Running Title: Antigen processing by metallopeptidases for MHC class I presentation

Sequential cleavage by metallopeptidases and proteasomes is involved in processing HIV-1 ENV epitope for endogenous MHC class I antigen presentation.[1]

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Summary

Antigenic peptides derived from viral proteins by multiple proteolytic cleavages are bound by MHC class I molecules and recognized by CTL. Processing predominantly takes place in the cytosol of infected cells by the action of proteasomes. To identify other proteases involved in the endogenous generation of viral epitopes, specifically those derived from proteins routed to the secretory pathway, we investigated presentation of the HIV-1 ENV protein p18 10mer ^{318}RGPGRAFVTI^{327} epitope to specific CTL in the presence of diverse protease inhibitors. Both metalloproteinase and proteasome inhibitors decreased CTL recognition of the p18 epitope expressed from either native gp160 or from a chimera based on the hepatitis B virus secretory core protein (HBe) as carrier protein. Processing of this epitope from both native ENV and the HBe chimeric protein appeared to proceed by a TAP-dependent pathway that involved sequential cleavage by proteasomes and metallo-endopeptidases; however, other protease activities could replace the function of the lactacystin-sensitive proteasomes. By contrast, in a second TAP-independent pathway we detected no contribution of metallopeptidases for processing the ENV epitope from the chimeric protein. These results show that, in the classical TAP-dependent MHC class I pathway, endogenous antigen processing of viral proteins to yield the p18 10mer epitope requires metallo-endopeptidases in addition to proteasomes.

Introduction

Newly synthesized viral proteins require proteolytic processing prior to MHC class I heavy chain-β_2m-peptide complex formation in the endoplasmic reticulum lumen (1;2). It is assumed that the proteasome is the primary proteolytic complex involved in the cytosolic generation of peptides (2). However, it is not so clear whether proteasomes fully process viral proteins to the peptides of 8 to 11 residues that are complexed with MHC class I molecules. Some studies show indirect evidence for non-proteasomal proteases in antigen processing *in vivo* (3-5). More direct evidence comes from the identification of signal peptidase (6), cystein proteases (7), leucyl aminopeptidase in vitro (8), and furin (9), as additional enzymes involved in processing of several protein antigens. The potential role of other proteases in MHC class I processing remains to be resolved. An attractive hypothesis is that the repertoire of presented peptides is expanded by profiting from some of the numerous cellular proteolytic systems and thus facilitates immunosurveillance.

The envelope gp160 glycoprotein of HIV-1, ENV, represents an interesting antigen for the identification of alternative processing pathways, as some of its epitopes

can be presented in a TAP-independent fashion by human MHC class I molecules, while others follow the classical TAP-dependent pathway (10). Of special interest is the gp160-derived p18 epitope[4], ^{318}RGPGRAFVTI^{327}, which is presented by various human: HLA-A2 (11), -A11 (12), -A3 (13), and -B27 (14), and murine MHC class I molecules: L^d (15), D^d (16), H-2^q, H-2^u, H-2^p (17). It was defined as an immunodominant epitope in the H-$2D^d$-restricted CTL response against the HIV-1 strain IIIB in BALB/c mice (H-2^d) (16;18;19) and its crystal structure in association with D^d has recently been determined (20). There are some suggestions that high processing efficiency of this ENV epitope leads to impaired presentation of other co-expressed epitopes in infected cells (López et al., manuscript submitted)[5], but the proteases involved remain to be characterized. Metallopeptidases, such as the angiotensin-converting enzyme (ACE), have been implicated in the extracellular cleavage *in vitro* of a 15mer synthetic peptide encompassing this epitope (21;22). However, little is known about the proteases involved in the endogenous ENV p18 processing pathway in living cells.

In this report, we studied processing and presentation of the p18 epitope in cells infected with recombinant vaccinia viruses (rVV) that express either the native gp160 protein or the immunodominant 10mer peptide inserted at the carboxyl-end of the hepatitis B virus secretory core protein (HBe). By using different protease inhibitors we demonstrate that, in addition to proteasomes, metallo-endopeptidases distinct from those that can be defined with the available specific inhibitors are involved in the TAP-dependent generation of the p18 10mer epitope from both native gp160 and HBe-chimeric proteins *in vivo*. Furthermore, we demonstrate that HBe chimeric proteins can be processed by a novel TAP-dependent pathway involving metallopeptidases, that is different from the previously described TAP-independent furin pathway (9).

Materials and Methods

Mice. BALB/c mice (H-2^d haplotype) were bred in the institutional colony.

Cell lines. Dr. H.-G. Rammensee (Tübingen) provided the P13.1 cell line, a derivative from mouse mastocytoma P815 cells (H-2^d) by transfection with the lacZ gene encoding β-galactosidase. Dr. P. Cresswell (New Haven) provided the TAP-deficient human lymphoblastoid T2 cells transfected with D^d. Both cell lines were cultured in IMDM supplemented with 10% FCS and 1% 2-ME.

Peptides. ENV-derived peptides 10ENV (RGPGRAFVTI) and 9ENV (GPGRAFVTI), as well as the CMV 9pp89 peptide (YPHFMPTNL) were synthesized in an Applied Biosystems peptide synthesizer model 431A, purified, and found homogeneous by HPLC analysis.

Inhibitors. Brefeldin A (BFA) and all protease inhibitors were from Sigma-Aldrich except leupeptin (Amersham-UBS), pepstatin (Boehringer Mannheim), Z-VAD.fmk (Enzyme System Products, CA, USA) and lactacystin (Dr. E. J. Corey, Harvard University). For control of activity of the protease inhibitors, P13.1 cells (1 x 10^8) were disrupted by sonication for 15 min at 4ºC, and centrifuged. A supernatant aliquot corresponding to 1 x 10^7 cells was directly frozen (non-degraded control). Equivalent aliquots were incubated in the presence of individual inhibitors at 200 μM and digestion by cellular proteases was allowed for 5 days at 37ºC in PBS. Inhibitors were renewed daily. A sample incubated without inhibitors was taken as the degraded control. After SDS-PAGE separation and Coomassie Blue staining of these samples, the overall protein content of each lane was quantitated by densitometry with the PCBAS 2.08e program (Isopenmeβgeräte, GmbH). % Inhibition of protein degradation caused

by each inhibitor was calculated as follows: 100 x (sample with inhibitor – degraded)/(non-degraded – degraded).

Recombinant vaccinia viruses. Generation of rVV-eC-10env with the H-2D^d-restricted HIV-1 ENV protein p18 10mer ^{318}RGPGRAFVTI^{327} epitope inserted into the carboxyl terminus of HBe has been described previously (López et al., manuscript submitted). rVV-ENV virus (vSC25) encoding full-length gp160 from HIV-1 strain IIIB was a kind gift of Dr. B. Moss (23). rVV-sA encodes a secreted, glycosylated 86 amino acid long protein construct containing 18 amino acids comprising an L^d-restricted murine hepatitis virus nucleocapsid epitope, which are linked to a stretch of 67 residues of the HIV gp160 IIIB V3 loop comprising the p18 epitope. It is similar to the published A recombinant (24), except that it is preceded by the adenovirus E19 signal sequence (5). Two other recombinants used as controls were those encoding hepatitis B virus e-protein, rVV-HBe (25), and murine CMV 9pp89 immunodominant epitope biterminally flanked by alanines and inserted into the amino terminus of HBe, rVV-eN-A9pp89A (26). All rVV-encoded proteins relevant to this study contain their respective signal sequences for endoplasmic reticulum translocation. All foreign genes cloned into the rVV used in this study are under the control of the vaccinia early-late promoter 7.5k.

T cell lines and cytolytic assays. Polyclonal pp89-monospecific CTL were generated by immunization of mice with murine CMV and cultured as described previously (7). BALB/c mice were immunized by i.p. injection of 5 x 10^7 plaque-forming units of rVV-ENV. Polyclonal ENV-specific CTL were generated from splenocytes obtained 3 weeks post-immunization that were stimulated *in vitro* in the presence of 10^{-6} M 9ENV peptide in α-MEM supplemented with 10% FCS and 1% 2-ME. Recombinant human interleukin 2, generously provided by Hoffmann-LaRoche, was added 5 days later at 15 U/ml. Long-term cultures were restimulated weekly with mitomycin C-treated spleen cells pulsed with 10^{-4} M 9ENV peptide, and cultured in medium supplemented with 60 U/ml interleukin 2 and 10^{-6} M 9ENV. Following this protocol, no vaccinia-specific CTL were selected. Polyclonal CTL were used as effector cells in standard 4-hr cytolytic assays (27). Over a period of several years, CTL thus prepared recognized peptide-pulsed cells with half-maximal lysis at 10ENV or 9ENV peptide concentrations between 4 x 10^{-10} and 8 x 10^{-9} M. Infected cells were used as target cells after a 3- to 5-hr infection with rVV as described (9). All inhibitors were added together with the virus and kept at a 5-fold higher concentration during the 1-hr adsorption period than throughout the infection. After washing the virus inoculum, the inhibitors were kept at the concentrations indicated for individual experiments throughout infection and ^{51}Cr labelling. BFA was then added to stop further endogenous presentation and kept until the end of the CTL assay. Inhibitors were not toxic at the indicated concentrations. The data are mean values of at least two experiments.

Results

Partial block of the endogenous processing of p18 epitope by the proteasome inhibitor lactacystin.

Endogenous expression of the gp160 protein of the HIV-1 IIIB strain results in efficient presentation of the p18 epitope by the MHC class I molecule D^d to gp160-specific CTL (18;19). The 10mer sequence ^{318}RGPGRAFVTI^{327}, as well as shorter sequences, are recognized by ENV-specific CTL when used as synthetic peptides and thus represent the epitope core (16). Processing and presentation of gp160 by H-2D^d-

positive P13.1 target cells following infection with rVV-ENV was confirmed by specific lysis using p18-specific CTL lines (Fig. 1A, lines). The level of recognition was similar to that obtained with exogenously added 10ENV peptide (75% specific lysis versus 0% without peptide at an E:T ratio of 5:1). These CTL lines recognized peptide-pulsed cells with high sensitivity (half-maximal lysis was at 5 x 10^{-9} M 10ENV). A multiplicity of infection was used that does not have any measurable effect on P13.1 host cell macromolecular synthesis (28). In addition, no antigen presentation to CTL could be detected when the cells were solely loaded with the input virus, without allowing the infection to proceed (data not shown).

Furthermore, presentation to CTL also took place when the 10mer sequence was included in the carboxyl end of the heterologous HBe secretory protein. Lines in Fig. 1B show efficient recognition of target cells infected with rVV-eC-10env expressing the epitope-tagged chimeric protein. These results confirm the validity of HBe as a carrier protein for CTL epitopes (9;26) and show that the isolated expression of the HIV epitope sequence in a different protein context allows its effective processing and presentation.

To demonstrate that the HIV ENV epitope requires endogenous processing, presentation was analyzed in the presence of BFA. This drug blocks class I export beyond the cis-Golgi compartment (29), thus preventing surface expression of newly assembled class I-peptide complexes from endogenous origin. Exogenously added peptides are not affected by BFA, and thus no inhibition was found when rVV-HBe infected cells pulsed with 10^{-4} M 9ENV peptide were treated with BFA (data not shown). By contrast, the full block of specific lysis caused by the addition of BFA during infection (open bars in Fig. 1) demonstrated that the epitope in both ENV constructs was indeed generated from proteins endogenously processed in infected cells.

The involvement of proteasomes in the presentation of p18 was tested by treatment of P13.1 target cells with the proteasome-specific inhibitor lactacystin, a *Streptomyces* metabolite (30;31) (Table I) , and infection with either rVV encoding the epitope. Although an inhibitory effect of lactacystin was clearly evident in either infected target, it was not complete (Fig. 1, open bars, LC). Specific inhibition was 48 $\pm$ 8% (n=6) in rVV-ENV-infected targets and 40 $\pm$ 9% (n=6) in rVV-eC-10env-infected cells. No further inhibition was observed when a higher concentration of lactacystin was used, 100 μM, while its effect was lost if it was lowered to 10 μM. A further control of the inhibitory potential of lactacystin was revealed by similar experiments with target cells infected with rVV-eN-A9pp89A (26) and treated with identical concentration of lactacystin, 50 μM, which completely failed to present the CMV-derived 9pp89 epitope to its specific CTL (inhibition of 83 $\pm$ 17%, n=4, see Fig. 3B). We also have the experience that this range of lactacystin concentrations can reveal selective effects on antigen presentation (7). Thus, the marked, but only partial inhibition of antigen presentation revealed by these data implicates a role for proteasomes in MHC class I processing of the p18 10mer *in vivo*, but also suggests the contribution of other proteases in this endogenous antigen processing.

Metalloproteinases inhibitor specifically blocks p18 10mer antigen presentation

In order to characterize proteases distinct from proteasomes that contribute to HIV epitope antigen processing, experiments with several specific protease inhibitors were carried out. They were chosen to cover a wide range of protease classes. The inhibitors leupeptin, pepstatin and 1,10-phenanthroline specific for different families of

proteases (Table I) were initially tested. Open bars in Fig. 2 show that both leupeptin and pepstatin had no effect on the specific recognition of target cells infected with either rVV-ENV (panel A), or rVV-eC-10env (panel B). In contrast, in the same experiments, target cells treated with the inhibitor 1,10-phenanthroline failed to present the p18 epitope to specific CTL. Whereas specific inhibition of rVV-ENV-infected cells was 70 $\pm$ 8% (n=5) (Fig. 2, panel A), it reached 96 $\pm$ 4% (n=4) using rVV-eC-10env-infected targets. (Fig. 2, panel B). These results clearly demonstrate that p18 10mer epitope presentation is predominantly metalloproteinase-dependent *in viv*o.

We wanted to exclude that the inhibitory effects of lactacystin or 1,10-phenanthroline were due to toxic effects on target cells or on rVV replication, rather than to a specific block of the respective proteases. To this end, similar experiments to those shown in Figs. 1 and 2 were performed in parallel using the rVV-sA, which codes for a recombinant 86 amino acid long protein comprising the HIV epitope, and rVV-eN-A9pp89A, which codes for the murine CMV 9pp89 immunodominant epitope biterminally flanked by alanines and inserted into the amino terminus of HBe. As is the case with the other two recombinant proteins, both sA and eN-A9pp89A are also preceded by a signal sequence for insertion into the endoplasmic reticulum. Fig. 3A (lines) shows specific recognition by ENV-specific CTL of target cells infected with the sA virus and not with the negative control rVV-HBe, and Fig. 3B represents similar data with rVV-eN-A9pp89A and pp89-specific CTL. Furthermore, antigen presentation from both constructs requires a functional endogenous MHC pathway, as was the case for native gp160, as revealed by the sensitivity of ENV and pp89 epitopes presentation to BFA (open bars in Fig. 3). In contrast, sA-infected target cells incubated with lactacystin or 1,10-phenanthroline were efficiently recognized by ENV-specific CTL and no inhibition was detected (open bars in Fig. 3A). Limiting conditions of sA presentation to CTL were used, concerning both time and multiplicity of infection. Thus, lack of susceptibility to these inhibitors could not be ascribed to very high peptide content in sA-infected cells. Different to the situation with sA, the addition of lactacystin but not of 1,10-phenanthroline to target cells totally blocks antigen presentation from the pp89 construct (Fig. 3B). These data indicate that inhibition of the two other ENV constructs by addition of lactacystin (Fig. 1) or 1,10-phenanthroline (Fig. 2) is formally due to specific blockage of the respective proteases and not to a block in rVV replication. In addition, they show that processing of the sA protein does neither require proteasomes or metallopeptidases.

Endopeptidases are involved in p18 10mer antigen processing

Although most metallopeptidases are located at the cell membrane and in the extracellular matrix, previous studies have identified about half a dozen different functional metallopeptidases in the cytosol and a similar number in other compartments related to the MHC class I presentation pathway, such as the ER and the trans-Golgi network (39). Any of these enzymes may play a role in the endogenous pathway of antigen processing.

On the basis of cleavage mechanism, metallopeptidases comprise aminopeptidases, carboxypeptidases, carboxy-dipeptidases, and endopeptidases, among others (reviewed in (40)). Some of these groups can be distinguished by the action of different specific inhibitors (summarized in Table I). To more precisely identify the metallopeptidase group involved in p18 antigen processing, H-2D^d-positive target cells were infected with either rVV-ENV (Fig. 4, panel A) or rVV-eC-10env (Fig. 4, panel B)

and treated with different individual group-specific inhibitors (Table I). All these compounds were found to be active in partially preventing protein degradation in cell extracts, with the exception of bestatin (Table I). The caspase-1-specific inhibitor z-VAD.fmk was also included in view of the sensitivity of this cystein protease to 1,10-phenanthroline. Remarkably, none of the different inhibitory compounds used prevented antigen presentation of any construct to specific CTL (Fig. 4). As phosphoramidon is more active in inhibiting bacterial endopeptidases, but does not block all metallo-endopeptidases of mammalian origin, the most likely explanation to our results is that mammalian metallo-endopeptidases that are not blocked by phosphoramidon are involved in p18 processing. This points at enzymes of the matrixins and astacin families of metallo-endopeptidases as candidates for processing the ENV epitope, since these are the only two families among the some fifty families of metallopeptidases that are not sensitive to phosphoramidon (39). Proteases in these families are neither sensitive to the other inhibitors used. Unfortunately, drugs that collectively and specifically block the endoproteolytic activity of these groups of enzymes have not been described. Since over fifteen well-characterized higher vertebrate different proteases belong to these two families, positive identification of the peptidase involved awaits further molecular and cellular biology work.

Previous reports (21;22) show that *in vitro* processing of a 15 amino acid long synthetic peptide encompassing the p18 epitope requires the carboxy-dipeptidase ACE found in extracellular FCS, as deduced from its sensitivity to captopril. Our data show, in contrast to these studies, that the endogenous processing of gp160 protein was blocked by 1,10-phenanthroline but not by captopril. It thus appears that in vitro results refer to a different situation to the one studied here in living cells, where we conclude that ACE-like metallopeptidases are not required for the endogenous generation of the p18 epitope.

Processing of p18 epitope in a HBe chimeric protein context proceeds via both a TAP-independent and a classical TAP-dependent pathway

Previous studies have reported antigen presentation by a TAP1/2-independent pathway of some HLA-restricted gp160-epitopes, in addition to the general TAP-dependent antigen processing pathway (10). It is not known, however, whether such a TAP-independent pathway also occurs in mouse cells, particularly for presentation of the p18 epitope by murine MHC. TAP dependency of p18 presentation from the secreted precursor proteins was therefore tested in TAP-deficient T2/D^d target cells. Fig. 5 demonstrates that rVV-ENV-infected cells are not recognized by ENV-specific CTL. Thus, the p18 10mer epitope generated from the native protein is only presented by the TAP-dependent pathway.

On the other hand, efficient specific lysis by CTL was detected on T2/D^d cells infected with rVV-eC-10env virus, indicating that the 318-327 epitope included into the HBe carrier protein was efficiently presented by a TAP-independent pathway (Fig. 5) (Gil-Torregrosa, B. C. et al., manuscript submitted)[6]. This pathway appears to co-exist with the classical TAP-dependent pathway revealed by the partial inhibition of presentation by lactacystin (see Fig. 1).

We next explored the TAP-dependency of the novel pathway defined by the metallo-endopeptidases. To this end, inhibition assays with 1,10-phenanthroline were carried out in T2/D^d target cells infected with rVV-eC-10env. Fig. 6 shows no inhibition of specific lysis in the presence of the metallopeptidase inhibitor. Thus,

metalloproteinases are not involved in the TAP-independent processing pathway of the HBe chimeric protein. TAP-independent presentation of a similar chimeric recombinant virus comprising a CMV epitope was previously reported in our laboratory (9). That study demonstrated that the trans-Golgi network protease furin is involved in antigen processing of CMV 9pp89 epitope. However, because we show here that the metalloproteinases that process the HIV epitope are not part of the TAP-independent processing pathway, they represent a different route not linked to the furin antigen processing pathway previously described (9).

Discussion

Diversity of proteases and parallel processing pathways involved in antigen recognition

Our results for D^d-restricted p18 epitope presentation are consistent with the model depicted in Fig. 7. Processing of the HIV p18 epitope from both native ENV and the HBe-based chimeric protein appears to proceed via a pathway involving metallo-endoproteinases and proteasomes. Because 1,10-phenanthroline treatment was more potent in inhibiting antigen presentation than lactacystin, especially for the epitope tagged HBe precursor, metallo-endoproteinases must constitute the major processing pathway operating in TAP-positive P13.1 cells. Thus, proteasomes and metallopeptidases must represent two stages of this same pathway. However, because inhibition by lactacystin was only partial, a product similar to that generated by lactacystin-sensitive proteasomes must also be produced by other uncharacterized protease activities. It is not excluded that this proteolytic activity is the lactacystin-resistant post-acidic specificity of proteasomes (32). Thus, the most likely explanation to our results on processing of these two ENV constructs is a branched TAP-dependent pathway, where the relative order of the different protease activities and TAP is still unknown (Fig. 7).

In addition, processing studies of the ENV epitope in the HBe chimeric protein revealed that the additional TAP-independent pathway was not affected by lactacystin or 1,10-phenanthroline; this pathway may involve the trans-Golgi network protease furin (Gil-Torregrosa, B. C. et al., manuscript submitted)[6] (Fig. 7).

A third construct containing the same ENV epitope, the sA recombinant protein, appears to have a different fate. Its processing remains to be characterized, since it was resistant to both lactacystin and 1,10-phenanthroline, unlike the other two ENV constructs. Although unlikely, we cannot exclude a contribution of proteasomes and metallopeptidases in processing the sA construct. But if these enzymes were involved, there must still be an important parallel pathway that still can provide enough peptide for target cell formation even if the putative metallopeptidase-proteasome route is blocked by inhibitors. It may seem surprising to invoke an additional pathway for processing of this 86-residue-long sA recombinant, which shares some 60 amino acids with the over 500-residue-long full-length ENV. However, it has been reported that less drastic differences in ENV primary sequence, such as deletion of the native signal sequence, produce rapid degradation and more efficient intracellular processing and presentation to ENV-specific CTL (41). Also, generation of presented peptides from an oligopeptide precursor can be different than from a protein (42). In addition, a precedent for more than one processing pathway operating for a given protein antigen has been reported previously even for a 19 amino acid long minigene product (7). The

demonstration of a TAP-independent route for the HBe chimera involving heterologous proteases confirms the existence of additional parallel processing pathways. Multiple proteolytic mechanisms, albeit all TAP-dependent, are also implicated in processing of native ENV, since neither inhibitor completely blocked presentation. This adds to the idea that multiple processing pathways can co-exist, and for given antigens some may prevail in the final aim of providing a peptide that can be presented and recognized as foreign by the immune system.

On the other hand, it may seem puzzling that two essentially diverse constructs, that only share the ENV epitope, appear to be processed by the same pathway. This should be no problem with proteasomes, because they are multicatalytic enzymes and thus can deal with a multitude of different protein substrates. The metallopeptidases involved in ENV antigen processing may similarly have broad sequence specificity. However, low efficacy cleavage of any of these constructs by enzymes with preference for other substrates might well be enough for optimal antigen presentation. The possibility is also open that different specific enzymes that share the same drug sensitivity are involved in processing either ENV construct. It is probable that this first analysis using inhibitors only unveils the possibility that full processing of any given protein can profit from the concerted action of several proteases. Further characterization of these activities in this and other systems may reveal other critical parameters, such as the effect of ubiquitination, unfolding, and protein context.

The two proteins studied here co-translationally enter the secretory pathway, but the sensitivity of their processing to lactacystin indicates that at least a part of the pathway occurs in the cytosol. The results with BFA and TAP-deficient cells further reinforce the endogenous nature of the sequential pathway involving proteasomes and metallo-endopeptidases. The subcellular localization of the metallopeptidases, a point which might shed some light on the issue of which protease acts first on the protein substrates, remains an open question. Surprisingly, many epitopes derived from proteins targeted to the secretory pathway are TAP dependent. Both direct proteolysis of newly translated protein products and proteolysis involving retrograde transport of proteins from the endoplasmic reticulum back to the cytoplasm have been implicated in this process (43-46)**.** Processing in vesicular compartments combined with retrograde movement of proteins or their proteolytic fragments to the cytoplasm (47) may enhance further degradation and provide an explanation for the apparent multiple processing pathways. A final processing step at the plasma membrane performed by ACE-like metallopeptidases, similar to what was described *in vitro* (22;48), seems unlikely, because we did not find any inhibition by captopril. It is likely that the HBe chimeric protein does not assemble into capsids (49), so that a pathway involving its secretion and exposure to the numerous membrane metalloproteinases followed by capsid phagocytosis and delivery to the cytosol (47) can probably be excluded. This route would not apply either to the membrane-anchored native ENV, which should also follow a conventional endogenous pathway.

We have described several proteolytic enzymes involved in processing of this epitope located in the V3 variable loop of HIV ENV protein. Our work concentrated on the processing pathway in mouse cells for presentation by murine MHC class I molecules. One can expect that most of these proteolytic systems be conserved between mouse and human cells. Thus, it is well possible that equivalent pathways also exist in human cells, where they may contribute to the previously described presentation of this epitope by a number of HLA molecules (11-14). It is possible that the promiscuous presentation of this epitope by almost a dozen different class I molecules may be related

to the diversity of processing pathways that the cell can use to generate this antigenic peptide.

In conclusion, our work demonstrates that for some epitopes the proteasome is not the only source of antigenic peptides bound to MHC class I molecules. Rather, it profits from the activity of other proteases in the same sequential pathway. The possibility of taking advantage of the many cellular proteolytic systems should help enlarge the repertoire of possible epitopes T lymphocytes can see, particularly in those instances where proteasomal degradation leads to epitope destruction (50-52). The reverse face of the picture would be that alternative proteolytic systems might in some cases destroy potential epitopes generated by proteasomes. Similar positive and negative outcomes have been reported for the action of γ-interferon (53-55). Our previous results (7;9) and those reported here highlight the diversity of proteases involved in antigen recognition. Further studies are needed to delineate the increasingly complex antigen processing pathways.

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Footnotes

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[4]Abbreviations used in this article: ACE: angiotensin-converting enzyme; BFA: brefeldin A; HBe: hepatitis B virus secretory core protein; p18: HIV IIIB ENV epitope ^{318}RGPGRAFVTI^{327}; rVV: recombinant vaccinia virus.

[5]HIV ENV protein expression inhibits cytomegalovirus MHC class I peptide presentation *in vitro* and *in vivo*. Daniel López, Yolanda Samino, Ulrich H. Koszinowski, and Margarita Del Val.

[6]Furin-mediated proteolytic maturation of antigenic proteins in the secretory pathway as a general Major Histocompatibility Complex class I antigen presentation pathway. Beatriz C. Gil-Torregrosa, A. Raúl Castaño, Daniel López, and Margarita Del Val.

Figure legends

Fig. 1. Recognition by CTL of recombinant viruses encoding ENV epitope.

P13.1 (H-2D^d-positive) target cells were infected for 5 hr with rVV-ENV (squares, panel A) at a multiplicity of infection of 20 plaque-forming units/cell or rVV-eC-10env (squares, panel B) at a multiplicity of 30, and tested with ENV-specific CTL in a cytolytic assay. rVV-HBe was used as negative control (circles) at the same multiplicity as ENV viruses. Percentage specific lysis in the absence of drugs at each E:T ratio is shown on the right vertical axis and plotted with lines. Percentage specific inhibition obtained by the addition of 5 μg/ml BFA or 50 μM lactacystin (LC), as indicated, was calculated for each E:T ratio as:

$$\% \text{ Specific Inhibition} = 100 - \frac{[(\text{Lysis rVV-Antigen} + \text{Inhibitor}) - \text{Lysis rVV-HBe}]}{\text{Lysis rVV-Antigen} - \text{Lysis rVV-HBe}} \times 100$$

and the mean $\pm$ SD of all E:T ratios is plotted as open bars and shown on the left vertical axis.

Fig. 2. Recognition by CTL of ENV p18 10mer epitope presented in the presence of general protease inhibitors.

Cells infected as described in Fig. 1 were treated with 100 μM leupeptin (LEUP) (trypsin-like and cystein proteases inhibitor), 50 μM 1,10-phenanthroline (1,10PHE) (metallopeptidases inhibitor), or 100 μM pepstatin (PEPST) (aspartic proteases inhibitor), as indicated, before the CTL assay. Identical codes as in Fig. 1 are used. Percentage specific inhibition was calculated as in Fig. 1.

Fig. 3. Specificity of the effect of lactacystin and 1,10-phenanthroline on MHC class I antigen presentation.

P13.1 cells were infected at a multiplicity of 10 with rVV-sA (panel A) or rVV-eN-A9pp89A (panel B) (squares) or rVV-HBe negative control (circles) and tested in a CTL assay. Lines represent specific lysis in the absence of inhibitors, as in Fig. 1. Open bars represent inhibition in the presence of 5 μg/ml BFA, 50 μM lactacystin (LC), or 100 μM 1,10-phenanthroline (1,10PHE), as indicated, calculated as in Fig. 1.

Fig. 4. Recognition of target cells infected in the presence of metalloproteinase subfamilies inhibitors.

Cells infected as described in Fig. 1 were treated with 100 μM captopril (ACE-like metallopeptidases inhibitor), 100 μM benzyl-succinic acid (BENZYL) (inhibits metallo-carboxypeptidases), 100 μM bestatin (metallo-aminopeptidases inhibitor), 50 μM 1,10-phenanthroline (1,10PHE) (inhibits all metallopeptidases), 100 μM phosphoramidon (PHOSP) (bacterial metallo-endopeptidases inhibitor), or 100 μM Z-VAD.fmk (blocks caspases) as indicated, before the CTL assay. Identical codes as in Fig. 1 are used. Percentage specific inhibition is represented by the open bars and was calculated as in Fig. 1.

Fig. 5. TAP-dependent recognition of ENV epitope from the native gp160 protein but TAP-independent presentation from the HBe-chimeric protein.

TAP^- T2/D^d target cells were infected at a multiplicity of 40 with rVV-ENV (triangles), rVV-eC-10env (circles), or rVV-HBe (diamonds, negative control), and tested with ENV-specific CTL.

Fig. 6. 1,10-Phenanthroline fails to block TAP-independent recognition.

TAP$^-$ T2/D^d cells were infected as in Fig. 5 with rVV-eC-10env (squares), or rVV-HBe (circles, negative control). Lines represent specific lysis by CTL of cells infected in the absence of inhibitors. Open bars represent inhibition in the presence of 50 μM 1,10-phenanthroline (1,10PHE). % Specific inhibition was calculated as in Fig. 1.

Fig. 7. Diversity of proteases and parallel processing pathways involved in HIV ENV p18 epitope presentation.

The model shows the components involved in each of the proposed pathways, but the relative order of the different steps is hypothetical. Involvement of proteasomes is deduced from the sensitivity of the pathway to lactacystin. Involvement of metallopeptidases is defined by 1,10-phenanthroline. The TAP-independent pathway is revealed in TAP$^-$ cells, but may represent a minor route in the TAP$^+$ cells used in this study.

Table I

General Specificity and Activity of Protease Inhibitors used in this Study

Inhibitor	Specificity	Reference	% Inhibition of Degradation[a]
Lactacystin	Proteasome chymotryptic and tryptic activities	(30-32)	n. d.
Leupeptin	Trypsin-like proteases and Cystein proteases	(33)	18
Pepstatin	Aspartic proteases	(22;33)	10
1,10-Phenanthroline	All Metalloproteases and Caspase-1	(22;34;35)	21
Bestatin	Metallo-aminopeptidases	(22;36)	-1
Benzyl-succinic acid	Metallo-carboxypeptidases A & B	(22)	24
Captopril	ACE and ACE-like Metalloproteases	(22)	4
Phosphoramidon	All bacterial Metallo-endopeptidases but few of mammalian origin	(22;37)	30
Z-VAD.fmk	Caspases	(38)	n. d.[b]

a. Activity of these inhibitors was measured as their ability to prevent proteolytic degradation in cellular extracts. The amount of protein still present after incubation was 1 relative unit in the case of the degraded control sample (that was considered as 0 % inhibition of degradation) and 77 relative units in the non-degraded unincubated sample (which was taken as 100 % inhibition). Data are mean of at least two independent experiments. Negative values indicate that there was enhanced degradation in the presence of the compound. n. d., not done.

b. The compound was found to block apoptosis (data not shown).

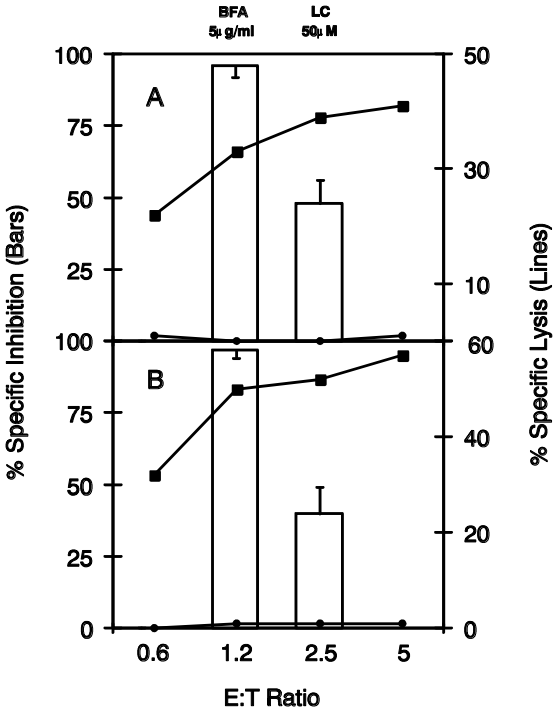
BFA
5μ g/ml
LC
50μ M
A
B
100
75
50
25
0
50
30
10
60
40
20
0.6
1.2
2.5
5
E:T Ratio
% Specific Inhibition (Bars)
% Specific Lysis (Lines)

Figure 1

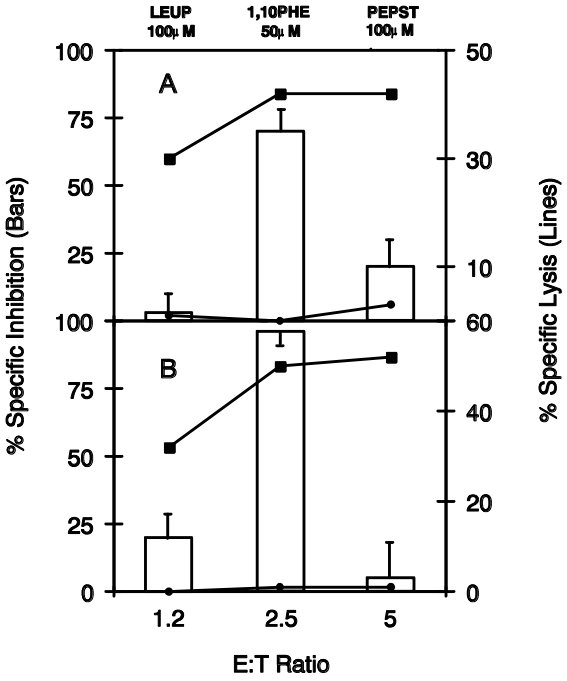
LEUP
100μ M
1,10PHE
50μ M
PEPST
100μ M
A
B
100
75
50
25
0
50
30
10
60
40
20
% Specific Inhibition (Bars)
% Specific Lysis (Lines)
1.2
2.5
5
E:T Ratio

Figure 2

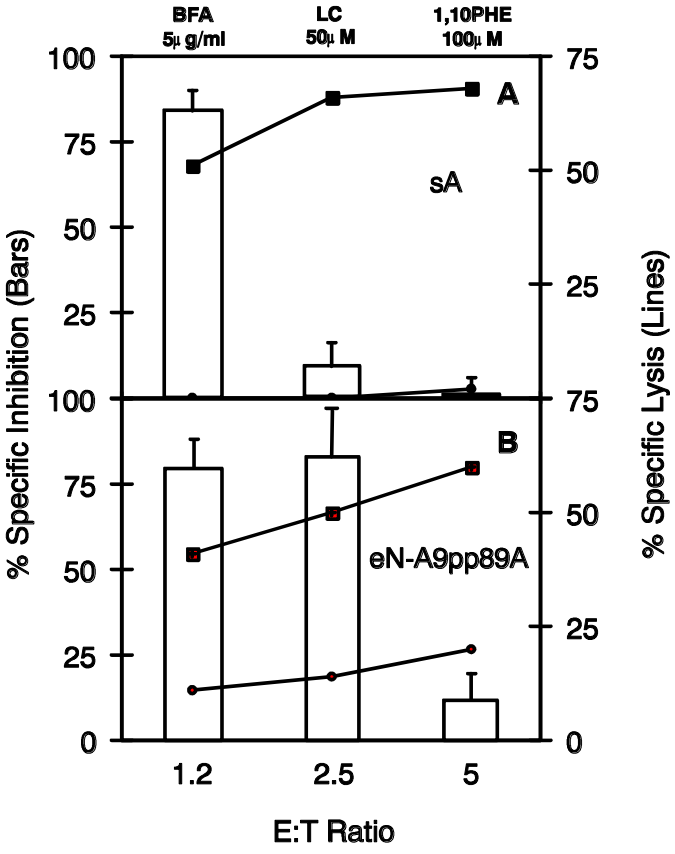
BFA
5μ g/ml
LC
50μ M
1,10PHE
100μ M
100
75
50
25
100
75
50
25
0
75
50
25
75
50
25
0
A
sA
B
eN-A9pp89A
1.2
2.5
5
E:T Ratio
% Specific Inhibition (Bars)
% Specific Lysis (Lines)

Figure 3

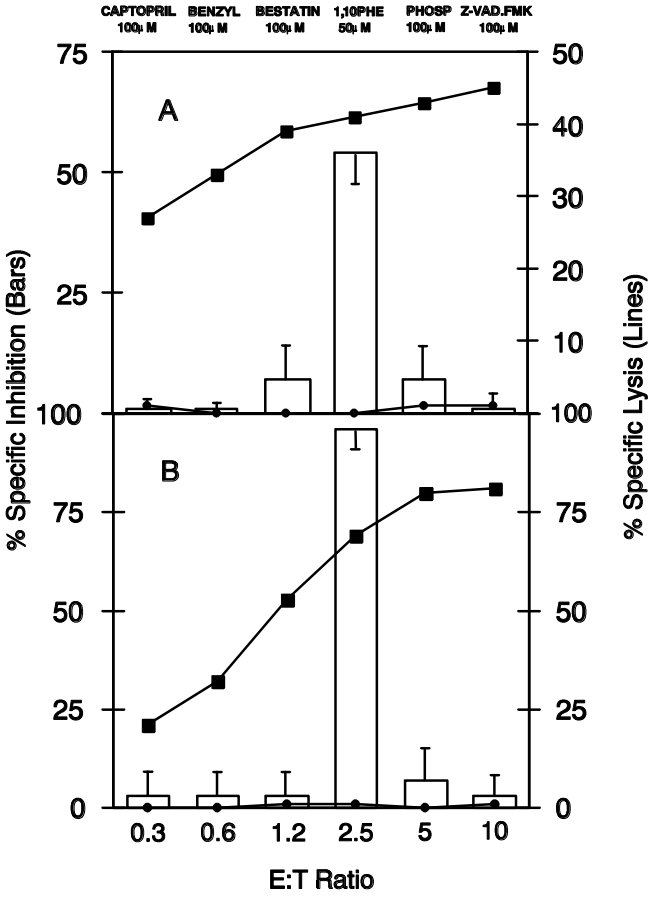
CAPTOPRIL 100µ M
BENZYL 100µ M
BESTATIN 100µ M
1,10PHE 50µ M
PHOSP 100µ M
Z-VAD.FMK 100µ M
A
B
% Specific Inhibition (Bars)
% Specific Lysis (Lines)
E:T Ratio
0.3
0.6
1.2
2.5
5
10

Figure 4

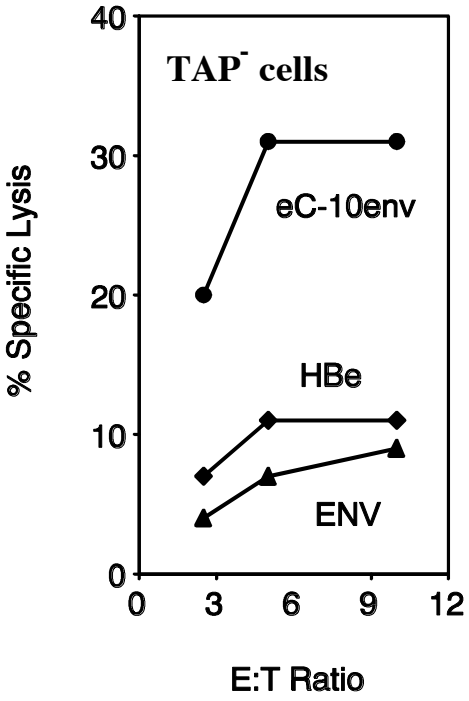
40
30
20
10
0
TAP- cells
eC-10env
HBe
ENV
% Specific Lysis
0
3
6
9
12
E:T Ratio

Figure 5

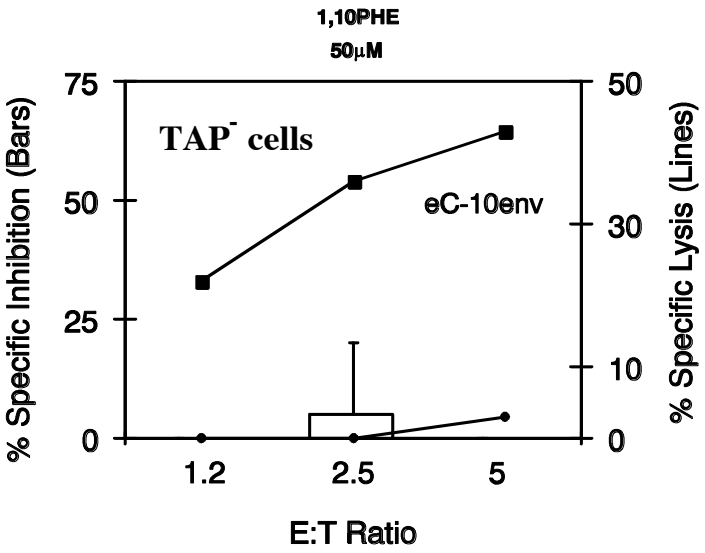
1,10PHE
50μM
TAP- cells
eC-10env
% Specific Inhibition (Bars)
% Specific Lysis (Lines)
75
50
25
0
50
30
10
0
1.2
2.5
5
E:T Ratio

Figure 6

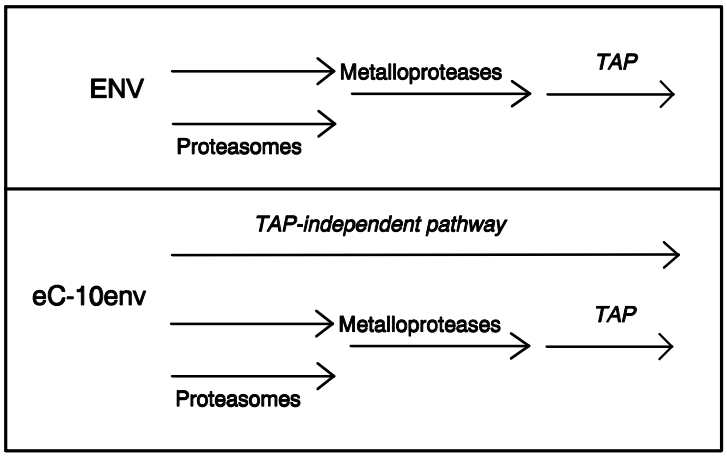
ENV
Metalloproteases
TAP
Proteasomes
TAP-independent pathway
eC-10env
Metalloproteases
TAP
Proteasomes

Figure 7