I
Mechanics of Antigen Processing

Immunodominance: The Choice of the Immune System. Edited by Jeffrey A. Frelinger

ISBN: 3-527-31274-9

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Class I MHC Antigen Processing

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1.1
Introduction

T-cell recognition of a peptide bound to class I major histocompatibility complex (MHC) molecule requires proper processing of that peptide by a cell's antigen presentation machinery. In many cases, only one peptide from a protein provides an epitope that elicits a T-cell response [1–6]. Any discussion of immunodominance must include how peptides are removed from larger precursors and loaded onto MHC. This chapter will focus on the mechanism of peptide processing and the properties of class I MHC molecules that influence those mechanisms.

1.2
Properties of MHC

1.2.1
Structure of MHC

The MHC is the most polymorphic gene family known in vertebrates. Interestingly, the crystallographic structures of class I MHC molecules are similar regardless of the allotype or species of origin [7–16]. Class I MHC molecules are heterotrimeric complexes comprised of a ~44-kDa heavy chain, a noncovalently bound 12-kDa serum protein, β_2-microglobulin (β_2m), and a small peptide (Figure 1.1a). All class I MHC molecules use their α1 and α2 domains to create a peptide-binding cleft. The cleft is flanked by two alpha helices and has a floor composed of a large beta sheet (Figure 1.1b). The α3 domain of class I MHC has an immunoglobulin fold and noncovalently associates with β_2m. The entire MHC protein is anchored to the membrane by a small transmembrane segment and is completed with a small intracellular tail.

The fact that the structures of class I MHC molecules are the same seems to be at odds with the observation that the MHC is the most polymorphic gene family in vertebrates. Interestingly, the distribution of polymorphic residues is not randomly scattered through the molecule; they are located in the peptide-binding cleft (Figure 1.1c).

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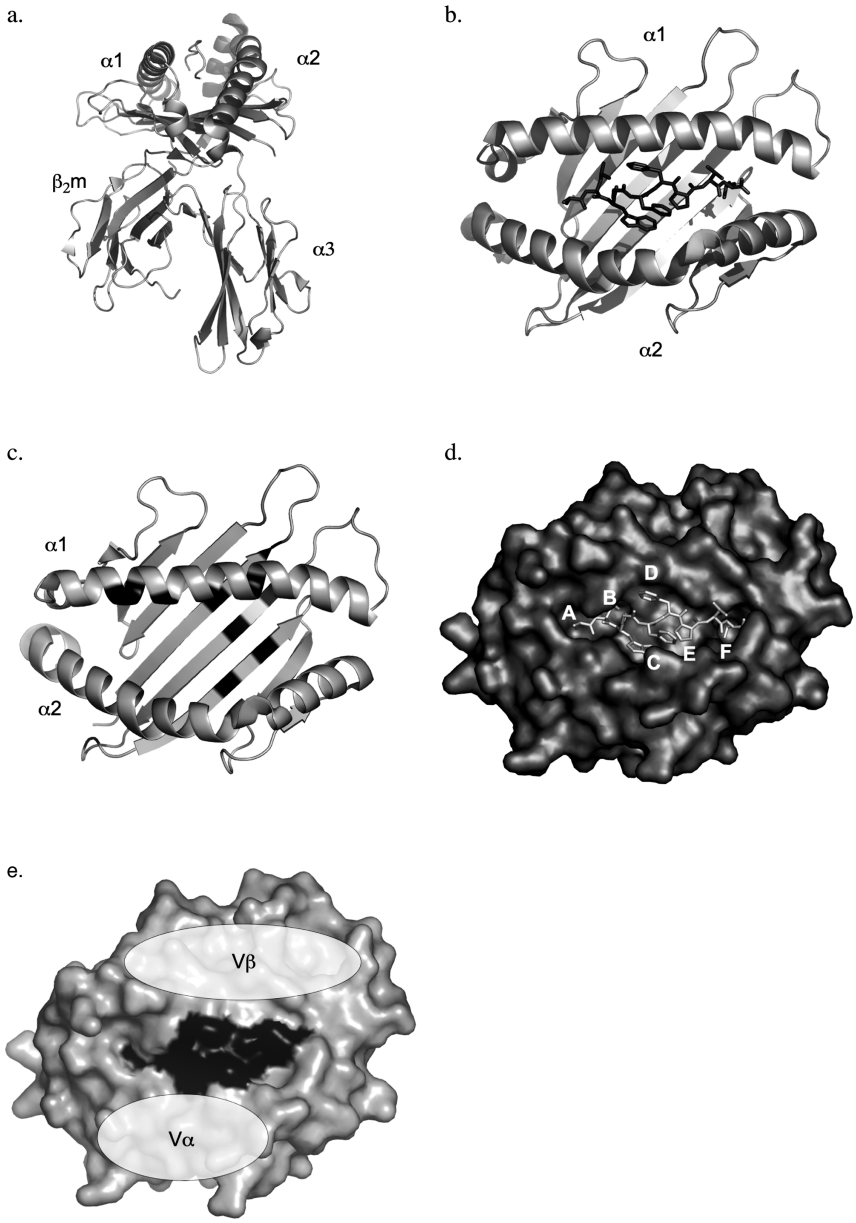


Class I MHC molecules are constitutively expressed on the plasma membrane and, in an unaltered cell, present endogenous self-peptides. In an unaltered cell, the complexity of peptides bound to class I MHC is high [17]. The presence of class I MHC molecules with those endogenous peptides on the surface of the cell is required for $CD8^+$ T-cell homeostasis [18]. The first peptides eluted from human

Figure 1.1 Class I MHC molecule. (a) Heterotrimeric complex composed of MHC class I heavy chain, β_2m, and peptide. The peptide-binding cleft created by the α1 and α2 helices and the beta-sheet floor is easily seen from the side. (b) A top view of the peptide-binding cleft of the class I MHC molecule. Bound peptide, ALWGFFPVL, is colored black. (c) The polymorphic regions of the class I MHC molecule, indicated in black, reside primarily within the α1 helix and beta-sheet floor of the peptide-binding cleft. (d) The chemical and spatial composition of the peptide-binding cleft creates six binding pockets in HLA-A2, labeled A–F. Pockets A, B, and F are especially prominent and accommodate the amino terminus, the side chain of P2, and the carboxyl-terminal leucine, respectively. (e) The molecular surface of the peptide–MHC complex is dictated by the bound peptide and the conformation of the MHC heavy chain. Peptide is highlighted in black. Ellipses represent the binding regions of TCR domains that make contact with the MHC heavy chain based on crystal structures of pMHC–TCR complexes with the Vα binding on the α2 domain and the Vβ binding more diffusely on the α1 domain. Figures were generated with PyMOL [236].

leukocyte antigen (HLA)-B27 were found to be fragments of proteins expressed at high levels in the cell, such as heat shock proteins, ribosomal proteins, histones, and HLA-B27 itself [19]. Thus, the initial hypothesis about which peptides get processed and presented was that they were linked to the expression level of the parental protein in the cell [20]. This has since proven to be simplistic, as will be described later.

1.2.2 Polymorphic Residues Generate Specificity Pockets

A close examination of the structure of HLA-A2 showed that some of the peptide side chains bound in pockets inside the peptide-binding cleft. Six binding pockets were described within the peptide-binding cleft of the class I MHC molecule (Figure 1.1d). Two pockets are comprised of conserved residues that bind the invariant positively and negatively charged peptidic termini. Pocket A is composed of conserved residues that act as hydrogen bond acceptors for the NH_3^+ of the peptide backbone. The entrance to the F pocket provides conserved hydrogen bond donors for the negatively charged carboxyl terminus of the peptide [21]. Pockets B–E and the base of pocket F are formed by a small set of conserved residues in conjunction with the polymorphic residues in the MHC. These pockets are used variably to bind peptide side chains, depending on the identity of the MHC molecule and the peptide. A comparison of HLA-A2 with HLA-B27 and HLA-Aw68 showed that the chemical nature of the pockets is complementary to the chemical nature of the side chain of the bound peptide; thus, the binding sites were described as specificity pockets [9, 13]. The primary sequence of a peptide that binds to a particular MHC molecule is a direct result of the chemical and spatial composition of these specificity pockets [22–24]. Although the majority of these polymorphic residues make contact with the bound peptide [22], the remaining polymorphic residues are solvent exposed and available for interaction with the T-cell receptor.

1.3 Properties of Peptides

1.3.1 Peptides That Bind Are Not Random Sequences

Peptides that bind to class I MHC molecules may be extracted from the MHC binding cleft by acid treatment. The peptides may be recovered by filtering the preparation through a very small pore filter to capture the peptide-sized material. Edman degradation of the eluted material showed that some of the positions in the peptide were not random distributions of amino acids [19, 24–27]. These positions were enriched for particular amino acids. Later experiments with single peptides showed that substitutions of amino acids at certain positions typically reduced peptide binding to the class I MHC molecule; therefore, these enriched positions were termed anchors [27, 28].

As technology improved, the sequences of individual peptides were determined by collision-induced dissociation mass spectrometry [17]. This allowed for a more careful determination of the distribution of bound peptides, including length. Peptides from murine H-2K^d, H-2D^b, and human HLA-A2 are all typically nonamers, whereas H-2K^b peptides are typically octamers. All peptides have a hydrophobic carboxyl-terminal end, with a predominance of leucine, isoleucine, or valine. Each allelic peptide tends to have an additional anchor residue besides the one found at the carboxyl terminus [25]. In addition to a branched hydrophobic, or aliphatic, side chain residue at the carboxyl terminus, K^d peptides tend to have a tyrosine at position 2 [25, 29]. D^b most often binds peptides with asparagine at position 5, a phenylalanine or tyrosine is present at position 5 in K^b, and A2 prefers leucine or methionine at position 2 [17, 25]. HLA-B27-bound peptides are nonameric, with a predominance of positive or hydrophobic residues at the carboxyl terminus, or position 9 (P9), and an anchor position at P2 dominated by arginine [19].

1.3.2 Peptide-binding Motifs

As a result of their specificity pockets, different MHC proteins bind different subsets of peptides. Some MHC molecules are very restrictive; B27 can bind only an arginine at position 2 [7]. Others are less restrictive and allow a larger selection, such as the P9 pocket for A2 (leucine, isoleucine, methionine). This propensity for particular amino acids at particular positions allows one to predict the types of peptides that may bind to a particular MHC. For example, a peptide that will bind to HLA-A2 should have a leucine or methionine at P2 and a valine, isoleucine, leucine, or methionine at P9. This type of description is the peptide-binding motif for HLA-A2. Table 1.1 lists a representative set of peptide-binding motifs. A complete listing up through 1994 was compiled by Rammensee et al. [28].

Table 1.1 Peptide-binding motifs of common MHC alleles.

	Position								
Allele	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8/9/10**	**Reference**
HLA-A1	–	T, S	D, E	–	–	–	–	Y	231
HLA-A2	–	L, M	–	–	–	–	–	V	17,25
HLA-A3	–	V, L, M	–	–	–	–	–	K	231
HLA-A11	–	T, V	–	–	–	–	–	K	231,232
HLA-A24	–	Y	–	–	–	–	–	F, L	231
HLA-B27	–	R	–	–	–	–	–	K	19
H-2K^{d}	–	Y	–	–	–	–	–	L, I	25,29
H-2D^{b}	–	–	–	–	N	–	–	M, I	17,25
H-2K^{b}	–	–	–	–	F, Y	–	–	L	17,25
H-2L^{d}	–	P	–	–	–	–	–	L, F, M	233
H-2D^{d}	–	G	P	–	–	–	–	L, I, F	234

1.3.3 Peptide Length Is Limited in Class I MHC Peptides

Peptides greater than nine amino acids long typically bind poorly to class I MHC molecules. Peptides longer than nine residues bound to H-2D^{b} have an approximately 100-fold greater off-rate [30]. Additionally, it was determined that peptides of 9–10 amino acids in length induce folding of free class I heavy chain *in vitro*, while peptides of greater length could not [31]. This strict requirement in length led to early predictions of class I peptides bulging in order to fit into the binding pocket of the class I MHC molecule [32]. This characteristic bulge has been confirmed by a number of crystal structures of peptide–MHC complexes [7–16]. High-affinity long peptides have been found [33–35], but the mechanism of this tight binding is not known.

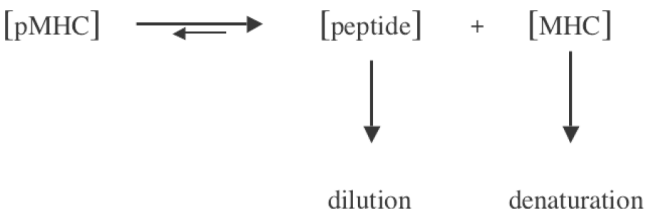
1.3.4 Binding Affinity

Peptides have been reported to bind to class I MHC molecules with moderate to high affinity (K_Ds around 10^{-8} to 10^{-7} M [30, 36]) and extremely slow off-rates (tens to hundreds of hours at 37 °C [22, 30, 37]). However, there are a number of problems with describing this system as a simple equilibrium. The structure suggests that a conformational change would be required to bind or release peptide. This

hypothesis was confirmed by binding experiments with an iodinated anchor residue ^{125}I-SV9. These experiments demonstrated that initial binding (on-rate) to murine H-2K^{b} was similar to non-iodinated wild-type peptide, but the rate of dissociation of ^{125}I-SV9 increased with temperature [38]. These data suggest that initially the heavy chain is open and can accommodate both peptides. Once binding occurs, a conformational change is induced and the MHC heavy chain closes over the peptide, trapping it in the groove. The wild-type peptide SV9 stabilizes the peptide–MHC (pMHC) complex, while the iodinated pMHC complex is unstable [38]. We can deduce then that an appropriate fit into the specificity pockets is required for the conformational change to occur. The amino and carboxyl termini of the peptide also play a crucial role in inducing a conformational change in the heavy chain and stabilizing the peptide–MHC complex [39].

A number of these earlier investigations utilized indirect measurements of peptide binding, such as competition assays [21, 30, 37, 40] and thermal denaturation [41, 42]. More recent investigations report direct measurements of peptide binding by utilizing fluorescence resonance energy transfer (FRET) [43] and fluorescence anisotropy [36]. Both studies concluded that peptide binding to and dissociation from class I MHC molecules is a biphasic, or second-order, process. The first phase is proposed as a conformational change in the MHC heavy chain from a peptide-inaccessible to peptide-accessible form, most likely induced by the association of β_2m. Rate constants (k_1) reported by these studies are 0.003 s^{-1} at 20 °C [43] and 0.008 s^{-1} at 31 °C [36]. The second phase reflects binding of peptide to the peptide-accessible heterodimer. The rate constants (k_{on}) reported for this phase are 2×10^6 M^{-1} s^{-1} at 37 °C [43] and 1×10^4 M^{-1} s^{-1} at 31 °C [36].

Peptides play an important structural role in the class I MHC molecule. They are essential in stabilizing the heavy chain during assembly of the MHC molecule in the endoplasmic reticulum (ER) [44, 45]. In their absence, class I MHC molecules can form, but they are unstable at physiological temperatures [46] and exhibit properties of molecules in a molten globule state [41]. Generally, peptides that confer the greatest stability also have the highest affinity. The fact that peptide-free MHC is extremely unstable and rapidly denatures is also a reason that it is not proper to discuss peptide binding to class I MHC as a standard equilibrium system. If the system were in simple equilibrium, Le Chatelier's principle would



Scheme 1.1

govern. Scheme 1.1 shows that peptide-bound complex (pMHC) would be rapidly depleted because of the loss of peptide-free MHC to denaturation and free peptide to dilution.

1.3.5 Molecular Recognition

Cytotoxic T lymphocytes (CTLs) recognize the molecular surface that is formed by a combination of atoms from the peptide and the class I MHC molecule (Figure 1.1e). Mutations in both the sequence of the peptide and in the MHC molecule have significant effects on recognition. One cannot simply classify a residue as either TCR contact or MHC binding in a particular peptide. Peptide side chains bound to their complementary specificity pockets also influence the reactivity of CTLs [47–51]. Additionally, amino acid mutations in the other five pockets of both human and murine class I MHC molecules also have a significant effect on activation of CTLs [48, 52]. Presumably, these mutations cause a slight conformational change in the peptide that alters the molecular surface of the pMHC complex and, hence, recognition by CTLs. This result, combined with the fact that peptides are buried so deeply in the groove that only 100–300 $Å^2$ is exposed outside the class I MHC molecule to the CTL receptor [8], confirms that the molecular recognition of pMHC by CTL is extremely sensitive to small structural changes. A small conformational change derived from a single amino acid substitution can turn an agonist peptide into an antagonist [53].

The relationship between immunodominance and binding of peptide to MHC has been investigated extensively. Experiments using the immunodominant ovalbumin $(OVA)_{257–264}$ (SIINFEKL) determinant versus the subdominant OVA determinant $OVA_{55–62}$ (KVVRFDKL) showed that the k_{on} for $OVA_{257–264}$ was ~10-fold faster and the k_{off} was twofold slower than the equivalent rates for the subdominant peptide. Interestingly, the anchor residues in these peptides are the same (F at P5 and L at P8) [54]. These findings and others indicate that each residue of the peptide, not just anchors, contributes to the affinity and stability of the peptide–MHC complex and aids in determining immunodominance [54–56]. However, increased immunogenicity is not guaranteed by increased affinity. Once a certain affinity threshold is reached, there is no longer a correlation between affinity and CTL reactivity [56–58].

1.3.6 Epitope Prediction

The wealth of experimental peptide-binding data has been used to create algorithms, which can predict the relative binding strengths of peptides, find peptide-binding motifs for a given MHC allele, or predict an epitope from a protein amino acid sequence. The first such algorithm was based on measuring binding of peptides using dissociation of β_2m from the complex as a marker for whether the peptide was bound (β_2m in complex) or free (β_2m free) [59]. Utilizing their experimen-

tal data for HLA-A2 (many more alleles are now available), a table of 180 coefficients (20 amino acids × 9 positions) was created. Each coefficient represents the apparent contribution that each amino acid makes toward binding at each of the nine positions. The coefficients are then multiplied together to determine a theoretical binding stability [59]. A similar approach is used by SYFPEITHI [60]. Later, computational groups created two peptide-binding prediction algorithms: the polynomial method and a neural network method [61]. A more recent Web server, ProPred1, goes one step further by utilizing the original binding database, as well as taking into account how peptides are generated in order to produce more accurate epitope predictions [62]. These prediction algorithms are powerful tools that will become increasingly reliable as more experimental data are gathered. Unfortunately, they are still unsuccessful at predicting CTL response and immunodominance of an epitope.

1.4 Cytosolic Processing

MHC-associated peptides are generated by cleavage of cellular proteins by the proteasome and other peptidases [63, 64]. These peptides are derived from proteins that are degraded at the end of their useful lifespan as part of normal protein turnover. Consistent with this thinking, the ubiquitin-dependent proteolytic pathway plays a major role in the production of peptides for MHC class I–restricted presentation [65]. The peptide pool also includes defective ribosomal products, or DRiPs, that result from errors in protein translation [20, 66, 67]. In the sections that follow, we will discuss the role that each part of the peptide-processing machinery plays in MHC peptide loading. A key factor in selection of a peptide for MHC presentation is the peptide's ability to be liberated from its precursor by proteolysis [58]. The peptide sequence must possess protease-recognition sites flanking its amino and carboxyl termini [68–70] and must lack internal cleavage sites in order to be processed successfully [71, 72].

1.4.1 The Proteasome

The proteasome is a multicatalytic proteinase complex consisting of five known proteolytic components that hydrolyze peptide bonds on the carboxyl side of basic, acidic, aromatic, branched-chain, and small amino acids. These activities are designated as trypsin-like, chymotrypsin-like, peptidyl-glutamyl peptide hydrolyzing, branched-chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAPP) [73–77]. The proteasome contains only endoprotease activity, yielding peptides of discrete length and not single amino acids [77]. The proteasome is composed of a 20S catalytic core and a PA28 (11S) or PA700 (19S) regulator. The 20S core of the proteasome is composed of four homoheptameric rings: two composed of α-subunits and two composed of β-subunits (Figure 1.2a).

The β-subunits β1, β2, and β5 are responsible for catalytic activity (reviewed in Ref. [78]). The entrance to the 20S core is closed by amino-terminal tails of the α-subunits [79, 80] (Figure 1.2b) unless opened by regulator particles [81], as is discussed below.

A role for the proteasome in generating MHC peptides was demonstrated using peptide aldehydes to inhibit the peptidase activities of the 20S and 26S (20S core with 19S regulator) particles of the proteasome. These inhibitors blocked presentation of an ovalbumin-derived peptide by class I MHC molecules [82]. Similar studies were repeated using a more efficient proteasome inhibitor, adamantine-

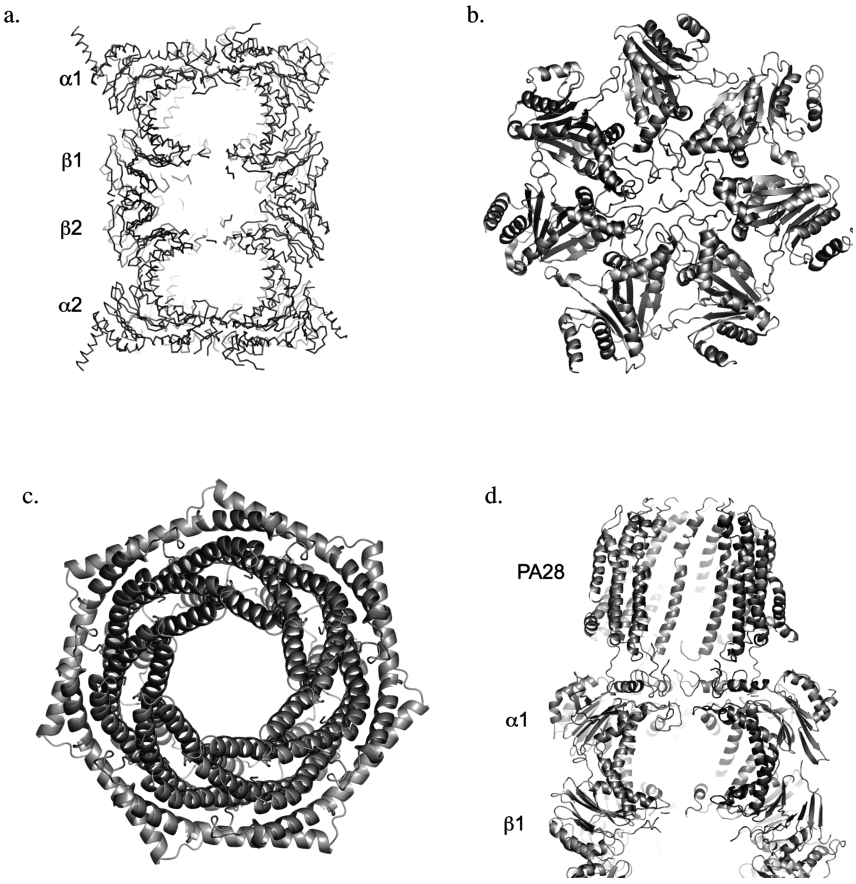


Figure 1.2 The proteasome. (a) Vertical cross-section of the 20S particle composed of four homoheptameric rings, two of α subunits and two of β subunits. The catalytic core is created by the β rings, while the α rings close the entrance to the particle. (b) Looking down on the α heptameric ring, the amino-terminal tails of the α subunits create a gate. (c) The heteroheptameric ring of the PA28 regulator is already in an open conformation. (d) PA28 associates with the α heptameric ring and binds the amino-terminal tails, opening the gate and allowing for polypeptide substrates to enter. Figures were generated with PyMOL [236].

acetyl-(6-aminohexanoyl)3-(leucinyl)3-vinyl-(methyl)-sulfone (Ada-Ahx_3-Leu_3-VS), which targets all three catalytic units of the 20S proteasome core without inhibiting cytosolic proteases. Treatment of cells with Ada-Ahx_3-Leu_3-VS did not influence cell growth characteristics, but it reduced the expression levels of class I molecules on the cell surface and inhibited presentation of endogenous tumor antigens on H-$2D^b$ [83]. This suggests that the cytosolic proteases are able to maintain the housekeeping functions required to survive but that generation of peptides for class I MHC presentation is largely handled by the proteasome.

1.4.2 The Immunoproteasome

In a pathogen-infected cell, peptide generation is increased and the peptide sequences generated are different because of the expression of an altered proteasome, the immunoproteasome. The immunoproteasome contains some of the constitutively expressed subunits of the proteasome as well as unique subunits, the low-molecular-weight proteins (LMPs). LMPs assemble into a complex that resembles the 20S proteasome particle in size and composition. LMP2 and LMP7 were mapped to the class II region of MHC in mice [84]. Similar to other MHC-encoded gene products, LMP proteins are polymorphic and conserved across species [85] and are upregulated by interferon-γ (IFN-γ) [86]. Low basal levels of LMP2 and LMP7 exist in IFN-γ-deficient mice, but IFN-γ is essential for upregulation during infection [87]. Another identified LMP, MECL-1 (LMP10), is not encoded within the *MHCI* region like LMP2 and LMP7, but MECL-1 is upregulated by IFN-γ [88, 89].

LMP2 (β1i) replaces β-type subunit Y (β1 or δ or 2) of the constitutive 20S particle. Subunit Y of the constitutive proteasome promotes cleavage on the carboxyl-terminal side of acidic residues. Incorporation of LMP2 reduces cleavage following acidic residues and increases cleavage after basic residues without affecting hydrophobic activity. LMP7 (β5i) replaces β-type subunit X (β5 or MB1 or 10 or ε). Subunit X does not seem to have a clear catalytic activity, but overexpression reduces proteolysis following hydrophobic and acidic residues. However, studies have shown that incorporation of LMP7 specifically increases cleavage after hydrophobic and basic residues without affecting acidic proteolysis [90, 91]. MECL-1 (β2i) replaces its homologous 20S subunit Z (β2) [88, 89]. RING10 and RING11 have been identified as LMP homologues in humans [92, 93].

The normal 26S proteasome is able to cleave after basic and hydrophobic residues, but not as efficiently as the proteasome with LMP2 and LMP7 [94]. Treatment of wild-type cells with IFN-γ resulted in increased proteolytic cleavage following hydrophobic and basic residues but reduced cleavage after acidic residues compared to mutants lacking LMP genes. The amount of LMP2 and LMP7 subunits in both the 20S and 26S proteasome particles increased after IFN-γ treatment, confirming that the proteasome composition changes following IFN-γ exposure [94].

The sequences of peptides recovered from the class I MHC molecules of healthy cells are different from those of peptides recovered from cells infected with Epstein-Barr virus (EBV). EBV-infected cells displayed peptides containing a glutamate residue at P2 on HLA-B40, while such peptides were missing from healthy cells. These *in vitro* findings support the idea that glutamyl activity in the proteasome is decreased during infection as a result of replacement of subunit Y by LMP2 [95].

In order to be incorporated into an immunoproteasome, the LMPs must be processed from a precursor form [96, 97]. The molecule PI31 was found to negatively regulate immunoproteasome formation by interfering with that processing. It is thought that this helps to maintain levels of constitutive proteasomes in cells when there is no infection [98]. LMP7 also appears to play a role in processing pre-LMP2 and pre-MECL1, as it is required for their incorporation into the proteasome [96].

Assembly of both the constitutive and immunoproteasome appears to be cooperative. When more X subunit is incorporated into the proteasome, then incorporation of Y is favored over LMP2, suggesting that the presence of one subunit influences the use of the other. Interestingly, IFN-γ does not suppress transcription of subunits X and Y [99]. This suggests that the use of the immuno-subunits is preferred over the constitutive subunits X and Y as the proteasome assembles [96, 99]. In addition, IFN-γ leads to reduced levels of phosphorylation of two α-subunits of the 26S proteasome. This destabilizes the constitutive proteasome complex, allowing for easier incorporation of the upregulated LMPs and the PA28 regulator [100]. Together, these data indicate that the mixing of X and Y with LMPs during proteasome assembly is disfavored [96].

1.4.3 Opening the Immunoproteasome

The catalytic core of the proteasome is closed off, and proteins or peptides may not enter the catalytic core until an 11S or 19S regulator binds to the α-heptameric ring and opens it. The 19S regulator, or PA700, is typically associated with the constitutive (housekeeping) proteasomes, while the 11S regulator, or PA28, associates with the immunoproteasome. Figure 1.2b shows that the entrance to the catalytic core is occluded by the heptameric ring of α-subunits of the 20S particle. Binding of the PA28 regulator (Figure 1.2c) or the 19S regulator induces a conformational change in the α-heptameric ring, allowing substrates to enter the catalytic core [81] (Figure 1.2d). Importantly, the 19S regulator, an ATPase, actively unfolds and guides full-length protein substrates into the proteasome in an ATP-dependent fashion, while PA28 permits shorter polypeptides to enter the catalytic core by diffusion, independent of ATP [101].

Like other members of the immunoproteasome, PA28 is upregulated by IFN-γ [102]. PA28 binds equally well to the constitutive 20S particle and the immuno-20S particle [81, 103]; however, it may aid with the incorporation of the immunosubunits, as LMP2, LMP7, and MECL-1 do not displace X, L, and Z subunits in

the absence of PA28 [104]. Incorporation of the LMP subunits into the 20S particle and binding of PA28 both help to increase the variation in peptides produced for antigen presentation by class I MHC molecules and are most effective when employed coordinately [91, 105]. It was first believed that PA28 had no influence on the proteolytic activity inside the proteasome core but only enhanced uptake and release of peptides by the proteasome [91, 103]. However, $PA28^{-/-}$ cells produce 70% fewer peptides possessing a hydrophobic residue at the carboxyl terminus, and PA28 knockout (KO) mice have impaired CTL responses [104], showing that PA28 does influence the types of peptides generated. Perhaps binding of PA28 to the proteolytic core induces a conformational change that not only opens the gate but also alters the accessibility of the active sites and, consequently, the efficiency of peptide generation [106–108]. Upon infection by lymphocytic choriomeningitis virus or *Listeria monocytogenes,* liver cells in mice were found to completely replace constitutive proteasomes with immunoproteasomes and PA28 [109]. However, an immunoproteasomal pathway involving PA28 is not required for all presented peptides. PA28 KO mice launched a normal immune response against infection with influenza A virus [110].

Kinetic studies suggest that PA28 induces double cleavage of peptide substrates, allowing for the increased rate of processed antigen by the proteasome. The identified peptide products were typically 7–12 amino acids long, which fits the class I MHC ligand size, or are slightly longer, which would allow them to serve as precursors [111].

1.4.4 Peptide Trimming

The proteasome generates peptide lengths with a range of 3–22 amino acids, and less then 15% of those peptides are eight or nine residues in length [106]. In the cytoplasm, some peptides are trimmed by aminopeptidases [112] such as puromycin-sensitive aminopeptidase, bleomycin hydrolase [113], and tripeptidyl-peptidase II (TPPII) [114]. TPPII has been shown to be essential for the generation of epitopes for HLA-B51 [115], HLA-A3, and HLA-A11 [114]. However, a great deal of amino-terminal trimming takes place in the ER.

The transporter associated with antigen processing (TAP) translocates a substantial quantity of peptides into the ER that are longer than the canonical nonamer [116–120]. Delivery of a 40-amino-acid influenza peptide to the ER using a signal sequence resulted in a specific CTL response to the final nonamer in the peptide. It could not have been generated without trimming [121]. Peptides with amino acids flanking both the amino-terminal and carboxyl-terminal ends may be delivered to the ER, but only amino-terminal-flanked antigenic peptides are presented by class I molecules to CTLs [122]. Two ER aminopeptidases have recently been identified. Leukocyte-derived arginine aminopeptidase (L-RAP) has been found to cleave precursor peptides in the ER with a preference for arginine and seems to be upregulated by IFN-γ [123]. However, the primary aminopeptidase in the ER seems to be the ER aminopeptidase associated with antigen processing

(ERAAP), or ERAP1, which is also upregulated by IFN-γ [124, 125]. ERAP1, unlike other aminopeptidases, seems to limit trimming to create minimally eight-residue peptides [126]. There are a number of class I MHC allotypes that prefer peptides with proline at P2 [28, 127]; however, peptides with the X-P-X_n motif are poorly transported by TAP [117, 128]. As ERAP1 trims, it halts cleavage as it comes to X-P, resulting in an accumulation of peptides with proline at P2 [122], which can then be loaded into their respective class I MHC molecules. These trimmed peptides must then reenter the peptide-loading complex. This may occur in the ER by competing with newly translocated peptides, or these trimmed peptides may be transported out into the cytoplasm through SEC61-mediated retrotranslocation [129] and then may be transported into the ER again via TAP [130]. These studies suggest that some class I MHC molecules are able to directly bind peptides generated by the proteasome and transported via TAP, while the peptides for other class I MHC molecules require additional processing in the ER prior to loading.

1.4.5 Association of the Proteasome with the Endoplasmic Reticulum

Peptides generated in the cytosol via the constitutive proteasome, immunoproteasome, or other cytosolic proteases must be translocated into the ER for loading into a class I MHC molecule. The most prevalent pathway is via the transporter associated with antigen presentation (TAP). Early observations noted an unknown molecule (later identified as TAP) that co-immunoprecipitated with the LMP complex (later known as the immunoproteasome), suggesting a physical interaction *in vivo* [131]. The *Lmp-2* gene is physically close to *Tap1*, suggesting that both these gene products are important in antigen processing [132]. Yeast two-hybrid experiments showed that TAP interacts with both the constitutive 26S proteasome subunit X and its replacement, LMP7, of the immunoproteasome [133]. Fluorescently tagged anti-LMP2 and anti-LMP7 antibodies showed that immunoproteasomes are primarily associated with the endoplasmic reticulum in cells treated with IFN-γ, while constitutive proteasomes are evenly distributed throughout the cytosol and nucleus [134]. These results also correlate well with cellular fractionation studies [135, 136]. Taken together, these data show that upon IFN-γ stimulation, immunoproteasomes are formed and associate with TAP at the ER membrane.

1.5 Peptide Transport

The loading of peptide onto folded class I MHC molecules occurs in the ER [45]. The primary means for peptides generated in the cytosol to enter the ER is by way of the transporter associated with antigen presentation (TAP). TAP is a subunit of the peptide-loading complex (PLC). The PLC is comprised of TAP, tapasin, Erp57, calreticulin, and class I MHC molecules, each of which will be discussed individually.

1.5.1 Transport via TAP

TAP1 and TAP2, encoded within the MHC, are members of the ATP-binding cassette (ABC) family of membrane transporters [137–140]. The TAP heterodimer spans the ER membrane, allowing for movement of peptides from the cytoplasm to the lumen of the ER [141]. Mutant cells with deletions in a portion of the *MHCI* locus encompassing both *Tap* and *LMP* genes (RMA-S in mice [142], 721.174/T2 in humans [143]) do not efficiently load peptides onto class I MHC and as a result do not express stable class I MHC molecules on their surface. It was shown that presentation of peptide could be restored by transfecting these RMA-S and T2 cells with both *Tap1* and *Tap2* [144, 145] but not individually, suggesting that TAP1 and TAP2 work coordinately as a heterodimer. This association was confirmed by co-immunoprecipitation [146, 147].

Typically, ABC transporters are found as dimers, and each possesses a hydrophilic nucleotide-binding domain (NBD) and hydrophobic domain consisting of six transmembrane segments. These nucleotide-binding domains contain highly conserved sequence motifs found in many ATPases, including Walker A and B motifs [148, 149]. Like other ABC transporters, transport of peptides across the ER membrane requires ATP and/or possibly GTP [150–154]. The binding of cytosolic peptides to TAP is ATP independent [119, 150], but stabilization of the TAP dimer by association with ATP or ADP has been shown to increase affinity of TAP for peptides [153, 154]. Hydrolysis of the bound nucleotides by both TAP1 and TAP2 induces a conformational change in the transmembrane domains, which results in the transport of peptide across the membrane [153, 155–157]. The X-ray crystallographic structure of the cytoplasmic NBD of TAP1 complexed with Mg^{2+} and ADP provided support for the model in which TAP1 and TAP2 dimerize in the presence of ATP [237].

1.5.2 TAP Selectivity

TAP does not transport every peptide with the same efficiency. The selectivity of TAP is dependent on the sequence and length of peptide, not just overall charge or hydrophobicity [150–152]. The presence of proline at P1, P2, or P3 seems to be most problematic with respect to transport for TAP. Immunodominant epitopes with a proline at P3 (LCMV NP [FQPQNGQFI], Ad10 [SGPSNTPPEI], M8 [KSPWFTTL], SV9 [FAPGNYPAL]) are transported very poorly. However, if naturally occurring flanking residues are added to the amino terminus, transport of these peptides increases significantly [117].

Polymorphism present in TAP results in different transport selectivities [158]. For example, the rat expresses TAP2A and TAP2B, which display different peptide specificities [159, 160]. TAP2A is more permissive of residues at the carboxyl terminus of peptides it transports. However, TAP2B is very selective for hydrophobic residues at the carboxyl terminus [161]. This polymorphism and the subsequent

properties of TAP2 affect the surface expression of the rat class I MHC molecule RT1.A^a, a phenomenon called class I modification (*cim*) [162]. RT1.A^a preferentially binds peptides with an arginine at the carboxyl terminus [163]. If a rat is homozygous for TAP2B, or *cim*^{b}, then peptide binding to RT1.A^a is severely impaired [164]. These biochemical data were confirmed by the crystal structures of the rat class I MHC molecules RT1.A^a and RT1.A1^c, which have substantially different F-pocket properties, leading to their preferred peptide carboxyl-terminal residues, arginine and leucine, respectively [165].

The ideal length of peptides binding to TAP is 8–11 residues, which is similar to the ideal length for binding to class I MHC molecules and seems to be the most important factor in transport. Peptides up to 25 amino acids can be transported, but TAP becomes much more selective and transport is less efficient [119]. A separate study also found that adding up to four amino acids to the amino terminus of melanoma-associated peptides improved transport into the ER [118]. These data suggest that while TAP transports peptides ready to be loaded into the class I MHC molecule, it also transports longer amino-terminally extended peptide precursors that are processed to their final length in the ER.

After length, the greatest determinant for TAP selectivity is the carboxyl-terminal residue. Peptides containing a basic or hydrophobic residues at the carboxyl terminus are translocated most effectively [119, 159, 166]. The relative importance of other residues in the peptide was determined by evaluating the affinity of TAP for over 250 peptides 9–16 residues long. Again, having hydrophobic or basic residues at the carboxyl terminus was found to be most beneficial, followed by hydrophobic residues at P3 and hydrophobic or basic residues at P2 [128]. Based on the selectivity that TAP exhibits for peptide binding and transport, it is not surprising that TAP influences peptides presented by MHC molecules to T cells [166, 167].

1.5.3 TAP-independent Peptide Transport

1.5.3.1 Endogenous Peptides

TAP is recognized as the primary transporter of peptides into the ER, but other means of peptides entering the ER in order to bind class I molecules have been identified. Some class I MHC molecules are expressed on the surface of TAP-deficient cells (T2), namely, H-2D^p, H-2K^b, and HLA-A2 [168, 169]. Peptide sequencing by mass spectrometry determined the most abundant peptide bound to HLA-A2 to be a fragment of a hydrophobic amino-terminal ER signal sequence of a constitutively expressed protein, IP30. Other peptides have been shown to be presented via class I MHC without a signal sequence. Therefore, additional TAP-independent pathways must exist for loading peptides onto class I MHC molecules [168, 170].

Another proposed alternate pathway for peptides entering the ER is via P-glycoprotein. Like TAP, P-glycoprotein is a member of the ABC transport family and

was originally identified and characterized in tumor cells as a multiple drug resistance efflux pump [171]. Recent evidence suggests that it is capable of binding and transporting peptides within the size range of MHC-bound peptides with basic or hydrophobic residues at the carboxyl termini [172, 173]. P-glycoprotein has also been shown to associate with subunit X of the constitutive 26S proteasome [133].

Polymorphism between MHC alleles has been shown to affect peptide repertoire with respect to transport via TAP. HLA-B*4402 and HLA-B*4405 differ by only one amino acid in the F pocket, Asp116 and Tyr116, respectively. In addition to binding different peptides preferentially, HLA-B*4402 cell surface presentation is TAP dependent, while HLA-B*4405 presentation is TAP independent. It appears that the tyrosine residue in HLA-B*4405 causes a structural change that inhibits incorporation of the protein into the peptide-loading complex (PLC). HLA-B*4405 and other MHC alleles that are TAP independent may have an evolutionary advantage over TAP-dependent alleles during an infection that interferes with TAP activity [174].

1.5.3.2 **Exogenous Peptides**

For the most part, peptides bound to class I MHC molecules are generated endogenously or through protein synthesis occurring within the cell. This is critical because the outcome of T-cell recognition is death of the presenting cell. It would be inappropriate to lyse bystander cells that happen to pick up peptides from their neighbor. However, it is advantageous for professional antigen-presentation cells, like dendritic cells, to pick up antigen without becoming infected themselves. This process is called cross-presentation (reviewed in Ref. [175]).

Using fluorescence probes, exogenous peptides were shown to be taken up by cells in vesicles and delivered to the ER [176]. Transport into the cytosol may occur via SEC61-mediated retrotranslocation [177]. SEC61 is thought to be present in the phagosome membrane as a result of ER and cell membrane fusion during early phagocytosis [178]. If the proteins are exported from the phagosome, these exogenous molecules can then be degraded by proteasomes [179–181]. If degraded in the phagosome, these peptides may be exported to the cytosol and translocated into the ER via TAP [179]. However, because it is partly derived from ER, the phagosome may contain the same peptide-loading machinery as the ER [182–184]. Therefore, these peptides may bind to class I MHC molecules in the phagosome and shuttle to the surface through a still unknown pathway.

Endosomal compartments contain proteases that degrade proteins and are involved in class I MHC peptide presentation pathways. One such endopeptidase, cathepsin S, has been shown to play a major role in generating peptides for cross-presentation in the endosomal compartments [185]. These peptides are loaded into class I MHC molecules in post-Golgi compartments. The source of the class I MHC molecules may be the traditional endogenous pathway from the ER, or it may actually be class I MHC that has been recruited from the surface of the cell by endocytosis [186].

1.6
Class I MHC Maturation and Peptide Loading

TAP facilitates loading of peptides directly onto class I MHC molecules by its association in the ER lumen with a large complex of proteins including nascently formed class I MHC (with β_2m) [187, 188], tapasin, calreticulin, and ERp57 [20]. Peptide loading and all the molecules involved in the process are diagramed in Figure 1.3. Before peptides can be loaded onto the class I MHC molecule, they must be processed through a complicated path involving the protein-folding machinery in the cell.

1.6.1
ER Chaperones: Calnexin, Calreticulin, ERp57, and Tapasin

1.6.1.1 Calnexin

Calnexin (CNX) and its soluble relation calreticulin (CRT) are both chaperones found in the ER that promote correct folding of glycosylated proteins including class I MHC heavy chain [189–194]. Immediately following translocation of class I heavy chain into the ER membrane, the new chain acquires an N-linked oligosaccharide at asparagine 86. Calnexin, a 65-kDa membrane-bound chaperone of the ER, associates with the heavy chain almost immediately [195–198] and aids in forming the heterodimer of heavy chain– β_2m [199, 200]. As a chaperone, calnexin protects monomeric MHC heavy chains in the ER from aggregation and subsequent degradation. Calnexin also impedes egress of peptide-deficient MHC heterodimers as well as free heavy chain from the ER [201], suggesting that it contributes to the efficient expression of peptide-bound MHC molecules on the cell surface. The crystallographic structure of the lumenal portion of calnexin shows that it consists of two domains: a globular domain, which contains an oligosaccharide recognition site; and a long arm containing proline-rich repeats (P-domain), which recruits the thiol reductase ERp57 [202, 203]. This long, flexible arm may be what allows calnexin to interact so well with misfolded glycoproteins.

1.6.1.2 Tapasin

Tapasin is a 48-kDa glycoprotein encoded within the *MHCI* locus. Sequence analysis places it in the immunoglobulin family [204]. Tapasin co-immunoprecipitates with TAP and is described as the bridge between TAP and class I molecules [205]. It is a type I transmembrane glycoprotein with an ER retention signal and is essential for peptide loading of class I MHC molecules. Cells that lack expression of tapasin have no detectable class I molecules, calreticulin, or ERp57 associated with TAP [204–208]. The carboxyl-terminal 128 residues of tapasin are essential for interaction with TAP, while deletion of the amino-terminal 50 residues abrogates the ability of tapasin to stabilize the class I heavy chain–β_2m–calreticulin–ERp57 heterocomplex [209]. Mutational analysis shows that tapasin binds to the

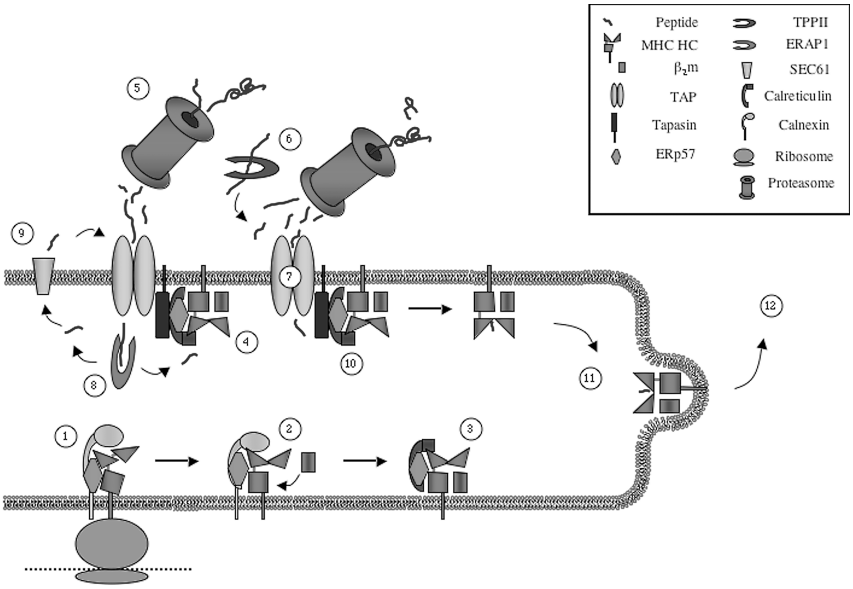


Figure 1.3 Production of peptide-loaded class I MHC. Class I MHC heavy chain and β_2m are translocated separately into the ER. Immediately when accessible, Asn86 of the heavy chain is glycosylated, allowing for recognition by the chaperone calnexin. (1) Calnexin also recruits Erp57, which mediates disulfide bond formation in α3 and α2 of class I heavy chain. (2) Once the heavy chain is folded and the disulfide bond in α3 is formed, soluble β_2m binds to class I heavy chain. While the MHC class I molecule is being formed (cycling on and off calnexin), newly translated tapasin associates with calnexin and ERp57, both of which facilitate its folding and disulfide bond formation. Tapasin with calnexin–ERp57 then binds and stabilizes the TAP1/2 dimer. (3) Upon binding of β_2m, calnexin dissociates and is replaced by calreticulin. The class I heavy chain–β_2m–calreticulin–ERp57 heterocomplex then associates with the tapasin–TAP–calnexin–ERp57 heterocomplex. ERp57 remains part of the peptide-loading complex (PLC) until peptide has been loaded, but it is not known whether ERp57 comes with tapasin or the class I molecule. (4) The PLC now consists of TAP, tapasin, heavy chain, β_2m, calreticulin, and ERp57. (5) Proteasomes in the cytosol degrade substrates, generating peptides of varying length. (6) Large proteasomal products can be further cleaved in the cytosol by aminopeptidases such as TPPII. (7) Peptides with affinity for TAP will bind, and ATP-induced conformational changes in TAP will transport it through the ER membrane. (8) ERAP1 performs amino-terminal trimming in the ER. (9) These peptides may go directly into the PLC, or they may be retrotranslocated back into the cytosol through SEC61 and have to enter the ER again via TAP. (10) If the amino acid motif of the peptide matches the class I MHC molecule, it will then bind in the peptide groove, inducing a conformational change that stabilizes peptide binding. ERp57 will mediate the disulfide formation in α2, "locking" the peptide in. (11) If the correct conformational change is induced, all chaperone molecules will dissociate. (12) The trimer of class I heavy chain–β_2m–peptide is shuttled from the ER to the Golgi apparatus and subsequently to the plasma membrane of the cell. (This figure also appears with the color plates.)

α2 domain of class I heavy chain, an observation that is conserved between H-2D^d, H-2L^d, and HLA-A2 [210]. The stoichiometric level of tapasin to other PLC components is unknown. It could be a 1:1 ratio of TAP-1/2 and tapasin [206], or four tapasin molecules associate with TAP-1/2 [204], potentially allowing up to four class I peptide-loading complexes to associate with TAP. Tapasin-deficient mice show low cell surface expression of empty class I molecules or class I molecules bound with suboptimal, low-affinity peptides, affecting CTL response [211–214]. Tapasin-expressing cells present stably bound peptide with a long half-life, while tapasin-deficient cells present peptides with a reduced half-life [215]. Presumably, tapasin accomplishes this by selecting peptides with the greatest half-life upon conformational change of class I heavy chain [215]. The tapasin–calnexin complex also recruits the thiol reductase, ERp57, which may remain to assist with loading the class I MHC molecule [216].

1.6.1.3 **ERp57**

ERp57 is responsible for facilitating disulfide bond formation in the α2 and α3 domains of the class I MHC heavy chain [217, 218]. After being recruited by calnexin to the class I heavy chain, ERp57 remains as part of the PLC until peptide is loaded [219]. ERp57 forms an interchain disulfide bond with tapasin [220, 221]. It appears that the isomerization of the disulfide in the α2 domain of class I heavy chain is controlled by tapasin–ERp57 and occurs only once the proper peptide has been loaded in the groove [221]. ERp57 may have a role in quality control in the ER by reducing improperly folded class I heavy chains [222], which could be translocated back into the cytoplasm for degradation by the proteasome [223]. Following proper disulfide bond formation in the class I MHC heavy chain and association with β_2m, calnexin dissociates and calreticulin binds the MHC heterodimer [205, 218, 224].

1.6.1.4 **Calreticulin**

Calreticulin, a 46-kDa soluble protein found in the ER, displays high sequence homology to the lumenal domain of calnexin, including the P-domain [225], and functions to keep class I heavy chain–β_2m in a peptide-receptive state [226]. Calreticulin remains associated with the PLC until proper peptide loading. Deleting calreticulin resulted in reduced efficiency of peptide loading by 50–80% and impaired CTL activation [227]. Calreticulin not only binds to class I heavy chain, but its P-domain also binds ERp57 in a manner similar to that of calnexin [228], keeping ERp57 in the class I heterocomplex until peptide is loaded.

1.6.2
Peptide Loading

The timing of the events that produce a peptide-loading MHC molecule is likely to be very carefully regulated, especially during inflammatory responses. Table 1.2

shows the timing of many of the steps in antigen presentation. The rate-limiting step appears to be folding of the class I MHC molecule from the nascent chain. Once the protein is folded properly, it is not clear exactly how peptide is passed from TAP to the peptide-binding cleft to allow for the final step in maturation to occur. TAP will bind to a peptide and transport it through the ER membrane. If necessary, ERAP1 will perform any additional amino-terminal trimming if the peptide is too long. If the amino acid motif of the peptide matches the class I MHC allele, it will then bind in the peptide cleft, inducing a conformational change that stabilizes peptide binding. ERp57 will mediate the disulfide formation in $\alpha 2$, "locking" the peptide in. Tapasin then somehow evaluates the affinity and/or half-life of the peptide. If the peptide has the appropriate motif and the correct conformational change is induced, all chaperone molecules will dissociate and the trimer of class I heavy chain–β_2m–peptide will be shuttled from the ER to the Golgi apparatus and subsequently to the surface of the cell.

Table 1.2 Estimated timing of steps in the formation of peptide-loaded MHC.

Molecule	Activity	Copies per cell	Reference
Proteasome	Substrates degraded per minute per proteasome	800,000	235
Cytosolic peptidases	1 N-terminal residue per 2–3 seconds	n.d.	112
TAP	2–5 peptides per second per TAP	~10,000	151
MHC–β_2m	4 minutes post-translation of heavy chain	n.d.	200
MHC–β_2m–TAP	5–10 minutes post-translation of heavy chain	n.d.	187,200
MHC–peptide (k_{on})	2×10^6 M^{-1} s^{-1}	1000–5000 in ER	43

1.7 Immunodominance and Class I MHC Peptide Processing

Peptide processing may be the primary filter for epitope selection within the cell. Both the constitutive proteasome and the immunoproteasome selectively cleave on the carboxyl-terminal side of specific residues. Upon infection, the immunoproteasome is more adept at producing peptides preferred by TAP and class I MHC molecules: those with hydrophobic or positively charged carboxyl-terminal residues. It is likely that the proteasome, TAP, and class I MHC molecules have coevolved to generate and select peptides with similar characteristics, namely, length and carboxyl-terminal properties.

Although there have been correlations between pMHC stability or peptide binding and immunodominance, most studies have shown that the relationship between antigen processing and immunodominance is a passive one. However, if the peptide is not produced by the antigen-presenting machinery, the number of responding T cells may be significantly reduced. LMP2 knockout mice produce 50% fewer T cells to specific influenza immunodominant peptides [229]. Conversely, other studies have shown that efficient T-cell recognition of an immunodominant ovalbumin epitope remains after deliberate frameshift mutations such that the antigen-presenting cells expressed very low levels of the correct protein sequence [230]. This suggests that regardless of whatever is translated and processed, the immunodominant epitope, even when at very low levels, is still immunodominant. Ultimately, it appears that immunodominance is defined by the T-cell repertoire in the host.

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