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Role of Metalloproteases in Vaccinia Virus Epitope Processing for Transporter Associated with Antigen Processing (TAP)-independent Human Leukocyte Antigen (HLA)-B7 Class I Antigen Presentation*[S]

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Background: Individuals with nonfunctional transporter associated with antigen processing (TAP) present HLA class I ligands generated by TAP-independent processing pathways.
Results: Different subsets of metalloproteinases generate two vaccinia-derived TAP-independent epitopes.
Conclusion: Various proteolytic systems contribute to the antiviral cellular immune response, thereby facilitating immunosurveillance.
Significance: This may explain why TAP-deficient individuals live normal life spans without any increased susceptibility to viral infections.

The transporter associated with antigen processing (TAP) translocates the viral proteolytic peptides generated by the proteasome and other proteases in the cytosol to the endoplasmic reticulum lumen. There, they complex with nascent human leukocyte antigen (HLA) class I molecules, which are subsequently recognized by the CD8$^+$ lymphocyte cellular response. However, individuals with nonfunctional TAP complexes or tumor or infected cells with blocked TAP molecules are able to present HLA class I ligands generated by TAP-independent processing pathways. Herein, using a TAP-independent polyclonal vaccinia virus-polyspecific CD8$^+$ T cell line, two conserved vaccinia-derived TAP-independent HLA-B*0702 epitopes were identified. The presentation of these epitopes in normal cells occurs via complex antigen-processing pathways involving the proteasome and/or different subsets of metalloproteinases (amino-, carboxy-, and endoproteases), which were blocked in infected cells with specific chemical inhibitors. These data support the hypothesis that the abundant cellular proteolytic systems contribute to the supply of peptides recognized by the antiviral cellular immune response, thereby facilitating immunosurveillance. These data may explain why TAP-deficient individuals live normal life spans without any increased susceptibility to viral infections.

Newly synthesized viral proteins are recognized constantly by CD8$^+$ lymphocytes as short peptides bound to human leukocyte antigen (HLA) class I molecules at the cell surface of infected cells (1). Proteolysis by the proteasome and other cytosolic proteases generates most of the peptides presented by HLA class I molecules. These peptides are transported into the endoplasmic reticulum (ER)[2] by the transporter associated with antigen processing (TAP), and subsequent N terminal trimming by the metallo-aminoproteases ERAP1 and −2 is often required (2, 3). Viral peptides assembled with newly synthesized β_2-microglobulin and HLA class I heavy chain generate stable peptide-HLA complexes that are exported to the cell membrane (reviewed in Ref. 4).

Mutations in the TAP genes that generate nonfunctional TAP complexes have been described in both humans (5) and mice (6). This HLA class I deficiency implies a reduced functional CD8$^+$ population but does not correlate with any increased susceptibility to viral infections or neoplasms. Thus, TAP-deficient patients live normal life spans with only a limited susceptibility to chronic respiratory bacterial infections. Therefore, their immune systems must be reasonably efficient, and antibodies, NK cells, CD8$^+$ $\gamma\delta$ T cells, and the reduced cytolytic CD8$^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may all contribute to immune defenses that protect against severe viral infections in these individuals. Some viruses block TAP expression or function to prevent cellular immune responses from identifying infected cells (reviewed in Ref. 7). Therefore, TAP-independent pathways must be important for killing cells infected with these viruses. TAP-independent pathways of antigen presentation of various pathogenic epitopes by MHC class I molecules have previously been reported (reviewed in Refs. 7–9).

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[S] This article contains supplemental Tables S1 and S2.
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[2] The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; BEN, benzyl succinyl acid; BES, bestatin; CTL, cytotoxic T lymphocyte; ICS, intracellular cytokine staining; LC, lactacystin; LeuSH, leucine thiol; PHE, 1,10-phenanthroline; TAP, transporter associated with antigen processing; VACV, vaccinia virus; ERAP, ER aminopeptidase.

Cross-protective vaccination with orthopoxviruses, first with an empirically developed vaccine against cowpox virus and later through the massive worldwide administration of vaccinia virus (VACV), achieved the eradication of smallpox, a pandemic disease caused by variola major virus (10). The role of cellular responses in this cross-protection is well documented (11, 12). The cowpox protein CPXV12 inhibits peptide translocation by TAP, thereby interfering with MHC class I-peptide complex formation (13). Thus, the identification of TAP-independent epitopes conserved among orthopoxviruses could be relevant to the study of the mechanisms of early empirical vaccination against smallpox disease performed with cowpox virus. In this study, using a TAP-independent polyclonal vaccinia virus-polyspecific $CD8^+$ T cell line, we identified two VACV-derived TAP-independent epitopes that are conserved among the Orthopoxviridae family, including cowpox virus.

EXPERIMENTAL PROCEDURES

Mice—H-2 class I double knock-out HLA-B*0702 transgenic mice (14) were bred in our animal facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish "Comisión Nacional de Bioseguridad" of the "Ministerio de Medio Ambiente y Medio Rural y Marino" (accreditation number 28079–34A). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Health "Carlos III" (Permit Number: PI-283). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell Lines—The mouse cell lines RMA (TAP-positive) and RMA-S (TAP-negative) stably expressing HLA-B*0702 $\alpha1\alpha2$ domains plus the mouse H-$2D^b$ $\alpha3$ transmembrane and cytoplasmic domains have been previously described (14). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5 μM β-mercaptoethanol.

Synthetic Peptides—Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC. The correct molecular mass of the peptides was established by MALDI-TOF MS, and their correct composition was verified by quadrupole ion trap micro-HPLC.

Inhibitors—Brefeldin A (BFA) and all protease inhibitors were purchased from Sigma, with the exception of leupeptin (Amersham Biosciences), pepstatin (Roche Applied Science), benzyloxycarbonyl-VAD-fluoromethyl ketone (Enzyme System Products, CA), and lactacystin (Dr. E. J. Corey, Harvard University). The specificity and activity of inhibitors used in this study are summarized in Table 1. As a control for the activity of protease inhibitors that do not block antigen presentation, RMA-HLA-A*0201 cells (1×10^8) were disrupted by sonication for 15 min at 4 °C and centrifuged as reported previously (15). A supernatant aliquot corresponding to 1×10^7 cells was directly frozen (nondegraded 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 37 °C 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 TINA 2.09e program (Isopenmessgeräte, GmbH, Germany). Percent inhibition of protein degradation caused by each inhibitor was calculated as follows: 100 × (sample with inhibitor − degraded)/(nondegraded − degraded).

Ligand Prediction—The on-line program SYFPEITHI was used to predict HLA-B*0702-specific ligands of VACV as described previously (16).

Ex Vivo Intracellular Cytokine Staining (ICS)—Intracellular cytokine staining assays were performed as described previously (17). Spleen cells were obtained from HLA-B*0702 transgenic mice at 7 days (acute response) or up to 30 days (memory response) post-intraperitoneal (intraperitoneal) infection with 1×10^7 pfu of VACV-WR as described previously (18). After harvest, cells were stimulated for 2 h with RMA HLA-B*0702 cells infected with VACV-WR and incubated for 3 h in the presence of 5 μg/ml BFA. Later, cells were incubated with FITC-conjugated anti-CD8 mAb (ProImmune, Oxford, UK) for 30 min at 4 °C, fixed with Intrastain kit reagent A (Dako-Cytomation, Glostrup, Denmark), and incubated with phycoerythrin-conjugated anti-IFN-γ mAb (Pharmingen) in the presence of Intrastain kit permeabilizing reagent B for 30 min at 4 °C. Results were acquired on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Biosciences).

T Cell Lines, Cytotoxicity Assays, and ICS—The TAP-independent polyclonal VACV-polyspecific $CD8^+$ T cell line was generated by immunizing mice intraperitoneally with 1×10^7 pfu of VACV-WR. Splenocytes from immunized mice were restimulated weekly *in vitro* with mitomycin C-treated VACV-infected RMA or RMA-S HLA-B*0702 cells as antigen-presenting cells. Also, uninfected mitomycin C-treated spleen cells of allogeneic BALB/c (H-2^d haplotype), C3H (H-2^k haplotype), and SJL (H-2^s haplotype) mice were alternately used as feeder cells. This allogeneic system prevents cross-presentation of TAP-dependent HLA-B*0702-restricted peptides in the cell culture. The $CD8^+$ T cell line was re-stimulated with VACV-infected RMA-S HLA-B*0702 cells and was cultured in α-minimal essential medium supplemented with 10% FBS and 1% β-mercaptoethanol and was used after five re-stimulations as effector cells in standard 4-h cytolytic assays (18) or ICS staining similarly to *ex vivo* ICS.

Polyclonal SIINFEKL or VACV peptide-monospecific CTLs were generated by immunizing mice intraperitoneally with 1×10^7 pfu of VACV-OVA(257–264) encoding the miniprotein MSIINFEKL or VACV-WR as described previously (15, 19), respectively. Splenocytes from immunized mice were restimulated *in vitro* with mitomycin C-treated spleen cells pulsed with 10^{-6} M of the respective peptide and cultured in α-minimal essential medium supplemented with 10% FBS, 1×10^{-7} M peptide, and 1% β-mercaptoethanol. Recombinant human interleukin-2 was generously provided by Hoffmann-La Roche for the long term propagation of all $CD8^+$ T cell lines. ICS assays to detect the recognition of infected cells by polyclonal $CD8^+$ T cell lines were performed as described previously (17). $CD8^+$ T cell lines were stimulated for 4 h with RMA HLA-B*0702 target cells that had been infected with VACV or

VACV-OVA(257–264) overnight and in the presence of 5 μg/ml BFA. When protease inhibitors were used, all drugs were added 15 min before the virus and kept at a 5-fold higher concentration during the 1-h adsorption period than that used throughout the infection. After the virus inoculums were washed, the inhibitors were kept at the concentrations indicated for the individual experiments. The inhibitors were not toxic at the indicated concentrations because they did not affect antigen presentation of either the J6R(303–311) or D1RL((807–817)) epitopes (see below) or the VACV infection when the Omnitope antiserum with specificity for VACV proteins from purified virions was used (ViroStat Inc., Portland, ME) (supplemental Table 1). ICS with polyclonal $CD8^+$ T cell lines was performed similarly to *ex vivo* ICS. The percentage of specific inhibition obtained by the addition of the inhibitors was calculated as shown in Equation 1,

$$\%\ \text{specific inhibition} = 100 - \frac{((\text{ICS VACV} + \text{inhibitor}) - (\text{ICS without infection}))}{(\text{ICS VACV} - \text{ICS without infection})} \times 100 \quad \text{(Eq. 1)}$$

Statistical Analysis—To analyze statistical significance, an unpaired Student *t* test was used. p values < 0.01 were considered to be significant.

RESULTS

*Identification of Two VACV-derived TAP-independent HLA-B*0702 Epitopes*—Spleen cells from HLA-B*0702 transgenic mice were primed with VACV and re-stimulated with the TAP-negative RMA-S cell line transfected with HLA-B*0702 to specifically recognize VACV-infected target cells. We observed 45% specific lysis *versus* 1% without virus in a standard ^{51}Cr-release assay. By intracellular cytokine staining (ICS) assays, we found that 32% of IFN-γ secreting cells had a specific response *versus* 1% without virus. Later, this $CD8^+$ cell line was used to identify TAP-independent epitopes with target cells previously pulsed with the VACV synthetic peptides previously reported as HLA-B*0702 epitopes identified from either HLA-B*0702 transgenic mice (A34R(82–90), D1R(808–817), and J2R(116–124)) (20) or human vaccines (AC1L(97–106), D1R(686–694), F4L(6–14), and J6R(303–311)) (21, 22). In addition, 14 HLA-B7 potential ligands from a VACV proteome-based *in silico* prediction of high binding were also included in the study to identify new epitopes (supplemental Table 2). In a preliminary ^{51}Cr-release assay, only two synthetic peptides, D1R(808–817) and J6R(303–311), were recognized by the TAP-independent CTL cell line (Fig. 1*A*, *arrows*). Additional ICS experiments with all synthetic peptides confirmed that only the D1R(808–817) and J6R(303–311) peptides specifically stimulated the production of IFN-γ in the $CD8^+$ T cells specific for VACV ligands (Fig. 1*B*). Fig. 2 shows that D1R(808–817) peptide was recognized 10-fold less efficiently in TAP-deficient cells *versus* TAP-sufficient cells. Both viral ligands were conserved among the *Orthopoxviridae* family, including cowpox virus (NCBI data base (/blast.ncbi.nlm.nih.gov). In summary, these results indicate that two conserved TAP-independent HLA-B7 epitopes were present in the TAP-deficient VACV-infected cells.

*Partial Interspecies Overlap in the $CD8^+$ Repertoire against HLA-B*0702 Viral Epitopes*—Previously, the D1R(808–817) viral epitope was immunogenic in the HLA transgenic mouse model (20), whereas the J6R(303–311) peptide was recognized by peripheral blood mononuclear cells of an HLA-$B7^+$ donor immunized with VACV (21). No interspecies overlap in the $CD8^+$ repertoire against these two VACV epitopes or the other five HLA-B*0702 epitopes has been reported (20–22). Because the HLA-B*0702 transgenic mice used in this study (14) have a different origin from those used the previous study (20), the VACV-specific $CD8^+$ acute and memory responses in our HLA-B7 transgenic model was evaluated using *ex vivo* ICS assays. A strong acute response ($6.1 \pm 0.4\%$ of IFN-γ-secreting cells) specific for the D1R(808–817) synthetic peptide was detected (Fig. 3). The A34R(82–90) and D1R(686–694) viral peptides were also immunogenic, with 3.0 ± 0.7 and $1.2 \pm 0.4\%$ $CD8^+$ IFN-γ^+ cells, respectively. Additionally, a small fraction of VACV-specific $CD8^+$ T lymphocytes recognized the J6R(303–311) peptide ($0.3 \pm 0.05\%$ of IFN-γ^+ cells, Fig. 3). When the VACV memory response was analyzed (Fig. 3, *filled bars*), an epitope hierarchy similar to the acute response was found, except that the percentage of IFN-γ secreting cells was slightly higher with the J6R(303–311) peptide ($0.7 \pm 0.2\%$) than with the D1R(686–694) epitope ($0.5 \pm 0.1\%$). None of the other 17 VACV peptides tested (for list see Fig. 1 and supplemental Table 1) stimulated the production of IFN-γ in the VACV-specific $CD8^+$ T cell acute or memory response (data not shown). Thus, two epitopes from both previously described transgenic mouse models (D1R(808–817) and A34R(82–90)) and human donors (D1R(686–694) and J6R(303–311)) are responsible for this specific $CD8^+$ response against VACV. The same *ex vivo* epitope hierarchy was also found using a TAP-dependent polyclonal VACV-polyspecific $CD8^+$ T cell line generated by re-stimulation *in vitro* with mitomycin C-treated VACV-infected RMA (TAP^+) HLA-B*0702 cells as antigen-presenting cells (data not shown). By contrast, in the previous study with the HLA transgenic model, the epitope hierarchy was A34R(82–90) > J2R(116–142) > D1R(808–817) (20). These results show both the quantitative and the qualitative differences between the two HLA-B*0702 transgenic mouse models available, although only the $CD8^+$ response of the HLA-B7 model used in our study showed partial overlap in $CD8^+$ repertoire with the study of human vaccines (21). In addition, these data show that half of the HLA-B7-restricted viral epitopes detected in a normal TAP-dependent T cell response could also be presented in a TAP-independent manner in the HLA-B7 transgenic model.

*Endogenous Processing of TAP-independent HLA-B*0702 Epitopes*—To study all antigen-processing pathways involved in the endogenous generation of the D1R(808–817) and J6R(303–311) viral epitopes, polyclonal $CD8^+$ T cell lines monospecific for the two TAP-independent HLA-B7 viral epitopes were generated. Later, we investigated the presentation of these epitopes to respective specific CTLs in the presence of diverse protease inhibitors in VACV-infected TAP-proficient cells. To test whether these HLA-B7-restricted epitopes

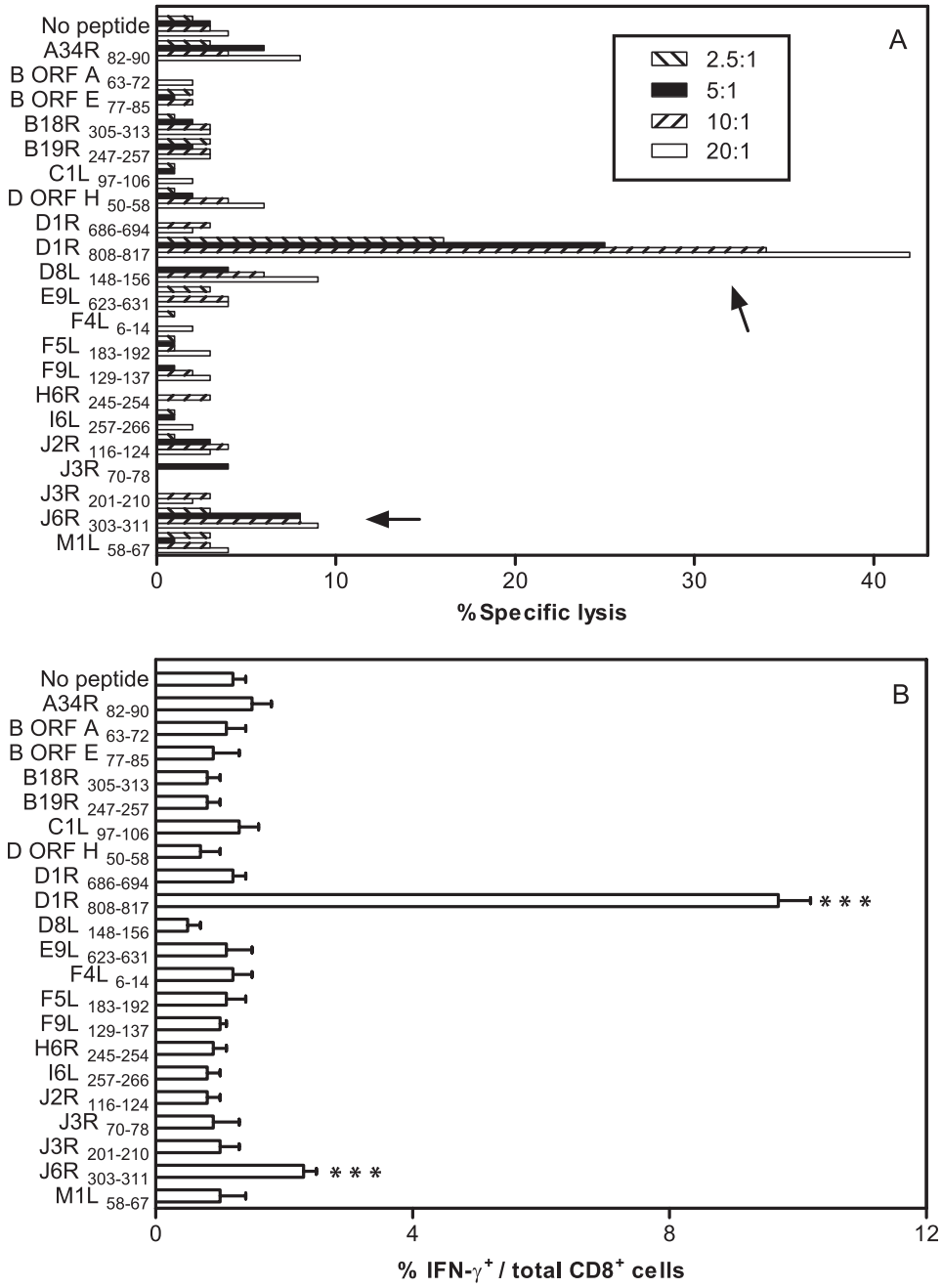

FIGURE 1. **Recognition of HLA-B7-restricted synthetic peptides by a VACV-specific TAP-independent CD8$^+$ T cell line.** RMA-HLA-B*0702 target cells pre-pulsed with 10^{-5} M of the indicated VACV-synthetic peptides were tested in a standard cytolytic assay (*A*) or by ICS for CD8$^+$ T cell activation (*B*) with VACV-specific CD8$^+$ T cells. The VACV-specific CD8$^+$ T cells were obtained from HLA-B*0702-transgenic mice immunized with VACV up to 30 days prior and restimulated *in vitro* with the TAP-negative RMA-S cell line transfected with HLA-B*0702. *Arrows* in *A* indicate the synthetic peptides detected with 2-fold higher specific lysis than the negative control (no peptide) in three *E*/*T* ratios. The data are the mean of four independent experiments ± S.D. (*B*). Significant *p* value, ***, $p < 0.001$.

require endogenous processing, we analyzed their presentation in the presence of BFA. This drug blocks class I export beyond the cis-Golgi compartment (23, 24), preventing the surface expression of newly assembled class I-peptide complexes of endogenous origin (Table 1 summarizes the specificity of all inhibitors used in this study). We observed complete inhibition of specific secretion of IFN-γ in the two specific CD8$^+$ T cell lines by the addition of BFA during infection (Fig. 4), demonstrating that these epitopes were indeed generated from proteins endogenously processed in VACV-infected cells.

*Proteasome Inhibitor Differentially Affects the Antigen Presentation of TAP-independent HLA-B*0702 Viral Ligands*—Lactacystin (LC), a bacterial metabolite (Table 1) (25–27), was used to study the role of the proteasome in the presentation of these epitopes. LC partially blocks (45 ± 15%) the specific recognition of target cells infected with VACV by J6R(303–311)-specific CD8$^+$ T cells (Fig. 4). By contrast, in the same experiment, this drug had no effect on the presentation of the D1R(808–817) epitope (3 ± 12%) (Fig. 4). We observed complete inhibition of infected cell recognition by another VACV-specific

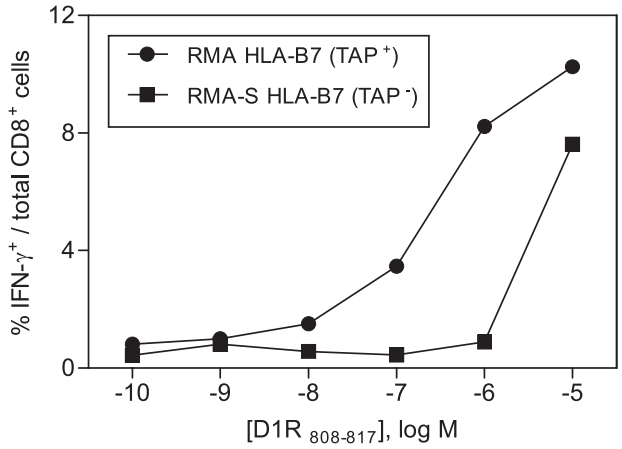

FIGURE 2. **Recognition of TAP$^+$ and TAP$^-$ cell lines by VACV-specific CD8$^+$ T lymphocytes.** Recognition by ICS for CD8$^+$ T cell activation of titration curves of D1R(808–817) synthetic peptide in HLA-B*0702 TAP$^+$ (*RMA*, *circles*) and TAP$^-$ (*RMA-S*, *squares*) cells. The VACV-specific CD8$^+$ T cells were obtained as Fig. 1. Results are the mean of three experiments.

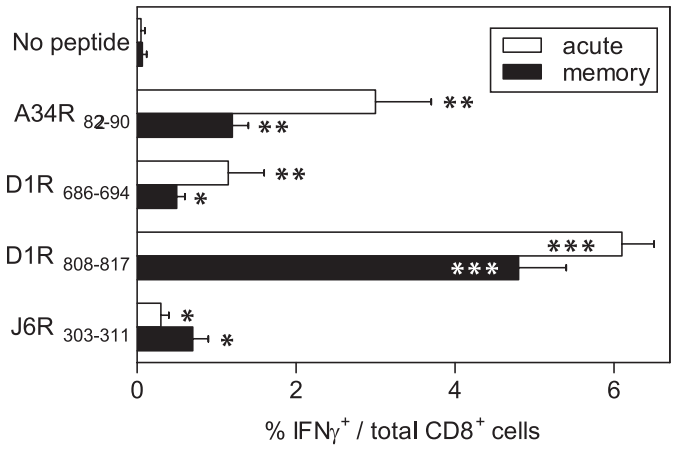

FIGURE 3. **Immunogenicity of VACV-derived HLA-B*0702-restricted peptides in HLA-B*0702 transgenic mice.** RMA-HLA-B*0702 target cells prepulsed with the indicated VACV synthetic peptides in Fig. 1 were analyzed by ICS for CD8$^+$ T cell activation with VACV-specific splenocytes obtained from HLA-B*0702 transgenic mice immunized 7 days (*acute* response, *open bars*) or up to 30 days *(memory*, *closed bars*) post-infection. The results are calculated as the mean of three or four independent experiments ± S.D. Significant *p* values are as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TAP$^+$ CD8$^+$ T cell line with LC treatment (96 ± 4%),[3] indicating that LC-mediated inhibition of proteasome activity is not absolutely required for antigen processing of the D1R(808–817) epitope. This further suggests that the proteasome partially contributes to the generation of the J6R(303–311) peptide in infected cells.

*A Metallopeptidase Inhibitor Specifically Blocks the Recognition of HLA-B*0702 Epitopes*—To characterize proteases distinct from proteasomes that may contribute to the processing of HLA-B*0702-restricted ligands, experiments with several specific protease inhibitors were performed. Leupeptin (28), pepstatin (28, 29), 1,10-phenanthroline (PHE) (29, 30), and E64 (31) inhibitors were initially tested because they are specific for different protease families (Table 1) and cover a wide range of protease classes. Puromycin (32) has also previously been suggested to generate pathogen-derived peptides; thus, the possible role of this enzyme in endogenous presentation of TAP-independent viral epitopes was studied using a specific inhibitor (Table 1). Four of five inhibitors had no effect on the specific recognition of target cells infected with VACV with the two specific CD8$^+$ T cell lines tested (Fig. 5). Thus, the enzymes inhibited by these drugs are not formally involved in the generation of TAP-independent ligands.

In contrast, PHE inhibited the recognition of infected cells by J6R(303–311) (42 ± 12%)- and D1R(808–817) (72 ± 20%)-specific CD8$^+$ T cells (Fig. 5). We wanted to exclude the possibility that the inhibitory effect of PHE was due to toxic effects on target cells or on VACV replication rather than to a specific block of the respective proteases. To this end, experiments similar to those shown in Fig. 5 were performed in parallel using VACV-OVA(257–264)-infected target cells. These infected cells were efficiently recognized by the SIINFEKL-specific CD8$^+$ T cell line. and no inhibition was detected (10 ± 4%, see Fig. 5). These data indicate that inhibition of specific recognition in both CD8$^+$ T cell lines by addition of PHE is formally due to specific blockage of the specific proteases not to a block in VACV replication (see also supplemental Table 1). In summary, these results indicate that either a metallopeptidase or different metalloproteases are involved in the generation of these two HLA-B*0702 epitopes.

*Metallocarboxyprotease and Metalloaminoproteases but Not ERAP Are Differentially Involved in the Generation of TAP-independent HLA-B*0702 Epitopes*—A variety of functional metallopeptidases are located in the cytosol or in other compartments related to the MHC class I presentation pathway, such as the ER and the trans-Golgi network (reviewed in Ref. 33). Any of these enzymes may play a role in the endogenous pathway of antigen processing. These enzymes can be grouped into aminopeptidases, endopeptidases, carboxypeptidases, and carboxydipeptidases, among others, based on their respective cleavage mechanism (reviewed in Ref. 34). Some of these groups can be distinguished by the use of different specific inhibitors (summarized in Table 1).

To more specifically identify the metallopeptidase group involved in antigen processing of both D1R(808–817) and J6R(303–311) viral peptides, VACV-infected target cells were treated with specific subfamily inhibitors (Table 1). The caspase-1-specific inhibitor benzyloxycarbonyl-VAD was also included due to the sensitivity of this cysteine protease to PHE (30). None of the inhibitory compounds used, except for leucine thiol (LeuSH), prevented antigen presentation of VACV-infected cells to the CD8$^+$ T cell line specific for the J6R(303–311) viral peptide (Fig. 6, *open bars*). When similar experiments were carried out with D1R(808–817)-specific CD8$^+$ T cells, complete blockage of antigen recognition (84 ± 11%) was also detected in the presence of the LeuSH inhibitor. The inhibitory effect of LeuSH was specific to these two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of this compound (12 ± 10%, see Fig. 6). Thus, these data implicate ERAP or other metallo-aminopeptidases in the generation of the two VACV epitopes studied.

[3] E. Lorente and D. López, manuscript in preparation.

TABLE 1
Specificity and activity of the inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference	Concentration	% inhibition of degradation[a]
Brefeldin A	BFA	Vesicle transport	23, 24	5 μg/ml	ND
Lactacystin	LC	Proteasome	25, 26	10 μM	ND
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	28	100 μM	38 ± 18
Pepstatin	PEP	Aspartic proteases	28, 29	100 μM	50 ± 5
1,10-Phenanthroline	PHE	Metalloproteases and caspase-1	29, 30	50 μM	ND
E64	E64	Cysteine proteases C1	31	100 μM	ND
Puromycin	PUR	Dipeptidyl-peptidase II and PSA	61	0.5 μg/ml	ND
Captopril	CAP	ACE and ACE-like proteases	29	100 μM	25 ± 2
Benzyl succinyl acid	BEN	Metallo-carboxypeptidases A and B	29	100 μM	−10 ± 8
Bestatin	BES	Most of metallo-aminopeptidases	29	50 μM	ND
Phosphoramidon	PHO	All bacterial metallo-endopeptidases but few of mammalian origin	29, 62	100 μM	15 ± 4
Leucine thiol	LeuSH	Metallo-aminopeptidases including ERAP	57	30 μM	ND
Benzyloxycarbonyl-VAD-fluoromethyl ketone	Z-VAD-fmk	Caspases	63	100 μM	ND[b]

[a] Activity of these inhibitors was measured as their ability to prevent proteolytic degradation in cellular extracts as in Ref. 15. The amount of protein still present after incubation in the case of the degraded control sample was considered 0% inhibition of degradation, and the nondegraded sample was taken as 100% inhibition. Data are means of two independent experiments. Negative values indicate that there was enhanced degradation in the presence of the compound. ND indicates not done.
[b] The compound was found to block apoptosis (data not shown).

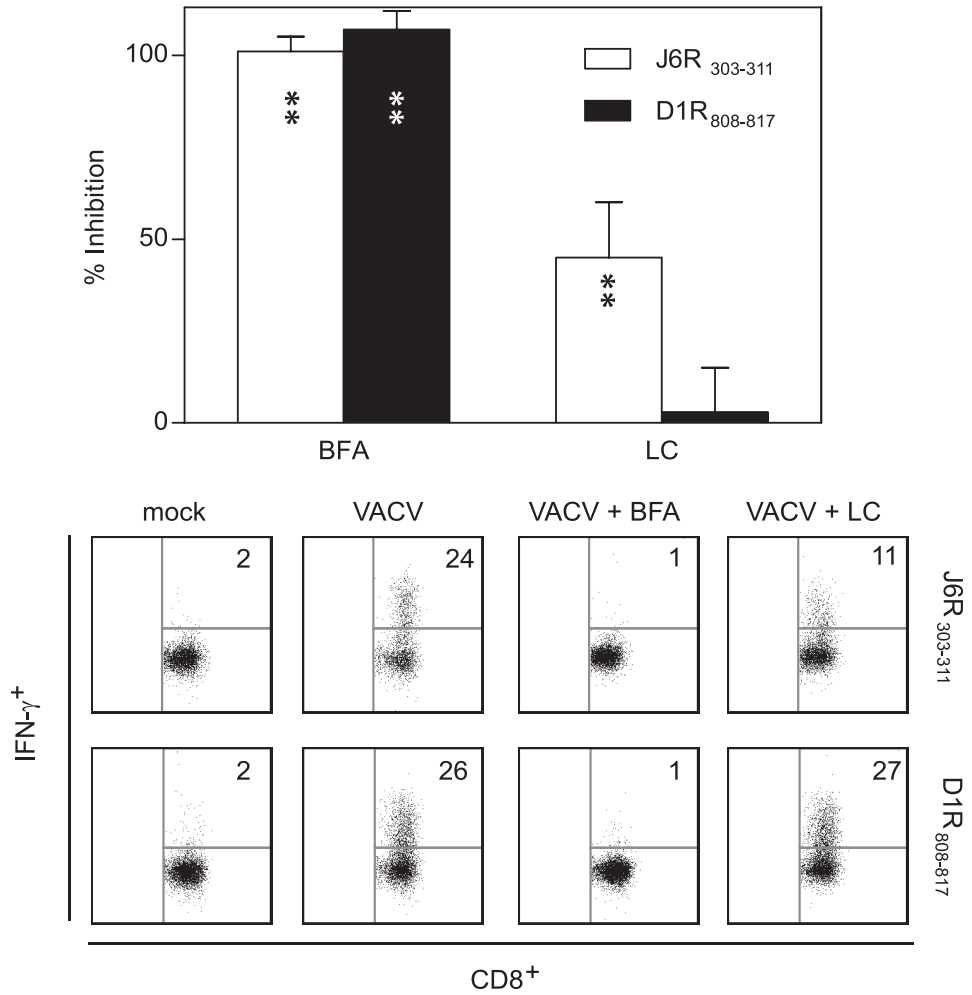

FIGURE 4. **Effect of BFA and a proteasome inhibitor on the recognition of the J6R(303–311) or the D1R(808–817) viral epitopes.** RMA-HLA-B*0702 target cells infected for 16 h with VACV at a multiplicity of infection of 40 plaque-forming units/cell were treated with BFA or LC. An ICS assay was used to test for recognition by the J6R(303–311)- (*open bars*) or D1R(808–817)-specific (*closed bars*) $CD8^+$ T cell lines. The data are expressed as percentage of inhibition ± S.D. as determined by ICS in the presence of the indicated inhibitors and are means of three to four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with J6R(303–311)- and D1R(808–817)-specific $CD8^+$ T cell lines were depicted in the *middle* and *bottom panels*, respectively. The percentages of IFN-γ-expressing $CD8^+$ T cells are indicated in each dot plot.

In addition, as shown in Fig. 6, a partial but specific blockage of the endogenous processing of the D1R(808–817) epitope by either the metalloaminoprotease inhibitor bestatin (BES) or the metallocarboxypeptidase inhibitor benzyl succinyl acid (BEN) was detected (44 ± 15% of specific inhibition with BES and 42 ± 16% with BEN). Thus, both metalloaminoprotease and metal-

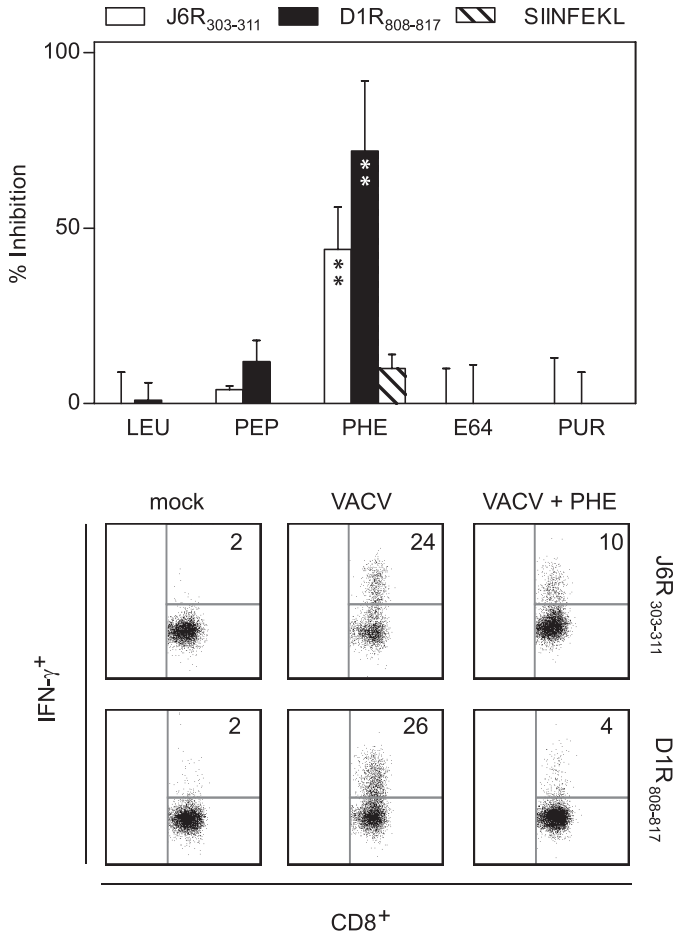

FIGURE 5. **Recognition of VACV-infected cells by J6R(303–311)- or D1R(808–817)-specific CD8$^+$ T cells in the presence of general protease inhibitors.** Cells infected as described in Fig. 4 were treated with leupeptin (*LEU*) (trypsin-like and cysteine protease inhibitor), pepstatin (*PEP*) (aspartic protease inhibitor), PHE (metallopeptidase inhibitor), E64 (cysteine protease C1 inhibitor), or puromycin (*PUR*) (dipeptidyl-peptidase II and PSA), as indicated, before the ICS assay. J6R(303–311)- (*open bars*), D1R(808–817)- (*closed bars*), or SIINFEKL-specific (*hatched bars*) CD8$^+$ T cell lines were used. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three or four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with J6R(303–311)- and D1R(808–817)-specific CD8$^+$ T cell lines were depicted in the medium and bottom panels, respectively. The percentages of IFN-γ-expressing CD8$^+$ T cells are indicated in each dot plot.

locarboxyproteases are involved in the antigen processing of the D1R(808–817) epitope.

J6R(303–311) Epitope May Be Processed in Parallel Either by Proteasomes or by Metallopeptidases Independently—The inhibition of antigen recognition by either LC (Fig. 4) or PHE (Fig. 5) indicates that both proteasomes and metalloproteases are involved in the antigen presentation of the J6R(303–311) epitope. The identical partial inhibition of VACV-infected cell recognition detected with both reagents (45 ± 15 and 42 ± 12% in the presence of LC or PHE, respectively) is compatible with two possible explanations. First, this epitope could be processed by proteasomes and metallopeptidases in a sequential pathway, and other uncharacterized proteases may be responsible for the other half of the antigen. Alternatively, these epitopes could be processed in parallel by proteasomes or by metallopeptidases independently, meaning that these two antigen-processing pathways would need to be inhibited at the same time to fully abrogate the specific recognition by J6R(303–311)-specific CD8$^+$ T cells. To test these hypotheses, the effects of the combination of both inhibitors on antigen presentation in vaccinia-infected cells were analyzed. A total block of presentation (97 ± 3%) was observed in target cells treated simultaneously with LC and PHE (Fig. 7). The inhibitory effect of LC and PHE was specific to these two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of these drugs (5 ± 10%, see Fig. 7). These results demonstrate that proteasomes and metallopeptidases are involved in two different antigen-processing pathways that contribute independently to the presentation of the J6R(303–311) epitope.

Sequential Cleavage by Amino- and Carboxy-metallopeptidases Is Involved in Antigen Processing of the D1R(808–817) Epitope—Like the J6R(303–311) epitope, the partial block of D1R(808–817) epitope recognition detected in the presence of BES or BEN (44 ± 15 and 42 ± 16%, respectively) is compatible with either sequential cleavage by amino- and carboxy-metallopeptidases or the activity of these enzymes in two different antigen-processing pathways to produce the D1R(808–817) epitope. The incubation of VACV-infected target cells with a mixture of both reagents produced a partial inhibition of antigen presentation (46 ± 4%, see Fig. 7), similar to two single inhibitors (Fig. 6). The inhibitory effect of the two metallopeptidase inhibitors was specific to the two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of BEN plus BES (7 ± 9%, see Fig. 7). Thus, both metalloaminoprotease and metallocarboxyproteases contribute to D1R(808–817) epitope cleavage in the same antigen-processing pathway. In addition, another uncharacterized protease is responsible for the BEN plus BES-resistant antigen processing detected with D1R(808–817)-specific CD8$^+$ T cells.

Diversity of Proteases Involved in Antigen Recognition of Vaccinia HLA-B7-restricted Epitopes—Table 2 summarizes the various inhibition patterns for antigen recognition of the J6R(303–311) and D1R(808–817) epitopes obtained with the drugs used in this study (Table 1). The inhibition observed with BFA indicates that both viral peptides were endogenously processed. The block with LC and PHE indicates that both proteasomes and metalloproteases are used in the processing of the J6R(303–311) epitope. Also, the LeuSH inhibitor impaired antigen recognition of target cells by J6R(303–311)-specific CD8$^+$ T cells. The second epitope, D1R(808–817), shows a different inhibition pattern. LC had no effect on the presentation of this epitope, and thus, LC-mediated inhibition of proteasome activity is not absolutely required for antigen processing of the D1R(808–817) epitope. By contrast, PHE, BEN, BES, and LeuSH significantly decreased antigen presentation of the D1R(808–817) epitope; thus, a variety of metalloproteases are involved in the generation of this TAP-independent epitope.

DISCUSSION

This study was undertaken to identify TAP-independent HLA-B*0702 epitopes from the vaccinia virus and to study their antigen presentation pathways. First, our results indicate that two of four epitopes detected in the standard antiviral response

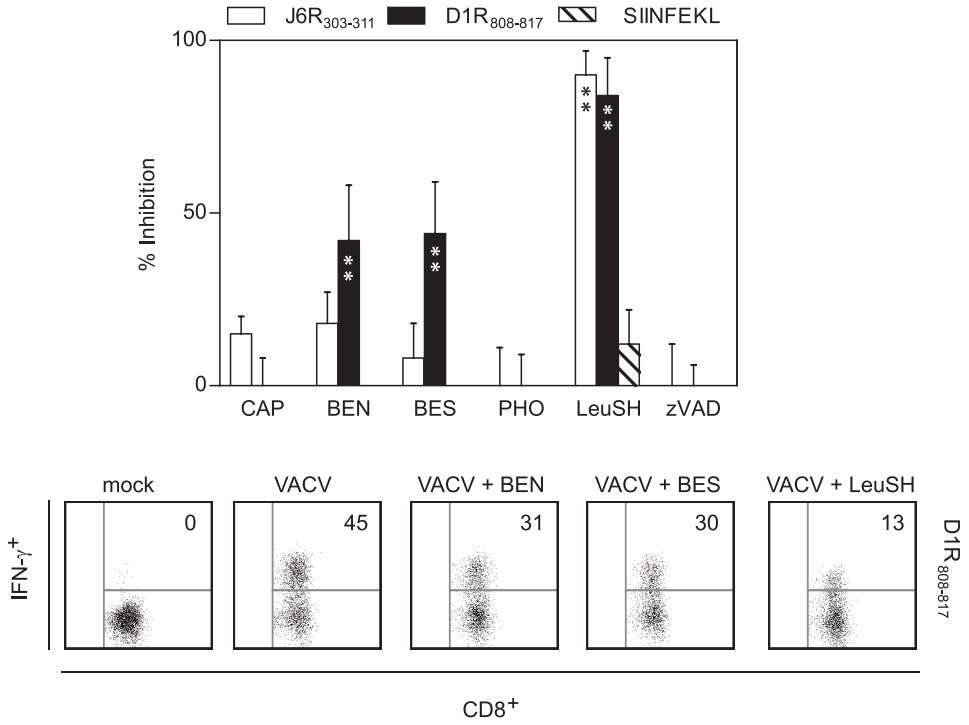

FIGURE 6. **Recognition of target cells infected in the presence of metalloproteinase subfamily inhibitors.** Cells infected as described in Fig. 4 were treated with captopril (*CAP*) (ACE-like metallopeptidase inhibitor), BEN (inhibits metallo-carboxypeptidases), BES (metallo-aminopeptidase inhibitor), phosphoramidon (*PHO*) (bacterial metalloendopeptidase inhibitor), LeuSH (mainly ERAP and other metallo-aminopeptidase inhibitor), or benzyloxycarbonyl-VAD (*zVAD*) (blocks caspases) as indicated, before the ICS assay. The figure is labeled as in Fig. 5. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three to four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with D1R(808–817)-specific $CD8^+$ T cell lines are depicted in the *bottom panels*. The percentages of IFN-γ-expressing $CD8^+$ T cells are indicated in each dot plot. *ACE*, angiotensin converting enzyme.

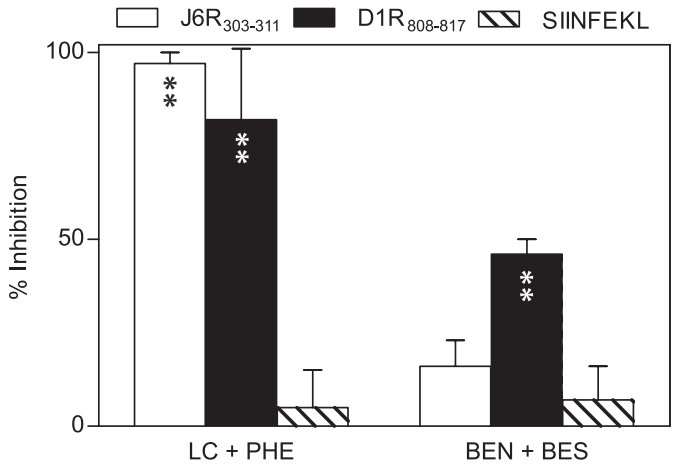

FIGURE 7. **Effect of combinations of inhibitors in the recognition of VACV-infected cells.** Cells infected as described in Fig. 4 were treated with the combination of either LC and PHE or BEN and BES, as indicated, before the ICS assay. The figure is labeled as in Fig. 5. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three or four independent experiments. Significant *p* value, **, $p < 0.01$. *ACE*, angiotensin converting enzyme.

from the H-2 class I double knock-out HLA-B*0702 transgenic mice (14) are presented by TAP-independent pathways as follows: the immunodominant D1R(808–817) epitope and the J6R(303–311) vaccinia peptide detected in human donors. Thus, TAP-independent HLA-B*0702 antigen presentation is sufficient to control vaccinia virus infection in the absence of a functional TAP complex. If these data are typical for all HLA class I molecules, this may help explain why individuals with unusable TAP complexes do not seem particularly susceptible to viral infections and may appear asymptomatic for much of their lives (reviewed in Ref. 5).

The sources of the two TAP-independent epitopes identified were the vaccinia J6R and D1R proteins. The J6R protein is a component of the viral RNA polymerase complex (35). The D1R protein is the large subunit of the viral mRNA capping enzyme (36) and is needed for early transcription termination (37). Presumably, the RNA polymerase carries the capping enzyme along as it transverses the template as a transcription elongation complex (38). Presentation of cytosolic proteins in cells lacking TAP has been previously reported (39, 40). This presentation of peptides could occur by passive diffusion (41), hydrophobic peptides with the ability to traverse membranes (42), or unidentified transport. Thus, either these VACV proteins or their respective ligands could be accessible to HLA-containing compartments with resident proteases for their TAP-independent HLA class I antigen processing and presentation.

Several proteases have been implicated in the processing of endogenously synthesized antigens independent of the classical proteasome pathway as follows: signal peptidase (43, 44); furin (45, 46); tripeptidyl peptidase II (47–49); lysosomal chloroquine-sensitive enzymes (50, 51); and caspases (52, 53). In this study, diverse proteolytic activities are required to generate the two HLA-B7-restricted epitopes studied. Our results using various protease inhibitors (summarized in Table 2) are consistent with the models depicted in Fig. 8. The block with LC indicates that the proteasome plays a role in the processing of the

TABLE 2
Summary of inhibition patterns

Epitope	BFA[a]	LC	PHE	BEN	BES	LeuSH	LC + PHE	BEN + BES
J6R(303–311)	+++[b]	+	+	−	−	+++	+++	−
D1R(807–817)	+++	−	++	+	+	+++	+++	+

[a] For specificity of different inhibitors see Table 1.
[b] −, +, ++, and +++ indicate % inhibition <20, 40–60, 61–80, and >81% respectively. All + inhibitions show significant *p* values ($p < 0.01$) *versus* controls without an inhibitor.

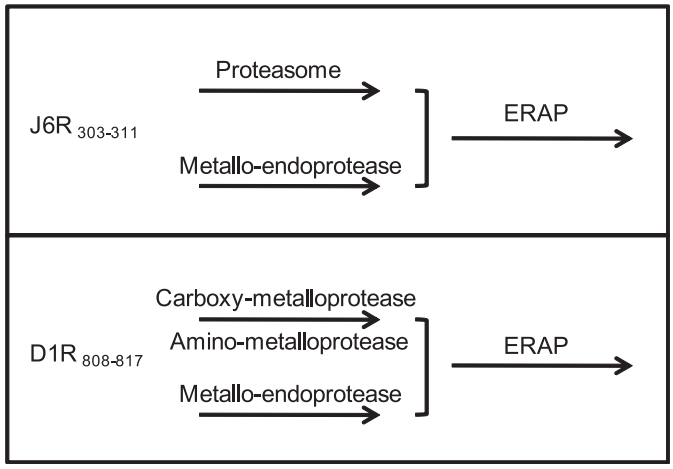

FIGURE 8. **Diversity of proteases and processing pathways involved in J6R(303–311) or D1R(808–817) epitope presentation.** The models show the components involved in each of the proposed pathways for the J6R(303–311) (*upper panel*) or D1R(808–817) (*lower panel*) epitopes. The role of proteases is deduced from the sensitivity of respective $CD8^+$ T cells to the various inhibitors (see Table 2).

J6R(303–311) epitope but not the D1R(808–817) viral ligand. The presentation of both vaccinia peptides was dependent on PHE-sensitive proteases, indicating that metalloprotease activity is required to process these epitopes. The LeuSH inhibitor impaired antigen recognition of target cells by both D1R(808–817) and J6R(303–311)-specific $CD8^+$ T cells. Because the pan-specific metalloprotease inhibitor PHE and the inhibitor of general aminoprotease activities BES did not block or had very little effect on ERAP (54, 55), the inhibition of D1R(808–817)- or J6R(303–311)-specific recognition requires both ERAP and other similar metalloproteases. In addition, the partial but selective impaired recognition of VACV-infected cells by D1R(808–817)-specific T cells in the presence of BEN or BES, which was not increased when both drugs were added together, implies that amino- (different from ERAP) and carboxy-metallopeptidases contribute sequentially to D1R(808–817) antigen processing. By contrast, the higher inhibition detected in the presence of LC and PHE *versus* single inhibitors demonstrated that the antigen processing of the J6R(303–311) epitope requires proteasomes and metallopeptidases independently.

Last, the recognition of VACV-infected cells by J6R(303–311)-specific $CD8^+$ T cells was partially blocked by PHE but not by the inhibitors of metalloproteases used in this study. Because phosphoramidon very efficiently inhibits bacterial metalloendopeptidases but does not block multiple higher vertebrate metallo-endoproteases, the most likely explanation for PHE-specific inhibition of J6R(303–311) recognition is that some mammalian metalloendopeptidases that are not blocked by phosphoramidon are involved in the antigen processing of this viral ligand. Similarly, there was higher inhibition of the antigen recognition of the D1R(808–817) epitope with PHE than with the combination of BEN and BES, suggesting additional metallo-endoprotease activity. More than 100 different well characterized higher vertebrate metalloendopeptidases are resistant to this reagent (33), and drugs that collectively and specifically block the endoproteolytic activity of this group of enzymes have not been described. Therefore, positive identification of the peptidase involved in the processing of these TAP-independent vaccinia epitopes awaits further characterization.

In summary, the J6R(303–311) product appears to be processed in parallel either by proteasomes or by metalloendopeptidases independently. Later, ERAP trims the final epitope. The processing of the D1R(808–817) epitope is more complex, involving a branched pathway in which metallo-endoproteases and a sequential cleavage of both metalloaminoprotease and metallocarboxyproteases are required to generate this epitope. As preceding epitope J6R(303–311), the trimming by ERAP generates the final D1R(808–817) epitope.

Metallopeptidases are among the hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule previously activated by a divalent metal cation (33). These proteases are allocated to 14 clans and subdivided into 62 families (MEROPS data base) (56). Based on their respective cleavage mechanism, these enzymes can be grouped into aminopeptidases, endopeptidases, carboxypeptidases, and carboxy-dipeptidases, among others (reviewed in Ref. 33). The ER resident amino-metallopeptidase ERAP plays an essential role in the trimming of different MHC class I ligands (3, 54, 57). In our study, we found that an ERAP-specific inhibitor blocks antigen presentation of both TAP-independent vaccinia ligands studied. Other amino-metallopeptidases also travel by the secretory pathway to their destination organelle or to the extracellular medium (33) and could be responsible for the BES-mediated inhibition observed in the antigen processing of the D1R(808–817) epitope. Also, carboxy-metallopeptidases abound in the secretory pathway and in vesicular compartments accessible to HLA class I molecules (33). Currently, no individual carboxypeptidases have been implicated in antigen processing in the vesicular pathway, but indirect evidence has been reported in two cases. First, the proteolytic action of furin in the secretory pathway is required to generate the antigenic CMV pp89 epitope located in the sHBe chimera (46). After this cleavage, nine C-terminal residues must be trimmed from the precursor peptide to generate the optimal 9pp89 epitope, and thus carboxypeptidases may be involved. Second, various signal sequence-derived peptides generated by signal peptidase complexes have C-terminally extended residues compared with the optimal HLA-bound epitope isolated (58–60); thus, carboxypeptidases may be involved in antigen processing of these

epitopes. This study directly implicates carboxy-metallopeptidases in the antigen processing of the vaccinia D1R(808–817) epitope. Finally, unknown metalloendopeptidases are involved in the processing of an HIV-1 epitope in a sequential TAP-dependent pathway that also implicates cleavage by proteasomes (15). In our study, proteasomes and uncharacterized metalloendopeptidases were found to be involved in J6R(303–311) cleavage in two different antigen-processing pathways.

In conclusion, the results reported here highlight the diversity of proteases involved in antigen recognition, and they uncover the complexity of antigen-processing pathways.

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