

The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues

Ian A. York¹, Shih-Chung Chang², Tomo Saric^{2,*}, Jennifer A. Keys¹, Janice M. Favreau¹, Alfred L. Goldberg² and Kenneth L. Rock¹

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Endoplasmic reticulum (ER) aminopeptidase I (ERAP1) appears to be specialized to produce peptides presented on class I major histocompatibility complex molecules. We found that purified ERAP1 trimmed peptides that were ten residues or longer, but spared eight-residue peptides. *In vivo*, ERAP1 enhanced production of an eight-residue ovalbumin epitope from precursors extended on the NH₂ terminus that were generated either in the ER or cytosol. Purified ERAP1 also trimmed nearly half the nine-residue peptides tested. By destroying such nine-residue peptides in normal human cells, ERAP1 reduced the overall supply of antigenic peptides. However, after interferon- γ treatment, which causes proteasomes to produce more NH₂-extended antigenic precursors, ERAP1 increased the supply of peptides for MHC class I antigen presentation.

Major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (ER) bind peptides derived from most cellular and viral proteins for transport and display on the cell surface. This process allows CD8⁺ T cells to identify and eliminate cells bearing mutations or viral infections^{1–3}. For most proteins, the first step leading to the generation of an MHC-binding peptide is degradation by proteasomes into oligopeptides^{2,4}. MHC class I binds peptides of eight to ten amino acids, depending on the MHC molecule. However, <15% of the peptides produced by purified proteasomes are of the correct length to bind to MHC class I⁵; another 15% are ten residues or longer⁵ and, thus, in principle, can serve as precursors for MHC class I-presented peptides if trimmed by cellular peptidases. The COOH-termini of MHC class I-presented peptides are directly generated by proteasomes^{6,7}, as mammalian cells lack carboxypeptidases that might trim COOH-extended epitopes^{6,8,9}. However, cells contain several aminopeptidases, both in the cytosol and the ER, that may trim NH₂-extended proteasome products to the mature epitopes.

NH₂-terminal trimming of longer precursors by aminopeptidases can generate MHC class I-binding peptides *in vivo*^{2–4}. NH₂-extended antigenic precursors introduced into cells are efficiently processed to mature epitopes and presented on the cell surface^{6,7,10}. This process does not rely on proteasomes because derivatization of the NH₂-terminal residues of the injected precursor peptides, which prevents trimming by aminopeptidases but not proteasomes, blocks peptide presentation by MHC class I⁷. Thus, cellular aminopeptidases are required for processing these precursors. In principle, aminopeptidases may also destroy antigenic peptides, as shortening peptides below the minimal length required for binding to MHC class I would prevent their presentation. Destruction of some antigenic peptides by cytosolic aminopeptidases

has been demonstrated in cell extracts^{4,9,11}. The extent to which aminopeptidases either help generate or destroy antigenic peptides *in vivo* is unknown.

Because most MHC class I peptides and their precursors are produced by proteasomes in the cytosol and are subsequently transported into the ER by the transporter associated with antigen processing (TAP), precursor peptides could be trimmed either in the cytosol or in the ER. The relative contribution of trimming in either compartment is unknown. Several cytosolic aminopeptidases have been implicated in NH₂-terminal trimming of antigenic precursors^{8,12}; one, leucine aminopeptidase, is induced by interferon- γ (IFN- γ)⁸, a cytokine which stimulates antigen presentation. NH₂-terminal trimming of peptides can also occur in the ER; for example, cells *in vivo* present MHC class I epitopes derived from NH₂-extended peptides targeted by a signal sequence to the ER^{6,13–16}. Also, isolated microsomes can import and trim NH₂-extended peptides to the MHC class I-presented epitopes^{11,17–20}. Peptides longer than ten residues readily enter the ER²¹, and some NH₂-extended precursors are transported by TAP more efficiently than the mature peptides^{22–24}. However, the identity of the enzyme in the ER that trims such precursors *in vivo* has remained unclear.

Saric *et al.*, in this issue, isolated an aminopeptidase from the microsomal lumen; they showed that it catalyzed the sequential removal of diverse NH₂-terminal residues from NH₂-extended antigenic peptides in order to generate the ovalbumin-derived epitope SIINFEKL from an NH₂-extended precursor²⁵. This enzyme was shown to be identical to a previously described peptidase of unknown function^{26,27} and has been renamed ER aminopeptidase I (ERAP1)²⁵—to indicate its localization and function—and appears to participate in antigen presentation because it is inhibited by the same agents that block the trimming of

¹Department of Pathology, University of Massachusetts Medical Center, Worcester, MA 01655, USA. ²Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA. *Present address: ATABIS GmbH, Cologne, Germany. Correspondence should be addressed to I.A.Y. (Ian.York@umassmed.edu).

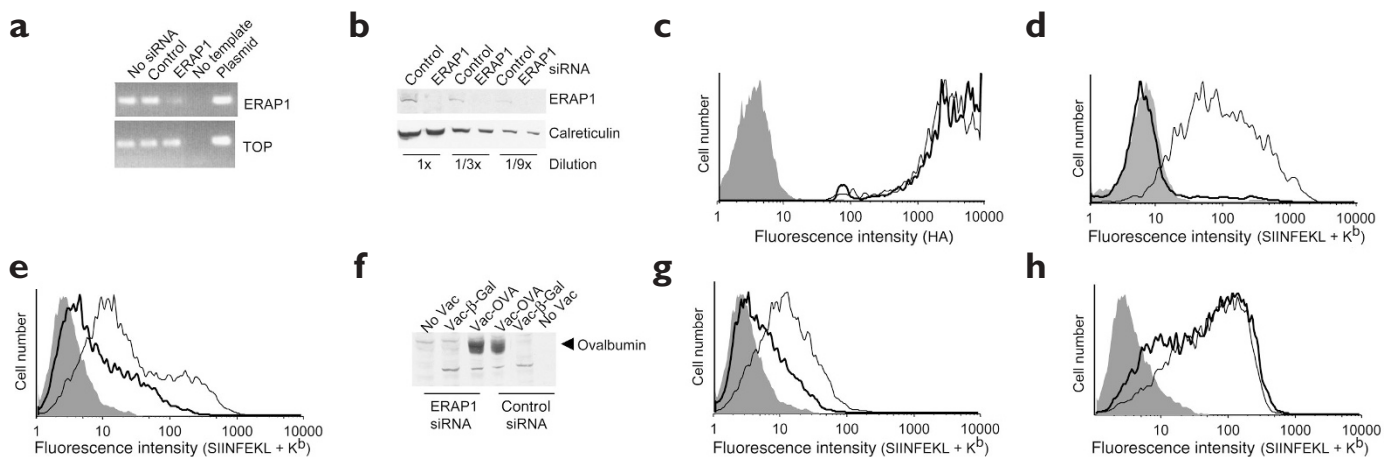


Figure 1. Elimination of ERAP1 with siRNA reduces presentation of SIINFEKL. (a,b) HeLa.K^b cells were treated with siRNA for ERAP1 or the irrelevant gene encoding mouse TOP (mTOP), which acted as a control, for 4 days. Cell viability, as assessed by Trypan blue exclusion, was similar (93.7 ± 4.8% for control siRNA; 89.7 ± 7.3% for ERAP1 siRNA in five independent experiments). (a) RNA was extracted and 30 cycles of RT-PCR were done with primers for ERAP1 or human TOP. (b) Serial threefold dilutions of detergent lysates from siRNA-treated cells were separated on SDS-PAGE and probed with antibodies to ERAP1 or calreticulin. (c) Two days after siRNA treatment, HeLa.K^b cells were transfected with plasmids expressing GFP and influenza HA. After a further 2 days GFP⁺ cells were stained with the mAb H36.4.5 (anti-HA) and analyzed by flow cytometry. MFIs were ERAP1 siRNA, 4306; control siRNA, 3890; background (cells transfected with pTracerCMV vector alone and stained with H36.4.5 or 25.D1.16), 16.2. (d) Experiments were done as in c, except that cells were transfected with LEQLESIINFEKL (the mature epitope SIINFEKL is underlined) targeted to the ER by an NH₂-terminal signal sequence (ss-N5-SIINFEKL) and stained with 25.D1.16 (anti-SIINFEKL-H-2K^b). MFIs were 32.4 for ERAP1 siRNA; 208 for control siRNA; and 14.7 for background. Three days after siRNA treatment, cells were infected for 16 h with recombinant vaccinia virus expressing β-galactosidase or (e) SIINFEKL with a single NH₂-terminal extension (MSIINFEKL), (f,g) full-length ovalbumin or (h) MIINFEKL. (e,g,h) Cells were stained with 25.D1.16 and analyzed by flow cytometry. Background was 25.D1.16 staining of cells infected with control vaccinia expressing β-galactosidase. MFI were as follows: (e) background, 4.2; ERAP1 siRNA, 17.2; control siRNA, 70.3; (g) background, 4.2; ERAP1 siRNA, 7.6; control siRNA, 18.4; and (h) background, 4.3; ERAP1 siRNA, 69; control siRNA, 84. (f) Detergent lysates from uninfected cells (No Vac), from cells infected with vaccinia expressing β-galactosidase (Vac-β-gal) or full-length ovalbumin (Vac-Ova) were separated on SDS-PAGE and probed with anti-ovalbumin. Data are representative of at least three independent experiments. (c–e,g,h) Thick lines, ERAP1 siRNA; thin lines, control siRNA; shaded histograms, background.

antigenic peptides in isolated microsomes and, when transfected, it increases the MHC class I presentation of an NH₂-extended precursor of SIINFEKL targeted to the ER²⁵. Another recently published report has described the same peptidase as “ER aminopeptidase associated with antigen processing” (ERAAP)²⁸.

Another indication that this enzyme is important in antigen presentation *in vivo* is that it is induced by IFN-γ^{25,28}. IFN-γ also induces alternate forms of the proteasome (termed immunoproteasomes), which—in degrading ovalbumin—produce substantially more NH₂-extended peptides compared to constitutive proteasomes²⁹. Such NH₂-extended peptides appear to be more resistant to degradation by cytosolic peptidases than mature epitopes^{4,7}; they may be preferentially transported by TAP^{22,23}, which is also induced by IFN-γ. Taken together these findings suggest that precursor trimming by ERAP1 may be of increased importance for antigen presentation upon stimulation by IFN-γ or other cytokines during infection.

We wanted to clarify the role played by ERAP1 and ER trimming in antigen presentation under different conditions *in vivo*. By reducing ERAP1 expression with small interfering RNA (siRNA), we have shown that ERAP1 is the major aminopeptidase active in trimming a model NH₂-extended precursor targeted to the ER for antigen presentation. ERAP1 also processed precursors of this peptide that were generated by proteasomes in the cytosol. By studying the activity of recombinant ERAP1 in response to many peptides, we have shown it has the distinct property of trimming longer peptides to products of either nine or eight residues. Consequently, under basal conditions, ERAP1 trims many peptides to below the minimal size needed for presentation on MHC class I molecules that bind nine-residue peptides and limits antigen presentation. However, in cells treated with IFN-γ, ERAP1 is particularly important in generating epitopes and increasing MHC class I presentation.

Results

ERAP1 is essential for trimming precursors in the ER

We used siRNA³⁰ to specifically eliminate ERAP1 from HeLa cells stably expressing the murine MHC class I molecule H-2K^b. ERAP1 mRNA, but not mRNA for an unrelated peptidase gene encoding mouse thimet oligopeptidase (TOP), was almost completely eliminated in ERAP1 siRNA-treated cells, as assessed by reverse transcription–polymerase chain reaction (RT-PCR) analysis (Fig. 1a). ERAP1 protein was undetectable in extracts of ERAP1 siRNA-treated cells analyzed by immunoblotting, but it was present in lysates of cells treated with a control siRNA (Fig. 1b). Expression of a control ER protein, calreticulin, was not decreased by ERAP1 siRNA treatment (Fig. 1b). Thus, siRNA treatment caused a highly selective reduction in ERAP1 protein by at least 90%.

Cells treated with ERAP1-specific siRNA remained viable, excluded Trypan blue dye (data not shown) and synthesized TOP mRNA (Fig. 1a) and MHC class I proteins (see below) normally, although they grew about 20% more slowly than controls. Because ERAP1 hydrolyzes peptides in the ER, we determined whether loss of ERAP1 might affect ER functions. The siRNA-treated HeLa cells were transfected with a plasmid expressing the influenza membrane protein hemagglutinin (HA) and the amount of mature HA at the cell surface was measured by flow cytometry. ERAP1 and control siRNA-treated cells expressed similar amounts of mature HA, which indicated that the loss of ERAP1 did not affect the synthesis and transport of a nascent protein into the ER or its subsequent transport to the cell surface (Fig. 1c). Therefore, we could eliminate ERAP1 from HeLa cells in order to study its function in antigen presentation.

After treatment with siRNA, HeLa cells stably transfected with H-2K^b (referred to as HeLa.K^b cells) were transfected with a plasmid

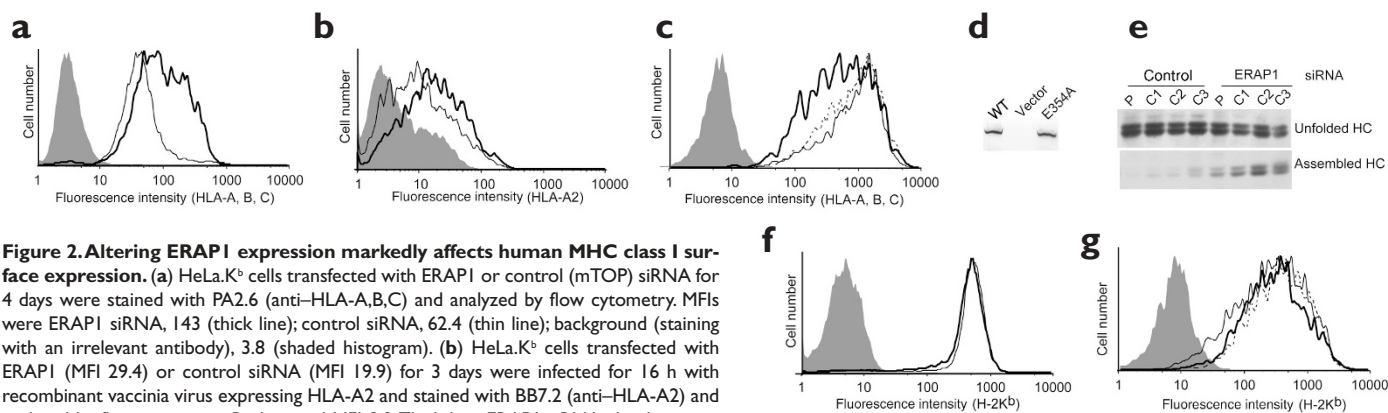


Figure 2. Altering ERAP1 expression markedly affects human MHC class I surface expression. (a) HeLa.K^b cells transfected with ERAP1 or control (mTOP) siRNA for 4 days were stained with PA2.6 (anti-HLA-A,B,C) and analyzed by flow cytometry. MFIs were ERAP1 siRNA, 143 (thick line); control siRNA, 62.4 (thin line); background (staining with an irrelevant antibody), 3.8 (shaded histogram). (b) HeLa.K^b cells transfected with ERAP1 (MFI 29.4) or control siRNA (MFI 19.9) for 3 days were infected for 16 h with recombinant vaccinia virus expressing HLA-A2 and stained with BB7.2 (anti-HLA-A2) and analyzed by flow cytometry. Background MFI 8.0. Thick line, ERAP1 siRNA; thin line, control siRNA; shaded trace, background (cells infected with vaccinia expressing β -galactosidase and stained with BB7.2). (c) COS.K^b cells were transfected with pTracerCMV and plasmids expressing ERAP1 (MFI 896, thick lines), an inactive ERAP1 with an E354A mutation (MFI 1264, dashed line) or with empty vector (MFI 1195, thin line) for 2 days; GFP⁺ cells were stained with PA2.6 and analyzed by flow cytometry. Shaded histogram, background as in a (MFI 10.5). (d) An ERAP1 immunoblot on lysates of transfected cells showed expression of mutant E354A ERAP1. (e) HeLa.K^b transfected with siRNA were pulsed with [³⁵S]methionine and cysteine for 10 min (P) and chased for 10 (C1), 20 (C2) or 40 (C3) min before detergent lysis. Lysates were immunoprecipitated with unfolded HC—HC10 (HLA-B and HLA-C not associated with β_2 M) plus HCA2 (HLA-A not associated with β_2 M) or assembled HC—W6/32 (HLA-A,B,C assembled with β_2 M). Samples were separated on SDS-PAGE and exposed to x-ray film. (f) Experiments were done as in a, and cells were stained with Y3 (anti-H-2K^b). MFIs were background, 5.5; ERAP1 siRNA, 513; control siRNA, 560. (g) Experiments were done as in c, and cells were stained with Y3. MFIs were background, 9.4; vector, 497; ERAP1 plasmid, 518; E354A ERAP1 mutant plasmid, 588. Data are representative of at least three independent experiments.

expressing green fluorescent protein (GFP)—to detect transfected cells—and LEQLESIIINFEKL (the mature epitope SIINFEKL is underlined)—the H-2K^b-binding epitope SIINFEKL from ovalbumin, with a five amino acid NH₂-terminal extension—targeted to the ER with a signal sequence (referred to as ss-N5-SIINFEKL). This precursor peptide, which is a substrate for ERAP1²⁵, is cotranslationally transported into the ER where the extra NH₂-terminal residues must be removed to generate the presented SIINFEKL peptide. Transfected cells were stained with 25.D1.16, a monoclonal antibody (mAb) that specifically recognizes SIINFEKL in combination with H-2K^b (ref. 31) and analyzed by flow cytometry. In the absence of ERAP1, presentation of SIINFEKL was almost undetectable in the ss-N5-SIINFEKL-transfected cells (Fig. 1d). Thus, ERAP1 was essential for trimming this NH₂-extended precursor in the ER, and other ER-luminal peptidases in HeLa cells could not serve this function. This finding enabled us to use ERAP1 siRNA to assess the overall importance of peptide trimming in the ER to antigen presentation.

Trimming of peptides generated in the cytosol

The majority of mature and NH₂-extended antigenic peptides are generated in the cytosol by proteasomes. However it is not known to what extent the trimming of precursor peptides occurs in the cytosol or in the ER after transport by TAP. To examine the relevance of ERAP1 in trimming peptides originating in the cytosol, we used a recombinant vaccinia virus that expresses SIINFEKL with a single extra NH₂-terminal amino acid (MSIINFEKL). ERAP1 siRNA treatment reduced the generation of peptide-MHC complexes from MSIINFEKL to ~20% of controls (Fig. 1e), although expression of protein by recombinant vaccinia viruses was not affected (Fig. 1f). Thus, MSIINFEKL was transported as an NH₂-extended precursor from the cytosol into the ER, where it was trimmed by ERAP1. However, the partial reduction in presentation of MSIINFEKL upon ERAP1 elimination contrasted with the near-complete inhibition that was observed with the ER-targeted ss-N5-SIINFEKL construct (Fig. 1d) and indicated that some of the MSIINFEKL was trimmed by peptidases in the cytosol. These results established that peptidases both in the ER (specifically ERAP1) and in the

cytosol contribute to the trimming of NH₂-extended peptides and to antigen presentation, at least from SIINFEKL minigene constructs.

Purified 26S proteasomes generate from full-length ovalbumin some of the final epitope SIINFEKL, but primarily yield NH₂-extended precursors²⁹ that must be trimmed by aminopeptidases before presentation on H-2K^b molecules. Whether proteasomes also generate primarily NH₂-extended precursor peptides *in vivo* has not been examined. We expressed full-length ovalbumin from vaccinia virus recombinants in HeLa.K^b cells treated with ERAP1 or control siRNA. In agreement with the results we obtained with NH₂-extended peptides expressed from minigenes, elimination of ERAP1 reduced the generation of SIINFEKL-H-2K^b complexes from full-length ovalbumin (Fig. 1g) without affecting expression of ovalbumin itself (Fig. 1f). Similar results were obtained when ovalbumin was expressed from a transfected plasmid (data not shown). Therefore, many NH₂-extended peptides must be generated *in vivo* by proteasomes and subsequently transported into the ER where they are trimmed by ERAP1.

To show that the effects of ERAP1 elimination on antigen presentation were due to the loss of peptide trimming and not to some other effect of ERAP1, we expressed in the cytosol of HeLa.K^b cells an eight-residue peptide, MIINFEKL, that requires no further trimming for presentation. This peptide binds to H-2K^b with an affinity similar to SIINFEKL, and MIINFEKL-H-2K^b complexes are recognized by the mAb 25.D1.16 almost as efficiently as are SIINFEKL-H-2K^b complexes (data not shown). In contrast to the marked reductions in presentation of NH₂-extended precursors that require trimming, ERAP1 elimination only slightly reduced the presentation of MIINFEKL on H-2K^b (Fig. 1h). Thus, the elimination of ERAP1 reduced antigen presentation because NH₂-extended precursors were not trimmed.

ERAP1 destroys many antigenic peptides

Although ERAP1 enhanced antigen presentation by generating epitopes from NH₂-extended precursors in the ER, the potential also exists for antigen presentation to be inhibited by trimming some peptides to products too small to bind to MHC class I. For example, if ERAP1 were to cleave even one residue from MIINFEKL, it would destroy this

Table 1. ERAP1 shows length preferences when degrading substrate peptides

Sequence	Length (residues)	MHC binding	Degradation after 1 h (%)	Degradation after 2 h (%)
<u>GLEQLESIINFEKL</u>	14	H-2K ^b (precursor)	40	70
<u>HGEFAPGNYPAL</u>	12	H-2K ^b (precursor)	75	100
<u>QLESIINFEKL</u>	11	H-2K ^b (precursor)	70	98
FIAGNSAYEYV	11	HLA-A2	29	51
<u>EFAPGNYPAL</u>	10	H-2K ^b (precursor)	60	85
KLGEFYNQMM	10	HLA-A2	44	78
LLDVPTAAV	9	HLA-A2	80	98
FAPGNYPAL	9	H-2K ^b	80	100
<u>ESIINFEKL</u>	9	H-2K ^b (precursor)	51	80
GLVRLNAFL	9	HLA-A2	40	64
YMNGTMSQV	9	HLA-A2	28	57
ELRSLVPFV	9	HLA-A2	18	36
GLQTIVKSL	9	HLA-A2	8	10
YILEETSVM	9	HLA-A2	7	10
YVLDHLIVV	9	HLA-A2	6	8
NLVPMVATV	9	HLA-A2	6	7
TLNAWVKVV	9	HLA-A2	3	4
ILDKKVEKV	9	HLA-A2	2	4
YTVKYPNL	8	H-2K ^b	18	ND
MIINFEKL	8	H-2K ^b	16	22
SIINFEKL	8	H-2K ^b	15	20
SALDFHKV	8	H-2K ^b	15	ND
AVYNFATC	8	H-2K ^b	14	ND
EQYKFYSV	8	H-2K ^b	3	ND
RGYNYEKL	8	H-2K ^b	3	ND
SIYRYYGL	8	H-2K ^b	2	ND

Synthetic peptides (150 nmol/ml) were incubated at 37 °C with purified ERAP1. After 1 and 2 h the concentration of the original peptide was determined by RP-HPLC. For precursors, the mature epitopes are underlined. ND, not determined.

epitope because the product, IINFEKL, is too short to bind to H-2K^b. In that case, elimination of ERAP1 would increase the presentation of MIINFEKL. However, elimination of ERAP1 did not increase the presentation of MIINFEKL, which indicated that this peptide is not destroyed *in vivo*.

To examine the contribution of ERAP1 to overall antigen presentation, we investigated the effect of eliminating ERAP1 on MHC class I assembly and surface expression. Newly synthesized MHC class I complexes are unstable and are retained in the ER until they bind peptide, after which the stable complexes are transported to the cell surface¹⁻³. The generation of stable MHC class I and their expression on the cell surface therefore depends on the supply of peptides in the ER and can be used to monitor overall peptide supply.

HeLa.K^b cells were treated with ERAP1 or control siRNA and the amount of mature MHC class I on the cell surface was quantified with an antibody that reacts with the human MHC class I molecules HLA-A, HLA-B and HLA-C. Elimination of ERAP1 from HeLa cells resulted in an increase in the overall number of human MHC class I molecules on the cell surface (Fig. 2a). In similar experiments, the amount of recombinant HLA-A2 molecules expressed from vaccinia (Fig. 2b) or plasmid (data not shown) vectors on the surface of ERAP1 siRNA-treated cells also increased above control amounts. On the other hand, overexpression of ERAP1 in COS cells reduced the surface expression of MHC class I (Fig. 2c,d), which was exactly the opposite of that observed when ERAP1 was eliminated (Fig. 2a). No such increase in surface MHC class I was seen upon overexpression of an inactive version of ERAP1 in which the catalytic zinc-binding site was mutated²⁵ (Fig. 2c,d). Thus, endogenous ERAP1 expression in HeLa and ERAP1 overexpression in COS cells reduced overall surface

amounts of human or monkey MHC class I.

How ERAP1 affects MHC surface expression was investigated by radiolabeling siRNA-treated cells with [³⁵S]methionine and following the synthesis and assembly of MHC class I by immunoprecipitation. Synthesis of MHC class I heavy chains was unchanged in ERAP1 siRNA-treated cells (Fig. 2e, upper panel). However, these heavy chains assembled into stable heterodimers more rapidly and to a greater extent in the ERAP1 siRNA-treated cells than in controls (Fig. 2e, lower panel), which showed that eliminating ERAP1 increased the number of peptides available to MHC class I. Thus, ERAP1 destroyed many epitopes and limited the overall supply of peptides for human MHC class I. However, many class I molecules were still assembled and transported to the surface of cells even when ERAP1 was overexpressed (Fig. 2c,d). Thus, whereas some peptides were destroyed by ERAP1, many others were resistant to, or escaped destruction by, this peptidase.

Because ERAP1 enhanced antigen presentation from SIINFEKL precursors on H-2K^b, we did similar experiments analyzing the effects of ERAP1 elimination and overexpression on this murine MHC class I molecule. Elimination of ERAP1 in HeLa.K^b cells with siRNA (Fig. 2f) and overexpression of ERAP1 in COS.K^b cells (which were COS-7 cells stably transfected with H-2K^b) (Fig. 2g) had no effect on H-2K^b surface expression. This difference between human and mouse MHC class I was not due to expression from different promoters, as ERAP1 did not affect the synthesis of either H-2K^b (data not shown) or human MHC class I heavy chains (Fig. 2e), and surface expression of a transfected human MHC class I molecule, HLA-A2, increased in the absence of ERAP1 (Fig. 2b).

Hydrolysis of peptides to eight or nine residues

The peptides that bind to H-2K^b are mostly eight residues in length, whereas most human MHC class I molecules predominantly bind peptides of nine or ten amino acids. The specific sequences bound by H-2K^b are also different to those bound by human MHC class I³². The finding that ERAP1 reduced peptide supply to human MHC class I but not mouse H-2K^b molecules suggested that ERAP1 may preferentially hydrolyze a portion of the peptide sequences that binds to human MHC class I. To test this hypothesis we examined the ability of recombinant human ERAP1 to hydrolyze a number of epitopes that bind to H-2K^b or human MHC class I and their extended precursors.

A panel of eight-residue H-2K^b-binding antigenic peptides was incubated individually with purified ERAP1, and their degradation was measured at different times by reverse-phase high performance liquid chromatography (RP-HPLC). All of these H-2K^b-binding peptides were hydrolyzed slowly, <20% per hour (Table 1). One of these poorly degraded eight-residue peptides was MIINFEKL, in accordance with our results showing that elimination of ERAP1 from cells had little effect on the presentation of MIINFEKL expressed from a minigene (Fig. 1h). The finding that ERAP1 weakly degraded other H-2K^b-binding epitopes also explained our *in vivo* findings that ERAP1 did not reduce the overall supply of H-2K^b-binding peptides.

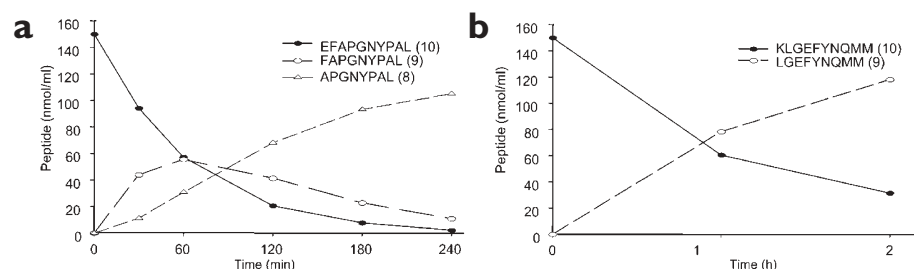


Figure 3. Purified ERAP1 efficiently hydrolyzes peptides to eight or nine residues. Synthetic peptides (a) EFAPGNYPAL or (b) KLGEFYNQMM (150 nmol/ml) were incubated with purified ERAP1 at 37 °C. At various intervals samples were processed as described (see Methods) and analyzed by RP-HPLC to determine concentrations of the original peptide or its derivatives.

We performed a similar analysis on 12 different nine-residue peptides (Table 1). Seven of these peptides were trimmed slowly by ERAP1, which was similar to what we observed with the H-2K^b-binding eight-residue peptides; however, five of these peptides were degraded more rapidly, but only to eight-residue peptides. These observations correlated with our finding that ERAP1 partially reduces the supply of peptides to human MHC class I in unstimulated HeLa cells. In fact many of these nine-residue peptides are ones that bind HLA-A2, an MHC class I molecule for which ERAP1 partially limits peptide supply (Fig. 2b).

In contrast to the results with eight- and nine-residue peptides, we found that peptides that were 10–14 amino acid long were all rapidly trimmed by purified ERAP1. The NH₂-terminal residue of the ten-residue peptide EFAPGNYPAL was rapidly cleaved to yield the nine-residue peptide FAPGNYPAL, which accumulated but then was further processed to the eight-residue peptide APGNYPAL, which was not further degraded (Fig. 3a). This pattern was similar to that observed for QLESINFEKL²⁵. Another ten-residue peptide, KLGEFYNQMM, was rapidly trimmed to LGEFYNQMM (nine-residues long), which was stable (Fig. 3b). In each case, ERAP1 trimmed the longer peptide rapidly to eight- or nine-residue products and then decreased or ceased its hydrolysis. These results suggested that ERAP1 trims longer peptides to a minimal size that is precisely the size range required for high-affinity binding to MHC class I.

ERAP1 enhances presentation in IFN- γ -treated cells

When the peptides supplied to the ER are predominately the nine-residue peptides that bind to human MHC class I, ERAP1 should trim some of

these epitopes and reduce the peptide supply to MHC class I. However, when the supply of NH₂-extended precursor peptides is increased in the ER, ERAP1 should help in generating more nine-residue peptides by trimming these longer versions to mature epitopes. Although some of these nine-residue peptides may be further trimmed and destroyed, others are resistant to ERAP1 (Table 1). Those that transiently accumulate *in vitro* (for example, FAPGNYPAL, Fig. 3a) may bind *in vivo* to MHC class I and be protected from further hydrolysis. The supply of peptides requiring NH₂-terminal trimming should increase after IFN- γ treatment because IFN- γ induces the production of immunoproteasomes and increases expression of TAP, which will transport more precursors. Thus, although causing a net destruction of MHC class I-binding peptides under basal conditions, ERAP1—which is also induced by IFN- γ ^{25,28}—might enhance epitope production in IFN- γ -treated cells.

To test this hypothesis, we compared the effect of ERAP1 elimination on human MHC class I expression in HeLa.K^b cells with or without IFN- γ treatment. In IFN- γ -treated cells, ERAP1 mRNA was up-regulated (Fig. 4a), but the ERAP1-specific siRNA reduced ERAP1 mRNA levels (Fig. 4a) and reduced ERAP1 protein expression by >90% (Fig. 4b). As expected, IFN- γ treatment increased surface expression of MHC class I (compare Figs. 4c to d and 4e to f). However, when ERAP1 was eliminated from IFN- γ -treated cells, expression of surface HLA-A, HLA-B and HLA-C molecules either did not increase or decreased (Fig. 4d), and H-2K^b expression was reduced (Fig. 4f). In other words, after IFN- γ treatment, ERAP1 appeared to play a key general role in generating epitopes for antigen presentation, whereas in the absence of IFN- γ , ERAP1 destroyed many peptides and limited their presentation.

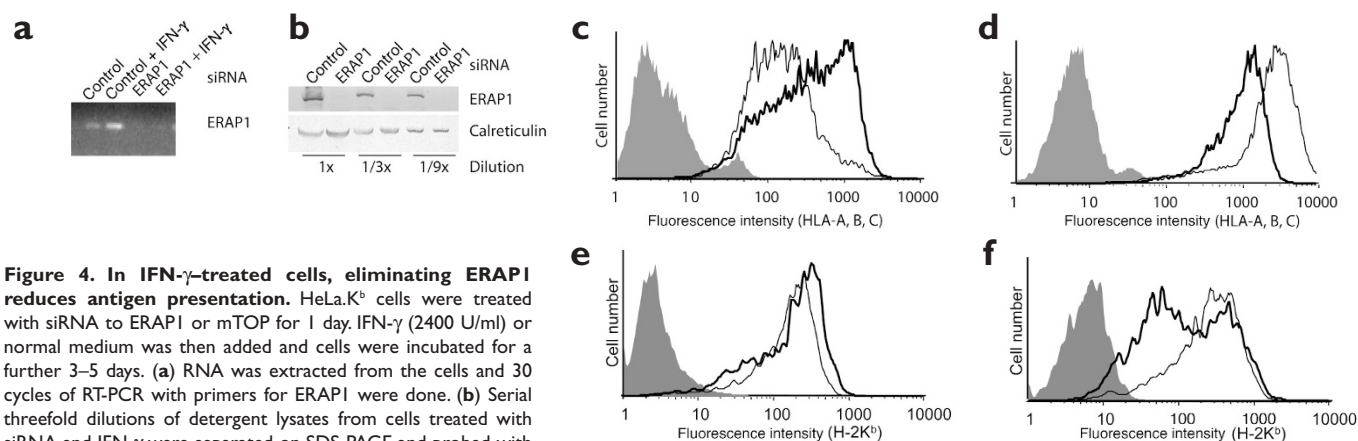


Figure 4. In IFN- γ -treated cells, eliminating ERAP1 reduces antigen presentation. HeLa.K^b cells were treated with siRNA to ERAP1 or mTOP for 1 day. IFN- γ (2400 U/ml) or normal medium was then added and cells were incubated for a further 3–5 days. (a) RNA was extracted from the cells and 30 cycles of RT-PCR with primers for ERAP1 were done. (b) Serial threefold dilutions of detergent lysates from cells treated with siRNA and IFN- γ were separated on SDS-PAGE and probed with anti-ERAP1 or anti-calreticulin. (c) Cells not exposed to IFN- γ were stained with PA2.6 (anti-HLA-A,B,C) or irrelevant antibody (background). MFIs were ERAP1 siRNA, 596 (thick line); control siRNA, 246 (thin line); background, 7.1 (shaded histogram). (d) Experiments were done as in c, except that cells were treated with IFN- γ for 3 days. MFIs were background, 8.1; ERAP1 siRNA, 1189; control, 2898. (e) Experiments were done as in a, except that cells were stained with Y3 (anti-H-2K^b). MFIs were background, 3.6; ERAP1 siRNA, 223; control siRNA, 194. (f) Experiments were done as in e, except that cells were treated with IFN- γ for 5 days. MFIs were background, 6.9; ERAP1 siRNA, 246; control siRNA, 343. Data are representative of at least three independent experiments.

Discussion

By eliminating ERAP1 from cells with siRNA, we have demonstrated here a major role for this enzyme in the trimming of MHC class I precursor peptides in the ER and addressed several outstanding questions about antigen processing. Although several aminopeptidases have been detected in microsomal extracts²⁵, ERAP1 was the major activity that trims an NH₂-extended SIINFEKL construct that was targeted into the ER with a signal sequence. A published report has reached a similar conclusion and termed the enzyme ERAAP²⁸. The small amount of residual SIINFEKL presentation by this construct after siRNA treatment may be due to residual ERAP1, other ER aminopeptidases or trimming in the cytosol of precursors that were not transported or retro-transported out of the ER. Elimination of ERAP1 also affected the overall supply of antigenic peptides in the ER, as shown by changes in assembly and transport of MHC class I. Therefore, ERAP1 must process many antigenic precursor peptides in the ER, and no other ER aminopeptidases can efficiently substitute for ERAP1.

Although both the ER and cytosol contain aminopeptidases that can trim antigenic precursors, the relative importance of these two compartments in this process and the specific enzymes involved were unknown. Because elimination of ERAP1 reduced by ~80% the presentation of an NH₂-extended peptide synthesized in the cytosol from a minigene, the majority of these precursors must be transported into the ER and trimmed by ERAP1. Residual presentation is probably due to trimming in the cytosol, although some other ER aminopeptidase may be involved. However, cytosolic trimming of this antigen is not the major source of antigenic peptides. This finding also fits with the observation that the production of SIINFEKL from a SIINFEKL-ornithine decarboxylase fusion in a cell-free system required the presence of ER vesicles²⁰.

The relevance of ERAP1 in the presentation of any specific antigen must depend on how many peptides of the correct size and how many NH₂-extended precursors are generated by proteasomes, trimmed by peptidases in the cytosol and transported into the ER. During the degradation of ovalbumin, purified 26S proteasomes produce some SIINFEKL but more NH₂-extended versions of this epitope²⁹, and immunoproteasomes generate even more NH₂-extended precursors. Because SIINFEKL presentation from full-length ovalbumin is reduced in cells lacking ERAP1, proteasomes *in vivo* must also generate considerable amounts of NH₂-extended precursors that are transported into and trimmed in the ER. Whether proteasomes degrade other proteins to the mature epitopes³³ or NH₂-extended precursors remains to be established. The importance of ERAP1 in MHC class I presentation probably varies with the antigen and the physiological state of the cell.

A distinct feature of ERAP1 is that it cleaves NH₂-terminal residues from peptides that are ten residues or longer and from many nine-residue peptides, but has much less activity against peptides that are eight-residues long. ERAP1 thus appears to have evolved to generate peptides of eight or nine residues from longer precursors. No other peptidase generates peptides of specific lengths; in fact, several peptidases tend to digest only very small peptides to amino acids. The activity of other aminopeptidases is typically influenced by the nature of the NH₂-terminal residues on the substrate. However, the susceptibility of peptides to ERAP1 cannot be explained simply by the nature of their NH₂ termini because several of the rapidly trimmed substrates (ESIINFEKL, EFAPGNYPAL, GLVRLNAFL and GLEQLESINFEKL) have the same NH₂-terminal residue as the poor substrates (EQYKFYSV and GLQTIVKSL). Because ERAP1 hydrolyzes some nine-residue peptides much faster than others, sequence as well as size must influence its substrate specificity. It will be useful to define

further the substrate specificity of ERAP1 and the molecular basis of this length preference, as these properties can determine the efficiency of MHC class I presentation.

It was unexpected that the size of peptides produced by ERAP1 (eight to nine residues) was precisely that required for stable binding to MHC class I. It is possible that the peptide-binding sites of ERAP1 and MHC class I coevolved to have similar size preferences, and perhaps they share structural features. It will be useful to determine whether special mechanisms have evolved to transfer the trimmed products from ERAP1 to the groove in MHC class I molecules. In any case, ERAP1's ability to generate peptides of appropriate size makes it ideally suited to participate in antigen presentation.

ERAP1's specificity can help explain many of our findings about how ERAP1 affects this antigen presentation *in vivo*. Because purified ERAP1 hydrolyzed poorly all the eight-residue peptides we examined, it is unlikely to destroy these epitopes *in vivo* and thereby reduce the supply of peptides for a MHC class I molecule that specifically binds eight-residue peptides. Accordingly, the loss of ERAP1, or its overexpression, had little impact on the overall supply of peptides to H-2K^b molecules, which predominately bind eight-residue peptides, nor did these treatments alter presentation of the eight-residue peptide MIINFEKL.

It was also unexpected that the loss or overexpression of ERAP1 did not affect the supply of peptides for H-2K^b because *in vivo* NH₂-extended peptides were generated from ovalbumin and trimmed by ERAP1. These findings may imply that SIINFEKL is an exception and that most peptides transported into the ER of HeLa cells, under basal conditions, are already eight-residues long and do not need to be trimmed by ERAP1. Alternatively, the production of some H-2K^b-binding epitopes from longer precursors by ERAP1 may be masked by its tendency to destroy other epitopes. In fact, H-2K^b does bind to a number of nine-residue epitopes³², which may be trimmed to eight-residue peptides and thus destroyed by ERAP1, as we demonstrated for the H-2K^b-binding nine-residue peptide FAPGNYPAL. It is also possible that, under basal conditions, peptide supply for H-2K^b does not limit its expression in HeLa.K^b cells, in which case the production of additional peptides by ERAP1 would have little or no effect on H-2K^b expression and assembly.

Purified ERAP1 rapidly trimmed ten- and some nine-residue peptides and, accordingly *in vivo*, ERAP1 destroyed a portion of the epitopes for human MHC class I molecules, such as HLA-A2, that specifically bind peptides of nine and ten residues. However, antigen presentation was only partially reduced when ERAP1 expression was increased, presumably because ERAP1 destroys only a fraction of the nine-residue peptides and this destruction is probably offset by the increased generation of some epitopes from even longer precursors. In addition, peptides in the ER are presumably subject to a kinetic competition between destruction by ERAP1 and protection by binding to MHC class I.

Because ERAP1 only destroys a subset of nine-residue peptides and because different MHC class I molecules bind different types of peptides, ERAP1 probably has different effects on antigen presentation by different MHC class I molecules. Whether ERAP1 promotes or limits antigen presentation should vary in different cells and depend on the particular MHC class I alleles (for example, whether they bind eight-, nine- or ten-residue peptides), whether the cell contains immunoproteasomes or constitutive proteasomes, the activities of their cytosolic aminopeptidases and probably the sequence flanking the epitopes of the cellular proteins that are being degraded (for example, whether epitopes are produced as NH₂-extended precursors or mature forms). In either case, ERAP1 is an

important factor that influences which peptides get presented.

The destruction of many MHC class I epitopes in the ER may seem wasteful. However, most cells express only low amounts of peptide–surface MHC class I complexes in the absence of inflammation, which may help protect the organism from untoward immune responses such as autoimmunity. In other words, peptide destruction may play a useful role in helping to damp-down the immune system in the absence of infection. In the presence of infection, however, proinflammatory cytokines such as IFN- γ are produced and stimulate the expression of all components of the antigen-presenting pathway, including ERAP1. IFN- γ causes cells to produce immunoproteasomes, which generate more NH₂-extended precursor peptides from ovalbumin²⁹ and peptides of different sequences^{34,35}. IFN- γ also induces PA28³⁶, which—by opening the central pore in the α rings of the proteasome—may also increase the production of longer peptides^{37–39}. These longer peptides appear to be less susceptible to degradation in the cytosol⁴ and may be preferentially transported by TAP^{22,23}. Therefore, after IFN- γ treatment, more NH₂-extended antigenic precursors should be transported into the ER and trimmed by ERAP1. As a result, more epitopes should be generated rather than destroyed. Therefore, in an infection, when proinflammatory cytokines are produced, ERAP1 must help to increase antigen presentation and aid in immune surveillance.

Together, these data indicate that ERAP1 is a key component of the MHC class I antigen-presentation pathway. Its capacity to trim longer peptides to eight- or nine-residue products is distinct and makes this aminopeptidase highly suited to generating epitopes for MHC class I molecules, but also allows it to limit the production of certain epitopes. In either case, ERAP1 is a key factor that greatly influences the repertoire of peptides available for presentation to the immune system.

Methods

Plasmids and viruses. cDNA for human ERAP1 (provided by M. Tsujimoto, RIKEN, Wako, Japan); MSIINFEKL, CD16OVA and ss-LEQLESIINFEKL (described in⁶); and influenza A/PR8/34 HA were subcloned into pTracerCMV (Invitrogen, Carlsbad, CA). Construction of an inactive ERAP1 with the active site E354A mutation was as described²⁵. Cells were transfected with plasmids with the use of Fugene6 (Roche, Indianapolis, IN), according to the manufacturer's directions. Vaccinia ovalbumin, vaccinia MSIINFEKL and vaccinia A2 were as described^{40,41} and were provided by J. Yewdell (NIAID/NIH, Bethesda, MD). Vaccinia MIINFEKL was constructed according to a similar procedure with the use of synthetic DNA oligonucleotides to generate the recombination plasmid.

Cells. COS.K^b cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum and G418 in the presence of 5% CO₂. HeLa.K^b cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and G418 in the presence of 10% CO₂. In some experiments, cells were treated with 500–2400 U/ml of human IFN- γ (Biogen, Cambridge, MA) for 3–6 days.

siRNA and RT-PCR. siRNA for ERAP1 (AACGUAGUGAUGGGACACCAUdTdT and AUGGUGUCCCAUCACUACGdTdT, base pairs 186–208 of human PILS-AP) and mTOP1 (CCUCAACGAGGACACCACCTdTdT and GGUGUGUCCUCGUUGAGGdTdT, base pairs 689–707 of mouse TOP) were from Dharmacon (Lafayette, CO) or synthesized at the University of Massachusetts Center for AIDS Research Core Services (Worcester, MA) and annealed as described³⁰. Cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's directions, except that the transfection was repeated after 4 h.

RNA was prepared from cells with the RNeasy kit (Qiagen, Valencia, CA) and cDNA was prepared with MMLV-RT or Superscript II (both from Invitrogen). Thirty cycles of PCR with the primers GGGAGCTGGAGAGAGGCTAT and CTTGCTTTGAAGGACGGTTC for ERAP1 or ACATGAACACAGGTGGAGGAG and CCGGTACAGGTCCAGGTAGA for human TOP was done with Taq polymerase (Invitrogen).

Antibodies. Rabbit anti-ERAP1⁴² (previously termed anti-A-LAP) was provided by M. Tsujimoto (RIKEN, Wako, Japan). Anti-calreticulin was from Stressgen (Victoria, Canada). Horseradish peroxidase–conjugated goat anti-rabbit was from Jackson ImmunoResearch (West Grove, PA). The mAbs W6/32⁴³ and PA2.6⁴⁴ both recognize HLA-A, HLA-B and HLA-C alleles only in association with β_2 -microglobulin (β_2 M) and cross-react with African Green Monkey (COS cell) A, B and C alleles. HC10 and HCA2⁴⁵

recognize unfolded HLA-B and HLA-C and unfolded HLA-A, respectively. H36.4.5 (anti-influenza HA) was a gift of W. Gerhard (The Wistar Institute, University of Pennsylvania).

Immunoblotting and immunoprecipitation. For immunoblotting, cells were lysed in 1% NP-40, 0.5% deoxycholate and 1 mM EDTA supplemented with phenylmethylsulfonyl fluoride (PMSF); 4 $\times 10^5$ cell equivalents per lane were run on SDS-PAGE and transferred to nitrocellulose membranes. After blocking with PBS, 5% nonfat milk and 0.2% Tween 20, the membranes were probed with specific antibodies followed by horseradish peroxidase–conjugated goat anti-rabbit; the blot was developed with enhanced chemiluminescence (Pierce, Rockford, IL).

For immunoprecipitations, 4 days after siRNA treatment, cells were starved for 1 h in cysteine–methionine–negative medium, then labeled with [³⁵S]cysteine and [³⁵S]methionine for 10 min and chased for various times by adding a tenfold excess of normal medium. Cells were lysed in 1% NP-40, 0.5% deoxycholate and 1 mM EDTA with PMSF. Insoluble material was removed with SpinX tubes (Corning, Corning, NY) and the lysate mixed with antibody bound to protein A–agarose beads (Repligen, Waltham, MA) for 2 h. The beads were washed with 1% NP-40, 0.5% deoxycholate and heated in Laemmli's buffer⁴⁶ for 10 min, separated on 10% SDS-PAGE and exposed to x-ray film.

Flow cytometry. Cells were incubated with primary antibody, washed with PBS, incubated with secondary antibody tagged with phycoerythrin or Cy5 (Jackson ImmunoResearch) and analyzed by flow cytometry on a FACSCalibur apparatus (Becton Dickinson, San Jose, CA) with CellQuest (Becton Dickinson) or FlowJo (Tree Star, San Carlos, CA) software. In some cases (data not shown), directly conjugated primary antibody was used to confirm that changes in mean fluorescence intensity (MFI) were similar to those obtained with a secondary antibody approach. When cells were transfected with GFP-expressing plasmids (pTracerCMV and derivatives), electronic compensation was used to prevent spectral emission from GFP from interfering with the emission of other fluorophores, and gates for GFP⁺ cells were used to limit the analysis to transfected cells.

Peptide-trimming assays. Peptide-trimming assays were done as described²⁵. Briefly, synthetic peptides (150 nmol/ml) were incubated with purified ERAP1 at 37 °C in 50 μ l of buffer (50 mM Tris-HCl at pH 7.8 and 0.5 μ g/ml of bovine serum albumin (Sigma, St. Louis, MO)). Reactions were terminated with trichloroacetic acid or trifluoroacetic acid, precipitated protein was removed by centrifugation and peptide-containing supernatant was analyzed by RP-HPLC on a C18 column with a linear gradient of 7–31.5% acetonitrile²⁵. The identity of peptides peaks was determined by comparing their retention times to pure synthetic peptides. The amount of each peptide present was calculated by the integration of peptide peaks.

Genbank accession numbers. Accession numbers were AF183569 for human PILS-AP and BC031722 for mTOP1.

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Competing interests statement

The authors declare that they have no competing financial interests.

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