Trimming of Antigenic Peptides in an Early Secretory Compartment

By Heidi Link Snyder, Jonathan W. Yewdell, and Jack R. Bennink

From the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Summary

Major histocompatibility complex (MHC) class I molecules bind peptides of 8–10 residues in the endoplasmic reticulum (ER) and convey them to the cell surface for inspection by CD8-expressing T cells (T_{CD8+}). Antigenic peptides are predominantly derived from a cytosolic pool of polypeptides. The proteolytic generation of peptides from polypeptides clearly begins in the cytosol, but it is uncertain whether the final proteolytic steps occur before or after peptides are transported into the ER by the MHC-encoded peptide transporter (TAP). To study the trimming of antigenic peptides in the secretory pathway in the absence of cytosolic processing, we used an NH_2-terminal signal sequence to target to the ER of TAP-deficient cells, "tandem" peptides consisting of two defined T_{CD8+} determinants arranged from head to tail. We find that in contrast to cytosolic proteases in TAP-expressing cells, which are able to liberate antigenic peptides from either end of a tandem peptide, proteases (probably aminopeptidases) present in an early secretory compartment preferentially liberate the COOH-terminal determinant. These findings demonstrate that proteolytic activities associated with antigen processing are not limited to the cytosol, but that they also exist in an early secretory compartment. Such secretory aminopeptidases may function to trim TAP-transported peptides to the optimal size for binding to class I molecules.

Antigenic peptides are predominantly derived from cytosolic or nuclear proteins (reviewed in references 1, 2), and they usually consist of 8–10 residues (3). Peptides of 8–15 or more residues are transported from the cytosol into the endoplasmic reticulum (ER) by the MHC-encoded TAP, acronymic for transporter-associated with antigen processing (4–8). Loosely assembled class I α chains and β_2 microglobulin are retained in the ER until peptide binding induces a conformational alteration, resulting in their release and rapid transport through the Golgi complex to the cell surface. Although the proteolytic generation of antigenic peptides from polypeptides clearly begins in the cytosol, it is uncertain whether all processing events occur in the cytosol, or whether peptides can be trimmed once they have been exported into the secretory compartment.

To study the proteolytic processing of antigenic peptides in the secretory pathway, we used the TAP-deficient cell line T2 (9, 10). T2 cells present cytosolic peptides to T_{CD8+} at low or undetectable levels. The presentation defect can, however, be bypassed by appending a hydrophobic NH_2-terminal signal sequence to target peptides to the ER (11). Such peptides are presumably liberated from their NH_2-terminal signal through the action of signal peptidase. Signal peptidase is intimately associated with the proteinacious channel used to convey signal-containing proteins to the ER, and it is believed to act cotranslocationally (reviewed in reference 12).

The dependence of peptide association with class I molecules in T2 cells on signal sequence targeting of peptides enables examination of proteolytic events that occur exclusively in the secretory pathway. We previously demonstrated that mouse T_{CD8+} specific for viral peptides can lyse T2 cells coinfected with recombinant vaccinia viruses (rVV) expressing mouse class I molecules and antigenic peptides routed to the ER via NH_2-terminal signal sequences (13, 14). In this paper, we use this system to examine the efficiency with which viral peptides are generated in the secretory pathway from rVV-encoded polypeptides consisting of an NH_2-terminal signal sequence followed by "tandem peptides" composed of residues 52–59 from vesicular stomatitis virus nucleocapsid protein (N) (designated P_1, restricted by K^b) (15) and residues 147–155 from influenza virus nucleoprotein (NP) (designated P_2 restricted by K^d) (16). The biochemical nature and intracellular site of proteolytic generation of the P_2 determinant were determined by HPLC analysis of acid-soluble peptides associated with K^d, as well as a form of K^d retained in the ER by replacement of its cytosolic tail with that of adenovirus E3/19K glycoprotein (17).

Materials and Methods

Cell Lines. T2 cells (generously provided by P. Cresswell, Yale University, New Haven, CT) (9, 10) were maintained in IMDM supplemented with 7.5% FBS (vol/vol). L929 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM

Table 1. *Sequence of Tandem Peptides*

M-P_1P_2

Met **Arg Gly Tyr Val Tyr Gln Gly Leu** Thr Tyr Gln Arg Thr Arg Ala Leu Val

S-P_1P_2

Met Arg Tyr Met Ile Leu Gly Leu Leu Ala Leu Ala Ala Val Cys Ser Ala *Ala* **Arg Gly Tyr Val Tyr Gln Gly Leu** Thr Tyr Gln Arg Thr Arg Ala Leu Val

S-P_2P_1

Met Arg Tyr Met Ile Leu Gly Leu Leu Ala Leu Ala Ala Val Cys Ser Ala *Ala* Thr Tyr Gln Arg Thr Arg Ala Leu Val **Arg Gly Tyr Val Tyr Gln Gly Leu**

Amino acid sequence of tandem peptides encoded by rVV. Sequences of precursor peptides expressed by rVV containing the class I determinants P1 (**bold**) and P2 (underlined) preceded by either an initiating Met (*M*) or the adenovirus E3/19 K signal sequence (*S*) with an additional Ala (*italicized*) at the COOH terminus of the signal. These sequence data are available from EMBL/GenBank/DDJB under accession numbers Influenza PR/8/34 Nucleoprotein J02147, VSV (Indiana) Nucleocapsid J02431, and Adenovirus E3 19K S57052.

supplemented with 7.5% FBS (vol/vol). L929 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 7.5% FBS. Both cell lines were grown at 37°C in an air/CO_2 atmosphere (91%/9%).

Mice. 6–8-wk-old BALB/cByJ and C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were immunized intravenously with 10^7 PFU of rVV in balanced salt solution supplemented with 0.2% BSA (wt/vol) (BSS/BSA).

Viruses. The influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) was grown in 10-d-old embryonated chicken eggs and used as infectious allantoic fluid. VSV Indiana strain was grown in baby hamster kidney cells and used as a cell-free supernatant. rVV were grown in thymidine kinase–deficient human 143B osteosarcoma cells. Construction of rVV expressing full-length influenza NP (18), VSV N-protein (19), H-$2K^b$ (14), H-$2K^d$ (14), and ER-retained H-$2K^d$ ($EC_{15}K^d$) (17) have been described. For simplicity and clarity in this paper, the signal sequence of the E3/19K glycoprotein of adenovirus 5, denoted as ES in our previous publications, has been replaced with *S* denoting the signal sequence. In construction of rVV expressing nontargeted minigenes, an initiation Met (*M*) precedes each minigene product. Recombinant VV expressing N_{52-59} residues (*M*-P_1 and *S*-P_1), and $NP_{147-155}$ residues (*M*-P_2, *S*-P_2) have also been described (13, 14). rVVs containing tandem T cell determinants were constructed in a manner similar to that described (13, 14). Briefly, synthetic oligonucleotides containing the appropriate nucleotide sequences were inserted into modified pSC11 or modified pSC11 containing the adenovirus 5 E3/19K signal sequence. Ligation of DNA encoding P_1P_2 or P_2P_1 into the NotI sites of the modified pSC11 plasmid resulted in the insertion of an additional Ala between *S* and the first class I determinant (14). Construction of P_1P_2 included an initiating *M* codon (*M*-P_1P_2). The fidelity of genetic engineering procedures was confirmed by DNA sequence analysis of plasmid inserts. Genes were inserted into the TK locus of VV by homologous recombination as described (20).

Cytolytic T Cell (CTL) Assay. Target cells (2 × 10^6) were infected with rVV (2 × 10^7 PFU) for 1 h in BSS/BSA followed by an additional 3-h incubation in IMDM. Cells were subsequently labeled with $Na^{51}CrO_4$ (10 μCi) for 1 h at 37°C, and were extensively washed in IMDM. Radiolabeled target cells were suspended in IMDM and incubated with NP- or VSV-specific T_{CD8+} cells generated from splenocytes primed with rVV expressing either NP (originally termed V69) (21) or N (originally termed IN N-Vac) (22), and were stimulated for 7 d in vitro by autologous spleen cells infected with PR8 or VSV, respectively. Effector cells were incubated with target cells for 6 h, after which 100 μl culture supernatant was harvested, and the amount of released ^{51}Cr was determined by γ counting. The percent-specific release was calculated from the formula: {[experimental release − spontaneous release (no splenocytes)]/[detergent release − spontaneous release]} × 100.

Peptide Extraction. T2 cells (10^9) were infected with rVV-*S*-P_1P_2 and either VV-K^d- or VV-$EC_{15}K^d$. Single rVV infections of rVV-*S*-P_1P_2 and rVV-$EC_{15}K^d$ were controlled for by coinfection with wild-type VV. Cells were infected at a multiplicity of infection of 10 for 1 h in BSS/BSA, and were then incubated for 15 h at 37°C in IMEM medium supplemented with 7.5% FBS (vol/vol). Peptides were extracted from T2 cells as described (16, 17), with the exception that cellular material <3,000 kD was collected by passing extracts through filters (Macrosep; Filtron Technology Corp., Northborough, MA) (3,000 kD cut-off). One fifth of the acid extract was resuspended in H_2O, vacuum dried, resuspended in 300 μl PBS, and assayed directly as described below. The remaining extract was separated by reverse-phase HPLC (Waters 600; Waters

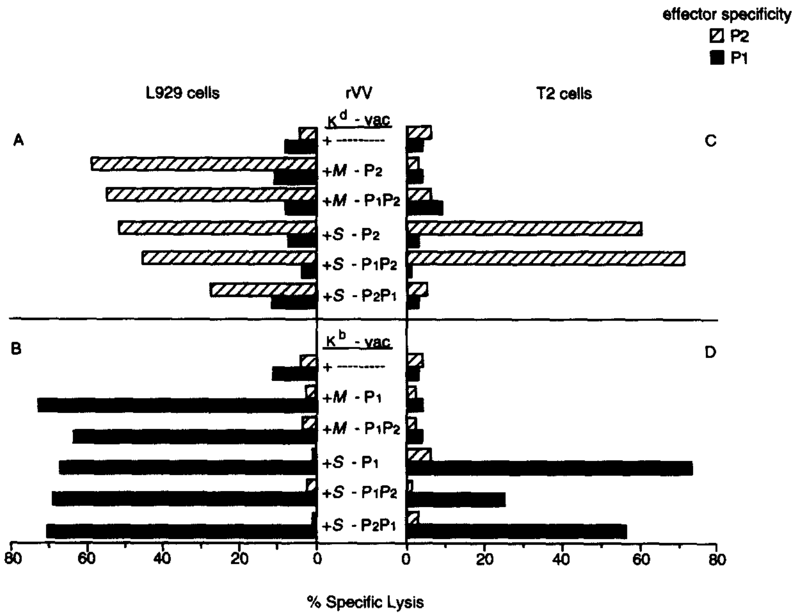

Figure 1. Trimming of antigenic peptides in the secretory pathway. L929 (*A* and *B*) or T2 (*C* and *D*) cells were coinfected with rVV expressing K^b or K^d, and rVV expressing either *M*-P_1, *S*-P_1, *M*-P_2, *S*-P_2, *M*-P_1P_2, *S*-P_1P_2 or *S*-P_2P_1. After ^{51}Cr labeling, cells were incubated with splenocytes containing T_{CD8+} specific for P_1 or P_2. Effector/target ratios: K^d-restricted, P_2-specific effectors (*hatched bars*) = 36.1; K^b-restricted, P_1-specific effectors (*solid bars*) = 12:1.

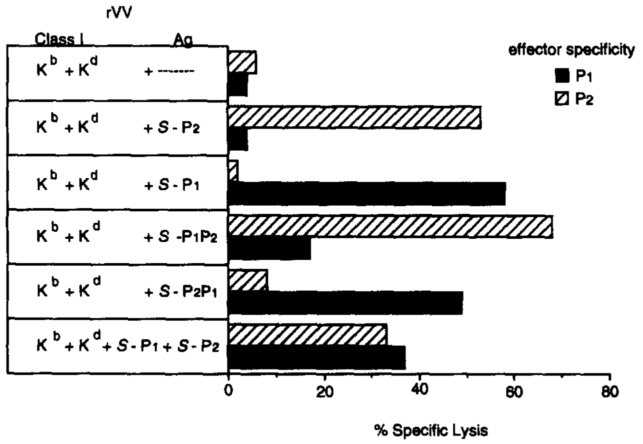

Figure 2. Trimming is not effected by coexpression of K^b and K^d. T2 target cells (*A* and *B*) were coinfected as indicated with rVV expressing K^b or K^d or both recombinants, and either *M*-P_1, *S*-P_1, *M*-P_2, *S*-P_2, *M*-P_1P_2, *S*-P_1P_2, or *S*-P_2P_1. After ^{51}Cr or labeling, cells were incubated with splenocytes containing T_{CD8+} specific for P_1 or P_2. Effector/target ratios: K^b-restricted, P_1-specific effectors (*solid bars*) + 60:1; K^d-restricted, P_2-specific effectors (*hatched bars*) = 60:1.

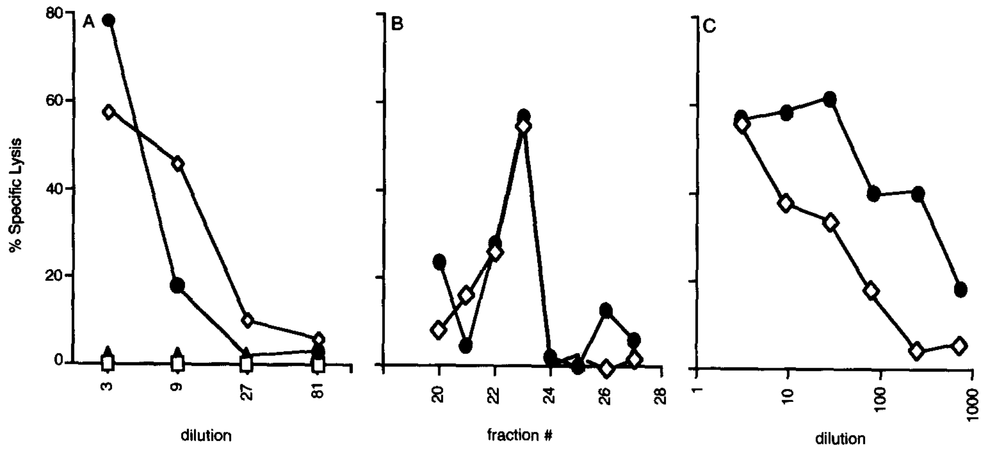

Figure 3. P_2 is trimmed from *S*-P_1P_2 in the ER. (*A*) ^{51}CR-labeled P815 (H-2^d) cells were incubated with threefold dilutions of TFA extracts of material <3 kD from T2 cells infected with rVVs as indicated and incubated with splenocytes containing T_{CD8+} specific for P_2. (*B*) Material present in extracts derived from cells coinfected with *S*-P_1P_2 and either VV-K^d or VV-$EC_{15}K^d$ was fractionated by HPLC and tested for the ability to sensitize ^{51}Cr-labeled P815 cells for lysis by P_2-specific T_{CD8+}. (*C*) Threefold dilutions of peptide isolated in fraction 23 in *B* were tested for the ability to sensitize ^{51}Cr-labeled P815 cells for lysis by P_2-specific T_{CD8+}. -□-, *S*-P_1P_2 + VV; -▲-, VV + EC15-K^d; -◇-, *S*-P_1P_2 + EC15-K^d; -●-, *S*-P_1P_2 + K^d.

Chromatography Division, Milford, MA), as described (16, 17). Fractions were vacuum dried and resuspended in 300 μl PBS. NP peptide antigenic activity was detected in the ^{51}Cr release assay by incubating threefold dilutions of each chromatographed fraction with ^{51}Cr-labeled P815 target cells preincubated overnight at 26°C to increase the expression of peptide binding MHC class I molecules. Cells were labeled at 26°C with $Na^{51}CrO_4$, and were incubated with peptide for 1 h at 26°C before the addition of H-2^d–restricted, NP-specific splenic effector cells at an effector/target ratio of 20:1. Effector and target cells were incubated at 37°C for 7–8 h and harvested as described above.

Results

Presentation of ER-targeted Tandem Peptides. Minigenes expressing peptide precursors of 17 residues were constructed from two class I–binding peptides, N_{52-57} (P_1) and $NP_{147-155}$ (P_2) arranged head-to-tail in both orientations, preceded by the adenovirus 5 E3/19K signal sequence (*S*) as shown in Table 1 and as described in the Materials and Methods. rVV were generated to express the tandem peptides, *S*-P_1P_2, *S*-P_2P_1 and also *M*-P_1P_2. The latter peptide lacks a signal sequence to control for TAP-independent peptide presentation. Since neither T2 cells nor L929 cells (used as TAP-expressing control cells) express the K^d or K^b class I molecules that present, respectively, the P_1 and P_2 peptides to T_{CD8+}, cells were coinfected with rVVs expressing antigenic peptides and rVVs expressing class I α chains. Presentation of P_1 and P_2 determinants was assessed in standard ^{51}Cr release assays using a secondary in vitro–stimulated splenocyte populations containing T_{CD8+} specific for P_1 or P_2.

As seen in Fig. 1, *A* and *B*, in L929 cells, both P_1 and P_2 determinants were liberated from *S*-P_1P_2 and *S*-P_2P_1, as well as from *M*-P_1P_2. The specificity of T_{CD8+} recognition of these determinants is demonstrated by the reciprocal pattern of peptide specific lysis by T_{CD8+}. The ability of L929 cells to present peptides from each of the rVVs tested provides functional demonstration of the integrity of the rVVs used to study presentation by T2 cells below.

TAP-deficient T2 cells (Fig. 1, *C* and *D*) demonstrated a more restricted pattern of presentation than L929 cells. Only the COOH-terminal determinant from either *S*-P_1P_2 or *S*-P_2P_1 was presented at similar levels to cells infected with rVVs expressing ER-inserted single determinants. Neither peptide was presented after infection with *M*-P_1P_2 which demonstrates that presentation of the *S*-tandem peptides in TAP-deficient cells is signal dependent. The ability of T2 cells to present P_1 from *S*-P_1 and P_2 from *S*-P_2 demonstrates that signal peptidase is perfectly capable of cleaving each of the antigenic peptides from the E3/19K signal sequence in an antigenic form. Thus, it is very likely that each of the tandem peptides is liberated from the signal sequence with its NH_2 terminus intact. Most striking was the complete failure of T2 cells to present P_2 from the *S*-P_2P_1 polypeptide. This finding was consistently reproduced in a large number of experiments. The presentation of the P_1 determinant from *S*-P_1P_2 peptide at intermediate levels was routinely observed, indicating that secretory proteases demonstrate some capacity to liberate this peptide from its flanking sequences.

Coexpression of K^b and K^d with precursor peptides in T2 cells did not alter the pattern of peptide presentation, since cells coinfected with VV-K^d, VV-K^b, VV-*S*-P_1, and VV-*S*-P_2 presented both determinants to T_{CD8+} (Fig. 2). This demonstrates that cells infected with even four rVVs express each foreign gene product at sufficient levels for T_{CD8+} recognition, although the decreased levels of presentation likely stem from reduced expression of individual gene products.

Based on these findings, we conclude (*a*) that optimal processing of antigenic peptides from longer polypeptides within the secretory pathway entails that the determinant be located at the extreme COOH terminus; and (*b*) that this selective presentation of COOH-terminal peptides is not influenced by the presence or absence of a class I molecule able to bind the NH_2-terminal peptide.

Intracellular Localization of the Site of Peptide Liberation. The dependence of peptide presentation on a NH_2-terminal signal sequence demonstrates that proteolytic processing of the peptide occurs in the secretory pathway. To localize the site of proteolysis to the early (ER, *cis*-Golgi network) or late (medial and *trans*-Golgi complex, post-Golgi transport vesicles) portions of the secretory pathway, we used a rVV expressing a K^d-molecule whose cytosolic domain was replaced with that of the E3/19K glycoprotein (17). We previously reported that L929 cells coinfected with this rVV (termed VV-$EC_{15}K^d$) and an rVV expressing full-length NP do not present $NP_{147-155}$ to T_{CD8+} (17). HPLC purification of TFA-soluble peptides from these cells demonstrated that $EC_{15}K^d$ bound similar quantities of antigenic peptides as unmodified K^d. Based on immunocytochemical and biochemical evidence, the poor efficiency of $EC_{15}K^d$ presentation of $NP_{147-155}$ can be attributed to its retention in the ER.

NP-specific T_{CD8+} were unable to lyse T2 cells coinfected with VV-$EC_{15}K^d$ and either VV *S*-P_2 or VV *S*-P_1P_2 (not shown), demonstrating that $EC_{15}K^d$ is also retained in the early secretory pathway of T2 cells. To determine whether intracellular $EC_{15}K^d$ contained antigenic peptides, cells were homogenized in 0.1% TFA, and serial dilutions of unfractionated material of <3 kD were tested for ability to sensitize P815 cells (H-2^d) for lysis by P_2-specific T_{CD8+}. As seen in Fig. 3 *A*, recovery of antigenic peptide in acid extracts required the coexpression *S*-P_1P_2 and $EC_{15}K^d$, once again demonstrating the requirement of class I molecules in the recovery of antigenic peptides (16). Similar amounts of peptides were recovered from cells expressing $EC_{15}K^d$ and unmodified K^d, indicating that retention of K^d in the ER has little, if any, effect on the efficiency of peptide generation or association. Antigenic peptides extracted from $EC_{15}K^d$ and K^d were chromatographed by reverse-phase HPLC, and the fractions were tested for antigenic activity. Antigenically active peptides were recovered in a single peak that coeluted with a synthetic peptide corresponding to the P_2 peptide, $NP_{147-155}$ (Fig. 3 *B*). This is consistent with the possibility that proteases in the early secretory pathway liberate the COOH-terminal nonamer from *S*-P_1P_2, but more precise chemical analysis of the antigenically active peptide is required to be certain that the peptide does not contain a few additional NH_2-terminal residues.

Discussion

In the present study, we show that proteases present in the early secretory pathway contribute to the processing of peptides delivered to the ER in a signal sequence–dependent manner. It is striking that the COOH-terminal peptide is presented much more efficiently than the NH_2-terminal peptide. This cannot be attributed to chemical differences between NH_2- and COOH-terminal peptides, since similar patterns were observed with rVVs encoding reciprocal peptide precursors. The more efficient presentation of the COOH-terminal determinant possibly reflects the critical nature of the interaction of COOH-terminal residues of antigenic peptides with class I molecules. The COOH-terminal residue serves as both an "anchor" residue whose side chain makes an important interaction with pockets in the class I molecule, and also provides critical binding energy through the interaction of main chain atoms with the binding groove. Extension of COOH termini by even a single residue often results in a drastic drop in peptide binding to class I molecules (16). We favor the idea that some portion of the tandem peptide is tethered to class I molecules via its COOH terminus, while the NH_2-terminal portion is trimmed by an aminopeptidase. The initial ligand might be the entire precursor peptide, or a fragment produced by endopeptidase activity.

The presence of endopeptidases in the early secretory pathway is consistent with the partial presentation of P_1 from S-P_1P_2. The inefficient nature of this presentation might indicate a low level of endopeptidase cleavage of the precise P_1P_2 junction, or inefficient trimming of a longer peptide by carboxypeptidases. It is important to emphasize that the very large difference in efficiency of presentation of P_1 and P_2 by cells expressing S-P_2P_1 points very strongly towards the action of an aminopeptidase that creates the P_1 peptide from either P_1P_2 or a COOH-terminal fragment of P_1P_2 created by endopeptidase activity.

Our findings implicating secretory aminopeptidases in antigen processing are, of course, limited to peptides delivered to the ER in a signal sequence–dependent manner. The possibility that these proteases can also contribute to the processing of TAP-transported peptides is, however, supported by recent findings regarding the specificity of TAP. Using a permeabilized cell system, TAP has been directly shown to transport peptides of up to at least 13 residues (7). Peptides of up to 30 or so residues have been recovered from a subset of HLA-B27 reactive with a specific mAb. The cell surface binding of this mAb to TAP-deficient cells was greatly reduced relative to TAP-expressing cells, suggesting that TAP is capable of transporting even very long peptides (23). Findings using permeabilized cells or isolated microsomes indicate that TAP preferentially transports peptides with the types of COOH-terminal residues favored by class I molecules (5, 6). Similarly, it has been reported that proteasomes (a multicatalytic cytosolic protease possibly involved in antigen processing) preferentially produce peptides with similar COOH termini, while cleavage at NH_2 termini is much less selective (24–26). These findings are consistent with the idea that many antigenic peptides are delivered to the ER with the proper COOH terminus, but with extensions at the NH_2 terminus that can be removed by ER-aminopeptidases. Furthermore, these findings imply that the presence of a carboxypeptidase in the early secretory pathway might constitute a serious threat to antigenic peptides. Notably, a deficiency in ER carboxypeptidases is supported by our previous observation that expression of an exotic secreted carboxypeptidase is needed to facilitate presentation of a peptide extended by two residues from the natural COOH terminus (13).

T2 cells efficiently produce antigenic peptides from HIV-I gp160, probably through the action of proteases located in an early secretory compartment (27). The processing of intact membrane or secretory proteins in the ER of T2 cells would appear to be the exception rather than the rule, however, since neither integral membrane glycoproteins from measles (28) nor influenza viruses, nor ovalbumin, nor a secreted full-length form of influenza virus NP (our unpublished observations) is detectably processed in the secretory pathway of these cells. Perhaps the proteolytic processing of gp160 in T2 cells is initiated by the formation of aggregates of GP160 with CD4, which are disposed of by the ER degradative system used to prevent the accumulation of defective proteins (29). It will be of interest to examine the relationship between the proteases that contribute to the presentation of gp160 and our peptide precursors.

The identities of the secretory proteases involved in antigen processing remain to be established by biochemical methods. The possible involvement of class I molecules in serving as a template for the appropriate proteases, or as a protease itself, as suggested in explanation for the dependence of class I molecules on the isolation of antigenic peptides from cells (30), also remains as a subject of further investigation. The reagents described in this report should prove useful in the biochemical characterization of the secretory proteases involved in antigen processing.

We thank Beth Buschling and Judy Stephens for excellent technical assistance.

Address correspondence to Dr. Heidi Link Snyder, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

Received for publication 25 April and in revised form 30 August 1994.

References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
2. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to MHC class I molecule-restricted T lymphocytes. *Adv. Immunol.* 52:1.
3. Rammensee, H.-G., K. Falk, and O. Rötzschke. 1993. Peptides naturally presented by MHC class I molecules. *Annu. Rev. Immunol.* 11:213.
4. Shepherd, J.C., T.N.M. Schumacher. P.G. Ashton-Rickardt, S. Imaeda, H.L. Ploegh, C.A. Janeway, Jr., and S. Tonegawa. 1993. TAP-1-dependent peptide translocation *in vitro* is ATP dependent and peptide selective. *Cell.* 74:577.
5. Schumacher, T.N.M., D.V. Kantesaria, M.-T. Heemels, P.G. Ashton-Rickardt, J.C. Shepherd, K. Fruh, Y. Yang, P.A. Peterson, S. Tonegawa, and H.L. Pleogh. 1994. Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. *J. Exp. Med.* 179:533.
6. Neefjes, J.J., F. Momburg, and G.J. Hämmerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science.* 261:769.
7. Mombrug, F., J. Roelse, G.J. Hämmerling, and J.J. Neefjes. 1994. Peptide size selection by the major histocompatibility complex–encoded peptide transporter. *J. Exp. Med.* 179:1613.
8. Androlewicz, M.J., and P. Cresswell. 1994. Human transporters associated with antigen processing possess a promiscuous peptide-binding site. *Immunity.* 1:7.
9. Salter, R.D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:943.
10. DeMars, R., C.C. Chang, S. Shaw, P.J. Reitnauer, and P.M. Sondel. 1984. Homozygous deletions that simultaneously eliminate expression of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. *Human Immunol.* 11:77.
11. Anderson, K., P. Cresswell, M. Gammon, J. Hermes, A. Williamson, and H. Zweerink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I–restricted cell-mediated lysis. *J. Exp. Med.* 174:489.
12. Dev, I.K., and P.H. Ray. 1990. Signal peptidases and signal peptide hydrolases. *J. Bioenerg. Biomembr.* 22:271.
13. Eisenlohr, L.C., I. Bačík, J.R. Bennink, K. Bernstein, and J.W. Yewdell. 1992. Expression of a membrane protease enhances presentation of endogenous antigens to MHC class I-restricted T lymphocytes. *Cell.* 71:963.
14. Bačík, I., J.H. Cox, R. Anderson, J.W. Yewdell, and J.R. Bennink. 1994. TAP-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino but not carboxy terminus of the peptide. *J. Immunol.* 152:381.
15. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-$2K^b$ molecule. *Nature (Lond.).* 348:213.
16. Falk, K., O. Rötzschke, K. Deres, J. Metzger, G. Jung, and H.-G. Rammensee. 1991. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *J. Exp. Med.* 174:425.
17. Lapham, C.K., I. Bačík, J.W. Yewdell, K.P. Kane, and J.R. Bennink. 1993. Class I molecules retained in the endoplasmic reticulum bind antigenic peptides. *J. Exp. Med.* 177:1633.
18. Smith, G.L., J.Z. Levin, P. Palese, and B. Moss. 1987. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. *Virology.* 160:336.
19. Mackett, M., J.K. Yilma, and B. Moss. 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. *Science (Wash. DC).* 227:433.
20. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5:3403.
21. Bennink, J.R., J.W. Yewdell, G.L. Smith, and B. Moss. 1986. Recognition of cloned influenza virus hemagglutinin gene products by cytotoxic T lymphocytes. *J. Virol.* 57:786.
22. Yewdell, J.W., J.R. Bennink, M. Mackett, L. Lefrancois, D.S. Lyles, and B. Moss. 1986. Recognition of cloned vesicular stomatitis virus internal and external gene products by cytotoxic T lymphocytes. *J. Exp. Med.* 163:1529.
23. Urban, R.G., R.M. Chicz, W.S. Lane, J.L. Strominger, A. Rehm, M.J.H. Kenter, F.G.C.M. Uytde-Haag, H. Ploegh, B. Uchanska-Ziegler, and A. Ziegler. 1994. A subset of HLA-B27 molecules contains peptides much longer than nonamers. *Proc. Natl. Acad. Sci. USA.* 91:1534.
24. Driscoll, J., M.G. Brown, D. Finley, and J.J. Monaco. 1993. MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. *Nature (Lond.).* 365:262.
25. Gaczynska, M., K.L. Rock, and A.L. Goldberg. 1993. Gamma-interferon and expression of MHC genes regulate hydrolysis by proteasomes. *Nature (Lond.).* 365:264.
26. Boes, B., H. Hengel, T. Ruppert, G. Multhaup, U.H. Koszinowski, and P.-M. Koetzel. 1994. Interferon γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J. Exp. Med.* 179:901.
27. Hammond, S.A., R.C. Bollinger, T.W. Tobery, and R.F. Siliciano. 1993. Transporter-independent processing of HIV-1 envelope protein for recognition by CD8+ T cells. *Nature (Lond.).* 364:158.
28. van Binnendijk, R.S., C.A. van Baalen, M.C.M. Poelen, P. de Vries, J. Boes, V. Cerundolo, A.D.M.E. Osterhaus, and F.G.C.M. Uytde-Haag. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I– and class II–restricted cytotoxic T cell recognition. *J. Exp. Med.* 176:119.
29. Bonifacino, J.S., and J. Lippincott-Schwartz. 1991. Degradation of proteins within the endoplasmic reticulum. *Curr. Biol.* 3:592.
30. Falk, K., O. Rötzschke, and H.-G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.).* 348:248.