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Review

Peptide trimming by endoplasmic reticulum aminopeptidases: Role of MHC class I binding and ERAP dimerization

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ABSTRACT

Presentation of short peptides, produced through intracellular proteolysis, by MHC class I molecules (MHC-I) is the basis of adaptive immune surveillance and responses by cytolytic $CD8^+$ T lymphocytes. In the principal pathway of peptide processing for MHC-I that operates in all nucleated cells, MHC-I-binding peptides are produced through stepwise proteolysis starting with source protein degradation by cytosolic proteasome complexes. Among the fraction of proteasome products reaching the lumen of the endoplasmic reticulum, a significant proportion is thought to have a length exceeding that adapted to MHC class I binding and requires N-terminal trimming. This is carried out by one murine and two human endoplasmic reticulum aminopeptidases, the ERAP enzymes. While the critical role of ERAP for producing a ligandome optimized for MHC-I is well documented, it remains unclear how this is mechanistically achieved. In this review, we will discuss the evidence supporting the alternative "MHC template" and "molecular ruler" models that have been proposed to explain how ERAP activity adapts to the ligand requirements of MHC-I. We will also review evidence for dimerization of the two human ERAP enzymes and its potential functional relevance.

1. Introduction

Research in the last 25 years has produced a fairly complete picture of the so-called endogenous antigen processing pathway for MHC-I (for a detailed review see Blum et al. [1]). Cellular proteins, or in dendritic cells also internalized proteins, are first degraded in the cytosol by the proteasome. While most proteasome products are further degraded to amino acids by a panoply of cytosolic peptidases, a fraction of the proteasome output enters the endoplasmic reticulum (ER) through the dedicated heterodimeric TAP transporters. Because the length of fragments produced by the proteasome is not optimized for MHC-I presentation, several steps of length selection are necessary to produce MHC-I ligands. The transport step includes an initial peptide selection, since only peptides with 8 to about 16 amino acids are efficiently transported by TAP [2,3]. Some peptides arriving in the ER therefore can be immediately suitable for MHC-I binding but a large proportion and probably the majority requires an additional step of length adaptation. This is carried out by the ER aminopeptidases first identified 17 years ago [4].

2. The ERAP enzymes

The mouse genome encodes one enzyme, according to author preference referred to as ERAP or ERAAP, and the human genome two complementary enzymes, ERAP1 and ERAP2. All are M1 family aminopeptidases and belong to the oxytocinase sub-family, named according to a third enzyme, oxytocinase or IRAP (insulin-regulated aminopeptidase) that carries out peptide trimming in endocytic compartments [4]. A large body of evidence has demonstrated the substantial impact of the ERAP enzymes on the ligandome of MHC-I and their requirement for efficient antigen presentation. To summarize key findings briefly, the generation of individual MHC class I ligands can be decreased (ERAP-dependent epitopes), not affected, or increased ("over-trimmed" epitopes) in the absence of ERAP [5–10]. Genetic deletion of ERAP results in a reduction of MHC-I at the cell surface by about 20% which is due to reduced stability and presumably increased

Abbreviations: MHC-I, major histocompatibility class I molecule(s); ERAP, endoplasmic reticulum aminopeptidase; TAP, transporter associated with antigen processing; wt, wild-type; ko, knock-out; PLC, peptide-loading complex; TAPBPR, TAP-binding protein related

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internalization and degradation of cell surface MHC-I, while MHC-I assembly and export through the secretory pathway proceed with normal kinetics [6,11,12]. ERAP-deficiency results in a shift to longer peptides among class I ligands [13]. In the mouse model, ERAP-deficient cells present peptides that are antigenic for the $CD8^+$ T cell repertoire of a wild-type (wt) mouse [5,14,15]. Comparing peptide repertoires of ERAP knock-out (ko) and wt cells, it has been suggested that ERAP deficiency, rather than eliminating substantial parts of the wt peptide repertoire, mainly creates an additional peptide repertoire. The antigenicity of this repertoire may rely to a significant extent on its presentation by non-classical MHC molecules [13]. In the mouse, a dedicated repertoire of $CD8^+$ T cells recognizing specific peptides presented by the class Ib molecule Qa-1b may even act as sentinels recognizing and eliminating ERAP-deficient cells [14].

3. ERAP and autoimmunity

Collectively the data discussed identify the ERAP proteins as essential players in the MHC-I antigen processing pathway. The biological importance of these enzymes is underlined by the finding that polymorphism in human ERAP1 and ERAP2 can confer increased risk to a series of MHC-I associated autoimmune and inflammatory diseases [16–19]. These include ankylosing spondylitis (ERAP1 and 2), psoriasis (ERAP1 and 2), Behçet's disease (ERAP1), birdshot retinopathy (ERAP1 and 2) and other diseases. The effect of ERAP polymorphism on the peptidomes presented by the different disease-associated HLA-I allomorphs has been studied in detail mainly by the laboratory of J.A. Lopez de Castro and has recently been reviewed by him [20]. Substitutions in the ERAP proteins generally affect trimming activity and in many cases disease-associated alleles possess high enzymatic activity. However, the effect of an individual substitution may vary according to the peptide substrate analyzed; moreover, at least in one case, an allele protecting from one disease (ankylosing spondylitis) confers increased risk for another (Behçet's disease) [20]. While it is hoped that the frequently epistatic association of ERAP with disease will help to decipher the pathogenetic mechanisms underlying the latter, more work will be required for this.

4. Two mechanistic models of peptide trimming

While the effect of ERAP on the MHC-I ligandome is well established, the molecular context in which ERAP exerts its trimming activity is less well understood. The "challenge" for the ERAP enzymes, as for the entire antigen processing pathway, is to produce peptides adapted to the MHC-I binding site thereby conferring optimal stability and potential for $CD8^+$ T cell stimulation to them. Concerning ERAP, this imposes efficient recognition and processing of potential MHC-I ligand precursors, ability to remove a large variety of chemically variable residues, and preferential avoidance of "over-trimming", i.e. shortening of precursors to a length inferior to the minimum for MHC-I binding. In the case of human ERAP1/mouse ERA(A)P, recognition of potential ligand precursors is ensured by the preference for peptides with a length not exceeding about 16 residues [21], corresponding to the length limit for efficiently TAP-transported peptides [3,22]. Removal of, with the exception of Proline [7], essentially all types of amino acids is possible due to the relaxed specificity of ERA(A)P/ERAP1 and to the additional expression of human ERAP2 with preference for basic residues [10,23–25]. It is mainly the third requirement, preferential production of peptides with an MHC-I-adapted length, that can be matched by two distinct molecular mechanisms proposed in the literature and that relate to alternative mechanistic models of ERAP function. One model, developed mainly for human ERAP1, proposes that the structure of ERAP1 imposes specificity for peptides with a length of 8–16 residues, such that peptides with the minimal length for MHC-I binding are poor ERAP1 substrates. The second model proposes that precursor peptides are trimmed while they are bound to MHC-I molecules, so that substrates become protected from ERAP once they reach the optimal length for MHC-I. Note that the two models are not necessarily mutually exclusive, as discussed below. In this brief review, we will discuss the biochemical, structural and cellular evidence supporting each of the models. We also refer the interested reader to a related recent review by Stratikos and colleagues who discuss the two models in depth, including detailed structural and biochemical considerations [26].

5. The molecular ruler model

The ability of the ERAP enzymes to digest peptides in the absence of MHC-I has been documented by many authors including us [8–10,23,27–29]. However, the concept that ERAP1 does this according to a "molecular ruler" was first proposed by Goldberg and associates who studied processing of synthetic peptides by recombinant human ERAP1 [21]. Analyzing processing of 29 individual peptides as well as a series of peptides with repetitive N-terminal extensions, these authors found that substrates with a length of 9 to 16 residues were preferred by ERAP1, while 8- and 18-mers were trimmed modestly, and 7 and 20-mers not at all. Note the remarkable alignment of this length preference with that of the human TAP transporter [3,30]. ERAP2 did not display a similar length preference and produced smaller peptides, a finding later confirmed by other studies [24,31]. ERAP1 also displayed a preference for large hydrophobic residues at the C terminus, while charged and especially basic residues were poorly trimmed. Given that various human MHC-I alleles bind peptides with basic C-terminal anchors, trimming by ERAP2 may be required to complement ERAP1 for such peptides. ERAP1 also displays some preference for internal peptide residues [23].

Although peptide digestion by other authors tend to confirm the arrest of ERAP1 trimming for peptides with optimal length for MHC-I binding, ERAP1 clearly can "over-trim" i.e. degrade some 8- or 9-mers and destroy some MHC-I ligands [5,32,33]. Moreover, studying ERAAP, the mouse homologue of human ERAP1, Kanaseki and associates did not observe product lengths consistent with a molecular ruler [28]. However, structural studies have provided evidence consistent with the molecular ruler hypothesis.

Multiple ERAP1 and ERAP2 structures are available, revealing highly similar organization in four domains of the two proteins [31,34–37]. Domain II contains the active site and typical M1 peptidase motifs (GAMEN, $HExxH_{18}E$) while domain I binds the peptide N-terminus and caps off the active site. The large domain IV contains a concave surface facing toward the active site and is linked by the small domain III to domain II. Remarkably, an unusual deep cavity extends from domain II to domain IV, providing a path of up to 36 Å potentially accommodating a long peptide [34,36]. Within domain IV of ERAP1, this path may contain a site binding the peptide C-terminus and activating the catalytic site in an allosteric manner. In support of this hypothesis, Stern and colleagues demonstrated activation of the cleavage of a small substrate by long peptides, with an optimum for an 8-mer, presumably the distance between the site binding the C-terminus and the catalytic site [34]. In a different study, Oppermann et al. proposed a deep domain IV pocket as putative secondary binding site [36]. Interestingly, this pocket is absent in the structure of ERAP2 [37], consistent with more efficient destructive processing of MHC-I ligands by this enzyme [31], and thus providing indirect support for a molecular ruler property of ERAP1. A paper reporting the structure of the isolated domain IV also identified a hypothetical binding site for the C-terminus of a poly-histidine peptide, although the poor trimming of peptides with basic C-termini by native ERAP1 limits the significance of this finding [35]. In conclusion, the molecular ruler model is supported by peptide digestion studies with recombinant human ERAP1 and can be accommodated with structural data on ERAP1. However, confirmation of the model will require obtaining ERAP1 structures with long peptides demonstrating not only the secondary binding site but also allosteric

activation of peptide cleavage, a feat that remains to be accomplished.

6. The MHC-I template model

The concept that proteolytic production of MHC-I ligands might be governed by the MHC molecules themselves as templates was first proposed by Rammensee and colleagues, 13 years before the ERAP enzymes were identified [38]. These authors observed that MHC-I ligands could only be detected in peptide extracts of antigen presenting cells if the correct MHC-I molecule was present, a finding consistent with binding of precursors to MHC-I and trimming "on-site". An alternative explanation, also proposed by the authors, was that the ligands were simply protected from destruction by MHC binding, a possibility difficult to rule out in this study as well as in various subsequent studies [38].

The group of N. Shastri, also responsible for first cloning and knocking out mouse ERAAP, has undertaken a series of elegant studies over 15 years that provided evidence consistent with the template model. The group devised an ingenious system allowing to detect precursors of the model ovalbumin epitope SIINFEHL (SHL8, a modification of the native SIINFEKL sequence) by trypsinization of cell lysates. In a first study, these authors found that an SHL8 precursor (K-SHL8) was transported by TAP into the ER where it presumably bound to a chaperone in the absence of the presenting MHC molecule (H-2K^b) but was converted to SHL8 in the presence of K^b [39]. In a subsequent study using microsomes rather than cells, these investigators were able to immunoprecipitate H-2K^b molecules associated with the precursor peptide LK-SHL8 in the presence of an aminopeptidase inhibitor. When the inhibitor concentration was reduced, the precursor was rapidly replaced by minimal SHL8 [40]. Similar results were obtained in a third study analyzing the trimming of a ligand for H-2L^d [28]. Collectively these observations led the authors to conclude that ERAAP acts on intermediate complexes of N-terminally extended peptides bound to MHC molecules. However and in spite of the elegant design of these studies, it is difficult to completely rule out that MHC-I acted not as template during trimming but to protect peptides after trimming of free peptides in solution.

7. Direct evidence supporting the template model

The basic tenet of the template model is that complexes between MHC-I and precursor peptides are substrates for the ERAP enzymes. We and another laboratory have employed covalently linked precursor-MHC-I complexes to examine the template model directly. While James and colleagues studied the effect of ERAP expression on such complexes expressed in transfected cells [41,42], M. Bouvier, in collaboration with us, chose an *in vitro* approach in which precursor-MHC complexes were incubated with recombinant dimeric ERAP1/ERAP2 complexes [43]. Use of ERAP dimers was based on the finding made previously in our group that a fraction of the two human ERAP enzymes can be detected as heterodimers in B lymphocyte microsomes [10]. Given the complementary preferences of the enzymes for removing hydrophobic versus basic residues, this observation had suggested enhanced trimming efficacy through dimer formation, a conclusion corroborated by antigen presentation and biochemical experiments as discussed below. In the experiments performed by Bouvier together with us, HLA-B8 molecules were loaded with 15- or 13-mer precursors of an influenza nucleoprotein epitope and incubated with heterodimeric complexes of human ERAP1 and ERAP2 that were stabilized by complementary jun/fos zipper tags [44]. After a 10-hour incubation, the precursors had been trimmed to the 9-mer minimal epitope, whereas incubation of the free 15-mer resulted in products ranging from 4- to 13-mers within 80 min. Similarly, ERAP1/2 trimming of three other precursors resulted in production of the final epitopes when the precursors were bound to HLA-B8 while a variety of products including short fragments were produced by trimming of the free precursors. These results suggested that ERAP was able to trim MHC-bound precursors. To rule out the possibility that fast on-off precursor dissociation and re-association allowed for trimming in solution, we performed additional experiments with a precursor covalently bound to the HLA molecule that corroborated this conclusion. Interpreting these experiments, we proposed that protrusion or temporary dissociation of 5 to 6 residues out of the groove rendered N-terminal extensions of precursor peptides accessible to ERAP trimming, with the C-terminus retaining the peptide in the MHC-I binding groove [43].

Janes and colleagues used a cellular model to interrogate trimming of MHC-I-bound peptides and arrived at conclusions very much in line with our study. These authors transfected ERAP-deficient cells with a single chain pMHC (H-2K^b) molecule in which the SHL8 epitope or a precursor were not only covalently linked to β2-microglobulin but also anchored at their C-terminus by a disulfide bridge to the MHC-I residue Tyr84. In a first report, they found that co-expression of wt ERAP1 increased stimulation of T cells recognizing SHL8 by cells expressing a single chain molecule containing a precursor peptide. Conversely, co-expression of an ERAP1 variant with very high activity (R725Q/Q730E) reduced T cell stimulation by transfectants expressing the single chain linked to the minimal epitope, indicating over-trimming [41]. More direct evidence for a role of N-terminal peptide dissociation was provided in a second study in which three single chain molecules with mutations in the MHC-I A pocket interacting with the peptide N-terminus were analyzed. T cell stimulation by transfectants expressing SHL8 linked to wt H-2K^b was not affected by co-expression of wt ERAP1, while co-expression of ERAP1 with one of the pocket A mutants reduced T cell stimulation. Structural analysis combined with molecular dynamics simulations suggested that N-terminal peptide moieties can dissociate from an otherwise normally conformed peptide binding domain within a sub-microsecond timescale, exposing the N-terminus for trimming, while a deeply buried peptide C-terminus retains the peptide in the MHC-I groove [42]. Together with our work, these studies provide clear evidence that trimming of MHC-bound epitope precursors can occur and propose a mechanistic model involving fast transient dissociation of the peptide N-terminus. However, before ERAP trimming of MHC-bound peptides can be validated as broadly applicable scenario, experimental evidence concerning ERAP and MHC-I structure will have to be reconciled with this concept.

8. Evidence inconsistent with the template model

Although at least some MHC-I bound precursors can be trimmed by ERAP, clearly not all MHC-I-bound "long peptides" undergo trimming. Peptides with a length exceeding the standard 8–10 residues can be eluted from MHC-I not only in ERAP-deficient but also in wt cells and can be highly immunogenic [13,45]. Such long peptides can "bulge" out of the MHC-I groove between the N- or C-terminus, as demonstrated in structural analysis [46], or more rarely at the N-terminus [47]. A particularly well-documented example was studied by Lopez and associates. The HIV gp160 protein contains a 9- and a 15-mer ligand for the MHC-I allomorph H-2L^d, with the latter being more abundant than the former in cell extracts and H-2L^d immunoprecipitates [48]. While the free 15-mer was converted completely to the minimal 9-mer epitope within one hour of incubation with ERAP *in vitro*, 15-mer/L^d complexes were entirely resistant to trimming, indicating protection from ERAP trimming by MHC-I binding. Importantly, the MHC-I binding affinity of the 15-mer was similar to that of the 9-mer [48,49]. A possible interpretation of these findings is that long peptides with high MHC-I affinity, possibly involving particularly efficient interactions in the A and B pockets of the MHC-I groove, will not undergo fast dissociation cycles and therefore not be accessible for ERAP.

Indirect evidence inconsistent with the template model can be derived from studies of the peptide loading complexes (PLC) formed by MHC-I molecules together with TAP and the chaperones tapasin, calreticulin and ERp57. ERAP was absent from the recent structure of the

PLC determined by Tampé and colleagues [50]. Moreover, the calreticulin P-loop reaching over the MHC molecule in that structure may be an obstacle to ERAP interaction with MHC-I. Although Tampé et al did not indicate whether the Burkitt lymphoma cells used to purify the PLC expressed ERAP, this likely was the case as lymphoid cells usually express significant amounts of ERAP1 and ERAP2 [51]. The template model would also suggest that ERAP and MHC-I can interact physically, however no published evidence documenting such interactions is available, despite efforts of several investigators searching for it. ERAP trimming appears to proceed efficiently in cells lacking TAP or tapasin, raising the question of whether trimming may occur after exit of MHC-I from the PLC [52]. Another fairly indirect argument inconsistent with the template model could be derived from the recent high-resolution structure of an MHC-I complex with the peptide editor TAPBPR (TAP-binding protein-related). This structure shows that a “scoop-loop” of TAPBR competes with binding of the peptide C-terminus to the F-pocket of the MHC-I groove [53]. This led the authors to speculate that MHC-I-peptide interaction is initiated by binding of the peptide N-terminus, a notion in apparent conflict with the template model but also with the experimental findings of James and colleagues unless it is assumed that ERAP trimming precedes peptide editing or (less likely) follows it. Finally, the slow kinetics (10 h) [43] of MHC-I-bound precursor trimming *in vitro* is not consistent with the kinetics of peptide-MHC assembly in cells [1] and suggests that either co-factors accelerating trimming were missing in the assay or that trimming of MHC-bound precursors is not a very efficient way of producing pMHC complexes in a cell.

The available structures of the ERAP enzymes also do not show enzyme conformations that are readily consistent with the template model. In the structure of “open” ERAP1 determined by Stern and colleagues, the sequestered active site in domain II, as well as arrangements of domains I and IV around its mouth, indicate that MHC-I could not approach the active site by less than 20 Å, corresponding to about 6 residues [34]. However transient dissociation of the peptide N-terminus might liberate a sufficient number of residues to match this requirement. More difficult to reconcile with the template model are the conclusions of Oppermann and colleagues who crystallized ERAP1 in one closed and several open conformations. These authors concluded that ERAP1 is inactive in the open state since the S1 pocket accommodating the residue preceding the cleavage site is not properly formed in this state [36]. It is not easy to conceive how an MHC-I molecule could maintain binding to a peptide accommodated in the closed active ERAP1 enzyme.

9. ERAP2 expression

Next to the issue concerning the role of MHC-I in trimming by ERAP, the relative roles of the two human ERAP enzymes are insufficiently understood and a second subject of this review. As mentioned above, the two human enzymes, ERAP1 and ERAP2, show different specificities for the substrate length and sequence [10,21,23,31]. This distinct and complementary specificity extends the range of antigenic epitope precursors that can be processed and adapts it to the peptidome presented by human cells. Although initially ERAP2 tended to be dismissed because of its more restricted specificity and more limited expression than ERAP1, the finding of its association with autoimmune pathologies rekindled interest for this enzyme. In EBV-immortalized B cell lines from healthy donors, both enzymes are usually highly expressed but some lines express only ERAP1 [51]. The latter pattern could be attributed to a splicing variant in the ERAP2 gene that leads to mRNA nonsense-mediated decay. As a result, 25% of individuals do not express ERAP2 [54]. Interestingly, a recent study showed that an allelic variant in the region between ERAP1 and ERAP2 correlates with an inverse expression of the two genes, coupling ERAP2 down-modulation with ERAP1 up-regulation [55]. In tumor cell lines, ERAP1/2 expression is highly variable, and, in some cases, expression is almost undetectable [51]. Malignant transformation of cells tends to result in imbalanced expression of the two enzymes, sometimes in gain but more often in loss of ERAP1 and ERAP2 expression [56,57].

10. Concerted trimming by ERAP1 and ERAP2

Since mice lack ERAP2, ERAP2 has been less studied than ERAP1. Nevertheless, the published reports provide a good functional understanding of the enzyme. In an initial study by our group [10], the two ERAP proteins were found not only to colocalize perfectly with each other but also to partially form heterodimeric complexes, as observed by immunoprecipitation. ERAP1 and ERAP2 coeluted in chromatographic microsome fractionation and were both found in a 230-kDa fraction in density gradient centrifugation that corresponded to the weight of a heterodimer. In addition, a mixture of the two enzymes was able to produce a natural MHC I epitope of HIV much more efficiently than each enzyme alone *in vitro*. Knockdown of the ERAP enzymes reduced MHC-I presentation of the HIV epitope expressed as precursor. Depending on the adaptation of the N-terminal epitope extensions to the specificity of the two enzymes, down regulation of an individual enzyme or of both affected presentation. This study was the first to highlight the functional role of ERAP2 in antigen presentation and the concerted action of the two human enzymes.

ERAP1/2 complexes were later shown to be able to produce other HIV epitopes, with the trimming rates determining the abundance of each epitope and therefore affecting the cytotoxic T lymphocyte hierarchy [58]. This effect was largely dependent on the HIV subtype with production of optimal epitopes being possible only for certain HIV subtypes [59]. However, comparison of epitopes presented in cell lines expressing ERAP1 alone or ERAP1 together with ERAP2 revealed highly similar peptidomes, suggesting that ERAP1 is frequently the dominant trimming enzyme whereas ERAP2 may have a secondary role [60]. Nevertheless, in another study examining the production of five HLA-B27 epitopes *in vitro*, the two enzymes acting in a concerted manner were shown to be much more efficient than each enzyme alone and, in some cases, both required to produce the epitopes [61]. As mentioned above, ERAP1/2 dimers have been used to demonstrate trimming of MHC-bound precursor peptides in vitro [43].

11. Potential ERAP1-ERAP2 dimer structure

Although the evidence discussed above suggests that ERAP heterodimers can be formed, conclusive evidence for the existence of dimers would require their structural characterization. ERAP1-ERAP2 dimers have not been crystallized so far. However, the crystal structure of ERAP2 provides useful information on the potential sites of interaction of the two proteins. ERAP2 crystallized as a homodimer [37]. The interface between the two ERAP2 monomers involves mainly the domain I that is relatively highly conserved in ERAP1 as are most of the residues involved in the dimer interface. As noted by Mavridis and colleagues, a number of additional observations suggest that the ERAP2 homodimer could represent a valid model for the heterodimer [37]. The large extent of the surface between the monomers suggests a high probability of a physiologically relevant interaction. Moreover, the topology of the dimer is such that peptides can access both active sites, that the hinge movement around domains III is unhindered, and that the two catalytic sites face each other, facilitating peptide exchange between the monomers. Interestingly, despite the absence of an ER retention signal, both ERAP1 and ERAP2 are retained in the ER. ER retention of ERAP1 has been proposed to rely on interaction of the sequence encoded by exon 10 with the chaperone ERp44 [62]. An exon of similar length encoding a loop stabilized by a disulfide bond is found in ERAP2 and has been suggested to act as a structural template providing a potential protein-protein interaction module [31].

12. Effect of ERAP dimer formation on function

The evidence cited above and obtained in *in vitro* digestions suggested that ERAP1-ERAP2 mixtures could trim some peptides more efficiently than the individual enzymes but did not reveal whether dimer formation had a functional effect. We addressed this question by producing enzyme dimers stabilized by the interaction of complementary jun/fos zippers [44]. Comparison of the trimming rates of a mixture of ERAP1 and ERAP2 unable to interact with each other with that of stabilized heterodimers showed that the latter produced antigenic epitopes more efficiently. Interestingly, the stable interaction improved only ERAP1 and not ERAP2 trimming activity, suggesting an allosteric effect of dimer formation that increases ERAP1 affinity for substrates. In addition, consistent with physiological formation of heterodimers in antigen-presenting cells, proximity ligation assays suggested physical interaction of the enzymes *in situ* that was enhanced upon IFN-γ stimulation [44].

13. Concluding remarks

As should be clear from the discussion above, the issue of whether the action of the ERAP enzymes is coordinated with MHC-I binding requirements through the template or the ruler model is far from being settled. While at first sight one might think that identifying the correct model is of purely academic interest since the end-product of trimming is identical between the two models, how peptide trimming is adapted to MHC-I likely has functional consequences. The molecular ruler model predicts that recognition of precursors by ERAP1/ERAAP and specifically sensing of precursor length is critical for efficient production of MHC-I ligands, thereby emphasizing interaction of the precursor peptide C-terminus with ERAP. Conversely, the template model emphasizes interaction of this C-terminus with the MHC molecule. In the absence of detailed knowledge about the kinetics of trimming and MHC-I binding *in cellulo*, it is difficult to assess the relative efficacy of the two models. If precursor binding to MHC-I is fast and efficient, then the template model might be the optimal way of capturing the largest number of potential ligands. However, if, as not unlikely, ERAP trimming of peptides in solution is fast, then the molecular ruler model might be the best way to protect precursors from destruction.

Whatever the correct or dominant model, perhaps the necessity of coordination between MHC-I binding and trimming should not be overestimated given that even ERAP2, an enzyme not respecting any molecular ruler with a tendency of epitope destruction [31], impacts the MHC-I ligandome as evidenced by its association with autoimmune diseases [20]. Unless ERAP2 acts strictly on MHC-I bound precursors and/or as activator of ERAP1 in heterodimers, its role in antigen processing and autoimmunity likely indicates that MHC-I molecules succeed in picking enough peptides out of a fairly random peptide mixture produced by an enzyme little adapted to MHC-I selectivity. On the other hand, and if coordination of ERAP trimming with MHC-I selectivity is critical, the two models may not be mutually exclusive. For example, peptides binding particularly well with their C-terminal moieties to the MHC-I groove and/or peptides with longer extensions may be efficient substrates for trimming while MHC-I bound. Conversely, precursors with poor MHC-I affinity and/or peptides produced particularly efficiently as precise final ligands by ERAP enzymes may be trimmed in solution according to the molecular ruler feature of ERAP1, or by ERAP1 activated through dimerization with ERAP2. Among the challenges for the future, providing plausible structural models explaining how MHC-I bound precursors can undergo trimming will be primordial. Producing ERAP1-ERAP2 dimers for structural analysis could also contribute to understanding how peptide trimming shapes the HLA-I ligandome and predisposes to autoimmunity.

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Appendix A. Supplementary data

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