Selective Involvement of Proteasomes and Cysteine Proteases in MHC Class I Antigen Presentation[1]

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Running title : Distinct minigene processing by proteasomes and cysteine proteases

Summary

CTL recognise peptides derived from protein antigens bound to MHC class I molecules. Proteasomes probably participate in the generation of these peptide epitopes. We investigated the role of proteasomes in presentation of endogenously-synthesised short viral proteins. To this end, we employed proteasome and cysteine protease inhibitors, and two closely related recombinant vaccinia viruses that code for 17- and 19-amino-acid-long products encompassing murine CMV 9pp89 epitope. Presentation of both minigene products required processing to shorter peptides and was independent of ubiquitination. Proteasomes were necessary for processing the 17mer product, and cysteine proteases were not required. In contrast, the 19mer product could be processed in parallel either by proteasomes or by cysteine proteases independently. These results highlight the diversity of alternative processing pathways even for short peptidic antigens, provide evidence for the involvement of cysteine proteases in MHC class I presentation, and show that cleavage by cysteine proteases is governed by sequences flanking the epitope.

Introduction

Recognition of infected cells by CTL requires prior proteolytic processing of viral proteins in the cytosol. This degradation generates peptides that are translocated to the endoplasmic reticulum (ER)[3] lumen by TAP, and then assemble with β_2-microglobulin and MHC class I heavy chain (1,2).

Some evidences suggest that proteasomes are involved in antigen processing. MHC expression and antigen presentation is closely related to expression and activity of proteasome subunit LMP7 (3). Also, synthetic peptides are degraded in vitro by purified 20S proteasomes to 5-15-amino-acid-long fragments. In some cases, low amounts of immunodominant nonamers are found among these products (4,5). In infected cells, it is thus assumed that proteins undergo proteolytic processing by proteasomes. However, it is not so clear what would be the fate of intermediate -or final- products of this reaction, that is, longer peptides with more amino acids than the optimal epitope sequence. One possibility is that they are cleaved to final optimal size by proteasomes (4). Alternatively, they may be transported by TAP (6), and still be presented by MHC, albeit with lower affinity (7), or be trimmed in the ER to optimal size and then efficiently bind to MHC (1,2,8,9).

Lactacystin has been characterised recently as a potent inhibitor of the three distinct peptidase activities of the multicatalytic complex proteasome. This inhibition seems to be proteasome-specific because none of five other proteases tested were inhibited (10,11). This reagent appears to modify covalently the conserved N-terminal threonine of mammalian proteasome subunit MB1 (10). Z-L_3VS is another covalent and irreversible inhibitor of all three activities of the proteasome (12), and has also been reported as an extremely potent and specific cysteine protease inhibitor (13). E-64 is a classical and effective irreversible inhibitor of cysteine proteases (14,15).

In the immune response against murine CMV, the nonapeptide ^{168}YPHFMPTNL^{176} (9pp89) has been reported as the immunodominant and protective epitope restricted by the

H-2L^d molecule in BALB/c mice (16-21). In addition, insertion of the 9pp89 sequence into the hepatitis B virus e-protein (HBe) (22) as well as construction of minigenes coding for peptides 17- and 19-amino-acids-long encompassing this epitope (5) revealed that local flanking amino acid residues influence antigen processing and presentation.

In this work, we studied antigen processing from recombinant vaccinia viruses (rVVs) encoding the two minigenes encompassing murine CMV pp89 immunodominant epitope. By using the protease inhibitors, we defined the role of proteasomes in endogenous antigen processing of short antigens, as well as the differential processing of these two closely related 17- and 19-amino-acid-long products by proteasomes and cysteine proteases.

Materials and Methods

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

Cell lines. All cell lines were cultured in IMDM supplemented with 10% FCS and 1% 2-ME. Cells transfected with L^d were used : L/L^d cells were obtained from Dr. U. H. Koszinowski (17) and $T2/L^d$ cells were provided by Dr. Peter Creswell (23).

Recombinant vaccinia viruses. The rVVs encoding the minigenes, rVV-m17 and rVV-m19, have been described previously (5). Two other recombinants were used as controls, those encoding hepatitis B virus e-protein, rVV-HBe (24), and murine CMV 9pp89 immunodominant epitope biterminally flanked by alanines and inserted into the amino terminus of HBe, rVV-HBe/N/$A_5$9pp89A_5 (22).

T cell lines and cytolytic assays. Polyclonal pp89-monospecific CTL were generated by immunisation of mice with murine CMV as described previously (17,25). Splenocytes thereof were restimulated in vitro with mitomycin C-treated spleen cells pulsed with 10^{-6} M 9pp89 peptide, and cultured in a-MEM supplemented with 10% FCS, $4x10^{-8}$ M 9pp89 and 1% 2-ME. These cells were used as effector cells in standard 4-6 hr cytolytic assays (17). Transfected cells were used as target cells after overnight infection with rVVs as described (17). The data are mean values of at least two experiments. Recombinant human interleukin 2 for the long-term propagation of pp89-specific CTL lines was generously provided by Hoffmann-LaRoche.

Reagents. Lactacystin and Z-L_3VS were gifts from Drs. S. Omura and H. Ploegh, respectively. Cysteine proteases inhibitor L-trans-epoxysuccinyl-leucylamido(4-guanidio)butane (E-64) was from Boehringer Mannheim, Germany. 9pp89 peptide was synthesised in an Applied Biosystems peptide synthesiser model 431A, purified, and found homogeneous by HPLC analysis.

Results and Discussion

To determine the involvement of proteasomes in antigen processing, two related minigenes encoded by rVVs were used (5). One minigene (m17) codes for a 17mer peptide representing the immunodominant murine CMV 9pp89 epitope and the local flanking amino acids of HBe carrier protein (MDIG**YPHFMPTNL**GDPY). The second minigene (m19) codes for a 19mer peptide (MDIGA**YPHFMPTNL**AGDPY) that differs from the previous minigene by a biterminal alanine spacer between 9pp89 core and HBe flanking residues. Antigen presentation from these minigenes has been previously described and found to be of different efficacy (5). Direct detection of the minigene products was not feasible even by using SDS-PAGE tricine gels for resolution of low-molecular-weight peptides and high sensitivity Western blotting (data not shown), that nevertheless resolved and detected the equivalent synthetic peptides. This presumably indicates that low amounts of both minigene products were present in infected cells. Fig. 1 shows the recognition by pp89-specific CTL of cells expressing each minigene product in comparison with the full-size HBe protein containing 9pp89 epitope, in rVV-HBe/N/$A_5$9pp89A_5. As shown in this figure, presentation of both 17- and 19mer products was TAP-dependent.

Lactacystin has been described as the first proteasome-specific inhibitor (10,11). L/L^d cells were treated with lactacystin and infected with rVVs encoding either minigene. Target cells infected with rVV-m17 and treated with lactacystin failed to present 9pp89 epitope to specific CTL as shown in Fig. 2. The same was true for rVV-HBe/N/$A_5$9pp89A_5 (data not shown). By contrast, in the same experiment, lactacystin had no effect on the specific recognition of target cells infected with rVV-m19 (Fig. 2). Lactacystin concentrations up to 100 μM produced the same selective inhibition (data not shown). Thus, although proteasomes may be involved in antigen processing of the 19-aa-long product, these data suggest that the lactacystin-inhibitable proteasome activity is not absolutely required. On the other hand, this selective block in antigen presentation of rVV-m17 directly implied proteasomes in antigen processing of a 17-aa-long product in vivo.

Previous characterisation of products of in vitro degradation by 20S proteasomes of the 17mer synthetic peptide showed partial cleavage of this peptide to yield a 13mer peptide, while the 9mer core was not detected (5). We extend this observations to demonstrate the in vivo requirement of proteasomes for processing the 17mer minigene product. Controversial data have suggested a role for the ubiquitin-activating enzyme E1 in antigen presentation by MHC class I molecules (26,27). Whereas one would expect that full-length proteins such as HBe/N/$A_5$9pp89A_5 might be susceptible to ubiquitin-dependent proteasome-mediated degradation, 17mer and 19mer products lack lysine residues important for ubiquitination (28). Thus, our data show in vivo ubiquitin-independent processing of a 17mer peptide by proteasomes. A recent study (29) demonstrates that lactacystin inhibits the specific presentation of epitopes to specific CTL only when these are included in a complete protein but not when they are expressed as optimal antigenic size nonamers from minigenes encoded in rVVs. The latter are directly translocated by TAP to the ER lumen and may bind to MHC class I molecules without processing. Our data show that the presentation of one minigene product was blocked by lactacystin and thus proteasome-dependent degradation is implied in the cytosolic generation of the corresponding epitope. Therefore, when a few amino acids flanked the minimal epitope, proteasome processing and TAP-dependent translocation were necessary for short peptides.

In addition, the 19mer minigene product was presented independently of proteasome activity. In order to study whether other proteases were involved in its processing, similar experiments were carried out with another recently described inhibitor of both cysteine proteases and proteasomes, Z-L_3VS (12,13). As opposed to the selectivity observed with lactacystin, recognition of both rVV-m17 and rVV-m19 was abolished by Z-L_3VS (Fig. 3). Both constructs behaved also equally at higher and lower concentrations tested, 10-100 μM (data not shown). None of the concentrations used resulted in any inhibition of antigen synthesis and accumulation, as controlled with rVV-HBe/N/$A_5$9pp89A_5 (data not shown). Since lactacystin does not have a detectable effect on various cysteine proteases tested

including calpain, cathepsin B, and papain (10), the inhibitory effect of Z-L_3VS, in contrast to that of lactacystin, demonstrated specific inhibition of a cysteine peptidase activity involved in processing of the 19mer minigene product. Multiple studies have defined different cysteine proteases in distinct compartments related to the MHC class I presentation pathway (ER lumen, cis-Golgi, trans-Golgi network, and plasma membrane) (30-36) and in the cytosol (37). A recent study (38) showed that in vitro processing of OVA with aspartic proteases generates the MHC class II-restricted $OVA_{322-336}$ epitope. In contrast, digestion of OVA with cysteine proteases destroyed this epitope. The authors suggested that mechanisms must exist for the protection of epitopes from destructive proteolysis in different compartments. Notably, the sequence of 9pp89 epitope YPHFMPTNL presents the N-terminal X-Pro sequence described as a structural protection motif against most frequent proteolytic degradation (39). Our data, in contrast to the cited study, show that cysteine proteases generated a class I-restricted epitope.

The proteasome-independent and cysteine-protease-dependent presentation of the 19mer minigene product can be explained by two hypotheses: first, cysteine proteases may be necessary to process the 19mer minigene product. As an alternative, both these proteases and proteasomes may be on their own sufficient. To discriminate between these possibilities, the effect on minigene antigen presentation of the cysteine-protease-specific E-64 reagent was tested. Presentation of both rVV-m17 and rVV-m19 was unaffected by E-64 (Fig. 4). Both constructs were equally resistant to inhibition in the range of 1-500 μM (data not shown). As opposed to the lack of effect on rVV-m19 recognition by CTL when each inhibitor was used alone, combination of E-64 and lactacystin blocked presentation of rVV-m19 (Fig. 5), confirming the results with Z-L_3VS. It is important to recall that combined inhibition of proteasomes and cysteine proteases did not result in any inhibition of antigen synthesis and accumulation, as controlled with Z-L_3VS. These data confirm that the 17mer product was processed by proteasomes and did not require cysteine proteases.

More interestingly, these results demonstrate that in vivo the 19mer peptide was

processed by cysteine proteases as well as by proteasomes. Digestion of 19mer synthetic peptide with 20S proteasomes in vitro yields as main cleavage product a 14mer and secondly the 9pp89 epitope core (5). Thus, our results confirm suggestions from in vitro experiments that proteasomes would be sufficient alone for processing the 19mer, and uncover an additional pathway operating in vivo. Because neither proteasomes nor cysteine proteases were absolutely required, our data showed that both cleavage pathways were independently processing the 19mer product for presentation by L^d. Thus, in the presence of lactacystin, cysteine proteases would process the peptide, whereas in the presence of E-64 proteasomes would be responsible for it, yielding in both cases targets recognised by specific CTL. Only when both activities were blocked by Z-L_3VS or by the combination of lactacystin and E-64, no presentation was detected, indicating that the proteasome and the cysteine protease pathways are the only two involved in processing the 19mer.

Processing of both minigene products by proteasomes in the cytosol would be followed by transport to the ER by TAP and binding and presentation by MHC class I molecules. The 19mer alternative cysteine protease processing pathway may proceed in the cytosol, in the secretory pathway, or in both, since this type of proteases have been described in several cellular compartments (30-37). In the first possibility, cytosolic cysteine proteases may process the 19mer minigene product prior to transport by TAP. As an alternative, the full-length 19mer would be translocated to the ER lumen by TAP, and cysteine proteases in the ER or in the secretory pathway would be responsible for its processing. Selective TAP-dependent transport to ER lumen of peptides longer than nonamers has been described (6). Also, limited antigenic processing in the ER has been described for both full-length proteins and short peptides (1,2,8,9). In the two pathways described in this report, cytosolic or ER trimming by proteases other than proteasomes and cysteine proteases is not excluded.

In summary, this study demonstrated selective involvement of proteasomes and cysteine proteases in differential processing of two very closely related short proteins, and

provided evidence for the involvement of cysteine proteases in class I antigen presentation. The efficiency of antigen processing and presentation from both long proteins (22) and minigene products (5) in vivo is governed by sequences that directly flank the epitope, and this has also been described for cleavage of peptides by proteasomes in vitro (5). We show that antigen processing by cysteine proteases is also governed by neighbouring sequences and that some flanking residues such as alanines determine the processing pathway to be followed in vivo.

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Footnotes

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[3]Abbreviations used in this paper: ER (endoplasmic reticulum), rVV (recombinant vaccinia virus).

Figure Legends

Figure 1.- Recognition of chimeric proteins with different flanking residues by pp89-specific CTL : minigene products presentation is TAP-dependent.

Left panel : L/L^d cells (TAP^+) were infected overnight at a multiplicity of infection of 5 plaque forming units per cell with either rVV-HBe/N/$A_5$9pp89A_5 (triangles), rVV-m17 (circles), rVV-m19 (diamonds), or control rVV-HBe (solid line). Right panel : T2/L^d cells (TAP^-) were infected overnight at a multiplicity of 40 with either rVV-HBe in the presence of 9pp89 peptide used as positive control (squares) or none (solid line) as negative control, rVV-m17 (circles) or rVV-m19 (diamonds).

Figure 2.- Lactacystin suppresses presentation of 17mer protein but not of the related 19mer related protein.

L/L^d cells were infected overnight at a multiplicity of 10 in the presence of 10 μM lactacystin, LC, (circles) or none (triangles) with rVV-m17(panel A) or rVV-m19 (panel B). rVV-HBe (diamonds) was used as negative control. Lactacystin was kept throughout infection and ^{51}Cr-labelling until the addition of pp89-specific CTL to target cells.

Figure 3.- Z-L_3VS inhibits recognition of both 17mer and 19mer minigene products.

L/L^d cells were infected overnight at a multiplicity of 10 in the presence of 50 μM Z-L_3VS (circles) or none (triangles) with rVV-m17(panel A) or rVV-m19 (panel B). rVV-HBe (diamonds) was used as negative control. Z-L_3VS was kept throughout infection and ^{51}Cr-labelling until the addition of CTL to target cells.

Figure 4.- E-64 fails to block recognition of both 17mer and 19mer minigene products.

L/L^d cells were infected overnight at a multiplicity of 10 in the presence of 100 μM E-64 (circles) or none (triangles) with rVV-m17(panel A) or rVV-m19 (panel B). rVV-HBe (diamonds) was used as negative control. E-64 was kept throughout infection and ^{51}Cr-labelling until the addition of CTL to target cells.

Figure 5.- Combined inhibitory effect of lactacystin and E-64 on 19mer presentation.

L/L^d cells were infected overnight at a multiplicity of 10 in the presence of 10 μM lactacystin, LC, and 50 μM E-64 (circles) or none (triangles) with rVV-m19. rVV-HBe (diamonds) was used as negative control. LC and E-64 were kept throughout infection and ^{51}Cr-labelling until the addition of CTL to target cells.

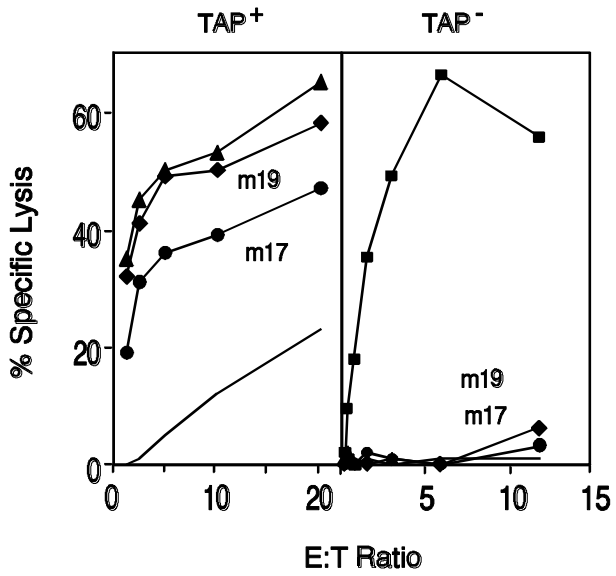

Fig. 1
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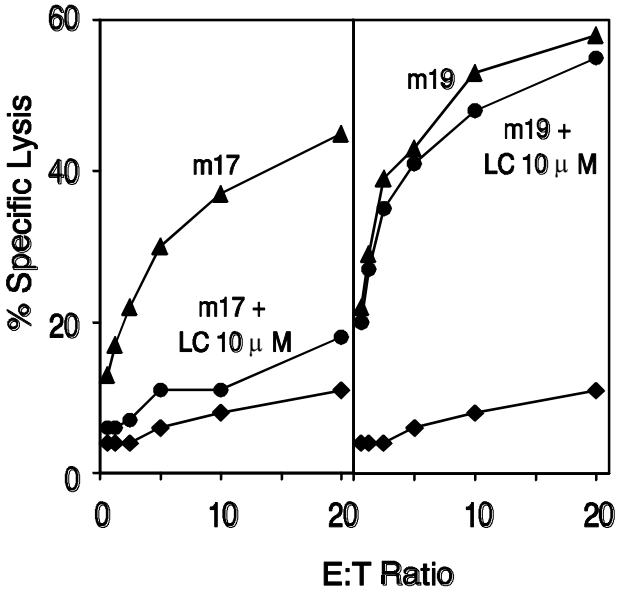

Fig. 2
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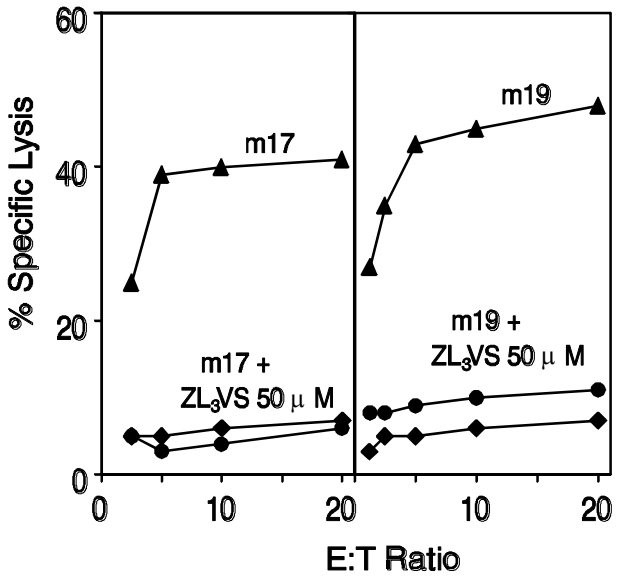

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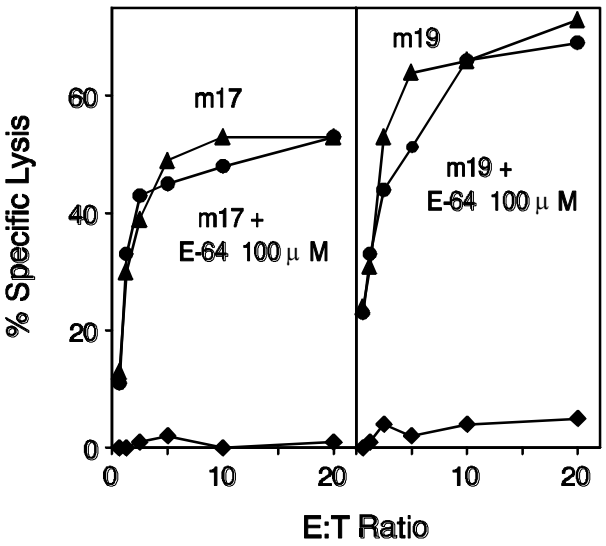

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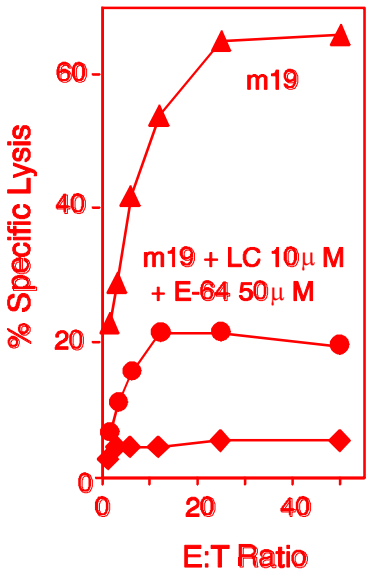

Fig. 5
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