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Cytosolic Aminopeptidases Influence MHC Class I-Mediated Antigen Presentation in an Allele-Dependent Manner

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Antigenic peptides presented by MHC class I molecules are generated mainly by the proteasome in the cytosol. Several cytosolic aminopeptidases further trim proteasomal products to form mature epitopes or individual amino acids. However, the distinct function of cytosolic aminopeptidases in MHC class I Ag processing remains to be elucidated. In this study, we show that cytosolic aminopeptidases differentially affect the cell surface expression of MHC class I molecules in an allele-dependent manner in human cells. In HeLa cells, knockdown of puromycin-sensitive aminopeptidase (PSA) by RNA interference inhibited optimal peptide loading of MHC class I molecules, and their cell surface expression was correspondingly reduced. In contrast, depletion of bleomycin hydrolase (BH) enhanced optimal peptide loading and cell surface expression of MHC class I molecules. We did not find evidence on the effect of leucine aminopeptidase knockdown on the MHC class I Ag presentation. Moreover, we demonstrated that PSA and BH influence the peptide loading and surface expression of MHC class I in an allele-specific manner. In the absence of either PSA or BH, the surface expression and peptide-dependent stability of HLA-A68 were reduced, whereas those of HLA-B15 were enhanced. The surface expression and peptide-dependent stability of HLA-A3 were enhanced by BH knockdown, although those of HLA-B8 were increased in PSA-depleted conditions. The Journal of Immunology, 2009, 183: 7379–7387.

MHC class I molecules present antigenic peptides to CD8+ T cells, thereby triggering the death of infected or transformed target cells. MHC class I molecules bind antigenic peptides derived from intracellular proteins in the endoplasmic reticulum (ER),3 wherein the MHC class I complex is assembled (1). Most antigenic peptides are generated by the proteasome in the cytosol and are translocated into the ER via the TAP. Upon binding to an appropriate peptide, the MHC class I molecule is dissociated from the peptide-loading complex (PLC) that regulates optimal peptide loading into the MHC class I peptide-binding groove. The peptide-loaded MHC class I molecule is then transported to the cell surface, where it presents the bound peptide to CD8+ T cells (2).

Thus far, the fate of mature epitope precursors that are released from proteasomes into the cytosol remains unclear. The bulk of proteasomal products (approximately two-thirds) are too short to be loaded onto MHC class I molecules (3). At most, ~10% of proteasomal products are of a suitable length to bind to MHC class I molecules (4–6). The remaining proteasomal products (10–15%) are N-terminally extended precursors that require trimming by peptidases for presentation by MHC class I molecules (4–6). Several cytosolic aminopeptidases, such as puromycin-sensitive aminopeptidase (PSA), bleomycin hydrolase (BH), and leucine aminopeptidase (LAP), are required for further trimming of proteasomal products (7–12). LAP (EC 3.4.11.1) is a Zn2+-binding metalloprotease (7) that is up-regulated by both IFN-γ (13) and IFN-α (14). A mature epitope, SIINFEKL (OVA8), is generated by LAP from extended versions of OVA-derived epitopes in vitro (7). LAP overexpression results in the rapid degradation of peptides available for MHC class I binding (14), and thus, LAP plays a role in the trimming of antigenic peptides during an immune response. PSA (EC 3.4.11.14) is also a Zn2+-binding metalloprotease that is highly conserved among species and is expressed ubiquitously (15). This enzyme has broad substrate specificity and exhibits some preference for hydrophobic and basic amino acids present at position 1 or 2 (10, 16). PSA is involved in the degradation of proteasomal products to amino acids (17, 18) as well as the trimming of antigenic peptides for MHC class I Ag presentation (10).

This peptidase can also participate in the N-terminal processing of human RU1 epitope precursors (8). BH (EC 3.4.22.40) is a neutral cysteine protease of the papain superfamily (19); it has been highly conserved throughout evolution and also has a broad specificity. BH has been implicated in MHC class I Ag processing (12). Purified PSA or BH, as well as cell extracts containing PSA or BH, can generate or destroy the mature epitope vesicular stomatitis virus nucleoprotein 52–59 from N-terminally extended precursors (8, 10). However, the distinct function of these cytosolic aminopeptidases in the context of human MHC (HLA) class I-mediated Ag presentation has been elusive.

In this study, our goal was to determine the specific role of each cytosolic aminopeptidase in Ag presentation by HLA class I molecules. We found that cytosolic aminopeptidases differentially regulate the cell surface expression of HLA class I molecules in an allele-specific manner through alterations in the repertoire of antigenic peptides loaded. In addition, we present evidence that cytosolic aminopeptidases may be helpful for one HLA class I allele...
and, at the same time, may be deleterious to the other HLA class I allele with regard to mature epitope generation.

Materials and Methods

DNA constructs

To clone siRNA of human PSA, BH, or LAP, each aminopeptidase-specific target sequence was predicted by the BLOCK-IT RNAi Designer using cDNA segments (Invitrogen Life Technologies). Selected targeting sequences were: PSA siRNAs, GCATGGTGATGCGACTACT (residue 2257–2275, no. 1) and AGCCGAGATGAGAAGC (residue 2694–2712, no. 2); BH siRNAs, AGATGGTGTCGCTTCCCT (residues 927–945, no. 1) and AGTTACCTGTCGATCAGC (residue 1213–1231, no. 2); and LAP siRNAs, CATCAGAGCTGCCTGCCG (residue 358–376, no. 1) and TGGGATCATCTCAGTGT (residues 732–750, no.2). The siRNA construct for each aminopeptidase was generated by cloning the specific target sequence into the pSUPER.retro.puro vector (OligoEngine). GFP siRNA (GGTATGACGAGAAGC) was used as a control and cloned into the same vector. pCMV Ouabain² vector (BD PharMingen) was used to select transfected cells.

HLA class I alleles from HeLa cells were typed by sequencing-based typing kits following the manufacturer’s instructions (BioSsewom), resulting in the identification of HLA-A*0802, -B*1503, and -Cw*1203. To clone and express HLA-A*0802 and -B*1503 alleles, we extracted total RNA from HeLa cells and obtained full-length cDNA, which was amplified by RT-PCR using the following allele-specific primers: HLA-A*6802, 5'-CCAGACTACGCTGGATCCGGCTCCCACTCCATG-3' and 5'-CATG GAGTGGGAGCCGGATCCAGCGTAGTCTGGGAC-3' of each HLA class I allele. Primers used for the overlap extension PCR were 5'-CCCCAGCTTATGGCCGTCATGGCGCCCCGA-3' and 5'-CC GGAATTCCTCACATTTACAACGTCGGAGAAG-3'; HLA-B*1503, 5'-CCGGGATCTCGGGTGAGAAGACAGCAGTG-3' and 5'-CCGAAGCTTACAGGTGATGGCACAGGAGAC-3'. The amplified products were cloned into pcDNA3.1 expression vector (Invitrogen Life Technologies) using BamHI and EcoRI restriction sites. We also used allele-specific primers for amplification and cloning of HLA-Cw*1203, but unfortunately this was unsuccessful.

DNA constructs encoding N-terminal hemagglutinin (HA)-tagged HLA class I alleles were generated by overlap extension PCR using two fragments: one as a reverse oligo containing the signal sequence of HLA-A*0201 followed by the HA epitope (YPYDVPDYA), while the other was the PCR product encompassing the region from a1 to the cytosolic domain of each HLA class I allele. Primers used for the overlap extension PCR were 5'-CCCAAGCTTATGGCCGTCATGGCGCCCCGAAG-3' and 5'-CATG GAGTGGGAGCCGGATCCAGCGTAGTCTGGGAC-3' of each HLA class I allele. The amplified products were ligated into pcDNA3.1 expression vector using the HindIII and EcoRI restriction sites. All generated constructs were confirmed by DNA sequencing.

Abs and peptides

The mAb W6/32 specifically recognizes MHC class I heavy chains associated with β2m. Anti-PSA goat polyclonal Ab (sc-26023), mouse monoclonal anti-β2m Ab (sc-13565), and rabbit polyclonal anti-HA Ab (sc-805) were purchased from Santa Cruz Biotechnology. Mouse polyclonal LAP Ab and mouse monoclonal BH Ab were purchased from Abnova Corporation and Abcam, respectively. Mouse monoclonal anti-HA Ab (no. 2367) was purchased from Abcam, as well as rabbit polyclonal anti-HA Ab (no. 2367). Cells were radiolabeled with [35S]-methionine for 10 min and chased for the indicated times. Cells were lysed with 1% TX-100 in PBS with a protease inhibitor mixture for 1 h at 4°C. The membrane was washed three times with PBS containing 0.1% Tween 20 and incubated with HRP-conjugated secondary Ab for 40 min. After washing the membrane three times, the immunoblots were visualized with ECL detection reagent (Pierce).

Flow cytometry

The expression of cell surface MHC class I was determined using a FACSCalibur flow cytometer (Becton Dickinson Biosciences). Cells were harvested, washed twice with cold PBS containing 1% BSA, and incubated for 1 h at 4°C with a saturating concentration of the Ab. The isotype control was used as a negative control for each experiment. Cells were washed twice with cold PBS containing 1% BSA and then stained with FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. A total of 10,000 gated events were collected and analyzed with CellQuest software (Becton Dickinson Biosciences). To measure the cell surface peptide-dependent stability of MHC class I, cells were treated with 5 μg/ml BFA during the indicated times, stained with W6/32, and then analyzed.

Immunoblotting

Cells were lysed with 1% Triton X-100 (TX-100; Sigma-Aldrich) in PBS with a protease inhibitor mixture for 1 h at 4°C, and the lysates were centrifuged at 13,000 rpm at 4°C for 30 min. The supernatants were harvested, quantified by Bradford assay, and incubated in SDS sample buffer for 10 min. Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, blocked with 5% skim milk in PBS with 0.1% Tween 20 for 1 h, and probed with the appropriate Ab overnight. The membrane was washed three times with PBS containing 0.1% Tween 20 and incubated with HRP-conjugated secondary Ab for 40 min. After washing the membrane three times, the immunoblots were visualized with ECL detection reagent (Pierce).

Metabolic labeling and immunoprecipitation

Cells were starved for 30 min in medium lacking methionine and cysteine, labeled with 0.1 μCi/ml [35S]-methionine (Translabel; NEN Life Sciences), chased in normal medium for the indicated times. Cells were lysed using 1% TX-100 in PBS with a protease inhibitor mixture for 1 h at 4°C. After preclearing lysates with protein G Sepharose (PGS) or protein A Sepharose (APS) (Amersham Pharmacia Biotech), lysates were quantified by the Bradford assay. Abs and PGS or PAS were added to the lysates and incubated at 4°C for 1 h. The beads were washed three times with 0.5% TX-100 in PBS. Immunoprecipitated proteins were eluted from the beads by boiling in an SDS sample buffer and separated by SDS-PAGE. For serial immunoprecipitation, radioabeled cells were lysed for 1 h in 1% TX-100 in PBS or for 2 h in 1% digitonin (Kalbchitin) in buffer A containing 50-mM HEPEs (pH 7.2), 20 mM CaCl₂, and 300 mM NaCl with a protease inhibitor mixture at 4°C. The lysates were immunoprecipitated as above (first immunoprecipitation; 1° IP) and immunoprecipitated proteins were eluted from the beads with 50 μl PBS containing 2% SDS by boiling for 20 min. The denatured eluates were added to 700 μl 1% TX-100 in PBS and reimmunoprecipitated with the indicated Ab overnight at 4°C (second immunoprecipitation; 2° IP). PGS or PAS was added to the lysates, which were incubated at 4°C for 1 h. The beads were washed three times with 0.5% TX-100 in PBS, and immunoprecipitated proteins were eluted from the beads by boiling in an SDS sample buffer for 10 min and then separated by SDS-PAGE. The gel was dried, exposed to BAS film, and then analyzed with the Phosphor Imaging System BAS-2500 (Fuji Film).

Endoglycosidase H (EndoH) treatment

Cells were radioabeled with [35S]-methionine for 10 min and chased for the indicated times. Cells were lysed and MHC class I was immunoprecipitated using the W6/32 Ab. For HA-HLAs, the eluted material was reimmunoprecipitated with an anti-HA Ab. The immunoprecipitated materials were eluted by boiling for 10 min with buffer B (50-mM sodium acetate (pH 5.6), 0.3% SDS, and 150-mM 2-ME). The eluate was treated with EndoH at 37°C overnight, boiled in SDS sample buffer for 10 min, and resolved by SDS-PAGE.
Thermostability assay

The thermostability assay was performed as previously described (25). In brief, cells were radiolabeled for 20 min using 0.1 mCi/ml [35S]-methionine as described earlier, and lysed with 1% TX-100 in PBS using a protease inhibitor mixture for 1 h at 4°C. The precleared lysates were aliquoted, and each aliquot was incubated at a different temperature for the indicated time. Each lysate was added to 700 μl 1% TX-100 in PBS and immunoprecipitated with W6/32 Ab. For HA-HLA transfectants, eluted material was reimmunoprecipitated with an anti-HA Ab and the second eluates were resolved by SDS-PAGE. To examine the stabilization of HA-HLA class I molecule by peptides, HA-HLA transfectants were radiolabeled for 30 min and lysed in 1% TX-100 buffer for 30 min in the absence or presence of 100 μM of the relevant peptide. After the lysates were centrifuged and precleared, each aliquot was incubated at a different temperature for 20 min. Each lysate was added to 500 μl 1% TX-100 in PBS and immunoprecipitated with W6/32 Ab. Eluted materials were reimmunoprecipitated with an anti-HA Ab and the second eluates were resolved by SDS-PAGE.

Results

Cytosolic aminopeptidases differentially influence the cell surface expression of MHC class I molecules

To investigate the distinct role of cytosolic aminopeptidases in Ag presentation, we used a small interfering RNA (siRNA) approach and specifically targeted each aminopeptidase. We designed two siRNA sequences for an individual aminopeptidase (siRNA no. 1 and no. 2) and used GFP-specific siRNA (siGFP) as a control. To enrich cells expressing each siRNA, we cotransfected GFP, PSA-, BH-, or LAP-specific siRNAs and a mock vector encoding the ouabain-resistance gene (mock-Ouα) into HeLa cells. Sixty hours after transfection, we treated cells with ouabain for efficient selection and measured the cell surface expression of MHC class I molecules by flow cytometry using W6/32 Ab, which recognizes the β2-m-MHC class I H chain complex. PSA knockdown resulted in a 70–80% decrease in PSA protein levels in cells transfected with siPSA no. 1 and siPSA no. 2 (Fig. 1A, top), but did not affect the steady-state levels of BH and LAP (Fig. 1C, right). BH knockdown increased the surface expression of MHC class I molecules (Fig. 1B, right). Because defects in PLC components such as tapasin and TAP are closely linked to the down-regulation of cell surface MHC class I molecules (26, 27), we investigated whether the absence of PSA affected the levels of PLC components and found that the amount of TAP1 and tapasin remained unchanged in PSA-depleted cells (Fig. 1B, right). BH knockdown increased the surface expression of MHC class I molecules (Fig. 1A, middle). BH knockdown did not affect the surface expression of MHC class I molecules (Fig. 1A, bottom) or the steady-state levels of PSA, BH, or PLC components (Fig. 1D). These results indicate that these three cytosolic aminopeptidases differentially affect the cell surface expression of MHC class I molecules.
Knockdown of PSA and BH influences the quality of peptides bound to MHC class I molecules

A decrease or an increase in MHC class I cell surface expression could be attributable to an alteration of either the quantity or the quality of the antigenic peptide supplied. To examine whether the differential effect of each aminopeptidase on the surface expression of MHC class I molecules reflected a qualitative change in the peptide repertoire bound to MHC class I molecules, we analyzed the lysates using a well-established “thermostability assay,” which correlates thermostability with the affinity of loaded peptides on MHC class I molecules (25). Concurrently, we monitored the effect of aminopeptidase depletion on the maturation of MHC class I molecules by the EndoH assay. There was no significant difference in MHC class I maturation rates in PSA-, BH-, or LAP-depleted cells compared with control cells (Fig. 2A). However, the MHC class I molecules exhibited significantly less thermostability in PSA-depleted cells and were considerably more thermostable in BH-depleted cells (Fig. 2B). Knockdown of LAP had little effect on thermostability of MHC class I molecules in our system (Fig. 2B). These thermostability data suggest that the differential effect of cytosolic aminopeptidases on the surface expression of MHC class I molecules reflects a qualitative change in the peptide repertoire bound to MHC class I molecules.

Cell surface MHC class I molecules contain peptides with a broad spectrum of affinity; their stability on the cell surface largely depends on the association between the peptide and their peptide-binding groove (28). The half-life of MHC class I molecules at the surface can be determined by the stability of the trimolecular complex comprising the MHC class I molecule, β2m, and the peptide (MHC class I-β2m-peptide complex). Thus, the quality of peptide bound to the MHC class I molecule can be examined by measuring the dissociation rates of preformed MHC class I complexes on the cell surface (29). To further confirm alterations in peptide-dependent stability of MHC class I molecules due to PSA and BH, we treated HeLa cells expressing siRNAs for each aminopeptidase with brefeldin A (BFA), which reorganizes the structure of the ER and Golgi complex (30). Because BFA treatment blocks the ER exit of newly assembled peptide-MHC class I complexes, we were able to exclusively analyze the MHC class I complex on the cell surface (29). We stained BFA-treated cells with a W6/32 Ab after the indicated chase time and then measured the peptide-dependent stability of MHC class I molecules on the cell surface by flow cytometry. HeLa cells stably expressing ICP47 protein, an immediate early gene product of herpes simplex virus that inhibits TAP function (31–33), were used as a negative control (Fig. 2C, F). The dissociation rate of preformed MHC class I complexes on the cell surface...
surface was faster in PSA-depleted cells (Fig. 2C, ▲) than in control cells (Fig. 2C, □). However, MHC class I complex dissociation on the cell surface was not affected by LAP depletion (Fig. 2C, ○) and the rates were slower in BH-depleted cells (Fig. 2C, ■) than in control cells. Consistent with the thermostability results, these findings indicate that the quality of antigenic peptides bound to MHC class I molecules deteriorates upon PSA depletion and improves upon BH depletion. These data seem to support the notion that a change in peptide-dependent stability of the MHC class I complex could result from the generation and/or destruction of optimal peptides by cytosolic aminopeptidases.

Characterization of the HA-tagged HLA class I molecules

We demonstrated that cytosolic aminopeptidases contributed specifically to the surface expression of MHC class I molecules by affecting antigenic peptides bound onto MHC class I molecules qualitatively in HeLa cells. Next, we proceeded to test whether the effect of aminopeptidases was allele-specific. We characterized HLA class I alleles from HeLa cells using sequencing-based typing kits according to the manufacturer’s instructions. HLA-A*6802, -B*1503, and -Cw*1203 were identified (data not shown). We attempted to clone those alleles from HeLa cells with allele-specific primers and successfully cloned HLA-A*6802 and -B*1503, but failed to amplify and clone HLA-Cw*1203. Therefore, HLA-A*6802 and HLA-B*1503 were chosen to examine the effect of cytosolic aminopeptidases on the surface expression of these two alleles. Unlike PSA and BH, LAP did not appear to affect the surface expression of MHC class I molecules, at least in HeLa cells (Fig. 1A, bottom), and therefore we limited our analysis to PSA and BH. Due to the lack of an allele-specific Ab, we tagged HA at the N terminus of HLA-A*6802 (HA-A68) and HLA-B*1503 (HA-B15) after the signal sequence.

To ensure that the HA-tagged version of the HLA class I allele displays a general phenotype comparable to that of the wild-type allele, we examined the expression of HA-HLAs at the cell surface. HeLa cells transiently transfected with mock vector, HA-A68 or HA-B15 cDNA were stained with an anti-HA Ab and analyzed by flow cytometry. Cell surface expression of HA-HLAs was normal (Fig. 3A). To investigate whether HA-HLAs could be incorporated into the PLC, we lyzed radiolabeled transfectants expressing HA-HLAs using 1% digitonin. The lysate was first immunoprecipitated with an anti-TAP1 or anti-tapasin Ab, and the resultant immunoprecipitates were reimmunoprecipitated using the indicated Abs. HA-A68 (data not shown) and HA-B15 associated with β2m as well as with the PLC components TAP1 and tapasin (Fig. 3B). The maturation of HA-HLAs appeared to be normal, as evidenced by EndoH analysis (Fig. 3C). Analysis using the thermostability assay also showed that the thermostability of HA-A68 and HA-B15 (Fig. 4A) was similar to that of the wild-type MHC class I molecules. In addition, to validate the stabilization of HA-HLA class I molecule by peptides (25), radiolabeled HA-HLA transfectants were lysed in 1% TX-100 buffer for 30 min in the absence or presence of the relevant peptide, before heating and immunoprecipitation of HA-HLAs as described in Materials and Methods. Index peptides for a specific HLA class I allele enhanced the thermostability of HA-HLAs, compared with the control set with no peptide. In contrast, low affinity peptides failed to improve the thermostability of HA-HLAs, but rather decreased the thermostability of HA-HLAs, compared with control (Fig. 4B). Based on these observations, we concluded that HA-HLAs have the same general characteristics as their wild-type counterparts.

Effect of aminopeptidases on MHC-mediated Ag processing and allele-specific presentation

Having validated the biological activity of HA-HLAs, we then set out to determine the allele specificity of PSA or BH. We first transiently transfected HA-A68 or HA-B15 cDNAs into HeLa cells. After 24 h, cells were replated and cotransfected with siRNA

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**FIGURE 3.** HA-tagged HLA class I molecules normally associate with PLC components and express on the cell surface. A, Cell surface expression of HA-HLAs in HeLa cells. HeLa cells were transiently transfected with either of the mock vectors, HA-A68, or HA-B15 cDNAs into HeLa cells transiently transfected with mock vector, HA-A68, or HA-B15 cDNA, stained with anti-HA Ab and analyzed by flow cytometry. B, Association of HA-HLAs with β2m and PLC components. Radiolabeled HeLa cells expressing HA-B15 were lysed with 1% digitonin and the precleared lysates were immunoprecipitated with anti-TAP1 or anti-tapasin Ab. The coimmunoprecipitated materials were dissociated and denatured with SDS. A one-fifth aliquot was analyzed directly as an input of the first immunoprecipitation and the remainder was diluted into 1% TX-100 buffer and immunoprecipitated with anti-HA, anti-β2m, anti-tapasin, or anti-TAP1 Ab. C, Maturation test for HA-HLAs. HeLa cells were transfected with either of the mock vectors, HA-A68, or HA-B15 cDNA, radiolabeled for 10 min, chased for 2 h, then lysed with 1% TX-100. The lysates were initially immunoprecipitated with W6/32 Ab followed by re-immunoprecipitation with anti-HA Ab. The second immunoprecipitates were treated with EndoH and resolved by SDS-PAGE.
Materials and Methods

A

Thermostability of HA-tagged HLA class I molecules correlates with the binding affinity of peptides. A, Thermostability of HA-HLAs. HeLa cells were transfected with either of the mock vectors, HA-A68 or HA-B15 cDNA, radiolabeled for 10 min, then lysed with 1% TX-100. The precleared lysates were divided into four aliquots, which were incubated at 4, 25, 37, or 50°C for 1 h. After addition of 700 μl 1% TX-100 in PBS and immunoprecipitated with W6/32 Ab. Eluted materials were reimmunoprecipitated with anti-HA Ab and the second eluates were resolved by SDS-PAGE. Result in A is a representative of two experiments.

B

Thermostability of HA-HLAs. HeLa cells were transfected with either of the mock vectors, HA-A68 or HA-B15 cDNA, radiolabeled for 30 min in the presence of 100 μM ouabain for 30 min and lysed in 1% TX-100 buffer for 30 min in the presence or absence of the relevant peptide. HA-HLA transfectants were radiolabeled for 30 min and lysed in 1% TX-100 for 30 min in the presence of 100 μM of the relevant peptide. After the lysates were centrifuged and precleared, each aliquot was incubated at a different temperature for 20 min. Each lysate was added to 500 μl 1% TX-100 in PBS and immunoprecipitated with W6/32 Ab. Eluted materials were reimmunoprecipitated with anti-HA Ab and the second eluates were resolved by SDS-PAGE. Result in B is a representative of two experiments.

Discussion

In this study, we showed that cytosolic aminopeptidases play a distinct role in MHC class I surface expression. We observed that depletion of PSA led to alterations in the quality of peptides loaded onto MHC class I molecules.

Disparity among HLA class I alleles with regard to aminopeptidase dependence

To test whether our observations could be generalized to other HLA class I alleles, we further analyzed eight different HLA class I alleles. For clarity, we renamed HLA-A*0101 as A1, HLA-A*0201 as A2, HLA-A*0301 as A3, HLA-A*1101 as A11, HLA-A*2402 as A24, HLA-B*0801 as B8, HLA-B*3501 as B35, and HLA-B*4402 as B44.

In the absence of PSA, cell surface expression of HA-A2 and HA-B8 (Fig. 6D) was enhanced by 50–60%; the surface level of HA-B44 also increased, albeit to a lesser extent. Conversely, surface levels of HA-B35 and HA-A1 were reduced by 10–20%. PSA knockdown had little effect on the surface expression of HA-A24 (Fig. 6D). In BH-depleted cells, cell surface expression of HA-A2 (88%) and HA-A3 (46%) was significantly augmented, whereas the surface level of HA-A1 was reduced by 10%. Depletion of BH modestly affected the other HLA class I alleles (Fig. 6E).

Because the surface expression of HA-A3 and HA-B8 was significantly affected by PSA and BH (Fig. 6A, D, and E), we further assessed the thermostabilities of HA-A3 and HA-B8. HA-B8 was more thermostable in PSA-depleted cells than in control cells (Fig. 6B, top, compare lanes 2 and 5), whereas HA-A3 was more thermostable in BH-depleted cells than in control cells (Fig. 6B, bottom, compare lanes 2 and 5). These results show that the thermostability of HA-B8 and HA-A3 correlates with their surface expression. Thus, it is highly probable that allelic specificity of cytosolic aminopeptidases is a general phenomenon among HLA class I alleles.
onto MHC class I molecules, thereby resulting in down-regulation of cell surface MHC class I expression in HeLa cells. BH depletion caused increased thermostability of MHC class I molecules and subsequent up-regulation of MHC class I molecules at the cell surface. LAP knockdown did not exert a considerable effect on the MHC class I surface expression, at least in our system. It was previously demonstrated that deficiency of LAP in HeLa cells has no effect on the surface presentation of H-2Kb/OVA8 (11); however, the surface level of HLA class I molecules was not analyzed in the same conditions. To our knowledge, our study is the first to examine the effect of LAP deficiency on the surface expression of human MHC class I molecules. We did not detect any defect in the surface expression of human MHC class I molecules in LAP-deficient HeLa cells; however, because we did not test the effect of LAP deficiency on HLA class I alleles in detail, we could not exclude the possibility that LAP affects the surface expression of HLA class I molecules in an allele-dependent manner. If LAP has an allelic dependency, like PSA and BH, the phenotype we observed in Fig. 1A (bottom) may be ascribed to a zero-sum situation; LAP might decrease the surface level of one allele and increase that of another allele. Alternatively, our findings could be explained by the irrelevance of LAP in human MHC class I Ag presentation as described in LAP-deficient mice (11). Finally, we provide evidence of a differential effect of cytosolic aminopeptidases PSA and BH on different HLA class I alleles.

A number of chemical inhibitors have been used to study the role of aminopeptidases in MHC class I Ag processing (7, 9, 18). A “model Ag,” a “model peptide precursor” (i.e., OVA), and a well-known viral epitope have been applied to study the enzymatic activity of cytosolic peptidases (11, 12, 36–38). However, the function of certain peptidases in the presentation of MHC class I Ags in vitro and in vivo systems, as well as the model Ags used in each case, have been controversial (8, 10, 12, 36, 39, 40). Rock and colleagues showed that the trimming of antigenic peptides in LAP-, BH-, or PSA-deficient mice was not reduced and that there was no significant difference in the presentation of OVA8 from N-terminally extended precursors or full-length OVA (11, 12, 36). This suggests that these aminopeptidases may not be essential for production of antigenic peptides, but rather, that they may have functional redundancy. As proposed by those authors, it remains possible that these aminopeptidases make a small portion of antigenic peptide, or contribute to the response to unique situations (11, 12, 36). Our results show slight inconsistencies with earlier reports indicating that LAP, BH, or PSA are not essential for

![FIGURE 5. Effect of PSA or BH on the cell surface expression of HA-A68 and HA-B15. A, HeLa cells expressing HA-A68 or HA-B15 were cotransfected with siRNA specific for PSA, BH, LAP, or GFP and mock-Out, selected by treatment with 100 μM ouabain for 12 h, stained with an anti-HA Ab, and analyzed with flow cytometry (left, HA-A68; right, HA-B15). HA-A68/siGFP or HA-B15/siGFP cells were stained with isotype-matched control Ab as a negative control (M1). M2 indicates a population of transfectants expressing both HA-HLAs and specific siRNAs. The extent of the effect was identified by the peak channel value of each FACS profile. The vertical line in M2 in each histogram represents the peak channel of HA-A68/siGFP (left) or HA-B15/siGFP (right). The number in each histogram reflects the relative value of the peak channel. B, The effect of PSA or BH depletion on the peptide-dependent stability of HA-A68 and HA-B15. Transfectants produced as in A were analyzed by thermostability assay (top) as described in Materials and Methods. The thermostability at 37°C was plotted (bottom). Error bars in B represent the SD of three independent experiments. C, Target gene depletion was confirmed by immunoblot analysis of the lysate of PSA- or BH-depleted cells. A, B (top), and C show one representative experiment that was independently performed three times, all with similar results.](http://www.jimmunol.org/ Downloaded from)
production of antigenic peptides (11, 12, 36). The apparent discrepancy might be due to species-specific differences, methodological differences, or differences in the model Ags analyzed.

Using human cells, we analyzed the effect of cytosolic aminopeptidases on Ag presentation by not only a total pool of HLA class I molecules, but also by an individual allele. Interestingly, we found that cytosolic aminopeptidases influence the surface expression of HLA class I molecules in an allele-dependent manner. Thermostability analysis of 10 different HLA class I alleles, including two endogenous alleles from HeLa cells, revealed that PSA or BH depletion causes changes in the quality of antigenic peptides loaded and, as a consequence, differentially affects the peptide-dependent thermostability and the cell surface expression of individual HLA class I alleles. These observations suggest that specific cytosolic aminopeptidases could promote the production of optimal peptides for one HLA class I allele, while simultaneously inhibiting the production of optimal peptides for the other HLA class I allele. Given the allelic diversity among human populations, our data support the notion that the lack of detectable effect of a particular aminopeptidase on the cohort level of HLA class I molecules expressed at the cell surface does not necessarily mean that the aminopeptidase is dispensable for HLA class I-mediated Ag presentation.

How might cytosolic aminopeptidases be involved in MHC class I-mediated Ag processing? First, the ER aminopeptidase associated with Ag processing (ERAAP in mice; human ortholog, ER aminopeptidase (ERAP1)) is involved in the generation of mature epitopes from antigenic peptide precursors in the ER (41, 42). Loss of ERAAP decreases the stability of the peptide-MHC class I complex and diminishes the CD8+ T cell response (29, 43). Cytosolic aminopeptidases may directly participate in a trimming of proteasomal products. In this scenario, cytosolic aminopeptidases might provide the peptide pool that is preferred for ERAP1.

Second, cytosolic aminopeptidase may protect proteasomal products by chaperoning them. Free antigenic peptides are very susceptible to hydrolysis by a variety of peptidases that exist in the cytosol. In support of this idea, it has been reported that group II chaperonin TCP-ring complex- and heat shock protein 90α-associated proteostatic products are protected from destruction and therefore serve a key function in the Ag processing pathway (44, 45). Finally, BH and PSA are predominantly detected in the cytosol in a soluble form, although a membrane-associated form is also found in some tissues (46, 47). Although the exact function of the membrane-bound form of BH or PSA is not yet clear, their association with the membrane might bring peptides into close proximity with TAP (8).

In summary, in this study we demonstrate that cytosolic aminopeptidases differentially contribute to the HLA class I Ag presentation in an allele-specific fashion by affecting the quality of antigenic peptides loaded onto HLA class I molecules. These data suggest that one aminopeptidase may be productive for generating optimal peptides for a particular HLA class I allele, and simultaneously the other aminopeptidase may be destructive for the same HLA class I allele. In this study, we examined the functional specificity of cytosolic aminopeptidases in a single aminopeptidase-depleted condition. It will be crucial to investigate whether the HLA class I allele-specific effect of each aminopeptidase is observed in the specific aminopeptidase-overexpressed situation. In addition, it is very important to study whether changes of the pool of cytosolic peptides is occurred by alterations in the expression level of cytosolic aminopeptidase and whether these changes correlate with the HLA class I allele-specificity.

**Disclosures**

The authors have no financial conflict of interest.
References