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MULTI-AUTHOR REVIEW

Post-proteasomal and proteasome-independent generation of MHC class I ligands

Peter van Endert

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Abstract Peptide ligands presented by MHC class I molecules are produced by intracellular proteolysis, which often involves multiple steps. Initial antigen degradation seems to rely almost invariably on the proteasome, although tripeptidyl peptidase II (TPP II) and insulin-degrading enzyme (IDE) may be able to substitute for the proteasome in rare cases. Recent evidence suggests that the net effect of cytosolic aminopeptidases is destruction of potential class I ligands, although a positive role in selected cases has been documented. This may apply particularly to the trimming of long precursors by TPP II. In contrast, trimming of ligand precursors in the endoplasmic reticulum is essential for the generation of suitable peptides and has a substantial impact on the repertoire of ligands presented. Trimming by the ER aminopeptidase (ERAP) enzymes most likely acts on free precursors and is adapted to the needs of class I molecules by way of a molecular ruler mechanism. Trimming by ERAP enzymes also occurs for cross-presented ligands, which can alternatively be processed in a special endosomal compartment by insulin-regulated aminopeptidase.

P. van Endert
Institut National de la Santé et de la Recherche Médicale, Unité 1013, Paris 75015, France

P. van Endert
Université Paris Descartes, Faculté de Médecine René Descartes, Paris 75015, France

P. van Endert (✉)
Inserm U1013, Hôpital Necker, 149 rue de Sèvres, 75743 Paris Cedex 15, France
e-mail: peter.van-endert@inserm.fr

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Introduction

Essentially all ligands presented by MHC class I molecules are derived by intracellular proteolysis [1, 2]. This is partly due to the intracellular quality control mechanisms that prevent the egress of empty or unstable class I molecules to the cell surface, thus necessitating their intracellular acquisition of peptide. The loading of a class I molecule with a suitable peptide is accomplished with the assistance of different chaperones that, together with class I molecules, constitute peptide-loading complexes, which are primarily localized to the endoplasmic reticulum (ER). Optimally loaded peptide-class I molecules that reach the cell surface are highly stable and resistant to peptide exchange. Furthermore, since the cell surface lacks proteolytic enzymes that favor the production of peptides adapted to MHC class I ligand binding sites, the responsibility for generating suitably trimmed ligands for MHC class I binding falls upon the intracellular proteases.

Among the multiple cellular proteases involved in production of MHC class I ligands, two stand out with dominant roles: cytosolic proteasome complexes with an essential role in the initial degradation of most source proteins for class I ligands (reviewed by [3] in this issue), and trimming ER aminopeptidases (ERAPs), which have been the subject of intense research in the last 5 years. This review will focus both on trimming peptidases acting downstream of the proteasome in the cytosol or in the ER, and on proteases suggested to produce ligands in a

proteasome-independent manner in the context of alternative proteolytic pathways. The emphasis will be on progress made over the last 5 years, since earlier data have been discussed in detail in previous reviews [4, 5]. The review will focus on peptides produced in the standard endogenous pathway, i.e., by proteolysis in the cytosol followed by trimming in the ER. Peptide production from cellular proteins in the secretory pathway and endolysosomal compartments is discussed by del Val in this issue [6]. Given that the proteases involved in cross-presentation of exogenous ligands by dendritic cells (DCs) are thought to be largely identical to those involved in the endogenous cytosolic pathway, I shall also discuss briefly antigen degradation in cross-presentation.

The difficult notion of proteasome independence

Following the initial demonstration of an essential role for the proteasome in MHC class I ligand production [7], it did not take long for observations interpreted as evidence for proteasome-independent pathways to emerge (reviewed in detail in [4]). Three major types of experimental evidence suggested the existence of such pathways: (1) enhanced presentation of some epitopes in the presence of proteasome inhibitors; (2) absence of effect or paradoxical effect of proteasome inhibitors on cell surface expression of some class I allomorphs such as HLA-A3 [8]; (3) relatively efficient MHC class I loading with peptides in long-term proteasome inhibitor-adapted cells [9]. Given that most proteasome subunits are essential, all of this evidence was obtained using pharmacological proteasome inhibition. However, interpretation of experimental results using proteasome inhibitors turns out to be complicated by various issues.

First, many commonly used proteasome inhibitors such as epoxomicin or MG132 primarily inhibit the chymotrypsin-like proteasome activity. Thus, rather than being inactive, "inhibited" proteasome complexes may still degrade substrates cleaving exclusively in a trypsin- and caspase-like manner, but sparing hydrophobic residues. In this scenario, production of epitopes containing internal hydrophobic residues (normally cleaved by the chymotrypsin-like activity) but ending with a basic residue (preferred by the trypsin-like activity), features applying to many HLA-A3 presented ligands, would be expected to be favored by such inhibitors. This example illustrates that apparent "proteasome independence" can reflect a shift in proteasome specificity. A similar scenario might occur even for inhibitors targeting all three catalytic proteasome subunits, since some epitopes that tend to be destroyed by internal cleavage may be produced more efficiently by proteasome complexes with reduced activity. It has become clear that both constitutive and immunoproteasome complexes can destroy epitopes. For example, in a recent study, Chapiro et al. studied three tumor epitopes and found that two of them were destroyed by immuno- and another by constitutive proteasome complexes [10]. The complexity inherent in the use of proteasome inhibitors was also demonstrated by Gavioli et al. who found that inhibitors increased presentation of some HLA-A2-restricted epitopes while reducing overall A2 expression, whereas the same inhibitors decreased presentation of some HLA-A3-restricted epitopes without affecting global A3 expression [11].

Interpretation of experiments using proteasome inhibitors is further complicated by feedback mechanisms induced by these chemicals [12]. Thus incubation of myeloma cells in the presence of the inhibitor bortezomib for 8 h resulted in strong upregulation of the α and ß proteasome subunits as well as components of the 19S cap [13]. Similarly, short-term incubation of rat vascular smooth muscle cells with lactacystin or MG-132 resulted in upregulation of 26S proteasome mRNAs, enhanced expression of the proteasome maturation protein POMP, and increased de novo biogenesis of proteasomes [14]. More recently Krüger et al. identified the transcriptional control loop counteracting proteotoxic stress caused by proteasome inhibition, which is activated by accumulation of oxidant-damaged proteins [15]. A compensatory adaptation of the ubiquitin-proteasome system also occurs in transformed cells grown for extended periods in the presence of proteasome inhibitors. Thus leukemia, myeloma, and lymphoma lines adapted to growth in the presence of bortezomib show increased expression of the catalytic ß1, ß2, and ß5 proteasome subunits and of the 11S activator [16]. Together with a reduced rate of protein synthesis, this adaptation is sufficient to maintain residual proteasome activity and avoid accumulation of poly-ubiquitinated proteins. It is likely that these adaptations explain much of the phenomena originally reported by Glas et al. and interpreted as evidence for the existence of a "backup system" able to replace the proteasome in the production of antigenic peptides [9]. Consistent with this reasoning Princiotta et al. showed that cells adapted to the inhibitors also used by Glas et al. contained active proteasome complexes [17]. In conclusion, experiments relying exclusively on proteasome inhibitors are poorly suited to providing definitive evidence for the existence of "proteasome independent" pathways of MHC class I ligands, and positive evidence is more relevant than negative evidence in this field of research.

Although the considerations discussed above suggest caution in interpreting of experiments using proteasome inhibitors, some intriguing recent data reported by Marcilla et al. indicate that some subsets of MHC class I ligands

may indeed be produced in alternative proteolytic pathways [18]. Using quantitative mass spectrometry to examine peptide ligands eluted from HLA-B27 molecules, these authors observed that about one-third of all eluted ligands were still produced in the presence of 0.15 or 2.5 μM epoxomicin. At these concentrations, the chymotrypsin-like proteasome activity was reduced by 85 and 98% and the trypsin-like by 28 and 87%, respectively, while the caspase-like activity was not affected. Given that the caspase-like activity is unlikely to carry out C-terminal cleavages to produce suitable HLA-B27 ligands (which have hydrophobic or basic C-terminals), partial proteasome inhibition cannot explain these observations. "Proteasome-independent" ligands were not different from proteasome-dependent ligands with respect to sequence motifs and flanking sequences or subcellular localization of source proteins. However, all but one of the independent ligands were derived from small (6–16.5 kDa) basic (pI $>$ 7.0) proteins, which correspond to only 6.6% of the human proteome, while the source proteins of proteasome-dependent ligands showed a normal distribution of molecular weight and pI. These results suggest that one or several proteases preferentially cleaving small basic substrates may be involved in the production of HLA-B27 ligands; however, the protease involved remains to be identified. Alternatively, residual proteasome activity may be sufficient to cleave small basic proteins but not larger proteins.

The controversial role of tripeptidyl peptidase II

The protease most studied as the candidate enzyme mediating "proteasome-independent" production of MHC class I ligands is tripeptidyl peptidase II (TPP II), a very large cytosolic protease of the subtilisin type [19]. TPP II removes tripeptides from the aminoterminus of substrates, displaying little sequence specificity, and also has low endoprotease activity with a trypsin-like specificity [20]. TPP II is a large, 6 MDa structure comprising two segmented twisted strands, each composed of 10 dimers made up of identical 138 kDa subunits [21]. The recent elucidation of its 3.2 Å structure revealed how stacking of these dimer subunits leads to the priming and sequestration of the catalytic sites into chambers [22]. In inactive dimers, the substrate binding site is occluded by a three-residue loop, the positioning of which displaces the active-site serine. Upon dimer assembly into stacks, the three-residue loop is repositioned to the roof of the catalytic chamber allowing the active-site serine to be repositioned, thus activating the protease. At the same time, a network of chambers providing multiple access pathways to the active site is formed. Assembled TPP II can be compared to a "molecular sponge" with a very high local concentration of active sites [22].

TPP II has attracted the attention of cancer biologists because of its upregulation and function in tumor cells [23]. Hong et al. reported that long-term incubation of EL-4 lymphoma cells with proteasome inhibitor induced TPP II expression, which could also be obtained by amino acid starvation. A similar phenotype was observed in some tumors displaying resistance to apoptosis due to the compromised degradation of IAP (inhibitor of apoptosis) proteins [24]. Other reports described that TPP II induces genetic instability by overriding mitotic checkpoints [25] and that TPP is required for a normal response to DNA damage, although the latter point is controversial [26, 27]. Mice with a genetic deletion of TPP II are viable but display degenerative alterations such as a decreased life span, premature immuno-hematopoietic senescence, and aggravated apoptosis [28]. Thus, the protease has important functions in cellular metabolism and apoptosis; however, the molecular targets and substrates involved remain unknown.

In the field of MHC class I antigen processing, TPP II made headlines on two occasions, each of which was followed by controversy and by the eventual conclusion that the initial data had probably been misinterpreted (reviewed in detail in [29]). First Glas et al. reported that cells adapted to growth in the presence of proteasome inhibitors upregulated a large protease activity hydrolyzing the tripeptide AAF-AMC, soon after being identified by another group as TPP II [20], and suggested that this activity compensated for the loss of proteasome activity in cellular metabolism and in production of MHC class I ligands [9]. As discussed above, it soon turned out that the cells studied retained significant residual proteasome activity [17], presumably because of both incomplete proteasome inhibition and compensatory upregulation of proteasome production and maturation [12]. Further evidence arguing against a "backup" role for TPP II was obtained by two groups examining TPP II-deficient mice. Firat and associates reported that MHC class I levels on the surface of TPP II knockout (ko) cells were increased up to 1.4-fold and that generation of the H-2K^b-restricted ovalbumin (OVA)-epitope SIINFEKL (S8L), both in the endogenous pathway and upon cross-presentation of OVA/anti-OVA immune complexes or of OVA-coated beads, was enhanced in the absence of TPP II [30]. However, TPP II ko had no effect on CD8$^+$ T cell responses to three LCMV epitopes. Kawahara et al., who studied gene-trapped mice that might have retained some residual TPP II activity, similarly observed slightly increased MHC class I expression and normal anti-LCMV responses [31]. Thus, the global effect of TPP II on the production of MHC class I ligands is negative, suggesting that TPP II destroys many potential epitopes.

The second controversy concerning TPP II was raised by a publication that studied TPP II as a cytosolic trimming peptidase [32], consistent with its specificity in vitro [19, 20]. Reits et al. reported that, in the cytosol, epitope precursors with a length of ≥15 residues were trimmed mainly by TPP II. Similar conclusions were reached by York et al. who used RNA interference to reduce TPP II expression and found that the requirement of TPP II for efficient presentation increased with the length of cytosolic precursor peptides [33]. Thus, there is consensus that TPP II has a nonredundant role as a trimming peptidase specializing in longer precursors with a maximum length of 30–40 residues [19], which cannot be trimmed by other cytosolic aminopeptidases. It was the relative importance of this function that raised disagreement. Observing that a combination of TPP II knock-down and pharmacological inhibition resulted in substantial reduction of MHC class I complex formation that could not further be enhanced by proteasome inhibition, Reits et al. concluded that the trimming function of TPP II was as essential as the antigen degrading function of the proteasome, or in other words, that all proteasome-produced epitopes had to undergo trimming by TPP II [32]. Although increased MHC class I expression by TPP II ko cells evidently argues strongly against this conclusion, it could be argued that these cells might upregulate compensatory proteases. However, results obtained with RNA interference, in which compensatory phenomena are unlikely to play a role, confirm that reduced TPP II expression has no effect on class I expression and in presentation of most epitopes [33, 34]. TPP II also does not play a role in the generation of "proteasome-independent" B27 ligands derived from small basic proteins [18]. Using both pharmacological inhibition and RNA interference, Marcilla et al. found that reduced TPP II activity had no effect on B27 expression [35] and also could not reproduce the previously reported dramatic effects in MelJuSo cells [32]. Collectively, these data indicate that long epitope precursors in the cytosol are dispensable as a source of MHC class I ligands and that TPP II, although able to trim such precursors, globally acts to destroy epitopes.

Although of very limited relevance in bulk epitope production, TPP II has been shown to produce epitopes directly in some cases. The first example of such an epitope was reported by Seifert et al. who found that an HLA-A3-restricted HIV-nef epitope, the presentation of which was insensitive to proteasome inhibition, could be produced by TPP II [36]. More recently, conflicting results were reported on proteolytic production of the "proteasome-independent" influenza nucleoprotein epitope 147–55 [37, 38]. Two groups reported that epitope presentation was inhibited by AAF-CMK, a serine protease inhibitor with high affinity but limited specificity for TPP II. However, while Wherry et al. found that butabindide, a more specific but highly unstable TPP II inhibitor, and siRNA knock-down of TPP II did not affect presentation, including in long-term proteasome inhibitor-adapted cells [38], the same approaches efficiently blocked presentation in the hands of Guil et al. [37]. More efficient use of siRNA and butabindide is one possible explanation for this discrepancy, as suggested by Guil et al. [37]. However, in vitro proteasome digestion data obtained by Wherry et al., who observed that the epitope was destroyed by fully active proteasome complexes but produced (in the form of precursors) in the presence of inhibitors, suggest an alternative scenario, namely that changed proteasome specificity in the presence of inhibitors results in increased presentation, thus explaining the apparent "proteasome independence." A similarly complex scenario may underlie the findings of Diekmann et al. who described two epitopes derived from an EBV antigen, the presentation of which was upregulated by proteasome inhibition or knockdown of the maturation factor POMP, while TPP II inhibition or knockdown reduced presentation [39]. TPP II, which, as discussed above, limits cross-presentation of OVA, may also produce some epitopes derived from internalized antigens. Schnurr et al. described that the melanoma antigen NY-ESO was presented in a proteasome-dependent manner when internalized by DCs as immune complexes but became "proteasome-independent" when formulated and internalized with Iscomatrix adjuvant [40]. Somewhat confusingly, immune complexes trafficked to lysosomes, while Iscomatrix complexes were rapidly translocated from endosomes to the cytosol. Here TPP II seemed to be involved in processing, since AAF-CMK and butabindide inhibited cross-presentation of the latter antigen form. However, it needs to be underlined that none of the studies reporting TPP II-dependent epitope production demonstrated that the epitope under study could actually be produced from the full length antigen in vitro or that TPP II was important for the degradation of the source antigen in cells. Considering the low endoproteolytic activity of TPP II [20], it is therefore likely that, even in the case of entirely TPP II-dependent epitopes, its action was preceded by other proteases producing larger fragments.

New players in cytosolic antigen processing

The very limited role played by TPP II in class I antigen processing prompted several groups to search for additional proteases that might be involved in "proteasome-independent" ligand production. These studies have very recently led to the discovery of multiple proteases that complement the proteasome or may even act fully independently in epitope production. Lopez and associates found that

caspases 5 and 10, which are activated in apoptotic cells, could produce a vaccinia virus-expressed CMV epitope [41]. In infected cells, both the proteasome and the caspases produced the epitope, so that only inhibition of both proteases blocked presentation fully. The authors suggest that caspase-mediated epitope production might enhance the immunogenicity of apoptotic cells; however, this hypothesis remains to be confirmed.

Parmentier et al., in collaboration with my laboratory, found that a proteasome-independent HLA-A1-restricted epitope from the cytosolic tumor antigen MAGE-A3 was produced by insulin-degrading enzyme (IDE; synonyms: insulinase, insulysin), a ubiquitous endoprotease [42]. Insulinase is a mainly cytosolic metalloprotease with high affinity for insulin but also cleaves a number of other substrates with low molecular weight ($\leq$12 kDa), such as glucagon, insulin-like growth factor, or atrial natriuretic peptide [43]. In contrast to most other proteases, IDE seems to recognize substrate conformation rather than sequence, cleaving primarily beta sheet structures in substrates that are prone to forming amyloids [44], including amyloid ß. Parmentier et al. demonstrated that IDE could directly produce the final epitope in vitro from a 20-mer precursor. Moreover, cytosol fractionation and depletion experiments, as well as inhibitor and RNA interference experiments showed that IDE was responsible for epitope production in tumor cells. Interestingly, examination of MAGE-A3 protein degradation in cells suggested that the antigen is degraded in two parallel cytosolic protein degradation pathways, implicating the proteasome and IDE, consistent with the fact that other MAGE-A3 epitopes are known to be proteasome-dependent [42]. This result is important because it suggests that IDE might indeed be autonomous in degrading a cytosolic antigen and thus be the only protease other than the proteasome capable of producing a class I ligand truly independently. It is conceivable that specific protein features, e.g., the folding state, could determine which of these pathways is used for degradation of a given protein molecule. Parmentier et al. did not address the broader relevance of IDE for MHC class I loading. We have observed that IDE knockdown in various human cell lines as well as IDE ko in the mouse does not affect net MHC class I peptide loading and cell surface expression (S. Culina, P. van Endert, unpublished observation).

Very recently, Kessler and associates were the first to describe a cytosolic pathway for the proteasome-independent production of epitope C-termini [45]. Starting from the observation that the proteasome cleaves with poor efficiency after lysine residues, they searched for a protease cleaving at the C-terminus of an epitope derived from the tumor antigen PRAME, where no cleavage by the proteasome occurred in in vitro digestions. In vitro digestion, inhibitor, and RNA interference studies revealed that nardilysin, a ubiquitous cytosolic endoprotease, cleaving before or between two basic residues and not previously implicated in antigen processing, was required for generation of the epitope C-terminus. Generation of the final epitope further required removal of 2–4 C-terminal amino acids by thimet oligopeptidase (TOP), another ubiquitous cytosolic endoprotease previously shown to play a major role in cytosolic peptide degradation [46], while the N-terminus was generated by the caspase-like activity of the proteasome [45]. Of note, demonstration of the role of the proteasome in generation of the PRAME epitope required the use of a specific inhibitor targeting this activity, while general proteasome inhibitors had no effect, thus providing another illustration that caution should be applied before concluding that an epitope is "proteasome-independent." Screening of an epitope database for dibasic motifs adapted to cleavage by nardilysin suggested that the protease may play a larger role in class I antigen processing, since a substantial percentage of epitopes presented by alleles such as HLA-B27, B35, HLA-A3, and A68 carry such motifs either at the N- or C-terminus. Consistent with this hypothesis, the authors obtained data suggesting that presentation of an EBNA3C epitope required N-terminal cleavage by nardilysin and presentation of an HLA-A2-restricted MART-1 epitope required C-terminal trimming by TOP. However, analysis of HLA class I expression by cells with increased or reduced nardilysin or TOP expression suggested that both proteases have a net destructive effect for most alleles including HLA-A2 and A3, while the expression of HLA-B27 and B35 was slightly decreased after knockdown. In conclusion, Kessler et al. revealed the existence of a cytosolic pathway in which proteasome-produced fragments and precursor peptides are processed by nardilysin and/or TOP to produce epitope C-terminals and/or N-terminals. Although this pathway clearly has the potential to produce a significant number of epitopes presented by selected class I allomorphs, the small and predominantly negative effects of knockdown on the bulk expression of alleles such as HLA-A3 and B27 suggest that this pathway alone is unlikely to underlie the reported relative "proteasome independence" of these and other alleles [4, 18].

Cytosolic aminopeptidases

Although the precise percentage of HLA class I ligands derived from N-terminally extended precursors produced by the proteasome is impossible to establish, it is likely that a major proportion of ligands are produced in this form. To "cope with this imperfection" [47], cells can resort to various aminopeptidases localized in the cytosol and in the

ER. It has previously been suggested that many of these peptidases participate in the highly efficient and rapid degradation of free peptides in the cytosol, in which TOP plays an important role [46, 48]. A role for cytosolic aminopeptidases in trimming MHC class I ligands was suggested by several authors studying presentation of defined $CD8^+$ T cell epitopes (reviewed in [4]). Prompted by these observations, the group of K. Rock has systematically knocked out most of these peptidases, namely leucine aminopeptidase (LAP), bleomycin hydrolase (BH), puromycin-sensitive aminopeptidase (PSA), and TPP II [31, 49–51]. Collectively, these studies come to the conclusion that cytosolic aminopeptidases either have no or a net negative effect on MHC class I ligand production.

As discussed above, evidence obtained by several laboratories demonstrated that TPP II specializes in trimming long precursors, although its net effect is clearly negative [30–33]. Induction of expression by interferon-γ, a hallmark of many proteins involved in antigen processing, initially suggested an important role for LAP [52]. However, LAP-deficient mice displayed normal responses to seven viral epitopes, while trimming and presentation of extended peptides and of full-length antigen, as well as the half-life of peptides in the cytosol, were identical to wild type (wt) cells, whether cells were treated with interferon-γ or not [49]. LAP deficiency also did not increase class I expression, in contrast to deficiency for other peptidases (see below). The physiologic role of its interferon-γ induction remains unclear. Similarly, BH-deficient mice display normal expression of class I molecules, normal presentation of full length antigens and extended epitope precursors, and unchanged $CD8^+$ T cell responses to eight different epitopes from three viruses [51]. The response to one LCMV epitope was increased, suggesting that BH can also destroy epitopes. Mice deficient for PSA, a highly abundant house-keeping peptidase with a role in degradation of poly-glutamine sequences [53], also mounted normal $CD8^+$ T cell responses to nine different epitopes and displayed normal peptide degradation in the cytosol and presentation of precursor peptides. Interestingly, MHC class I expression on DCs of PSA ko mice was increased by 25%, while no difference was observed for B or T lymphocytes and for epithelial fibroblasts [50]. It is unclear why PSA limits antigen presentation in DCs but not other cell types. Although examination of PSA and BH ko mice did not provide evidence for an important role for these enzymes in antigen processing, Kim et al. observed allele-specific negative and positive effects on HLA class I expression using RNA interference [54].

A common feature of all cytosolic and ER aminopeptidases mentioned so far is their inability to cleave before or after a proline residue. This fact has been suggested to be one of the reasons for the relatively high number of MHC class I alleles using proline as an anchor in position N+1 [55]. Recently, Geiss-Friedlander et al. were the first to show that peptides carrying N-terminal proline residues can undergo trimming and destruction in the cytosol [56]. These authors showed that dipeptidyl peptidase 9 (DPP9), a relatively abundant cytosolic enzyme removing Xaa-Pro dipeptides from the N-terminus of peptides and proteins, is the main activity in HeLa cell lysates degrading the substrate Gly-Pro-AMC. DPP9 also accepts Lys, Trp, and Val in substrate position 1, whereas Asp is fairly resistant to cleavage. Knockdown of DPP9 increased presentation of the tumor epitope $RU1_{34–42}$, suggesting that the enzyme can destroy epitopes and is rate limiting for cytosolic degradation of Xaa-Pro peptides [56].

In summary, the global effect of cytosolic aminopeptidases is characterized both by redundancy and by a more or less manifest trend to epitope destruction, consistent with the short half-life of peptides microinjected into the cytosol [48]. Thus, one might say that, for a candidate epitope produced by the proteasome, it is most imperative to escape rapidly from the cytosol, rather than getting trimmed to its final, MHC-adapted size in it. In fact there is little "need" for final trimming in the cytosol, given that the TAP pumps transport extended peptides very efficiently [57] and that the ER is equipped with (a) peptidase(s) dedicated to precursor trimming [47].

Endoplasmic reticulum aminopeptidases

Relative to the cytosol, the ER contains little proteolytic activity. Next to signal peptidase and signal peptide peptidase, ER-resident peptidases are limited to a single aminopeptidase, ERAP1 or ERAAP, in the mouse and to two highly related enzymes, ERAP1 and ERAP2, in humans. Carboxypeptidase activity is absent from the ER. The essential role of ERAP1/ERAAP in the trimming of MHC class I ligands was discovered 9 years ago [58, 59] and that of ERAP2 3 years later [60]. The ERAP enzymes belong to the oxytocinase subfamily of M1 zinc-binding metalloaminopeptidases characterized by a GAMEN motif and a $HEXXH(X_{18})E$ catalytic site motif (reviewed in [61]). In humans, this family has three members, oxytocinase [synonym: P-LAP; identical to insulin-regulated aminopeptidase (IRAP)] and the two ERAP enzymes. ERAP1 is also known as ARTS-1, PILS, and A-LAP, while ERAP2 has also been termed L-RAP. However, only the term ERAP has been approved by the HUGO Gene Nomenclature Committee (personal communication by Dr. E. Bruford, HGNC) and should be used.

The expression of human and murine ERAP enzymes is upregulated by interferon-γ, while that of IRAP is not [58–60, 62]. The human enzymes are also induced by type I

interferon in hepatitis C virus-infected hepatocytes [63]. Regulation of basic ERAP2 expression implicates interferon regulatory factor (IRF) 2, while its interferon-γ-induced upregulation involves IRF-1 [64]. In neuroblastoma cells, NF-κB regulates ERAP1/2 expression together with that of MHC class I molecules [65].

Prior to the identification of its role in MHC class I antigen processing, the finding that ERAP1 inactivates angiotensin II and III and converts kallikrein to bradykinin led to the suggestion that it plays a role in the regulation of blood pressure (reviewed in [61]). Consistent with this, a polymorphism (Lys528Arg) more recently linked to ankylosing spondylitis was shown to be associated with high blood pressure [61]. Workers in the group of S. Levine suggested that ERAP1 mediates shedding of the ectodomains of three different cytokine receptors by binding to them. Binding of catalytically active ERAP1 promoted the shedding of IL-6Rα and IL-1RII, while catalytically inactive ERAP1 was sufficient to promote shedding of the TNFR1 [66–68]. The underlying mechanism has not been elucidated. Although these data remain to be confirmed by a second group, they suggest that ERAP1 may play a role in inflammatory responses, a hypothesis of interest in light of ERAP1's association with ankylosing spondylitis (see below).

The expression and tissue distribution of the ERAP enzymes was examined by Fruci and coworkers. In one study, these authors found that all 14 EBV-transformed B cell lines and 35 tumor lines examined expressed one or both of the enzymes; however, expression of the enzymes was coordinated only in the B lymphocyte lines [69]. Cell surface expression of HLA class I molecules correlated with ERAP1 but not ERAP2 levels, suggesting that ERAP1 has a larger impact on the global ligand supply to class I molecules. Both the broader substrate specificity of ERAP1 and the higher prevalence of N- and C-terminal residues able to be cleaved by it rather than by ERAP2 (see below) could explain the dominant role of this enzyme. In a subsequent study, Fruci et al. investigated ERAP1 and 2 expression in 38 normal tissue and cell types as well as in malignant tissue counterparts [70]. When the enzymes were expressed in normal tissues, it was always in the epithelial component. Eleven of 38 normal tissues were double positive and 14 double negative, with the rest showing discordant expression. Among tumor samples, only 21 and 33% expressed ERAP1 or ERAP2, respectively. These studies suggest that frequent loss of expression of ERAP enzymes by tumors may contribute to escape from immune surveillance. In line with this, Andrés et al. recently reported evidence supporting a global role of ERAP2 in MHC class I expression by lymphocytes. These authors described that a polymorphism inducing destruction of ERAP2 mRNA by nonsense-mediated decay and low to undetectable ERAP2 protein levels was associated with slightly reduced MHC class I expression by B lymphocytes [71]. ERAP1 is also expressed in HLA-class Ia negative human trophoblasts where it has been suggested to regulate surface expression of HLA-G molecules [72].

ERAP specificity and mode of action

Among the three murine and human ERAP enzymes, only human ERAP1 has been studied in detail with respect to its specificity. However, it is likely that ERAAP, its murine analog, displays similar specificity. Chang et al. reported that peptides with a length of 9–16 residues were optimal substrates for ERAP1 [73], a feature perfectly adapted to the identical length preference of human TAP transporters [57]. Shorter peptides were found to possess dramatically lower affinity, prompting the authors to propose that ERAP1 acts as a molecular ruler [73]. According to this model, ERAP1 is uniquely adapted to the production of final MHC class I ligands, most of which have a length of 8–10 residues. However, a review of published data indicates that ERAP1 trimming does not strictly follow a molecular ruler mode. The presentation of some epitopes is clearly increased in the absence of ERAAP, consistent with in vitro data showing that the enzyme can destroy various epitopes [55, 59, 74]. A recent study reported that ERAP1 destroyed four out of seven epitopes tested [75], consistent with our data showing that ERAP1 and ERAP2 frequently produce 7- or even 6-mers from longer precursors (M. Weimershaus, A. Iversen, S. Tenzer, P. van Endert, unpublished). Nevertheless, published data agree that at least a very substantial proportion of MHC class I ligands are directly produced by ERAP and protected from destruction, lending support to the molecular ruler model. The fact that the efficiency of trimming by ERAP1 varies according to the residues at both the N- and C-termini is also consistent with the model, as it is implies that ERAP1 interacts with both substrate terminals. A very recent structural and biochemical analysis of ERAP1 provided evidence for a regulatory site situated close to the catalytic site that activates substrate hydrolysis. Nguyen et al. suggested a mechanism for ERAP1's length-dependent trimming activity, whereby binding of long but not short substrates to the regulatory site induces a large conformational change that leads to the reorientation of a key catalytic residue towards the active site [76].

ERAP1 shows a strong preference for large hydrophobic residues such as Leu, Met, Phe, and Tyr at both the N- and C-termini of substrates, while substrates carrying charged residues in these positions are trimmed poorly (Arg, Lys) or very poorly (Glu, Asp) [73, 77]. Other residues such as Val, Asn, Gly, and Thr are also trimmed

with low efficacy [78]. The ERAP1 residue 181 plays a decisive role in this specificity since the substitution of the native Gln by Asp or Glu creates an enzyme preferring basic residues [79]. Like all other M1 family aminopeptidases, ERAP1 does not cleave before or after Pro. Cleavage by ERAP1 is also influenced by the internal substrate sequence, as shown by Evnouchidou et al. [77]. In 9- or 10-mer substrates, positions two, five, and seven were most important and showed inhibitory effects for negatively charged residues, while hydrophobic and positively charged residues promoted trimming. In humans, poor trimming of substrates with a basic C-terminus creates a problem for loading various HLA class I allomorphs such as A3, A68, or B27, which frequently present peptides containing a basic amino acid at the C-terminus. The requirement for trimming such peptides may explain why humans express a second enzyme closely related to ERAP1 but with different specificity, while mice, which do not express class I molecules preferring ligands with basic C-terminals, do not. Human ERAP2 displays strong preference for substrates with N-terminal basic residues and processes substrates with basic C-terminals efficiently [60, 80]. Human ERAP1 and ERAP2 can associate to form dimeric complexes, which display increased efficiency in removing longer extensions [60]. It remains to be seen whether dimer formation affects enzyme activity and/or specificity. The substrate specificity of ERAP2 has not been analyzed in detail. Comparing cleavage of 9-, 8-, and 5-mer versions of an epitope by ERAP1 and ERAP2, Chang et al. observed that ERAP1 cleaved only the 9-mer while ERAP2 cleaved both of the longer peptides but not the short one [73]. The authors concluded that ERAP2 does not show a length preference; however considering that this conclusion was based on a single substrate and that cleavage of 8-mers by ERAP2 is likely to differ according to substrate sequence as also shown for ERAP1 [74, 75], this conclusion seems premature. In summary, the substrate specificities of human and murine ERAP enzymes are very well adapted both to those of the TAP transporters and to the ligand binding requirements of MHC class I molecules. In the mouse, this is achieved by a single enzyme, while two enzymes are required to cover the broader spectrum of ligands presented by human class I molecules.

The setting in which ERAP trims peptides has been a matter of discussion. Two mutually nonexclusive models have been proposed: the molecular ruler model (see above) and the template model. The latter was inspired by a paper by Falk et al. published in 1990 that showed that the cellular peptide composition is governed by MHC class I molecules [81]. One of the models proposed by these authors postulated that peptides were trimmed after binding to MHC class I molecules ("the template model"), while another explained the phenomenon by protection of peptides bound to class I molecules. In support of the template model, Kanaseki et al. found that epitope precursor peptides protected from trimming by a Pro residue in position 2 could be precipitated bound to class I molecules [82]. Consistent with the original study by Falk et al., these authors also demonstrated that the final class I ligand was generated within cells and in vitro only in the presence of the appropriate class I molecules. In an earlier study, we had found evidence arguing against the template model. Peptidases in B lymphocyte microsomes trimmed precursors of HLA-A2 ligands with equally high efficiency regardless of whether these microsomes contained the appropriate class I molecules [83]. More recently, Infants and colleagues observed that a 15-mer, which was efficiently trimmed by ERAAP in its free form, could not be trimmed when bound to the murine class I molecule L^d [84]. Although the latter finding argues at first sight against the template model, it is conceivable that trimming of class I-associated precursors requires a specific "open" class I conformation that would depend upon the presence of the chaperones constituting the peptide loading complexes found in the ER, and therefore lacking in in vitro digestions. The fact that ERAP has so far not been reported to associate with ER peptide-loading complexes further weakens the template model.

Evidence against the template model also derives from structural ERAP1 analysis [76]. ERAP1 is organized into four domains. Domain 2 contains the catalytic site that is capped off by domain 1, which also binds the substrate N-terminus. Domain 4 forms a large concave bowl facing the active site. The active site is sequestered such that an MHC class I-bound precursor peptide would have to protrude from the class I peptide-binding site by more than six residues to be trimmed. Thus, the template model seems incompatible with the ERAP1 structure. Comparison of the structure of bestatin-bound ERAP1 in an open conformation obtained by Nguyen et al. (PDB accession: 3MDJ) with a second structure of ERAP1 in a closed conformation (PDB accession: 2XDT) reveals that ERAP1 undergoes a large conformational change upon closing, possibly triggered by peptide binding to the regulatory site (see above). Importantly, closed ERAP1 retains a large cavity sufficient to accommodate a 12- to 14-mer peptide [76]. In conclusion, current evidence suggests that class I molecules, rather than serving as templates during trimming, protect trimming products from degradation and that optimal coordination of trimming with the needs of class I molecules is achieved through a molecular ruler mechanism based on peptide interaction with a regulatory site within ERAP1.

Impact of ERAP in MHC class I antigen processing in the mouse model

The substantial impact of ERAP in MHC class I antigen processing became evident when gene-targeted mice were analyzed. Four groups independently produced ERAAP-deficient mice on an H-2^b background [74, 85–87]. All authors observed that expression of the K^b and D^b class I molecules was reduced by 20–40%. Hammer et al. also examined expression of L^d, an allomorph binding peptides with a Pro residue in position 2, which was reduced by 70% [86]. Since murine TAP transporters have low affinity for Xaa-Pro peptides [88], most peptides presented by this molecule are expected to enter the ER as N-terminally extended precursors, explaining the dramatic effect of ERAAP ko on this allomorph. Class I expression levels on heterozygous cells were intermediate, suggesting that ERAAP trimming is rate limiting for MHC class I peptide loading [87]. Although the biosynthetic maturation of class I molecules was normal, cell surface molecules decayed more rapidly as a result of loading with suboptimal peptides [86, 87].

Somewhat discordant results were obtained when antigen-specific $CD8^+$ T cell responses were examined. Although most authors observed compromised presentation of the OVA epitope S8L, the in vivo response to vaccinia-virus encoded OVA was normal [85]. Consistent with earlier results obtained using pharmacological ERAAP inhibition [59], $CD8^+$ T cell responses to viral and minor histocompatibility epitopes showed a mixed pattern ranging from negative to neutral or positive effects. This not withstanding, clearance of LCMV or influenza virus in vivo was normal [85]. Two papers also described evidence for an altered T cell repertoire, suggesting that precursor trimming is required for normal thymic $CD8^+$ T cell selection [86, 87]. ERAAP deficiency also compromised cross-presentation of particle-associated OVA or OVA/anti-OVA immune complexes [85, 87], an expected result given that the ER proteins involved in endogenous class I presentation are known to be involved in cross-presentation [89]. Collectively, these results demonstrate that ERAP has a profound and globally positive effect on the peptide repertoire presented by MHC class I molecules, although it can also act as a destructive force.

The important role of the ERAP enzymes in class I antigen processing suggested that ERAP deficiency and ERAP polymorphisms could have a biological impact in infectious diseases and autoimmunity. A unique example of the possible impact of ERAAP was described by Blanchard et al. [90]. It was known that H-$2L^d$-restricted $CD8^+$ T cell responses are important in the resistance of Balb/c mice to infection by the intracellular parasite *Toxoplasma gondii*. Blanchard et al. found that $CD8^+$ T cells recognizing a single epitope derived from the parasite protein Gra6 played an essential protective role in *T. gondii*-infected mice. Because this epitope was L^d-restricted, its generation was ERAAP-dependent, and ERAAP ko mice succumbed more easily to *T. gondii* infection. In this model both the fact that parasites, which reside in an intracellular vacuole, expose little antigenic material and that L^d-restricted epitopes are strongly dependent on trimming contributed to the dramatic impact of ERAAP deficiency [90].

To characterize the impact of ERAAP deficiency on the peptide repertoire presented by MHC class I molecules, Hammer et al. immunized wt mice with ERAAP ko splenocytes and vice versa [91] and observed strong $CD8^+$ T cell responses, similar to those elicited by MHC-mismatched antigen-presenting cells. Thus, ERAAP-deficient cells presented novel immunogenic peptide-MHC complexes, which were also formed by wt cells in the presence of ERAAP inhibitors. Immunization with ERAAP ko splenocytes also triggered production of pMHC-specific antibodies, suggesting that the novel complexes are structurally unique [91]. The nature of these peptides was characterized biochemically by Blanchard et al. [92]. Not surprisingly, MHC class I molecules of ERAAP ko cells presented a higher proportion of long peptides; 15% of K^b-presented peptides were 10-mers and 57% of D^b-presented peptides were 10-, 11-, or 12-mers. Sequencing of a large number of peptides revealed that the additional residues could be placed to both sides of the internal anchor residue, presumably resulting in peptides bulging in the N- or C-terminal moiety. However, the C-terminal anchor was strongly conserved. Analysis of $CD8^+$ T cell responses to a model epitope showed that, in ERAAP ko mice, responses to the canonical 9-mer epitope were substituted by responses to its N-terminally extended 10-mer variant [92]. This flexibility in T cell responses to epitope variants is presumably responsible for the limited effect of ERAAP deficiency in anti-viral responses in vivo [85].

Impact of ERAP in human immune responses to viruses and tumors, and in autoimmunity

In humans, ERAP deficiency has not been described so far. However, there is evidence that the selectivity and/or specificity of ERAP trimming not only affects human $CD8^+$ T cell responses against viruses and tumors, but also plays a role in susceptibility to autoimmune diseases. First anecdotal evidence was reported by Draenert et al. who suggested that an Ala to Pro substitution of the residue preceding an HLA-B57-restricted T cell epitope from HIV-gag contributed to viral escape from the immune response [93]. Indirect statistical evidence for a role of ERAP

trimming in anti-HIV responses was reported by Tenzer et al., who found that the efficiency of intracellular antigen processing, including ERAP1/2 trimming, was an important parameter in immunodominance of $CD8^+$ T cell epitopes [94]. Additional evidence for an impact of ERAP in anti-HIV responses derives from a genetic study by Cagliani et al. [95]. According to these authors, ERAP1 and ERAP2 lie in separate linkage disequilibrium blocks, so that the association of each enzyme with a condition can be studied independently. The study focused on one single nucleotide polymorphism (SNP) in each gene, with linkage to the Lys528Arg substitution in ERAP1, and to the Asn392Lys substitution in ERAP2, respectively. In a comparison between an HIV-exposed seronegative cohort and a control population, homozygosity for Lys392 in ERAP2 protected significantly from HIV infection [95]. Collectively, these results indicate that ERAP trimming has an impact in protection from HIV infection and in the immunodominance of $CD8^+$ T cell epitopes from HIV proteins. ERAP1 has also been reported to be associated with cervical cancer. Mehta et al. used immunohistology to study expression of antigen-processing proteins (TAP1 and 2, LPM2 and 7, tapasin, calreticulin, calnexin, ERAP1) in cervical tumors and found that downregulation of LMP7, TAP1 and ERAP1 was associated with decreased overall survival and that ERAP1 downregulation was an independent predictor for lower overall and disease-free survival [96]. In parallel genetic studies, the same group observed that ERAP1 was associated with an increased risk of cervical cancer and with accelerated tumor growth and lower survival [97, 98]. While ERAP2 was not examined in these studies, another recent study suggested that ERAP2 is associated with pre-eclampsia [99].

Both human trimming peptidases have been reported to affect susceptibility to autoimmune diseases. A genome-wide association study (GWAS) using >14,000 non-synonymous SNPs revealed a highly significant association of ERAP1 with ankylosing spondylitis, an autoimmune disease strongly associated with HLA-B27 [100]. In a North American population sample, ERAP1 polymorphism contributed as much as 26% to the genetic risk of disease. Additional GWAS and smaller studies as well as family studies confirmed this association in several ethnic groups [101–103] and identified an ERAP1/ERAP2 haplotype potentially associated with the disease [104]. Among the coding sequence polymorphisms associated with disease susceptibility, the substitution Lys528Arg occupies a prominent place. Interestingly, this substitution, which is also associated with essential hypertension as mentioned above, had previously been shown to have a striking effect on cleavage of the artificial substrate Leu-NA and to reduce cleavage of two peptide substrates by 70% [105]. In the ERAP1 structure, residue 528, as well as other disease-associated substitutions (Arg127Pro, Asp575Asn, Val674Ile), localizes to domain junctions and therefore could indirectly affect specificity or enzymatic activity by altering the conformational change between open and closed forms [76]. Other variants are either located close to the active site (Met349Val) or in the cavity accommodating the substrate (Arg725Gln, Gln730Glu) and could affect enzyme activity or specificity more directly. ERAP1 also confers susceptibility to psoriasis, an autoimmune disease previously known to be linked to HLA-C, specifically to the allelic variant Cw*0602. GWAS analysis of >2,000 patients and >5,000 controls revealed a strong interaction between HLA-C and ERAP1, such that ERAP1 polymorphism increased disease risk exclusively in individuals carrying risk-associated HLA-C alleles [106]. While it cannot be ruled out that the association between ERAP1 and ankylosing spondylitis may relate to the putative role of ERAP1 in the peptide shedding of cytokine receptors (see above), the strong interaction between HLA-C and ERAP1 in the case of psoriasis directly implicates the function of ERAP as a trimming enzyme in autoimmunity. A very recent study reported that ERAP2 is linked to Crohn's disease, a finding suggesting a role of $CD8^+$ T cells in this condition [107].

Bulk peptidase activities in the cytosol and the endoplasmic reticulum

Several groups examined bulk trimming activity in the cytosol and the ER. These studies provided additional insight on the relative impact of cytosolic and ER-luminal peptide trimming on epitope selection. Using a bioinformatics approach, Schatz et al. wondered whether the nature of the amino acids preceding the N-terminus of known epitopes showed evidence of selection and found that the frequency of amino acids in positions N-1–N-5 deviated significantly from expected random values, constituting an "N-terminal processing motif" [108]. This motif reflected the sequence preferences of the proteasome and the TAP transporters, consistent with the notion that many epitopes are produced as N-terminally extended precursors by the proteasome and transported by TAP. However, some amino acids such as Trp, Val, and Ile were underrepresented in positions N-1 and N-2 despite being preferred by the proteasome and TAP, suggesting that the specificity of the trimming activities also contributed to the processing motif. Analysis of the bulk trimming activity of ER and cytosol extracts showed some significant differences. Thus Pro and Trp were removed by cytosolic peptidases, while Gly, Leu, and Met were removed more efficiently by ER peptidases. A comparison between the bioinformatically derived processing motif and global ER and cytosol trimming activities revealed that the experimentally observed

ER trimming specificity strongly correlated with the predicted amino acids of motif positions N-1–N-3, while the cytosolic trimming specificity was associated with motif positions "further upstream." This suggests that most precursors are transported into the ER with extensions of 1–3 residues, while initial trimming of longer precursors occurs in the cytosol [108].

Hearn et al. examined the specificity of ER trimming in cells expressing ERAP1 but not ERAP2 and found that the observed preferences corresponded to the ERAP1 specificity profile observed in in vitro digestions [78]. A statistical analysis of the sequences preceding the N-termini of database epitopes globally confirmed the "N-terminal processing motif" proposed by Schatz et al. [108] for positions N-1–N-3. Notably, basic residues were not underrepresented in N-terminal extensions even though they could not be trimmed by ERAP1. This suggests that basic residues are either removed in the cytosol or trimmed in the ER, most likely by ERAP2 that has the appropriate specificity [78]. A subsequent study by the same authors also concluded that the ER and cytosol displayed significantly different trimming specificities and that a large proportion of presentation from cytosolic precursors is due to an ERAP1-independent component. Hearn et al. proposed that initial trimming in the cytosol is frequently followed by ER trimming, consistent with the proposition by Schatz et al. [108, 109].

Endosomal trimming of cross-presented MHC class I ligands

The proteases discussed above play a role not only in degradation of endogenous antigens synthesized in the cytosol but also in that of antigens internalized by professional antigen-presenting cells, especially DCs, through phagocytosis, receptor-mediated endocytosis, or pinocytosis. Internalized antigens first encounter endosomal and lysosomal proteases. Some antigens and antigen formulations, e.g., antigens coated on lactic acid beads, can be degraded by these proteases in a productive manner such that their degradation products can be loaded on recycling class I molecules [110, 111]. Cathepsin S may play a particularly important role in this TAP-independent pathway [112]. However, efficient cross-presentation of most internalized antigens appears to depend on escape from the late endosomal/lysosomal pathway so as to avoid degradation by highly active acid proteases. In DCs, this escape is favored by the delayed acidification of phagosomes [113, 114]. Nevertheless, limited degradation by acid proteases is required for cross-presentation of some antigens, possibly as a prerequisite for their translocation from phagosomes to the cytosol [115].

Once internalized antigens have been translocated to the cytosol, they are exposed to the complete set of proteases also processing endogenous antigens along the cytosol-to-ER-to-membrane pathway. However, it appears that, rather than being loaded on class I molecules in the perinuclear ER, peptides produced by cytosolic degradation of internalized antigens can be loaded on class I molecules in phagosomes and endosomes [116, 117]. Although this model is controversial and many details remain unclear (e.g., the source of the class I molecules involved has not been determined), this pathway presumably involves delivery of ER components including TAP to the vesicles.

Studying aminopeptidases involved in MHC class I antigen processing, my laboratory identified an exclusive role for IRAP in cross-presentation. IRAP is closely related to the two ER enzymes but possesses a 110-residue aminoterminal extension, which mediates its localization to a regulated endosomal storage compartment [118]. Proteins residing in this compartment, such as the Glut4 glucose transporter in adipocytes, are rapidly transported to the cell surface upon signaling through cell-specific receptors such as insulin receptors in adipocytes or IgE receptors in mast cells [118, 119]. In DCs, IRAP resides in a specific endosomal compartment where it colocalizes and associates with endocytosed MHC class I molecules [62]. IRAP is rapidly recruited to phagosomes while ERAP is not. Cross-presentation of particulate and soluble, receptor-targeted antigens by IRAP-deficient DCs is compromised in vitro and in vivo though not abolished. ERAP deficiency similarly results in reduced but not abolished cross-presentation [62, 85, 87]. Collectively, these observations suggest that cross-presented MHC class I ligands can undergo final N-terminal trimming in several parallel pathways. Among these, the IRAP-dependent pathway almost certainly involves trimming in an endosomal or phagosomal compartment, while the ERAP-dependent pathway may involve the perinuclear ER and/or endosomal or phagosomal compartments [62].

The specificity of IRAP has been shown by several teams to be compatible with a role as a trimming enzyme. Matsumoto et al. found IRAP to display broad substrate specificity, including cleavage of N-terminal hydrophobic and basic residues situated next to cysteines with intramolecular disulfide bonds, which are present in hallmark substrates such as oxytocin and vasopressin [120]. IRAP also was able to trim a 15-mer to the 9-mer final epitope with the same efficiency as ERAP1 and ERAP2 combined [62]. Georgiadou et al. undertook a more comprehensive analysis of IRAP specificity and found that IRAP has equal or higher activity toward peptide antigenic ligand precursors than ERAP1 [75]. Interestingly, IRAP has a broader pH optimum (70% activity at pH 6 or 8) than ERAP1 (30% activity), rendering the enzyme more suitable for trimming

in an endosomal environment. When trimming of seven 10-mer precursors was studied, both enzymes produced the final ligand in some but not all cases (six for ERAP, four for IRAP) and at similar rates and both were able to destroy ligands in four out of seven cases. The authors concluded that the specificities of IRAP and ERAP, although somewhat different, are both adapted to trimming of MHC class I ligands [75].

Outlook

With the recent discovery and characterization of the ERAP enzymes following on that of the cytosolic aminopeptidases, it seems likely that the major players in post-proteasomal antigen processing in the standard endogenous pathway have now been mapped. Indeed, analysis of bulk cytosolic and ER peptidase activities suggested that most or all observed activities can be accounted for by known enzymes [77, 105, 106]. However, the impact of trimming enzymes in immunity to infectious pathogens, in transformed cells, and on self-determinants is only starting to be examined. Elucidating the association of ERAP1 with autoimmune diseases such as ankylosing spondylitis and psoriasis, and possibly other pathologies, will be of particular interest and may, in the case of the former disease, be instrumental in answering the long-standing question whether HLA-B27 acts as a standard antigen-presenting molecule or in another manner.

The recent identification of the roles of IDE and nardilysin in cytosolic antigen processing suggests that the issue of "proteasome-independent" pathways, after several years of stagnation and disappointment, still has life in it. The general impact of these new players remains to be determined. Furthermore, the relative "proteasome-independence" of some MHC class I allomorphs or groups of class I ligands (e.g., B27 ligands derived from small basic proteins) remains somewhat mysterious and cannot be explained by the new discoveries, suggesting that additional players and mechanisms may remain out there. Thus, post-proteasomal and proteasome-independent generation of MHC class I ligands may still have some surprises in store.

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