Major Histocompatibility Complex Class I Viral Antigen Processing in the Secretory Pathway Defined by the *trans*-Golgi Network Protease Furin

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Summary

Classical antigen presentation by major histocompatibility complex class I molecules involves cytosolic processing of endogenously synthesized antigens by proteasomes and translocation of processed peptides into the endoplasmic reticulum (ER) by transporters associated with antigen presentation (TAP). Alternative pathways for processing of endogenous antigens, generally involving the ER, have been suggested but not fully proved. We analyzed the potential for class I presentation of proteolytic maturation of secretory antigens in the exocytic pathway. We found that hepatitis B (HB) virus secretory core protein HBe can efficiently deliver COOH-terminally located antigenic peptides for endogenous class I loading in the absence of TAP. Antigen presentation to specific cytotoxic T lymphocytes correlates with protein maturation at the COOH terminus, since modification of maturation and transport of HBe through the secretory pathway alters antigen presentation. Both maturation and a necessary processing step occur in the Golgi or post-Golgi compartment. Antigen presentation is independent of proteasome activity, but inhibitors of the *trans*-Golgi network resident protease furin inhibit both HBe maturation and antigen presentation. These results define a new antigen processing pathway located in the secretory route, with a central role for proteolytic maturation mediated by the subtilisin protease family member furin as an efficient source for antigen presentation.

Key words: antigen processing • major histocompatibility complex class I • secretory pathway • furin • virus

Major histocompatibility complex (MHC) class I molecules display a repertoire of endogenously processed peptides to $CD8^+$ T lymphocytes. These perform an immunological surveillance in search of possible antigenic peptides to destroy those presenting cells synthesizing foreign or abnormal proteins. Peptides bound by class I molecules have a typical size of 8–10 amino acids because their NH_2 and COOH termini are anchored in pockets that establish an extensive network of hydrogen bonds and contribute significantly to the overall affinity and thermal stability of the complex (1, 2). The majority of peptides are generated during degradation of proteins in the cytosol and are transported into the endoplasmic reticulum (ER)[1] by the MHC-encoded peptide transporter associated with antigen processing (TAP), where they bind nascent class I molecules (3, 4). The quality control machinery of the ER determines that only those complexes with the proper conformation and thermal stability travel through the exocytic route to the cell membrane.

It is currently agreed that cytosolic proteasomal degradation is the major source of peptides for class I binding and presentation to CTLs. The proteasome is a large 26S complex that degrades ubiquitinated proteins and contains the 20S proteolytic core, a large multicatalytic complex of 700 kD composed of 14 different subunits (5). In vitro experiments show that proteolytic digestion of full-length proteins or synthetic peptide precursors by the proteasome can, in some cases, generate the same antigenic peptides presented in vivo, in conjunction with many other products (6, 7). The differential efficacy of in vivo processing of related substrates can be mimicked in vitro with purified proteasomes (8). Natural modulators or synthetic inhibitors of proteasomes enhance or prevent in vivo antigen presentation, respectively, by MHC class I molecules (9–11). Lactacystin, a yeast metabolite that specifically inhibits proteasome by covalent binding to the conserved NH_2-terminal threonine of β catalytic subunits without inhibiting other proteases (12), inhibits antigen presentation to CTLs, although it does not fully prevent stable MHC class I mole-

[1]*Abbreviations used in this paper:* BFA, brefeldin A; ER, endoplasmic reticulum; HBV, hepatitis B virus; rVV, recombinant vaccinia virus; TAP, transporter associated with antigen processing; TGN, *trans*-Golgi network.

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cule assembly. In addition, the majority of antigenic peptides are generated in the cytosol, since cell mutants lacking TAP transporters for peptide translocation into the ER have severely reduced levels of class I molecules at the cell surface (13). Thus, generation of most antigenic peptides appears to require cleavage by proteasomes in the cytosol. What remains to be demonstrated is the implication of other cytosolic degradative pathways in antigen generation, and whether proteasomes generate the final antigenic peptides or intermediate precursors that must be trimmed to the optimal length. The latter may well be the case, as TAP efficiently translocates 9–13 amino acid–long peptides, although efficacy rapidly decreases for longer peptides (14). Recent studies with minigenes encoding epitope-containing oligopeptides show that trimming to actual epitope size may occur without the involvement of proteasomes (15–17). Trimming may take place in the cytosol or inside the ER (18), or along the secretory pathway (19).

Cytosolic peptides do not constitute the only source of peptides for class I binding as seen in the TAP^- mutants. Bound peptides in these cells derive from signal sequences that have direct access to the ER, where they are liberated by signal peptidase without involvement of proteasomes (20). In addition, ER-targeted polypeptide constructs containing natural epitopes can be trimmed effectively, probably by action of aminopeptidases, to generate the appropriate peptide for class I loading (21, 22), but full-length proteins could not be processed. Additional evidence for alternative processing is given by presentation in TAP^- mutant cells of epitopes corresponding to transmembrane regions of multiple membrane-spanning proteins (23), but it is unknown if these peptides are generated in the ER or in the cytosol, having access to the reticulum by a TAP-independent mechanism. In one case, an ER resident protein inserted in the membrane through an unknown mechanism independent of the translocon is able to provide antigenic peptides in a TAP-independent way, generated either in the ER, probably by action of signal peptidases, or, in some special constructs, by an unknown mechanism in the cytosol (24). The only additional full-length protein that appears to be processed in the ER-secretory pathway for antigen presentation is the HIV-1 env protein, although the proteases involved are unknown and therefore formal proof for processing in this pathway is lacking (25).

In this study, we have analyzed antigen processing of a protein known to mature in the secretory pathway in a process that involves proteolytic release of its COOH-terminal region. Endoproteolytic cleavage of proproteins is a common posttranslational modification of many peptide hormones, neuropeptides, growth factors, viral glycoproteins, etc., in the exocytic transport route, involving in most cases proteases with specificity for dibasic residues (26). We examined if this processing could be a source for generation of peptides appropriate for class I loading and antigen presentation. The natural occurrence of the hepatitis B virus (HBV) core protein both in the exocytic pathway and in the cytosol due to alternative usage of a precore signal sequence allowed us to compare processing in the secretory compartment with the classical pathway for class I presentation. In this way, we minimized artificial mistargeting of proteins to a different compartment, which may result in rapid degradation of the mislocated protein, and we thus provide a good control for studying antigen processing under normal conditions (27, 28). We used recombinant vaccinia viruses (rVV) encoding chimeric proteins that contain a murine CMV epitope at two different positions within the protein, both in the cytosolic and ER-targeted forms, to demonstrate efficient antigen processing in the secretory pathway, and for the first time identify proteases of the exocytic route involved in this new pathway of class I presentation.

Materials and Methods

Materials. Tunicamycin, brefeldin A (BFA), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). Pepstatin was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Lactacystin was either a gift from Dr. S. Omura (Kitasato Institute, Tokyo, Japan) or purchased from E.J. Corey (Harvard University, Cambridge, MA). The decanoyl-peptidyl-chloromethylketone decRVKR-CMK was a gift from Dr. W. Garten (Marburg University, Germany [29]). 9pp89 peptide was synthesized in a peptide synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA), purified, and analyzed by reversed-phase HPLC.

Cell Lines. The P13.1 cell line, a derivative from P815 mastocytoma cells ($H\text{-}2^d$) by transfection with the lacZ gene encoding β-galactosidase, was provided by Dr. H.G. Rammensee (Tübingen University, Germany [30]). The TAP-deficient human lymphoblastoid cell line T2 was provided by Dr. G. Hämmerling (German Cancer Research Center, Heidelberg, Germany). Murine Ltk^- fibroblasts ($H\text{-}2^k$) were obtained from Dr. U.H. Koszinowski (Munich University, Germany). L^d gene transfectants $T2/L^d$ and L/L^d were provided by Dr. P. Cresswell (Yale University, New Haven, CT [31]) and Dr. U.H. Koszinowski (32), respectively. All cell lines were maintained in IMDM supplemented with 10% FCS and 1% 2-ME, and incubated at 37°C under 5% CO_2.

rVV and Viral Infections. The rVV cC-A9A and sC-A9A encode chimeric proteins containing the murine CMV antigenic nonamer 9pp89 (YPHFMPTNL) flanked by penta-alanine and inserted at position 179 at the COOH terminus of the HBV precore protein. The chimeric protein cC-A9A (named HBc/C/$A_5$9A_5 in reference 33) is expressed in the cytosol, as it lacks an NH_2-terminal signal sequence. The wild-type signal sequence of the HBV precore protein was replaced with the one from influenza virus hemagglutinin (denoted s) in rVV sC-A9A, sN-9, and sN-9S. The rVV sN-9 and sN-9S express chimeric proteins containing 9pp89 at position 3 at the carrier protein NH_2 terminus. The rVV sN-9S differs from sN-9 by an exchange of a Gly residue next to the pp89 epitope for Ser that generates a glycosylation site, YPHFMPTNLS. The rVV eN-A9A (named HBe/N/$A_5$9A_5 in reference 33) encodes a chimeric protein containing 9pp89 flanked by penta-alanine and inserted at position 3 of the carrier protein with the wild-type signal sequence (denoted e). All rVV were generated according to Del Val et al. (33). The generation of rVV that encode the hemagglutinin signal sequence has been described (34).

$T2/L^d$ cells were infected as described (19) for 1 h with rVV at 40 PFU/cell at a concentration of 10^7 cells/ml in PBS with 0.2% BSA. After adsorption, cells were washed three times to eliminate virus inoculum and then were diluted tenfold in IMDM plus

7.5% FCS. This was followed by an additional 12-h incubation for CTL assays or a 15-h incubation for Western blot analysis. For CTL assays, P13.1 cells were infected for 3 h as described (19). For Western blot analysis, infected P13.1 cells were incubated for 5 h. To study chimeric protein glycosylation, tunicamycin was added to cells at a final concentration of 5 μg/ml after viral adsorption. To study the effect of BFA, infected cells were incubated with BFA after adsorption, at a concentration of 1 μg/ml for cytolysis or 0.5 μg/ml for Western blot analysis. To study the effect of lactacystin, P13.1 cells were treated with 30 or 100 μM lactacystin after viral adsorption. Because T2/L^d cells showed toxic effects at higher lactacystin concentrations and longer infection times, they were pretreated for 30 min and infected for 1 h in the presence of 5 μM lactacystin and then incubated with 10 μM lactacystin during 4 h infection (24). To analyze protein maturation, cells were incubated with pepstatin or decRVKR-CMK after viral adsorption.

Cytolytic Assays. Polyclonal 9pp89-specific CTLs were generated from mice immunized with murine CMV as described previously (16). Recombinant human IL-2, used for the long-term propagation of 9pp89-specific CTL lines, was provided by Hoffmann-La Roche (Basel, Switzerland). Infected cells were labeled for 1 h with $Na^{51}CrO_4$, washed, and incubated with CTLs at known E/T ratios in a standard 3–5 h chromium release assay (32). For controls with synthetic 9pp89, peptide was incubated with target cells during ^{51}Cr labeling. When cells had been treated with BFA during infection, the CTL assay was performed in the presence of BFA 0.5 μg/ml. All other CTL assays were performed in the absence of inhibitors. Coculture experiments were performed as described previously with minor modifications (22). In brief, cold T2/L^d cells infected with the testing rVV were mixed for 2 h with equal amounts of ^{51}Cr-labeled T2/L^d cells infected with control rVV, and CTLs were then added. Cycloheximide at a final concentration of 10^{-4} M was included during the coculture and CTL assay to prevent coinfection with the coculturing virus.

Western Blot. Chimeric proteins in the infected cells and secreted to the medium were analyzed by Western blot. Cell pellets or supernatant medium concentrated by Centricon 30 (Amicon Inc., Beverly, MA) corresponding to 10^6 cells were loaded onto polyacrylamide gels. After SDS-PAGE separation, proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and developed with a rabbit polyclonal anti-HBe serum by standard ECL procedures according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The antiserum was produced by repeated injection in Freund's adjuvant of purified HBV core particles (a gift of Dr. H.-J. Schlicht, Ulm University, Germany), and recognized both HBe and HBc.

Isolation and Quantitation of Naturally Processed Peptides. T2/L^d and T2 cells (2×10^8) were infected with rVV at a multiplicity of infection of 40 PFU/cell for 1 h. For L/L^d and Ltk^- cells (2×10^8), a multiplicity of infection of 10 was used. Cells were then incubated overnight in IMDM plus 7.5% FCS. Naturally processed peptides were extracted from whole cells with TFA and purified essentially as described previously (33). Macrosep 10K (Filtron Technology Corp., Northborough, MA) centrifugal concentrators were used for the isolation of low molecular weight peptides. Reverse-phase HPLC was performed using a Smart system (Amersham Pharmacia Biotech, Inc.) equipped with a C18 column, and peptides were eluted with a 20–37% CH_3CN gradient in 0.1% TFA. As internal standards in all HPLC runs, two pp89-unrelated peptides were included. Fractions of HPLC runs were tested in triplicate with pp89-specific CTLs by incubating with P13.1 target cells that had been cultured overnight at 26°C to maximize binding of exogenous peptide. Serial dilutions of positive fractions of HPLC runs were further tested.

Results

TAP-independent Presentation of an HBe-inserted Epitope. The C gene of human HBV codifies for a core protein that is naturally found in two forms: a secretory HBe form and a cytosolic HBc form (see scheme in Fig. 3 *A*). Alternate initiation codon usage adds a 29 amino acid–long pre-C signal to the HBc p21c protein that targets it to the ER (p25 form), where removal of its 19 amino acid–long signal peptide generates the immature HBe (p22) protein with an extended 10 amino acid–long NH_2 terminus compared with HBc. HBe is processed in the secretory pathway by an unknown mechanism involving removal of 34 residues of its COOH-terminal region, generating a secretory p17e form that is secreted to the external medium (35). This secretion is specific and occurs concomitantly with biosynthesis. Other forms are also found in the external medium after long periods of culture or high levels of protein expression (36). Under some experimental conditions, translocation to the ER is not complete and some HBe protein can be found in the cytosol (37). Substitution of the pre-C signal sequence by the influenza virus hemagglutinin signal sequence, generating a final product after signal peptidase cleavage of the same length as HBe but differing at the 10 NH_2-terminal amino acids (hereafter sHBe), results in the complete translocation of HBe to the ER, where the same processing machinery generates the homologous p17 secretory protein (34).

The existence of these two natural forms of a protein that differ in their intracellular location and maturation provided the opportunity to analyze the influence in antigen presentation by MHC class I molecules of processing in the secretory pathway. We were interested in studying if proteolytic maturation of a protein in the exocytic pathway could be a mechanism of antigen processing for class I presentation. For that purpose, we used a set of rVV that codify for HBe-related chimeric proteins consisting of either the cytosolic or the secretory proteins with the immunodominant 9-mer epitope of the murine CMV pp89 protein (YPHFMPTNL, or 9pp89; references 3, 32, and 33) flanked on both sides by five alanine residues (denoted A9A) and located at position 179 close to the COOH terminus (named C-A9A). Polyclonal CTLs specific for the 9pp89 epitope presented by L^d were used to analyze antigen processing and presentation by P13.1 target cells infected with the different rVV. As seen in Fig. 1, both cytosolic and secretory forms of the chimeric proteins were properly processed to the antigenic form of the 9pp89 epitope, since they were recognized by the L^d-restricted CTLs. On the contrary, on TAP^- T2/L^d cells, both proteins showed a different capacity to provide 9pp89 peptide to L^d (Fig. 1). As expected, 9pp89 in the cytosolic cC-A9A protein was not presented, as it cannot be transported to the ER. Surprisingly, cells infected with the secretory rVV construct sC-A9A were recognized and lysed by CTLs.

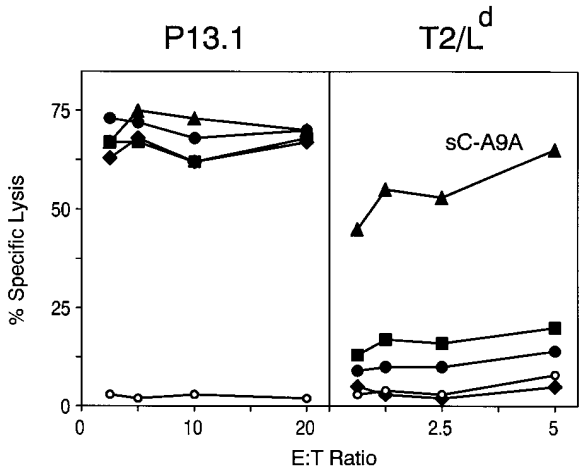

Figure 1. TAP-independent presentation to CTLs of secreted chimeric HBe proteins. P13 TAP^+ or $T2/L^d$ TAP^- cells infected with rVV encoding sC-A9A (*filled triangles*), cC-A9A (*filled circles*), sN-9 (*filled diamonds*), eN-A9A (*filled squares*), or HBe (*open circles*) were confronted in a ^{51}Cr-release assay with 9pp89-specific CTLs restricted by H2-L^d. See Materials and Methods for rVV nomenclature.

When we used rVV codifying for either secretory or cytosolic proteins with 9pp89 located at the NH_2 terminus, a region not affected by proteolytic maturation, TAP^- infected cells were not recognized. Both types of chimeric proteins were properly processed in TAP^+ P13.1 cells independently of their intracellular targeting (Fig. 1, and data not shown). Thus, 9pp89 in the ER-targeted sC-A9A protein is correctly processed and gains access to the secretory pathway for L^d binding and presentation without the need for peptide transporters, suggesting that antigen processing occurs in the secretory pathway.

Endogenous Processing of the TAP-independent Epitope. A potential alternative explanation to the generation of antigenic peptide in the exocytic pathway is that some sC-A9A molecules are processed in the cytosol or in other cellular compartments to a form that secretes 9pp89 or precursor antigenic peptides to the external medium that subsequently may bind to L^d at the cell surface. To exclude this possibility, we first checked that antigenic peptide was absent in the rVV inoculum. rVV–sC-A9A inoculum was adsorbed to $T2/L^d$ cells for 1 h just before ^{51}Cr labeling for the T cell assay. $T2/L^d$ cells treated in this way were not sensitized (Fig. 2 *A*, *top bar*), whereas cells incubated with synthetic 9pp89 peptide were readily lysed (*second bar*). Second, we incubated uninfected labeled target cells with the supernatant from cultures of rVV-infected $T2/L^d$ cells and tested for CTL recognition. As shown in the third bar, $T2/L^d$ cells incubated with culture medium from rVV–sC-A9A–infected cells were not lysed, and neither were $T2/L^d$ cells infected with rVV expressing the native secretory HBe protein or the cytosolic cC-A9A chimera (*fourth bar*, and data not shown). It could be argued that extracellular free peptide has a short life without being bound to L^d, but that accumulation of complexes during the time of infection with a continuous supply of free peptide would yield a sufficient number of L^d–9pp89 complexes for CTL recognition. To rule out these possibilities, ^{51}Cr-labeled target cells infected with native HBe-expressing rVV were coincubated with cold rVV–sC-A9A–infected cells for 2 h before the CTL assay. If there were extracellular antigenic peptides, cold rVV–sC-A9A–infected bystander cells should sensitize target cells. However, lysis was not observed (*seventh bar*). The relevant control showing no interference of cold cells with the CTL assay was provided by the recognition of labeled rVV–sC-A9A–infected targets at similar levels in the presence or absence of cold rVV-HBe–treated cells (*fifth* and *sixth bars*). To further prove that the epitope recognized in rVV-sC-A9A–infected cells derives from endogenously processed proteins, we used BFA, a drug that blocks class I export beyond the *cis*-Golgi compartment (38) and prevents surface expression of class I–peptide complexes of endogenous origin. The addition of BFA during infection completely blocked presentation in sC-A9A–infected $T2/L^d$ cells, whereas it did not affect sensitization by exogenous peptide (Fig. 2 *B*). These experiments show compelling evidence that the pp89 peptide presented by $T2/L^d$ infected with rVV-sC-A9A is derived from endogenous processing of the ER-targeted chimeric protein.

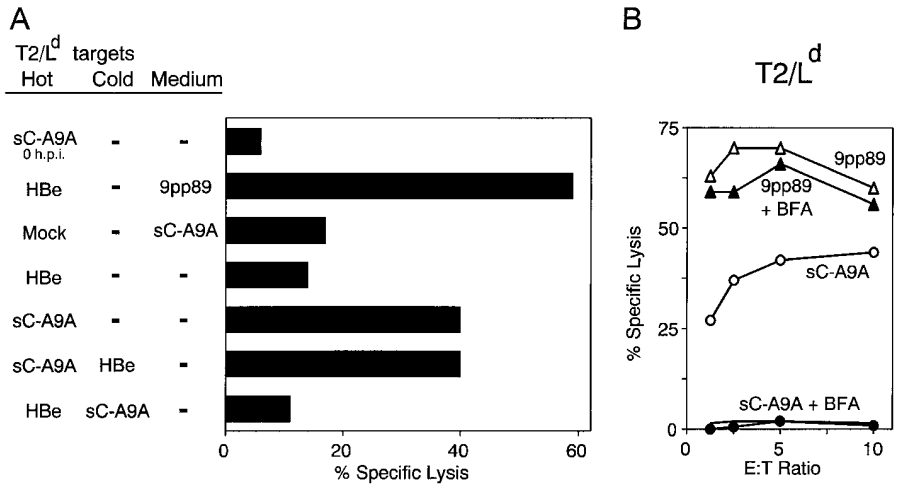

Figure 2. TAP-independent recognition of 9pp89 by CTLs requires endogenous processing of sC-A9A chimeric protein. (*A*) ^{51}Cr-labeled $T2/L^d$ target cells infected with either rVV–sC-A9A or rVV-HBe or mock-infected were incubated with 40 nM 9pp89 peptide, supernatant from rVV–sC-A9A–infected $T2/L^d$ cells, or cocultured with rVV-infected cold cells as indicated, and tested in a CTL assay. Recognition of rVV-HBe– and rVV–sC-A9A–infected controls without further treatment and of rVV–sC-A9A–infected targets immediately after infection (0 h) are shown. Specific lysis at an E/T ratio of 5:1 is shown. (*B*) Effect of BFA (*filled symbols*) on recognition of 9pp89 peptide–loaded (*open triangles*) and rVV–sC-A9A–infected (*open circles*) $T2/L^d$ cells by specific CTLs. The negative control (*solid line*) was provided by rVV-HBe–infected $T2/L^d$ cells.

HBe and Derived Chimeric Proteins Mature in the Secretory Pathway. Processing of HBe protein in the secretory pathway generates four major molecular species that are readily detected by Western blot with anti-HBe–specific antiserum. Although not fully characterized, the heaviest p25 form represents the full-length protein with the leader peptide, intermediate p23 and p22 correspond to two forms of the full-length protein lacking the signal sequence, and the lightest p17e represents the final secretory form (Fig. 3 *A*; reference 34). Some partially processed intermediates can also be detected, with different uncharacterized cleavage points in the COOH-terminal region depending on the cell type and the amount of protein being synthesized. Exchange of the natural for the hemagglutinin-derived signal sequence introduces a glycosylation signal at Asn_{-3} that allowed monitoring transport of the different forms through the secretory pathway at steady state. To check processing of the chimeric proteins, cell lysates of rVV-infected P13.1 cells were subjected to SDS-PAGE, transferred to a membrane, and probed with an anti-HBe antiserum (Fig. 3 *B*). Some differences can be observed in the relative amounts of maturation intermediates of HBe upon introduction of the A9A oligopeptide at the COOH-terminal region, such as an increase in the molecular weight of some of the forms and in the number of species due to partial glycosylation of the signal sequence. However, the complex electrophoretic pattern of the different intracellular molecular species detected with anti-HBe antiserum was unchanged overall. Similarly, introduction of the pp89 epitope at the NH_2-terminal region did not significantly change the electrophoretic pattern of molecular species at steady state, taking into consideration differences in the expected molecular weights of the final and intermediate maturation forms (see sN-9 chimera). Tunicamycin treatment of sC-A9A and sN-9 during rVV infection confirms that glycosylation at residue −3 is only partial, affecting a minority of the ER-translocated protein, probably due to its location at the very NH_2 terminus. It also decreases the level of proteolytic maturation, both in nonglycosylable native HBe and in glycosylable chimeras, perhaps by affecting proteases involved in their processing. Introduction of a second glycosylation site in the 9pp89 epitope of a chimeric secretory molecule by including an Ser residue as a linker with the carrier protein (sN-9S) increased the molecular weight of all sHBe forms, indicating that glycosylation at this second site was complete and that all forms detected had passed through the secretory pathway. Treatment with tunicamycin confirms that essentially all of the sN-9S detected forms had been glycosylated. As a control, equivalent cytosolic HBc NH_2-terminal chimeras (not shown) and cC-A9A were found to be single molecular species, without evidence of further processing.

T2 cells infected with the different rVV were also analyzed to check the maturation pathway of the different chimeras. As in P13.1 cells, multiple forms of the protein were found in the steady state by Western blot, differing only in relative amounts between different chimeras. The signal level of processed bands detected by Western blot of sC-A9A protein in T2 cells was consistently lower than in P13.1 (~10 versus 50%). This cannot be attributed to increased transport through the secretory pathway, as secreted processed forms' signals were also decreased in T2 cell extracellular media (data not shown). It is more probably due to a decreased level of proteolytic maturation. Glycosylation of sC-A9A and sN-9 chimeras was also partial, although more effective than in P13.1, and inhibitable by tunicamycin, which also inhibited the maturation process, reducing most of the total protein to a single nonglycosylated form. sN-9S chimera was completely glycosylated at the second site, demonstrating that all secretory chimeric proteins were translocated into the ER, where they proteolytically mature. Thus, all detectable species in sHBe-derived proteins had been subjected to the glycosylation machinery of the secretory pathway in both P13.1 and T2 cell lines, without any evidence of cytosolic resident forms, which are otherwise stable, as can be detected in rVV-HBc–infected cells.

Alteration of Maturation and Transport of HBe Through the Secretory Pathway Affects Class I Presentation. We have shown that the ER-targeted sHBe chimeric protein is processed in the secretory pathway and that it is able to generate the 9pp89 epitope for class I–restricted antigen recognition. We reasoned that altering maturation and transport of the sHBe protein through the secretory pathway would affect the outcome of 9pp89 presentation.

First, we checked the influence of BFA in maturation of HBe-derived proteins to delimit the compartment in which proteolysis of the COOH-terminal region may oc-

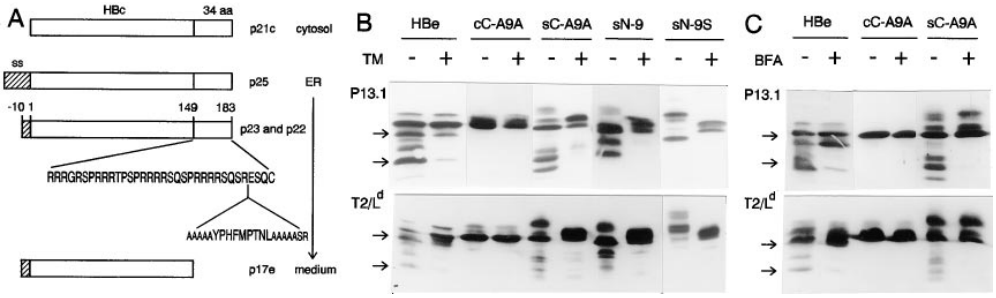

Figure 3. Maturation of HBe and derived chimeric proteins. (*A*) Schematic representation of main HBe and HBc forms during the maturation process. Localization of 9pp89 epitope (YPHFMPTNL) with flanking residues in sC-A9A construct is shown. *aa*, Amino acids. *ss*, Signal sequence. (*B*) Western blot analysis of HBe-derived chimeras in rVV-infected cells. Complete cellular extracts of rVV-infected P13 cells and rVV-infected T2/L^d cells in the presence or absence of tunicamycin (*TM*) or BFA (*C*) were separated by SDS-PAGE, transferred to a membrane, and detected with an anti-HBe antiserum. *Arrows*, Positions of p22 and p17 HBe forms.

cur. BFA destroys *cis*-Golgi compartmentalization by fusing it with the ER, thus avoiding transport through the exocytic pathway (38) and preventing antigen presentation (Fig. 2 *B*). As seen in Fig. 3 *C*, BFA treatment blocks maturation of HBe-derived proteins, reducing by >90% the amount of processed protein detected at the steady state, both in P13.1 and T2 cells, without affecting the cytosolic cC-A9A chimera. Thus, we conclude that proteolytic maturation of HBe proteins requires transport of the immature protein to the Golgi compartment and that it must occur in the Golgi or a post-Golgi compartment.

Second, we checked the influence of pepstatin in the recognition by specific CTLs of T2/L^d cells infected with sC-A9A. It has been previously shown that pepstatin, an inhibitor of aspartyl proteases which participate in either processing of inactive proproteins to mature polypeptides in the secretory pathway or which are active in lysosomal compartments (39), is able to inhibit secretion of processed HBe (36) with accumulation of intermediate processing forms. As seen in Fig. 4 *A*, 100 μM pepstatin completely abrogates secretion of processed forms of sC-A9A in T2/L^d cells while only partially inhibiting secretion of complete forms (~40%, as revealed by densitometric analysis of electrophoretic bands). Analysis of the intracellular protein pool shows a concomitant dose-dependent increase of partially and fully processed species relative to the nonprocessed forms. The former reach 30% of the amount of full-length forms' signal detected instead of the usual 10% in the steady state of nontreated cells. In addition, treatment with pepstatin produced a general decrease in the total amount of intracellular sC-A9A proteins detected, reaching a 50% decrease at 100 μM, which parallels the diminished levels of extracellular full-length proteins.

This alteration of the biochemical pathway of HBe maturation had direct consequences for the generation of 9pp89 epitope. T2/L^d target cells treated with pepstatin presented more L^d–9pp89 complexes at the cellular surface, as indicated by increasing dose-dependent levels of CTL recognition (Fig. 4 *B*). This effect was specific for the generation of the antigenic epitope, as recognition of peptide-loaded target cells was not affected. Therefore, intracellular accumulation of intermediates of processing of the COOH-terminal region of the chimeric protein in the secretory pathway increased class I presentation by providing more antigenic peptide for class I loading, strongly suggesting that the peptide epitope is being generated in the secretory pathway. On the other hand, no connection was found between the full-length proteins detected in the extracellular medium and recognition by CTLs.

Proteasomes Are Not Involved in 9pp89 Processing in the Secretory Route. It has recently been shown that many proteins that were supposed to be degraded in the ER are in fact digested in the cytosol by the general cytosolic ubiquitin-dependent degradative machinery of proteasomes, probably after retrograde transport of the polypeptide through the protein-conducting translocon channel (40, 41). In our case, it would be difficult to explain why such cytosolic processing of sC-A9A would yield a product able

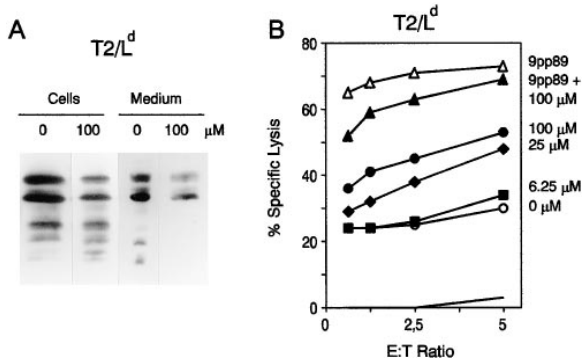

Figure 4. Effect on maturation and antigen presentation of alteration of the secretory pathway by pepstatin. (*A*) Cell extracts and supernatant culture medium of T2/L^d cells infected with rVV–sC-A9A and treated with the indicated amounts of the aspartic protease inhibitor pepstatin were analyzed by Western blot with HBe-specific antiserum. (*B*) CTL recognition of T2/L^d cells infected with rVV–sC-A9A (*open circles*) and treated with 100 μM (*filled circles*), 25 μM (*filled diamonds*), and 6.25 μM (*filled squares*) pepstatin. A control of 9pp89 peptide–loaded target cells (*filled triangles*) treated or untreated (*open triangles*) with pepstatin is included. The negative control (*solid line*) was provided by rVV-HBe–infected T2/L^d cells.

to be translocated back into the ER by a TAP-independent mechanism, when such a product is not produced from the otherwise identical, except for the lack of the 10 NH_2-terminal amino acids, cytosolic cC-A9A protein. Despite this, we decided to test if proteasomes could be involved in generating the 9pp89 epitope from the secretory protein sC-A9A. Lactacystin is perhaps the most specific and efficient inhibitor of proteasomes, as it covalently binds the β catalytic subunits (12). T2/L^d cells were extremely sensitive to lactacystin, and toxic effects occurred upon long exposure or at high concentrations. For this reason, to test the possible involvement of proteasomes in 9pp89 processing, T2/L^d cells were preincubated with lactacystin and then infected for a shorter time period in the presence of 10 μM lactacystin. As shown in Fig. 5 *A*, lactacystin treatment did not affect T cell recognition of sC-A9A–infected T2/L^d cells. Addition of BFA at the time of CTL assay to prevent additional antigen presentation did not alter the result (data not shown). The effect of proteasome inhibition was additionally tested in P13.1 cells infected with rVV encoding the secretory or cytosolic chimeras. These cells were also slightly sensitive to lactacystin treatment, as recognition of 9pp89 peptide–loaded uninfected cells was subtly inhibited, to the same extent as sC-A9A infected cells (not shown). Nevertheless, recognition of cC-A9A–infected cells was completely abrogated even at concentrations 1/3 lower than for sC-A9A (Fig. 5 *B*), indicating specific involvement of proteasomes in antigenic processing of the cytosolic chimera. Similarly, presentation of cytosolic versus secretory chimeras was also selectively sensitive to lactacystin treatment in L/L^d transfectants (data not shown). Therefore, we concluded that most of the 9pp89 peptide presented by L^d in sC-A9A–infected TAP^+ and TAP^- cells is generated independently of the general cytosolic processing pathway of proteasomes.

Proteases of the Secretory Pathway Are Involved in Antigen Presentation. We have shown conclusive evidence that

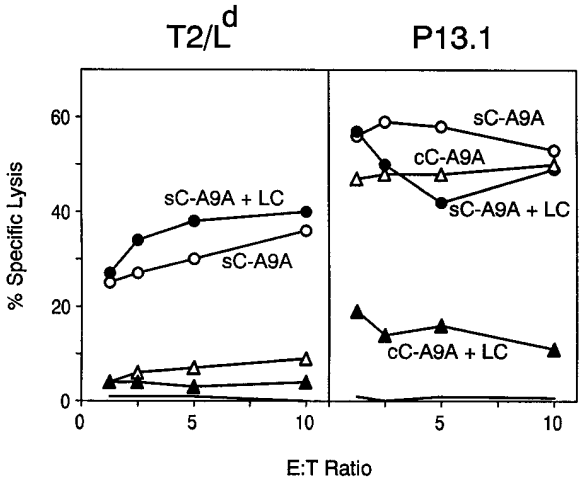

Figure 5. Effect of the proteasome inhibitor lactacystin (*LC*) on antigen presentation of HBe-derived chimeric proteins. Recognition by 9pp89-specific CTLs of cell lines T2/L^d and P13.1 infected with rVV–sC-A9A (*open circles*) or rVV–cC-A9A (*open triangles*) treated with lactacystin (*filled symbols*) 10 μM in the case of T2/L^d cells, 30 μM for rVV–cC-A9A–infected P13.1, and 100 μM for rVV–sC-A9A–infected P13.1 cells is shown. The negative control (*solid line*) was provided by rVV-HBe–infected cells.

processing for class I loading of the secretory sHBe core protein takes place in the secretory pathway. Which proteases could be involved in this new pathway? The 193 amino acid–long HBe core protein has a peculiar COOH-terminal region characterized by an accumulation of 16 basic Arg residues in the 150–179 segment (Fig. 3 *A*). This region is clipped off during maturation in the secretory pathway, generating the final secretory p17 form that encompasses amino acids −10 to 147/9. Several proteases specific for dibasic residues are known to participate in proteolysis of protein precursors to generate their mature forms (26). Furin, a *trans*-Golgi network (TGN) resident protein known to participate in processing of several proproteins, such as peptide hormones and viral glycoproteins, has a specificity for an RXR/KR motif (42), which occurs three times at the COOH-terminal region of the HBV core protein. We speculated that furin could be involved in the processing of HBe for generation of both the secreted forms and the 9pp89 epitope located after this region in the chimeric sC-A9A protein. The peptidyl chloromethylketone decRVKR-CMK, a furin-specific inhibitor due to its mimicry of the four amino acid binding motif (29), was used to assess the involvement of this protease in sC-A9A processing. Incubation of rVV–sC-A9A–infected T2/L^d targets with the furin inhibitor produced a specific dose-dependent inhibition of CTL recognition (Fig. 6 *A*), without toxic effects to the peptide-loaded control targets (data not shown). Further, if we also added the inhibitor during the CTL assay, complete abrogation of CTL recognition could be observed at a 10-fold lower dose than when only added during the infection phase (compare 8 μM* with 75 μM). This is consistent with the reversible and unstable nature of the inhibitor, and did not unspecifically inhibit CTL recognition of peptide-loaded targets (data not shown). Abrogation of antigen presentation correlated with inhibition of proteolytic maturation of the HBe protein in the secretory pathway. Fig. 6 *B* shows that decRVKR-CMK inhibited maturation of sC-A9A both in T2 and P13.1 cells in a dose-dependent manner. In T2/L^d, decRVKR-CMK is more effective, inhibiting appearance of final processing products even at low doses. As a consequence, secretion of the processed forms to the external medium was also abrogated by inhibitor activity, as was proteolytic maturation of native HBe and NH_2-terminal secretory chimeras (data not shown). In P13.1 cells, generation of all processed products was partially inhibited at low doses (8 μM concentration), but high doses (75 μM) were necessary to prevent detection of all processed forms (at 25 μM, processed lower bands' signal still constitutes ~4% of the total intracellular detected protein). Therefore, higher doses of inhibitor would be needed to prevent generation of the 9pp89 epitope in P13.1 cells. This would explain why treatment of P13.1 targets did not essentially affect the level of CTL recognition (data not shown). Alternatively, sC-A9A might be processed by either pathway in P13.1 cells, and the presence of one of them alone might suffice for antigen presentation. Thus, proteolytic cleavage of the COOH-terminal region of HBe protein by furin or other proteases of similar specificity is absolutely necessary for processing the 9pp89 epitope from the chimeric protein in the secretory pathway for class I presentation. We do not know which cleavage points are involved, if furin action involves a progressive proteolysis at the different potential sites, or if all or some sites are affected independently during HBe processing. Whichever COOH-terminal peptides are clipped off, the action of additional proteases on both sides of the 9pp89 epitope might be required to produce an antigenic peptide suitable for L^d binding and CTL recognition.

Nature and Quantity of 9pp89 Processed Peptides Generated by TAP-dependent and -independent Routes. We have seen

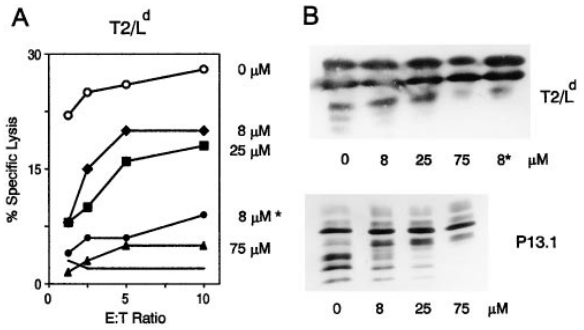

Figure 6. Furin inhibitor decRVKR-CMK inhibits maturation and antigen presentation of sC-A9A protein. (*A*) CTL recognition of rVV–sC-A9A–infected T2/L^d cells untreated (*open circles*) or treated with furin inhibitor (*filled symbols*) at the indicated concentrations. 8 μM* (*filled circles*) signifies that a second addition of 8 μM decRVKR-CMK was performed before CTL assay. The negative control (*solid line*) was provided by rVV-HBe–infected T2/L^d cells. (*B*) Cell extracts from rVV–sC-A9A–infected T2/L^d and P13.1 cells treated with the indicated amounts of decRVKR-CMK during infection were analyzed by Western blot with an anti-HBe antiserum. In the 8* lane, a second addition of 8 μM decRVKR-CMK was performed 3 h before cell harvesting.

how 9pp89 in HBe-derived chimeric proteins can be processed and presented by L^d by the normal TAP-dependent pathway or by a new, TAP-independent route, depending on its intramolecular location and the compartment targeting of the carrier protein. It is possible that different antigenic epitopes with the 9pp89 core sequence would be generated by the two routes. To test this possibility, we prepared acid extracts of rVV-infected cells and fractionated them by reversed-phase HPLC. Individual fractions were then used to sensitize P13.1 cells and were confronted with 9pp89-specific CTLs. Fig. 7 *A*, *left*, compares the chromatographic profile of peptide cell extracts from T2/L^d and L/L^d cells infected with either rVV–sC-A9A or rVV–cC-A9A. As expected, in T2/L^d cells expressing the cytosolic cC-A9A chimera, antigenic peptide could not be detected. In contrast, when expressing the secretory sC-A9A protein, two peaks of CTL activity were found, the same peaks as in TAP^+ L/L^d cells expressing either chimera. Previous work in our laboratory has demonstrated that the peak at fraction 69 coelutes with the pp89 optimal 9-mer YPHFMPTNL epitope, whereas the first peak corresponds to the oxidized form of the peptide at the methionine residue (3). This oxidation does not result in any decrease in antigenicity, as assessed with oxidized synthetic peptide (data not shown). In summary, the two processing pathways generated peptides of the same chromatographic mobility recognized by CTLs, strongly suggesting that both routes generate the same final optimal epitope.

But although the same final products were generated by both pathways, they differed in their need for L^d for stabilization, supporting our claim that they are produced in different environments. This was revealed when an equivalent peptide extraction experiment was performed in cells that do not express L^d (Fig. 7 *A*, *right*). In T2 and Ltk^- cells, although no antigenic material was present in the cytosolic cC-A9A, both antigenic peaks were unexpectedly detected for the ER-targeted chimera. Quantitative analysis testing dilutions of the active fractions in the CTL assay shows that peptide yield was some 25-fold higher in L/L^d than in T2/L^d cells for the secretory chimera, probably due to the difference in cell types, whereas the reverse ratio was found for the L^{d-} cells (Fig. 7 *B*). The antigenic peptide derived from cC-A9A in L cells was rescued from cytosolic degradation and stabilized by L^d by more than 2,000-fold (Fig. 7 *B*). In contrast, the peptide generated by the secretory route from sC-A9A was only slightly more abundant in T2/L^d than in T2 cells, indicating that binding to L^d was not significantly required to stabilize this free peptide. This is consistent with our previous experiments suggesting that sC-A9A is processed in the exocytic pathway, a less degradative environment than the cytosol (43), and thus could be partially stable in the absence of L^d. Since in TAP^- cells L^d molecules would be avidly awaiting the appropriate peptides for binding, it is reasonable to assume that most of the 9pp89 peptide generated in the secretory pathway was bound to L^d. Analogously, 9pp89 is also found in Ltk^- cells expressing the secretory sC-A9A but not the cytosolic cC-A9A chimera, further confirming the compartmental origin of the epitope even in TAP^+ cells. In contrast to T2 cells, in TAP^+ L cells, ~700-fold more sC-A9A–derived peptide was detected in the L^d transfectant (Fig. 7 *B*), indicating that it was stabilized by L^d more than in T2 cells but certainly less than the cytosolic peptide in these same L cells (see above). This suggests that the degradative environment for the secretory 9pp89 peptide might be much stronger in TAP^+ Ltk^- than in TAP^- T2 cells. Alternatively, both the secretory and the cytosolic pathways might be operating in processing sC-A9A in the TAP^+ cells, so that what we see is a combination of both. In summary, based on these findings, we conclude first that the final product of processing in the secretory pathway is the same as in the cytosolic route, and second that 9pp89 produced in the secretory pathway as a result of COOH-terminal processing of the full-length HBe protein is relatively protected from degradation in a class I–independent manner, as it does not have direct access to the cytosolic degradation machinery.

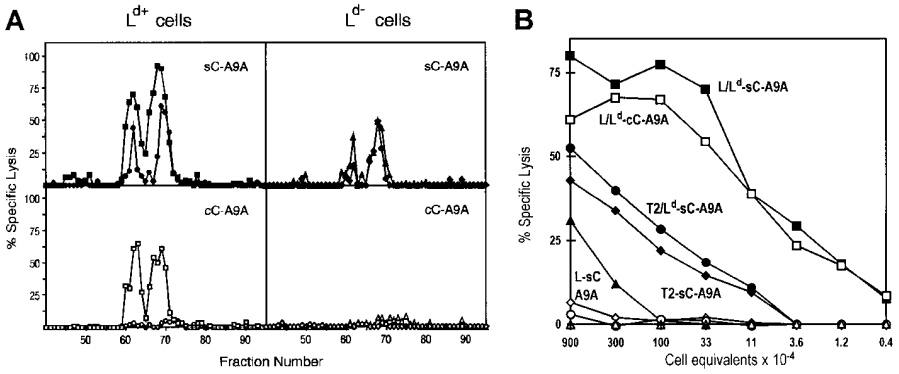

Figure 7. Characterization and quantitation of naturally processed antigenic peptides in cell extracts. Acid-soluble extracts prepared from T2/L^d (*filled circles*), T2 (*filled diamonds*), L/L^d (*filled squares*), or Ltk^- (*filled triangles*) cells infected with rVV–sC-A9A (*filled symbols*) or rVV–cC-A9A (*open symbols*) and fractionated by reverse-phase HPLC were tested for their ability to sensitize P13 cells for lysis by 9pp89-specific CTLs. (*A*) Detection of peptides by 9pp89-specific CTLs after HPLC fractionation of whole cell extracts. Each test well contained 9×10^6 cell equivalents. (*B*) Peptide quantitation by CTL recognition of serial dilutions of antigenic fractions. The average of specific lysis of fractions 62 and 69, the peaks of the two CTL-reactive peptide forms, is shown. 10% average specific lysis was chosen as the minimum level for considering positive T cell recognition and comparison among different rVV and lines.

Discussion

It is generally agreed that the vast majority of endogenous peptides presented by MHC class I molecules derive from proteins degraded in the cytosol by proteasomes and transported into the ER by the TAP heterodimer. In addition, some studies have shown the potential existence of alternative, proteasome/TAP-independent pathways for antigen presentation of full-length proteins (24, 25). Because the mechanisms involved remain uncharacterized to date, the question is open on the possibility of antigen processing in the ER and secretory route.

In this report, we address that question. We describe a mechanism that allows antigen processing and presentation in the secretory pathway of a proprotein that is naturally processed to a secreted form. Maturation in the exocytic route of the HBV HBe core protein results in a form that is specifically secreted to the medium, and involves proteolytic cleavage of its 24 COOH-terminal amino acids (35). We have examined antigen presentation by class I L^d molecules of the reporter epitope 9pp89 engineered in the COOH terminus of an HBe-derived chimeric protein. The epitope in an endogenously synthesized ER-targeted chimera is presented by TAP^- cells, whereas chimeras with the epitope located at the NH_2 terminus or cytosol-targeted chimeras are not presented. TAP-independent antigen presentation to CTLs occurs via an endogenous pathway that is independent of lactacystin-sensitive proteasomes, and the ER-targeted chimeras effectively enter the ER as they are fully glycosylated. Inhibition by BFA and correlation of correct protein maturation with effective antigen presentation locate the point of antigen processing at the Golgi or a post-Golgi compartment. The use of a furin-specific inhibitor identifies the TGN-resident protease furin, or proteases with similar specificity for polybasic residues, as critical for liberating intermediate COOH-terminal peptides that are eventually further processed to the final antigenic optimal 9pp89 peptide, probably by exopeptidase trimming, thus characterizing this novel mechanism of antigen processing in the exocytic pathway.

This route is not an exception to common rules of proprotein and antigenic processing. In fact, many proteins are cleaved during their maturation process in the exocytic pathway, generally at two or more contiguous dibasic residues by proteases of the subtilisin family (26). Among them, seven viral glycoproteins have been described to mature by this mechanism (42), which would make them available for antigen processing by this route. The advantage of HBe for antigen presentation is that cleavage occurs at a point close to the COOH-terminal end of the protein, generating relatively small peptides that could be processed to antigenic forms by mere trimming by exopeptidases. Trimming capacity in the ER or the secretory route has been suggested by a number of experiments, generally involving signal sequence–mediated targeting of minigenes or truncated proteins with an antigenic epitope to the ER (21, 22, 44). The signal peptidase liberates the epitope from minigene products in the compartment where peptide loading normally occurs, leading to very effective antigen presentation. If that epitope is accompanied by flanking regions besides the signal sequence, antigen presentation will occur with different efficiency, following the COOH-end rule, which suggests that trimming to the optimal antigenic size is more effective if the epitope is located at the COOH end of the oligopeptide rather than at the NH_2 terminus (17, 21). In our case, the antigenic epitope is located at position 179 of the sHBe chimera, flanked by five alanine residues on both sides and six extra residues at the COOH end. Peptides generated by the proteolytic action of furin in the secretory pathway would have a maximum of 50 and a minimum of 29 amino acids, with 9pp89 located at 11 residues from the COOH end in all cases. Therefore, a minimum of 9 NH_2-terminal residues and 11 COOH-terminal residues must be trimmed from the precursor peptide to generate the optimal 9pp89 epitope, which is generated by this alternative as well as by the classic proteasome/TAP-dependent route, and thus both amino- and carboxy-peptidases may be involved.

It is somewhat surprising that the same final antigenic peptide is produced by the cytosolic proteasome-dependent pathway and by this new secretory route. Although data (not shown) with other related constructs from our laboratory reveal that these polyclonal 9pp89-specific CTLs are able to detect other endogenous uncharacterized 9pp89-related peaks in HPLC fractions, they are probably biased to better detect the optimal 9pp89 peptide and thus might miss precursor intermediates generated with different efficiency by each pathway. Alternatively, the same final peptide would be generated by both routes if similar enzymes were involved in the final trimming of potential precursor peptides in the exocytic route.

Degradation of ER-resident or targeted proteins has been proven to occur not in the ER but in the cytosol, under the control of the ubiquitin-proteasome pathway (41). Recruitment of cytosolic ubiquitinating enzymes to the ER membrane is necessary not only for proteasome degradation of ER proteins, but also for retrograde transport out of the ER of the lumenal substrate to be degraded (45). This pathway is most probably not involved in processing of 9pp89 in the ER-targeted sC-A9A chimera. First, in our case, antigen presentation is not related to degradation of the protein, but rather to maturation to shorter secretable forms, liberating a short peptide suitable for further trimming, as deduced from the pepstatin and furin inhibitor experiments. Second, degradation of ER proteins is completely inhibited by the proteasome-specific inhibitor lactacystin, whereas no influence has been detected in CTL recognition of rVV–sC-A9A–infected TAP^+ or TAP^- cells. Third, there is no obvious explanation for why processing of retrograde translocated ER-targeted chimeras would generate a peptide able to trespass the ER membrane in a TAP-independent fashion, while the same does not apply to the equivalent cytosolic cC-A9A chimera, which differs only in 10 amino acids 179 residues away from the epitope.

In addition, ER-targeted chimeras with A9A located at the NH_2-terminal region cannot be recognized in T2/L^d cells, implying that this putative TAP-independent peptide cannot be generated in the cytosol in other types of constructs. Most probably, the fact that the NH_2 terminus of the protein is not affected by proteolytic maturation explains the absence of CTL recognition, as indicated by the inhibition of presentation of the COOH-terminal chimera by the furin inhibitor. Thus, processing of 9pp89 in this new pathway conforms to all expectations for class I presentation, and is fully explainable by proteolytic mechanisms previously described for the exocytic route.

Could other compartments be involved in further processing the precursor peptides liberated by the proteolytic activity of furin? It is possible that precursor peptides can be transferred to endocytic or lysosomal vesicles where further processing could occur. Agents that inhibit lysosomal degradation by increasing pH, such as chloroquine, also affect furin activity (29), which is active at neutral and mildly acidic pH, and therefore inhibit 9pp89 presentation in T2/L^d cells (data not shown). Leupeptin, an inhibitor of many lysosomal proteases, did not influence CTL recognition (data not shown), although this result does not discard involvement of minor lysosomal proteases. Again, liberation to the cytosol of a peptide with the capacity to be translocated into the ER in a TAP-independent fashion should be postulated. Alternatively, the final antigenic peptide generated in the lysosomal pathway would reach recycling L^d molecules in endocytic vesicles and be bound and reexported to the cell membrane (46).

The question arises as to where in the secretory route loading of 9pp89 to L^d occurs. Two possibilities are suggested. The first is that a retrograde gradient of 9pp89 peptide or precursor peptide is generated in the secretory pathway from its generation point at the TGN. Retrograde transport of peptides from the TGN to the ER has been demonstrated to be specific and dependent on retrieval signal KDEL (47). Recently, constitutive retrograde transport to the ER of Golgi-localized or even TGN-resident proteins lacking retrieval signals has been demonstrated (48). Thus, specific or constitutive retrograde transport may occur, and sufficient amounts of 9pp89 could travel to the traditional loading compartment for class I molecules in the ER, where binding to nascent L^d molecules could occur. Alternatively, the small proportion of functionally empty class I molecules that travel through the exocytic pathway to the cell membrane bind peptides generated in the TGN. In our case, these peptide-empty molecules, named L^dalt for their different conformation (49), would bind either fully processed optimal 9pp89 epitope or the 9pp89 precursor liberated by furin that would be trimmed in its bound conformation.

Surprisingly, quantitative analysis of the peptide content of cell extracts shows that 9pp89 peptide from the secretory sC-A9A chimera is stable in the absence of L^d in both T2 and Ltk^- cells, whereas it cannot be detected in cells expressing the cytosolic cC-A9A protein. This evidence strongly suggests that 9pp89 from sC-A9A is produced in a compartment where it is at least partially protected from the degradative machinery of the cytosol, such as the secretory pathway (43). This is supported by the fact that T2 cells expressing L^d have analogous amounts of 9pp89 peptide as L^{d-} cells. 9pp89 is significantly more abundant in T2 than in L cell lines in the absence of L^d, despite the fact that L cells produce more peptide as deduced from comparison of L^d-expressing L and T2 cells. This can be accounted for by the fact that lack of TAP transporters in T2 may also affect chaperones of the secretory pathway specialized in peptide binding, such as gp96, protein disulfide isomerase, or BiP, which would also be deprived of their normal peptide supply (50–53). Thus, even in the absence of stabilizing class I molecules, most of the 9pp89 peptide generated in the secretory pathway in T2 cells could be stabilized by resident chaperones which otherwise would be occupied by TAP-dependent peptides, as occurs in L cells. Another explanation might be that TAP not only participates in importing peptides from the cytosol, but also exports peptides in a less specific and effective way, as demonstrated recently for Sec61 translocon (40). This would effectively diminish the amount of free 9pp89 peptide in the ER of L cells without applying to T2 cells. In any case, the different behavior of cytosolic and secretory chimeras strongly supports our conclusion that 9pp89 is generated by processing in the secretory pathway without intervention of more degradative compartments (43).

We do not know the significance of this alternative processing route in TAP^+ cells. The fact that T2 and T2/L^d cells have analogous amounts of 9pp89 peptide strongly suggests that all peptide presented by T2/L^d is produced in the secretory route. Unfortunately, furin inhibitor completely blocks neither protein maturation nor antigen presentation in P13.1 cells, probably due to the high level of sC-A9A maturation, precluding us from testing the contribution of this route to antigen presentation in TAP^+ cells. It is possible that conventional processing by the proteasome machinery of aberrant or mistargeted sC-A9A protein would provide enough antigenic 9pp89 peptide for CTL recognition, but this is clearly a minor contribution, as lactacystin treatment does not influence antigen presentation from sC-A9A while it prevents recognition of cytosolic chimeras, despite both producing similar amounts of 9pp89 peptide. Therefore, although both routes probably coexist, we feel that the contribution of processing in the secretory pathway is more relevant to presentation of 9pp89 epitope from the secretory protein.

We have shown that an antigenic epitope localized at a region of an ER-targeted protein cleaved off during maturation can be efficiently presented in the absence of TAP peptide transporters. This new route, which we describe for the first time, might not be exclusive of HBe protein, but probably applies to other secretory molecules known to mature in the secretory pathway and may be responsible for presentation of some natural viral antigens, as a number of viral glycoproteins do proteolytically mature in the exocytic route (29, 42). The strategy described in this report may constitute a novel and efficient means of vaccinating for subdominant epitopes, as it would bypass the processing

hierarchy of the proteasome–TAP system that is often unable to generate sufficient amounts of a class I–specific peptide to mount a primary response, although it may provide enough peptide for T cell recognition and secondary responses. Thus, it would be possible to induce a more diverse response against a given antigen using several epitopes, increasing the chances of a more effective effector phase, which would lead to a more efficient immune response.

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