Current Topics

Aminopeptidases in Health and Disease

Processing of Antigenic Peptides by Aminopeptidases

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Antigenic peptides presented to major histocompatibility complex (MHC) class I molecules are generated in the cytosol during degradation of cellular proteins by the ubiquitin-proteasome proteolytic pathway. Proteasome can generate N-extended precursors as well as final epitopes, and then the precursors are processed to mature epitopes by aminopeptidases. Both cytosolic peptidases (*i.e.* puromycin-sensitive aminopeptidase, bleomycin hydrolase and interferon- γ -inducible leucine aminopeptidase) and recently identified metallo-aminopeptidase located in the endoplasmic reticulum (*i.e.* adipocyte-derived leucine aminopeptidase/endoplasmic reticulum aminopeptidase 1 and leukocyte-derived arginine aminopeptidase) can generate final epitopes from precursor peptides. Some of these aminopeptidases are also considered to destroy certain antigenic peptides to limit the antigen presentation. Taken together, it is getting evident that aminopeptidases located in the cytosol and the lumen of endoplasmic reticulum play important roles in the generation of antigenic peptides presented to MHC class I molecules.

Key words aminopeptidase; antigen processing; major histocompatibility complex (MHC) class I; antigen presentation; proteasome; protein degradation

The adaptive immune system has evolved to protect organisms against pathogens. Central to maintaining immunity is cell-surface presentation of antigenic peptides to specific receptors on CD8⁺ cytotoxic T lymphocytes by class I major histocompatibility complex (MHC) molecules on infected cells. This cell-mediated recognition process results in the elimination of infected cells and is critically important to maintain immunity against virus and tumors.

By recent numerous studies, the comprehension how MHC class I ligands are generated has increased. Especially, during the last years, it has been clarified that several aminopeptidases can contribute to the processing of antigenic peptides presented to MHC class I molecules. In this review, the current knowledge about the role of aminopeptidases in the antigen generation is summarized.

CYTOSOLIC PROCESSING OF ANTIGENIC PEPTIDES

It is well established that cytosolic proteolysis is the major source of MHC class I bound ligands.¹⁾ Most of these 8—11 residue peptides are derived from peptides generated in the cytosol or nucleus during protein degradation by the ubiquitin-proteasome pathway.^{2—4)} Accordingly, inhibitors of proteasome block MHC class I antigen presentation.²⁾ Most of peptides degraded by proteasome are hydrolyzed quickly to amino acids by cytosolic peptidases, while some peptides escape from further degradation, transported into the endoplasmic reticulum (ER) and presented on MHC class I molecules.

While proteasome plays the major role in the generation of antigen peptides, involvement of other proteases in this process has been also demonstrated. Glas *et al.* exposed membrane permeable proteasome inhibitor, NLVS, to EL-4 lymphoma and cloned resistant cell line which termed adapted cells.⁵⁾ Although the activity of proteasome was markedly decreased in the cells, hydrolytic activity to alanyl-

alanyl-phenylalanyl-7-amino-4-methylcoumarin (AAF-AMC) which is a substrate for trypsin protease, a large protease complex in *Thermoplasma*, was increased, suggesting that AAF-AMC cleaving activity in adapted cells could compensate for the loss of proteasome function in antigen presentation. One of the responsible enzymes for AAF-AMC hydrolytic activity was co-purified with 26S proteasomes and identified as tripeptidyl peptidase (TPP) II.⁶⁾ TPP II possesses predominantly trypsin-like endoproteolytic activity as well as aminopeptidase activity that removes tripeptides sequentially from free N-termini of peptides.^{6,7)} TPP II and proteasome cleaved 41-mer polypeptide products derived from ovalbumin differently from each other. Furthermore, it was suggested that in human dendritic cells TPP II can generate immunodominant human immunodeficiency virus (HIV) epitope, HIV-Nef (73—82), independently from proteasome. These observations suggest that TPP II can degrade certain antigen proteins to peptide fragments distinct from that generated by proteasome, increasing the complexity of the MHC class I peptide repertoire.

TRIMMING AND DESTROY OF ANTIGENIC PEPTIDES IN THE CYTOSOL

Ligands for the MHC class I molecules are composed of peptides of 8—11 a.a. in length. Proteasome degrades the bulk of intracellular proteins and generates peptides ranging from 2 to 25 a.a. in length.⁸⁾ Whereas some antigenic peptides are directly produced by the proteasome in their final forms, others are produced as precursor peptides. In regard to precursors of epitopes, Craiu *et al.* examined which termini of precursor of antigenic peptide might be generated by proteasome.⁹⁾ It was revealed that proteasome inhibitor blocked the presentation of a chicken ovalbumin-derived antigenic epitope, SIINFEKL, with a C-terminal extension, but not with an N-terminal extension. These results suggest that

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while proteasome mediates proper C-terminal cleavage for the generation of several immunodominant class I-presented peptides, precursors of antigenic peptides with N-terminal extensions require further processing to final epitopes by some aminopeptidases after the proteasome action.^{9,10)}

In efforts to identify trimming aminopeptidases, leucine aminopeptidase (LAP) was first identified by Beninga *et al.*¹¹⁾ They tested the effects of interferon (IFN)- γ , which enhances several important steps of antigen presentation, on the degradation of ovalbumin-derived antigenic peptide precursor QLESINFEKL in the cytosol. IFN- γ enhanced degradation of the precursor peptide and generation of final epitope SIINFEKL in HeLa and U937 cells. They also examined the hydrolytic activity toward various amino acid-AMC in soluble fraction from HeLa and U937 cells to investigate the aminopeptidases involved in the trimming of antigenic peptide precursors. In IFN- γ -stimulated cells, hydrolytic activity to several substrates such as Leu-, Lys-, Met-, Cys- and Phe-AMC were enhanced, indicating the presence of IFN- γ -inducible aminopeptidases in the cytosol. Since the substrate specificity of IFN- γ -inducible aminopeptidase was similar to that of LAP purified from porcine kidney, LAP was considered as an IFN- γ -inducible aminopeptidase involved in the antigen processing.¹²⁾ In addition, LAP can generate a final antigen epitope SIINFEKL *in vitro*. Taken together, it is concluded that LAP can contribute to the trimming process of antigenic peptide in the cytosol.

Two other aminopeptidases were also identified as trimming enzymes of precursor peptides in the cytosol. It was shown that the class I epitope of vesicular stomatitis virus nucleoprotein (VSV-NP) was first processed through both proteasome-dependent and -independent proteolytic steps and subsequently generated by some aminopeptidases.¹³⁾ AAF-chloromethylketone (CMK) inhibited the cleavage of the precursor of VSV-NP epitope with N-terminal flanking 5 amino acids, SLSDLRGYVYQGL, to the final epitope RGYVYQGL. In an attempt to purify the enzyme responsible for SLSDL-AMC hydrolytic activity sensitive to AAF-CMK, puromycin sensitive aminopeptidase (PSA) and bleomycin hydrolase (BH) were identified from cytosol fraction of a human EBV-transformed B cell line.¹⁴⁾ PSA is a member of aminopeptidase belonging to the M1 family of metallopeptidases⁸⁾ and the first candidate in this group of peptidases which function as a trimming enzyme of antigen peptides. It has been suggested that PSA is required for regulation of cell cycle, normal growth and behavior associated with anxiety and pain.^{15,16)} However, physiological substrates are still elusive. Another trimming peptidase, BH, is a cysteine protease and originally discovered as an enzyme that can inactivate anticancer drug bleomycin.¹⁷⁾ Its expression level is associated with sensitivity to bleomycin in tumor cells. PSA and BH are widely distributed in many tissues and their gene structures are in common with housekeeping genes.^{18,19)} Furthermore, the enzymes were shown to generate VSV-NP epitope from N-extended minigenes *in vitro*. From these results, it is concluded that PSA and BH can act as trimming enzymes which generate the class I epitopes in the cytosol. In addition, the possibility that TPP II and PSA can act sequentially on N-terminal processing of human RU1 epitope was also reported.²⁰⁾

Peptides generated by proteasome are generally hydrolyzed into amino acids rapidly. Antigenic peptides and their precursors are also thought to be degraded to amino acids efficiently. Saric and colleagues searched the metallopeptidases which might degrade antigenic peptides and found that cytosolic peptidases such as PSA and thimet oligopeptidase are involved in antigen degradation both *in vitro* and *in vivo*.^{21,22)} In addition, some chaperones such as Hsp 70, Hsp 90 and group II chaperonin TRiC can protect proteasome products from further degradation.^{23,24)} It is considered that the regulation of antigenic peptide degradation by these proteins is an important factor to determine the efficiency of antigen presentation.

PROCESsing OF ANTIGENIC PRECURSORS IN THE ER LUMEN

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Antigenic peptides generated in the cytosol are transported into the ER lumen by transporters associated with antigen processing (TAP) heterodimer, TAP1 and TAP2, and loaded onto newly synthesized MHC class I molecules.²⁵⁾ It was shown that some mature epitopes presented efficiently to MHC class I molecules often show very low affinities to TAPs. On the other hand, N-extended precursors of these epitopes reveal high affinities to TAPs and presented efficiently to cytotoxic T lymphocytes.²⁶⁾ These findings suggest that TAP preferentially transport antigenic peptides as N-extended precursor forms into the ER lumen and transported precursors are then trimmed by some ER aminopeptidases.

To elucidate the molecular nature of the trimming peptidase in the ER lumen, enzymatic properties of ER aminopeptidases were characterized. Komlosi *et al.* showed that the conversion of ESIINFEKL, a precursor of ovalbumin-derived antigenic epitope, to SIINFEKL was completely inhibited by the metallo-chelator, 1,10-phenanthroline, suggesting that metalloenzyme(s) is involved in the antigen processing in the ER.²⁷⁾ Other aminopeptidase inhibitors such as leucine thiol, Leu-CMK and bestatin also reduced the class I antigen presentation. Substrate specificity of luminal trimming peptidase was examined using a library of N-terminally extended precursors of SIINFEKL epitope with five amino acid residues.²⁸⁾ Trimming activity in the ER lumen was specifically blocked if proline is located at P2 position, leading the accumulation of X-Pro-X_n peptide. In fact, peptide with the X-Pro-X_n motif is a large fraction (*ca.* 20%) of peptides presented to some MHC class I molecules. Although TAPs show lower affinity to peptides containing proline at P2 position, longer precursors with N-terminal flanking residues can be efficiently transported into the ER by TAPs. Thus, trimming by ER aminopeptidases seems to be an essential step for the generation of antigen epitopes with the X-Pro-X_n motif.

The first identification of a candidate protein which trim precursors to final epitopes was reported by Menoret *et al.*²⁹⁾ They demonstrated that an abundant ER luminal chaperone, gp96/glucose-regulated protein 94 (GRP94), purified to homogeneity had aminopeptidase activity toward several synthetic substrates such as Leu-*p*-nitroanilide and Ala-*p*-nitroanilide. The activity was eliminated by both aminopeptidase inhibitors (*i.e.* bestatin and amastatin) and a serine protease inhibitor, PMSF. When Ser⁶⁵⁵, a putative PMSF targeting residue, was mutated, the aminopeptidase activity of gp96/GRP94 was eliminated. In addition, gp96/GRP94-me-

diated trimming of K^b-binding VSV epitope was also shown. From these results, they concluded that gp96/GRP94 is responsible for the antigen trimming in the ER.

Identification of ER aminopeptidases responsible for the trimming was independently reported by two groups through biochemical approaches. They purified an aminopeptidase localized in the ER lumen, and named it ER aminopeptidase associated with antigen processing (ERAAP)³⁰⁾ or ER aminopeptidase 1 (ERAP1).³¹⁾ ERAP1/ERAAP is identical to previously described aminopeptidase named adipocyte-derived leucine aminopeptidase (A-LAP),³²⁾ puromycin insensitive leucine specific aminopeptidase (PILS-AP)³³⁾ and aminopeptidase regulator of tumor necrosis factor receptor type I shedding (ARTS-1).³⁴⁾ A-LAP/ERAP1 was originally cloned by us as a homologous protein of oxytocinase/placental leucine aminopeptidase (P-LAP)³⁵⁾ using expression sequence-tags (ESTs) constructed from human adipose tissue cDNA library. A-LAP/ERAP1 contains HEXXH(X)₁₈E gluzincin consensus zinc-binding motif and is classified into the M1 family of zinc metalloaminopeptidases. Immunocytochemical analysis showed that the enzyme was co-localized exactly with immunoreactivity of anti-KDEL antibody against the ER-retention signal sequence KDEL, indicating that A-LAP/ERAP1 is localized in the luminal side of the ER. Although recombinant A-LAP/ERAP1 showed limited specificity toward synthetic substrates, it could release various amino acids from N-terminal end of peptide hormones and precursors except when proline is located at the P2 position, leading the generation of epitopes with X-Pro-X_n motif.^{31,36)} These features of the A-LAP/ERAP1 fulfill the criteria for an antigen trimming aminopeptidase in the ER lumen as described above. In addition, like other proteins involved in antigen presentation, expression of A-LAP/ERAP1 was markedly enhanced by IFN- γ . When overexpressed,

A-LAP/ERAP1 enhanced the presentation of ovalbumin-derived epitope from N-extended precursors in the ER of intact cells. On the other hand, reduction of the enzyme expression through RNA interference prevents presentation of the ovalbumin-derived epitope to MHC class I molecules on the cell surface.³⁷⁾ These results indicate that A-LAP/ERAP1 is essential for the presentation of SIINFEKL epitope.

Like A-LAP/ERAP1, we found sequences similar to P-LAP in leukocyte EST library and cloned another ER aminopeptidase, leukocyte-derived arginine aminopeptidase (L-RAP). L-RAP and A-LAP/ERAP1 differ in their specificity toward synthetic substrates. While A-LAP/ERAP1 revealed a preference for leucine, L-RAP hydrolyzed Arg-AMC most efficiently, followed by Lys-AMC. Arg hydrolytic activity was indeed found in the luminal fraction of the ER by Saric *et al.*³¹⁾ Since L-RAP can trim several antigenic precursor peptides such as HIV-Nef antigen and melanoma-associated protein gp100 antigen, we concluded that the enzyme is the second metallopeptidase which contributes to the processing of the MHC class I ligands.³⁸⁾ Indeed, L-RAP also fulfills the criteria for ER trimming peptidase, *i.e.* sub-cellular localization, susceptibility to inhibitors, generation of antigenic peptide and up-regulation by IFN- γ . However, there remains to elucidate the *in vivo* role of the enzyme in the future studies.

From genomic analysis, we have shown that P-LAP, A-LAP/ERAP1 and L-RAP form distinct subfamily of the M1 aminopeptidase family.³⁸⁾ We proposed that they should be classified into the oxytocinase subfamily of M1 aminopeptidases.³⁹⁾ It is tempting to speculate that the recent diversion of ER aminopeptidase genes reveals a linkage between the gene evolution of these enzymes and the acquisition of the adaptive immune system.

Fig. 1. Processing Pathway of Antigenic Peptides Presented to MHC Class I Molecules

CONCLUDING REMARKS

It is now getting evident that aminopeptidases both in the cytosol and ER play important roles in the generation of antigenic peptides presented to MHC class I molecules (Fig. 1). Further elucidation of the molecular mechanisms of antigenic peptide processing will allow the development of new therapeutic approaches to several pathological conditions such as virus infection, tumors and auto-immune diseases.

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REFERENCES

- 1) Rock K. L., Goldberg A. L., *Annu. Rev. Immunol.*, **17**, 739—779 (1999).
- 2) Rock K. L., Gramm C., Rothstein L., Stain R., Dick L., Hwang D., Goldberg A. L., *Cell*, **78**, 761—771 (1994).
- 3) Yewdell J. W., Bennink, J. R., *Curr. Opin. Immunol.*, **13**, 13—18 (1999).
- 4) Goldberg A. L., Cascio P., Saric T., Rock K. L., *Mol. Immunol.*, **39**, 147—164 (2002).
- 5) Glas R., Bogoy M., McMaster J. S., Gaczynska M., Ploegh H. L., *Nature* (London), **392**, 618—622 (1998).
- 6) Geier E., Pfeifer G., Wilm M., Lucchiari-Hartz M., Baumeister W., Eichmann K., Niedermann G., *Science*, **283**, 978—981 (1999).
- 7) Barret A. J., Rawlings N. D., Woessner J. F., "Handbook of Proteolytic Enzymes," Academic Press, London, 1998.
- 8) Kisselev A. F., Akopian T. N., Woo K. M., Goldberg A. L., *J. Biol. Chem.*, **274**, 3363—3371 (1999).
- 9) Craiu A., Akopian T., Goldberg A. L., Rock K. L., *Proc. Natl. Acad. Sci.*, **94**, 10850—10855 (1997).
- 10) Mo X. Y., Cascio P., Lemerise K., Goldberg A. L., Rock K. L., *J. Immunol.*, **165**, 5851—5859 (1999).
- 11) Beninga J., Rock K. L., Goldberg A. L., *J. Biol. Chem.*, **273**, 18734—18742 (1998).
- 12) Harris C. A., Hunte B., Krauss M. R., Taylar A., Epstein L. B., *J. Biol. Chem.*, **267**, 6865—6869 (1992).
- 13) Stolze L., Dick T. P., Deeg M., Pommerl B., Rammensee H.-G., Schild H., *Eur. J. Immunol.*, **28**, 4029—4036 (1998).
- 14) Stoltze L., Schirle M., Schwarz G., Schroter C., Thompson M. W., Hersch L. B., Kalbacher H., Stevanovic S., Rammensee H.-G., Schild H., *Nat. Immunol.*, **1**, 413—418 (2000).
- 15) Constam D. B., Tobler A. R., Rensing-Ehl A., Kemler I., Hersch L. B., Fontana A., *J. Biol. Chem.*, **270**, 26931—26939 (1995).
- 16) Osada T., Ikegami S., Takagi-Hayashi K., Yamazaki Y., Katoh-Fukui Y., Higashinakagawa T., Sakaki Y., Takeuchi T., *J. Neurosci.*, **19**, 6068—6078 (1999).
- 17) Sebt S. M., Mignano J. E., Jani J. P., Srimatkandada S., Lazo J. S., *Biochemistry*, **28**, 6544—6548 (1989).
- 18) Thompson M. W., Tobler A., Fontana A., Hersch L. B., *Biochem. Biophys. Res. Commun.*, **258**, 234—240 (1999).
- 19) Ferrando A. A., Pendas A. M., Llano E., Velasco G., Lidereau R., Lopez-Otin C., *J. Biol. Chem.*, **272**, 33298—33304 (1997).
- 20) Levy F., Burri L., Peitrequin A.-L., Levy N., Bachi A., Hellman U., Van den Eynde B. J., Servis C., *J. Immunol.*, **169**, 4161—4171 (2002).
- 21) Saric T., Beninga J., Graef C. I., Akopian T. N., Rock K. L., Goldberg A. L., *J. Biol. Chem.*, **276**, 36474—36481 (2001).
- 22) York I. A., Mo A. X. Y., Lemerise K., Zeng W., Shen Y., Abraham C. R., Saric T., Goldberg A. L., Rock K. L., *Immunity*, **18**, 429—440 (2003).
- 23) Srivastava P. K., *Annu. Rev. Immunol.*, **20**, 395—425 (2002).
- 24) Kurosawa J., Shastri N., *Mol. Cell*, **12**, 565—576 (2003).
- 25) Brigitte L.-B., Tampe R., *Physiol. Rev.*, **82**, 187—204 (2002).
- 26) Lauvau G., Kakimi K., Niedermann G., Ostankovitch M., Yotnda P., Firat H., Chisari F. V., Van Ender P. M., *J. Exp. Med.*, **190**, 1227—1239 (1999).
- 27) Komlos A., Momburg F., Weinschenk T., Emmerich N., Schild H., Nadav E., Shaked I., Reiss Y., *J. Biol. Chem.*, **276**, 30050—30056 (2001).
- 28) Serwold T., Gaw S., Shastri N., *Nat. Immunol.*, **2**, 644—651 (2001).
- 29) Menoret A., Li Z., Niswonger M., Altmeyer A., Srivastava P. K., *J. Biol. Chem.*, **276**, 33313—33318 (2001).
- 30) Serwold T., Gonzalez F., Kim J., Jacob R., Shastri N., *Nature* (London), **419**, 480—483 (2002).
- 31) Saric T., Chang S.-C., Hattori A., York I. A., Markant S., Rock K. L., Tsujimoto M., Goldberg A. L., *Nat. Immunol.*, **3**, 1169—1176 (2002).
- 32) Hattori A., Matsumoto H., Mizutani S., Tsujimoto M., *J. Biochem.*, **125**, 931—938 (1999).
- 33) Schomburg L., Kollmus H., Friedrichsen S., Bauer K., *Eur. J. Biochem.*, **267**, 3198—3207 (2000).
- 34) Cui X., Hawari F., Alsaaty S., Lawrence M., Combs C. A., Geng W., Rouhani F., Maskinis D., *J. Clin. Invest.*, **110**, 515—526 (2002).
- 35) Rogi T., Tsujimoto M., Nakazato H., Mizutani S., Tomoda Y., *J. Biol. Chem.*, **271**, 56—61 (1996).
- 36) Hattori A., Kitatani K., Matsumoto H., Miyazawa S., Rogi T., Tsu-ruoka N., Mizutani S., Natori Y., Tsujimoto M., *J. Biochem.*, **128**, 755—762 (2000).
- 37) York I. A., Chang S.-C., Saric T., Keys J. A., Favreau J. M., Goldberg A. L., Rock K. L., *Nat. Immunol.*, **3**, 1177—1184 (2002).
- 38) Tanioka T., Hattori A., Masuda S., Nomura Y., Nakayama H., Mizutani S., Tsujimoto M., *J. Biol. Chem.*, **278**, 32275—32283 (2003).
- 39) Tsujimoto M., Hattori A., *Biochim. Biophys. Acta* (2004) in press.