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ERp57 interacts with conserved cysteine residues in the MHC class I peptide-binding groove

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Abstract The oxidoreductase ERp57 is a component of the major histocompatibility complex (MHC) class I peptide-loading complex. ERp57 can interact directly with MHC class I molecules, however, little is known about which of the cysteine residues within the MHC class I molecule are relevant to this interaction. MHC class I molecules possess conserved disulfide bonds between cysteines 101–164, and 203–259 in the peptide-binding and α3 domain, respectively. By studying a series of mutants of these conserved residues, we demonstrate that ERp57 predominantly associates with cysteine residues in the peptide-binding domain, thus indicating ERp57 has direct access to the peptide-binding groove of MHC class I molecules during assembly.

Keywords: Immunology; Major histocompatibility complex; ERp57; Antigen presentation/processing

1. Introduction

Major histocompatibility complex (MHC) class I molecules bind and present short peptides to $CD8^+$ T lymphocytes, allowing the detection and elimination of infected cells. During assembly in the endoplasmic reticulum (ER), MHC class I molecules interact initially with calnexin and BiP [1,2], before forming the peptide-loading complex (PLC), which contains MHC class I molecules, calreticulin, tapasin, ERp57, protein disulfide isomerase (PDI) and TAP [3–10]. Within the PLC, class I molecules acquire an optimal peptide subset [11,12].

Disulfide-bonded intermediates between the MHC class I heavy chain and ERp57, and also between MHC class I and PDI have been reported [9,13,14], potentially representing transient intermediates which would be expected to form during attempts to correctly fold MHC class I molecules. In the case of PDI recent data suggest that it directly controls the oxidation of the peptide-groove disulfide bond between Cys101 and Cys164 of MHC class I molecules within the PLC, thereby also regulating peptide optimisation [9].

In contrast, the function of ERp57 within the PLC still remains unclear. In ERp57-deficient B cells there is a lack of optimal peptide loading and an absence of MHC class I molecules within the PLC [15]. Furthermore, in cells expressing low levels of ERp57 by RNA interference the initial folding events of MHC class I in the ER are delayed [16].

To date it has not been determined which of the cysteine residues within MHC class I heavy chains interact with ERp57 during the early events in assembly. We have studied a series of mutant HLA-A*0201 molecules in which the conserved cysteine residues that contribute to intrachain disulfide bonds have been replaced with serine residues. Our data indicate that the cysteine residues in the peptide-binding groove interact with ERp57 during the assembly of MHC class I molecules.

2. Materials and methods

2.1. Cell lines and DNA

The rat C58 thymoma [17] and human .220.B8 (a gift from P. Lehner, University of Cambridge, UK) cell lines were maintained in RPMI 1640 supplemented with 5% FCS. The single cysteine mutant C58 transfectants have been described previously [18]. All constructs contain a C-terminal v5 epitope tag to aid detection. The double cysteine mutants in vector pCR3 (Invitrogen) were generated using Quickchange (Stratagene) methodology and sequenced to confirm mutagenesis. C58 cells were transfected by electroporation (180 V, 975 μF, Biorad Genepulser) and selected in medium containing 1 mg/ml G418.

2.2. Antibodies

Monoclonal antibodies (mAbs) and antisera used were as follows: pK (anti-V5 tag, present on the C-terminus of HLA-A*0201) is referred to as anti-MHC I heavy chain in the text and figures for simplicity, HC10 (recognising partially folded HLA-B molecules), BB7.2 (anti-HLA-A2), anti-calnexin antiserum (a gift from D. Williams, Toronto, Canada), anti-ERp57 antiserum (a gift from N. Bulleid, Manchester, UK), anti-BiP antiserum (Stressgen, SPA-826).

2.3. Immunoprecipitations and immunoblotting

Cells were pretreated with 20 mM *N*-ethylmaleimide (NEM, Sigma) on ice for 15 min and lysed in 1% Nonidet P-40 lysis buffer (1% v/v NP40, 150 mM NaCl, 10 mM Tris pH 7.5, 1 mM PMSF, and 10 mM NEM). Protein A-Sepharose precleared lysates were immunoprecipitated with anti-MHC class I heavy chain mAb. Washed beads were resuspended in non-reducing sample buffer and analysed on 8% SDS–PAGE gels. Immunoblots were probed with the indicated mAb or antiserum at 1:10000 dilution in PBS containing 0.1% Tween 20 (Sigma), followed by HRP conjugated anti-mouse or rabbit IgG antisera as

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Abbreviations: PDI, protein disulfide isomerase; MHC, major histocompatibility complex; PLC, peptide-loading complex

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required (Sigma), and developed by chemiluminescence (Supersignal West, Perbio). Pulse-chase experiments were performed as previously described [19]. Briefly 5×10^6 cells were labelled with 3.7 Mbq Trans label (ICN) for 15 min, and chased for the indicated times in normal medium. Samples were lysed and immunoprecipitated as above, then digested with 1 mU endoglycosidase H (Roche) for 1 h at 37 °C. Reduced samples were analysed by SDS–PAGE, and autoradiography.

2.4. Flow cytometry

Cells, in PBS supplemented with 1% FCS, were stained with antibodies for 30 min at 4 °C. After washing, cells were stained with FITC conjugated anti-mouse IgG, rewashed and analysed using Cellquest software on a Facscan cytometer (Becton Dickinson).

3. Results

3.1. Assembly and cell surface expression in the absence of conserved cysteine residues

We have previously reported a disulfide-bonded intermediate between ERp57 and MHC class I [14]. To determine which of the conserved cysteine residues in MHC class I molecules are responsible for this interaction we have studied a series of mutants wherein the conserved cysteine residues at positions 101, 164, 203 and 259 (Fig. 1A) of the HLA-A2 heavy chain have been mutated to serine. The mutants were C-terminally epitope-tagged to permit isolation of a non-conformational dependent pool of MHC class I molecules, and were expressed as stable transfectants in the rat C58 cell line.

Mutation of the conserved cysteine residues to serine prevented cell surface expression at 37 °C, as determined by flow cytometry (Fig. 1B). Pulse-chase and immunoprecipitation experiments also revealed that, whilst the wildtype A2 acquired partial endoglycosidase H (endo H) resistance, the mutants remained fully sensitive to endo H digestion during a three-hour chase period, indicating a failure to exit from the ER (Fig. 1C). Cell surface biotinylation and immunoprecipitation also revealed no detectable surface expression (data not shown). Several of the mutants, A2.C164S for example, displayed clear evidence of ER degradation after 3 h chase.

When extended pulse-chase experiments were performed at reduced temperature (26 °C), significant acquisition of endo

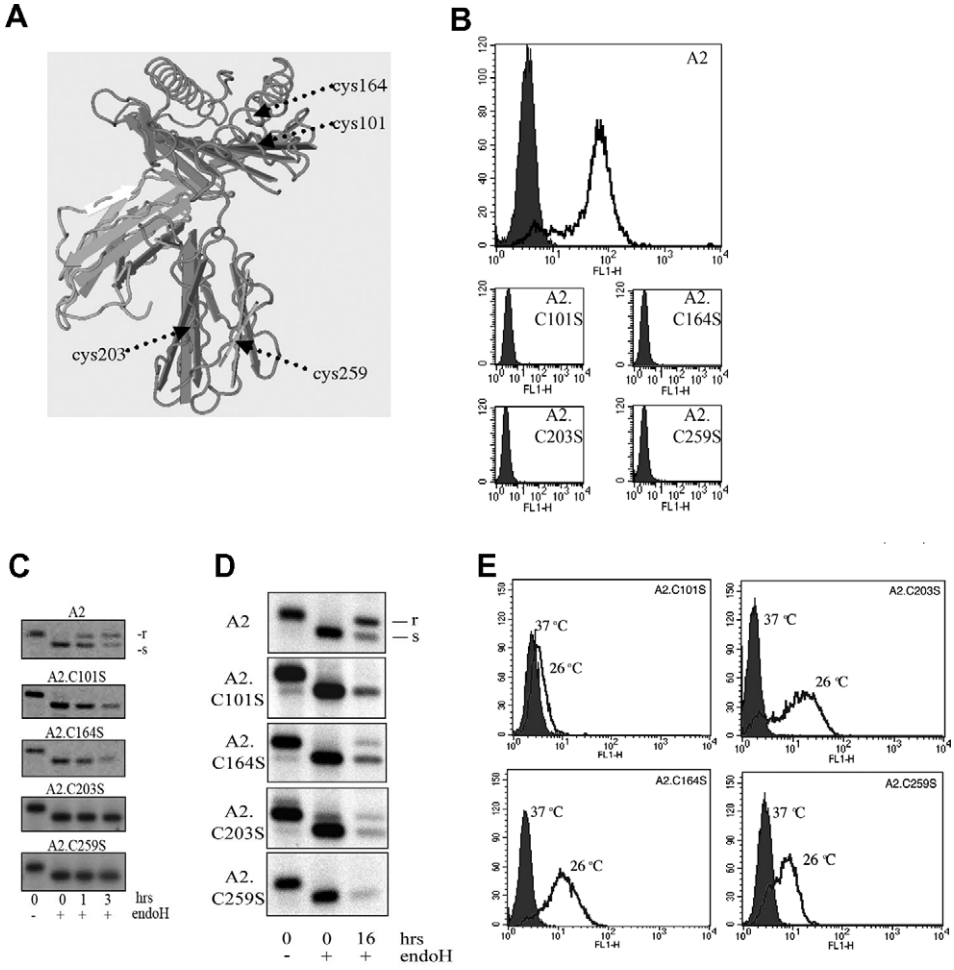

Fig. 1. Lack of conserved cysteines can be compensated for by incubation at reduced temperature. (A) Location of the conserved cysteine residues in MHC class I molecules that form disulfide bridges. (B) C58 rat thymoma cells expressing wildtype or mutant A2 molecules were analysed by flow cytometry using mAb BB7.2. Filled curve in the top panel: second stage FITC anti-mouse IgG alone. Solid line indicates BB7.2. Filled curves in the small panels indicates BB7.2 staining. (C) Pulse-chase analysis. MHC class I molecules were immunoprecipitated at the indicated chase timepoints, and samples digested with or without endo H. Endo H sensitive (-s) and resistant (-r) species are indicated. (D) Cells were radiolabelled and chased for 16 h at 26 °C, lysed and MHC class I immunoprecipitated. (E) The indicated cells were incubated overnight at 37 °C or 26 °C and then analysed by flow cytometry using mAb BB7.2.

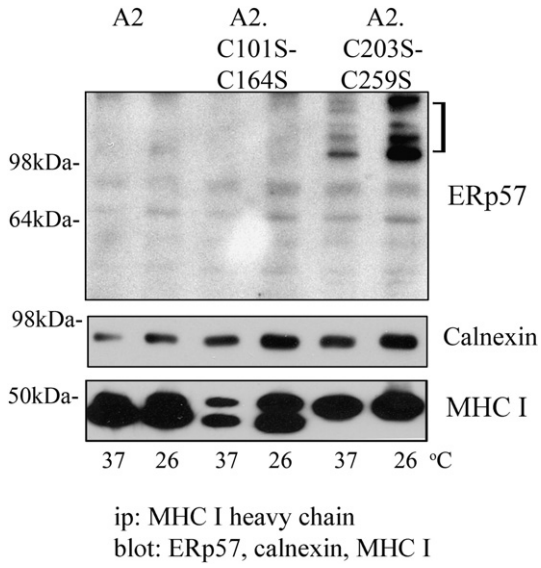

Fig. 2. ERp57 associates with A2 molecules lacking both conserved cysteines in the α3 domain. C58 cells expressing wildtype A2 molecules, or the double cysteine mutants A2.C101S-C164S and A2.C203S-C259S were incubated overnight at the indicated temperatures of 37 °C or 26 °C, and then lysed, immunoprecipitated with anti-MHC class I heavy chain and immunoblotted for ERp57, calnexin and MHC class I.

H resistance was observed for several of the mutants, suggesting partial rescue of assembly and delivery to the cell surface (Fig. 1D). Flow cytometry confirmed surface expression (Fig. 1E), correlating with the pulse-chase observations. Cell surface biotinylation and immunoprecipitation confirmed the expression of B2m-associated A2 molecules at the cell surface (not shown). Thus incubation at reduced temperature can partially compensate for the lack of a conserved disulfide bond in both the peptide-binding groove and the α3-domain.

3.2. Detection of ERp57-MHC class I intermediates

The ability of the cysteine mutant molecules to fold and be delivered to the cell surface at reduced temperature led us to ask whether we could analyse ERp57-MHC class I interactions in these cells. We generated C58 transfectant lines expressing HLA-A2 cDNA mutants lacking either both cysteine residues in the peptide-binding groove (annotated A2.C101S-C164S), or in the α3 domain (A2.C203S-C259S). Anti-MHC I heavy chain immunoprecipitations were performed on cells that had been incubated overnight at 26 °C or 37 °C. Non-reduced samples were immunoblotted for ERp57, which revealed the presence of ERp57-class I heavy chain complexes in A2.C203S-C259S cells, i.e. in the A2 molecule that still possessed cysteine residues in the peptide groove (Fig. 2). This interaction was enhanced at reduced temperature. No interaction was detected in cells expressing A2.C101S-C164S, suggesting that ERp57 does not associate significantly with the α3 domain cysteine residues. Further immunoblotting of the immunoprecipitates revealed the presence of the chaperone calnexin at similar levels in all immunoprecipitated samples.

3.3. ERp57 association with cysteine residues in the peptide-binding groove

Based on the above findings, we next studied which of the two conserved cysteine residues in the peptide groove were capable of interacting with ERp57. We made use of the single conserved cysteine mutants of A2 expressed in C58 cells. As above, anti-MHC class I heavy chain immunoprecipitation of cell lysates was performed and non-reduced samples immunoblotted for ERp57, calnexin and MHC class I heavy chain. As shown in Fig. 3A, ERp57-class I heavy chain complexes were detected primarily in A2.C101S and A2.C164S cells, and only very weakly in A2, A2.C203S and A2.C259S cells. Furthermore, the different relative gel mobilities of the ERp57-A2.C101S and the ERp57-A2.C164S complexes appear to correlate with the two main species detected in ERp57-A2.C203S-C259S cells (Fig. 3A), suggesting that in the double mutant A2.C203S-C259S cells, ERp57 can interact with both peptide-groove cysteines. To confirm that the observed ERp57-MHC class I interactions were not due to the xenogeneic expression of a human MHC class I molecule in a rat cell line, we also studied the tapasin deficient .220 cell line transfected with HLA-B8. Immunoprecipitation

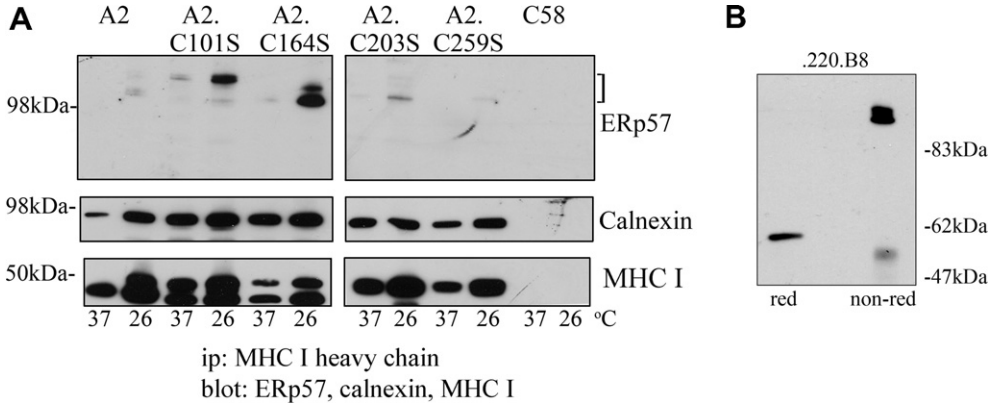

Fig. 3. (A) ERp57 associates with A2 molecules lacking one of the two conserved cysteine residues in the peptide-binding groove. Untransfected C58 cells, or cells expressing wildtype A2, or the single cyteine mutants A2.C101S, A2.C164S, A2.C203S and A2.C259S, were incubated overnight at the indicated temperatures and then lysed, immunoprecipitated with anti-MHC class I heavy chain and immunoblotted for ERp57, calnexin and MHC class I. (B) .220.B8 cells were lysed, immunoprecipitated with HC10, and reduced and non-reduced samples analysed by SDS–PAGE and immunoblotting for ERp57.

with HC10, followed by immunoblotting with ERp57 revealed similar complexes to those observed in rat C58 cells (Fig. 3B).

4. Discussion

To assist the dissection of the early events in MHC class I folding, in this study we have initiated an approach to determine which of the conserved cysteine residues in MHC class I molecules interact with ERp57. Oxidoreductase-substrate intermediates are often short-lived and constitute a relatively small proportion of the protein population undergoing folding. Furthermore, to isolate ERp57-class I heavy chain intermediates we have avoided the use of mAbs that depend on the conformation of the class I molecule. Instead, our approach has been to utilise an epitope tag inserted at the C-terminus of the class I heavy chain, permitting isolation irrespective of folding status.

By removing both cysteine residues in either the peptide-binding groove (C101S-C164S), or the α3-domain (C203S-C259S) our data demonstrate that ERp57-class I heavy chain complexes can be detected when the peptide-groove cysteines are retained. In the single cysteine mutants the ERp57-class I heavy chain interactions were detected when we disrupted the normal disulfide bond formation in the peptide-binding groove. The noted differences in gel mobility in the single cysteine mutants (Fig. 3A), which correlate with the species seen in the α3-domain (C203S-C259S) mutant (Fig. 2), suggest considerable conformational differences exist between the A2.C101S-ERp57 and A2.C164S-ERp57 species. However, at this stage we cannot distinguish whether these differences reside in the MHC class I heavy chain or ERp57, although alterations in the MHC class I heavy chain seem more likely. Taken together our data point to ERp57 being able to interact with the most membrane-distal region of MHC class I molecules, the peptide-binding groove.

Calnexin and calreticulin interact with proteins through their lectin-binding activity [20], with the crucial glycan site on MHC class I molecules being asparagine at position 86, located at the end of the peptide-binding groove on the α1 helix [21,22]. From this location the extended calnexin/calreticulin P-domain arm, which interacts with ERp57 at its tip [23], would conceivably allow ERp57 access to the nascent peptide-binding groove.

In ERp57 deficient B cells there appears to be no gross defect in the redox state of MHC class I molecules [15]. Such data indicate that oxidation of MHC class I molecules during assembly can occur in the absence of ERp57, and indeed recent data ascribe this function to PDI [9]. However, peptide optimisation is disrupted in such circumstances. Therefore, ERp57 may indeed have a direct role, in combination with PDI, in MHC class I assembly.

In the experiments reported here we did not set out primarily to determine the disulfide interactions occurring within the PLC, and for this reason we utilised a detergent that would not preserve the PLC, but would be able to preserve interactions with calnexin. Thus our observations may relate to early stages of MHC class I assembly. Experiments are now underway to determine the relative contributions of ERp57 and PDI in the PLC.

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