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

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Signal sequences of human MHC class I molecules are a unique source of epitopes for newly synthesized nonclassical MHC 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 recognition of surface class I molecules by the killer Ig-like receptors on NK cells (7), the HLA-E signal peptide fragment, HLA-E-peptide complexes indirectly provide an additional level of control to the direct presentation 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 preprolactin (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 Soll/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, 17, 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'-CTCTCTCTCTACCTCTGTGGTGCCTCTGCTGCGCCAG ACCTGGGCGGG-3' and the antisense primer 5'-CCGGCCAGTTCT GGGCCAGAGACAGGAGAAGACGCATTAGCAGGAGGAG-3'. It resulted in pSV-Sport1/HLA-Aspnt. To generate a mutant of HLA-A*0301 with an extended signal peptide (HLA-Asp**), see Fig. 1 for sequence) the coding region of pSV-Sport1/HLA-A*0301 was amplified by PCR using the sense primer 5'-AGTCAGGTGCGACATGGCCAAGACACAGCAAG GTCGGCGCTATAGGGCGGCCCCG-3', which included a Soll restriction site and codons for the six additional amino acids (underlined). A standard T7 primer was used as reverse primer. The Soll/HindIII fragment of the resulting PCR product was transferred into pSV-Sport1 to generate pSV-Sport1/HLA-Asp**. 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**, the respective coding region was amplified with PCR using Puu DNA polymerase (Stratagene), SP6 primer, and a reverse primer, starting with 5'-NNNNGNNNNNCTA, 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 m3G(5'ppp5')G CAP analog (New England Biolabs, Beverly, MA) (12).
Translations of mRNA coding for HLA-A*0301/124 and HLA-Aspmt/124 were performed in 25 μl of reticulocyte lysate (Promega, Madison, WI) containing [35S]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-methyamine (13) and 5 μM (Z-LLE)3-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 Na2CO3, pH 11.3 (13). Translations of mRNA coding for the signal peptides were translated in 25 μl 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-methoxy succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (250 μM, 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

Peptides and proteins were analyzed by SDS-PAGE using Tris-bicine gels (14). Membrane pellets and proteins precipitated with (NH4)2SO4 or tri- chloroacetic 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 x 80 x 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 μg DNA at 270 V with a capacitance of 1500 μF (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'), (Sigma-Aldrich) (2).

TAP transport

721.221 cells were permeabilized according to Jodot et al. (16), except that digitonin (0.006%) was used instead of saponin. For TAP transport (17), 3 x 10^5 permeabilized cells were incubated in 25 μl of 50 mM HEPES-KOH (pH 7.6), 150 mM KOAc, 5 mM Mg(OAc)2, 250 mM sucrose, and 1 mM DTT, and 60 mM 125I-labeled RRYQNSTEL (9 Ci/mmol) and 2 μl of 125I-labeled SPPase inhibitor (9 Ci/mmol) and 2 μl of ATP mix (12.5 mM ATP, 3.5 μM creatine kinase, and 110 mM creatine phosphate). ATP was depleted by the addition of 0.3 U of hexokinase and 20 μmol 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-N erti cleavage product, which was labeled by the methionine- and B-) (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-LLE)-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 (SPP34) with six additional residues (MGKNSKVAVM...) at the N terminus was applied in the assay. SPP34 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 SPP34 (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-Aspmt/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-Aspmt was not processed by SPPase and remained 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 1. Processing of the HLA-A*0301 signal peptide. A, Signal sequences of HLA-A*0301 and p-Prl. 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 N-terminal 124 aa of HLA-Aspmt (lane 1) in the presence of microsomes (lanes 2–5) and (Z-LL)2-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 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 ER-derived microsomes (lanes 2–5) and (Z-LL)2-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 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-Asm, 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 125I-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 IC50 value of ~0.4 μM (Fig. 4B). With the exception of the 14-residue-long signal peptide fragment ending with a serine, all other peptides were transported efficiently as well and reached IC50 values of 0.7–3 μM in the competition assay (Fig. 4B). The slightly reduced transport efficiency of the 14-mer (IC50 ~ 8 μM) 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 μM as previously shown (1). However, binding at the lower concentrations of 3 and 0.3 μM (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).
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/NKG2 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 portion 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.

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