

Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum

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The generation of many HLA class I peptides entails a final trimming step in the endoplasmic reticulum that, in humans, is accomplished by two 'candidate' aminopeptidases. We show here that one of these, ERAP1, was unable to remove several N-terminal amino acids that were trimmed efficiently by the second enzyme, ERAP2. This trimming of a longer peptide required the concerted action of both ERAP1 and ERAP2, both for *in vitro* digestion and *in vivo* for cellular antigen presentation. ERAP1 and ERAP2 localized together *in vivo* and associated physically in complexes that were most likely heterodimeric. Thus, the human endoplasmic reticulum is equipped with a pair of trimming aminopeptidases that have complementary functions in HLA class I peptide presentation.

Major histocompatibility complex (MHC) class I molecules present degradation products of cellular proteins that are supplied by the antigen-processing machinery of the presenting cell. This machinery 'co-opts' cellular proteolytic systems for producing peptides for MHC class I molecules¹. The initial degradation of antigenic proteins is accomplished by cytosolic proteasome complexes, whose cleavages create the C-terminal ends of peptides presented by MHC class I because of the absence of carboxypeptidase activities in the MHC class I processing pathway². In contrast, aminopeptidases with the potential to trim precursors of MHC class I peptides have been identified both in the cytosol and in the endoplasmic reticulum (ER)¹. In the cytosol, proteasome products relevant for antigen presentation may often have a length greater than 15 residues and require trimming by tripeptidyl peptidase II to render them suitable for further handling by the processing system³. Enzymes acting 'downstream' presumably increase the variety of peptides available for MHC class I presentation and/or help to adapt them to the binding requirements of MHC class I molecules. ER trimming enzymes are also essential when final peptides presented by HLA class I are poorly translocated into the ER; this may occur often for some HLA class I 'allomorphs' that bind peptides with anchor positions conferring low affinity for transporter associated with antigen processing (TAP)^{4,5}. In such cases, transport of precursor peptides with higher affinities for TAP, followed by N-terminal trimming by luminal peptidases, is required for efficient peptide presentation^{4,6}.

Considerable progress has been made in the elucidation of peptide trimming in the ER. A metallopeptidase that has been described in other contexts with various designations^{7–9} has been proposed to represent the principal ER enzyme for the trimming of MHC class I ligand precursors^{10–12}. Mouse ER aminopeptidase associated with antigen processing (ERAAP), also known as ER aminopeptidase 1 (ERAP1) in humans, localizes together with ER markers, is induced by interferon- γ (IFN- γ) and shows an unusual preference for peptides with a length of nine or more residues. Experiments using RNA interference have suggested that ERAP1 may be involved in the formation of about one third of peptide–MHC class I complexes^{10,11}. Nevertheless, the net effect of ERAP1 on antigen presentation reportedly varies according to the cell type or epitope studied. 'Knockdown' of ERAP1 mRNA in untreated HeLa cells increases cell surface HLA class I expression, whereas the same treatment of IFN- γ -induced cells reduces it^{10,11}. Given that the enzyme has been reported to trim peptides with little sequence specificity, other than poor cleavage of the X-Pro bond (where 'X' is any amino acid), the reasons for these phenomena remain unclear^{12,13}. A second putative human trimming peptidase has been identified based on its homology with ERAP1 (ref. 14). Like ERAP1, this leukocyte-derived arginine aminopeptidase (L-RAP) is localized in the ER and is induced by IFN- γ . *In vitro* trimming of several synthetic precursor peptides by recombinant L-RAP suggested that the enzyme might also be involved in precursor trimming in the ER¹⁴. The identification of L-RAP

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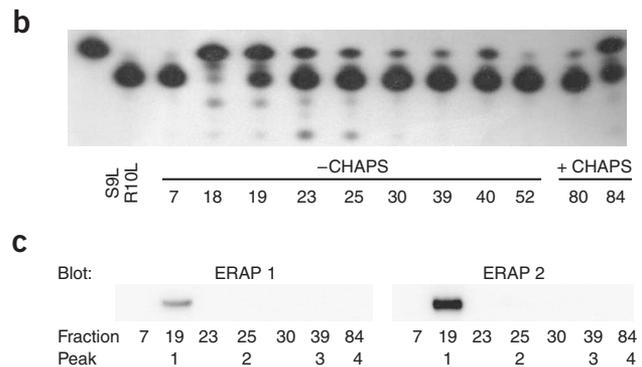
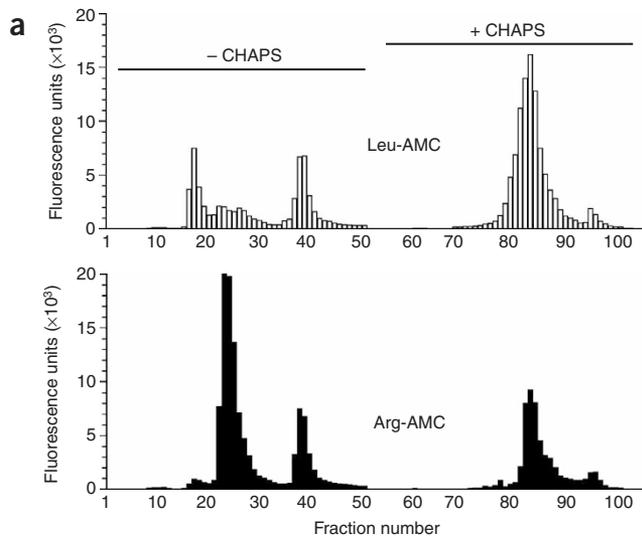


Figure 1 Multiple aminopeptidase activities are associated with human B cell microsomes. **(a)** Aminopeptidase activity of fractionated ER samples. Microsomes prepared from 2×10^9 MGAR B cells were digested with proteinase K to remove extraluminal peptidases, were lysed in PBS with 1% CHAPS and were injected onto an anion-exchange column. Proteins were eluted sequentially by NaCl gradients in the absence (– CHAPS) and presence (+ CHAPS) of 0.5% CHAPS. A portion (150 μ l) of each fraction was tested for activity against Leu-AMC substrate (top) or Arg-AMC substrate (bottom). **(b)** TLC analysis of 7.5 μ l of the fractions obtained in **a** (fractions, below lanes), after incubation for 10 min with 0.5 μ l of 125 I-labeled R10L. Far left, undigested labeled reference peptides S9L and R10L. **(c)** Immunoblot analysis of 10% of the fractions obtained in **a** (fractions, below lanes). Samples were concentrated and were analyzed by immunoblot with mAbs specific for ERAP1 (6H9) or ERAP2 (3F5). Data are representative of at least ten experiments (**a,b**) or of two experiments (**c**).

raised the question of why the human ER is equipped with two trimming aminopeptidases.

An ER activity has been described that removes the N-terminal Arg residue from peptide RSLYNTVATL (R10L), a nonamer epitope derived from human immunodeficiency virus (HIV) gag protein that elicits immunodominant cytotoxic T cell (CTL) responses in HLA-A2⁺ patients infected with HIV¹⁵. In an effort to identify the underlying enzyme, we isolated the two ER enzymes described above as proteins purifying together. We found that the two enzymes formed complexes and combined their restricted respective specificities to remove complex N-terminal extensions, both *in vitro* and in a cellular peptide presentation assay. Therefore, the human ER has a nonredundant system of physically associated aminopeptidases that ensures highly efficient trimming of diverse precursor peptides.

RESULTS

Aminopeptidase activity in human microsomes

To identify the peptidase(s) responsible for trimming of R10L, we fractionated luminal proteins from human microsomes. We used microsomes from a human B lymphoblastoid cell line (MGAR), because such cells have high expression of IFN- γ -regulated proteins linked to antigen processing¹⁶. Using aminoacyl-aminomethylcoumarin (aminoacyl-AMC) substrates to test the aminopeptidase activity of fractionated ER contents, we detected four activity peaks reproducibly (Fig. 1a). The peak 1 activity preferentially hydrolyzed Leu-AMC (fraction 18) and the peak 2 activity hydrolyzed Arg-AMC (fraction 23), whereas the peak 3 activity eluting without CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate hydrate; fraction 39) and the peak 4 activity eluting with CHAPS (fraction 84) hydrolyzed both substrates.

We also tested all fractions for hydrolysis of peptide R10L using thin-layer chromatography (TLC)¹⁵ (Fig. 1b). In multiple experiments, peak 1 activity hydrolyzed peptide R10L to SLYNTVATL

(S9L) with the greatest efficiency. Fractions within peak 4 also had substantial trimming activity, whereas fractions containing peaks 2 and 3 were much less active.

Consequently, we focused on identifying the peptidase(s) contained in peak 1. We used the following techniques sequentially: native polyacrylamide gels overlaid with Leu-AMC substrate, standard SDS-PAGE separation of the proteins in the fluorescent band and analysis of tryptic fragments by tandem mass spectrometry. A single protein band isolated by SDS-PAGE yielded two peptides corresponding to two human aminopeptidases: YQFSLSTEK (GenBank accession number NP_057526) and LNIPTDVLK (GenBank accession number NP_071745). The former corresponds to the enzyme designated ERAP1 (ref. 10) and the latter corresponds to L-RAP¹⁴. Based on the results we present here, we propose use of ‘ERAP1’ and ‘ERAP2’, respectively. We conclude that peptide R10L was trimmed by one or both of the ERAP enzymes.

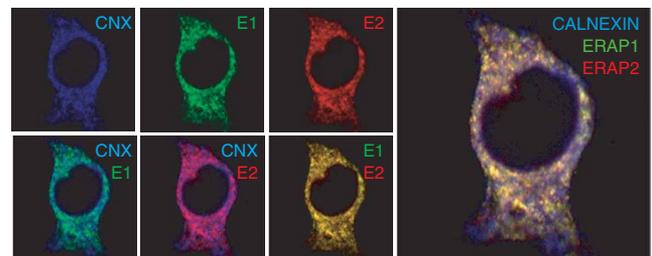
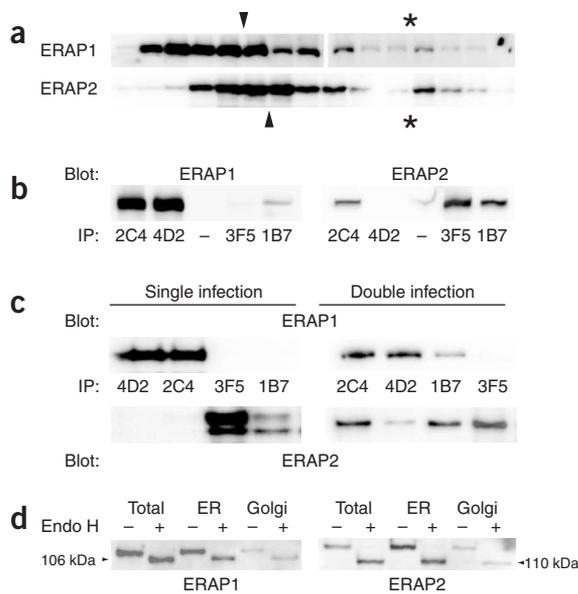


Figure 2 ERAP1 and ERAP2 localize together in the ER. A HeLa cell treated for 48 h with 1,000 U/ml of IFN- γ was stained with antibodies to calnexin (CNX), ERAP1 (E1) and ERAP2 (E2). Top row (left), individual staining; bottom row (left), merging of two fluorescent signals at a time; far right (main image), merging of all three signals. Data represent one of two experiments.



To study ERAP1 and ERAP2 further, we produced recombinant peptidases, which we used to immunize mice for the production of monoclonal antibodies (mAbs). We selected three mAbs for further study of ERAP1; two of these (2C4 and 4D2) recognized native enzyme, whereas mAb 6H9 recognized denatured protein and was used for immunoblots. Both mAbs selected for the study of ERAP2 (3F5 and 1B7) recognized both native and denatured enzyme. We extensively tested all selected mAbs to ensure exclusive recognition of the aminopeptidase used as immunogen.

Given that multiple R10L-trimming activities were associated with B cell microsomes, we screened the four activity peaks for ERAP1 and ERAP2 by immunoblot (Fig. 1c). That analysis demonstrated proteins of the expected molecular weights (about 120 kilodaltons (kDa) for glycosylated ERAP1 and 130 kDa for glycosylated ERAP2) exclusively in peak 1. Thus, human B cell microsomes contain additional, as-yet-unidentified aminopeptidases; at least one of these (the activity in peak 4) can trim peptide R10L.

We analyzed the regulation of the identified aminopeptidase activities by IFN- γ , a hallmark of proteins involved in MHC class I antigen processing. By quantitative PCR and immunoblot analysis, ERAP1 and ERAP2 were both strongly induced after treatment of HeLa cells with IFN- γ (mRNA induction by a factor of 10.5 for ERAP1 and 7.2 for ERAP2, compared with that of untreated cells; data not shown), confirming published reports^{11,12,14}.

ERAP1 and ERAP2 form complexes

ERAP1 and ERAP2 have each been reported to localize together with ER markers^{10,12,14}. However, localization of the two enzymes together with each other has not been reported to our knowledge. We stained IFN- γ -treated HeLa cells simultaneously with antibodies to calnexin (a protein localized mainly in the ER), ERAP1 and ERAP2 (Fig. 2). Both peptidases localized to a large extent, although not completely, together with calnexin. However, immunodetection of ERAP1 and ERAP2 showed complete localization together with each other, suggesting identical subcellular distribution.

These data suggested that the peptidases might interact physically. To address this possibility, we first analyzed the molecular sizes of the peptidases in microsome extracts by zonal sedimentation analysis (Fig. 3a). Although the bulk of the enzymes sedimented in the

Figure 3 ERAP1 and ERAP2 form ER-resident complexes. **(a)** Immunoblot analysis of the distribution of ERAP1 and ERAP2. Digitonin-solubilized microsomal proteins from 2.5×10^7 MGAR B cells were separated according to molecular weight in a sucrose gradient, followed by immunoblot. Arrowheads, aldolase (158 kDa); asterisks, catalase (232 kDa). **(b)** Microsomes from 3×10^8 MGAR B cells were solubilized in 1% digitonin, and peptidases were immunoprecipitated (IP) with mAbs specific for ERAP1 (2C4 and 4D2) or ERAP2 (3F5 and 1B7). The precipitates were divided, followed by immunoblot with unrelated mAbs specific for ERAP1 or ERAP2. **(c)** Sf9 insect cells (5×10^6) were infected with a baculovirus encoding ERAP1 or ERAP2 (Single infection) or with both viruses simultaneously (Double infection). After 36 h, peptidases were immunoprecipitated and were detected by immunoblot as described in **b**. **(d)** ER- and Golgi-enriched fractions from 2×10^7 MGAR B cells were lysed in 1% digitonin and were incubated with immobilized mAb 2C4 specific for ERAP1. The precipitates were digested with endoglycosidase H and were analyzed by separate immunoblots for the detection of ERAP1 and ERAP2. Left and right margins, molecular sizes. Data represent one of two **(a,d)**, three **(c)** or four **(b)** experiments.

fractions corresponding to the molecular weight of a single peptidase molecule of about 120 kDa, a small proportion of each enzyme had a molecular size of about twice this value. The higher-molecular-weight forms most likely correspond to heterodimers of ERAP1 and ERAP2, but may also correspond to homodimers of each enzyme or reflect association of the enzymes with unidentified 'third-party' proteins.

To address the possibility of heterodimer formation directly, we did coimmunoprecipitation experiments in the presence of the mild detergent digitonin. Of two mAbs immunoprecipitating ERAP1, one precipitated a substantial amount of ERAP2 from B cell microsomes (Fig. 3b). In the reciprocal experiment, one of the two mAbs precipitating native ERAP2 also reproducibly precipitated a small but reproducible amount of ERAP1. To confirm those results and to rule out the possibility of weak cross-recognition of the peptidase precipitated together by the mAb used for precipitation, we did similar experiments using insect cells expressing a single peptide or both peptidases (Fig. 3c). In cells expressing a single peptidase (Fig. 3c, left), each of the four mAbs used precipitated only the enzyme it had been raised against. In contrast, those mAbs precipitating a peptidase complex from human microsomes also did so with insect cells infected with both constructs.

To obtain evidence for the subcellular localization of ERAP complexes, we did coprecipitation using sucrose gradient-fractionated organelles of MGAR B cells. ERAP1-ERAP2 complexes precipitating together partitioned into the ER-enriched fraction and carried N-linked carbohydrate moieties fully sensitive to digestion by endoglycosidase H, confirming localization in the ER or cis-Golgi (Fig. 3d). Notably, the percentage of ERAP2 precipitated together with ERAP1 was higher than vice versa (maximum of 20–30% of total ERAP2 versus 10–15% of total ERAP1; Fig. 3b and data not shown). However, this result was compatible with the formation of heterodimeric complexes, as the total protein amount of ERAP2 was about half that of ERAP1 (0.75 ng versus 1.5 ng per 1 μ l of microsomal lysate, respectively). Thus, both human B cell microsomes and insect cells expressing ERAP1 and ERAP2 contained a distinct population of peptidase complexes that could be precipitated together by mAbs specific for either peptidase. These results suggest strongly that ERAP1 and ERAP2 form heterodimeric complexes.

Cooperative peptide trimming by ERAP1 and ERAP2

Limited and distinct specificities of ERAP1 and ERAP2 would be one explanation for the phenomenon that the human ER contains the two trimming enzymes. We studied the specificity of the enzymes for

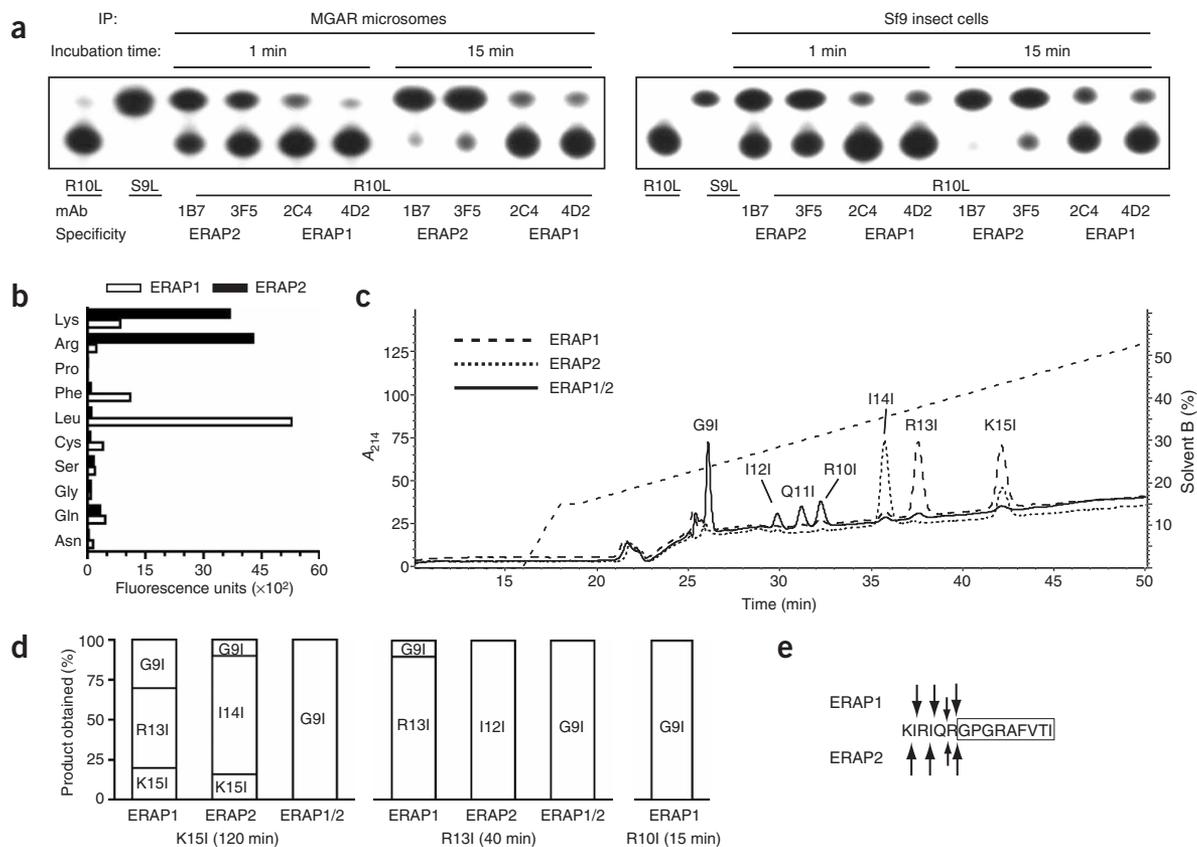


Figure 4 Specific and cooperative peptide trimming by ERAP1 and ERAP2. **(a)** TLC analysis of peptidases immunoprecipitated from MGAR microsomes (left) or Sf9 insect cells expressing a single peptidase (right). Peptidases precipitated from MGAR microsomes (12 ng; left) or recombinant peptidases from Sf9 insect cells (30 ng; right) were incubated with iodinated peptide R10L, followed by TLC. **(b)** Peptidase specificity in aminoacyl-AMC digestions. Peptidases immunoprecipitated from insect cells were incubated with aminoacyl-AMC substrates (vertical axis). **(c)** *In vitro* trimming of peptide K15I. Purified recombinant ERAP1 (3 μ g), immunoprecipitated recombinant ERAP2 (3 μ g) or both together were incubated for 15 min with 5 μ g of peptide K15I. Digestion products were resolved by cation-exchange chromatography. The elution times of the indicated digestion products were determined with synthetic peptides and were confirmed by mass spectrometry. Peptide elution by increasing concentrations of solvent B (dashed line; Solvent B (%), right vertical axis) was monitored by ultraviolet absorbance at 214 nm (A_{214} , left vertical axis). Data represent one of two **(b)**, three **(a)** or four **(c)** experiments. **(d)** Summary of other digestions. For each digestion product, the percentage relative to the total peptide material is plotted. Percentages correspond to the mean values of two, three or four digestions. **(e)** Summary of the results of the digestion of peptide K15I. Each arrow corresponds to a major cleavage site of the peptidase. The sequence of the minimal epitope G9I is boxed.

synthetic AMC substrates and precursors of HLA class I peptides (Fig. 4). In agreement with published work^{8,14}, ERAP2 showed a strong 'preference' for the basic residues Arg and Lys, whereas ERAP1 'preferred' Leu-AMC (Fig. 4b). Next we sought to determine whether the enzymes showed similar specificity for peptide precursors of HLA class I ligands. To answer the initial question of which peptidase trims peptide R10L, we immunoprecipitated the two enzymes from human B cell microsomes and used TLC to study the trimming of labeled R10L (Fig. 4a, left). There was substantial R10L trimming to S9L after 1 min of incubation with ERAP2, and trimming was essentially complete after 15 min. In contrast, ERAP1 was incapable of efficient R10L trimming even with 15 min of incubation. Similar experiments with peptidases expressed in insect cells (Fig. 4a, right) confirmed unequivocally that ERAP2 and not ERAP1 is the peptidase that accounts for the highly efficient trimming of R10L that has been described before¹⁵. Thus, examination of peptide substrate R10L suggested that precursor trimming by the peptidases shows a selectivity matching the spectrum noted with fluorescent substrates.

If this conclusion were correct, it would be conceivable that the two peptidases act jointly to remove longer N-terminal extensions

containing hydrophobic residues, favored by ERAP1, together with basic residues, favored by ERAP2. We studied the *in vitro* trimming of a peptide, KIRIQRGPGRAFVTI (K15I), derived from the HIV IIIIB envelope (env) protein (Fig. 4c). The C-terminal GPGRAFVTI (G9I) nonamer and RGPGRAFVTI (R10I) decamer within K15I are natural ligands for mouse H-2D^d molecules¹⁷. The natural N-terminal extension of K15I (KIRIQR) is composed of a mixture of residues 'preferred' by the two peptidases.

During a 15-minute incubation, ERAP1 processed 50% of the starting substrate K15I to the 13-residue RIQRGPGRAFVTI (R13I), with an N-terminal Arg residue. Accumulation of K15I and R13I suggested that removal of the N-terminal basic residues from these peptides by ERAP1 was inefficient (Fig. 4c). Even after 2 h of digestion, about 20% of the starting peptide K15I remained undigested, whereas 50% had been converted to R13I and 30%, to G9I (Fig. 4d). Similarly, peptide R13I was trimmed very inefficiently by ERAP1. In contrast, peptide R10I, also with an N-terminal Arg, was never detected as a product of K15I or R13I digestion and, when digested directly, was converted completely to G9I (Fig. 4d). Thus, factors other than the N-terminal residue also influence the efficiency

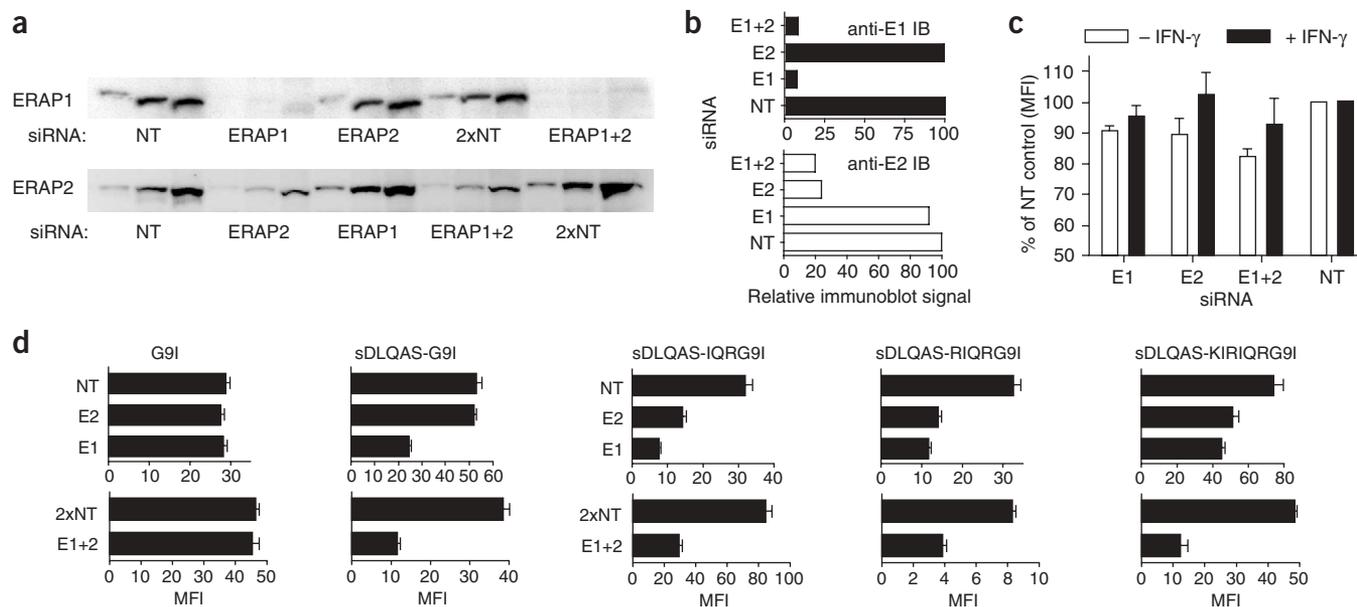


Figure 5 Effect of ERAP knockdown on HLA class I expression and epitope presentation. **(a)** Immunoblot analysis of ERAP expression. HEK 293–H-2D^d cells were transfected with one or two pools of siRNA. After 48 h, IFN- γ was added to cells, followed by immunoblot analysis of 5, 15 or 30 μ g of cell lysate protein after another 48 h. Data represent one of three experiments. NT, nontargeting siRNA; 2xNT, twice the amount of nontargeting siRNA; ERAP1+2, ERAP1 and ERAP2. **(b)** Quantitative evaluation of the blot in **a**. Signal intensities are expressed as percentage of signals obtained for nontargeting siRNA. E1+2, ERAP1 and ERAP2; E1, ERAP1; E2, ERAP2; anti-E1, antibody to ERAP1; anti-E2, antibody to ERAP2; IB, immunoblot. Data represent means of values for each of the three protein amounts. **(c)** Flow cytometry of cell surface MHC class I complexes. HeLa cells were transfected with siRNA pools targeting ERAP1, ERAP2 or both, followed 4 d later by incubation with (+) or without (–) IFN- γ and flow cytometry with mAb W6/32. Equal concentrations of a nontargeting pool were used as controls. Mean fluorescence intensity (MFI) is expressed as percent of the intensity for NT or 2xNT controls. Means and standard deviations derive from seven (ERAP1), four (ERAP2) and three (ERAP1 plus ERAP2) experiments. **(d)** CTL stimulation. HEK 293–H-2D^d cells transfected with siRNA pools (vertical axes) were transfected with plasmids expressing the various epitope forms (top), then were infected with a vaccinia virus encoding ICP47 to block TAP transport (except for G9I), and then were used to stimulate CTLs. CTL stimulation was measured by intracellular staining of CTLs for IFN- γ . Data represent the mean fluorescence intensity and s.d. of triplicate samples.

of digestion by ERAP1. Conversely, when we incubated K15I with ERAP2, the 14-residue intermediate IRIQRGPGRAFVTI (I14I) accumulated after 15 min and after 2 h, again suggesting that an ‘unfavorable’ residue was inefficiently removed (Fig. 4c,d). Similarly, trimming of peptide R13I did not proceed beyond the intermediate IQRGPGRAFVTI (I12I) with an N-terminal Ile (Fig. 4d).

The result of digestion of K15I with a mixture of the two enzymes was very different: within 15 min, most of the peptide was converted to the nonamer product, where digestion stopped (Fig. 4c); after longer digestion of peptides K15I and R13I, there was complete trimming to peptide G9I (Fig. 4d). Therefore, efficient trimming of an extension containing hydrophobic and basic residues required the joint action of ERAP1 and ERAP2, which together are able to generate *in vitro* a natural MHC class I viral ligand (Fig. 4e).

ERAP requirement in cellular antigen presentation

Given that the ER may harbor additional trimming activities, we examined whether the function of ERAP2 noted by *in vitro* digestion and the concerted action of ERAP1 and ERAP2 were vital for antigen presentation in living cells. We used RNA interference to determine whether cooperative trimming by the two peptidases was required for cellular processing of the model HIV env epitope. We obtained reproducible highly efficient knockdown of ERAP1 and ERAP2 mRNA and proteins after transfection of RNA duplex pools (sequences, Supplementary Table 1 online) into HeLa and HEK 293 cells (Fig. 5). The abundance of ERAP RNA was reduced by more than 80% and up to 99% after transfection of one ERAP

RNA pool or of both together, as measured by quantitative PCR in many experiments. ERAP protein expression was also considerably reduced (Fig. 5a,b).

HeLa cells have been used to examine the effect of ERAP1 knockdown on cell surface MHC class I expression, which reflects supply of high-affinity peptides for assembly with MHC class I molecules in the ER¹¹. Simultaneous knockdown of both peptidases reduced cell surface MHC class I expression by about 20%, whereas down-regulation of a single peptidase had only half of this effect ($P < 0.025$ for single and double knockdown; Student’s *t*-test; Fig. 5c). However, the same treatment did not have a significant effect when the antigen-processing machinery including ERAPs was upregulated by IFN- γ treatment ($P > 0.25$).

We also studied presentation of the H-2D^d-restricted HIV env epitope, analyzed above by *in vitro* digestion, by HEK 293 cells with reduced ERAP1 and/or ERAP2 expression. For these experiments we used a polyclonal CTL line producing IFN- γ after recognition of peptides R10I or G9I in the context of H-2D^d (ref. 17). We introduced the epitopes and precursors by transient transfection of HEK 293–H-2D^d antigen-presenting cells with plasmids in which ‘minigenes’ were linked to a CD4 marker by an internal ribosome entry site. This system allowed us to select cells with almost identical expression of CD4, and thus epitopes, for presentation assays. Knockdown of one or both peptidases had no effect on presentation of the minimal nonamer epitope form G9I when it was expressed in the cytoplasm, demonstrating that ERAP knockdown did not affect HLA class I presentation of epitopes not requiring trimming (Fig. 5d, top left).

To study the effect of ERAP knockdown on ER trimming of G9I precursors, we expressed epitopes and precursors downstream of a signal peptide whose removal by signal peptidase left the N-terminal epitope extension DLQAS. To focus the analysis on ER aminopeptidases and to limit recycling of peptides between the ER and the cytoplasm^{18,19}, we blocked TAP transport in antigen-presenting cells using the TAP inhibitor ICP47 (ref. 20).

Knockdown of ERAP1 alone reduced the presentation of all ER-targeted epitope forms substantially (between 42% and 75%; **Fig. 5d**). Knockdown of ERAP2 alone had a similar effect (a reduction of 39–60%) on the presentation of all epitope forms except precursor DLQAS-G9I. Simultaneous knockdown of both peptidases reduced the presentation of two precursors to an extent similar to that obtained by knockdown of a single peptidase, but it had additive effects for the two other precursors. Specifically, double ERAP1-ERAP2 knockdown reduced presentation of the precursor DLQAS-KIRIQRG9I by 75%, compared with approximately 40% for single peptidase knockdown (**Fig. 5d**). These experiments were consistent with the results of the *in vitro* digestion studies and provide strong evidence of an important function for both ER peptidases in the trimming of the model epitope precursors.

DISCUSSION

We set out to identify the R10L-trimming peptidase in the ER and report here strong evidence that ERAP2 not only is the luminal enzyme responsible for trimming this epitope but also has a general function in ER trimming, in conjunction with that of ERAP1. The previous description of production of the HLA-A2 ligand S9L in B cell microsomes by a highly efficient, HLA class I-independent ER peptidase can now be interpreted as a demonstration of the potential of ERAP2 to trim precursors of HLA class I ligands¹⁵. Analysis of a second substrate, peptide K15I, suggested that ERAP2 is required mainly for the removal of basic residues and confirmed that human ERAP1 is poorly suited to remove some extensions better adapted to and trimmed efficiently by human ERAP2. Thus, the human ER is equipped with a pair of aminopeptidases sharing very similar structure, each of which is likely to have an essential but nonoverlapping function in trimming of HLA class I ligands. Identification of ERAP2 as the peptidase trimming R10L also offers the possibility of reconciling conflicting reports on the function of MHC class I molecules for ER trimming^{15,21}: ERAP1 may be MHC class I dependent and ERAP2 may be MHC class I independent. However, interaction of ERAP1 with MHC class I molecules has not been reported so far^{10–12}.

In contrast to previous studies leading to the identification of ERAP1 and ERAP2 (refs. 10,12,14), the protocol we used here resulted in the identification of the two peptidases together. This was probably due to our choice of human microsomes for peptidase identification. In extensive searches of the mouse and rat genomes²², we have not been able to identify ERAP2 homologs. Therefore, the choice of rodent liver microsomes^{10,12} in the previous search strategies probably precluded the identification of ERAP2. In accordance with those studies, we found that both enzymes were strongly induced by IFN- γ and resided in the ER. Using newly developed mAbs, we have shown here complete localization of the two peptidases together. Moreover, we have demonstrated that human B cell microsomes contain several additional, as-yet-unidentified peptidase activities acting on peptide R10L. Although these enzymes may not necessarily be associated with the ER, IFN- γ induction of two additional activities (data not shown) suggests involvement in antigen processing.

We have reported here that ERAP1 and ERAP2 form complexes. This conclusion is based most directly on coprecipitation experiments.

Identification of the two enzymes together from a single ion-exchange fraction and native gel band, perfect localization together by confocal analysis, and the demonstration of a 230-kDa form of each enzyme provide further support for the idea of the existence of ERAP1-ERAP2 complexes. The results obtained in coprecipitation experiments may suggest that only a minor fraction (less than 30%) of both enzymes is engaged in complex formation. However, given that noncovalent protein associations are easily disrupted during lengthy experiments in the presence of detergents, it is difficult to ascertain the proportion of the enzymes forming complexes. Notably, oxytocinase, the enzyme most closely related to ERAP1 and ERAP2 among human peptidases, has been suggested to form homodimers²³. The extremely high homology between ERAP1 and ERAP2 (more than 50% sequence identity) may facilitate heterodimer formation.

Contrary to published suggestions that ERAP1 and ERAP2 hydrolyze peptides without sequence specificity^{12,14}, we found evidence of sequence specificity for both enzymes. Although these specificities are consistent with the preferences noted with aminoacyl-AMC substrates, our results suggest that parameters beyond the nature of the N-terminal residue also affect cleavage. Thus, ERAP1 was unable to remove Lys in the first position and Arg in the third position of K15I but removed the N-terminal Arg efficiently from the decamer substrate R10I. In contrast, the Arg residue at the N terminus of the other decamer substrate tested, R10L, was not removed efficiently by ERAP1. These observations suggest that both the peptide length and the sequence downstream of the N-terminal residue affect the efficiency of cleavage by ERAP1.

RNA interference experiments allowed us to assess the effect of ERAP2 on cellular antigen processing. The effect of ERAP2 knockdown on cell surface MHC class I expression and epitope presentation was of a magnitude similar to that of ERAP1 knockdown, suggesting equivalent functions for the two peptidases in the cells studied. The greater effect of the double knockdown in some cases suggests that each enzyme can function independently, so that their effects are additive, a conclusion consistent with the functionality of recombinant isolated enzymes *in vitro*. The twofold higher expression of ERAP1 than ERAP2 in the cells studied and the presumably more limited expression pattern of ERAP2 relative to that of ERAP1 (ref. 14) are additional challenges to the idea of an absolute requirement for ERAP1-ERAP2 complexes for a functional ER trimming system.

RNA interference experiments also demonstrated that the observations made by *in vitro* digestion of peptide K15I reflected cellular antigen processing; that is, that ERAP1 and ERAP2 trimmed K15I in a concerted way in living cells. However, our studies do not prove that their physical association is also required. By bringing the active sites of the two enzymes in proximity, cooperative peptide trimming should be enhanced and accelerated. However, complexes may also serve other or additional purposes such as allosteric modification of the activity of one or both enzymes or modulation of the subcellular location of one enzyme by association with the 'sister' enzyme carrying a targeting or retention signal.

The effect of single ERAP1 knockdown on surface MHC class I expression we noted here was lower than that reported before¹¹. In part, this may be due to clonal variation in HeLa cells that have been described as ERAP2 negative^{14,24}, whereas we readily detected ERAP2 expression. ERAP2-deficient HeLa cells may rely more heavily on ERAP1 trimming and should be more sensitive to ERAP1 knockdown. Moreover, whereas we noted reduced MHC class I expression only in untreated cells, an effect of ERAP1 knockdown has been noted before only in IFN- γ -treated cells¹¹. The reason for this discrepancy is unclear. It is not entirely unexpected that ERAP knockdown may

have smaller effects in cells that overexpress the enzymes because of treatment with IFN- γ .

It seemed puzzling at first that ERAP2 has no analog in the mouse. Whereas ERAP1, ERAP2 and oxytocinase are encoded in a 200-kilobase segment on the human chromosome 5q21, mouse ERAP1 is encoded by a sequence near a putative breakpoint on chromosome 13, and oxytocinase is encoded on chromosome 17. Mouse ERAP2 may therefore have been destroyed by a recombination event; alternatively, human ERAP2 may have evolved by a relatively recent duplication of ERAP1. Whatever the reason for the lack of ERAP2 in the mouse, the enzyme is apparently not required for efficient trimming in that species. It is possible that a broader specificity of the mouse enzyme, which may have evolved to compensate for the loss of the second enzyme, is the explanation. Alternatively, peptides with N-terminal extensions including Arg and Lys, which are strongly 'preferred' by human TAP transporters^{25,26}, may be imported more frequently into the ER in humans than in mice.

Our results together with previously published work indicate the following picture of trimming in the human ER. ERAP1, the enzyme with wider tissue distribution and possibly also wider substrate specificity^{8,11,12,27}, may represent the dominant trimming enzyme. However, for trimming of complex or longer extensions or of many extensions composed of basic amino acids, ERAP2 may be required. ERAP2 may also be the dominant trimming enzyme in tissues with low ERAP1 expression¹⁴. Additional ER enzymes may complement ERAP1 and ERAP2 or may provide 'backup' trimming activity in cells lacking both known ERAP enzymes. Finally, in cells devoid of all ER trimming enzymes, cytoplasmic trimming activities, acting partly on peptides exported from the ER and reimported by TAP into the ER^{18,19}, may provide yet another 'backup' mechanism for the production of HLA class I-adapted peptides. However, many MHC class I ligands are likely to be destroyed by aminopeptidases^{28,29}. Given the unique preference of ERAP1 (and potentially ERAP2) for peptides with nine or more residues¹¹, the ER may be the only compartment in which productive trimming outweighs epitope destruction by aminopeptidases.

METHODS

Microsome preparation and fractionation. Microsomes prepared by sucrose gradient centrifugation³⁰ from 2×10^9 MGAR homozygous human lymphoblastoid B cells or from 6×10^8 HeLa cells, were suspended in a sucrose-Tris buffer at a final volume of 4 ml (2 ml for HeLa cells)³⁰. For initial identification of peptidases, and where indicated (Fig. 1a), vesicles were digested with 0.4 mg/ml of proteinase K (30 min at 4 °C), followed by washing and incubation with 2 mM phenylmethylsulfonylfluoride (30 min at 4 °C) for proteinase K inactivation, to remove extraluminal proteins before further processing; microsomes used for immunoprecipitation of peptidases and for linear sucrose gradients were not predigested. For the preparation of luminal protein extracts, vesicles were lysed in PBS with 1% CHAPS (Sigma-Aldrich), were clarified by centrifugation and were stored at -80 °C or were used immediately. A portion of the extracts (2 ml) was diluted in chromatography buffer A (10 mM HEPES, pH 7.0, 1 mM dithiothreitol (DTT), 0.1 mM zinc acetate and 5% glycerol) and was injected on a Q HyperD20 anion exchange column (Biosepra) at a flow rate of 0.5 ml/min using a Beckman System Gold 126 module (Beckman-Coulter). For initial peptidase identification, proteins were eluted with two sequential gradients of buffer B (buffer A plus 1 M NaCl): 0–18% over 100 min and then 18–35% over 48 min. For the experiment in Figure 1, the column was eluted with a gradient from 0 to 30% of buffer B over 100 min. For recovery of additional proteins, the column was then equilibrated in buffer A with 0.5% CHAPS and re-eluted with a gradient of 0% to 30% of buffer B with 0.5% CHAPS run over 60 min. Eluate was collected in 0.5-ml fractions.

Peptidase assays. For fluorescence assays, 150 μ l from each high-performance liquid chromatography fraction was mixed with 50 μ l of buffer A containing

2 mM aminoacyl-AMC (Bachem). After 1 h at 37 °C, the reaction was stopped by the addition of 100 μ l acetic acid and fluorescence was measured with a Versafluor fluorometer (BioRad) with excitation at 380 nm and emission at 440 nm. For measurement of the activity of peptidases immunoprecipitated by specific antibodies immobilized on Sepharose 4B beads, 10 μ l of beads was added to 100 μ l of 50 mM Tris buffer, pH 7.5, containing 1 mM DTT and 0.5 mM aminoacyl-AMC. In these experiments, 1 ng of immunoprecipitated recombinant ERAP1 reproducibly hydrolyzed 9 arbitrary units of Leu-AMC in a 40-minute assay and 1 ng of recombinant ERAP2 hydrolyzed 5 arbitrary units of Arg-AMC in equivalent conditions. Based on those specific activities, the fluorescence assay was used for normalization of the amount of peptidases in peptide digestions.

For TLC experiments, trimming of the substrate R10L, labeled with ¹²⁵I by the chloramine T method, was monitored as described¹⁵. In tests of high-performance liquid chromatography fractions, 100 μ l of eluate was added to 5 μ l of labeled peptide followed by incubation for 1–30 min at 37 °C. For the study of immunoprecipitated peptidases, 50 μ l of a buffer of 50 mM Tris and 2 mM DTT plus 5 μ l of ¹²⁵I[R10L] was added to a bead volume corresponding to a known amount of the peptidase (12 or 30 ng), as determined by immunoblot. The reactions were stopped by the addition of buffered phenol (50 μ l or 30 μ l, respectively), and 3 μ l was spotted onto plates (Silica gel 60 F254; Merck).

Purification and identification of aminopeptidases from MGAR microsomes. Fractions from ten column 'runs' containing aminopeptidase activity were pooled and concentrated in Microcon YM3 devices (Millipore). Concentrated fractions were loaded on 10% native acrylamide gels prepared and 'run' in TBE buffer (90 mM Tris, pH 8, 90 mM boric acid and 100 mM EDTA) with 2.5% sucrose. Proteins were separated by electrophoresis for 140 min at 300 V and 4 °C and gels were stained with Arg-AMC. Fluorescent bands were excised and loaded into the wells of standard 10% SDS polyacrylamide gels; proteins were separated by electrophoresis and were visualized by silver staining (Silver SNAP; PerBio Science). Major bands were excised, lyophilized, reduced and alkylated (10 mM DTT and 55 mM iodoacetic acid), were washed in 50 mM (NH₄)₂CO₃ and were trypsinized overnight with 0.5 mg porcine trypsin. Peptides were extracted from the gel matrix with a 60:40 (volume/volume) solution of acetonitrile and 0.1% TFA and were lyophilized and then were analyzed by 'nano' liquid chromatography–tandem mass spectrometry on a quadrupole time-of-flight instrument.

Expression and purification of ERAP1 and ERAP2. Full-length ERAP1 cDNA was amplified by PCR from vector pHA-LAP1 (a gift from M. Tsujimoto, RIKEN, Saitama, Japan) with high-fidelity polymerase (Advantage HF; BD Biosciences Clontech) and the following primers: 5' primer (*NcoI* site in italics), 5'-CCTAACCATGGTGTCTTCTGCCCTCAAATG-3'; 3' primer (*SmaI* site in italics), 5'-CATTACCCGGGCATACGTTCAAGCTTTTCACTTT-3'. Vector pIVEX2.3 (Roche), which contains a poly-His extension and in which the *Bam*HI site had been replaced by a *NotI* site, was digested with *NcoI* and *SmaI*; the PCR amplification product was cloned into the product of that digestion. Finally, ERAP1 was subcloned as an *NcoI-NotI* fragment, including the polyhistidine extension, into the product of the digestion of baculovirus transfer vector pVL1393 with *SmaI* and *NotI*. Full-length ERAP2 cDNA was amplified from plasmid pBacPAK9-hLori with the following primers: 5' primer (*PciI* site in italics), 5'-CCTAACCATGGTTCATTTCTGCAATGGTT-3'; 3' primer (*StuI* site in italics), 5'-CATTAAGGCCTAGTATTAACCATTAGCCAAGTCTCT-3'. After cloning of the PCR product into pCR-Blunt (Invitrogen), a polyhistidine-encoding sequence was joined to its 3' end by transfer of a *SmaI-HindIII* fragment from pIVEX2.3 into the product of the digestion of pCR-Blunt/ERAP2 with *StuI* and *HindIII*. Finally, the polyhistidine-tagged ERAP2 sequence was subcloned as a *StuI-SmaI* fragment into the baculovirus transfer vector pVL1393 (Invitrogen).

Recombinant baculoviruses encoding the two peptidases were produced by transfection of Sf9 insect cells with BaculoGold DNA (BD Pharmingen) together with the transfer plasmids described above. Peptidases were purified from Sf9 cells infected 4–5 d before with recombinant viruses. For purification of native ERAP1, cells were suspended in 20 mM Tris, pH 7.9, with 0.6% CHAPS and protease inhibitors and were lysed with a Dounce homogenizer

and by sonication. ERAP1 was then captured by nickel–nitrilotriacetic acid beads (ProBond; Invitrogen), eluted with 250 mM imidazole and dialyzed against PBS. Contaminants were removed by gel-filtration chromatography on a Superdex 200 column (Amersham-Pharmacia); fractions containing peptidase activity were pooled and concentrated in Amicon Ultra devices (Millipore). Purified ERAP1 resolved as a single band by SDS-PAGE. Although functional ERAP2 could be immunoprecipitated from infected Sf9 cells (discussed below), this peptidase could not be purified in native conditions with nickel–nitrilotriacetic acid resins. For mAb production, denatured ERAP2 was solubilized from insect cell pellets by 8 M guanidine hydrochloride and was bound to nickel–nitrilotriacetic acid beads. The guanidine hydrochloride was then removed by washing of the beads in reducing amounts of urea from 6 M to 0 M. The protein was eluted in 20 mM Tris, 500 mM NaCl, 10% glycerol, 0.2% SDS and 250 mM imidazole, was dialyzed in PBS and was adjusted to 0.06% SDS for further use.

Production of mAbs. BALB/c mice were immunized three times (subcutaneously with complete Freund's adjuvant and then incomplete Freund's adjuvant, plus a final intraperitoneal injection without adjuvant) with recombinant purified protein in PBS with 0.02% SDS for ERAP1 and in PBS with 0.06% SDS for ERAP2, and hyperimmune splenocytes were fused to Ag8.653 myeloma cells. The mAbs obtained were screened sequentially by enzyme-linked immunosorbent assay on plates coated with recombinant proteins and by immunoblot; in both tests, ERAP1 was used as control for ERAP2 and vice versa. ERAP1-specific mAbs were also tested for immunoprecipitation of functional recombinant peptidase. Five mAbs recognizing ERAP1 were selected; three (4D2, 2C4 and 3B6) recognized native protein and three (3B6, 6H9 and 8F2) 'reacted' in immunoblots. Three mAbs recognizing ERAP2 (3F5, 1B7 and 5G7) were used for this study; all 'reacted' in immunoblots, and two (3F5 and 1B7) recognized native protein.

Immunoprecipitation and immunoblots. MGAR microsomes (1 ml) or Sf9 insect cells (7×10^6) infected 60 h before with one or two peptidase-encoding baculovirus(es) were lysed at 4 °C in 0.5 ml of 50 mM Tris, pH 7.5, and 150 mM NaCl with 1% digitonin (Sigma). After clarification and overnight preclearing of lysates, peptidases were immunoprecipitated by incubation for 2 h with 20 μ l of immobilized mAb to peptidase (coupled at a concentration of 5 mg mAb per 1 ml of beads). After three washings, beads were suspended in peptidase assay or gel-loading buffer, as required. Where indicated (Fig. 3d) precipitated proteins were then digested for 12 h at 37 °C with 2 mU of endoglycosidase H (Roche Diagnostics) in 20 mM sodium acetate buffer, pH 5.2, with 0.1% SDS. For immunoblot analysis, proteins were separated by 7% SDS-PAGE and were transferred at 70 V for 90 min (ERAP1) or 40 min (ERAP2) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Sigma) with 10% methanol. Immunoblots were stained by incubation with mAb 6H9 for ERAP1 or 3F5 for ERAP2 at a concentration of 1.5 μ g/ml, followed by a peroxidase-coupled secondary antibody and ECL-Plus (Amersham-Pharmacia) luminescent substrate. Fractions obtained by ion-exchange chromatography or from linear sucrose gradients were concentrated, generally tenfold, for immunoblot analysis by precipitation with the methanol-chloroform method³¹.

Sucrose gradients. ER and Golgi fractions from mechanically lysed MGAR cells were enriched by discontinuous sucrose gradients according to published protocols³² with layers of 0.8 M, 1.2 M, 1.4 M and 1.6 M sucrose and centrifugation for 5 h at 100,000g. The ER-enriched fraction was collected at the interface between 1.4 M and 1.6 M and the Golgi-enriched fraction, between 1.2 M and 0.8 M sucrose. The molecular size of the peptidases was determined by zonal sedimentation analysis on a sucrose density gradient. MGAR microsomes (50 μ l) lysed in 1% digitonin were loaded on a continuous 15–30% sucrose gradient and were fractionated at 190,000g for 12 h. For calibration of the gradient, 250 μ g aldolase and 250 μ g catalase (Amersham) were loaded on duplicate separate gradients and were sedimented in the same ultracentrifuge 'run' as the microsomal proteins.

Confocal microscopy. HeLa cells grown for 48 h in 1,000 U/ml of IFN- γ (Peprotech) were fixed in 4% paraformaldehyde, were permeabilized in PBS with 0.2% BSA and 0.2% saponin (Sigma) and were stained sequentially with rabbit serum to calnexin (1:100 dilution; Stressgen), indocarbocyanine-labeled

donkey antibody to rabbit (1:500 dilution; mAb 3F5 at a concentration of 15 μ g/ml; Jackson ImmunoResearch), indocarbocyanine-labeled goat antibody to mouse (1:200 dilution; Jackson) and, finally, mAb 4D2 labeled with Alexa 488 (30 μ g/ml; Molecular Probes). Images were acquired on a Zeiss LSM510 microscope (Zeiss).

Digestion of peptide K15I. Recombinant purified ERAP1 (3 μ g) and/or recombinant ERAP2 (3 μ g) immobilized on Sepharose beads coupled to mAb 3F5 were (was) incubated for the indicated periods (Fig. 4c,d) at 37 °C with synthetic peptide K15I (5 μ g) in 50 μ l of 50 mM Tris, pH 7.4, and 1 mM DTT. The reaction was stopped and then peptides were separated by cation-exchange chromatography on an S HyperD 10 column (BioSeptra), with 7 mM sodium acetate buffer, pH 4, using 30% acetonitrile as a starting buffer and a gradient of 150–700 mM NaCl over 45 min for elution (0.5 ml/min). Calibration experiments showed that all breakdown products of K15I, produced by stepwise removal of the first six N-terminal residues, could be resolved in these conditions. The identity of peptides in the various peaks was confirmed by quadrupole ion-trap nano-electrospray tandem mass spectrometry in an LCQ Deca XP mass spectrometer (Finnigan Thermoquest).

Quantitative PCR. Total RNA was purified from 2×10^6 cells with the QuickPrep kit (Amersham) and was used to generate cDNA with the Superscript II kit (Invitrogen). A Prism 7700 Seq system (Applied Biosystems) was used for quantitative PCR analysis, with SYBR Green master mix (ABI) and standard PCR conditions. The primers used were as follows: ERAP1 forward, 5'-AACCATGATGAACACTTGGA-3' and reverse, 5'-TCGGATTTGCTGGTGATGAA-3'; and ERAP2, forward, 5'-TGACTTGTGGAGCAGTCTGT-3' and reverse, 5'-GGAGAGTCCATGTAGTCATC-3'. Two 'housekeeping' genes were used for normalization of ERAP-specific PCR: glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-TGCACCACCAACTGCTTAGC-3'; reverse, 5'-GGCATGGACTGTGGTCATGAG-3') and β -actin (forward, 5'-CTGGAACGGTGAAGGTGACA-3'; reverse, 5'-AAGGGACTTCTGTAAACAATGCA-3').

RNA interference experiments. Pools of four specific RNA duplexes for each peptidase and a pool of nontargeting duplexes (as a control; SmartPool; Dharmacon) were used for ERAP knockdown experiments. Oligonucleotides were transfected by electroporation at a concentration of 100 nM for each oligonucleotide; when pools of ERAP1 and ERAP2 small interfering RNA (siRNA) were transfected together, the control pool was used at a concentration of 200 nM per oligonucleotide. Knockdown rates were measured by real-time PCR at 24 h for RNA and by immunoblot at 96 h for ERAP proteins. According to quantitative PCR, the average reduction in mRNA abundance relative to that of cells transfected with nontargeting control duplexes was 88.5% for ERAP1 (range, 80–95; $n = 7$) and 89.9% for ERAP2 (range, 80–95; $n = 7$) for single transfections and 81.4% for ERAP1 (range, 75–86) and 91.2% for ERAP2 (range, 83–99) for double transfections ($n = 5$). Pools of ERAP1 siRNA had no effect on ERAP2 mRNA abundance and vice versa. For study of the effect of ERAP knockdown on MHC class I expression, HeLa cells were used. At 48 h after transfection, cells were detached and replated in six-well plates at a density of 3×10^5 or 5×10^5 cells per well for untreated or IFN- γ -treated cells (1,000 U/ml), respectively. Another 48 h later, cells were detached, were stained with mAb W6/32 and fluorescein isothiocyanate-labeled secondary antibody to mouse immunoglobulins, and were analyzed by flow cytometry.

Minigene expression vectors. Epitope G9I and its precursors were expressed in pMACS4-IRESII (Miltenyi Biotech). Initially, a *Bgl*III-*Eco*RI fragment encoding the gp64 insect cell protein signal peptide was removed from a published pAcUW51-based plasmid³³ and was ligated to pMACS-IRESII opened with *Eco*RI and *Eco*RV. Then, sequences encoding epitope G9I or a precursor were joined to the 3' end of the gp64 sequence by PCR, with a 5' primer including the *Nco*I site upstream of the signal peptide, and a 3' primer encoding the epitope and an *Eco*RI site as overhang. PCR products were cloned into pCR Blunt (Invitrogen) and sequenced and were transferred as *Nco*I-*Eco*RI fragments into pMACS-IRESII. A plasmid encoding epitope G9I without signal peptide was produced similarly but with empty pMACS-IRESII as the PCR template.

CTL assays. Polyclonal CTL lines recognizing the HIV-1-derived, H-2D^d-restricted epitopes consisting of HIV IIIB env, amino acids 318–327 and 319–327 (R10I and G9I), were produced by immunization of BALB/c mice with a recombinant vaccinia virus encoding HIV env, as described¹⁷. Lines were maintained by weekly restimulation with irradiated splenocytes pulsed for 3 h with 10 μ M peptide G9I and were grown in RPMI medium with 10% FCS and 8% T cell growth factor.

For study of the effect of ERAP knockdown on epitope presentation, HEK 293 transfectants expressing H-2D^d were detached 72 h after transfection of siRNA and were transfected in duplicate or triplicate with 20 μ g of a pMACS-IRESII minigene expression construct. Then, 24 h later, cell surface expression of CD4 was measured by flow cytometry and transfectants with similar expression of CD4 (less than 10% variation) were selected for use as antigen-presenting cells. Selected cells were incubated in triplicate for 5 h at a density of 2×10^5 cells/sample with an equal number of CTLs in complete DMEM with 10 μ g/ml of brefeldin A (Sigma-Aldrich). Then the cells were washed, were stained with allophycocyanin-conjugated rat antibody to mouse CD8 at a concentration of 4 μ g/ml (Becton Dickinson) and were fixed for 20 min on ice in 0.2 ml Cytofix buffer, followed by permeabilization by two 5-minute washes in Cytoperm buffer (buffers from Becton Dickinson), and were finally stained with phycoerythrin-labeled rat antibody to mouse IFN- γ (4 μ g/ml; Becton Dickinson). IFN- γ staining of CD8⁺ cells was analyzed with a FACSCalibur cytometer (Becton Dickinson). In some CTL assays, 12–18 h after pMACS-IRESII transfection, HEK 293 cells were infected with a vaccinia virus encoding the viral TAP inhibitor ICP47 (a gift from H. Hengel, Robert Koch Institut, Berlin, Germany) at a multiplicity of infection of 20. After vaccinia infection for 1 h, peptide–MHC class I complexes on the cell surface were removed by acid stripping (incubation for 90 s in 90 μ l of 131 mM citric acid and 66 mM Na₂HPO₄, pH 3, followed by neutralization in 100 volumes of DMEM with 10% FCS). Cells were used for CTL assays 2 h after acid stripping.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

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