells is empty of peptides, as has been demonstrated for the complex between calreticulin and MHC class I in human cells [5]. It has been suggested that linking the activities of calnexin, calreticulin and ERp57 could be one method of controlling the folding of glycoproteins [4]. As ERp57 shares some homology with protein disulfide isomerase (PDI) [2], it may have a role in the formation or control of disulfide bonds within peptide-receptive MHC class I molecules. Alternatively, as PDI has been shown to interact with peptides transported by TAP [7,8] and ERp57 has been shown to have proteolytic activity [3], ERp57 might be directly involved in the loading or 'trimming' of peptides.

Materials and methods

C58 [9], CRNK16 [10] and T1 cell lines were maintained in RPMI 1640 medium plus 5% fetal calf serum (FCS). For metabolic labelling, cells were starved in methionine-free medium for 30 min then labelled for 5 or 15 min with 3.7 MBq 35Trans-label (ICN). Lysis and immunoprecipitation in digitonin-containing or NP40-containing buffers was as previously described [11]. Castanospermine (Boehringer Mannheim) was included at the indicated concentrations for the starvation and labelling steps. TAP was immunoaffinity purified from 4×10^8 C58 cells lysed in 0.5% digitonin with 20 μg sheep anti-TAP2 antisera. The sample was heated in non-reducing sample buffer to prevent immunoglobulin heavy chain contaminating the 50 kDa region. Western blots were performed as previously described [11]. Anti-calreticulin antibody was from Stressgen, anti-calnexin antibody was a gift from D. Williams, anti-ERp57/ER60-protease reagents were gifts from M. Kito, R. Urade and Tom Wileman, OX18 was a gift from G. Butcher and anti-rat TAP1 and TAP2 were previously described [11]. Coomassie-stained gel pieces were digested *in situ* with trypsin [12]. Samples were analysed using a thin film matrix of 4-hydroxy-a-cyanocinnamic acid : nitrocellulose (2:1) in a Perceptive Biosystems Elite STR mass spectrometer in reflectron mode. Spectra were internally calibrated with matrix and trypsin autolysis ions and the peptide masses were database searched using the MS-FIT program at the Ludwig Institute (London) with a mass error set at 50 ppm.

Acknowledgements

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