A New Role for Zn(II) Aminopeptidases: Antigenic Peptide Generation and Destruction

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Abstract: During the last few years a novel role for previously known Zn(II) aminopeptidases has emerged, attracting a great deal of scientific interest to these molecules. Aminopeptidases appear now to play a key role in the last, yet crucial, proteolytic steps that generate small peptides for presentation onto MHC class I molecules so that the mature MHC-peptide complexes can be recognized by cytotoxic T-lymphocytes. In that context, ER aminopeptidases have been shown to strongly affect the adaptive immune response. ER aminopeptidase 1 (ERAP1) has been demonstrated to be a critical determinant of the immune response by generating mature antigenic epitopes from peptide precursors that arrive into the ER originating primarily from intracellular proteins degraded by the proteasome. At least one more related aminopeptidase, renamed ERAP2, appears to have important yet distinct roles in antigenic peptide generation. This review discusses recent findings that help to unravel the role of ER aminopeptidases in the immune response as well as the molecular properties that underlie this role. Determining the exact role and mechanism of action of these aminopeptidases will potentially provide tools for the pharmaceutical manipulation of the immune response on a subtle and qualitative level leading to novel therapeutic opportunities for the treatments of diseases ranging from autoimmunity to cancer.

ANTIGENIC PEPTIDES PLAY THE CENTRAL ROLE IN THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune response has the difficult task of differentiating between healthy and abnormal cells so that it can proceed to eradicate the latter. Central to this task are cytotoxic T-lymphocytes (CTLs) a class of white-blood cells that 'screen' other cells for signs of infection or malignant transformation. The primary molecular interaction that is responsible for the recognition of abnormal cells by CTLs is the binding of a CTL surface receptor called T-cell receptor (TCR) to surface receptors that are expressed on all cells and belong to the Major Histocompatibility Complex I (MHCI molecules) [1, 2]. MHCI molecules are heterodimers, consisting of a large α-chain and a smaller β-chain (β-microglobulin) that come together to form an extracellular domain with a large binding groove facing towards the exterior of the cell [3]. This binding groove is occupied by small peptides, usually 8-9 amino acids long that are derived from intracellular proteins [4, 5]. The peptide is bound in such conformation that some of its side chains are buried in binding clefts within the MHCI and some extend outwards giving the MHCI-peptide complex unique structural features depending on the sequence of the bound peptide [6]. Structural features arising from the MHCI molecule itself and the bound peptide are recognized by the TCR [7]. Successful recognition leads to further molecular interactions between surface receptors of the two cells and to the formation of a large interface between the two cells, called the immunological synapse [8-12]. A cascade of molecular events ensues, leading to the transfer of specialized proteins from the CTL to the target cell and triggering apoptosis and target cell lysis [13-15].

The peptides that are bound onto MHCI and presented on the cell surface eliciting a CTL-mediated immune response are called antigenic peptides and are derived from specific sites on antigenic proteins called epitopes. The MHCI molecules are polymorphic; hundreds of different alleles exist within a population, differing often in only a few amino acids that contribute to the shape of the binding site [16]. As a result, each MHCI allele can bind different sets of peptides that represent a small subset of the very large number of possible peptides that can be produced by the MHCI peptide processing pathway [17, 18]. Each MHCI allele has stringent restrictions on which peptides it can bind. Length is the most common one: MHCI molecules bind peptides that are 9 amino acids long, although 8 or 10 amino acids are common. In few cases longer peptides up to 14 amino acids have been found bound onto certain MHCI alleles [19, 20]. Sequence determinants are equally crucial. Specific side-chains on the peptide can interact with specificity pockets on the MHCI binding site to allow for tight binding [3, 21]. The binding preferences of the MHCI alleles carried by an individual can shape their immune response by biasing the presentation of certain peptide sequences.

THE PROTEASOMAL PATHWAY OF ANTIGENIC PEPTIDE GENERATION

The pathway that leads to the generation of antigenic peptides is schematically depicted in Fig. (**1**). The first step occurs in the cell's cytosol where intracellular proteins are degraded by a large cytosolic particle called the proteasome

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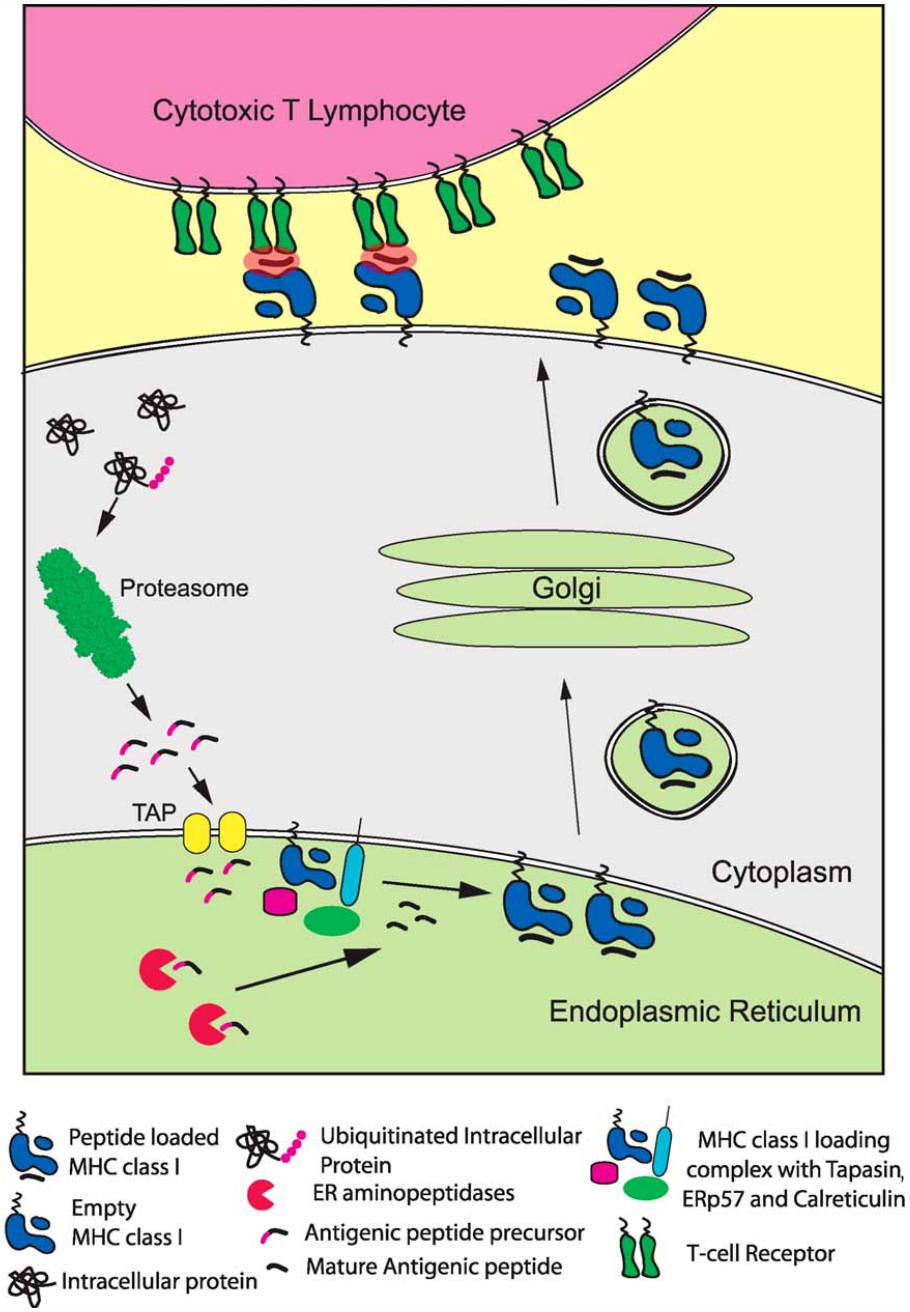

Fig. (1). Schematic representation of the MHC class I antigen processing and presentation pathway. Intracellular proteins are degraded by the proteasome to peptides that are translocated into the ER by TAP. ER resident aminopeptidases further trim the peptides to mature antigenic epitopes that are loaded onto nascent MHC class I molecules by the aid of the MHCI loading complex. The MHCI-peptide complex proceeds along the secretory pathway to the cell surface where it is recognized by the TCR on cytotoxic T-lymphocytes.

[22]. The proteasome is responsible for the majority of proteolytic events in the cytosol and proteins are primarily targeted to it after being tagged by a small protein called ubiquitin [23]. However, incorrectly folded cytosolic proteins, often the product of misfolding during synthesis (Defective Ribosomal Products, DRiPs), are also degraded by the proteasome [24, 25]. Overall, virtually every cytosolic protein is at some point liable to proteasomal degradation and as a result, this pathway is continuously "sampling" the cell's protein content. The proteasome contains three proteolytic activities and can cleave peptide bonds within almost every peptide sequence. The proteasomal products are peptides between 3 and 16 amino acids long that can be further degraded by other proteases in the cytosol for amino acid recycling [26, 27]. A small subset of the peptides generated by the proteasome survives further proteolysis and is transported into the ER by a specialized transporter called Transporter Associated with Antigen Processing (TAP) [28, 29]. Many of the peptides that arrive into the ER by this pathway are extended versions of the final antigenic peptides

and are called antigenic peptide precursors [26]. A characteristic of these antigenic peptide precursors is that they carry the same C-terminus as the mature antigenic peptide and are therefore extended only at their N-terminus. This N-terminal extension is presumably important for the peptides to survive the cytosolic peptidases before they are transported into the ER. Interestingly, inflammatory cytokines such as interferon-γ (INF-γ) that up-regulate several components of the MHC class I antigen processing and presentation pathway including MHCI molecules, also control the expression of specific proteasome subunits that slightly alter the proteasome structure giving rise to a different version of the proteasome called the immunoproteasome [30-32]. The immunoproteasome has a slightly altered proteolytic specificity and gives rise to a greater number of N-terminally extended peptides than the proteasome, essentially boosting the number of antigenic peptide precursors that arrive into the ER [26]. In the ER, the N-terminal residues of the antigenic peptide precursors are sequentially excised by ER-resident aminopeptidases that trim them down to smaller lengths [19, 33]. The products of this trimming, the mature antigenic peptides, bind onto newly synthesized MHCI molecules depending on their sequence and MHCI binding preferences. The MHCI molecules are inherently unstable in the absence of a bound peptide and are retained in the ER by chaperons or destroyed [34-39]. Binding of antigenic peptides stabilizes MHCI molecules and allows them to be transported via the secretory pathway to the cell surface for presentation to CTLs. Which peptides are ultimately loaded onto MHCI is a crucial factor for the correct function of the immune response.

DISCOVERY OF ERAP1

Up to 2002 it was well established that antigenic peptide precursors are finally trimmed down to mature antigenic peptides by unknown aminopeptidase activities in the ER [19, 33]. Three independent research groups led by Dr. Nilabh Shastri, Dr. Kenneth Rock and Dr. Alfred Goldberg were the first to identify one of these aminopeptidases, naming it ERAAP (Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing) or ERAP1 (ER-aminopeptidase 1) [40-42]. Shastri and co-workers discovered a peak with aminopeptidase activity that was detected by ion exchange chromatography of the soluble fraction of microsomes derived from mouse liver and spleen. The activity of this peak was inhibited by leucine-thiol, a classic aminopeptidase inhibitor. Analysis of the fractions by SDS-PAGE indicated a single band migrating with an apparent molecular weight of 100 kDa. Submission of this band to tryptic digestion, followed by mass spectrometry of the peptide fragments was used to identify the unknown protein. The band corresponded to an already identified protein named A-LAP (Adipocyte-derived Leucine Amino-Peptidase). They decided to rename it as ERAAP, a name that highlights its sub-cellular localization, as well as its peptide trimming function in the MHC class I antigen processing and presentation pathway [41].

Goldberg's group detected four different aminopeptidase activities after ion exchange chromatography of ER proteins [40]. The highest aminopeptidase activity for an antigenic precursor corresponded to the peak that showed activity for L-leucine 7-amido-4-methyl-coumarin (L-AMC), a typical leucine aminopeptidase fluorigenic substrate. After isolating and characterizing the protein, they also identified it to be the previously named A-LAP and proposed the new name ERAP1, highlighting the fact that it was only a single of 4 aminopeptidase peaks detected. The group also studied ERAP1's subcellular localization by using confocal microscopy, and demonstrated that ERAP1 is located in the ER.

ERAP1 exists in all tissues but its expression is much higher in tissues that express higher concentrations of MHCI molecules such as liver, lungs, spleen and thymus. In addition, ERAP1 expression is induced by IFN-γ, a pre-inflammatory cytokine that induces the expression of various molecules that participate in the MHC class I antigen processing and presentation pathway [40-42].

Shastri's research group performed siRNA experiments to examine the effect of ERAP1 down-regulation. Using flow cytometry they detected down-regulation of MHCI molecules on the cell surface of siRNA treated cells. That result implicated ERAP1 in antigen presentation because MHCI molecules that are not loaded with peptides are unstable and their expression on the cell surface is reduced [34, 37-39, 43]. Another significant observation was that ERAP1 down-regulation led to decrease in the production of certain epitopes and to increase in the production of others. To explain this observation, it was hypothesized that ERAP1 is often essential to produce the final epitope, whereas in other cases it can destroy it. To further clarify the role of ERAP1 in antigenic peptide generation, Shastri and colleagues studied the effect of ERAP1 silencing on the production of specific antigenic epitopes. They constructed fibroblast cells that did not express TAP so that peptides generated in the cytosol could not enter the ER. Peptide precursors were introduced in the cell by mini-gene transfection and targeted directly into the ER by a signal-sequence at their N-terminus. One precursor contained the antigenic epitope alone and the other the epitope with an N-terminal extension of 7 amino acids. It was observed that the presence of ERAP1 only affected the epitope production from the N-terminally extended precursor. They also demonstrated that ERAP1 was unable to trim the N-terminus when the second residue of the peptide precursor is a proline. This is a fundamental characteristic of this particular aminopeptidase that may be related with the fact that many MHCI molecules present peptides with the "X-Pro-X_n" motif where X represents any amino acid [41].

Rock's group showed that ERAP1 is necessary for the production of the mature antigenic epitope SIINFEKL from its precursor that carries 5 extra residues at the N-terminus. When ERAP1 was silenced, SIINFEKL presentation was almost undetectable. As a result, they suggested that ERAP1 is the final step in the production of mature antigenic epitopes from their N-extended precursors. In contrast, ERAP1 silencing in HeLa cells increased the total number of MHCI on the cell surface, whereas ERAP1 overexpression in COS cells decreased this number, suggesting that ERAP1 has also a capacity to destroy antigenic peptides [42].

The activity of most aminopeptidases is primarily determined by the N-terminus of their substrates. An important observation that was made during the initial study of ERAP1

trimming of several substrates was that although it showed high specificity for dipeptides it didn't show the same specificity for longer peptides. Particularly, for dipeptides, ERAP1 trimmed L-AMC efficiently, M-AMC slower but could not degrade other substrates of the X-AMC form [44, 45]. This so limited specificity can not be consistent with ERAP1's role in processing many different sequences to produce mature antigenic peptides. By examining ERAP1's capacity to trim various N-extended epitope precursors, it was observed that it presented a much broader specificity. It could remove efficiently several amino acids from the N-terminus of many different precursors, contrary to the strong specificity it showed for dipeptide substrates [40]. In addition, ERAP1 was found to trim substrates with the same N-terminus at very different rates, suggesting that its specificity is probably not only determined by the N-terminus, but also by other factors such as the peptide length and sequence [40, 42].

Rock and Goldberg's groups studied the hydrolysis of several precursors and antigenic epitopes by ERAP1 *in vitro* and observed that ERAP1 displays a strong preference for the length of its substrates. Specifically, both groups demonstrated that ERAP1 trimmed peptides with length of 10-14 residues very efficiently. ERAP1 degraded these long precursors down to 8-9 residues long and further trimming was practically absent. Similarly, 9mer substrates were either hydrolyzed at very slow rates or hydrolyzed yielding only the 8mer as product but not any shorter products. Furthermore, all 8mers were very resistant to trimming. Length preference appears to be a characteristic of only this particular aminopeptidase because no other aminopeptidase produces peptides of a specific length and most of them tend to degrade very small peptides efficiently even down to single amino acids. Since most antigenic peptide precursors are 8-9 amino acids long, both groups concluded that ERAP1 trims precursors until they reach the appropriate length so that they can bind onto MHCI [40, 42].

PREVIOUSLY KNOWN FUNCTIONS ATTRIBUTED TO ERAP1

ERAP1 had been identified long before it was correlated with the MHC class I antigen processing and presentation pathway. Alternate names for ERAP1 are A-LAP (Adipocyte-derived Leucine AminoPeptidase), PILS-AP (Puromycin-Insensitive Leucyl-Specific Aminopeptidase) and ARTS-1 (Aminopeptidase Regulator of TNFR1 Shedding) [44-46]. ERAP1 had been proposed to be involved in the regulation of blood pressure [47-49]. Intracellular ERAP1 had been found to induce angiogenesis through endothelial integrin activation [50], whereas the secreted form of the enzyme suppresses angiogenesis through angiotensin II inactivation [51]. ERAP1 had also been shown to participate in the innate immune response by increasing the production of cytokine receptors [46, 52, 53]. From all the functions attributed to ERAP1, its association with the MHC class I antigen processing and presentation pathway seems to be the most clearly demonstrated and as a result it has essentially monopolized all research efforts regarding this enzyme since its discovery in 2002.

DISCOVERY OF ERAP2

ERAP2, previously characterized as human leukocyte-derived arginine aminopeptidase (LRAP), is the second aminopeptidase suggested to trim antigenic precursors in the ER and it was identified by Dr. Tsujimoto's group in 2003 [54]. Interestingly, ERAP2, unlike ERAP1, is only expressed in humans and not in mice. It was shown to prefer arginine and lysine as the N-terminus of dipeptide fluorigenic substrates assayed *in vitro*. It is localized in the ER and its expression is induced by IFN-γ in some cell types. ERAP2 has the capacity to produce mature antigenic epitopes from their precursors *in vitro* [54]. It has been demonstrated to efficiently cleave angiotensin and kallidin, indicating a potential role in the regulation of blood pressure, as it was formerly proposed for ERAP1 as well [55, 56].

Dr. Van Endert and co-workers identified ERAP1 and ERAP2 in the same peak with aminopeptidase activity after purification of the microsome fraction of Hela cells by anion-exchange chromatography [57]. The two aminopeptidases were found to co-localize with each other and have identical subcellular distribution in HeLa cells stained by specific monoclonal antibodies. Zonal sedimentation analysis of the microsome fraction suggested that a small percentage of each enzyme had double the molecular weight than expected. The group suggested that this band could correspond to an ERAP1/ERAP2 heterodimer or to a homodimer or even to a complex of each of these enzymes with an unidentified protein. Coimmunoprecipitation experiments showed that one out of two monoclonal antibodies that precipitated ERAP1 could also precipitate a small amount of ERAP2 and vice versa, indicating the presence of an ERAP1/ERAP2 heterodimer. The heterodimers were found to localize in the ER by co-immunoprecipitation of sucrose gradient fractions of MGAR B cells [57].

The same group went on to demonstrate that a precursor of an epitope of HIV IIIB *env* protein could be degraded efficiently yielding the mature epitope only when both aminopeptidases were present in the reaction mixture [57]. The epitope, whose precursor contained an extension of hydrophobic and basic residues, could not be efficiently produced by the action of only one of these aminopeptidases alone. Similarly to earlier reports [40-42], this research group also demonstrated that ERAP1 knock-down resulted in a reduction in overall MHCI expression. Interestingly, similar effects were seen when ERAP2 was knocked-down. When both ERAP1 and ERAP2 were silenced simultaneously, MHCI expression was further reduced by twofold. In addition, the simultaneous knock-down had an additive effect to the reduction of the presentation of two out of four precursor peptides tested. The group concluded that although ERAP1 appears to be the dominant trimming enzyme in the ER, it may not have the capacity to remove some extensions efficiently. These extensions can be presumably trimmed efficiently by ERAP2, giving this enzyme a secondary, or auxiliary role. Furthermore, ERAP1/ERAP2 heterodimer formation could induce peptide trimming by bringing the two active sites in close proximity to allow concerted trimming or affect activity by allosteric modifications of one or both active sites. However, there is no proof that a

physical association between the two enzymes is required in order for them to act co-operatively [57].

ERAP STRUCTURE - DOMAINS

ERAP1 and ERAP2 (ERAPs) are 100-110 kDa, monomeric, soluble zinc aminopeptidases that belong to the M1 family of metallopeptidases in the MA(E) peptidase clan [58]. They are not yet included in the IUBMB recommendations and they can be found as EC 3.4.11.x aminopeptidases [59]. In mammals, the M1 aminopeptidase family consists of nine different proteins, five of which are integral membrane proteins [60]. ERAPs were classified along with the placental leucine aminopeptidase (PLAP, EC 3.4.11.3) as the "Oxytocinase subfamily" of the M1 aminopeptidases [61]. They share the characteristic **H**EXX**H**$(X)_{18}$**E** zinc-binding motif of the gluzincins metalloproteases [62] and the exopeptidase GAMEN motif [63] in their active sites. A single zinc ion that binds to the two histidines and the second glutamic acid of the **H**EXX**H**$(X)_{18}$**E** motif is required for catalysis, which occurs most probably via a thermolysin-like mechanism [64]. The first glutamic acid acts as a general base, whereas a conserved active-site tyrosine residue may act as a proton donor. Recently, glutamine-181 was demonstrated to be crucial for ERAP1's enzymatic activity and specificity. Mutating glutamine 181 to aspartate altered the preference for the N-terminus of ERAP1's substrates from hydrophobic amino acids to basic amino acids. Interestingly, the corresponding reverse mutation in ERAP2, D198Q, resulted in improved trimming of hydrophobic residues but positively charged residues could still be trimmed effectively [65]. Overall it appears that glutamine-181 for ERAP1 and aspartate-198 for ERAP2 are critical residues for the enzymes' specificity.

Human ERAP1 and ERAP2 share 51% sequence identity (479/936) with 69% positives (650/936) and 2% gaps (19/936) as shown in Fig. (**2**). A BLAST search [66] in the Swissprot database revealed that PLAP displays 46% sequence identity (418/903) with ERAP1 (Positives=575/903 (63%), Gaps=38/903 (4%)), and 44% identity (4-1/906) with ERAP2 (Positives = 561/906 (61%), Gaps = 36/906 (3%)). It is interesting to note that PLAP possesses a significantly longer N-terminal domain (~80 residues) than both ERAP1 and ERAP2, which possibly contains a transmembrane region.

A BLAST search in the PDB [67] for available crystallized homologues revealed that ERAP1 shares 32% sequence identity (192/594) with the tricorn interacting factor F3 (TIFF3) from *Thermoplasma Acidophilum*, as well as 25% identity (82/326) with the human leukotriene A4 hydrolase (LTA4H), and 24% sequence identity (92/374) with aminopeptidase N from *E. coli*. Similarly, ERAP2 is found to share 30% sequence identity (181/590) with TIFF3, 24% identity (122/497) with aminopeptidase N and 26% identity (79/299) with LTA4H. A common sequence insertion of ~25–35 residues long is evident from the multiple alignment of ERAP1 and ERAP2 compared to their homologous

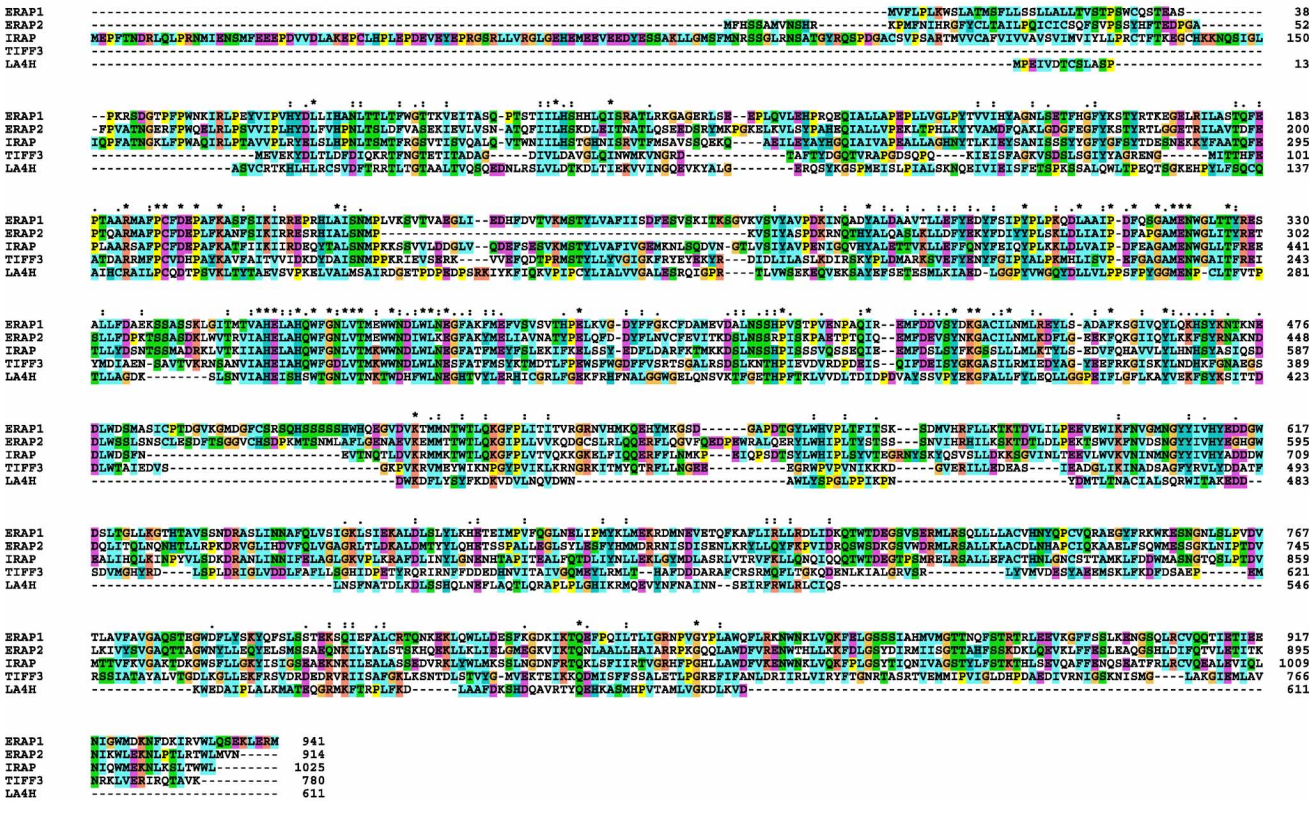

Fig. (2). Multiple sequence alignment of ERAP1, ERAP2, IRAP(PLAP), TIFF3 and LTA4H performed with ClustalW 2.0 [127] and illustrated with Jalview 2.4 [128].

aminopeptidases (Fig. **2**), which is encoded by exon 11 of the ERAP1 and ERAP2 genomic sequence [68].

ERAP1 residues 280–486 comprise the major part of the catalytic domain and display 43% sequence identity to the equivalent domain of TIFF3, with 84% identity at the core of the zinc-binding domain (residues 352-382). ERAP2 residues 298–503 comprise the enzymes catalytic domain, which shares 45% identity with the catalytic domain of TIFF3 and 74% identity at the zinc-binding core (residues 369–399). The sequence homology of both ERAP1 and ERAP2 with TIFF3 is adequate for the preparation of homology structural models based on the TIFF3 crystal structure [69]. Interestingly, TIFF3 has been crystallized in three different conformations that probably capture the molecular dynamics of this aminopeptidase [70]. The relative arrangement of the TIFF3 domains creates a hook-shaped structure with a deep cleft between two lobes, which accommodates the peptidic substrates. Its size varies within the different crystal forms, thus representing a more open and a more closed conformation (PDB ID: 1Z5H), as well as an intermediate form (PDB ID: 1Z1W). The latter has been used as the template for the construction of the homology model of ERAP1 as described in [71], as well as for the construction of the homology model of ERAP2 presented here. As illustrated in Fig. (**3**), the overall organization of ERAP1 and ERAP2 resembles that of TIFF3, with two lobes forming a deep cleft that can accommodate the enzyme's substrates. The central cleft of ERAP1 exhibits a strongly negative electrostatic potential and contains several hydrophobic pockets [71]. The electrostatic potential in the equivalent region of TIFF3 or ERAP2 is significantly weaker (not shown).

KNOCK-OUT MICE HIGHLIGHT ERAP1'S IMPORTANCE IN THE IMMUNE RESPONSE

To elucidate the exact role of ERAP1 in antigen presentation *in vivo* four independent research groups created transgenic mice lacking a functional ERAP1 gene ($ERAP1^{-/-}$). To create the knock-out mice the groups either deleted exon 4 of the ERAP1 gene that contains the sequence that codes for the zinc binding motif [72, 73] or they deleted a larger region that codes for the whole aminopeptidase active site [74, 75]. The knock-out mice were healthy, fertile, developed naturally and didn't show any visible defects. It was therefore concluded that ERAP1's gene is not necessary for survival [72-75].

In order to estimate the effect of ERAP1's absence in the MHC class I antigen processing and presentation pathway, MHCI expression on the cell surface was quantitated by flow cytometry. To perform this analysis, various cell types of the knock-out ($ERAP1^{-/-}$) as well as the control mice ($ERAP1^{+/+}$) were labelled with specific antibodies for particular MHC alleles. The cell surface levels for most MHCI alleles were found to be decreased. However, certain MHCI alleles were unaffected and others were actually enhanced [72-75]. On the contrary, MHCII expression was the same in control and knock-out mice, confirming that ERAP1 is only involved in the class I antigen processing and presentation pathway [73, 74].

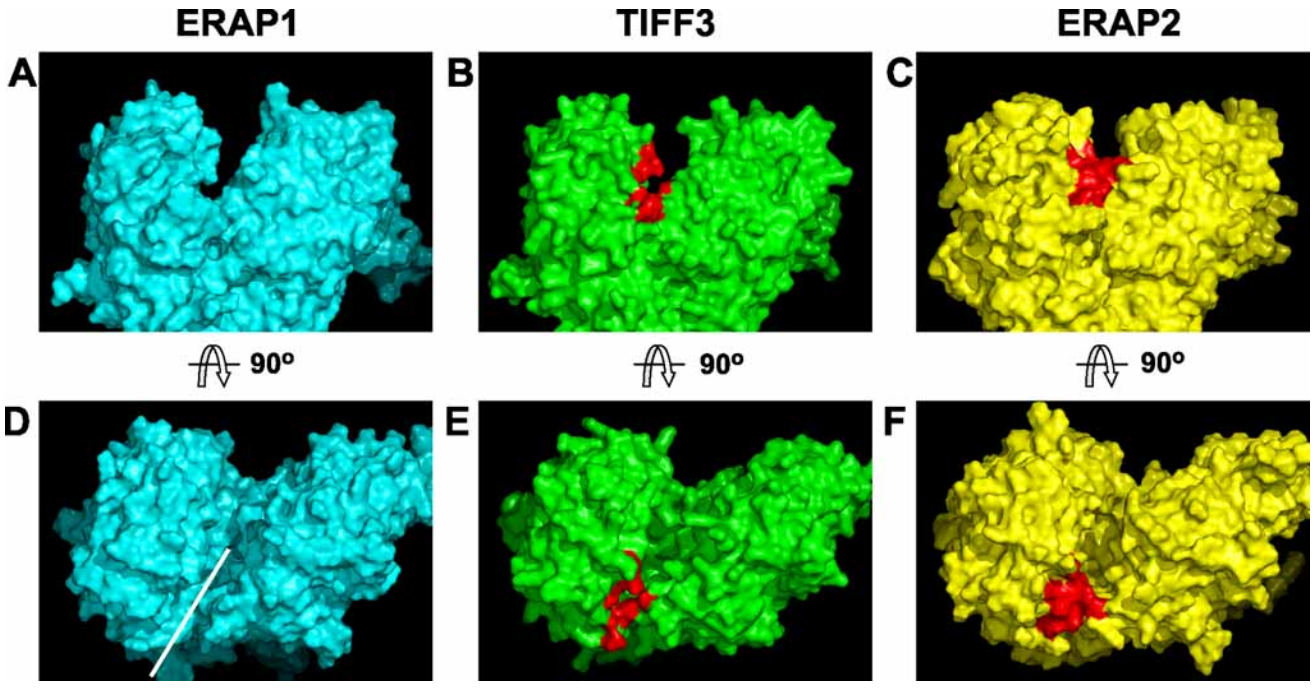

Fig. (3). Surface representation of the homology model of ERAP1 (in cyan, panels A and D), the crystal structure of TIFF3 (in green, panels B and E) and the homology model of ERAP2 (in yellow, panels C and F). Panels D-F are rotated by 90 degrees around the x-axis compared to panels A-C. Highlighted in red are the residues that constrict or block part of the exit to the solvent of the central cavity that leads to the catalytic site for the three proteins. Protein model pictures were created using PyMol 0.99 (http://www.pymol.org)

Rock and co-workers measured the response of $CD8^+$ T lymphocytes towards lymphocyte choriomeningitis virus (LCMV) infection and found important differences between knock-out and control mice. Mice were initially infected with LCMV and eight days later, their splenocytes were activated with LCMV antigenic epitopes, labelled with appropriate antibodies and analyzed by flow cytometry. The levels of $CD8^+$ T lymphocytes in knock-out mice were lower for most epitopes, for one epitope they remained unchanged and for another one were increased. These alterations resulted to a significant change in the immunodominance hierarchy of these epitopes. Immunodominance describes the phenomenon of only a few epitopes being immunogenic when many more are suitable for MHC binding. As a result, immune responses are actually mounted towards a relatively small number of epitopes. In wild-type mice a clear immunodominance hierarchy for LCMV epitopes is formed that remains the same between different individuals, different experiments and in different laboratories. In the ERAP1 knock-out mice a new immunodominance hierarchy was established and this was attributed to changes in antigenic peptide generation due to lack of processing by ERAP1 [75].

Niedermann and co-workers on the other hand observed reduced levels of $CD8^+$ T lymphocytes for only one of the LCMV epitopes they tested. Furthermore, the epitope's reduced presentation did not result in lower resistance to the virus and the ERAP1 knock-out mice cleared the virus as effectively as the wild type mice. When they studied the response to a secondary infection they found less efficient control of the virus only for knock-out mice primed with the epitope. Knock-out mice that were primed with the whole virus showed no significant differences compared to the wild-types. As a result, the group concluded that ERAP1's absence has little effect on the immunodominance hierarchy and antiviral immunity in the context of LCMV infection [73].

Shastri's and Van Kaer's groups also demonstrated that in ERAP1 knock-out mice cells, MHCI-peptide complexes that are presented on the cell surface were less stable. Both groups cultured splenocytes from wild type and knock-out mice in the presence of brefeldin A, so that nascent MHCI-peptide complexes could not migrate to the cell surface. The cells were then labelled with appropriate antibodies that recognize specific MHCI complexes and analyzed by flow cytometry. This experiment revealed a significant increase in MHCI degradation in $ERAP1^{-/-}$ cells. A possible explanation for this phenomenon was that in the absence of ERAP1 a big percentage of surface MHCI molecules binds sub-optimal peptides, leading to looser binding and to easier or faster degradation of MHCI-peptide complexes [72, 74]. A particularly clear example of the effect of ERAP1 came with the recent analysis of an immunodominant protective antigenic peptide from the parasite *Toxoplasma gondii*. In ERAP1 deficient mice, generation of this antigenic peptide is impaired, protective T-cell populations can not expand efficiently and the mice are more susceptible to toxoplasmosis [76].

Overall, the ERAP1 gene knock-out experiments established that ERAP1 is the dominant enzyme that trims precursor antigenic peptides in the ER and its absence cannot be compensated by another aminopeptidase's action. In the absence of ERAP1 precursor peptides that need further trimming cannot be hydrolyzed, and as a result some antigenic epitopes are not produced at all or are produced at very low levels. Epitopes that don't require further processing are presented at the same levels in wild type and ERAP1 knock-out mice. The fact that lack of ERAP1 leads to an increase of certain epitopes or to the presentation of epitopes that are not normally presented confirms the idea that ERAP1 also has the capacity to destroy some epitopes. Finally, several lines of evidence suggest that ERAP1's absence can lead to an altered immunodominance hierarchy, although this effect might depend on the specific system studied [72-75].

EDITING PROPERTIES OF ERAP1

Even from the initial identification of ERAP1 as the major aminopeptidase behind antigenic peptide precursor trimming in the ER, it became apparent that its effects on antigen presentation were complex. Different antigenic epitopes were either up- or down-regulated in the absence of ERAP1 whereas some epitopes remained unaffected [41]. A complex landscape of effects was also seen from the study of ERAP1 knock-out mice [72-75]. Specifically, in the knock-out mice the presentation of many of the studied epitopes was reduced where the presentation of other epitopes was enhanced. These effects have been interpreted in the context that ERAP1 is on the one hand crucial in the generation of many epitopes that arrive in the ER only in the form of N-terminally extended precursors but, on the other hand, ERAP1 can effectively destroy other epitopes by trimming them beyond their optimal binding length. Most of these studies could not evaluate the contribution of other ER amino-peptidases like ERAP2, because they were performed on murine models that lack ERAP2.

Recently, Hammer *et al.* systematically analyzed the presented peptide repertoire from ERAP1 knock-out mice. The researchers not only found that many peptides were completely missing from the repertoire but also detected many unstable and novel MHCI-peptide complexes [43]. Surprisingly, these novel complexes elicited strong T- and B-cell responses indicating that lack of ERAP1 can actually enhance overall immunogenicity. These findings, taken as a whole, have been interpreted to indicate that ERAP1 acts to shape the peptide repertoire and therefore modulates the immune response. This notion categorizes ERAP1 as an antigenic peptide editor along with previously proposed editors of the antigen presentation pathway HLA-DM and Tapasin [77].

We have recently demonstrated that ERAP1 trims peptides *in vitro* with very different kinetics depending on the peptide sequence [71]. This finding could provide an interesting twist on ERAP1's peptide editing properties that has not been thoroughly investigated yet. ERAP1 may trim antigenic peptide precursors based on their internal sequence, imposing a sequence bias on the generated peptides. This concept may help us understand why ERAP1 silencing can lead to different effects depending on the epitope studied; different antigenic peptide precursors have different sequences and are affected by ERAP1 activity to a

different degree. In that context ERAP1 may skew the peptide repertoire in a sequence dependent manner similarly to the effects of MHCI allele binding preferences.

OTHER AMINOPEPTIDASES IMPLICATED IN ANTIGENIC PEPTIDE PROCESSING

Insulin Regulated Aminopeptidase (IRAP) also named Placental Leucine Aminopeptidase (PLAP) or Oxytocinase is another M1 aminopeptidase that has been assigned to the Oxytocinase subfamily of M1 aminopeptidases along with ERAP1 and ERAP2 [61]. It shares high overall homology with ERAP1 (43%) and ERAP2 (49%) but contains an N-terminal extension that presumably defines an intracellular domain. Although PLAP can be detected inside the cell, its presence in the ER has not been established. PLAP's sequence resemblance to ERAP1 and ERAP2 and its ability to trim many peptide substrates *in vitro* in combination with the existence of non-identified aminopeptidase peaks from microsomal fractions [40] suggest that it may also play a role in antigenic peptide precursor trimming in the ER. However, this has not been demonstrated yet. Furthermore, the strong effects seen for antigenic peptide generation by ERAP1 possibly suggest that, at least for most epitopes, no more aminopeptidase activities in the ER are necessary.

The first step in the generation of antigenic peptides occurs in the cytosol by the action of the proteasome. Before the proteasome-generated peptides can be transferred into the ER for further processing by ERAP1 or ERAP2, they are exposed to a variety of aminopeptidase activities that could potentially trim the N-terminally extended peptides down to the mature epitope, making ER processing unnecessary. Several cytosolic aminopeptidases have been proposed to play a role in antigenic peptide generation, such as leucine aminopeptidase (LAP), bleomycin hydrolase (BH) and puromycin-sensitive aminopeptidase [78, 79]. Although these peptidases can convert antigenic peptide precursors to the mature epitopes in cell lysates, their role in antigen presentation in intact cells is either redundant or unproven [80]. Tripeptidyl peptidase II (TPPII) has been proposed to be essential for trimming precursors longer than 15 residues long in the cytosol and Thimet oligopeptidase has been demonstrated to destroy antigenic peptides in cell lysate assays [81-84]. However, silencing TPPII had only marginal effects in antigen presentation, suggesting that its effects are redundant [85]. Overall, the importance and necessity of post-proteasomal cytosolic peptidase events in antigen presentation has not been clearly established as of yet. In contrast, it appears that the simplest model of antigenic peptide generation involving the proteasome and ERAP1/2 is sufficient to explain the generation of most antigenic epitopes tested so far.

UNUSUAL PROPERTIES OF ERAP1 FIT ITS ROLE IN THE IMMUNE RESPONSE

Shortly after the initial identification of ERAP1 as the aminopeptidase responsible for the trimming of antigenic peptide precursors in the ER, it became apparent that ERAP1 had unusual properties compared to previously characterized aminopeptidases. Although ERAP1 had a strong preference for hydrophobic amino acids when degrading short fluorigenic substrates, it was able to excise a larger variety of amino acids from longer peptides. Trimming of longer antigenic peptide precursors resulted in the generation of mature antigenic peptides that accumulated as products and appeared to be resistant to further trimming [40, 42]. Analysis of trimming rates for a collection of unrelated peptides revealed a strong preference for peptides of 9-16 amino acids long, with shorter peptides being especially resistant to N-terminus trimming by ERAP1 [86]. This was in sharp contrast to other known aminopeptidases that were found to become more efficient the shorter the peptide gets. Finally, the nature of the C-terminus of the peptide appears to affect the rate of N-terminus trimming by ERAP1 so that peptides with hydrophobic or positively charged C-termini are preferred [71, 86]. Taken together, these observations appear to fit well with the proposed role of ERAP1 in antigen presentation: most antigenic peptide precursors are within the 9-16 amino acid range and most mature antigenic peptides are 8-9 amino acids long – as a result ERAP1 should efficiently trim precursors and spare mature epitopes. Murine and human TAP transports peptides with hydrophobic C-termini and murine and many human MHC class I molecules bind peptides with a hydrophobic C-terminus, both consistent with ERAP1's reported preferences.

In our laboratory we recently embarked on the systematic *in vitro* characterization of ERAP1 specificity. We have discovered that ERAP1 peptide trimming is not only affected by the peptide's N- and C-termini but also by several positions within the peptide sequence, exhibiting strong preferences for positively charged and hydrophobic residues [71]. By designing model peptide substrates based on library screens we were able to demonstrate trimming rates that vary within 5 orders of magnitude. Such strong sequence preferences have never been demonstrated by an aminopeptidase before. If these findings are corroborated *in vivo*, they would suggest that ERAP1 will trim peptides with a strong sequence bias. Such sequence preferences should probably be taken into account when interpreting ERAP1's complex role *in vivo*.

ERAP2 does not appear to share the unique trimming properties of ERAP1. Chang *et al*, demonstrated that ERAP2, contrary to ERAP1, could rapidly digest several 8mers and 9mers [86]. Moreover, ERAP2 does not appear to have any preference for the C-terminus of its substrates whereas ERAP1 was demonstrated to prefer hydrophobic or basic residues. As a result, ERAP2 may trim antigenic precursors with C termini that ERAP1 has difficulty in trimming, but following a distinct mechanism than that of ERAP1, since it does not show the same length preference [86]. Overall, these preliminary findings point towards a specific role for ERAP2 in antigen presentation, possibly one that complements ERAP1 [57]. However, more work is necessary to clarify this role as well as the specific molecular properties of ERAP2. No peptide repertoire editing properties have been assessed for ERAP2 as of yet. The lack of ERAP2 in mice makes the evaluation of its *in vivo* role difficult. It is possible that this fundamental difference between the murine and human aminopeptidase trimming activities is a hint to fundamental differences in qualitative aspects of antigen presentation between the two species.

SPECIFICITY OF ERAP1 AND MODELS OF ANTIGENIC PEPTIDE TRIMMING

Two models have been proposed to help explain the mechanism underlying ERAP1's role in antigen presentation. Goldberg and colleagues have proposed that the ability of ERAP1 to generate antigenic epitopes is inherent to the molecular mechanism of trimming by the enzyme. This hypothesis was based on the initial observation that ERAP1 trims longer peptides but not shorter ones, stopping when the mature epitopes have been generated [40, 42]. Further investigation, revealed that this length preference applies to a large pool of unrelated peptide sequences and it is unique amongst other known aminopeptidases including ERAP2 [86]. The peptide's C-terminus was also found to affect the rate of N-terminal trimming, a finding that led to the formulation of the "molecular ruler" model, according to which, the C-terminal side chain is recognized by the enzyme on a site distinct from the catalytic site. If the peptide is large enough for its N-terminus to reach to the catalytic site, trimming will occur rapidly – shorter peptides cannot bind their C-termini and N-termini at the same time and are not trimmed. According to this model, ERAP1 has the inherent capacity to produce correct length peptides carrying an appropriate C-terminus for binding onto MHCI [86].

An alternative model has been proposed by Shastri and colleagues based on the observation that larger, N-terminally extended peptides can be found to associate with MHCI. According to this model, ERAP1 trims the antigenic peptide precursors while they are bound onto MHCI and all length and sequence restrictions in the final product are brought forth from the MHCI-peptide interaction: when the peptide is of the correct length it cannot be trimmed further because it is protected by the MHCI binding site [19, 20, 87]. In this model, ERAP1 does not need any special properties in peptide trimming but the weight of peptide selection is shifted onto the MHCI binding properties that are already pretty well characterized.

Our own findings that the peptide sequence can greatly affect trimming rates [71] are easier understood in terms of the Goldberg "molecular ruler" model, which they actually help to expand: we propose that the full length of the peptide sequence is recognized within an extended peptide binding site on ERAP1. This modification of the "molecular ruler" model, "burdens" ERAP1 with selection properties on antigenic peptide generation based on the precursor sequence, well before any MHCI binding takes place. However, the two models are not necessarily mutually exclusive and could be operating in parallel perhaps depending on the nature of the peptide and/or MHCI allele involved.

Unfortunately, no high resolution structure is available for ERAP1 as of yet which could provide answers on the mechanism of peptide trimming. To gain insight into the structural properties of ERAP1 and ERAP2 that may be related to antigenic peptide trimming we constructed two homology models based on the structure of the homologous aminopeptidase TIFF3 [70]. Not surprisingly, due to the relatively high homology between the 3 proteins, the gross structural features of the models of ERAP1 and ERAP2 resemble the structure of TIFF3 to a high degree. A surface representation of the two models and of TIFF3 is shown in Fig. (**3**). Visual inspection of the three structures reveals that all three molecules contain a deep cleft that leads to the zinc-containing catalytic site. The exit of this cleft towards the solvent is much wider in the case of ERAP1 (in cyan, panels A and D), especially compared to the model for ERAP2 (in yellow, panels C and F). Both TIFF3 and ERAP2 feature a "constriction" on the edge of the cleft. The residues that form this constriction are highlighted in red in Fig. **3**. As a result of the opening at the edge of the cleft of ERAP1, the cleft is wider and can easily accommodate a 10-15mer peptide as demonstrated by simple docking simulations, in a configuration where the peptide N-terminus is located adjacent to the Zn(II) atom and the C-terminus is extending outwards along the edge of the cleft (marked with a white line, panel D, Fig. **3**). Although this binding model is purely speculative at this point, it does highlight a potential difference between ERAP1 and ERAP2 that would be consistent with ERAP1's preference for longer peptide substrates [86]. This extended cleft of ERAP1 is also consistent with the "molecular ruler" model since it can contain the necessary binding sites for the peptide's side-chains. Regardless of the accuracy of the homology models in terms of detailed structural features, it is reasonable to assume that the overall structural organization of ERAP1 and ERAP2 is going to be highly similar to the crystal structure of TIFF3 given the reasonably high homology between the 3 proteins. It is notable that the zinc-containing catalytic site is buried deep inside the deep cleft making the approach of an MHCI-peptide complex problematic. Manual attempts to approach the N-terminus of an 11mer bound onto an MHCI allele to the Zn(II) atom of the model failed due to steric clashes between the MHCI side-chains and ERAP1; the closest *in silico* achievable distance between the N-terminus of the peptide while bound onto MHCI and the Zn(II) atom inside ERAP1 was about 40 Å, a distance that would make catalysis impossible. However, it is possible that in the actual structure of ERAP1, the cleft is wider, allowing the approach of an MHCI-peptide complex. For now, our modeling efforts appear to be more consistent with the Goldberg model of antigenic peptide generation. However, the determination of a high-resolution structure of ERAP1 will be necessary to clarify this issue.

ERAP1/ERAP2 POLYMORPHISMS IN AUTOIMMUNITY AND CANCER

Recent studies have linked genetic variations in ERAP1 and ERAP2 with human diseases such as ankylosing spondylitis, diabetes and cervical carcinoma. Ankylosing spondylitis is an autoimmune disease that belongs to spondyloarthritides. It causes characteristic inflammatory back pain that can lead to structural and functional restrictions in patients and consequently to a decreased life quality [88]. Two SNPs on chromosome 5 exceeded the 10^{-5}-10^{-6} threshold set for gene-based scans [89] for ankylosing spondylitis (rs27044: $P = 1.0 \times 10^{-6}$; rs30187: $P = 3.0 \times 10^{-6}$). These SNPs correspond to Q730E and K528R of ERAP1, respectively. ERAP1 association was found to be genuine and the population attributable risk was 26% [90]. In addition, two other studies showed that specific ERAP haplotypes are strongly associated with the disease. Specifically, rs27044/10050860/30187-CCT and rs30187/26618/

26653-CTG, were found to be associated with high and low risk of developing Ankylosing Spondylitis respectively, whereas high risk was also correlated with a third haplotype containing an ERAP2 SNP, rs27044/30187/2549782-GTT [91, 92]. More than 90% of the ankylosing spondylitis cases bear the HLA-B27 allele, so the association of ERAP1 and ERAP2 with the disease could give insight on the mechanism that links HLA-B27 with ankylosing spondylitis. Predisposition to another autoimmune disease, type 1 Diabetes, has been reported to be linked to SNPs in the 5q15 chromosomal region where the ERAP1 gene resides, providing further evidence for the role of ERAP1 polymorphisms in the pathogenesis of autoimmunity [93].

Cervical carcinoma is a cancer induced by the human papillomavirus (HPV). It occurs when oncogenic types of the HPV infect the uterine cervix and cause malignancy [94, 95]. Some individuals are more susceptible than others to persisting HPV infection and development of cervical carcinoma [96]. It has been hypothesized that this diversity between individuals is due to down-regulation of some components of the antigen processing machinery or variations in the genes that express these components [97-99]. It was recently demonstrated that there is a significant association of certain SNPs in these genes with an increased risk of developing cervical carcinoma. Two of those SNPs (rs27044: $P = 0.010$; rs30187: $P = 0.007$) were located in the ERAP1 gene. These SNPs correspond to Q730E and R127P of ERAP1, respectively. Presence of the minor allele at both of these loci was associated with increased risk ($P<0.001$). Presence of the minor allele at ERAP1-127 and ERAP1-730 together with presence of the major allele at TAP2-651 and LMP7-145, that are also components of the MHC class I antigen processing and presentation pathway, were significantly associated with a three-fold higher risk of developing cervical carcinoma. This haplotype combination was estimated to represent almost 12% of all cervical carcinoma patients [100]. In another study, ERAP1-56 and ERAP1-127 were significantly associated with decreased overall survival. Moreover, ERAP1-127 and the haplotype consisting of the major allele at ERAP1-56 and the minor allele at ERAP1-127 were associated with ERAP1 expression and overall survival and, interestingly, in both cases heterozygosity was associated with normal ERAP1 expression and therefore better survival [101].

The molecular mechanism linking disease predisposition to ERAP1 genetic variations is not clear. However, ERAP1 SNPs have been shown to affect enzymatic activity. Specifically, the polymorphism K528R reduces ERAP1's activity toward the peptide hormones angiotensin II and kallidin [102]. It is possible that SNPs associated with ankylosing spondylitis, diabetes or cervical carcinoma could lead to alterations in the enzyme's specificity and trimming capacity. These changes could alter the epitope repertoire presented, therefore leading to a defective immune response. No correlation between ERAP2 SNPs and enzymatic activity has been reported to date, but an ERAP2 genetic variation (rs2762) has been found to result to an eight-fold higher gene expression [103].

Expression levels of both ERAP1 and ERAP2 have been found to be greatly affected in malignant tissues. ERAP1 down-regulation was shown to be an independent predictor for decreased overall survival and disease-free survival in cervical carcinoma patients [104]. Partial loss of ERAP1 expression could result in preferential loading and presentation of tumour-unrelated peptides. Consequently, the phenotype would become less immunogenic allowing the tumour to grow further.

ERAP1 and ERAP2 expression has been found to be strongly down-regulated in the majority of tumour samples when assayed by immunohistochemical methods. Even when expression of ERAP1 and ERAP2 was detectable, it varied greatly for different types of cancer. Breast, kidney, ovary, lung and brain tumours showed no detectable levels of ERAP1 whereas ERAP2 was undetectable in kidney, ovary and stomach tumours. Interestingly, ERAP1 and ERAP2 expression levels were found to be greatly dys-coordinated in most malignant tissues [105]. Based on the established role of ERAP1 and ERAP2 in the immune response it is possible that their down-regulation in cancer cells is important for immune evasion.

Taken together, these recent findings establish a correlation of endoplasmic reticulum aminopeptidases with autoimmunity and cancer and open up novel exciting directions for pharmaceutical interventions. However, until the molecular mechanisms behind those effects are elucidated, any pharmaceutical interventions for autoimmunity and cancer based on ER aminopeptidase activity manipulation should be approached with great caution.

AMINOPEPTIDASE INHIBITION AS A TOOL FOR IMMUNE RESPONSE MANIPULATION

Very little information exists regarding potent and specific inhibitors for ERAP1 and ERAP2. However, a considerable amount of information exists for inhibitors of related aminopeptidases. Recently, there has been an increasingly large number of papers that report the development and use of aminopeptidase inhibitors, which are described in many recent reviews [106-117]. Generally, aminopeptidases are dependent on a single zinc ion for activity and therefore are inhibited by broad-range metal chelating agents, such as EDTA, leucinethiol and 1,10-phenanthroline, which are also inhibitors of many metallopeptidases [45]. By focusing on the M1 family of aminopeptidases, a search in the MEROPS database [118] revealed the potent inhibitors shown in Fig. (**4**). Bestatin (Ubenimex) was first described as inhibitor of aminopeptidase B [119], but is an effective inhibitor of a wide range of metalloaminopeptidases in several families and clans. The low toxicity of bestatin led to its evaluation for the treatment of cancers [119]. Amastatin, a product of actinomycetes, inhibits aminopeptidase N, aminopeptidase A and leucyl aminopeptidase, the latter complex with amastatin determined by x-ray crystallography [120]. Actinonin is also an antibiotic produced by actinomycetes, and is often used as a specific inhibitor of aminopeptidase N [121]. Captopril and RB 101(S) are thiol-containing compounds that inhibit metallopeptidases by binding the zinc ion via their sulphydryl group. Captopril is used as a drug for the control of blood pressure by inhibition of angiotensin-converting enzyme, but is also a weak inhibitor of LTA4H [122]. RB 101(S) is a potent

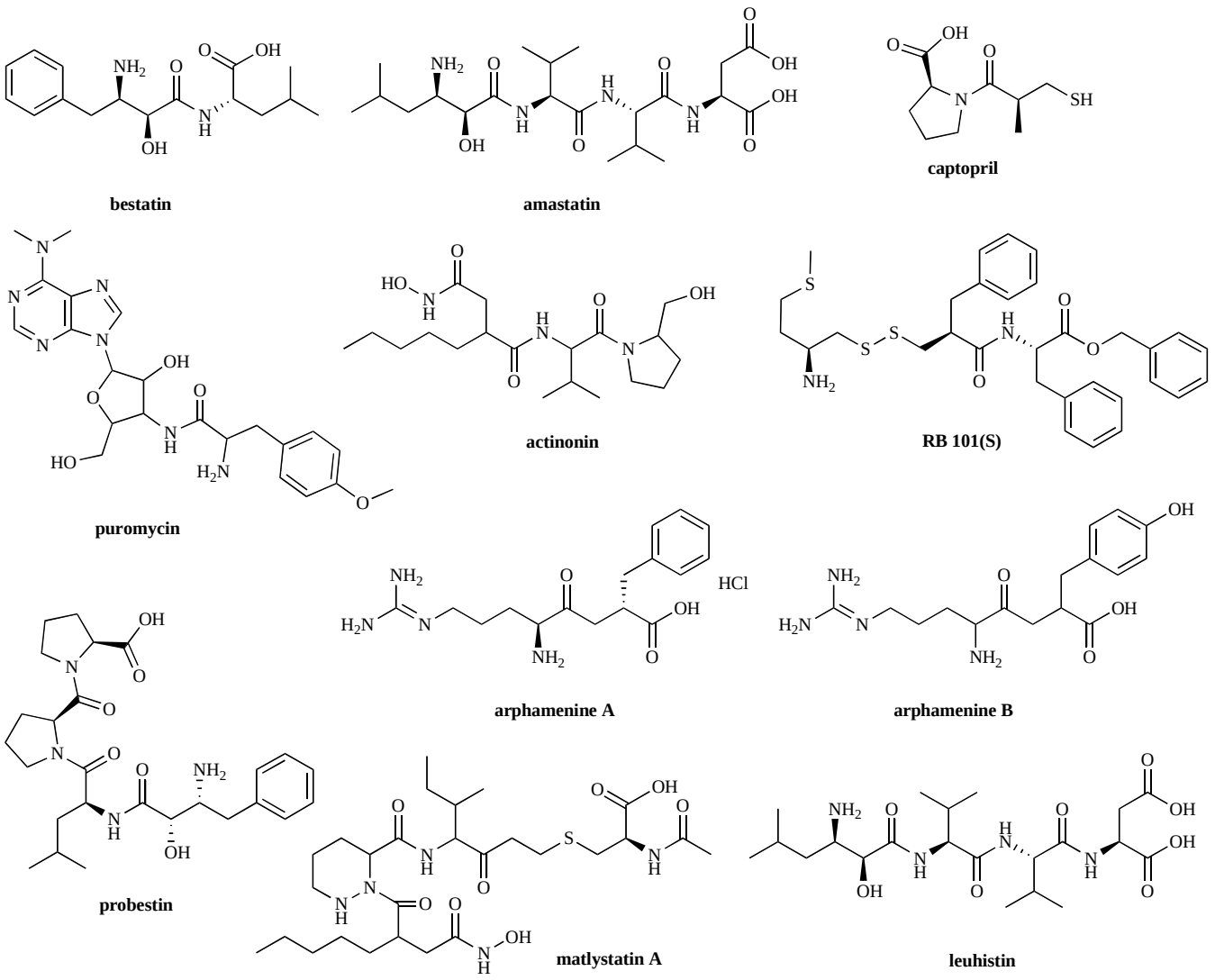

Fig. (4). M1 Aminopeptidase inhibitors classified in the MEROPS database [118].

inhibitor of enkaphalin degradation and a reversible inhibitor of aminopeptidase N. Arphamenines A and B are ketomethylene analogues of Arg–Phe and Arg–Tyr dipeptides, respectively, in which the scissile bond is replaced by the O=C–CH_2 group. They cause reversible inhibition by acting as transition state analogues of the N-terminal arginine residues that are the substrates of the affected peptidases [123]. Arphamenines A and B are selective inhibitors of aminopeptidase B, and inhibit LTA4H much more weakly. Puromycin has been used to distinguish active aminopeptidase M from inhibited cytosol alanyl aminopeptidase. Most other peptidases are unaffected by puromycin, and ERAP1 has been termed "puromycin-insensitive aminopeptidase" (PILS-AP), to distinguish it from cytosol alanyl aminopeptidase [45]. Probestin is a natural product that inhibits aminopeptidase N and also aminopeptidase A. Matlystatin A belongs to the large family of hydroxamate-based metallo-protease inhibitors, in which the hydroxamic acid group forms a bidentate complex with the active site zinc. Therefore, it is a reversible inhibitor of matrix metalloproteinases (MMPs), but inhibits aminopeptidase N [124], as well. Leuhistin is a natural product that is regarded as a specific inhibitor of aminopeptidase N, but also weakly inhibits aminopeptidase A and aminopeptidase B [125]. In a recent study, potent and specific inhibitors of IRAP were identified and shown to be active biologically as cognitive enhancers [126]. These compounds however, were found to show little inhibition versus ERAP1 or ERAP2.

Few of the above aminopeptidase inhibitors have been tested on ERAP1 or ERAP2. Leucinethiol and 1,10-phenanthroline can inhibit ERAP1 and ERAP2 but are by no means specific inhibitors [50, 54, 56]. Amastatin has been shown to be a potent inhibitor of ERAP1 and ERAP2, but bestatin is a very poor inhibitor of those enzymes [54, 56]. Furthermore, very little is known regarding the effects of ER aminopeptidase inhibitors on antigen presentation *in vivo*. Leucinethiol has been shown to affect ERAP1-mediated antigen presentation in cell-based assays [43, 74]. However, leucinethiol is not very specific and can target many intracellular and extracellular metallo-enzymes and a much more specific inhibitor would be needed in order to clearly

evaluate the *in vivo* effects of ERAP1 inhibition. Nonetheless, due to the antigenic peptide editing properties of ERAP1, specific inhibition of ERAP1 opens up the exciting possibility of regulating the immune response on the antigen presentation level. This could lead to a novel paradigm of immune response regulation that operates on a more subtle mechanistical level and manipulates immunodominance rather than shutting down the immune system. Partial or full inhibition of ERAP1 may be used to either silence the presentation of a peptide that contributes to autoimmunity or to enhance the presentation of a peptide that can facilitate recognition of tumour cells. The unique enzymatic properties of ERAP1 [86] can potentially be exploited for the rational design of highly specific inhibitors that can have an *in vivo* effect in antigen presentation. In our laboratory we have recently demonstrated very strong preferences (ranging over 5 orders of magnitude) by ERAP1 for residues distal to the N-terminus of the peptide [71]. These preferences, not seen before for other amino-peptidases, may be the key for the rational design of ERAP1 specific inhibitors. However, the effects of ERAP2 silencing have not been evaluated and the elucidation of the role and specificity of ERAP2 should be necessary in order to fully understand all the components of this pathway, before any pharmacological interventions are undertaken.

CONCLUDING REMARKS

The discovery of the importance of aminopeptidases in antigen presentation and the immune response has given new momentum to aminopeptidase research by providing a novel paradigm of aminopeptidase role in biology. The importance of ERAP1 in antigen presentation *in vivo* is now well established. A potential partner of ERAP1, named ERAP2 has also been proposed. Unique enzymatic properties of ERAP1 have been characterized that appear to fit well with its *in vivo* role. Many questions however remain unanswered. Are the activities of ERAP1 and ERAP2 sufficient to explain all ER aminopeptidase related effects in antigen presentation or more aminopeptidases are needed to supplement trimming of specialized antigenic precursors? Is the activity of ERAP2 necessary or redundant in supplementing ERAP1? Do the apparently different but poorly characterized enzymatic properties of ERAP2 designate a unique role for this molecule *in vivo*? What is the molecular mechanism by which ERAP1 and ERAP2 mediate antigenic peptide generation?

The pharmacological intervention on ER aminopeptidase activity is an exciting avenue for the near future. Although not tested on ERAP1 and ERAP2, many potent aminopeptidase inhibitors exist and considerable experience in the design of aminopeptidase inhibitors is available. The unique enzymatic properties of ERAP1 may offer a strategy for the design of highly specific inhibitors for this molecule. It has already been demonstrated, using the general aminopeptidase inhibitor leucinethiol, that the pharmacological manipulation of antigen presentation via the inhibition of ERAP1 is possible. The observation that ERAP1 activity has not only quantitative but also qualitative (editing) effects on the immune response opens up the exciting possibility of pharmacologically manipulating the immune response on a subtle level, circumventing side-effects associated with traditional immunosuppressive therapies. For example, targeted ERAP1 inhibition could potentially eradicate autoimmunity-sustaining epitopes without a complete shutdown of the immune response. Moreover, ERAP1 inhibition can lead to altered immunodominance hierarchies and enhanced immunogenicity thus providing a new tool in the anti-tumour arsenal. Still, further systematic research is necessary in order to fully understand the role of these molecules in diverse pathological contexts, but the potential for therapeutic possibilities appears to be worth the effort.

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