Extracellular 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.

Class I molecules bind peptide antigens derived by processing of endogenously synthesized cellular proteins and present these for recognition by CTL (1–6). Recent studies in which peptides have been extracted from the antigen binding grooves of class I molecules have revealed that most endogenous peptides are eight to nine residues in length (7–11). Cell surface class I molecules can also bind synthetic peptides that contain appropriate T cell determinants, and such peptides have been used successfully in the identification of numerous CTL epitopes (1–6, 12). Recent studies from this laboratory and others indicated that only unoccupied class I molecules on the cell surface are receptive to binding of exogenous peptides (13–17). The number of such empty molecules is increased in the presence of an exogenous source of β_2-microglobulin (β_2m)[1] such as serum. This significantly increases class I uptake of exogenous peptides as monitored by target cell lysis by antigen-specific CTL. In the course of these studies it was observed that in addition to β_2m, there appeared to be another component present in serum that greatly increased the ability of the influenza antigen nucleoprotein (NP) (147–158R$^-$) to bind the K^d molecule (13). This particular peptide represents an altered form of residues 147–158 of the influenza NP in which the arginine at position 156 is deleted. It had been reported that this alteration resulted in an increase of three orders of magnitude in the ability of the peptide to be recognized by CTL (12). Our results explain this paradox and could have important implications for previous studies in which T cell determinants have been identified through the use of synthetic peptides.

[1] *Abbreviations used in this paper:* ACE, angiotensin-converting enzyme; β_2m, β_2-microglobulin; NP, nucleoprotein; SF, serum free.

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 ^{51}Cr 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 pulsed 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 ^{51}Cr release assay. Target cells (10^6) were labeled in 0.15 mC Na ^{51}Chromate (DuPont Co., Wilmington, DE) in a final volume of 0.25 ml containing RPMI 1640 and the indicated additions. Unless stated otherwise, target cells were not exposed to media containing serum until washed free of unbound ^{51}Cr and peptide. Labeled target cells (10^4) plus the indicated number of CTL effectors were incubated at 37°C in round-bottomed microtiter plates containing 0.2 ml per well final volume of culture media containing 5% FCS. Culture supernatants were harvested after 4 h.

Peptides. Peptides used in these studies were synthesized by solid phase synthesis on an ABI 430-A automated synthesizer (Applied Biosystems, Inc., Foster City, CA).

Affinity Chromatography of Serum Activity. Human serum was depleted of β_2m and albumin by affinity chromatography on sepharose conjugated with the β_2m-specific mAb BBM.1 (19) obtained from the American Type Culture Collection (Rockville, MD),

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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 10^4 ^{51}Cr-labeled P815 (H-2^d) 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 centricon 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.). 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 (20). 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 were incubated in 15 μl SF media containing 0.8 μg/ml of HPLC-purified NP(147–158R$^-$), 70 μg/ml ACE inhibitor, and 10^4 ^{51}Cr-labeled P815 target cells in wells of a U-bottomed microtiter place. After incubation for 1 h at 37°C, 10^5 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^b 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 β_2m. 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 β_2m. To test this hypothesis, human serum was depleted of β_2m by affinity chromatography and then tested for its affect on peptide binding. As anticipated, the amount of peptide in the β_2m-depleted serum was similar to that obtained in SF media for VSV-N (47–63), and OVA (253–276), thus confirming that β_2m was required for optimal peptide binding (Fig. 1). In contrast, removal of β_2m did not reduce the ability of serum to facilitate class I binding of either NP(365–380) or NP(147–158R$^-$) (Fig. 1).

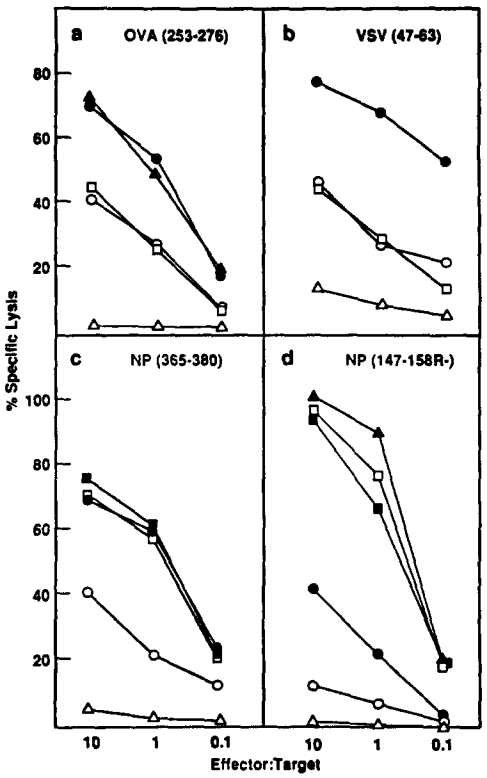

Figure 1. Peptide binding by cell surface class I molecules in serum depleted of β_2m. Target cells (10^6) maintained in SF media (Nutridoma; Boehringer Mannheim) were labeled 1–1.5 h with ^{51}Cr in a final volume of 0.25 μl in the absence (△) or presence (○) of peptide, or peptide plus the following additions: 3 μg/ml β_2m (human; Sigma Chemical Co.) (●); 3% human sera depleted of β_2m (□); 3% human sera depleted of β_2m plus 3 μg/ml β_2m (▲); sera obtained from β_2m-deficient transgenic mice (■). The indicated peptide antigens were present at the following concentrations: OVA(253–276), 10 μg/ml; VSV-N(47–63), 3 μg/ml; NP(365–380), 10 μg/ml; NP(147–158R$^-$), 10 μg/ml. Target cells were either EL4 (*a–c*) or the B cell lymphoma 439.4.2 (*d*), which expresses both H-2^b and H-2^d antigens. Effector cells used in these assays were the appropriate antigen-specific CTL described in Materials and Methods. It should be noted that unless indicated otherwise, target cells were not exposed to media containing serum until washed free of unbound ^{51}Cr and peptide.

Considering the β_2m-depleted serum retained ~5% of its β_2m, it was possible this residual material could foster binding by some peptides. We therefore obtained sera from transgenic mice that have disrupted β_2m genes and thus

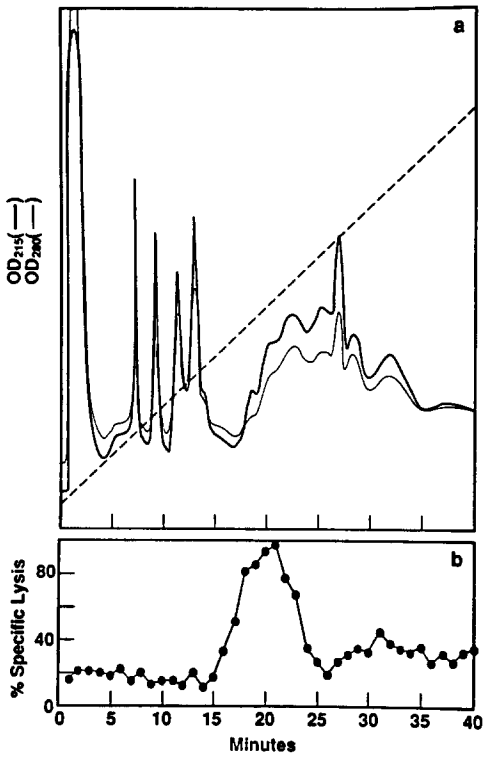

Figure 2. Fractionation of the serum activity responsible for class I binding by NP(147–158R⁻) by ion-exchange chromatography. (*a*) The serum activity was partially purified by ion-exchange chromatography on a Mono-Q column. (*b*) 5 μl of each fraction was assayed for its ability to enhance recognition of NP(147–158R⁻) by clone 9 as described in Materials and Methods.

could not express β_2m (21). Again, it was observed that the NP peptides bound class I efficiently in such β_2m-negative sera (Fig. 1). Taken together, these results suggested the presence in sera of a factor (different from β_2m) that was capable of enhancing peptide binding by some but not all peptides above the level observed in SF media.

In view of the complexity of serum, the relevant component was partially purified before attempting to define its mechanism of activity. Serum depleted of β_2m and albumin by affinity chromatography was subjected to fractionation on an ion exchange column, and fractions were tested for their affect on peptide binding by P815 cells in SF media. A major peak of activity was identified, which was utilized for further characterization (Fig. 2). Gel filtration analysis revealed it migrated with a molecular weight in excess of 100,000 (data not shown).

Considering that the serum activity enhanced T cell recognition for some peptides but not others, it was considered likely that it recognized the peptide rather than the class I 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. 3 *a*. Thus, the proteolytic activity was a COOH-terminal dipeptidase that cleaved NP(147–158R⁻) into a sequence with high affinity for K^d identical to the one recently reported as the endogenously processed product in H-2^d 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 a 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

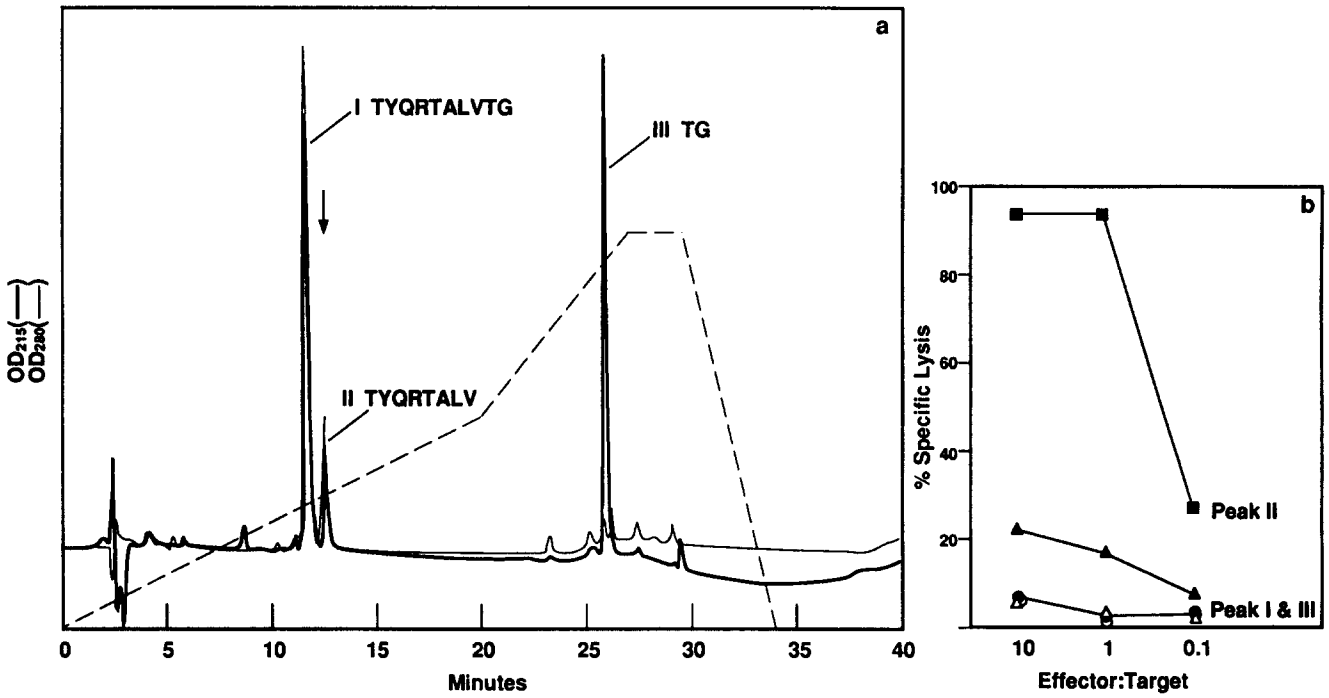

Figure 3. The serum component activates NP(147–158R⁻) by release of a COOH-terminal dipeptide. (*a*) HPLC analysis of 50 μg of NP(147–158R⁻) after incubation with the partially purified serum activity. Arrow denotes position of migration of the synthetic peptide NP(147–155) as determined in a separate experiment. (*b*) A portion (10%) of the material in the indicated peaks was lyophilized and reconstituted in 50 μl of PBS. ^{51}Cr-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	Percent specific lysis		
		–	+ 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	
		–	+ Anti-ACE	+ Anti-K^k
2	–	7	–	–
	Serum fraction	52	16	49

Recognition by clone 9 of ^{51}Cr-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 some of the controversy currently in the field concerning the efficacy of soluble peptide, or peptide-coated cells, as immunogens (24, 26, 27). For a peptide to be an effective immunogen it may need to bind large numbers of class I 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.

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