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TAP-Independent Presentation of CTL Epitopes by Trojan Antigens

Jun Lu,* Peter J. Wettstein,* Yuichiro Higashimoto, † Ettore Appella, † and Esteban Celis2*

The majority of CTL epitopes are derived from intracellular proteins that are degraded in the cytoplasm by proteasomes into peptides that are transported into the endoplasmic reticulum by the TAP complex. These peptides can be further processed into the optimal size (8–10 residues) for binding with nascent MHC class I molecules, generating complexes that are exported to the cell surface. Proteins or peptides containing CTL epitopes can be introduced into the cytoplasm of APCs by linking them to membrane-translocating Trojan carriers allowing their incorporation into the MHC class I Ag-processing pathway. The present findings suggest that these “Trojan” Ags can be transported into the endoplasmic reticulum in a TAP-independent way where they are processed and trimmed into CTL epitopes. Furthermore, processing of Trojan Ags can also occur in the trans-Golgi compartment, with the participation of the endopeptidase furin and possibly with the additional participation of a carboxypeptidase.

We believe that these findings will be of value for the design of CTL-inducing vaccines for the treatment or prevention of infectious and malignant diseases. The Journal of Immunology, 2001, 166: 7063–7071.

Cytotoxic T lymphocytes are probably the most effective mechanism that the immune system has to destroy “abnormal” cells such as those infected by intracellular pathogens or tumor cells. CTL recognize these abnormal cells via TCRs, which bind to peptide epitopes that are complexed onto cell surface MHC class I molecules (1). Most CTL peptide epitopes are derived from intracellular proteins such as viral and tumor-associated Ags that are synthesized within the cell and become processed in the cytoplasm into peptides, which are transported into the endoplasmic reticulum (ER)3 (2–5). Once in the ER, these peptides may be further “trimmed” into an optimal size of 8–10 residues that may associate with MHC class I molecules that will be exported to the cell surface of APCs to possibly interact with TCR (6–11). This process, known as the MHC class I Ag-processing pathway, is still not completely understood.

The requirement for intracellular cytoplasmic processing of Ags creates some constraints for the induction of CTL responses using conventional noninfectious vaccines, such as killed pathogens or recombinant proteins. The reason is that in general APC are not very efficient in processing exogenous Ags through the MHC class I pathway. To circumvent this problem, chimeric molecules, which we call here “Trojan Ags” (TA) have been designed to deliver exogenous noninfectious material containing CTL epitopes into the cytoplasm of APC (12, 13). These TA are composed of CTL epitopes, which are linked to membrane-translocating Trojan peptide causing these molecules across the plasma membrane into the cytoplasm (14–17). The two most commonly used carriers are both highly positively charged sequences derived from the HIVtat Ag or the Antennapedia homeodomain (AntpHD) protein. It has been reported that TA are indeed capable of delivering CTL epitopes into the MHC class I pathway, enabling the efficient recognition of APC by CTL (12, 13). These studies assumed that once in the cytoplasm, the TA are processed and transported into the ER to generate the corresponding CTL epitopes. In this study, we report that TA are able to generate CTL epitopes through a TAP-independent mechanism because they have the capacity to translocate into the ER and trans-Golgi compartments, where they undergo processing. Our results show that both TAP-competent and TAP-deficient cells are equally effective in generating peptide-MHC class I complexes derived from various TA. The present findings agree with previous reports that CTL peptide epitopes can be trimmed in the ER by an aminopeptidase (6, 7, 11) or, in other cases, by furin in the trans-Golgi network (18). We believe that these findings could be of relevance for the design of CTL-inducing vaccines for the treatment or prevention of infectious and malignant diseases.

Materials and Methods

Peptide synthesis

Synthetic TA were prepared by producing synthetic peptides containing the minimal CTL epitope joined to the HIVtat protein transduction domain (RKKRRQRRRR) using a triple-alanine spacer. All synthetic peptides were synthesized according to standard solid-phase synthesis methods using an Applied Biosystems (Foster City, CA) apparatus and were purified by HPLC. The purity (>95%) and identity of peptides were determined by analytical HPLC and mass spectrometry analysis. Peptides were dissolved at 10 mg/ml in DMSO containing 0.1% trifluoroacetyl or trifluoroacetic acid and were aliquoted in small volumes to be maintained frozen at −20°C until further use.

Recombinant TA

A recombinant DNA-derived TA containing the melanoma gp100 epitope IMDQVPSV, linked to the AntpHD translocating sequence RQIKIVFNPERRMKWK, was designed following a similar approach as described by Shutze-Redelmeier et al. (12). The CTL epitope was flanked with influenza nucleoprotein (NP) sequences, at the N terminus (AEIDL) and at the C terminus (LRTED), to facilitate the correct processing. To produce the final product (RQIKIFNPERRMKWKAEIDLIMDQVPSVLRTEDE), two synthetic oligodeoxynucleotide (ODN) primers were prepared: primer A, GAC

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‡ Abbreviations used in this paper: ER, endoplasmic reticulum; TA, Trojan Ag; Antennapedia homeodomain; NP, nucleoprotein; ODN, oligodeoxynucleotide; CEA, carcinoembryonic Ag; BFA, brefeldin A.
GAGCACAAGATGCGTGCAGATCAAGTCTGGTTCCGAGCGGCTGT
ATGAAATGCGAAAGGGAGAATGCTGGCTGTTG

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Column with an acetonitrile/0.1% trifluoroacetic acid gradient. Purity was determined in a standard 4–6-h 51 Cr release cytotoxicity assay mediated by untreated EL-4 T2/Kb cells and TAP-competent EL-4 mouse cell line transfected with the mouse H-2K b allele (19), and the TAP-deficient EL-4 H-2K b−/− (H-2b) and TAP-deficient T2 cells, transfected with the mouse H-2K b allele (20) human cell line were used as targets for the HLA-A2-restricted human CTL lines. The TAP-competent EL-4 mouse cell line (H-2b) and TAP-deficient T2 cells, transfected with the mouse HLA-A2 (19) and TAP-deficient HLA-A2-restricted human CTL lines. The TA peptide was purified by HPLC on a C4 reverse-phase column with an acetonitrile/0.1% trifluoroacetic acid gradient. Purity was determined by analytical HPLC and mass spectroscopy.

Cell lines

The TAP-competent 221.A2 cell line, which was derived from a MHCI class I-deficient cell line transfected with HLA-A2 (19), and the TAP-deficient T2 (20) human cell line were used as targets for the HLA-A2-restricted human CTL lines. The TAP-competent EL-4 mouse cell line (H-2b) and TAP-deficient T2 cells, transfected with the mouse H-2K b−/− (T2/Kb) were used as APC for those studies using the immunodominant H-2K b-restricted H-2K b-restricted HLA-A2-restricted CTL epitope for CEA (Table I). This construct, called OVA257-HIVtat (Table I), was compared with the minimal CTL epitope, CEA691 for their ability to sensitize TAP-deficient HLA-A2−/− targets for CTL lysis. In the present study, we first evaluated a TA composed of an HLA-A2-restricted chimera Biotech, Piscataway, NJ) for 1–2 h at 37°C. Various numbers of effectors were mixed with 2 × 10^4 labeled Targets in 96-well round-bottom plates in a final volume of 0.2 ml of complete medium. After 4–6 h incubation at 37°C, 30 µl of supernatant was collected from each well and the percentage of specific lysis was determined according to the formula: [(cpm of spontaneous release – cpm of spontaneous release)] × 100. The inhibitory effect of BFA was determined by preincubating the target cells with 1 µg/ml BFA for 1 h before peptide pulsing and during peptide incubation and then performing the cytotoxicity assay in the presence of 0.5 µg/ml BFA. Flow cytometry

The effects of protease inhibitors on Ag processing and presentation were evaluated by analysis of 25D1.16 staining on different Ag-pulsed EL-4 or T2/Kb cells using flow cytometric analysis. In brief, EL-4 or T2/Kb cells were harvested from OT-1 TCR-transgenic mice and restimulated in vitro with irradiated peptide-pulsed APC for 7 days before being used as effectors for the corresponding protease inhibitors. To exclude the influence of the protease component in serum, we used serum-free AIM-V medium during the peptide-pulsing process. After washing the cells twice with FACS buffer (PBS supplemented with 2% FCS and 0.02% sodium azide), peptide-loaded APC were stained with 25D1.16 Ab followed by FITC-labeled goat anti-mouse IgG for flow analysis. Untreated EL-4 and T2/Kb cells were pulsed with peptides and stained simultaneously as controls.

Immunofluorescence and confocal microscopy

T2/Kb or L-2/Kb cells were cultured overnight on polylysine-treated glass coverslips. The culture medium was discarded, and the cells were gently washed once with PBS. The cells were preincubated with serum-free AIM-V medium at 37°C for 30 min and then incubated with peptide-TA solutions at the appropriate concentrations for 12 h at 37°C. After peptide loading, cells were then washed and fixed with 2% formaldehyde for 20 min at room temperature. For indirect immunofluorescence microscopy, fixed cell monolayers were permeabilized with 0.2% Triton X-100 in PBS and incubated with 5% goat serum to block nonspecific protein binding. The cells were double-labeled with mouse mAb 25D1.16 specific to the OVA257-HIVtat complex and rabbit polyclonal Ab TGN38 (TGN marker) or rabbit anti-calreticulin (ER marker), followed by a 1-h staining with FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG as secondary Abs. After washing, the coverslips were mounted on glass slides using the Prolong antifade kit (Molecular Probes). The stained cells were examined and photographed in a Leica confocal laser-scanning microscope (Leica, Deerfield, IL).

Results

TA sensitizes TAP-deficient as well as TAP-functional targets

It has been reported that peptides or proteins containing CTL epitopes can be delivered into the cytoplasm of APC by linking these molecules with membrane-translocating proteins such as HIVtat or AntpHD. Once in the cytoplasm, these fusion peptides, which we call TA, are processed via the MHC class I pathway generating MHC-peptide complexes that are recognized by CTL. In the present study, we first evaluated a TA composed of an HLA-A2-restricted CTL epitope for CEA that we linked via its carboxyl-terminal end to the active portion of the membrane-translocating region of HIVtat (RKKRRQRRR). This construct, called CEA691-HIVtat (Table I), was compared with the minimal CTL epitope, CEA691, for its capacity to sensitize HLA-A2+ target cells for lysis by a human CTL clone that recognizes this epitope. The data shown in Fig. 1 indicate that peptides CEA691-HIVtat and CEA691 were equally effective in sensitizing the targets for CTL lysis. In theory, peptide CEA691 is likely to bind directly to the surface HLA-A2 molecules that are temporarily empty or to HLA-A2 molecules by displacing low-affinity binding peptide ligands. In contrast, we assumed that peptide CEA691-HIVtat would not bind to surface HLA-A2 molecules but needed to enter the MHC class I-processing pathway, which could involve some proteolysis in the cytoplasm, followed by transport of peptides into the ER by TAP.
not identical, activities (Fig. 1B). Furthermore, Ag dose responses using the TAP-deficient T2 cells as targets indicate that CEA691-HIVtat and CEA691 were equally effective in generating the CTL epitope (Fig. 1C).

**Target cell sensitization by TA require intracellular loading**

The ability of peptide CEA691-HIVtat to sensitize TAP-deficient T2 cells for CTL lysis could be explained if this peptide was able to translocate into the ER across intracellular membranes using the HIVtat carrier, where it would be processed into the optimal CTL epitope. Alternatively, it is also possible that peptide CEA691-HIVtat could be degraded extracellularly by serum or cell-derived proteases, generating the CEA691 minimal epitope that would then bind to surface HLA-A2 molecules. To explore the latter possibility, we tested the capacity of an irrelevant HLA-A2 binding peptide, HBC18-27, to block the binding of peptide CEA691 and the possible proteolytic fragment of CEA691-HIVtat to surface HLA-A2 molecules. As shown in Fig. 2A, peptide HBC18-27 was quite effective in inhibiting the sensitization of target cells for CTL lysis by peptide CEA691, but not the sensitization by the CEA691-HIVtat construct. Another approach to evaluate whether peptide CEA691-HIVtat must penetrate into the cell to generate the CTL epitope was to treat the peptide with trypsin, which cleaves at the carboxyl end of R or K residues, to destroy (or decrease) the carrier epitope. Alternatively, it is also possible that peptide CEA691-HIVtat carrier, where it would be processed into the optimal CTL epitope (Fig. 1C).

**Ag processing and trimming of TA**

To extend these observations to another CTL epitope and to a different carrier sequence, we prepared a TA construct containing the HLA-A2-restricted melanoma CTL epitope gp100209, which was attached to the active carrier portion of AntpHD (Table I). Furthermore, this construct was produced via a recombinant DNA

![FIGURE 1. TA sensitization of TAP-competent and TAP-deficient target cells for CTL lysis. The TAP-competent 221-A2 (A) or the TAP-deficient T2 cells (B) were incubated overnight with 10 μM of peptide CEA691 (○), construct CEA691-HIVtat (○), or medium alone (○). Next day the cells were washed and tested as targets in a 4-h 51Cr release cytotoxicity assay against a human CEA-specific CTL line. C. Curve-dose responses of peptide CEA691 (○) and construct CEA691-HIVtat (△) using TAP-deficient T2 cells. Results correspond to the means of triplicate samples where the SD of the means were consistently below 10% of the value of the mean.](http://www.jimmunol.org/)

### Table I. List of CTL epitope peptides and TA

<table>
<thead>
<tr>
<th>Ag</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA691</td>
<td>IMIGVLGVV</td>
</tr>
<tr>
<td>CEA691-HIVtat</td>
<td>RKKRRQRRRAAAIMIGVLGVV</td>
</tr>
<tr>
<td>HIIVtat-CEA691</td>
<td>IMIGVLGVAARAKRQRQRRAA</td>
</tr>
<tr>
<td>CEA691-KKK</td>
<td>IMIGVLGVKKK</td>
</tr>
<tr>
<td>CEA691-RRR</td>
<td>IMIGVLGVRRR</td>
</tr>
<tr>
<td>CEA691-RKK</td>
<td>IMIGVLGVRRR</td>
</tr>
<tr>
<td>OVA257</td>
<td>SIINFEKL</td>
</tr>
<tr>
<td>OVA257-HIVtat</td>
<td>SIINFEKLAAARAKRQRQRRAA</td>
</tr>
<tr>
<td>HIIVtat-OVA257</td>
<td>RKKRRQRRRAASIIINFEKL</td>
</tr>
<tr>
<td>gp100209</td>
<td>RQIKIWFNRMKWKKAEIDL</td>
</tr>
<tr>
<td>AntpHD-gp100209</td>
<td>FLPSDYFFSY</td>
</tr>
</tbody>
</table>

*Underlined residues correspond to the minimal CTL epitope.*
method instead of making a synthetic peptide (see Materials and Methods for details). The resulting TA (AntpHD-gp100209) also contained flanking sequences of influenza NP to facilitate Ag processing as suggested by Schutze-Redelmeier et al. (12). We observed that both TAP-competent (Fig. 3A) and TAP-deficient targets (Fig. 3B) were efficiently sensitized by peptide AntpHD-gp100-209. Moreover, dose-curve responses using T2 cells as targets also indicated that construct AntpHD-gp100-209 and peptide gp100209 had similar activities (Fig. 3C). These results illustrate that different types of TA can be quite effective in generating CTL epitopes in a TAP-independent manner.

As mentioned above, the capacity of TA to generate peptide-MHC complexes, which are recognized by CTL, in a TAP-independent manner, may be via the delivery of epitopes directly into the ER. Once in the ER, the TA may be trimmed allowing the formation of peptide-MHC complexes that would be exported via the Golgi to the cell surface. If this were to be the case, the process should be blocked by BFA, which inhibits the transport of products from the ER to the Golgi (22). The results in Fig. 4 show that indeed BFA is effective in blocking the sensitization of TAP-deficient T2 cells by HIVtat-CEA691 and AntpHD-gp100-209, but not by the minimal epitopes CEA691 and gp100209. However, less inhibition was observed at high concentrations of TA, indicating that these constructs may also deliver CTL epitopes to other cellular compartments where MHC-peptide complexes can be formed. Alternatively, the high concentration of TA peptide used may far exceed the inhibitory action of BFA in our system. The inhibitory effects of BFA were also studied with TA containing the immunodominant mouse H-2Kb-restricted CTL epitope, SIINFEKL, which is derived from OVA. For these experiments, this peptide epitope (OVA257) was linked to the HIVtat carrier either at the carboxyl- or amino-terminal end creating the OVA257-HIVtat and HIVtat-OVA257 constructs, respectively (Table I). As with the TA containing the human CTL epitopes, BFA treatment of APC resulted in the reduction of peptide-MHC complexes produced by the OVA257-HIVtat and HIVtat-OVA257 constructs, but not by the OVA257 peptide (data not shown).

Intracellular localization of TA and corresponding peptide-MHC complexes

The experiments presented so far indicate that TA may have the property of penetrating into intracellular compartments where they can be processed to generate peptide-MHC complexes corresponding to CTL epitopes. To assess the formation of these complexes intracellularly, we used the TA containing the OVA257 CTL epitope because of the availability of mAb 25D1.16, which specifically reacts with H-2Kb-OVA257 complexes. Constructs

### FIGURE 2. Requirement for intracellular loading of TA for CTL sensitization by TA. A. The HLA-A2 binding peptide HBc18-27 was tested for its capacity to compete for the binding of peptide CEA691 or construct CEA691-HIVtat to surface HLA-A2 molecules of T2 TAP-deficient cells. Radiolabeled T2 cells were preincubated with various amounts of HBc18-27 peptide for 30 min then pulsed with 1 μM CEA691 or CEA691-HIVtat, still in the presence of HBc18-27, for an additional 30 min. Target cells were washed, and cytotoxicity by a CEA-specific CTL line was determined at an E:T ratio of 20:1 in a 4-h assay (similar results have been obtained with TAP-competent target cells). B. Proteolysis by trypsin destroys the activity of TA to sensitize target cells for CTL lysis. Peptide CEA691 and construct CEA691-HIVtat (both at 1 μM) were incubated with 50 mg/ml trypsin or medium alone at 37°C for 4 h before sensitizing T2 target cells for CTL lysis. Cytotoxicity was determined as described in A. C. Target cell sensitization by various peptides and TA. TAP-deficient T2 cells were incubated overnight with 10 μM CEA691-HIVtat (●), HIVtat-CEA691 (○), and extended peptides CEA691-KK (▲), CEA691-RRR (▼), and CEA691-RKK (●) and the following day were used as targets for lysis by CEA-specific CTL. Cytotoxicity was determined as described in A. Results correspond to the means of triplicate samples, and the SD of the means were consistently below 10% of the value of the mean.

### FIGURE 3. Recombinant TA protein containing the AntpHD carrier is effective in sensitizing targets for CTL lysis. The recombinant DNA-derived TA, AntpHD-gp100209, was tested for its ability to sensitize TAP-competent 221-A2 cells (A) or TAP-deficient T2 cells (B) for lysis by a melanoma gp100-specific CTL clone. Target cells were incubated overnight with 10 μM of AntpHD-gp100209 (○), gp100209 (●), or no peptide (▲), followed by a 4-hr 51Cr release assay at various E:T ratios. C. Curve-dose responses of peptide gp100209 (□) and construct AntpHD-gp100209 (○) using TAP-deficient T2 cells. Results correspond to the means of triplicate samples, and the SD of the means were consistently below 10% of the value of the mean.
OVA<sub>257</sub>-HIVtat and HIVtat-OVA<sub>257</sub> were first compared with the OVA<sub>257</sub> minimal epitope (SIINFEKL) for their capacity to sensitize TAP-competent and TAP-deficient target cells for CTL lysis. The peptide titration curves presented in Fig. 5 indicate that both these TA were effective, and even 10- to 1000-fold more potent than OVA257, in sensitizing either cell type for lysis by Ag-specific CTL.

Using the mAb 25D1.16, which reacts with H-2K<sup>b</sup>-OVA<sub>257</sub> complexes, we proceeded to evaluate, using confocal microscopy, the formation and presence of this CTL epitope in permeabilized TAP-competent and TAP-deficient cells expressing H-2K<sup>b</sup>. The photomicrographs presented in Fig. 6 indicate that H-2K<sup>b</sup>-OVA<sub>257</sub> complexes (green) were abundant in TAP-competent cells that were incubated with either HIVtat-OVA<sub>257</sub> (Fig. 6A) or OVA<sub>257</sub> (Fig. 6D). These cells were also stained with an Ab specific for calreticulin (red), which is localized within the ER (Fig. 6, B and E). After superimposing the corresponding images, it is apparent that abundant H-2K<sup>b</sup>-OVA<sub>257</sub> complexes were found within the ER (bright yellow) in the cells that were incubated with peptide HIVtat-OVA<sub>257</sub> (Fig. 6C), but not in those cells incubated with OVA<sub>257</sub> (Fig. 6F). In experiments using TAP-deficient cells and observations using TA peptide, OVA<sub>257</sub>-HIVtat gave similar results (data not shown).

Next, we used an Ab specific for the trans-Golgi network (TGN38) to evaluate whether H-2K<sup>b</sup>-OVA<sub>257</sub> complexes could also be detected in this cell compartment of cells incubated with TA peptides. As shown in Fig. 7, high amounts of intracellular H-2K<sup>b</sup>-OVA<sub>257</sub> complexes were evident in TAP-deficient cells (T2/K<sup>b</sup>) that were incubated with OVA<sub>257</sub>-HIVtat (Fig. 7A). In these cells, Ab TGN38 stained brightly (red), a structure corresponding to the trans-Golgi (Fig. 7B). By superimposing these images, it is evident that the majority of these complexes colocalized within the trans-Golgi (Fig. 7C). In contrast, no colocalization of H-2K<sup>b</sup>-OVA<sub>257</sub> complexes could be observed in T2/K<sup>b</sup> cells that were treated with peptide OVA<sub>257</sub> (Fig. 7F). Furthermore, it is apparent that peptide OVA<sub>257</sub> mostly produced surface H-2K<sup>b</sup>-OVA<sub>257</sub> complexes under these experimental conditions (Fig. 7D). These observations reinforce the findings that TA peptides need to penetrate into the cells to create the MHC class I-peptide complexes that are observed in both the ER and trans-Golgi, even in TAP-deficient cells.

Effects of protease inhibitors in the processing and presentation of TA

So far, our results suggest that TA may be processed in various cellular compartments to produce smaller peptides, which then associate with MHC class I molecules that are subsequently exported to the cell surface. Ag processing may take place: 1) in the cytoplasm, via proteasomal degradation followed by TAP transport; 2) within the ER, where peptide trimming via an aminopeptidase could occur; 3) in the trans-Golgi, where processing of Ags through proteases such as furin has been reported; and/or 4) in early endocytic compartments, where peptides could be generated that could either bind to recycling MHC class I molecules or leak into other compartments of the MHC class I pathway. Using Ab 25D1.16, we studied the effects of various inhibitors of Ag processing on the formation of H-2K<sup>b</sup>-OVA<sub>257</sub> surface complexes by peptides OVA<sub>257</sub>-HIVtat and HIVtat-OVA<sub>257</sub>. As shown in Fig. 8, inhibition of proteasome activity using lactacystin (10 μM, the
highest concentration used without largely compromising the viability of the cells) did not have an effect on the amount of surface H-2Kb-OVA257 complexes expressed on TAP-competent EL-4 cells (Fig. 8, A and B). Similarly, this proteosomal inhibitor did not have an appreciable effect in the expression of these complexes induced by peptide HIVtat-OVA257 on TAP-deficient cells (Fig. 8D). Interestingly, lactacystin appeared to increase the level of peptide-MHC complexes resulting from the processing of the OVA257-HIVtat construct in TAP-deficient cells ~3-fold (Fig. 8C). These results suggest that a great proportion of the processing of these TA does not appear to take place in the cytoplasm, even in TAP-competent cells.

Next, we studied the effect of the endosomal aspartyl-protease inhibitor pepstatin A on the generation of H-2Kb-OVA257 complexes in TAP-competent and TAP-deficient cells. Interestingly, this inhibitor increased the amounts of surface peptide-MHC complexes resulting from the processing of the OVA257-HIVtat construct in TAP-deficient cells 3- to 5-fold (Fig. 8C). These results suggest that a great proportion of the processing of these TA does not appear to take place in the cytoplasm, even in TAP-competent cells.

It has been reported that some Ags can be processed in the trans-Golgi by the endopeptidase action of furin, or other similar enzymes, which cut at the C-terminal side of dibasic amino acid...
FIGURE 8. Effect of the proteasomal inhibitor lactacystin on the processing of TA by APC. Constructs OVA257-HIVtat (A and C) or HIVtat-OVA257 (B and D) were tested for their capacity to generate H-2Kb-OVA257 complexes in TAP-competent EL-4 cells (A and B) or TAP-deficient T2/Kb cells (C and D). APC were treated with lactacystin (dark lines) or not (gray shaded area). The isotype control is indicated by a thin line. APC were pretreated with 10 μM lactacystin for 1 h and then incubated with the TA overnight at 37°C in presence of the drug. The expression of H-2Kb-OVA257 complexes on the cell surface was analyzed by flow cytometry using the specific Ab 25D1.16.

FIGURE 9. Effect of endosomal protease inhibitor pepstatin on the processing of TA. Constructs OVA257-HIVtat (A and C) or HIVtat-OVA257 (B and D) were tested for their capacity to generate H-2Kb-OVA257 complexes in TAP-competent EL-4 cells (A and B) or TAP-deficient T2/Kb cells (C and D). APC were treated with pepstatin (dark lines) or not (gray shaded area). The isotype control is indicated by a thin line. APC were pretreated with 100 μM pepstatin for 1 h and then incubated with the TA overnight at 37°C in presence of the drug. The expression of H-2Kb-OVA257 complexes on the cell surface was analyzed by flow cytometry using the specific Ab 25D1.16.

Discussion

Because CTL play such a critical role in the control of intracellular pathogens and possibly in some malignancies, it is important to understand the mechanisms involved in the generation of peptide-MHC class I complexes. It is clear that this knowledge would be of value for the design of effective vaccination strategies aimed at eliciting CTL responses to treat or prevent infectious or malignant diseases. It is generally accepted that the majority of peptide-MHC class I complexes are derived from proteins that are degraded in the cytoplasm by proteasomes, resulting in peptides that are then transported into the ER by the TAP complex. Once in the ER, such peptides may be further processed into the optimal size (8–10 residues) required for binding to empty MHC class I molecules, which are then exported to the cell surface for interaction with CTL receptors. Thus, the formation of CTL epitopes from a particular Ag necessitates that the Ag somehow gains access to the cytoplasm for proteasomal degradation. Although proteins that are synthesized within the cell have easy access to proteasomes, most exogenously acquired proteins do not. Vaccines composed of proteins, which are in general effective in eliciting strong Ab and Th responses, are usually poor at inducing CTL. Thus, vaccines that...
are intended to trigger CTL responses are generally made of entities that mediate the intracellular synthesis of the Ag (e.g., infectious agents or DNA plasmids) or alternatively, small peptides that do not require extensive processing and may bind to surface MHC molecules.

TA have been reported to deliver T cell epitopes into the interior of APC, resulting in the formation of surface peptide-MHC class I complexes that are recognized by CTL (12, 13). Although the exact mechanism of how these proteins can translocate across the plasma membrane into the cytoplasm remains a mystery, it is clear that these molecules are internalized in a receptor- and energy-independent fashion. We have observed that target cells can be sensitized for CTL recognition when they are pulsed with TA for a short time in the cold (data not shown). These findings suggest that TA do not require an active process such as receptor-mediated endocytosis or pinocytosis to penetrate into the cells. Notwithstanding, it has been reported that, in some circumstances, exogenous peptides can be delivered via pinocytosis into the ER, where they can then bind to class I MHC molecules (23). We cannot eliminate the possibility that under physiological conditions (at 37°C) some of the TA molecules will be endocytosed and may end up in the ER following this pathway. Nevertheless, our results indicate that once inside of the cell, TA can translocate across organelle membranes into the ER and possibly into the trans-Golgi network, gaining direct access to the Ag-processing pathway in the secretory compartments. Our results show that the proteasomal inhibitor, lactacystin, was not able to inhibit the generation of surface peptide-MHC complexes by TA in TAP-competent cells (Fig. 8). Thus, it is possible that our TA, even though they are relatively long (20–35 residues) can be transported into the ER by TA without proteasomal degradation (24). However, this possibility is difficult to imagine in our system because the TA were highly effective in generating peptide-MHC complexes on TAP-deficient cells. Although lactacystin did not have an effect in TAP-competent cells, it appeared to slightly increase (~3-fold) the amounts of peptide-MHC complexes produced by TA in TAP-deficient cells (Fig. 8C). It is possible that lactacystin could be preventing some degradation of the TA in the cytoplasm, increasing their TAP-independent translocation into the secretory compartments for subsequent Ag processing. This effect could also be due in part to the fact that proteasome inhibitors such as lactacystin also block the translocation of proteins from the ER to the cytosol (25). Notwithstanding, the enhancement induced by lactacystin was not evident in TAP-competent cells, possibly because the proteasomal degradation products in these cells would be transported by TAP into the ER.

Others have reported that a TA prepared by chemically conjugating HIVtat synthetic peptide to OVA protein was effective in the generation of the OVA257 CTL epitope (13). However, in contrast to our findings, this OVA protein-HIVtat construct required the function of TAP because RMA-S cells (a TAP-deficient cell line) did not generate the H-2Kb-ova257 CTL epitope (13). It is unknown whether the OVA protein-HIVtat chemical conjugate was capable or not of translocating into the secretory pathway, but in either case it is apparent that CTL epitopes were not generated in these cell compartments. Therefore, it seems likely that the complex nature of the OVA protein-HIVtat construct required proteasomal degradation in the cytoplasm and TAP functionality to produce the CTL epitopes.

In a second report, several TA were prepared as recombinant proteins by linking the carboxyl-terminal end of the 60-aa AntpHD protein to another mouse CTL epitope (12). These studies concluded that the flanking regions of the peptide epitope are critical for the generation of the appropriate MHC binding peptides, but the role of TAP and proteasomal degradation was not addressed. For our studies we prepared a recombinant construct similar to the ones produced by this group, except that we only used 15 of the 60 residues of AntpHD, which are the ones responsible for the membrane translocation function of this protein (16). In addition, as suggested by Schutze-Redelmeier et al. (12), we flanked the peptide epitope at both ends with influenza virus NP residues. Our results show that the recombinant AntpHD-gp100209 construct, containing the same linkers described by this group, was effective in generating CTL epitopes (Fig. 3). However, for all the synthetic peptide-derived TA described here, we joined the CTL peptide sequences at either side with the HIVtat carrier, simply by using triple-alanine linkers (Table I) and all of these constructs were effective in generating the corresponding CTL epitopes. It is possible that flanking regions of CTL epitopes may be important for proteasomal processing, but these may not be that critical for Ag processing in the secretory compartments, where terminal-end peptides may play the principal role. Because AntpHD-gp100209 was active in both TAP-competent and TAP-deficient cells, it is evident that this particular construct will require the activity of both amino and carboxyl peptides to produce the appropriate CTL epitope.

In addition to amino and carboxyl peptides, our results show that when the CTL peptide is placed at the amino-terminal end of the TA carrier (HIVtat), the trans-Golgi-resident protease furin (or a similar endopeptidase) plays an important role in liberating the CTL epitope. Furin, which recognizes the RX(R/K)R motif (26), has been reported to participate in the TAP-independent processing of CTL epitopes in the secretory pathway. Using recombinant vaccinia virus, a CTL peptide sequence was inserted into the sequence of the secreted form of hepatitas B viral eAg, which is targeted to the ER (18). In these experiments, the furin inhibitor decRVKR-CMK blocked the production of the corresponding CTL epitope after infection with the recombinant virus. In addition to furin, both amino and carboxyl peptides were probably involved in the processing of this CTL epitope, because a five-alanine flanking region was placed at both ends of the epitope sequence (18). Our findings corroborate that peptide trimming can take place at both the amino- and the carboxyl-terminal ends in the secretory compartments allowing the generation of MHC binding peptides. The activity of ER-resident aminopeptidase(s) in Ag processing has been well studied by several groups (6, 7, 9, 27, 28), resulting in the “COOH-end rule,” which states that the ER has a high capacity to remove NH2-terminal residues from signal sequence peptides, liberating CTL epitopes found in the COOH end (29). In contrast, little information exists regarding the existence and function of a carboxypeptidase in the secretory compartments that may participate in Ag processing. Our results and those of Gil-Torregrosa et al. (18) indicate that peptide trimming at the carboxyl-terminal end probably takes place in the trans-Golgi. It is possible that one or more of the various membrane carboxypeptidases that reside in the trans-Golgi may function in the generation of MHC binding peptides after processing by furin. We do not know whether the peptides generated in the trans-Golgi by the combined action of furin and carboxypeptidases will bind to MHC molecules in the trans-Golgi or alternatively may travel in a retrograde fashion into the ER to bind to nascent MHC molecules. We are currently exploring this possibility.

The ability to deliver CTL peptide epitopes to secretory compartments (ER and trans-Golgi) may be advantageous for generating high numbers of peptide-MHC class I complexes. Accordingly, peptide sequences corresponding to CTL epitopes have been placed in the NH2-terminal signal sequences to translocate these products into the ER (6, 27, 30, 31). Another approach to deliver
exogenous proteins directly into the MHC class I Ag-processing pathway has been to prepare recombinant fusion products of bacterial toxins with CTL epitopes (32–38). However, these constructs do not translocate across the plasma membrane but gain intracellular access via surface receptors and travel in a retrograde manner into the secretory compartments, where apparently they are processed and CTL epitopes can be generated. As with TA, in some cases the bacterial toxin constructs do not require the participation of proteasomes or TAP to produce MHC class I binding peptides (32, 33). Although large numbers of specific peptide-MHC class I complexes can be generated using these methods, there are potential drawbacks for their use in vaccine development. The approach using signal sequences requires either the use of infectious agents or gene transduction techniques because these proteins have to be synthesized within the cell. It is likely that this method and the use of bacterial toxin constructs will introduce highly infectious agents or gene transduction techniques because these antigens expressed on solid epithelial tumors. As seen in TA, antigen processing can take place in the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. J. Immunother. 21:127.


