

**Luminex**  
complexity simplified.

## Guava® SARS-CoV-2 Multi-Antigen Antibody Assay

New assay for SARS-CoV-2 antibody detection on your flow cytometer  
For Research Use Only. Not for use in diagnostic procedures.



Learn More >



## Intramembrane Proteolysis of Signal Peptides: An Essential Step in the Generation of HLA-E Epitopes

This information is current as of September 18, 2021.

Marius K. Lemberg, Felicity A. Bland, Andreas Weihofen, Veronique M. Braud and Bruno Martoglio

*J Immunol* 2001; 167:6441-6446; ;  
doi: 10.4049/jimmunol.167.11.6441  
<http://www.jimmunol.org/content/167/11/6441>

**References** This article **cites 47 articles**, 22 of which you can access for free at:  
<http://www.jimmunol.org/content/167/11/6441.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

\*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2001 by The American Association of  
Immunologists All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Intramembrane Proteolysis of Signal Peptides: An Essential Step in the Generation of HLA-E Epitopes<sup>1</sup>

Marius K. Lemberg,<sup>2\*</sup> Felicity A. Bland,<sup>2†</sup> Andreas Weihofen,<sup>\*</sup> Veronique M. Braud,<sup>3†</sup> and Bruno Martoglio<sup>4\*</sup>

Signal sequences of human MHC class I molecules are a unique source of epitopes for newly synthesized nonclassical HLA-E molecules. Binding of such conserved peptides to HLA-E induces its cell surface expression and protects cells from NK cell attack. After cleavage from the pre-protein, we show that the liberated MHC class I signal peptide is further processed by signal peptide peptidase in the hydrophobic, membrane-spanning region. This cut is essential for the release of the HLA-E epitope-containing fragment from the lipid bilayer and its subsequent transport into the lumen of the endoplasmic reticulum via the TAP. *The Journal of Immunology*, 2001, 167: 6441–6446.

The MHC class I molecules HLA-A, -B, -C, and -G are expressed with a typical signal sequence for targeting to the secretory pathway. Their signal sequences contain a highly conserved segment that is eventually presented at the cell surface by the nonpolymorphic nonclassical MHC class I molecule HLA-E (1, 2). There, the HLA-E-peptide complexes can bind to CD94/NKG2A receptors on NK cells and inhibit NK cell-mediated lysis (3–5). Cells that fail to express MHC class I molecules on the cell surface, e.g., certain virus-infected cells and tumor cells, are thought to be eliminated by NK cells (6). Thus, via the signal peptide fragment, HLA-E-peptide complexes indirectly report the expression level of numerous polymorphic MHC class I molecules and provide an additional level of control to the direct recognition of surface class I molecules by the killer Ig-like receptors on NK cells (7).

The pathway that yields HLA-E-binding epitopes derived from MHC class I signal sequences is not known. During translocation of proteins through the translocons at the endoplasmic reticulum (ER)<sup>5</sup> membrane, signal sequences are usually cleaved off from the pre-protein by signal peptidase (8). At this stage, some liberated signal peptides are thought to span the ER membrane at their central hydrophobic region, with the N terminus facing the cytosol and the C terminus exposed toward the ER lumen (9). The conserved HLA-E binding epitope of polymorphic MHC class I molecules is

located in the N-terminal portion of their signal sequences (see Fig. 1A). We therefore hypothesized that the liberated N-terminal MHC class I signal peptides are initially released from the ER membrane toward the cytosol. This model would fit with the requirement of a functional TAP transporter for HLA-E cell surface expression (2, 10).

Using an in vitro system, we previously reported that the liberated signal peptide of the hormone prolactin (p-Prl) is processed within the hydrophobic portion by a signal peptide peptidase (SPPase) (11). This cut was found to be critical for the release of the N-terminal signal peptide portion from the membrane toward the cytosol. In this study, we show in vitro and in living cells that intramembrane proteolysis of MHC class I signal peptides by SPPase is essential for the generation of HLA-E-binding epitopes.

## Materials and Methods

### Plasmid construction

The *SalI/HindIII* fragment of pBK-CMV/HLA-A\*0301 was transferred into pSV-Sport1 (Life Technologies, Carlsbad, CA) under the control of the SP6 promoter to give pSV-Sport1/HLA-A\*0301. To generate the signal sequence mutant (HLA-Aspmt, see Fig. 2 for sequence), codons 14, 15, 16, 18, and 20 of the coding region were exchanged by the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the sense primer 5'-CTCCTCTGCTACTCGTGGTGCTCCTGCTCGCCAGACCTGGGCGGG-3' and the antisense primer 5'-CCGCCCCAGGTCTGGCGAGGAGCAGGAGCACCACGAGTAGCAGGAGGAG-3'. It resulted in pSV-Sport1/HLA-Aspmt. To generate a mutant of HLA-A\*0301 with an extended signal peptide (HLA-Asp<sup>ext</sup>, see Fig. 1 for sequence) the coding region of pSV-Sport1/HLA-A\*0301 was amplified by PCR using the sense primer 5'-AGTCAGGTCGACCATGGGCAAGAACAGCAAGGTGGCCGTCATGGCGCCCCG-3', which included a *SalI* restriction site and codons for the six additional amino acids (underlined). A standard T7 primer was used as reverse primer. The *SalI/HindIII* fragment of the resulting PCR product was transferred into pSV-Sport1 to generate pSV-Sport1/HLA-Asp<sup>ext</sup>. For stable transfections, the insert of pSV-Sport1/HLA-Aspmt was subcloned into the *BamHI* restriction site of pCDNA3 (Invitrogen, San Diego, CA).

### In vitro transcription, translation, and signal peptide processing

To prepare mRNA coding for HLA-A\*0301/124, HLA-Aspmt/124, and the signal peptides of HLA-A\*0301, HLA-Aspmt, and HLA-Asp<sup>ext</sup>, the respective coding region was amplified with PCR using Pfu DNA polymerase (Stratagene), SP6 primer, and a reverse primer, starting with 5'-NNNNNNNNNCTA, to introduce a TAG stop codon at the desired position. PCR-amplified DNA fragments were transcribed in vitro with SP6 RNA polymerase at 42°C in the presence of 500 μM m<sup>7</sup>G(5')ppp(5')G CAP analog (New England Biolabs, Beverly, MA) (12).

<sup>\*</sup>Institute of Biochemistry, Swiss Federal Institute of Technology (Eidgenössische Technische Hochschule), Zurich, Switzerland; and <sup>†</sup>Medical Research Council, Human Immunology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom

Received for publication August 1, 2001. Accepted for publication October 4, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by grants from Eidgenössische Technische Hochschule, Zurich and the Swiss National Science Foundation (to B.M.) and the Medical Research Council (to F.A.B.). V.M.B. was a Royal Society University Research Fellow.

<sup>2</sup> M.K.L. and F.A.B. contributed equally to this work.

<sup>3</sup> Current address: Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, 06560 Valbonne, France.

<sup>4</sup> Address correspondence and reprint requests to Dr. Bruno Martoglio, Institute of Biochemistry, Swiss Federal Institute of Technology (Eidgenössische Technische Hochschule), 8092 Zurich, Switzerland. E-mail address: bruno.martoglio@bc.biol.ethz.ch

<sup>5</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; p-Prl, prolactin; SPPase, signal peptide peptidase; wt, wild type.

Translations of mRNA coding for HLA-A\*0301/124 and HLA-Aspmt/124 were performed in 25  $\mu$ l of reticulocyte lysate (Promega, Madison, WI) containing [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) and, where indicated, two equivalents of nuclease treated rough microsomes prepared from dog pancreas and *N*-glycosylation inhibitor *N*-benzoyl-Asn-Leu-Thr-methylamide (13) and 5  $\mu$ M (Z-LL)<sub>2</sub>-ketone (11). Samples were incubated for 15 min at 30°C. Microsomes were extracted with 500 mM KOAc and prepared for SDS-PAGE as described previously (11). For extraction with alkali, KOAc-extracted microsomes were treated with 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 11.3 (13). Translations of mRNA coding for the signal peptides were translated in 25  $\mu$ l of wheat germ extract at 25°C for 15 min (13).

Signal peptide processing with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate-solubilized microsomal membrane proteins was performed as described elsewhere (11), except that the signal peptidase inhibitor *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (250  $\mu$ M; Sigma-Aldrich, St. Louis, MO) was added to the reaction mixture to prevent cleavage of the HLA-A signal peptides at a cryptic signal peptidase cleavage site.

### Electrophoresis

Proteins and peptides were analyzed by SDS-PAGE using Tris-bicine gels (14). Membrane pellets and proteins precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or trichloroacetic acid were dissolved in sample buffer containing 360 mM bis-Tris, 160 mM bicine, 1% SDS, 50 mM DTT, 15% sucrose, and 0.004% Serva blue. All samples were incubated for 15 min at 65°C. Proteins were finally separated on 15% T, 5% C, 8 M urea acrylamide gels (70  $\times$  80  $\times$  1 mm). Labeled proteins were visualized by a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### HLA-E cell surface expression

To prepare stably transfected cells expressing mutant HLA-A\*0301 (HLA-Aspmt), 721.221 were electroporated with 30  $\mu$ g of the respective plasmid DNA at 270 V with a capacitance of 1500  $\mu$ F (15). Stable transfected clones were obtained after 3 wk. Surface expression of HLA-A\*0301 and HLA-E was monitored by flow cytometry using GAP-A3 and DT9 Abs followed by PE-labeled anti-mouse (Fab')<sub>2</sub> (Sigma-Aldrich) (2).

### TAP transport

721.221 cells were permeabilized according to Jadot et al. (16), except that digitonin (0.006%) was used instead of saponin. For TAP transport (17), 3  $\times$  10<sup>5</sup> permeabilized cells were incubated in 25  $\mu$ l of 50 mM HEPES-KOH (pH 7.6), 150 mM KOAc, 5 mM Mg(OAc)<sub>2</sub>, 250 mM sucrose, and 1 mM DTT, and 60 nM [<sup>125</sup>I]-labeled RRYQNSTEL (9 Ci/mmol) and 2  $\mu$ l of ATP mix (12.5 mM ATP, 3.5 U/ $\mu$ l creatine kinase, and 110 mM creatine phosphate). ATP was depleted by the addition of 0.3 U of hexokinase and 20  $\mu$ mol of glucose during the assay. After the reaction, salt concentration was raised to 500 mM KOAc, and cells were separated by a 3-min centrifugation through a 100- $\mu$ l sucrose cushion (50 mM HEPES-KOH (pH 7.6), 500 mM KOAc, 2 mM MgOAc<sub>2</sub>, and 500 mM sucrose) at 48,000 rpm and 4°C in a Beckman TLA100 rotor (Beckman Coulter, Fullerton, CA). [<sup>125</sup>I]-Labeled peptide in the membrane fraction was quantified by gamma-counting and analyzed by SDS-PAGE as previously described (11). All values were determined by three independent assays.

### Peptide binding to HLA-E

HLA-E-binding assays were conducted as described previously (1). 721.221 cells were starved for 60 min in methionine-free medium followed by labeling with 100  $\mu$ Ci of [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech.) per 10<sup>7</sup> cells for 1 h. Cells were then washed in ice-cold PBS and lysed for 20 min at 4°C in lysis buffer (40 mM Tris-HCl (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM iodoacetamide, and 2 mM PMSF) in the presence or absence of the indicated concentrations of peptide or with the conformation-dependent Ab W6/32. Supernatants were heated for 2 min at 44°C before preclearing by the addition of 10% *Staphylococcus aureus* cells (Pansorbin; Calbiochem, San Diego, CA) overnight. Peptide-HLA-E complexes were recovered by immunoprecipitation using W6/32 and protein A-Sepharose beads (Sigma-Aldrich). Samples were analyzed by 1D-IEF (18) followed by autoradiography. Peptide binding was quantified by densitometry of the HLA-E H chain band using a FLA-2000 Image Analyzer (Raytek Scientific, Sheffield, U.K.). Results are expressed as a ratio of ODs (OD<sub>peptide</sub> - OD<sub>PBS</sub>/OD<sub>W6/32</sub> - OD<sub>PBS</sub>)  $\times$  100 and referred to as a percentage of binding to HLA-E. Each peptide was tested in three independent assays.

## Results

### The signal peptide of classical MHC class I molecules is processed by SPPase

Although MHC class I molecules are highly polymorphic, their signal sequences are relatively conserved. This is consistent with their role in providing a conserved peptide to HLA-E. To characterize how these peptides are generated, we investigated in vitro the fate of a representative MHC class I molecule, HLA-A\*0301. mRNA coding for the signal sequence plus 100 additional residues of HLA-A\*0301 (HLA-A/124) was translated in reticulocyte lysates in the presence of ER-derived rough microsomes (Fig. 1B). Microsomes were subsequently isolated and analyzed for [<sup>35</sup>S]methionine-labeled translation products. As expected, HLA-A/124 was translocated into the microsomes and yielded HLA-A/100 after cleavage of the signal sequence (Fig. 1B, lane 2). The 24-residue-long signal peptide was not found in the membrane fraction, suggesting it had been further processed.

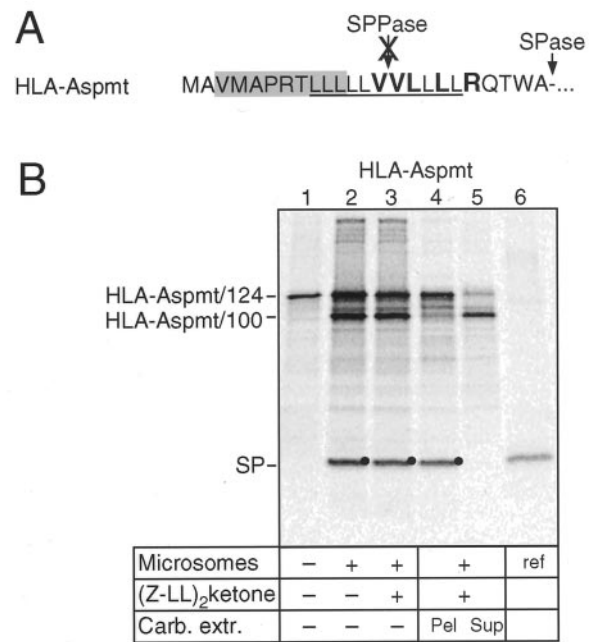
We have recently reported that in vitro the liberated signal peptide of p-Prl was rapidly processed by SPPase, and signal peptide fragments were released from the membrane. However, when SPPase was inhibited by the new specific inhibitor (Z-LL)<sub>2</sub>-ketone, the unprocessed signal peptide remained associated with the microsomes (11). To test whether SPPase was also involved in the processing of MHC signal sequences, the SPPase inhibitor was added to a reaction with HLA-A/124. The signal peptide of HLA-A\*0301 was found to be associated with the microsomes in the presence of the SPPase inhibitor (Fig. 1B, lane 3). Extraction with sodium carbonate revealed a tight interaction of the signal peptide with the lipid bilayer similar to typical transmembrane proteins (Fig. 1B, lanes 4 and 5) (19). These findings indicate that the HLA-A\*0301 signal peptide liberated from the pre-protein is further processed by SPPase, inducing the release of signal peptide fragments from the membrane.

In an attempt to locate the SPPase cleavage site, a protease assay was performed with in vitro-translated signal peptide and detergent-solubilized, partially purified SPPase (11). The HLA-A\*0301 signal peptide was cleaved (Fig. 1C, lane 2), but cleavage products were not detected, most likely because the expected short peptide could not be fixed on the gel. When (Z-LL)<sub>2</sub>-ketone was added, processing of the signal peptide was inhibited (Fig. 1C, lane 3). To identify cleavage products on the gel, an extended signal peptide (SP<sup>ext</sup>) with six additional residues (MGKNSKVAVM... ) at the N terminus was applied in the assay. SP<sup>ext</sup> was processed by SPPase like the wild-type (wt) signal peptide (Fig. 1C, lane 5). The observed cleavage product, which was labeled by the methionine residues in the N-terminal portion, had an electrophoretic mobility similar to a peptide corresponding to the N-terminal 20 residues of SP<sup>ext</sup> (Fig. 1C, lane 7). This result indicates that SPPase cleaves the peptide in the center of the hydrophobic region where the helix-breaking serine and glycine residues are located (Fig. 1A). The result is consistent with the previous finding that the signal peptide of p-Prl is cleaved by SPPase in the center of the hydrophobic region at the helix-breaking serine and asparagine residues (Fig. 1A) (11).

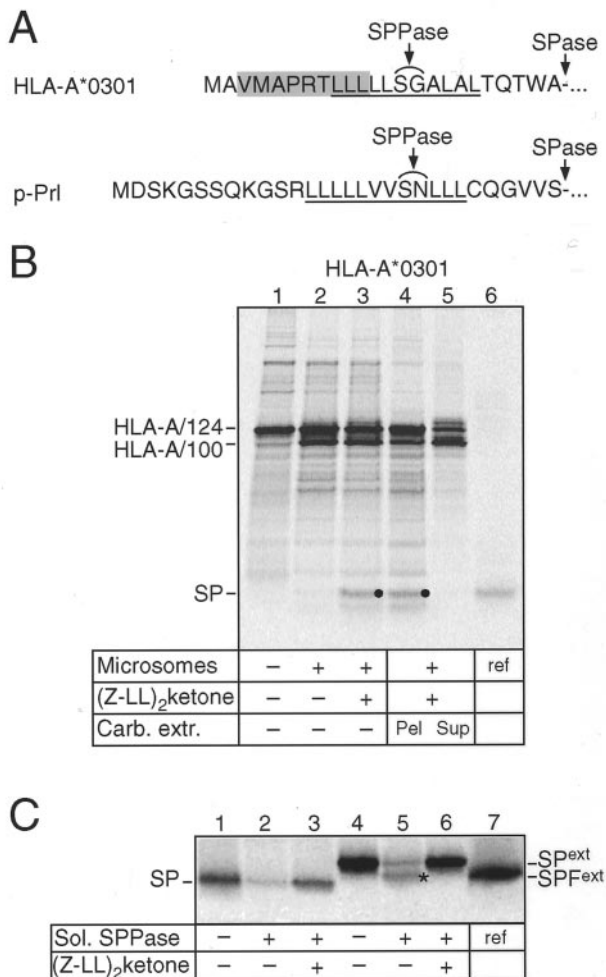
### The generation of HLA-E epitopes requires signal peptide processing by SPPase

To test whether signal peptide processing by SPPase is an essential step in HLA-E epitope generation, we prepared an HLA-A\*0301 signal sequence mutant (HLA-Aspmt) that cannot be processed by SPPase. Systematic studies with p-Prl and HLA-A\*0301 signal sequence mutants revealed that positively charged residues flanking the hydrophobic core inhibit signal peptide processing, and

central helix-breaking residues are essential for proteolysis in the transmembrane region (M. K. Lemberg and B. Martoglio, unpublished data). The helix-breaking motif in the center and the small residues in the C-terminal portion of the hydrophobic region were therefore replaced by amino acids with long hydrophobic side chains and an arginine was introduced at the end (Fig. 2A). In the *in vitro* translation/translocation experiment, HLA-A<sup>Aspmt</sup>/124 was translocated into microsomes and cleaved by signal peptidase like wt HLA-A/124 (Fig. 2B, lane 2). However, the liberated signal peptide of HLA-A<sup>Aspmt</sup> was not processed by SPPase and re-



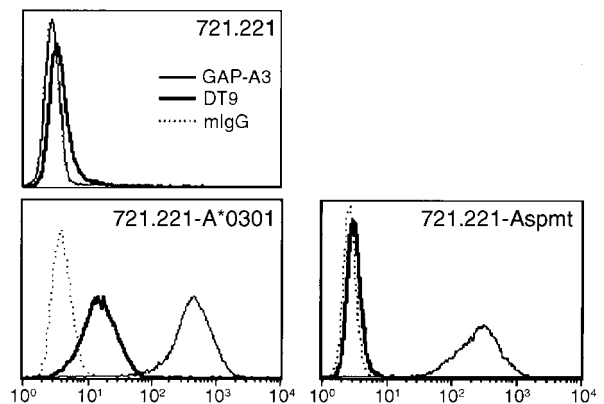
**FIGURE 2.** SPPase cleavage-deficient mutant of HLA-A\*0301 (HLA-Aspmt). *A*, Signal sequence of HLA-Aspmt. Mutated amino acid residues are indicated in bold. *B*, *In vitro* translation of mRNA coding for the N-terminal 124 aa of HLA-Aspmt (lane 1) in the presence of microsomes (lanes 2-5) and (Z-LL)<sub>2</sub>-ketone (lane 3) as described in Fig. 1 legend. One aliquot of microsomes was extracted with sodium carbonate and separated into pellet (Pel, lane 4) and supernatant (Sup, lane 5). Dots indicate the signal peptide (SP); lane 6 shows *in vitro*-translated reference signal peptide.



**FIGURE 1.** Processing of the HLA-A\*0301 signal peptide. *A*, Signal sequences of HLA-A\*0301 and p-PrI. The hydrophobic regions are underlined; arrows indicate the signal peptidase (SPase) and approximate SPPase cleavage sites; the HLA-E binding epitope is shaded. *B*, *In vitro* translation of mRNA coding for the signal sequence plus 100 aa residues of HLA-A\*0301 (HLA-A/124; lane 1) in the presence of ER-derived microsomes (lanes 2-5) and (Z-LL)<sub>2</sub>-ketone (lanes 3-5). After translation, microsomes were extracted with 500 mM KOAc and recovered by centrifugation through a sucrose cushion. One aliquot of microsomes was extracted with sodium carbonate and separated into pellet (Pel, lane 4) and supernatant (Sup, lane 5). Dots indicate the signal peptides (SP); lane 6 shows *in vitro*-translated reference signal peptide. *C*, Signal peptide processing with detergent-solubilized microsomal membrane proteins (lanes 2, 3, 5, and 6) in the presence of (Z-LL)<sub>2</sub>-ketone (lanes 3 and 6). The asterisk indicates the N-terminal signal peptide fragment (SP<sup>ext</sup>); lane 7, *in vitro*-translated reference peptide corresponding to the N-terminal 20 residues of SP<sup>ext</sup>.

mained entirely associated with the membrane in a carbonate-resistant manner (Fig. 2B, lanes 2-5).

To test whether the mutation of the HLA-A\*0301 signal sequence affects HLA-E cell surface expression, the HLA-A-, -B-, -C-, and -G-negative cells 721.221 were stably transfected and selected to express an identical level of either wt HLA-A\*0301 or mutant HLA-Aspmt (Fig. 3) (20). HLA-E surface expression, as measured by flow cytometry using the mAb DT9 (2), was only observed with cells expressing wt HLA-A\*0301 (Fig. 3). HLA-E



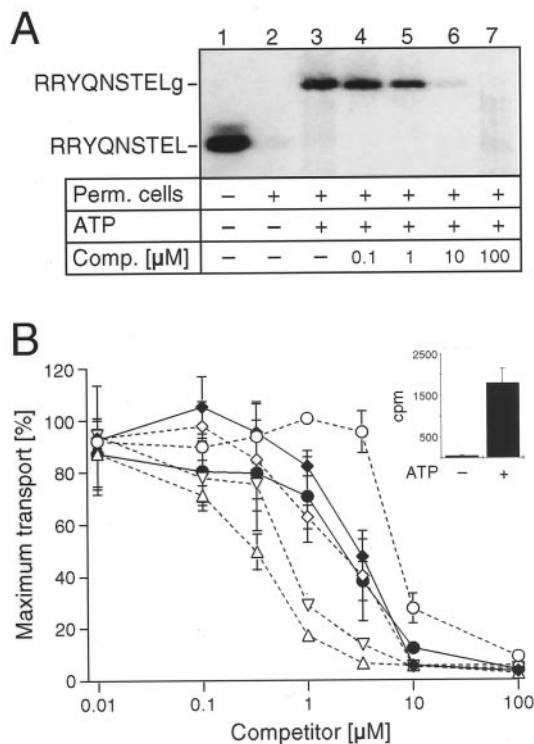
**FIGURE 3.** Processing of the HLA-A\*0301 signal peptide by SPPase is essential for HLA-E cell surface expression. 721.221 cells were stably transfected with HLA-A\*0301 or HLA-Aspmt. Cell surface expression of HLA-E and HLA-A\*0301 were monitored by flow cytometry using the mAbs DT9 and GAP-A3, respectively. Anti-trinitrophenol Abs were used as control.

surface expression could not be detected with cells expressing HLA-Aspmt, whose signal peptide cannot be processed by SPPase (Fig. 3). Cleavage by SPPase in the C-terminal portion of the hydrophobic region is therefore essential for the generation of HLA-E-binding epitopes.

#### N-terminal signal peptide fragments are substrates for TAP

HLA-E presents nonameric peptides derived from the signal sequence of classical HLA molecules, e.g., residues -22 to -14 of HLA-A\*0301 (Fig. 1A) (1, 2, 10). To generate the nonamer from the ~14-residue-long N-terminal signal peptide fragment produced by SPPase, the N and C termini have to be trimmed either before or after the peptide binds to HLA-E. HLA-E expression has been found to be TAP dependent (2, 10), suggesting that the N-terminal signal peptide portion of HLA-A\*0301 is released toward the cytosol.

To assess where the trimming occurs, a series of truncated N-terminal HLA-A\*0301 signal peptide fragments was synthesized and their transport by TAP and binding to HLA-E were tested. TAP transport into digitonin-permeabilized 721.221 cells was assayed using the  $^{125}$ I-labeled reporter peptide RRYQNSTEL with unlabeled synthetic HLA-A\*0301 signal peptide fragments as competitors (Fig. 4A) (17). The peptide corresponding to the nonameric epitope (VMAPRTLLL) was transported most efficiently and competed transport of the reporter peptide with an  $IC_{50}$

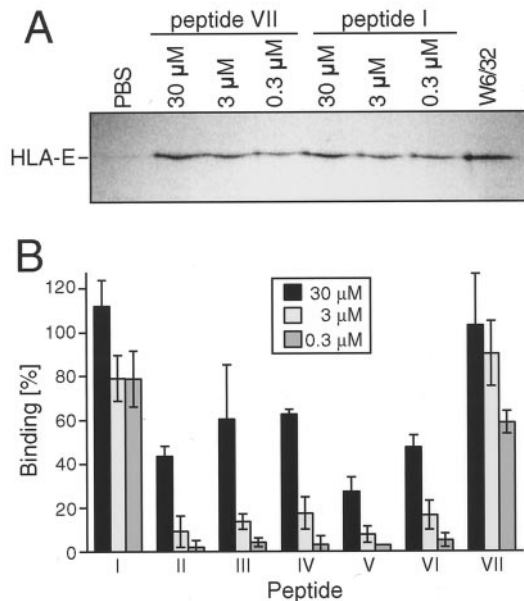


**FIGURE 4.** Transport of HLA-A\*0301 signal peptide fragments by TAP. *A*, Permeabilized 721.221 cells were incubated with the  $^{125}$ I-labeled reporter peptide RRYQNSTEL in the absence (lane 2) or presence of an ATP regeneration system (lanes 3–7). Synthetic MAVMAPRTLLLLLS was added at increasing concentrations (lanes 4–7). Cells were extracted with 500 mM KOAc, separated by centrifugation, and radiolabeled peptides recovered in the pellet were analyzed by SDS-PAGE. Lane 1, Unglycosylated  $^{125}$ I-labeled RRYQNSTEL. *B*, Competition of peptide transport with MAVMAPRTLLLLLSG (●), MAVMAPRTLLLLLS (○), MAVMAPRTLLLLL (◆), MAVMAPRTLLLL (◇), MAVMAPRTLLL (▽), and VMAPRTLLL (△). Values of transport with ATP and without ATP, respectively, are indicated in the bar graph.

value of  $\sim 0.4 \mu$ M (Fig. 4B). With the exception of the 14-residue-long signal peptide fragment ending with a serine, all other peptides tested were transported efficiently as well and reached  $IC_{50}$  values of 0.7–3  $\mu$ M in the competition assay (Fig. 4B). The slightly reduced transport efficiency of the 14-mer ( $IC_{50} \sim 8 \mu$ M) is most likely due to the C-terminal serine residue, which is known to reduce the binding affinity of peptides to TAP (21, 22). These results indicate that potential N-terminal signal peptide fragments of HLA-A\*0301 are all good substrates for TAP, but N- and C-terminal trimming can increase the efficiency of transport.

#### Efficient binding to HLA-E requires C-terminal trimming

The synthetic HLA-A\*0301 signal peptide fragments were next tested for binding to HLA-E as determined by stabilization of HLA-E molecules in cell lysates (Fig. 5) (1). Extension by two residues at the N terminus of the nonamer peptide did not significantly affect peptide binding affinity (Fig. 5, peptide VII). By contrast, extension at the C terminus did. Peptides with additional residues at the C terminus bound to HLA-E at 30  $\mu$ M as previously shown (1). However, binding at the lower concentrations of 3 and 0.3  $\mu$ M (Fig. 5B, peptides III–VI) was not significantly higher than binding of the negative control VTAPRTLLL, the epitope known to fail to up-regulate HLA-E at the cell surface (Fig. 5, peptide II)



**FIGURE 5.** Binding of HLA-A\*0301 signal peptide fragments to HLA-E is greatly increased when the correct C terminus is present. 721.221 cell lysates were incubated with PBS or peptide. Upon heating to 44°C, stabilized peptide-HLA-E complexes were isolated by immunoprecipitation with the conformation-dependent Ab W6/32. For the positive control, cell lysates were incubated in the presence of W6/32, and HLA-E stabilized by the Ab was recovered by immunoprecipitation upon heating. Analysis was by 1D-IEF (an example is shown in *A* for peptides I and VII). Each peptide was tested in three independent assays. *A*, Extension of the nonamer peptide at the N terminus (peptide VII, MAVMAPRTLLL) does not significantly affect binding to HLA-E when compared with the optimal nonamer peptide (peptide I, VMAPRTLLL). *B*, Peptide titrations of N-terminal HLA-A\*0301 signal peptide fragments binding to HLA-E. The data obtained from peptides I and VII, as shown in *A* is represented alongside the data of peptides II–VI: I, VMAPRTLLL; II, VTAPRTLLL; III, MAVMAPRTLLLLLSG; IV, MAVMAPRTLLLLLS; V, MAVMAPRTLLLLL; VI, MAVMAPRTLLLL; and VII, MAVMAPRTLLL. Percentage of binding was calculated as a ratio of ODs normalized to binding with W6/32.

(1, 2). These results show that the expected 14-residue-long N-terminal HLA-A\*0301 signal peptide fragment produced by SPPase has to be trimmed at its C terminus for efficient binding to HLA-E. Nevertheless, because the nontrimmed peptide can bind to HLA-E with low affinity, it remains open, whether some trimming can occur after binding and during transport of the peptide-HLA-E complex to the cell surface, as it was described for other MHC class I Ags (23).

## Discussion

The present study identifies signal peptide processing by SPPase as a new and essential step in the generation of HLA-E-binding epitopes derived from the signal sequence of polymorphic MHC class I molecules. Proteolysis by SPPase in the transmembrane region of signal peptides promotes the release of the fragment containing the epitope from the ER membrane. The resulting signal peptide fragments can then be transported into the ER lumen by TAP and subsequently bind to HLA-E, which, in turn, is transported to the cell surface and presents the epitope to CD94/NDG2 receptors on NK cells.

The functions of a signal sequence in protein targeting and membrane insertion are well established (24) but the fate of signal peptides after they have been cleaved off from the pre-protein by signal peptidase is hardly understood (25). As shown here in the *in vitro* translation/translocation system, the liberated signal peptide is anchored in the microsomal membrane in a carbonate-resistant manner, like a membrane protein. Cleavage within the hydrophobic transmembrane region by the SPPase promotes the release of signal peptide fragments from the lipid bilayer. This mechanism is reminiscent of the regulated intramembrane proteolysis described for an increasing number of eukaryotic or prokaryotic membrane proteins involved in a variety of cellular pathways (for review, see Refs. 26 and 27).

In higher organisms, MHC class I molecules present 8–10 residue peptides on the surface of virtually every nucleated cell, where they can serve as target Ags for cytotoxic T lymphocytes (28). The major proteolytic activities required for the generation of these peptides are the proteasome in the cytosol for protein fragmentation, and in some cases aminopeptidases in the cytosol or ER lumen for peptide trimming (29–34). Although it is possible to prevent the generation of most epitopes through the use of proteasome inhibitors (35, 36), others remain resistant to their effects. This suggested that nonproteasomal proteases might be responsible for the generation of a fraction of MHC class I ligands (37–39). It is speculated that the proteasome is not involved in the generation of HLA-E-binding peptides, as far as it can be deduced from experiments with the mouse functional homologue of HLA-E, Qa-1 (40). However, we show that the SPPase is required to release the peptide fragment containing the HLA-E epitope from the ER membrane. The peptide requires further trimming at both N and C termini to produce the nonamer epitope. Because extension at the C terminus dramatically reduces the peptide-binding affinity to HLA-E, it is likely that C-terminal trimming occurs in the cytosol. This would be consistent with previous reports suggesting that the generation of the Qdm peptide binding to Qa-1 involves cytosolic C-terminal trimming (41) and that ER-resident proteases can trim peptides at their N terminus and not at the C terminus (31–33, 36, 42). Conversely, N-terminal trimming could occur in the cytosol or the ER and may even take place after binding of the peptide to HLA-E since N-terminal extensions do not appear to affect peptide binding (30–33, 36, 43).

TAP is also required for the cell surface expression of signal peptide-derived HLA-E epitopes (2, 10). TAP dependency is consistent with the results presented here, which indicate that SPPase promotes the release of the epitope-containing signal peptide por-

tion from the ER membrane toward the cytosol. SPPase apparently produces TAP substrates from membrane-anchored signal peptides in analogy to the proteasome, which produces TAP substrates from cytosolic proteins (29). The generation of MHC class I epitopes via signal peptide processing may thus be an alternative route to the more common proteasome-dependent pathway of epitope production, and may guarantee a close correlation between the number of HLA-E-peptide complexes and synthesized MHC class I molecules. One aspect that still needs to be investigated is whether SPPase can generate both TAP-dependent and TAP-independent signal peptide fragments capable of binding to MHC class I molecules. Interestingly, removing the charged residue (Arg at position 7) in the N-terminal region of the mouse HLA class I signal sequence alters its insertion and induces TAP-independent presentation of the Qdm peptide (41, 44). The human cytomegalovirus glycoprotein UL40 (HCMV gpUL40) also provides such signal peptide in a TAP-independent manner (45, 46). Preliminary studies have however ruled out a role for SPPase in its generation (V. M. Braud, B. Martoglio, and collaborators, manuscript in preparation). It also remains to determine whether SPPase cleaves only a discrete number of signal peptides, which may have specific properties and functions beyond processing, or whether signal peptide processing is part of a default pathway to clear the ER membrane from the unwanted peptides by analogy to the involvement of the proteasome in the clearance of defective ribosomal products (47). The latter function seems more likely, but stresses the question of the fate of the released peptides. Are signal peptide fragments released into the cytosol generally substrates for TAP, as proposed above, or are they substrates for cytosolic proteases? Is there a selection, for example, HLA-E epitope-containing peptides and how are liberated signal peptides protected from degradation? One can speculate that chaperone molecules may be involved in such a process (25). Clearly the detailed characterization of the SPPase responsible for the cleavage of signal sequences and its role in peptide fragmentation and epitope generation will be a challenge for the future.

## Acknowledgments

We thank N. Cereb, S. Y. Yang, and M. Colonna for the plasmid pBK-CMV/HLA-A\*0301; K. Di Gleria for the synthesis of peptides; and M. F. van den Broek, P. Kreci, and C. Aeberle for iodination of RRYQN-STEL. We also acknowledge the critical discussions with A. Helenius, A. Williams, and C. A. Peh.

## References

1. Braud, V., E. Y. Jones, and A. McMichael. 1997. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur. J. Immunol.* 27: 1164.
2. Braud, V. M., D. S. J. Allan, D. Wilson, and A. J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* 8:1.
3. Braud, V. M., D. S. J. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NDG2A, B and C. *Nature* 391:795.
4. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NDG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187:813.
5. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. E. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NDG2A. *Proc. Natl. Acad. Sci. USA* 95:5199.
6. Ljunggren, H. G., and K. Karre. 1990. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol. Today* 11:237.
7. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359.
8. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67:835.
9. Paetzelt, M., R. E. Dalbey, and N. C. Strynadka. 1998. Crystal structure of a bacterial signal peptidase in complex with a  $\beta$ -lactam inhibitor. *Nature* 396:186.

10. Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160:4951.
11. Weihofen, A., M. K. Lemberg, H. L. Ploegh, M. Bogyo, and B. Martoglio. 2000. Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. *J. Biol. Chem.* 275:30951.
12. Nilsson, I. M., and G. von Heijne. 1993. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* 268:5798.
13. Martoglio, B., S. Hauser, and B. Dobberstein. 1998. Cotranslational translocation of proteins into microsomes derived from the rough endoplasmic reticulum of mammalian cells. In *Cell Biology: A Laboratory Handbook*, Vol. 2. J. E. Celis, ed. Academic, San Diego, CA, p. 265.
14. Wiltfang, J., A. Smirnov, B. Schnierstein, G. Kelemen, U. Matthies, H. W. Klafki, M. Staufenbiel, G. Huther, E. Ruther, and J. Kornhuber. 1997. Improved electrophoretic separation and immunoblotting of  $\beta$ -amyloid (A $\beta$ ) peptides 1–40, 1–42, and 1–43. *Electrophoresis* 18:527.
15. Brielmeier, M., J. M. Bechet, M. H. Falk, M. Pawlita, A. Polack, and G. W. Bornkamm. 1998. Improving stable transfection efficiency: antioxidants dramatically improve the outgrowth of clones under dominant marker selection. *Nucleic Acids Res.* 26:2082.
16. Jadot, M., M. W. Hofmann, R. Graf, H. Quader, and B. Martoglio. 1995. Protein insertion into the endoplasmic reticulum of permeabilized cells. *FEBS Lett.* 371:145.
17. Neefjes, J. J., F. Momburg, and G. J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. [Published erratum appears in 1994 *Science* 264:16.] *Science* 261:769.
18. Neefjes, J. J., I. Doxiadis, N. J. Stam, C. J. Beckers, and H. L. Ploegh. 1986. An analysis of class I antigens of man and other species by one-dimensional IEF and immunoblotting. *Immunogenetics* 23:164.
19. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97.
20. Adam, S. A., T. Nakagawa, M. S. Swanson, T. K. Woodruff, and G. Dreyfuss. 1986. mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. *Mol. Cell. Biol.* 6:2932.
21. Uebel, S., W. Kraas, S. Kienle, K. H. Wiesmuller, G. Jung, and R. Tampe. 1997. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proc. Natl. Acad. Sci. USA* 94:8976.
22. Daniel, S., V. Brusic, S. Caillat-Zucman, N. Petrovsky, L. Harrison, D. Riganelli, F. Sinigaglia, F. Gallazzi, J. Hammer, and P. M. van Endert. 1998. Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J. Immunol.* 161:617.
23. Gil-Torregrosa, B. C., A. Raul Castano, and M. Del Val. 1998. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the *trans*-Golgi network protease furin. *J. Exp. Med.* 188:1105.
24. Walter, P., and A. E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10:87.
25. Martoglio, B., and B. Dobberstein. 1998. Signal sequences: more than just greasy peptides. *Trends Cell Biol.* 8:410.
26. Brown, M. S., J. Ye, R. B. Rawson, and J. L. Goldstein. 2000. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100:391.
27. Hoppe, T., M. Rape, and S. Jentsch. 2001. Membrane-bound transcription factors: regulated release by RIP or RUP. *Curr. Opin. Cell Biol.* 13:344.
28. York, I. A., A. L. Goldberg, X. Y. Mo, and K. L. Rock. 1999. Proteolysis and class I major histocompatibility complex antigen presentation. *Immunol. Rev.* 172:49.
29. Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* 16:323.
30. Stoltze, L., M. Schirle, G. Schwarz, C. Schroter, M. W. Thompson, L. B. Hersh, H. Kalbacher, S. Stevanovic, H. G. Rammensee, and H. Schild. 2000. Two new proteases in the MHC class I processing pathway. *Nat. Immunol.* 1:413.
31. Serwold, T., S. Gaw, and N. Shastri. 2001. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat. Immunol.* 2:644.
32. Menoret, A., M. L. Niswonger, A. Altmeyer, and P. K. Srivastava. 2001. An ER protein implicated in chaperoning peptides to MHC class I is an aminopeptidase. *J. Biol. Chem.* 276:7.
33. Komlos, A., F. Momburg, T. Weinschenk, N. Emmerich, H. Schild, E. Nadav, I. Shaked, and Y. Reiss. 2001. A role for a novel luminal endoplasmic reticulum aminopeptidase in final trimming of 26S proteasome-generated major histocompatibility complex class I antigenic peptides. *J. Biol. Chem.* 276:23.
34. Cascio, P., C. Hilton, A. F. Kisseliev, K. L. Rock, and A. L. Goldberg. 2001. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J.* 20:2357.
35. Benham, A. M., M. Gromme, and J. Neefjes. 1998. Allelic differences in the relationship between proteasome activity and MHC class I peptide loading. *J. Immunol.* 161:83.
36. Rock, K. L., and A. L. Goldberg. 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* 17:739.
37. Glas, R., M. Bogyo, J. S. McMaster, M. Gaczynska, and H. L. Ploegh. 1998. A proteolytic system that compensates for loss of proteasome function. *Nature* 392:618.
38. Luckey, C. J., G. M. King, J. A. Marto, S. Venkateswaran, B. F. Maier, V. L. Crozter, T. A. Colella, J. Shabanowitz, D. F. Hunt, and V. H. Engelhard. 1998. Proteasomes can either generate or destroy MHC class I epitopes: evidence for nonproteasomal epitope generation in the cytosol. *J. Immunol.* 161:112.
39. Geier, E., G. Pfeifer, M. Wilm, M. Lucchiarri-Hartz, W. Baumeister, K. Eichmann, and G. Niedermann. 1999. A giant protease with potential to substitute for some functions of the proteasome. *Science* 283:978.
40. Bai, A., and J. Forman. 1997. The effect of the proteasome inhibitor lactacystin on the presentation of transporter associated with antigen processing (TAP)-dependent and TAP-independent peptide epitopes by class I molecules. *J. Immunol.* 159:2139.
41. Bai, A., C. J. Aldrich, and J. Forman. 2000. Factors controlling the trafficking and processing of a leader-derived peptide presented by Qa-1. *J. Immunol.* 165:7025.
42. Mo, X. Y., P. Cascio, K. Lemerise, A. L. Goldberg, and K. Rock. 1999. Distinct proteolytic processes generate the C and N termini of MHC class I-binding peptides. *J. Immunol.* 163:5851.
43. Beninga, J., K. L. Rock, and A. L. Goldberg. 1998. Interferon- $\gamma$  can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.* 273:18734.
44. Bai, A., J. Broen, and J. Forman. 1998. The pathway for processing leader-derived peptides that regulate the maturation and expression of Qa-1b. *Immunity* 9:413.
45. Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287:1031.
46. Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J. W. Ellwart, M. Pla, and E. H. Weiss. 2000. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol.* 164:5019.
47. Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404:770.