Extracellu lar Processing of Peptide Antigens That Bind Class I Major Histocompatibility Molecules

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Summary

One problem associated with the use of synthetic peptides as antigens in vivo is their susceptibility to inactivation by proteolytic degradation. A situation is described in which a serum protease, angiotensin-converting enzyme (ACE), is actually responsible for the class I binding activity of a commonly used influenza antigen, nucleoprotein (NP) (147-158R-). This peptide has been reported to be a highly efficient class I antigen. Evidence is presented that demonstrates that the peptide is inactive until cleaved by ACE, which is a normal constituent of serum. The enzyme removes a COOH-terminal dipeptide resulting in the sequence NP(147-155), which is identical to the naturally processed peptide. Such extracellular processing of peptides and proteins may occur for a variety of antigens both in vitro and in vivo, and could have important implications for the design of proteolytically resistant vaccines.

Materials and Methods

Animals. Mice used as a source of APC for stimulation of CTL clones included C57BL/6 and B10.D2, both of which were obtained from the breeding colony of The Scripps Research Institute.

Cell Lines. Cell lines used as targets for 51Cr release assays included EL4, P815, or 439.4.2 (13). Cells were maintained in RPMI 1640 supplemented with 5 × 10^{-5} M, 2-ME, and 2 mM L-glutamine (culture media), and either 10% FCS (HyClone Laboratories, Logan, UT) or 1% serum-free (SF) media (Nutridoma; Boehringer Mannheim, Indianapolis, IN).

CTL Clones. CTL lines and clones used in these studies include clone 9 specific for NP(147-158) (13); clone 34 specific for NP(365-380) (13); the OVA (253-276)-specific clone GA4 (18), and a VSV-N(47-63)-specific line (18). These were maintained by weekly stimulation with irradiated syngeneic spleen cells that had been pulse with the relevant peptide antigens as described previously (13, 18). Culture media was supplemented with 10% rat Con A supernatant and 10% FCS.

CTL Assays. Cytotoxicity was assessed in a standard 51Cr release assay. Target cells (10⁶) were labeled in 0.15 mCi Na ⁵¹Cr and incubated in RPMI 1640 supplemented with 5 × 10^{-5} M, 2-ME, and 2 mM L-glutamine for 3 h. After washing free of unbound ⁵¹Cr and peptide, labeled target cells (10⁶) plus the indicated number of CTL effectors were incubated at 37°C in round-bottomed microtiter plates containing 0.2 ml media (HyClone Laboratories, Logan, UT) or 1% serum-free (SF) media (Nutridoma; Boehringer Mannheim, Indianapolis, IN).
followed by Affi-Gel blue (Bio-Rad Laboratories, Richmond, CA). 0.4 ml of sera was diluted fourfold in 10 mM phosphate buffer, pH 7.5, and applied to a Mono-Q HR5/5 column (Pharmacia/LKB, Alameda, CA). Protein was eluted with a continuous gradient of 0–0.5 M NaCl in this same buffer over a period of 50 min at a flow rate of 1.0 ml/min.

5 μl of each column fraction was assayed by incubation for 1 h at 37°C with 106 51Cr-labeled P815 (H-2b) tumor cells in 15 μl SF media containing 1 μg/ml NP(147–158R-^-) before addition of clone 9 effector cells in SF media (final volume 0.2 ml). Incubation was continued for an additional 4 h and lytic activity assessed.

**HPLC Analysis of Peptides.** 50 μg of the NP(147–158R-^-) synthetic peptide purified by reverse phase HPLC using a Brownlee Aquapore RP300 column (Applied Biosystems, Inc.) was incubated with 100 μl of material obtained by further purification of Mono Q fractions 18–23 (see Fig. 2) by gel filtration chromatography on Toyo Soda TSK-3000SW (Beckman Instruments, Inc., Palo Alto, CA). After 3 h at 37°C, the low molecular weight material was recovered by spinning through a centricron 30 filter (Amicon Corp., Danvers, MA), and analyzed on a Brownlee Aquapore RP300 column, equilibrated with 0.1% TFA developed with a continuous gradient of 0–40% acetonitrile containing 0.08% TFA over a period of 20 min followed by 40–80% over the next 7.5 min with a flow rate of 300 μl/min. Amino acid analysis was performed on the indicated peaks. The hydrolyzed samples were analyzed using a high performance analyzer (6300; Beckman Instruments, Inc., Palo Alto, CA). Based on this composition analysis, the molar ratio of material in peaks I, II, and III in Fig. 3 was approximately 6:1:1, respectively, and therefore, the absorbance at 215 nm of peak III does not reflect the relative concentration of the dipeptide.

**Inhibition of Serum Activity or Purified Angiotensin-converting Enzyme (ACE) with ACE Inhibitor or Anti-ACE.** ACE (EC 3.4.15.2 peptidyl/dipeptide hydrolase) from rabbit lung was purchased from Sigma Chemical Co. (St. Louis, MO), as was ACE inhibitor. An mAb specific for human ACE was obtained from Dr. M. Rohrbach (Mayo Clinic, Rochester, MN) and used as culture supernatant on this antibody is known to inhibit the activity of human ACE.

Assays using ACE inhibitor were performed as follows. The indicated concentration of rabbit ACE or a pool of fractions 18–23 from the Mono Q-purified human serum was incubated in 15 μl SF media containing 0.8 μg/ml of HPLC-purified NP(147–158R-^-), 70 μg/ml ACE inhibitor, and 106 51Cr-labeled P815 target cells in wells of a U-bottomed microtiter plate. After incubation for 1 h at 37°C, 105 effector cells (clone 9) were added in a volume of 185 μl SF media, and incubation was continued for 4 h.

In assays using anti-ACE, the serum fraction and the indicated mAb were incubated for 40 min at 37°C before addition of target cells and peptide. mAbs were 10 μl of culture supernatant prepared by growing the indicated hybridoma (either the anti-ACE line 124.10 [20] or the anti-K^d^ monoclonal Y3 [13]) in SF media to avoid contamination by ACE from serum.

**Results and Discussion**

It was previously reported by this laboratory (13, 17) and others (14–16) that class I molecules on cells grown and pulsed with peptide in SF media bind peptide poorly as compared with cells pulsed in media supplemented with FCS, and that the efficiency of peptide binding in SF media could be increased significantly by the addition of the serum component β2m. This was observed to be true for each of four different peptides examined, including influenza NP(365–380), NP(147–158R-^-), OVA (253–276), and a peptide from the vesicular stomatitis virus nucleoprotein, VSV-N (47–63) (Fig. 1). It was therefore anticipated that the component in serum that enhanced peptide binding to class I was β2m. To test this hypothesis, human serum was depleted of β2m by affinity chromatography and then tested for its effect on peptide binding. As anticipated, the amount of peptide in the β2m-depleted serum was similar to that obtained in SF media for VSV-N (47–63), and OVA (253–276), thus confirming that β2m was required for optimal peptide binding (Fig. 1). In contrast, removal of β2m did not reduce the ability of serum to facilitate class I binding of either NP(365–380) or NP(147–158R-^-) (Fig. 1).

Considering the β2m-depleted serum retained ~5% of its β2m, it was possible this residual material could foster binding by some peptides. We therefore obtained sera from transgenic mice that have disrupted β2m genes and thus
peptide was incubated with the partially purified serum molecule or the cell. One type of mechanism that could explain these results would be cleavage of the relevant peptide into a form with higher affinity for class I or that was recognized more efficiently by the TCR. To test this possibility, peptide was incubated with the partially purified serum activity and the peptide separated from high molecular weight material by centrifugation through a filter that excludes material with a molecular weight >30,000, and thus would exclude the serum component. When compared with the untreated peptide, an equal volume of the recovered peptide was >100-fold more efficient in sensitizing cells for lysis, suggesting that the serum activity had in some way altered the peptide (data not shown).

To prove that proteolytic cleavage was indeed responsible for this increased activity, NP(147-158R-') was first purified by HPLC to remove minor contaminants. Surprisingly, the major peak, which was confirmed to be NP(147-158R-) by amino acid composition analysis, was unable to sensitize targets for lysis by NP-specific CTL, suggesting minor contaminants were responsible for the low activity originally observed using this synthetic peptide (data not shown). This purified material was next incubated with the partially purified serum activity and then rechromatographed by HPLC. As compared with chromatograms of NP(147-158R-) (which migrates at the position of peak I) and the serum fraction, two new peaks were observed (peaks II and III). Based on the mass of recovered material (as determined by amino acid analysis), peak II proved to be at least 1,000-fold more active than the original synthetic peptide, and peaks I and III had no detectable activity (Fig. 3). Amino acid analysis confirmed that peak I was NP(147-158R-'). The active peak (peak II) was missing two COOH-terminal residues, thr and gly. Peak III was found to contain this missing dipeptide. The identity of peak II was further confirmed by comparison of its position of migration with that of the synthetic peptide NP(147-155), which is indicated by the arrow in Fig. 3a. Thus, the proteolytic activity was a COOH-terminal dipeptidase that cleaved NP(147-158R-) into a sequence with high affinity for K4 identical to the one recently reported as the endogenously processed product in H-2d influenza-infected cells, NP(147-155) (7). In agreement with published reports (22), we observed that the synthetic peptide corresponding to the endogenously processed antigenic peptide, NP(147-155), is active in sensitizing targets at concentrations as low as 10^{-11} M.

COOH-terminal dipeptidases are rare and the only one known to exist in serum is ACE. This enzyme (mol wt, 170,000) converts angiotensin I into angiotensin II by removal of a COOH-terminal dipeptide (23). Its specificity has been studied extensively and it is known to be inhibitable by peptides that contain a penultimate proline. We therefore tested the ability of the active component from serum to be inhibited by an ACE inhibitor containing this sequence and also tested the effect of purified ACE on class I binding by NP(147-158R-'). As indicated by the data in Table 1 (Exp. 1), the serum component was completely inhibited by ACE inhibitor, and purified ACE was able to enhance binding by NP(147-158R-) (Table 1). Further evidence that the active component was indeed ACE was obtained through the use of an mAb that is specific for ACE and blocks its enzymatic activity (20). Incubation of the serum fraction with this mAb prevented it from producing an active form of the peptide (Table 1, Exp. 2). Taken together, these data strongly suggested that the active material was ACE.

As demonstrated in Fig. 1, serum also contained a factor
Figure 3. The serum component activates NP(147-158R-) by release of a COOH-terminal dipeptide. (a) HPLC analysis of 50 µg of NP(147-158R-) after incubation with the partially purified serum activity. Arrow denotes position of migration of the synthetic peptide NP(147-155) as determined in a separate experiment. (b) A portion (10%) of the material in the indicated peaks was lyophilized and reconstituted in 50 µl of PBS. 51Cr-labeled P815 cells were pulsed for 1 h with 6 µl from each peak or 0.6 µg of the unpurified synthetic peptide NP(147-158R-) (△) in a volume of 100 µl SF media. Targets were washed twice and then tested for recognition by clone 9.

capable of increasing the efficiency of binding of NP(365-380). ACE was tested for its ability to increase binding of NP(365-380) by appropriate target cells. No increase was observed, suggesting that a different serum component, presumably a different protease, was responsible for this activity (data not shown).

Our observations concerning the impact of serum peptidase on class I–peptide interactions is of significance with respect to identification of antigenic peptides. These results caution that in some cases the active form of peptide that actually binds to class I may differ significantly from the predominant synthetic peptide added to cultured cells, as recently reported (9, 10). Further processing may occur resulting in the observed class I–peptide interaction. This is certainly true of NP(147-158R-) and may be the case with NP(365-380). As a result, this can lead to identification of peptide sequences

Table 1. Inhibition of Processing of NP(147-158R-) by ACE Inhibitor

| Exp. | Addition     | -          | + ACE inhibitor |
|------|--------------|------------|----------------|
| 1    | -            | 0          | 0              |
|      | 0.75% serum  | 70         | 16             |
|      | Serum fraction | 54        | 9              |
|      | ACE (.002 U/ml) | 80       | 30             |
|      | ACE (.006 U/ml) | 91       | 70             |
| 2    | -            | 7          | -              |
|      | Serum fraction | 52       | 16             |
|      |              |            | + Anti-ACE     |
|      |              |            | + Anti-Kk      |

Recognition by clone 9 of 51Cr-labeled P815 cells pulsed with NP(147-158R-) preincubated with the indicated additions as described in Materials and Methods.
that contain an antigenic sequence, yet may themselves have no or only low affinity for class I. Attempts to use such suboptimal peptides as vaccines could prove ineffective, as was the case for NP(147–158R−) (24, 25). This may help explain no or only low affinity for class I. Attempts to use such suboptimal molecules with high efficiency. Thus, only those peptides that efficiently bind may prove effective as vaccines.

The stability of a peptide in vivo may also be an important factor for effective immunization. A great deal is known concerning the ability of ACE to cleave certain sequences (23). It is likely that ACE contributes significantly to peptide degradation in vivo. It may be possible to fashion peptides that withstand such enzymatic breakdown yet retain antigenic activity.

Our results present the possibility that ACE, either alone or in conjunction with other serum or cell-bound peptidases, may serve a role in antigen processing in vivo. It has been speculated that a specialized cell exists that has the ability to present exogenous antigen in association with class I (28–30). In this regard, it has been demonstrated that activated monocytes produce high levels of ACE on their surface (20, 23, 31). Indeed, it is this source of ACE that is responsible for the elevated levels of serum ACE that occur in association with certain diseases (23, 31). The mechanism of cleavage by this dipeptidase makes it an excellent candidate for an antigen processing enzyme, as it removes COOH-terminal dipeptides in a nonprocessive manner (32). Thus, there should be ample opportunity for successive products to attempt binding to cell surface class I. A role of ACE, or any extracellular enzyme, in antigen processing awaits verification that such a pathway is indeed utilized in vivo.

We thank David Rauet for providing sera from β2m-negative mice, Nancy Hosken for providing the VSV-N-specific CTL line, and Carol Wood for expert assistance in the preparation of this manuscript. We are particularly indebted to Dr. M. S. Rohrbach, who provided us with his anti-ACE monoclonal.

This work was supported by a grant from the National Institutes of Health.

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Received for publication 2 December 1991 and in revised form 7 February 1992.

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