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Review

Assembly of MHC class I peptide complexes from the perspective of disulfide bond formation

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Abstract. Assembly of functional major histocompatibility complex (MHC) class I peptide complexes within the endoplasmic reticulum is critically important for the development of an adaptive immune response. The highly regulated loading of peptides onto MHC class I molecules is controlled by a multi-component chaperone system called the MHC class I peptide loading complex. The recent identification of the thioredoxin family member ERp57 as a component of the loading complex led to an interesting question: Why is there a thiol-disulfide oxidoreductase inside a complex dedicated to inserting peptides into a receptor binding site? Most recently, specific ERp57-mediated disulfide bond rearrangements have been identified inside the loading complex. What these biochemical events mean for the peptide loading process remains a matter of conjecture. While several important questions wait to be answered, this review intends to summarize our current view of the oxidative folding of MHC class I molecules and addresses the question of how the receptor ligand interaction might be regulated by thiol-based redox reactions.

Key words. MHC class I; ERp57; tapasin; peptide loading complex; disulfide bond formation; disulfide isomerization; ER quality control.

Antigen processing and presentation

Upon entry of host cells, viruses and bacteria have evolved mechanisms to avoid direct contact with the immune system. To nevertheless expose the presence of microbes, pieces of foreign antigens are displayed on the surface of infected cells, a process termed antigen processing and presentation. Two major pathways of antigen processing have been recognized. Foreign proteins internalized by endocytosis are typically processed and presented by the so-called major histocompatibility complex (MHC) class II pathway [1]: the proteolytic environment of the endocytic pathway generates peptides that bind to peptide receptors known as MHC class II molecules. Following transport to the cell surface, MHC class II-peptide complexes are screened by CD4+ T cells for the presence of foreign antigens. In contrast, foreign proteins located in the cytoplasm are typically processed and delivered by the so-called MHC class I pathway [2, 3]: they are first degraded by the proteasome and other cytosolic proteases, giving rise to peptide fragments which are subsequently translocated into the endoplasmic reticulum (ER). Once inside the secretory pathway, suitable peptides are bound to a related class of peptide receptors, the MHC class I molecules. Receptors loaded with peptides then travel to the cell surface for presentation to CD8+ T cells, which in turn trigger effector mechanisms leading to destruction of the infected host cell. Antigen processing and presentation is a shared feature of jawed vertebrates [4] and the foundation of adaptive immunity.

Given the importance of antigen presentation for immune function, the process of loading peptides onto the MHC class I peptide receptor is expected to be tightly regulated. Peptide receptors are polymorphic, and different alleles

have different requirements for stable peptide binding. Depending on the specific MHC class I allele, peptides must possess certain chemical properties in order to fit tightly into the binding groove of a peptide receptor [5]. The process of peptide binding should select for high-affinity ligands, as peptides should not dissociate during transport to and on arrival at the cell surface. In general, it seems essential that nonfunctional or partially functional peptide receptors not reach the plasma membrane where their presence could give rise to erroneous signals. The quality control of peptide binding and receptor assembly is not yet fully understood, and this review will discuss some new evidence demonstrating a crucial role for disulfide bond isomerization in this process.

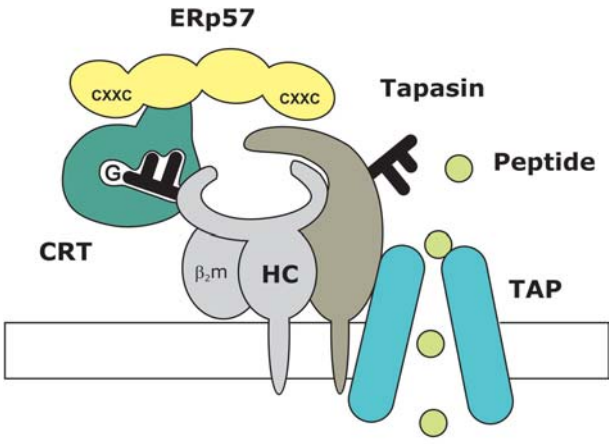

Figure 1. Composition of the MHC class I loading complex. Peptides are translocated into the lumen of the ER by the transporter associated with antigen processing (TAP). The type I transmembrane glycoprotein tapasin provides a bridge between TAP and the peptide receptor, which is composed of heavy chain (HC) and $\beta 2$-microglobulin (β_2m). While tapasin carries a deglucosylated glycan, the incompletely folded MHC heavy chain displays a glycan with a single terminal glucose residue (G). This specific glycan is bound by the lectin calreticulin (CRT), which in turn provides a binding site for the thiol-disulfide oxidoreductase ERp57. The two terminal domains of ERp57 harbor active sites (CXXC).

The peptide loading machinery

Peptide loading onto MHC class I receptors takes place inside the ER, a compartment specialized in the folding and assembly of secretory and membrane-bound proteins [6]. While waiting for suitable peptides to bind within the peptide binding groove, the MHC class I molecule is surrounded by several ER-resident proteins which facilitate the peptide loading process [7]. This multi-component machinery is called the MHC class I loading complex. After successful peptide binding, the loading complex disassembles and releases the loaded MHC molecule to continue its journey along the secretory pathway to the cell surface.

Thus far, seven different polypeptides have been recognized as components of the loading complex (fig. 1). The transporter associated with antigen processing (TAP) is a heterodimeric transmembrane protein specialized in translocating peptides from the cytosol into the ER [8]. It is in direct contact with tapasin, a type I transmembrane protein whose only known cellular function is in the context of MHC class I maturation [9]. Tapasin provides a physical bridge between the TAP transporter and the MHC class I molecule, thereby bringing the receptor in close proximity to the peptide source. In addition, tapasin is actively involved in the peptide loading process, making peptide loading much more efficient [10, 11]. However, the mode and mechanism by which tapasin modulates peptide binding to class I molecules remains to be understood. The MHC class I peptide receptor itself is composed of two separate chains, the glycosylated heavy chain (HC) and the associated β_2-microglobulin (β_2m). Inside the loading complex, class I HC is associated with the ER-resident lectin calreticulin (CRT) [12]. This interaction is mediated by the N-linked glycan, which provides a binding site for CRT [13]. Most recently, the thiol-disulfide oxidoreductase ERp57 has been recognized as an additional component of the loading complex [14–16]. ERp57, a member of the protein disulfide isomerase (PDI) family, is known to be recruited to incompletely folded glycoproteins by interaction with either CRT or calnexin (CNX) [17]. ERp57 is composed of four thioredoxin (TR) domains, two of which carry the active site motif CXXC [18]. Unlike tapasin, CRT and ERp57 are general chaperones of glycoprotein folding in the ER [19].

A role for thiol oxidation in peptide selection and loading?

The identification of ERp57 as a component of the loading complex led to an interesting question: Why is a thiol-disulfide oxidoreductase part of a complex dedicated to inserting peptides into a receptor binding site? Does ERp57 exercise redox activity in this complex? If yes, does it act on disulfide bonds in the MHC class I molecule? Like all newly synthesized membrane proteins, MHC molecules are cotranslationally inserted into the ER with their cysteine residues fully reduced. Productive folding and assembly inside the ER lumen is accompanied by formation of the correct disulfide bonds. An elaborate quality control system allows only correctly folded and assembled proteins to leave the ER and to proceed to the cell surface [19]. Accordingly, formation of the correct set of disulfide bonds is typically required to pass quality control [19]. It is, however, not clear why disulfide bond formation in the class I molecule should relate to peptide binding, since disulfide bond formation might

well be completed prior to the peptide selection and loading process. If disulfide bond formation is complete prior to peptide loading, the receptor would attain its native state upon peptide binding without any further redox chemistry taking place. Apart from the presence of ERp57, is there any other indication for a connection between thiol redox chemistry and peptide binding?

Disulfide bonds present in the mature MHC class I molecule

Assuming that ERp57 is acting on the MHC class I molecule within the loading complex, which of the cysteines or disulfide bonds might be targeted by its redox activity? The mature MHC heavy chain harbors two disulfide bonds, one located in the membrane-proximal α_3 domain and one in the α_2 domain which forms part of the peptide binding groove. β_2-Microglobulin contributes another disulfide bond to the heterodimeric peptide receptor (fig. 2A). Two of the three disulfide bonds are part of classical immunoglobulin (Ig) folds, namely those of β_2m (Cys25-Cys80) and the α_3 domain of heavy chain (Cys203-Cys259). Immunoglobulin domains are independent folding units, and their conserved disulfide bond becomes buried in the hydrophobic core [20, 21]. Once formed, these disulfide bonds are unlikely to participate in further thiol-disulfide exchange reactions.

Unlike the ubiquitous immunoglobulin fold, the fold characteristic of the MHC peptide binding groove (α1/α2 domain) has so far only been found in MHC molecules and their close relatives. As revealed by the crystal structure of MHC class I [22], the disulfide bond of the α_2 domain (Cys101-Cys164) is located at the fringe of the peptide binding groove, forming a link between the β-sheet floor and the α_2 helix bordering the groove (fig. 2B). The disulfide bond is in close proximity to the hydrogen-bonding network that anchors the N-terminal amino group of the peptide ligand in the peptide binding groove [23] (fig. 2C), and it seems likely that the overall arrangement of this crucial binding site would be significantly compromised if cysteines 101 and 164 were not involved in a disulfide bond. Therefore, the structure of MHC class I peptide complexes suggests that oxidation of the α_2 cysteines is a prerequisite for stable peptide binding. In fact, the importance of complete heavy chain oxidation for peptide binding has been observed experimentally. As measured by conformation-specific antibodies, peptide binding converts oxidized, but not reduced, L^d molecules to fully conformed complexes [24]. In vitro, assembly of

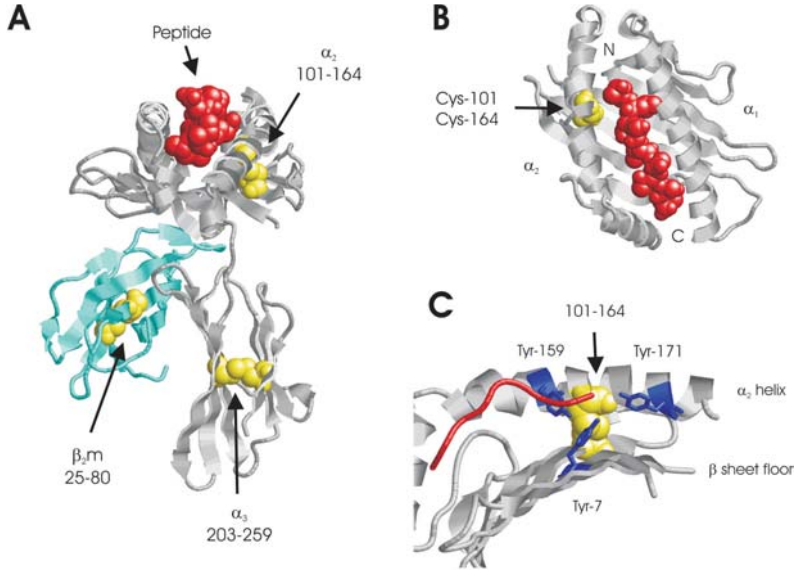

Figure 2. (*A*) Disulfide bonds in the mature MHC class I peptide complex. The heavy chain harbors two disulfide bonds, one located in the membrane-proximal α_3 domain (Cys203-Cys259) and one in the α_2 domain (Cys101-Cys164), which forms part of the peptide binding groove (peptide shown in red). β_2-Microglobulin contributes another disulfide bond to the heterodimeric peptide receptor (Cys25-Cys80). Two of the three disulfide bonds are part of classical immunoglobulin (Ig) folds, namely those of β_2-m and the α_3 domain of heavy chain. (*B*) Top-down view of the MHC peptide binding domain. The α_2 disulfide bond (Cys101-Cys164) is located at the fringe of the peptide binding groove, forming a link between the β-sheet floor and the α_2 helix bordering the groove. The N-terminal portion of the peptide (red) is in close proximity to the disulfide bond. (*C*) Proximity between the N-terminal hydrogen-bonding network and the α_2 disulfide bond. The N-terminus of the peptide ligand is typically hydrogen bonded to the hydroxyl groups of four tyrosine residues, three of which are shown in the figure (the α_1 helix has been omitted for clarity). Two of the tyrosine residues (Tyr-159 and Tyr-171) are located on the α_2 helix and close to Cys-164. It appears likely that the stereochemical arrangement required for the hydrogen-bonding network depends on the prior formation of the Cys101-Cys164 disulfide bond (shown in yellow and as a space-filling model). The figure has been created using PDB entry 1HHG (HLA-A2 complexed with a nonameric peptide from gp120).

recombinant class I molecules is most effective when disulfide bonds are formed prior to peptide binding [25]. The available MHC class I structures also demonstrate that the presence of the peptide ligand contributes to the burial of the α_2 disulfide bond. A peptide of appropriate affinity could therefore shield or make the Cys101-Cys164 disulfide bond less accessible to reduction by ERp57. This was further suggested by the in vitro observation that partially folded molecules are susceptible to reduction by ERp57, whilst fully folded or peptide-loaded MHC class I molecules were resistant to ERp57-mediated reductase activity [26]. Therefore, the spatial proximity between the α_2 disulfide bond and the peptide binding site suggests a possible relationship between thiol redox state and occupation of the peptide binding groove.

How is the maturation of MHC class I molecules affected when the Cys101-Cys164 disulfide bond is genetically disrupted? A somatic mutation of Cys-164 (C164Y) in the mouse K^b molecule was found to be responsible for the loss of surface expression of this allele in a tumor cell line [27]. A Cys-101 mutant (C101S) of the mouse H-2L^d molecule is poorly expressed on the cell surface and was found to be deficient in its interaction with the loading complex [24, 28–30]. Mutation of either Cys-101 (C101S) or Cys-164 (C164A) in HLA-A2 led to impaired maturation and reduced surface expression (5% of wild type) [31]. HLA-A2 cysteine mutants (C101A, C164A) also fail to integrate into the MHC class I loading complex [T. Dick and P. Cresswell, unpublished observations]. As a result, the α_2 cysteine mutants could not be used to study the role of the α_2 disulfide bond in the context of peptide loading. Unfortunately, double mutants lacking both cysteines have not been analyzed, and it remains unclear whether the observed defect in maturation is really the result of a missing disulfide bond or, alternatively, the creation of an unpairable cysteine.

Considering the deleterious effects of α_2 cysteine mutations for the maturation of class I molecules, it is not surprising that the α_2 disulfide bond is highly conserved. Both α_2 cysteines have been found in all class I molecules that have been sequenced, including representatives from cartilaginous fish, bony fish, amphibians, reptilians and mammals [32–34]. Interestingly, nonclassical MHC class I molecules also have a conserved Cys101-Cys164 disulfide bond. Inspection of the current Pfam database [35], entry PF00129 (class I histocompatibility antigen, domains alpha 1 and 2; 3786 entries, June 2003), reveals very few exceptions to this pattern. No sequence lacking both cysteines has been reported. There is only one sequence lacking cysteine-101, representing PD6, an expressed MHC molecule from the miniature swine (*Sus scrofa*) [36]. Another five sequences in the database carry substitutions in position 164. One of these sequences, a class I molecule from the chimpanzee, encodes a cysteine in position 168 instead of position 164 [37]. Since residue 168 is a close neighbor of residue 164 in the α_2 helix and is also oriented towards the β sheet, this MHC molecule possibly forms an alternative disulfide bond (Cys101-Cys168). It is, however, possible that some of the apparent exceptions are the result of sequencing errors or the sequencing of pseudogenes.

It is also interesting to compare the location of disulfide bonds between the peptide binding domains of MHC class I and class II molecules which are homologous and structurally comparable [38]. While the class I peptide binding domain folds from a single chain, the class II peptide binding domain is assembled from two separate chains (α and β). A comparison of class I and class II crystal structures shows that the class I α_1 domain corresponds to the α_1 domain of class II α chain and that the class I α_2 domain corresponds to the β_1 domain of the class II β chain. As expected by this assignment, two cysteines, in positions homologous to those of the class I α_2 domain, are found to be absolutely conserved in the MHC class II β_1 domain (see Pfam PF00969). Correspondingly, no such cysteines are present in the MHC class II α_1 domain (with the exception of DM molecules and α chains from bony fish, see Pfam PF00339).

Given the universal conservation of the class I α_2 disulfide and its homologous class II β_1 disulfide, bond formation could be an intrinsic requirement for the correct folding of the domain, independent of the nature of the ligand or the function of the resulting receptor. However, if the quality control machinery in the ER operates such that any MHC molecule with an unpaired single cysteine in one location is predominantly retained and degraded, any alteration is strongly selected against and loss of both cysteines would be extremely unlikely, no matter how important the actual disulfide bond is for folding or stability.

Mechanisms of disulfide bond formation in the ER

Oxidative protein folding, i.e. folding with simultaneous introduction of disulfide bonds, is the central function of the ER. According to the prevailing view, the purpose of disulfide bond formation is stabilization of native protein structure. It is reasoned that extracellular proteins are exposed to variable and disruptive environments, posing higher demands on protein stability. Disulfide-mediated conformational stability is also thought to increase resistance against extracellular proteases. In contrast, intracellular proteins are considered to be situated in more protected and homeostatic environments, posing reduced demands on protein stability. Our understanding of the mechanism of disulfide-mediated stabilization is, however, incomplete [39, 40].

Although there is no doubt that disulfide bonds have the potential to contribute to overall protein stability [41],

this does not necessarily mean that all disulfide bonds have this function. Certain disulfide bonds are required for in vivo protein folding, but nevertheless do not contribute to stability. For example, the disulfide bridges of the cystine knot motif, a domain found in various extracellular signaling molecules, do not contribute to the thermodynamic stability of the folded state [42]. Apart from restricting the conformational space during folding, disulfides have the potential to guide the ER quality control machinery directly, as exposed disulfide bonds or free cysteines frequently indicate malfolded or incompletely folded structures. In fact, cysteines exposed in the ER have been found to serve as signals for assembly, retention and degradation [43].

Until recently, oxidized glutathione (GSSG) was widely considered to be responsible for the de novo generation of protein disulfide bonds inside the ER. This conclusion was primarily based on the observation that the ratio of oxidized (GSSG) to reduced glutathione (GSH) is higher inside the ER (GSSG/GSH ~0.33–1) as compared with the cytosol (GSSG/GSH ~0.01–0.03) [44]. However, new evidence has led to a remarkable reevaluation of the role of glutathione in the ER. It has been recognized that the glutathione system does not participate in protein thiol oxidation [45] and, in opposition to previous belief, acts as a net reductant for disulfide bonds in the ER [46]. Instead, protein thiol oxidation is facilitated by specific protein-protein interactions [47]. Substrate proteins are first oxidized by the thiol-disulfide oxidoreductase protein disulfide isomerase (PDI), a member of the thioredoxin superfamily. In turn, PDI is reoxidized by Ero1, a lumenal protein associated with the ER membrane [48]. Ero1 was shown to couple disulfide bond formation directly to the consumption of oxygen [49].

In the ER there exist several additional thiol-disulfide oxidoreductases of the PDI family, including ERp57, ERp72, ERp44 and P5 [18]. Since PDI has been found to be responsible for the shuttling of electrons between Ero1 and folding substrates, what is the function of these other redox enzymes? Are their functions distinct or redundant? In the periplasmic thiol oxidation system of prokaryotes, different oxidoreductases are dedicated to distinct pathways, either oxidation or reduction and isomerization [50]. It is therefore reasonable to assume that the various eukaryotic PDI homologues are differentiated in terms of redox potential and/or specialized for the maturation of distinct sets of proteins or protein domains.

In fact, ERp57 operates in a distinct pathway together with CNX and CRT to facilitate folding of proteins containing N-linked glycans. This ERp57-specific pathway has been named the calnexin/calreticulin cycle and has been studied extensively [19, 51, 52]. Following transfer of the core oligosaccharide to a glycosylation site in the nascent chain, two glucoses are removed by glucosidases I and II. The resulting monoglucosylated glycoprotein then interacts with CNX or CRT [13, 53]. Either lectin in turn recruits ERp57 into the complex through an extended armlike domain which is termed the P-domain [54–56]. ERp57 interacts with the folding substrate and may engage in mixed disulfide intermediates. Cleavage of the remaining glucose by glucosidase II terminates the interaction. If the released protein is not yet properly folded, it will be recognized and reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (UGT), which acts as a sensor for unfolded protein structures [57]. Upon reglucosylation reassociation with CNX/ERp57 or CRT/ERp57 takes place and the cycle is restarted.

How is the glucosylation/deglucosylation cycle coupled to ERp57 redox activity? When mixed disulfide intermediates between ERp57 and its substrates are stabilized by ERp57 active site mutations (C60A, C409A), CNX and CRT remain stably associated with the trapped substrate proteins [58]. This observation might suggest that reduction of mixed disulfide intermediates is a requirement for the next step in the cycle to take place, trimming by glucosidase II. Accordingly, exposed cysteines might regulate the transition points of the deglucosylation/glucosylation cycle.

Most importantly, the nature of the in vivo redox activity of ERp57 remains unknown. Although it is clear that ERp57 is capable of forming mixed disulfide intermediates with substrate proteins [59], there are several possibilities for its specific function: (i) ERp57 may predominantly function to create new disulfide bonds. However, unlike PDI, ERp57 does not interact with Ero1 [60], but other reoxidation pathways may exist that have yet to be recognized. (ii) ERp57 might be dedicated to the reduction of exposed disulfide bonds, possibly to initiate retrotranslocation and degradation of malfolded proteins [26]. (iii) ERp57 might be dedicated to the reshuffling of existing disulfide bonds. (iv) ERp57 might engage in more than one kind of redox reaction and possibly use its two active TR-like domains in different ways.

Early oxidation of the MHC class I heavy chain

Returning to the specific case of the MHC class I molecule, what do we know about the generation of disulfide bonds in the class I heavy chain? Disulfide bond formation in the α_3 domain has been found to be fast and efficient [61]. It is, however, not clear which oxidoreductase catalyzes the formation of the α_3 disulfide bond. Nascent immunoglobulins have been found to interact with PDI [62], and oxidoreductase requirements for the folding of Ig domains might be conserved. Accordingly, the oxidation of the α_3 domain might be facilitated by electron transfer along the Ero1-PDI axis.

The formation of the α_2 disulfide bond has been studied in more detail. Based on the reactivity of domain-specific antibodies with class I molecules translated in vitro, it

was concluded that in the absence of β_2m the α_3 disulfide is formed but the α_2 disulfide is not [63]. In vivo studies with β_2m-negative cells confirmed that β_2m association is required for either the formation or maintenance of the α_2 disulfide [64]. The association of free heavy chain with β_2m later turned out to be synonymous with the assembly of the complete loading complex [12], and two studies have since provided evidence for the complete oxidation of heavy chain before its integration into the loading complex [24, 61]. The observation that some class I alleles form mature peptide complexes independent of tapasin [65] also implies that formation of the peptide loading complex is not a requirement for the complete oxidation of HC. Taken together, it seems that full oxidation takes place in free heavy chains, and that the α_2 disulfide remains highly unstable unless integrated into the loading complex. This suggests that the loading complex somehow protects the α_2 disulfide bond from reduction, either passively, by shielding it from reductants, or actively, by providing a reoxidizing activity (or both). Which agent might be responsible for reducing the α_2 disulfide after its initial formation?

Reduced glutathione is the main reductant of the ER, and it probably targets disulfide bonds as they become exposed in incompletely folded protein domains [46]. Therefore, the loading complex might primarily provide protection against GSH-mediated reduction, possibly by shielding the disulfide bond until it finally gets buried in the peptide-MHC complex. Tapasin, CRT or ERp57 might contribute to the spatial exclusion of GSH or other reductants. Alternatively, if the α_2 disulfide remains vulnerable to reduction inside the loading complex, ERp57 might actively facilitate its reoxidation. If GSH attacks the α_2 disulfide bond, a glutathione-heavy chain mixed disulfide would be generated. Might ERp57 be specialized in the regeneration of intrachain disulfides from mixed glutathione-protein disulfides? Alternatively, if the α_2 disulfide becomes fully reduced, ERp57 might engage in de novo oxidation.

If the HC is oxidized prior to its integration into the loading complex, how is the α_2 disulfide bond formed in the first place? Class I HC becomes associated with CNX shortly after its synthesis [66]. ERp57 is known to be recruited by CNX and has been detected in these early MHC class I folding complexes [15, 16, 67]. Hence, early involvement of ERp57 in conjunction with CNX might be responsible for the primary oxidation of the α_2 cysteines. In fact, all three redox states of heavy chain (0, 1 and 2 disulfide bonds) have been found to be associated with CNX [61]. Given the independent folding of the α_3 Ig domain, it is likely, though not proven, that the correct pairing of the cysteines has taken place at this stage. To date, the participation of PDI in α_2 disulfide formation cannot be excluded, but there are no indications for its involvement, either.

Disulfide bond isomerization inside the loading complex

To identify cysteines targeted by ERp57 inside the loading complex, a trapping method was applied [58]. The basic principle of trapping involves the expression of an affinity-tagged oxidoreductase lacking the downstream cysteine of the thioredoxin motif (CXXC), thereby allowing the enzyme to form mixed disulfide intermediates with target molecules (via the N-terminal cysteine), but preventing nucleophilic attack by the C-terminal cysteine that would normally break the mixed disulfide bond. In an independent approach, cells expressing wild type ERp57 were treated with the alkylating agent *N*-ethyl maleimide (NEM) prior to lysis to protect labile disulfide intermediates. Both approaches led to the identification of tapasin as a target of ERp57 redox activity inside the loading complex [68]. Under the same conditions, no disulfide intermediate with MHC class I could be identified.

Upon further analysis, it was seen that all the ERp57 and tapasin molecules inside the loading complex are linked to each other by a defined disulfide bond. This exclusive conjugation at steady state was rather unexpected because mixed disulfide intermediates usually are short-lived and only detectable in a small fraction of the molecules at any given time [59]. The most likely explanation for this phenomenon is that the class I loading complex represents a stalled folding intermediate with a 'frozen' transitional disulfide. Since the loading complex is waiting for an appropriate peptide to complete the folding process, it might be arrested in a transitional state until this happens.

Disulfide bonding with tapasin is specifically mediated by cysteine-57, the upstream cysteine of the TR motif in the N-terminal domain of ERp57 ($C_{57}XXC_{60}$). Interestingly, it was found that certain mutations in the C-terminal TR motif ($C_{406}XXC_{409}$), specifically replacement of Cys-406, abolish tapasin conjugation to the N-terminal domain [68]. This finding indicates cooperativity between the two TR domains of ERp57 and implies that the C-terminal domain participates in the disulfide bond exchange leading to tapasin conjugation at the N-terminal domain. Within tapasin, cysteine-95 was identified as the ERp57-conjugating residue. Cys-95 represents an unpaired cysteine, since the other two cysteines in the N-terminal domain (Cys-7, Cys-71) form a disulfide bond with each other, at least in those tapasin molecules outside the loading complex. A tapasin mutant of cysteine-95 (C95A) stably assembles with TAP, heavy chain-β_2m dimers, and CRT. However, interaction with ERp57 is not detectable in this complex. It appears that the tapasin-ERp57 mixed disulfide is a major cause for retention of ERp57 inside the loading complex. The partial loading complex (lacking ERp57) was found to be deficient in

class I peptide loading. Moreover, the HC associated with this complex was found to be partially reduced, indicating that the α_2 disulfide bond is prone to reduction in the absence of Cys-95 of tapasin and, ultimately, ERp57 [68].

Because heavy chains associated with the wild type loading complex are fully oxidized (as expected for peptide-receptive HC-β_2m dimers), the tapasin cysteine mutation and the concomitant loss of ERp57 association seem to inactivate the mechanism by which HC is kept in a fully oxidized state. If HC is actively reoxidized by either ERp57 or tapasin, mixed disulfide intermediates should exist. However, no other mixed disulfide intermediates involving HC have been identified inside the loading complex, neither ERp57-HC, nor tapasin-HC. Maybe these intermediates, if they exist, are extremely short-lived and difficult to detect.

In contrast, the detection of mixed disulfide intermediates between class I HC and ERp57 outside the loading complex has been reported [26, 69]. These conjugates most likely represent early folding intermediates of calnexin-associated heavy chain, rather than β_2m-associated dimers [3]. However, it remains to be shown that the formation of the reported disulfide-linked conjugates actually involves the α_2 cysteine residues Cys-101 or Cys-164. Several MHC class I alleles, including HLA-B27, HLA-Cw*01 and K^b, feature additional cysteines in their α_1 or α_2 domains, and it is therefore possible that formation of the reported conjugates depends on these additional cysteines.

Taken together, it is now established that disulfide bond isomerization takes place inside the class I loading complex and that it involves at least two participants: tapasin and ERp57. Several important questions remain unresolved: does the heavy chain α_2 disulfide bond engage in thiol-disulfide exchange with ERp57 or tapasin? If yes, how does isomerization of the α_2 disulfide bond relate to the regulation of peptide binding? If not, how would disulfide bond exchange between ERp57 and tapasin serve the selection and loading of peptide ligands?

Discussion and outlook

There is little doubt that the operational principle of the MHC class I peptide loading machine is a specific case of general ER quality control, guided by the same principles. This assumption has been reinforced recently by glycan analysis of the loading complex [70].

Peptide loading into MHC class I receptors is a situation similar to other temporarily interrupted assembly processes taking place inside the ER: incompletely folded domains with cysteine residues are waiting for their correct interaction partners, which may be distant domains in the same protein chain, different protein chains or other ligands. The triggering of MHC release by peptide binding is reminiscent of the pharmacological chaperone concept, which refers to small-molecule ligands that target a specific protein during folding in the ER [71]. Ligand binding stabilizes native-like conformers able to interact with the ligand and thereby shifts the equilibrium from incompletely folded to native forms of the protein. Typically there is a correlation between ligand-binding affinity and ligand-mediated rescue. For example, retinol-binding protein (RBP) is retained in the ER until it binds retinol [72]. Interestingly, retention and release of RBP has been associated with CNX association and thiol redox activity [73, 74].

Recent studies of cotranslational hemagglutinin folding indicate an approximate correlation between the location of glycans and critical cysteines along the protein chain [75]. Glycans seem to be strategically located in the vicinity of cysteine residues, which are in need of protection against inappropriate interactions or rearrangements. Lone cysteines or exposed disulfides appear to be shielded by CNX/ERp57 or CRT/ERp57 until other parts of the chain or specific cysteines become available as interaction partners. ERp57 may predominantly target free cysteines and recruit them into mixed disulfide intermediates using its TR domains. These mixed disulfides might then be converted into intradomain disulfide bonds (if another free cysteine is available nearby), or literally remain 'on hold' until a cysteine becomes available by interaction with another domain or protein. The continued engagement of ERp57 with 'unpairable' single cysteines might lead to very long-lived mixed disulfide intermediates and extensive folding delays. Only the appropriate interaction partner will then induce the continuation of the folding process: the interaction partner might either provide the 'missing' cysteine (e. g. for the formation of a long-loop interdomain disulfide within the same protein) or might induce conformational changes that will expose cysteines or disulfides required for further isomerization.

How could such a scheme relate to MHC class I peptide binding? In the early HC-CNX complex ERp57 would probably form very transient disulfide intermediates: those that lead to the formation of the intradomain α_2 disulfide bond. The subsequent association of oxidized HC with β_2m then leads to a replacement of CNX/ERp57 by CRT/ERp57 but also to formation of a binding site for tapasin. Tapasin carries a single unpaired cysteine (Cys-95) on its N-terminal domain and thereby entraps ERp57 in a long-lived mixed disulfide intermediate. Accordingly, the entire loading complex gets arrested in a state that requires an additional interaction partner to induce the continuation of disulfide isomerization. If the N-terminal portion of tapasin somehow binds into or on top of the peptide binding groove of the class I molecule, tapasin could be responsible for protecting the α_2 disulfide from nonspecific reduction. Incoming peptides would then have to compete with the N-terminus of

tapasin for access to the binding groove, possibly explaining the influence of tapasin on the selection of the class I peptide repertoire. Displacement of the N-terminal domain of tapasin by high-affinity peptides might then induce a conformational change that triggers further disulfide isomerization and leads to reduction of the tapasin-ERp57 disulfide bond. The peptide-triggered isomerization process might involve the other two cysteines in the N-terminal domain of tapasin (Cys-7 and Cys-71). Understanding of class I maturation is currently limited by our general understanding of oxidative protein folding and the associated quality control mechanisms. Beyond that, the thiol redox chemistry of MHC class I molecules poses additional questions: How are malfolded class I molecules reduced prior to retrotranslocation? Could the α_2 disulfide bond be a target for oxidoreductases on the cell surface or inside endosomal compartments? Might disulfide bond reduction and reoxidation play a role in peptide exchange? Finally, several members of the MHC superfamily encode additional conserved cysteine residues in their α_1 or α_2 domains. The function of these additional cysteines remains poorly understood, but it has become clear that they can bestow interesting new properties to MHC class I molecules on the cell surface [76, 77].

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1 Watts C. (1997) Capture and processing of exogenous antigens for presentation on MHC molecules. Annu. Rev. Immunol. **15:** 821–850
2 Pamer E. and Cresswell P. (1998) Mechanisms of MHC class I-restricted antigen processing. Annu. Rev. Immunol. **16:** 323–258
3 Antoniou A. N., Powis S. J. and Elliott T. (2003) Assembly and export of MHC class I peptide ligands. Curr. Opin. Immunol. **15:** 75–81
4 Flajnik M. F. and Kasahara M. (2001) Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. Immunity **15:** 351–362
5 Rammensee H. G. (1995) Chemistry of peptides associated with MHC class I and class II molecules. Curr. Opin. Immunol. **7:** 85–96
6 Stevens F. J. and Argon Y. (1999) Protein folding in the ER. Semin. Cell Dev. Biol. **10:** 443–454
7 Cresswell P. (2000) Intracellular surveillance: controlling the assembly of MHC class I-peptide complexes. Traffic **1:** 301–305
8 Schmitt L. and Tampe R. (2000) Affinity, specificity, diversity: a challenge for the ABC transporter TAP in cellular immunity. Chembiochem. **1:** 16–35
9 Momburg F. and Tan P. (2002) Tapasin-the keystone of the loading complex optimizing peptide binding by MHC class I molecules in the endoplasmic reticulum. Mol. Immunol. **39:** 217–233
10 Williams A. P., Peh C. A., Purcell A. W., McCluskey J. and Elliott T. (2002) Optimization of the MHC class I peptide cargo is dependent on tapasin. Immunity **16:** 509–520
11 Tan P., Kropshofer H., Mandelboim O., Bulbuc N., Hammerling G. J. and Momburg F. (2002) Recruitment of MHC class I molecules by tapasin into the transporter associated with antigen processing-associated complex is essential for optimal peptide loading. J. Immunol. **168:** 1950–1960
12 Sadasivan B., Lehner P. J., Ortmann B., Spies T. and Cresswell P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity **5:** 103–114
13 Helenius A. and Aebi M. (2001) Intracellular functions of N-linked glycans. Science **291:** 2364–2369
14 Hughes E. A. and Cresswell P. (1998) The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. Curr. Biol. **8:** 709–712
15 Lindquist J. A., Jensen O. N., Mann M. and Hammerling G. J. (1998) ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. EMBO J. **17:** 2186–2195
16 Morrice N. A. and Powis S. J. (1998) A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. Curr. Biol. **8:** 713–716
17 Oliver J. D., Roderick H. L., Llewellyn D. H. and High S. (1999) ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. Mol. Biol. Cell **10:** 2573–2582
18 Ferrari D. M. and Soling H. D. (1999) The protein disulphide-isomerase family: unravelling a string of folds. Biochem. J. **339 (Pt 1):** 1–10
19 Ellgaard L. and Helenius A. (2003) Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell. Biol. **4:** 181–191
20 Halaby D. M., Poupon A. and Mornon J. (1999) The immunoglobulin fold family: sequence analysis and 3D structure comparisons. Protein Eng. **12:** 563–571
21 Ioerger T. R., Du C. and Linthicum D. S. (1999) Conservation of cys-cys trp structural triads and their geometry in the protein domains of immunoglobulin superfamily members. Mol. Immunol. **36:** 373–386
22 Bjorkman P. J., Saper M. A., Samraoui B., Bennett W. S., Strominger J. L. and Wiley D. C. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. Nature **329:** 506–512
23 Matsumura M., Fremont D. H., Peterson P. A. and Wilson I. A. (1992) Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science **257:** 927–934
24 Smith J. D., Solheim J. C., Carreno B. M. and Hansen T. H. (1995) Characterization of class I MHC folding intermediates and their disparate interactions with peptide and beta 2-microglobulin. Mol. Immunol. **32:** 531–540
25 Ostergaard Pedersen L., Nissen M. H., Hansen N. J., Nielsen L. L., Lauenmoller S. L., Blicher T. et al. (2001) Efficient assembly of recombinant major histocompatibility complex class I molecules with preformed disulfide bonds. Eur. J. Immunol. **31:** 2986–2996
26 Antoniou A. N., Ford S., Alphey M., Osborne A., Elliott T. and Powis S. J. (2002) The oxidoreductase ERp57 efficiently reduces partially folded in preference to fully folded MHC class I molecules. EMBO J. **21:** 2655–2663
27 Zeff R. A., Nakagawa M., Mashimo H., Gopas J. and Nathenson S. G. (1990) Failure of cell surface expression of a class I major histocompatibility antigen caused by somatic point mutation. Transplantation **49:** 803–808
28 Harris M. R., Lybarger L., Yu Y. Y., Myers N. B. and Hansen T. H. (2001) Association of ERp57 with mouse MHC class I molecules is tapasin dependent and mimics that of calreticulin and not calnexin. J. Immunol. **166:** 6686–6692
29 Solheim J. C., Harris M. R., Kindle C. S. and Hansen T. H. (1997) Prominence of beta 2-microglobulin, class I heavy chain conformation and tapasin in the interactions of class I heavy chain with calreticulin and the transporter associated with antigen processing. J. Immunol. **158:** 2236–2241
30 Shiroishi T., Evans G. A., Appella E. and Ozato K. (1984) Role of a disulfide bridge in the immune function of major histo-

compatibility class I antigen as studied by in vitro mutagenesis. Proc. Natl. Acad. Sci. USA **81:** 7544–7548
31 Warburton R. J., Matsui M., Rowland-Jones S. L., Gammon M. C., Katzenstein G. E., Wei T. et al. (1994) Mutation of the alpha 2 domain disulfide bridge of the class I molecule HLA-A*0201. Effect on maturation and peptide presentation. Hum. Immunol. **39:** 261–271
32 Grossberger D. and Parham P. (1992) Reptilian class I major histocompatibility complex genes reveal conserved elements in class I structure. Immunogenetics **36:** 166–174
33 Okamura K., Ototake M., Nakanishi T., Kurosawa Y. and Hashimoto K. (1997) The most primitive vertebrates with jaws possess highly polymorphic MHC class I genes comparable to those of humans. Immunity **7:** 777–790
34 Flajnik M. F., Canel C., Kramer J. and Kasahara M. (1991) Evolution of the major histocompatibility complex: molecular cloning of major histocompatibility complex class I from the amphibian *Xenopus*. Proc. Natl. Acad. Sci. USA **88:** 537–541
35 Bateman A., Birney E., Cerruti L., Durbin R., Etwiller L., Eddy S. R. et al. (2002) The Pfam protein families database. Nucleic Acids Res. **30:** 276–280
36 Ehrlich R., Lifshitz R., Pescovitz M. D., Rudikoff S. and Singer D. S. (1987) Tissue-specific expression and structure of a divergent member of a class I MHC gene family. J. Immunol. **139:** 593–602
37 Kato T., Esumi M., Yamashita S., Abe K. and Shikata T. (1992) Interferon-inducible gene expression in chimpanzee liver infected with hepatitis C virus. Virology **190:** 856–860
38 Jones E. Y. (1997) MHC class I and class II structures. Curr. Opin. Immunol. **9:** 75–79
39 Wedemeyer W. J., Welker E., Narayan M. and Scheraga H. A. (2000) Disulfide bonds and protein folding. Biochemistry **39:** 4207–4216
40 Betz S. F. (1993) Disulfide bonds and the stability of globular proteins. Protein Sci. **2:** 1551–1558
41 Creighton T. E. (1988) Disulphide bonds and protein stability. Bioessays **8:** 57–63
42 Muller Y. A., Heiring C., Misselwitz R., Welfle K. and Welfle H. (2002) The cystine knot promotes folding and not thermodynamic stability in vascular endothelial growth factor. J. Biol. Chem. **277:** 43410–43416
43 Fra A. M., Fagioli C., Finazzi D., Sitia R. and Alberini C. M. (1993) Quality control of ER synthesized proteins: an exposed thiol group as a three-way switch mediating assembly, retention and degradation. EMBO J. **12:** 4755–4761
44 Hwang C., Sinskey A. J. and Lodish H. F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum. Science **257:** 1496–1502
45 Frand A. R. and Kaiser C. A. (1998) The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol. Cell. **1:** 161–170
46 Cuozzo J. W. and Kaiser C. A. (1999) Competition between glutathione and protein thiols for disulphide-bond formation. Nat. Cell Biol. **1:** 130–135
47 Frand A. R. and Kaiser C. A. (1999) Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol. Cell. **4:** 469–477
48 Tu B. P., Ho-Schleyer S. C., Travers K. J. and Weissman J. S. (2000) Biochemical basis of oxidative protein folding in the endoplasmic reticulum. Science **290:** 1571–1574
49 Tu B. P. and Weissman J. S. (2002) The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. Mol. Cell. **10:** 983–994
50 Collet J. F. and Bardwell J. C. (2002) Oxidative protein folding in bacteria. Mol. Microbiol. **44:** 1–8
51 Zapun A., Darby N. J., Tessier D. C., Michalak M., Bergeron J. J. and Thomas D. Y. (1998) Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. J. Biol. Chem. **273:** 6009–6012
52 Oliver J. D., van der Wal F. J., Bulleid N. J. and High S. (1997) Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. Science **275:** 86–88
53 Bergeron J. J., Zapun A., Ou W. J., Hemming R., Parlati F., Cameron P. H. et al. (1998) The role of the lectin calnexin in conformation independent binding to N-linked glycoproteins and quality control. Adv. Exp. Med. Biol. **435:** 105–116
54 Schrag J. D., Bergeron J. J., Li Y., Borisova S., Hahn M., Thomas D. Y. et al. (2001) The Structure of calnexin, an ER chaperone involved in quality control of protein folding. Mol. Cell. **8:** 633–644
55 Frickel E. M., Riek R., Jelesarov I., Helenius A., Wuthrich K. and Ellgaard L. (2002) TROSY-NMR reveals interaction between ERp57 and the tip of the calreticulin P-domain. Proc. Natl. Acad. Sci. USA **99:** 1954–1959
56 Leach M. R., Cohen-Doyle M. F., Thomas D. Y. and Williams D. B. (2002) Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. J. Biol. Chem. **277:** 29686–29697
57 Ritter C. and Helenius A. (2000) Recognition of local glycoprotein misfolding by the ER folding sensor UDP-glucose:glycoprotein glucosyltransferase. Nat. Struct. Biol. **7:** 278–280
58 Dick T. P. and Cresswell P. (2002) Thiol oxidation and reduction in major histocompatibility complex class I-restricted antigen processing and presentation. Methods Enzymol. **348:** 49–54
59 Molinari M. and Helenius A. (1999) Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. Nature **402:** 90–93
60 Mezghrani A., Fassio A., Benham A., Simmen T., Braakman I. and Sitia R. (2001) Manipulation of oxidative protein folding and PDI redox state in mammalian cells. EMBO J. **20:** 6288–6296
61 Tector M., Zhang Q. and Salter R. D. (1997) Beta 2-microglobulin and calnexin can independently promote folding and disulfide bond formation in class I histocompatibility proteins. Mol. Immunol. **34:** 401–408
62 Roth R. A. and Pierce S. B. (1987) In vivo cross-linking of protein disulfide isomerase to immunoglobulins. Biochemistry **26:** 4179–4182
63 Ribaudo R. K. and Margulies D. H. (1992) Independent and synergistic effects of disulfide bond formation, beta 2-microglobulin and peptides on class I MHC folding and assembly in an in vitro translation system. J. Immunol. **149:** 2935–2944
64 Wang H., Capps G. G., Robinson B. E. and Zuniga M. C. (1994) Ab initio association with beta 2-microglobulin during biosynthesis of the H-2Ld class I major histocompatibility complex heavy chain promotes proper disulfide bond formation and stable peptide binding. J. Biol. Chem. **269:** 22276–22281
65 Peh C. A., Burrows S. R., Barnden M., Khanna R., Cresswell P., Moss D. J. et al. (1998) HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. Immunity **8:** 531–542
66 Degen E. and Williams D. B. (1991) Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. J. Cell Biol. **112:** 1099–1115
67 Farmery M. R., Allen S., Allen A. J. and Bulleid N. J. (2000) The role of ERp57 in disulfide bond formation during the assembly of major histocompatibility complex class I in a synchronized semipermeabilized cell translation system. J. Biol. Chem. **275:** 14933–14938
68 Dick T. P., Bangia N., Peaper D. R. and Cresswell P. (2002) Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. Immunity **16:** 87–98
69 Lindquist J. A., Hammerling G. J. and Trowsdale J. (2001) ER60/ERp57 forms disulfide-bonded intermediates with MHC class I heavy chain. FASEB J. **15:** 1448–1450

70 Radcliffe C. M., Diedrich G., Harvey D. J., Dwek R. A., Cresswell P. and Rudd P. M. (2002) Identification of specific glycoforms of major histocompatibility complex class I heavy chains suggests that class I peptide loading is an adaptation of the quality control pathway involving calreticulin and ERp57. J. Biol. Chem. **277:** 46415–46423
71 Morello J. P., Petaja-Repo U. E., Bichet D. G. and Bouvier M. (2000) Pharmacological chaperones: a new twist on receptor folding. Trends Pharmacol. Sci. **21:** 466–469
72 Melhus H., Laurent B., Rask L. and Peterson P. A. (1992) Ligand-dependent secretion of rat retinol-binding protein expressed in HeLa cells. J. Biol. Chem. **267:** 12036–12041
73 Tosetti F., Ferrari N., Pfeffer U., Brigati C. and Vidali G. (1992) Regulation of plasma retinol binding protein secretion in human HepG2 cells. Exp. Cell Res. **200:** 467–472
74 Bellovino D., Morimoto T., Tosetti F. and Gaetani S. (1996) Retinol binding protein and transthyretin are secreted as a complex formed in the endoplasmic reticulum in HepG2 human hepatocarcinoma cells. Exp. Cell Res. **222:** 77–83
75 Daniels R., Kurowski B., Johnson A. E. and Hebert D. N. (2003) N-linked glycans direct the cotranslational folding pathway of influenza hemagglutinin. Mol. Cell. **11:** 79–90
76 Boyson J. E., Erskine R., Whitman M. C., Chiu M., Lau J. M., Koopman L. A. et al. (2002) Disulfide bond-mediated dimerization of HLA-G on the cell surface. Proc. Natl. Acad. Sci. USA **99:** 16180–16185
77 Kollnberger S., Bird L., Sun M. Y., Retiere C., Braud V. M., McMichael A. et al. (2002) Cell-surface expression and immune receptor recognition of HLA-B27 homodimers. Arthritis Rheum. **46:** 2972–2982