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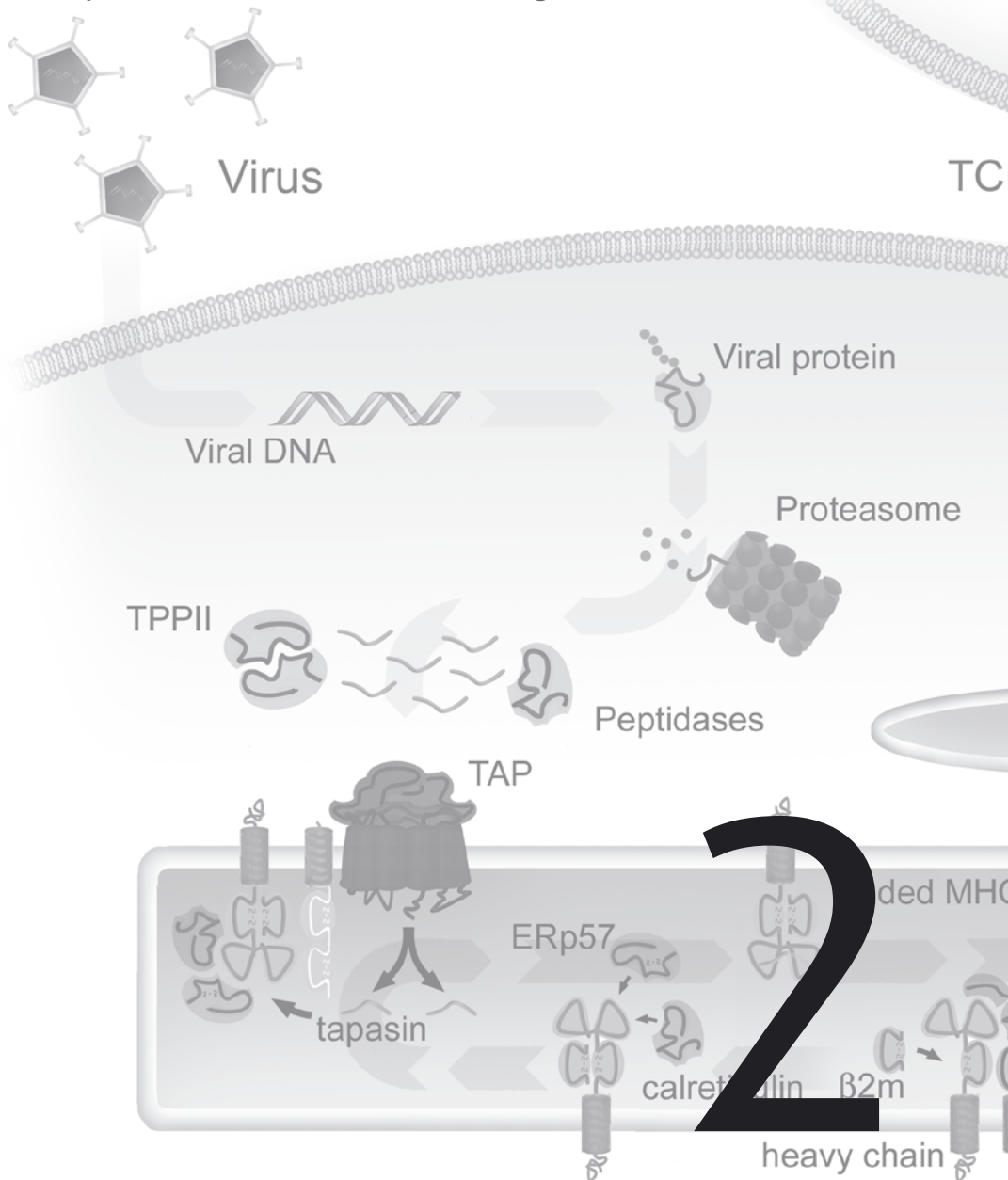
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MHC class I alleles and their exploration of the antigen-processing machinery

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MHC class I alleles and their exploration of the antigen-processing machinery

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At the cell surface, major histocompatibility complex (MHC) class I molecules present fragments of intracellular antigens to the immune system. This is the end result of a cascade of events initiated by multiple steps of proteolysis. Only a small part of the fragments escapes degradation by interacting with the peptide transporter associated with antigen presentation and is translocated into the endoplasmic reticulum lumen for binding to MHC class I molecules. Subsequently, these newly formed complexes can be transported to the plasma membrane for presentation. Every step in this process confers specificity and determines the ultimate result: presentation of only few fragments from a given antigen. Here, we introduce the players in the antigen processing and presentation cascade and describe their specificity and allelic variation. We highlight MHC class I alleles, which are not only different in sequence but also use different aspects of the antigen presentation pathway to their advantage: peptide acquaintance.

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Antigen presentation by major histocompatibility complex class I molecules, a multienzyme process

Major histocompatibility complex (MHC) class I molecules present small fragments from intracellular expressed proteins to the immune system. This presentation enables the immune system to monitor the intracellular protein content, albeit through the exposure of a snapshot of these proteins in the form of small peptides, usually 9 amino acids in length (1). The fact that fragments from intracellular proteins are presented at the plasma membrane can only be the result of a number of biochemical processes. These include proteolysis for the generation of protein fragments, folding/stabilization to prepare MHC class I molecules for peptide capture, and transport of peptide fragments to the site of association with MHC class I molecules.

The beginning of the end, substrate recognition for degradation

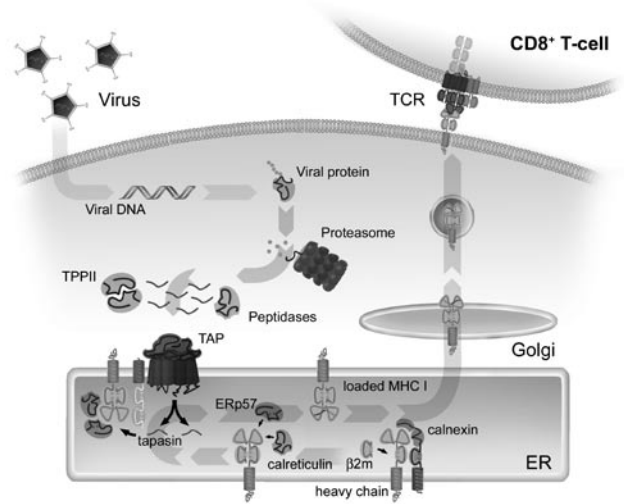
'Classical' MHC class I antigen presentation is a system more complex than that suggested above (Figure 1). It all starts with the recognition of a protein (self or foreign) by the intracellular degradation machinery and the transfer of multiple ubiquitin moieties to the substrate protein (2). Recognition of substrate proteins for ubiquitination is a complex mechanism, involving many diverse systems. During protein synthesis, chaperones are thought to be involved in the targeting of misfolded proteins for degradation (3). Recognition during other cellular processes (e.g. the cell cycle and posttranslational signaling) is dependent on other mechanisms including phosphorylation, destruction boxes, E2 and E3 protein complexes, and possibly more proteins, as not all mechanisms have been elucidated yet (2). Several protein families are able to transfer the ubiquitin to proteins after initial activation by the

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Figure 1. Classical pathway of major histocompatibility complex (MHC) class I antigen presentation. MHC class I molecules are assembled in the endoplasmic reticulum (ER) supported by the chaperones calnexin, calreticulin, and ERp57, after which they dock onto the ER-resident peptide transporter associated with antigen processing (TAP). This docking is facilitated by the specialized chaperone tapasin. TAP pumps peptides into the ER lumen. These peptides are from cellular or viral origin and are produced in the cytosol/nucleus by the proteasome, tripeptidyl peptidase II (TPPII), and other peptidases. Once in the ER, peptides can bind to MHC class I molecules that are subsequently released from the TAP–tapasin loading complex. MHC class I–peptide complexes can then leave the ER for transport to the plasma membrane. Here, they can be inspected by the T-cell receptor of CD8⁺ T cells.



E1-activating enzyme. This enzyme binds ubiquitin covalently at the cost of adenosine triphosphate (ATP), followed by transfer of the ubiquitin to an E2-conjugating enzyme. With the help of substrate-specific E3-ligating enzymes, the ubiquitin is then covalently coupled to the target protein (4). The family of E3-ligating enzymes can roughly be separated in two groups: homologous to E6-AP C-terminus (HECT) domain and RING finger complexes. Although the overall structure of the two groups is quite similar, the functional interactions are different. When complexed with E2 enzymes, a bended arm-like complex is formed, which facilitates transfer from the ubiquitin moiety to the target protein (Figure 2). HECT domain proteins contain, as their name implies, a HECT domain, in which a conserved cysteine is able to form a covalent thioester bond with ubiquitin before the latter is transferred to the substrate protein (5). RING finger complexes appear not to be able to form a covalent bond with ubiquitin, but they might facilitate the interaction between the substrate and the ubiquitin-bound E2 enzyme. The RING finger family can be subdivided into several subfamilies that differ in the number of adapter proteins incorporated in the complex. Some of these RING finger proteins function alone with the E2s (e.g. c-CBL), others (e.g. ROC1) need adapter proteins such as Cullins and F-box proteins to form SCF (Skp, Cullin, F-box), VBC (VHL-clongin, B-clongin, C-clongin), or APC (anaphase-promoting complex) complexes (5). In the latter subfamilies, the adapter proteins are involved in the recognition of the substrate, while the metal-binding

RING finger domains might be used to catalyze the transfer of ubiquitin.

The three families of enzymes (E1, E2, and E3) might even not be sufficient for complete polyubiquitination of a substrate protein, which is necessary for recognition by the proteasome and subsequent degradation (proteins can also be monoubiquitinated, but this post-translational modification is implicated in other cellular functions, among which are endocytic trafficking and DNA repair (6), and is beyond the scope of this review). Recently, E4 proteins have been identified in yeast with defined human orthologs (7, 8). These E4 proteins are reported to elongate the polyubiquitin tree and are able to bind the 19S cap. This process is essential for recognition and unfolding by the 19S cap of the proteasome that finds the substrate by simple diffusion and collision (9) (Figure 3).

There are other factors that complicate matters; substrate ubiquitination can be counteracted by deubiquitinating proteins (10). This family comprises about 50 members in humans, each recognizing different ubiquitinated substrates and stabilizing them by removal of the ubiquitin tag (11). Thus, tagging with ubiquitin is not necessarily the protein's end.

Obviously, protein degradation is not merely a simple process initiated by the addition of a ubiquitin moiety, but it is tightly controlled and highly regulated by a large set of enzymes. This complexity is perhaps most clearly illustrated by the fact that more than 1% of the

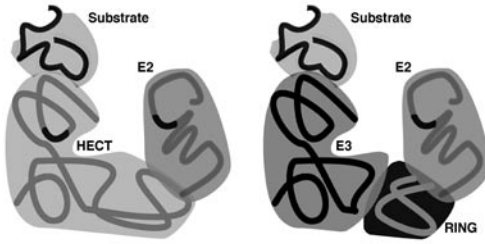


Figure 2. Ubiquitin ligation complexes. The two families of E3-ligating enzymes are shown. **(Left)** Homologous to E6-AP C-terminus (HECT) domain containing E3 ligases contain a HECT domain, which transfers ubiquitin from the E2 to the substrate following covalent binding of the ubiquitin to the HECT domain. **(Right)** RING finger complexes facilitate the transfer of ubiquitin from the E2 to the substrate but do not interact covalently with ubiquitin. RING finger complexes can contain multiple adapter proteins with different functions.

human proteome is involved in the recognition and control of degradation of all proteins.

Destined for retirement, the proteasome architecture

The proteasome is an abundant protein complex in all living cells. It is built of a central cylindrical barrel (the 20S part) and one or two optional caps (19S parts, see below) (12). The proteasome is a threonine protease

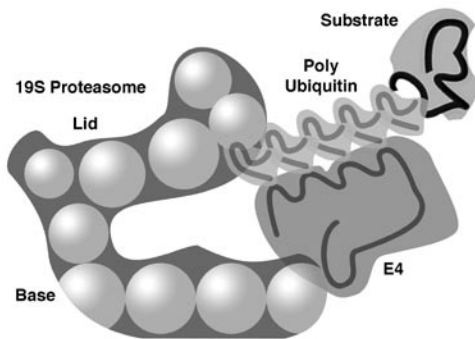


Figure 3. Binding of polyubiquitin chains to the 19S proteasome with the help of E4 proteins. E4 proteins elongate ubiquitin trees and facilitate binding of the polyubiquitinated substrate to the 19S cap of the proteasome. The 19S cap removes the ubiquitin moieties, unfolds the substrate, and upon proper binding to a 20S proteasome core facilitates the transfer of the substrate into the catalytic chamber of the proteasome.

(meaning that the amino acid threonine acts as the nucleophil donor for the chemical reaction to break the peptide bond, thus cleaving the substrate) (13). These threonines are positioned in the central chamber of the 20S cylinder (14, 15). Again, life is more complicated. Two forms of the central chamber can be distinguished representing the constitutive and the immunoproteasome (13). The differences between these two are the three β -subunits that donate catalytic threonines to the proteasome's central chamber. The β -1i, β -2i, and β -5i are strongly upregulated after interferon- γ treatment at the cost of their 'normal' β -subunit counterparts (β -1, β -2, and β -5, respectively) (16). The genes for two of these subunits (β -1i and β -5i) are located in the MHC locus, close to the transporter associated with antigen presentation (TAP) subunits (17). Upon interferon- γ treatment, new proteasomes will incorporate these alternative subunits and gradually replace (taking some 48 h) the old 'constitutive' proteasomes (12). The digestion pattern of substrate proteins may then change resulting in different peptides presented by MHC class I molecules (18, 19).

The proteasome is a self-compartmentalized protease, and only substrates accessing the central chamber will be digested. To get access to the 20S core of the proteasome, proteins have to pass a small pore of about 13Å (14, 15, 20). In other words, proteins have to be unfolded for entry in the 'digestion chamber', a task performed by the 19S cap of the proteasome (21). The 19S cap is a multisubunit complex composed of about 20 proteins that may be dynamically attached to either one side or both sides of the 20S core, but they can also diffuse as a solitary complex (22). Some subunits are involved in the recognition of polyubiquitin chains (i.e. S5a (23, 24)), some in the removal of the polyubiquitin tree from the substrate protein (i.e. Rpn11 (25)), and others in the unfolding of the substrate protein (the AAA ATPases, of which six members form a hexameric ring near the entrance of the 20S proteasome (26, 27)). Unlike the digestion process, substrate unfolding requires energy (ATP). Other activities (including nucleotide excision repair) have been attributed to both individual subunits and the complete 19S cap, but these will not be discussed here (28). Again, alternatives for the 19S cap are found expressed after interferon- γ treatment, especially two subunits (PA28 α and β that form the so-called activator complex (29). These proteins may alter proteasome activity and specificity, but how they do so is unclear (19, 30). PA28 complexes are unable to deubiquitinate and unfold protein substrates, and it is assumed to sup-

port 20S proteasomes with a 19S cap attached to the opposite end of the barrel (29). Mice deficient for the PA28 complex (like those for the immuno- β subunits) have only mild phenotypes (31). Importantly, most non-hematopoietic cells only express the constitutive 26S (= 20S + 2 x 19S) proteasomes, and most immune cells express a mixture of constitutive and immuno-proteasomes. As the two proteasome forms digest substrates somewhat differently, simultaneous expression of both forms automatically results in the generation of a wider variety of substrate peptides (19).

Imperfection as source, what defines proteasomal substrates?

Protein degradation is a tightly regulated process. Proteins are only degraded at defined moments (i.e. cell cycle proteins) and/or when approaching the end of their natural life. How the latter is monitored is unclear, but possibly some form of unfolding acts as an initiator. In both cases, however, the proteasome is perfectly capable to digest these substrates, as it is present in both the cytoplasm and the nucleus (9). More recently, another pool of proteasome substrates was identified, named defective ribosomal products (DRiPs) (32, 33). These are proteins degraded cotranslationally or very swiftly after translation, possibly as the result of protein misfolding, misassembly, or mistranslation/transcription, probably representing all proteins expressed in a cell. In fact, this pool of DRiPs is considerable, ranging from 20 up to more than 70% of all cellular translation products, depending on the cell type analyzed (34). Cells will do their best to prevent protein aggregation, and misfolded proteins (or proteins that followed an incorrect folding path after translation) will be either unfolded and then refolded (a very delicate process) or simply degraded, thus constituting the DRiPs.

DRiPs are interesting for antigen presentation by MHC class I molecules, because they couple antigen translation to antigen presentation. Degradation of a pool of proteins immediately following translation will generate peptides that are presented at the plasma membrane some 30 min later, even when the properly folded antigen is stable for many hours or even days. As viruses can leave the cells within hours after infection, a swift response is required, which the DRiPs and not the stable protein pool guarantee (35).

Fragmented results, what are the proteasomal products?

Proteins are degraded by the proteasome into small fragments. *In vitro* experiments have suggested that the fragments are between 3 and 20 amino acids in size, but predominantly octa- and nonamers (36, 37). By contrast, *in vivo* experiments suggest that the majority of the peptides produced by the proteasome are longer than 15 amino acids (38). These fragments are substrates for cytosolic peptidases, which appear to be exclusively aminopeptidases unable to remove C-terminal residues (37, 39). The proteasome is therefore the only candidate to generate the correct C-terminus of MHC class I-restricted epitopes (37), while the N-terminus can then be trimmed to the correct size by the various aminopeptidases. This picture is not fully correct as the peptidases tripeptidyl peptidase II (TPPII) (38), thymet oligopeptidase (TOP), and neurolysin (40) are able to generate 9-mer or longer peptides after recognizing the free N-terminus of the peptide. Still, the proteasome and the peptidases do not 'know' which peptides are preferred by MHC class I molecules. It will produce some and probably destroy many potential MHC class I-binding peptides by cleaving in instead of at the end of a potential class I-binding peptide (40, 41).

Recycling to amino acids, the role of TPPII and other cytosolic peptidases

Peptides released by the proteasome are deposited in a rather unfriendly environment, where they are exposed to various peptidases. Reits et al. (39) have studied the behavior of peptides in living cells by using fluorescence-bleaching techniques. They concluded that the majority of peptides are free rather than associated with larger proteins, including heat shock proteins that would have protected them from degradation. Peptides can associate with heat shock proteins, as shown in various vaccination studies (42), but they probably do so in a highly transient manner. The diffusion through the cell as free peptides makes them accessible substrates for cytosolic peptidases.

Like the proteasome, most peptidases are large protein structures. Electron microscopy images suggest that the peptidase TPPII is even larger than the 26S proteasome, and leucine aminopeptidase (LAP), for example, is a 300-kDa hexamer (43, 44). Various other peptidases have been identified, including neurolysin, TOP, puromycin-sensitive aminopeptidase, and bleo-

mycin hydrolase (44–49). The collective activity of the cytoplasmic peptidases ensures that peptides are degraded within seconds in living cells (39). But why are so many different peptidases expressed? The peptidases are probably subspecialized for substrates. TPPII prefers substrates over 15 amino acids in length (38), which represent most of the proteasomal substrates *in vivo*. It usually removes the first 2–3 N-terminal amino acids (50), but the same structure can also cleave more than 9 amino acids away from the N-terminus, thereby generating new C-termini for MHC class I-binding peptides (38, 43, 51, 52). It should be questioned whether TPPII has a genuine endoproteolytic activity, because it always requires a free unprotected substrate N-terminus (probably for docking in the enzyme's active site), even when long peptides are generated. TPPII is probably the only peptidase for substrates longer than 15 amino acids, and chemical inhibition or knockdown by short interfering RNA of TPPII results in a marked downregulation of MHC class I expression at the cell surface (38). This finding indicates that TPPII is a critical intermediate between the proteasome and other peptidases, TAP, and MHC class I molecules. TOP and its homolog neurolysin have complimentary activities to TPPII (40, 41). These peptidases cleave substrates of 8–17 amino acids that will include peptide products of TPPII. TOP and neurolysin have a docking site where the free N-terminus of the peptide is bound, with the active center located at some distance (53). Consequently, TOP and neurolysin cleave 4–10 amino acids away from the N-terminus, thereby destroying but also generating peptides for MHC class I molecules (40). Inhibition of TOP by RNAi showed a marked increase in MHC class I expression, suggesting that under normal conditions many peptides are destroyed by TOP (54). Complementarily, overexpression of LAP (another aminopeptidase) decreases peptide formation for MHC class I molecules (39). It is unclear whether the other peptidases are specialized in the products of TOP and neurolysin or whether they have largely overlapping activities (49). Finally, other peptidases should degrade the peptides to free single amino acids, a process critical for cell survival but less relevant for MHC class I antigen presentation.

Escape from degradation, the peptide transporter TAP and the MHC class I-loading complex

Peptides derived from cytosolic or nuclear antigens have to pass the endoplasmic reticulum (ER) membrane to interact with MHC class I molecules that are

retained there by chaperones. A peptide is the critical third subunit for MHC class I assembly and is required to release the MHC class I complex from the ER chaperones tapasin, ERp57, and calreticulin and for exit from the ER (55). Peptides do not spontaneously pass membranes; they are translocated by TAP (56, 57). TAP is a member of the ATP-binding cassette transporter family. It is a two-part transporter made of one TAP1 and one TAP2 subunit, which together form three subdomains: a multimembrane spanning part that contains the ER retention signals, followed by a peptide-binding domain, and two ATP-binding cassettes (58, 59). Hydrolysis of ATP by one of the ATP-binding cassettes, probably TAP1, is necessary for opening the transmembrane pore, and hydrolysis of ATP by the other closes it again (60). These alternating cycles of ATP hydrolysis result in major alterations in the TAP structure, as observed by following their diffusion in the ER using fluorescent recovery after photobleaching (FRAP) (32). Two viral inhibitors (US6 and UL49.5) bind to TAP in the ER and inhibit these conformational changes, thus preventing peptide import in the ER and antigen presentation (61–64). Comparing two rat TAP alleles with different substrate selectivity has identified the peptide-binding area (65–67). This study indicated that TAP2 is required for binding of the C-terminal amino acid in the peptide substrate.

TAP translocates peptides with a minimal size of 8 and prefers 9–12 amino acid long peptides, like MHC class I molecules, but it also handles peptides of up to 40 amino acids, albeit with reduced efficiency (68, 69). In addition, TAP has very broad substrate specificity (70–72), which is expected, as it has to feed many different MHC class I alleles with peptides. The peptide extremities (a free N- and C-terminus spaced by at least 8 amino acids) and correct orientation of the peptide bond are especially important for interaction (73). Human TAP differs from the murine form, as it allows effective transfer of peptides almost irrespective of the C-terminal amino acid. Murine TAP prefers exclusively hydrophobic or aromatic amino acids at that position (74, 75). This difference is also reflected in the peptides associated with MHC class I molecules, which in the murine case invariably are hydrophobic or aromatic at the C-terminal position (usually as an anchor residue), whereas human MHC class I molecules also allow basic amino acids at that position (76). Acidic amino acids have not been defined at this position. Only one other amino acid has a dominant effect on TAP recognition: peptides with

the amino acid proline at position 2 or 3 are poorly handled by TAP (70–72, 74, 77). Still, several MHC class I molecules have a proline as an anchor residue at these positions (i.e. HLA-B7 and HLA-B35), which appears a contradiction (76). These peptides are probably translocated into the ER by TAP with additional N-terminal amino acids, thereby repositioning the proline to another position (72, 77). These peptides are then trimmed by the ER aminopeptidase (ERAP1 or ERAAP) until it results in a proline at position 2, thus generating the correct MHC class I-binding peptide (78, 79). ERAP1 may thus be critical for particular MHC class I alleles because of its specificity to modify particular TAP substrates. Finally, TAP can translocate modified and extended side chains, such as glycosylated and phosphorylated peptides (80, 81). In fact, peptides with an extended side chain of approximately 70Å can be translocated (73). Larger peptide side chains form competitive inhibitors for TAP (73).

TAP also appears to be the center of a large complex where many (but not all) MHC class I alleles dock (82). Coupling peptide transfer by TAP to peptide capture may improve the efficiency of peptide loading of MHC class I molecules. This so-called MHC class I-loading complex consists of TAP, four molecules of a dedicated chaperone called tapasin, four MHC class I molecules lacking peptide, and four chaperones ERp57 and calreticulin (83, 84). Tapasin interacts with the peptide-binding groove of MHC class I molecules (positions 116 and 134 have been identified (85, 86)) and with ERp57 (87). ERp57 is probably important for supporting disulfide formation of the MHC class I complex (88–90). In addition, tapasin bridges TAP and partially unfolded MHC class I H-chain/ β 2-microglobulin heterodimers (90, 91). As a consequence, the stability and export of many MHC class I alleles is reduced in tapasin-deficient cells or mice (92). Still, many MHC class I molecules successfully present their cargo at the plasma membrane, implying that the interactions with tapasin and TAP are supportive but not essential for antigen presentation (85). Many human leukocyte antigen-B (HLA-B) alleles, including HLA-B13, B35, B44, B56, B60, and B62, are not at all using this complex for peptide acquaintance (85), implying that peptides find their MHC class I molecules outside of the MHC class I-loading complex. This process is not unexpected as, as discussed earlier, some MHC class I alleles bind peptides that are trimmed by ER peptidases outside this MHC class I-loading complex. So, how are peptides behaving in the ER lumen?

In and out again, peptides in the ER

Most TAP-translocated peptides that enter the ER probably will not bind MHC class I molecules, because TAP does not 'know' which MHC class I alleles have to be loaded (and TAP is not polymorphic). Two anchor residues in a (9-mer) peptide are usually required for MHC class I binding, and each anchor residue constitutes 1–2 of the 20 natural amino acids. In addition, three to six MHC class I alleles are expressed per cell. The last anchor residue is usually the most C-terminal amino acid. As cells lack cytosolic carboxypeptidase activity (39), the proteasome (with additional support of TPPII or TOP/neurolysin) usually generates the C-terminus. This formation suggests that at best 6% of the peptides (six alleles \times 2/20 \times 2/20 anchor residue/total amino acids) entering the ER have the capacity to bind MHC class I molecules.

Peptides that are not immediately captured by MHC class I molecules may be N-terminally trimmed by ERAP. ERAP seems to have some molecular ruler and trims the peptide to a minimal size of 8 amino acids but probably not beyond that size (93, 94). Still, many peptides will not bind MHC class I molecules. These can interact with ER chaperones such as gp96, gp170, ERp72, ERp57, calnexin, and BiP (95–97). Photoaffinity labeling experiments suggest that especially protein disulfide isomerase (PDI) binds peptides with high efficiency and some selectivity (97, 98). That chaperones interact with peptides may not be surprising, as they can be considered as unfolded protein (stretches), but some chaperones (PDI) are clearly better in peptide binding. A direct need for chaperones in peptide delivery to MHC class I molecules in the ER has not been uncovered. However, various peptide-chaperone complexes have been tested in tumor vaccines, with some success, and they are apparently able to deliver peptides to MHC class I molecules through a process called cross-presentation (42, 99, 100). How this delivery occurs exactly is still unclear.

Peptides that are not used by MHC class I molecules have to leave the ER at one point (otherwise the ER would become packed with crystalline peptide). ER peptides rapidly leave the ER in an ATP-dependent manner (57, 69). The same pathway as used for ER-associated degradation (ERAD), where ER proteins are retrotranslocated to the cytoplasm probably through the translocon (101), is used by peptides to leave the ER (102). BiP and possibly PDI play an im-

portant role as a lid on the translocon (thus controlling opening and closing) (103), and it is possible that peptides leave this lid for retrotranslocation. If so, it becomes apparent why BiP and PDI are peptide acceptors in the ER. Subsequently, peptides can be further trimmed in the cytoplasm, and those peptides escaping complete degradation may enter the ER again after TAP-mediated translocation (69). This peptide cycle over the ER membrane may be an alternative way to trim peptides to a correct size for binding to MHC class I molecules.

The overall picture of MHC class I antigen presentation

Our introduction into the MHC class I antigen presentation pathway has revealed that many proteins are involved to achieve a simple thing: presentation of a cytosolic peptide fragment at the plasma membrane by MHC class I molecules. The majority of these proteins are proteases involved in both the generation and destruction of the peptide (51). A smaller set of proteins is involved in the assembly of MHC class I molecules and only few proteins are selectively involved in the process of MHC class I antigen presentation. These proteins (TAP, tapasin, and MHC class I molecules) originated relatively late during evolution and used existing systems (proteasome, peptidases, translocon, and general chaperones) to successfully perform their function: presentation of a snapshot of the intracellular protein pool to the immune system.

Hide and seek, how to inhibit antigen presentation by MHC class I molecules?

MHC class I molecules present viral and tumor antigens to the immune system, which then responds by eliminating the cells expressing these antigens. It is obvious that viruses have developed stealth technologies during evolution to prevent presentation to the immune system by MHC class I molecules. Indeed, viral proteins interfering with proteins in the antigen presentation pathway that are non-essential for cell survival have been identified. Their targets are TAP, tapasin, and MHC class I molecules, but not the proteasome or general chaperones such as ERp57 or calnexin. Various viral proteins have been identified that inhibit TAP. The herpes simplex virus-encoded cytosolic protein ICP47 acts as a high-affinity peptide substrate for TAP and prevents binding and translocation of other peptides (104–106). Human cytomegalovirus (HCMV) encodes a protein called US6. This ER-located

protein binds to the pore of TAP and arrests TAP in a defined conformation (32), thus preventing peptide translocation (but not binding) (61, 62). A similar phenotype is found for the unrelated varicellovirus protein UL49.5, where the small ER-located protein segment also inhibits the conformational cycle of TAP and thus peptide translocation. In addition, UL49.5 also induces degradation of the TAP class I-loading complex (63). Other proteins, such as the adenoviral protein E319K, affect the organization of the MHC class I-loading complex or the interaction of TAP and tapasin (107). Finally, various proteins affect assembly of MHC class I molecules or induce their degradation. HCMV expresses proteins (US2 and US11) that send MHC class I complexes into the ERAD pathway (108); other proteins (UL18) rapidly induce internalization of cell surface MHC class I molecules for degradation in lysosomes (62, 109). Adenovirus expresses a protein called E19 that associates with MHC class I heavy chains and prevents further assembly (107, 110). Many other viruses are awaiting analysis, which will almost certainly lead to identification of new proteins affecting antigen presentation by MHC class I molecules. Still, the easiest way to inhibit antigen presentation is downregulating transcription of components in the MHC class I antigen presentation pathway, for example, by oncogenic adenovirus 12 E1A (111, 112).

Multiple roads to cross-presentation by MHC class I molecules

Antigenic fragments have to be presented by professional antigen-presenting cells in the lymph nodes, even antigens produced in other cells unable to move to these sites. This cross-presentation is required for cytotoxic T-lymphocyte (CTL) expansion, but the question is how antigenic information can be transferred from the interior of an infected cell to a dendritic cell (113, 114). This cross-presentation is essentially different from the classical MHC class I antigen presentation route, where intracellular antigenic fragments are exclusively presented by MHC class I molecules from the same cell. Various routes for cross-presentation have been proposed (Figure 4).

Intracellular antigens can be released from dead cells in a soluble form or in apoptotic bodies. These enter the endocytic route in dendritic cells, where the antigen may be loaded onto recycling MHC class I molecules. Peptides on MHC class I molecules can be easily exchanged at pH 4.5–5.5, which corresponds to the endosomal pH (115). Analogous to antigen pre-

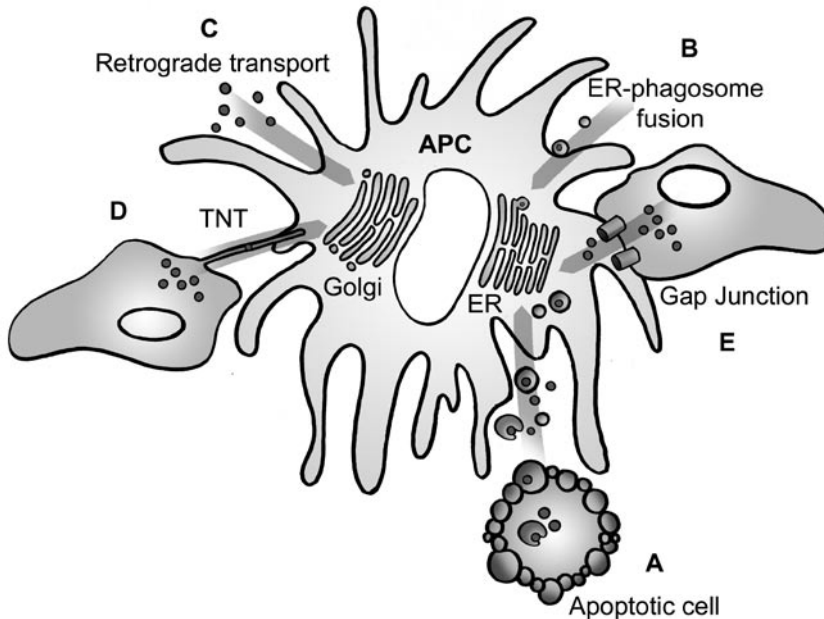


Figure 4. The alternative pathways for major histocompatibility complex (MHC) class I-mediated cross-presentation. (A) Apoptotic material. Intracellular antigens, which may be associated with stress proteins, can be released from dead cells in a soluble form or in apoptotic bodies. The antigens can enter the endocytic route of dendritic cells, and the antigen is loaded onto recycling MHC class I molecules or degraded by endosomal proteases such as cathepsin S. (B) Endoplasmic reticulum (ER)–phagosome fusion. In this model, the ER membrane forms the phagosomal membrane and extracellular antigens are subsequently released by the translocon, degraded in the cytoplasm, and can enter the MHC class I presentation route that is now in the phagosomal membrane. (C) Retrograde transport. Exogenous soluble antigens are endocytosed, move to the Golgi, and then follow the retrograde transport route to enter the ER. Here, they use the ER degradation pathway to enter the cytoplasm and then enter the classical MHC class I pathway involving peptidases, TAP, and MHC class I molecules assembled in the ER. (D, E) Direct transfer. Intracellular cytoplasmic antigens from one cell may be transferred directly into the cytoplasm of another (adjacent) cell. It has been shown that immunologically relevant peptides can diffuse through gap junctions, facilitating this immunological coupling of cells. An alternative for this pathway could be the transfer of antigens via tunneling nanotubes (TNT).

sensation by MHC class II, presentation by recycling MHC class I molecules is also dependent on acidic pH and inhibited by compounds such as NH_4Cl and chloroquine (115–117). In principle, antigens can enter the endocytic pathway as peptides associated with stress proteins, such as gp96, hsp70, and others, and deliver the peptide to MHC class I at that site (42). Alternatively, internalized proteins are degraded by endosomal proteases such as cathepsin S to peptides suitable for binding MHC class I molecules (118). In the latter case, peptides different from those made by the proteasome/TPPII may be generated from a defined antigen, possibly resulting in an activation of incorrect CTLs. How antigens are able to leave the interior of apoptotic bodies is unclear. However, such antigens should not be loaded directly in recycling MHC class I molecules, but they may also leave the

endocytic pathway to enter the classical pathway of MHC class I antigen presentation, as discussed below, and be presented by new MHC class I molecules.

More recently, a hybrid form of cross-presentation has been proposed (119, 120). Bead-induced phagosomes have been suggested to directly fuse to the ER. In fact, in this model the ER membrane forms the phagosomal membrane (121). Antigens from the bead are then released by the translocon, degraded in the cytoplasm, and pumped back as peptides in the phagosome by TAP (119, 120). The MHC class I molecules in the ER–phagosome finally transport the peptide to the plasma membrane (utilizing the recycling pathway). However, the fusion of ER and phagosomes has not been directly shown by immunoelectron microscopy with ER markers, and its concept imposes a number

of cell biological problems, including the emptying of ER–calcium stores and alterations in other essential elements of the ER environment such as ATP. In addition, the physiological equivalent of a bead is fairly unclear, and fusion of the ER with bacteria-induced phagosomes has not been observed in spite of thorough analyses (unpublished observations).

An alternative mechanism is more attractive. Here, exogenous antigens enter a route also used by various bacterial toxins (122, 123). These antigens are endocytosed, move from the endosome to the Golgi (at least a small fraction of it), and then follow the retrograde transport route to enter the ER. The antigens follow the ER degradation pathway to enter the cytoplasm. Finally, the antigens are degraded by the proteasome and follow the standard MHC class I pathway involving peptidases, TAP, ERAP1, and MHC class I molecules in the ER. Cytosolic rather than exogenous antigens (unless used in a vaccination protocol) are the main source of antigens for cross-presentation, and thus, they will not follow this pathway. Antigens may be transferred directly from one cell into another one. An option is tunneling nanotubes. These are actin-driven protrusions directed toward a neighboring cell. It has been reported that these very thin structures (50–200 nm in diameter) facilitate the transfer of membrane vesicles and organelles but, remarkably, not small molecules (124). The volume transferred, however, should be very small, and excessive amounts of antigens are required to successfully prime another cell using these nanotubes.

Most recently, an alternative mechanism has been identified where gap junctions transfer antigenic peptides from the cytoplasm of an infected cell directly into the cytoplasm of its neighbor (125). The neighbor could be an innocent bystander cell (then also recognized by CTLs) but also an activated monocyte or dendritic cell. Gap junctions are small channels allowing electric (126), metabolic, and also immunological coupling of cells to mediate cytosol-to-cytosol transfer. Peptides of up to 1800 Da are allowed to pass these gap junctions (125). This pathway explains how cytosolic antigenic information can be transferred from an infected cell to antigen-presenting cells. Gap junctions are abundantly expressed and found between various antigen-presenting cells and normal tissue, including Langerhans' cells and keratinocytes, intestinal dendritic cells and surrounding cells, and dendritic cells and thymic endothelial cells (125, 127). Coupling the antigen presentation pathways of neighboring cells

through gap junctional contact, the antigen-presenting cells continuously sample antigenic information that can be transferred and exposed in lymph nodes for T-cell activation and expansion.

Lost in action, the inefficiency of antigen presentation

It is tempting to think that the system of MHC class I antigen presentation is constructed to optimally generate peptides and load them onto MHC class I molecules. In fact, the opposite is true; this pathway is very inefficient. On average, a cell presents only 20,000–50,000 MHC class I molecules with fragments from 2 billion protein copies expressed in that cell; this excludes a full representation of all proteins as peptides in MHC class I molecules. Yewdell (128) has quantified the so-called 'economics of antigen presentation'. He showed that about 2 billion proteins per cell are expressed and turned over in approximately 6 h to maintain equilibrium. Consequently, few million proteins are degraded per minute per cell, and as the proteasome will digest a protein in many peptides, approximately 100 million peptides per minute per cell are generated. However, only a few hundred MHC class I molecules are made per minute in the same cell (35, 128). Pulse-chase experiments indicate that in spite of enormous apparent peptide excess, a large fraction of the MHC class I molecules fail to acquire a peptide (129). These class I molecules will be degraded partially by the ERAD and could be considered DRiPs. MHC class I loading with peptides is thus not a saturated process. This finding suggests that the majority of peptides are lost between the site of production (the proteasome) and loading (the ER lumen).

Cells appear to have excessive peptidase activities. In fact, a peptide has on average an *in vivo* half-life of a few seconds (39). They are destroyed by a large number of peptidases with different specificities, and more than 99% of the cytosolic peptides are destroyed before they encounter TAP (39). This number probably is even higher (expected to be around 99.99 or even 99.999%) (35). The important lesson from these data and calculations is that antigens will be presented by MHC class I molecules when expressed in minimally 10,000 copies or so per cell. Below this threshold, the statistical chance of having one peptide surviving the proteolytic attacks and binding successfully an MHC class I molecule is close to zero, and such antigens are ignored by the immune system.

Some MHC class I alleles are more equal than others: locus- and allele-specific differences

MHC molecules are unique, because they are polymorphic. This quality is reflected not only in a difference in the set of peptides presented by the different MHC class I alleles but also in their biochemical behavior (Table 1). Humans express three locus products, HLA-A, HLA-B, and HLA-C. HLA-C products are usually poorly expressed and/or poorly assembled in the ER, resulting in a low cell surface expression (129–131). HLA-A and HLA-B locus products are more efficiently expressed at the cell surface. Still, these differ in their dependency for transport on the associated N-linked glycan, which is required for the interaction with the ER chaperones calnexin and calreticulin during the early stages of folding. Many HLA-B locus products assemble poorly when N-linked glycosylation is blocked by the drug tunicamycin, whereas many HLA-A locus products are not affected (129). This variation probably represents a different dependency on the support of the lectin chaperones calreticulin and calnexin, but these details are unclear. MHC alleles are intriguing. In spite of their high sequence and structural similarity, different behaviors in almost every step in antigen processing for MHC class I presentation have been observed.

Table 1. Specificity of the components of the MHC class I pathway.

	HLA-A	HLA-B	HLA-C
Expression	++	++	+/-
Assembly efficiency	+	++	-
TAP/tapasin association	+	+/-	+
N-linked glycosylation	-	++	?
Transport rate	+	++	-
Antigen presentation	+	++	-

MHC class I alleles, the proteasome, and peptidases

The proteasome is critical in the generation of peptides, and MHC class I expression is strongly reduced following proteasome inhibition (132). Still, more extensive analysis revealed that presentation by and expression of certain MHC class I alleles (including HLA-A3, A11, B27, and B35) is not influenced by proteasome inhibition (133). These MHC class I alleles differed from the other alleles tested in this study, as they bind peptides with a basic C-terminal

anchor residue. The major proteasome activity is a chymotryptic one, i.e. cleaving behind hydrophobic and aromatic amino acids (37). Proteasome inhibition likely results in the generation of larger peptide fragments that require trimming by TPPII, which can make endocleavages behind basic amino acids (43), but whose exact specificity is unclear. The endoproteolytic activities of TPPII (43), and probably also TOP and neurolysin (40), could generate 9-mer peptides with C-terminal basic amino acids and may be responsible for the allelic differences observed upon proteasome inhibition.

MHC class I alleles, TAP, and the MHC class I-loading complex

TAP translocates peptides into the ER for binding to MHC class I molecules. Only few MHC class I alleles (HLA-A2 and HLA-E molecules) bind peptides in the absence of TAP (134, 135). These peptides represent signal sequence fragments that obviously do not require TAP for entry in the ER. As the various MHC class I alleles bind different peptides, TAP should have a broad specificity. Mouse TAP prefers peptides with a hydrophobic C-terminus, whereas human TAP does not have such selectivity (70). This difference is also reflected in the peptides binding to the different MHC class I alleles. Murine MHC class I alleles invariably require a hydrophobic/aromatic C-terminal anchor residue, where human MHC class I alleles use either hydrophobic/aromatic or basic C-terminal anchor residues (acidic amino acids have not been found, as they are poorly made by the proteasome) (76). Obviously, TAP specificity co-evolved with the MHC class I alleles, and TAP specificity corresponds to the C-terminal anchor residue preference for MHC class I molecules. However, it is unclear why such species differences exist.

TAP is the center of the MHC class I-loading complex used as a docking station for assembly of MHC class I molecules. However, various HLA alleles do not interact with this complex (about half of the HLA-B alleles tested) (85). Whereas TAP and tapasin support the proper loading and stability of the associated HLA molecules (note the phenotype of the tapasin-deficient mice and cell lines (92, 136)), the other HLA class I alleles are stable, despite the lack of this support. One curious observation associated with the absence of tapasin interaction is that these MHC class I alleles are more efficiently assembled and transported, suggesting that tapasin binding slows exit from the ER (92).

In fact, this is exactly the phenotype of the tapasin-deficient mice, where the MHC class I molecules are more rapidly transported but at the cost of optimal folding (92). The HLA alleles that do not require folding and stabilization supported by the TAP–tapasin complex may have evolved to rapidly present antigenic fragments at the plasma membrane. On average, these MHC class I alleles reach the cell surface 30–90 min before the ones formed in the MHC class I-loading complex (129).

MHC class I alleles, assembly and transport

Polymorphism has evolved to present different fragments from an antigen and thus guarantee the survival of the species. Whether some MHC class I alleles are better expressed because they obtain more peptides is unclear. However, the efficiency of assembly into proper MHC class I complexes differs markedly between the different locus and allelic products. HLA-C locus products assemble inefficiently, because they are more selective in binding peptides (131). Most HLA-A locus products assemble with an efficiency of 30–70%, and most HLA-B locus products assemble almost completely into MHC class I-peptide complexes that are transported to the plasma membrane (129). As assembly rate differs, the transport rate also varies. HLA-B locus products usually reach the plasma membrane faster than HLA-A or HLA-C molecules (129). Why this is different is unclear. It is possible, however, that these biochemical differences are the result of peptide supply and timing of presentation. MHC class I allelic preferences for resistance or susceptibility to diseases could then be the result. HLA-B27, for example, is strongly linked to Bechterew disease and Reiter's syndrome, although the causative agent is unknown (137). The study of population responses to massive viral infections such as human immunodeficiency virus have revealed strong allelic preferences (HLA-B8) for successful antigen presentation and consequently an increased representation of these alleles in the population (138). Whether the overrepresentation of HLA class I alleles such as HLA-A2 (approximately 50% in the Caucasian population) or HLA-B7 (> 15–44%, depending on geographic location) is the consequence of previous epidemics is unclear but not unlikely (www.allelefreqencies.net) (139).

Dinner is served, substrate specificity in the MHC class I antigen processing and presentation pathway

The specificity of various molecules involved in peptide generation and loading of MHC class I molecules has been studied in depth to arrive at reasonable predictions of the peptides presented from a defined antigen (140, 141). Recently, a number of new components have been added to the list of proteins involved, each with their own specificities, as summarized below.

The proteasome and TPPII

The first proteolytic step in the MHC class I route is degradation of a substrate protein into peptides of varying length. This process is normally performed by the proteasome, although proteolysis does occur in cells devoid of (most) functional proteasomes. It is then carried out by other protease activities, including TPPII (43). The constitutive proteasome displays chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues), and peptidylglutamyl (cleavage after acidic residues) peptide hydrolytic activities, which accounts for most of the naturally occurring 20 amino acids (142). But there are two proteasome types, the constitutive and the immunoproteasome, which differ in three active β -subunits. Although the three inducible immunosubunits all contain N-terminal threonine residues, like their normal counterparts, their kinetic properties and substrate specificities do differ. The inducible forms showed enhanced (chymo)tryptic activities (especially after branched residues) and decreased cleavage after acidic (aspartic acid) residues (143). This apparent small change may have marked effects on antigen presentation, as shown for some viral antigens (144) and other proteasomal target proteins (19). Note that preferred generation of one epitope may be at the cost of generation of another epitope, and immunoproteasome activity may not always be favorable for antigen presentation. The constitutive proteasome generates cleavages that differ in about half of the ones from the immunoproteasome form. Although the average length of peptides generated by either form is similar (*in vitro* between 7 and 9 amino acids), the C-termini may be more favorable for (many) MHC class I, thus resulting in better peptide generation from the same number of antigens.

When both proteasomal subsets are chemically inhibited, cells usually die within 24–48 h (145). Under these conditions, some proteasome activity remains, generating larger fragments that should then be handled by other proteases. Complete inhibition of the proteasome is invariably lethal to cells (146). One

enhanced proteolytic activity in cells surviving incomplete proteasome inhibition was identified as TPPII. TPPII, a >5-MDa serine protease, possesses both tripeptidyl peptidase activity and endopeptidase activity (43, 147). TPPII removes terminal bits of 2–3 amino acids, unless it finds a N-terminal proline (148). No other selectivity for the tripeptidyl activity has been identified. The endopeptidase activity is poorly defined. The proteasome is undoubtedly the main machinery responsible for protein degradation in the nucleus and cytoplasm, but there may be life after the proteasome. Under normal circumstances, however, the proteolytic activity of TPPII is necessary during the next step of protein degradation and antigen presentation: peptide trimming (38).

TOP and other cytosolic peptidases

At least two homologous peptidases are known to handle substrates generated by TPPII and the proteasome. These are TOP and its close homolog neurolysin; both prefer substrates of 8–16 amino acids (40), while TPPII is the only activity handling peptides longer than 15 amino acids (38). Like the proteasome and TPPII, TOP and neurolysin are ubiquitously expressed (40, 149). Both enzymes show a broad substrate specificity that can be explained by the plasticity of its binding cleft. The N-terminus of the peptide substrate is docked in the peptidase groove of the enzymes. The active centers of both TOP and neurolysin are positioned at slightly different distances from the docked N-terminus (53). Consequently, neurolysin cleaves on average one or two amino acids further away from the N-terminus than TOP. The enzymes thus generate different peptides from the same substrate. Both enzymes are able to generate peptides up to 10 amino acids in length, thus generating new C-termini in MHC class I epitopes (of > 8 amino acids). Not only the proteasome but also TPPII, TOP, and neurolysin are apparently able to generate the peptide's C-terminus. The specificity for this reaction by TOP or neurolysin is not defined, but it appears not to be highly restrictive (40). The aminopeptidase LAP has a clear preference for hydrophobic residues at the N-terminus (44, 45). However, not much is known about the specificities of the other aminopeptidases and their involvement in class I peptide generation (35).

The collective activity of intracellular peptidases is unclear (except the most relevant parameter), because their relative amounts and activities are undefined. Global analysis using internally quenched peptides

introduced into living cells revealed that peptides are very rapidly (within seconds) destroyed exclusively by aminopeptidases, because N-terminally protected peptides are perfectly stable (38, 39). Systematic amino acid variations at the N-terminal 1–3 amino acid positions did not reveal any obvious difference in substrate recognition, as all peptides were degraded at rates that differed no more than a factor of 3 (38). Apparently, the heterologous pool of peptidases has sub specialization for peptide size, without dramatically favoring particular sequences over others. The longest peptides (> 15 amino acids) are handled exclusively by TPPII (38), shorter ones (8–16 amino acids) by TOP, neurolysin (40), and maybe others, while the substrates of the latter two enzymes are handled by other peptidases.

The peptide transporter TAP

All different MHC class I alleles receive peptides translocated by non-polymorphic TAP molecules, and it is therefore no surprise that TAP has a very broad selectivity. The minimal size of peptides binding to TAP is 8 amino acids, corresponding to the minimal size of MHC class I-associated peptides (70). Furthermore, peptides of 9–12 amino acids are best translocated by TAP, and usually peptides of around 9 amino acids are found associated with MHC class I molecules. More rarely, longer peptides can be found; for example, a 4000 Da corresponding to approximately 33-mer peptide associated with HLA-B27 (150). TAP can handle these peptides as well, albeit considerably less efficiently (68, 70).

TAP has more aspects in common with MHC class I molecules. For example, it requires peptides with a free N- and C-terminus (probably to dock the peptide) (56). Consequently, the amino acid proline at position 1 in the peptide is not favored (70). More extensive analysis of peptides with variant sequences revealed more subtle differences with one exception, peptides with proline at position 2 or 3 (71, 72, 77). These peptides are very poorly translocated by TAP. They are probably translocated as longer (N-terminally extended) peptides to relocate proline to other positions and subsequently trimmed in the ER to the correct size for binding MHC class I molecules.

Amino acids at other positions may have additional effects, but analysis of direct translocation using a large set of systematically varied amino acids did not reveal differences of more than a factor of 3 in efficiency (71).

Such differences may contribute to the prevalence of peptides in MHC class I molecules. However, it is unclear whether such differences are additive, and it is thus difficult to use these rules in prediction analyses of sequences different from the ones tested, although some attempts have been made (72).

The last (C-terminal) amino acid of the peptide substrate is unusual. Murine TAP and a rat TAP allele preferred only non-charged hydrophobic and aromatic amino acids. Human TAP and another rat TAP allele did not show any selectivity (within a factor of 2–3) for amino acids at this position (70, 151). Various human and murine TAP alleles did not differ in selectivity as well (152, 153). This selectivity is reflected in the peptides found in MHC class I molecules from the respective species (76). Whereas mouse class I molecules almost invariably contain hydrophobic or aromatic C-terminal anchor residues, various human class I alleles also contain peptides with basic C-terminal anchor residues. Acidic C-terminal anchor residues have not been identified, which is not the result of TAP selectively but of the proteolytic activity and selectivity of the proteasome and TPPII for cleavage after such residues.

ER aminopeptidases

The activity of ER peptidases was first monitored in 1994. This study (69) revealed a relatively slow ER peptidase activity compared to cytosol, and many peptides were actively removed from instead of degraded in the ER. It subsequently took another 8 years before the enzyme responsible for this activity was isolated (79, 93, 154). This enzyme, called ERAAP or ERAP1, was identical to an aminopeptidase isolated before, called adipocyte-derived leucine aminopeptidase, and is strongly upregulated by interferon- γ . The activity of this enzyme was already characterized. It was found to have a broad specificity for amino acids (154). This finding was further tested using a large set of peptide substrates of different length and sequence (94). No obvious sequence selectivity could be defined, although substrates were degraded at different rates, implying that some form of sequence selectivity should exist. The most obvious characteristic appears to be that ERAAP/ERAP1 is unable to handle peptides with a proline residue at position 2 (78). Such peptides should be more stable in the ER, which may explain why a proline amino acid at position 2 is used as a preferred anchor residue for various MHC class I molecules, while TAP does not translocate such

peptides (71, 72, 77). Surprisingly, ERAP1 displays a marked size selectivity. Peptides of 8 or 9 amino acids are poor substrates for ERAP1 compared with longer substrates (93, 94). This finding suggests that ERAP1 trims peptides to ideal substrates for MHC class I molecules. Indeed, ERAP1 expression is important for MHC class I expression (79, 93).

Possibly other ER aminopeptidase activities exist as well, but these have been poorly characterized. The contribution of other activities to peptide trimming for MHC class I antigen presentation is unknown. Although the exact specificity of ERAP1 is unclear, definition is important for the prediction of the presented epitope. However, these predictions should also consider the specificity of the export machinery (69, 102) and possibly that of ER chaperones such as PDI and gp96 as well (95). The relative contribution of these activities to successful antigen generation or delivery to MHC class I molecules is unclear.

MHC class I alleles

MHC class I alleles differ in many biochemical properties. Their main distinguishing feature is the difference in sequence (polymorphism) that is usually clustered in and around the MHC class I peptide-binding groove (155). Consequently, different MHC class I alleles bind different peptides. These different peptides are usually of similar length (9 amino acids), because their N- and C-termini are associating with the respective ends of the peptide-binding groove of MHC class I molecules (156).

Two sets of data revealed how MHC class I polymorphism altered the set of peptides presented to the immune system. Firstly, structural analysis showed that the polymorphic residues in the peptide-binding groove of MHC class I molecules altered the surface of the groove. In most cases, at least two obvious pockets in the bottom of the peptide-binding groove were identified in this structure (157). Secondly, pool sequencing of peptides associated with a particular MHC class I allele revealed the existence of (usually) two 'conserved' amino acids (158). These so-called anchor residues fit the pockets composed by the polymorphic amino acids and are usually located between position 2–5 and at the last position of the peptide (76). Polymorphism thus shapes the peptide repertoire associated with an MHC class I molecule. As many anchor residues in the peptide sets associated with MHC class I alleles are now defined (76), this information can be

used to predict the peptides presented from an antigen by a particular MHC class I allele. The anchor residues for most human and mouse MHC class I alleles, as defined by pool sequencing, can be found at <http://www.syfpeithi.de> (159). Peptide binding to an MHC class I allele is the most specific event in the process of antigen presentation and is expected to add most predictive power. Still, it is not perfect, simply because the other processes also contribute to the efficiency of antigen presentation.

Combining specificities for improving the prediction of presented antigenic peptides

In principle, the combined activities of the proteasome, TPPII, and other cytosolic peptidases, TAP, ERAP1, and MHC class I alleles should be sufficient

to accurately predict the peptides presented from a linear sequence. The definition of anchor residues for defined MHC class I alleles has supported such predictions, which, however, are far from accurate. The definition of the TAP selectivity in combination with the proteasomal cleavage patterns will improve such predictions (18, 160–162). The peptidase activity may be a particularly important factor, as it destroys the majority of the peptides (35), and stable peptides are thus expected to be overrepresented in MHC class I molecules. This part of the antigen presentation cascade equilibrium is only beginning to be defined. Determining the specificity (already partially done) and relative contribution (in most cases unclear) should result in accurate predictions of the peptides presented by different MHC class I alleles.

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