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Immunology

Enhancement of peptide antigen presentation by a second peptide

ANTON I. P. M. DE KROON* AND HARDEN M. MCCONNELL†

Department of Chemistry, Stanford University, Stanford, CA 94305

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ABSTRACT **The influence of nonstimulatory "competitor" peptides on the binding of an antigenic peptide to a major histocompatibility complex (MHC) class II molecule was investigated. Using high-performance size-exclusion chromatography and fluorescein-labeled peptides, we show that the presence of the peptides dynorphin A-(1–13) and poly(L-lysine) results in enhancement rather than inhibition of the binding of hen egg lysozyme peptide-(107–116) [HEL-(107–116)] to the detergent-solubilized mouse class II molecule IE^d. In parallel, dynorphin A-(1–13) and poly(L-lysine) were found to enhance the specific activation of an IE^d-restricted T-cell hybridoma by HEL-(107–116). A molecular mechanism involving an intermediate two peptide–MHC class II protein complex is proposed to explain the enhancement of peptide binding to class II molecules by an irrelevant second peptide.**

Class II molecules of the major histocompatibility complex (MHC) play a central role in the immune response to pathogens. The class II MHC molecules, $\alpha\beta$ heterodimeric membrane proteins, form molecular complexes with pathogen-derived peptides, and these complexes are targets for recognition by $CD4^+$ T (helper) cells. The biological function of such complexes requires that an individual MHC molecule have the capacity to bind many different antigenic peptides but at the same time have the ability to retain each peptide in the binding site for long periods of time. This unusual dual requirement has led us to study the kinetics of MHC class II–peptide reactions.

It is generally accepted that the stoichiometry of a functional MHC class II–peptide complex is 1:1 (1). However, a number of observations in the literature suggest that the presence of a nonstimulatory peptide can play a role in the presentation of an antigenic peptide to the T-cell receptor other than by simply competing for the same MHC binding site. For example, Bhayani and Paterson (2) have described a nonstimulatory pigeon cytochrome *c*-(88–104) analogue differing at only one position from the native peptide, which enhances the stimulation of a specific IE^k-restricted T-cell hybridoma by the native peptide. A similar finding has been reported for human immunodeficiency virus (HIV)-derived peptides interacting with class I molecules (3).

Recently, the possibility of a second peptide binding site in class II molecules was suggested by the demonstration of energy transfer between two fluorescence-labeled full-length antigenic peptides bound to the so-called "floppy" conformation of IA^d, which can be identified on SDS gels (4). Energy transfers were also observed between labeled short peptides in both the floppy and "compact" conformations of IA^d. Experiments showing that the antigenic hen egg lysozyme peptide-(107–116) (HEL) bound to the class II molecule IE^d can be displaced by competitor peptides present in excess (5) argue in favor of the existence of an intermediate two-peptide complex.

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The binding of the antigenic peptide HEL to the mouse class II protein IE^d has been characterized in detail (6). The analysis of HEL analogues with single amino acid substitutions and the comparison with other IE^d-restricted antigenic peptides have led to the proposal that the presence of multiple positive charges is required for binding to IE^d molecules. This contention was verified by testing a number of (not necessarily antigenic) peptides that meet this requirement, such as the neuropeptide dynorphin A-(1–13) (Dyn), for binding to IE^d in competition studies (6–8). In a subsequent study, Dyn, among other positively charged peptides, was shown to accelerate the release of HEL from IE^d (5).

In work (to be published elsewhere) designed to elucidate the molecular mechanism of this displacement reaction, we have investigated the specificity and kinetics of the acceleration of the release of peptides from MHC class II proteins by second peptides. In the course of these experiments, we observed yet another kinetic effect, which is reported here: one peptide can act catalytically in facilitating the replacement of an endogenous peptide by a second peptide. Thus, peptide-enhanced binding of an antigenic peptide to the detergent-solubilized class II molecule IE^d and the parallel enhancement of T-cell stimulation are demonstrated. These results provide further evidence for reactions involving two peptide–MHC class II intermediates.

MATERIALS AND METHODS

Purification of Class II Molecules. IE^d was purified from a Nonidet P-40 (NP-40) lysate of A20-1.11 cells by affinity chromatography using the antibody 14.4.4S according to standard procedures (9). After replacement of NP-40 by the detergent *n*-dodecyl β-D-maltoside (DM; Sigma) IE^d was eluted from the affinity column with 0.5 M NaCl/1 mM DM/0.1 M Na_2CO_3, pH 11.5. The eluate was immediately neutralized to pH 7, concentrated, extensively dialyzed against 150 mM NaCl/1 mM DM/10 mM NaP_i, pH 7.0, and stored at 4°C. The protein concentration was determined by the Lowry method. IE^k and IA^d were purified accordingly from CH27 and A20 cells by using the antibodies 14.4.4S and MKD6, respectively.

Peptide Synthesis and Labeling. The peptides HEL (H-AWVAWRNRCK-OH) and Dyn (H-YGGFLRRIRPKLK-OH) were synthesized by standard 9-fluorenylmethoxycarbonyl chemistry on a model 431A (Applied Biosystems) peptide synthesizer, cleaved from the resin in trifluoroacetic acid containing the appropriate scavengers, and purified to >98% by reverse-phase HPLC. Dyn and HEL were labeled with fluorescein at the N terminus, to yield FDyn and FHEL,

Abbreviations: MHC, major histocompatibility complex; HEL, hen egg lysozyme-(107–116); FHEL, fluoresceinated HEL; DM, *n*-dodecyl β-D-maltoside; Dyn, dynorphin A-(1–13); FDyn, fluoresceinated Dyn; HPSEC, high-performance size-exclusion chromatography; IL-2, interleukin 2; FOva, fluoresceinated ovalbumin-(323–338)Y (where Y is an added C-terminal tyrosine).

*Present address: Centre for Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.
†To whom reprint requests should be addressed.

while still on the resin by reaction with a 2.5-fold molar excess of fluorescein-5-isothiocyanate and 5-(and 6-)carboxyfluorescein succinimidyl ester (Molecular Probes), respectively, in the presence of a 10-fold molar excess of diisopropylethylamine, using *N*-methylpyrrolidone as solvent. Alternatively, the cysteine residue of HEL was labeled with 5-iodoacetamidofluorescein yielding FCysHEL. The cysteine residue of unlabeled HEL and FHEL was carboxamidomethylated with iodoacetamide to prevent the formation of peptide dimers. N-terminally fluoresceinated ovalbumin-(323–338)Y (where Y is an added C-terminal tyrosine) (H-ISQAVHAAHAEINEAGY-OH) (FOva) was synthesized as described (9).

The identity of all peptides was established by mass spectrometry. Peptide stock solutions in water were stored in aliquots at −20°C. The concentrations of the peptide stock solutions (range 1–5 mM) were determined by quantitative amino acid analysis and confirmed by spectrophotometry. The concentration of the poly(L-lysine) (polymerization degree 14–19; Sigma) stock solution was calculated based on quantitative amino acid analysis, using an average polymerization degree of 16.5.

Peptide Binding Assay. Class II proteins were incubated at 0.3 μM with 5 μM fluoresceinated peptide in 150 mM NaCl/1 mM DM/10 mM NaP_i, pH 7.0 (HPSEC buffer) at 37°C. Peptide binding was assayed by high-performance size-exclusion chromatography (HPSEC) (10). At different times, 20-μl aliquots of the incubation mixture (6 pmol of class II) were injected onto a TSK gel G3000SW (Tosohaas, Montgomeryville, PA) size-exclusion column eluted with HPSEC buffer. The column was connected to a fluorescence detector and an absorbance detector set up in series. The fluorescence intensity associated with the αβ heterodimer [elution vol, 15 ml (11)] was normalized to the corresponding absorbance signal and converted into the amount of peptide bound per mol of IE^d by a calibration procedure that takes into account the changes in the fluoresceinated peptides' quantum yield upon binding to IE^d.

T-Cell Stimulation Assay. Mitomycin c-treated A20 cells were incubated at 10^5 cells per well together with 10^5 cells of the HEL-specific T-cell hybridoma D2-1E5 (12) in the presence of the indicated concentrations of peptide(s) in a total vol of 225 μl of RPMI 10% fetal calf serum at 37°C. After 24 h, the culture supernatants were harvested and assayed (in serial dilution) for their ability to sustain the growth of the interleukin 2 (IL-2)-dependent cell line HT2 for 18 h (2×10^4 HT2 cells in a total vol of 100 μl per well). IL-2 production was quantitated by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] assay (13), which was calibrated with an IL-2 standard.

RESULTS

Competition for Binding of HEL and Dyn to IE^d. By attaching a fluorescein label to the N terminus of both HEL and Dyn, the binding of these peptides to detergent-solubilized IE^d could be monitored directly by HPSEC. The fluorescein moiety present at the N terminus of HEL does not interfere with the ability of this peptide to stimulate the IE^d-restricted HEL-specific T-cell hybridoma D2-1E5 (unpublished data).

The slow kinetics of binding and the modest values of the occupancy observed for both FHEL and FDyn (Fig. 1) are typical for MHC class II–peptide systems (9, 11) and are due to the presence of a heterogeneous population of endogenous peptides in the IE^d preparation (14). As expected for specific binding, under conditions of excess peptide over IE^d, the presence of equimolar amounts of the corresponding unlabeled peptides reduces the binding of FDyn and FHEL by a factor of 2 (Fig. 1). An equimolar amount of unlabeled HEL

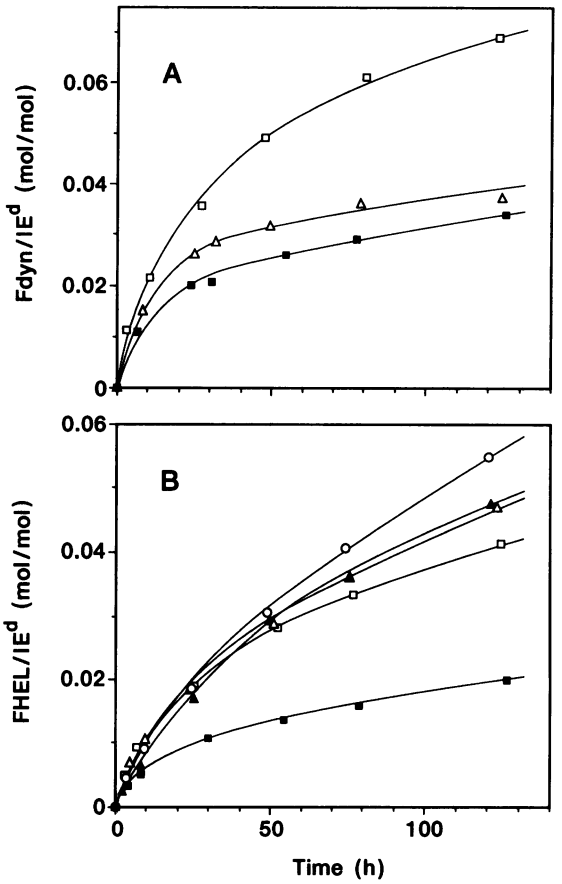

FIG. 1. Time course of binding to detergent-solubilized IE^d of 5 μM FDyn in the absence (□) and presence of an equimolar amount of unlabeled Dyn (■) and HEL (△) (*A*) and 5 μM FHEL in the absence (□) and presence (■) of 5 μM HEL and in the presence of 5 μM (▲), 15 μM (○), and 50 μM (△) Dyn (*B*). IE^d was incubated at 0.3 μM with the indicated peptides at 37°C in HPSEC buffer. Peptide binding to IE^d was determined by HPSEC as described.

reduces the binding of FDyn to IE^d to a slightly lesser extent than unlabeled Dyn (Fig. 1*A*). This result indicates that both peptides use the same binding site on IE^d and suggests that the affinities of Dyn and HEL for IE^d are similar. Surprisingly, the presence of unlabeled Dyn up to a molar ratio of 10 with respect to FHEL does not inhibit binding of FHEL to IE^d (Fig. 1*B*). Instead, there is an enhancement of FHEL binding, which reaches a maximum between concentrations of 5 and 50 μM Dyn. The T-cell stimulation assay carried out with Dyn present in the same concentration range (Fig. 2) reveals a parallel Dyn-induced increase of the response to HEL, the extent of which is dependent on the molar ratio of Dyn/HEL present. At higher Dyn/HEL ratios the displacement of (F)HEL by Dyn (5) probably starts to outweigh the induced increase in binding (Figs. 1*B* and 2).

The Effect of Poly(L-lysine) on Peptide Binding to IE^d. To investigate the role of positively charged amino acid residues in the interaction of peptides with IE^d, we studied the homopolymer poly(L-lysine) with a polymerization degree of 14–19. Peptide displacement experiments established that poly(L-lysine) accelerates the release of FHEL from detergent-solubilized IE^d, although less efficiently than Dyn. Fig. 3 shows the effect of poly(L-lysine) on the binding of FHEL to IE^d. The addition of 5 μM poly(L-lysine) is sufficient to double FHEL (also present at 5 μM) binding to IE^d; at 500 μM poly(L-lysine), the initial rate of FHEL binding to IE^d is ≈50% faster than that obtained at pH 5.0 (dashed line in Fig. 3). Acidic conditions are known to enhance both the extent and the rate of binding of peptides to class II proteins (15–17).

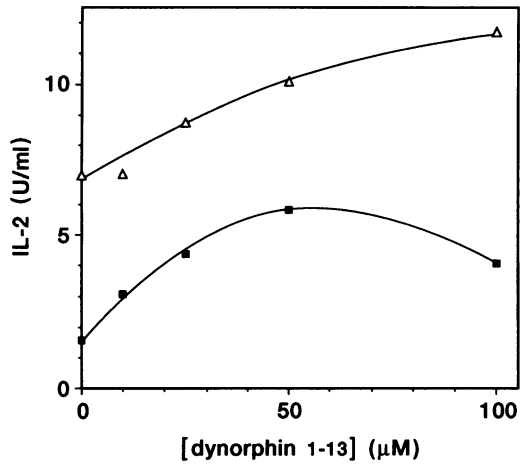

FIG. 2. IL-2 production of the IE^d-restricted HEL-specific T-cell hybridoma D2-1E5 upon incubation with A20-1.11 cells and 10 μM (■) or 25 μM (△) HEL in the presence of the indicated concentrations of Dyn. Estimated error in the data is ±1 unit/ml. Control experiments showed that Dyn itself does not elicit IL-2 production by the D2-1E5 cells and that this peptide does not affect the viability of the HT2 cells in the concentration range used.

At pH 5, it takes more poly(L-lysine) to enhance the binding of FHEL to IE^d—e.g., 500 μM poly(L-lysine) enhances the extent of binding by some 20% (data not shown). The presence of 3.3 mM L-lysine [the monomer equivalent of 200 μM poly(L-lysine)] does not affect FHEL binding to IE^d (Fig. 3).

The poly(L-lysine) effect on binding to IE^d is not unique for FHEL as shown in Fig. 4. FCysHEL, the nonstimulatory fluoresceinated HEL derivative, shows the same extent of binding to IE^d as FHEL, yet the enhancement of its binding

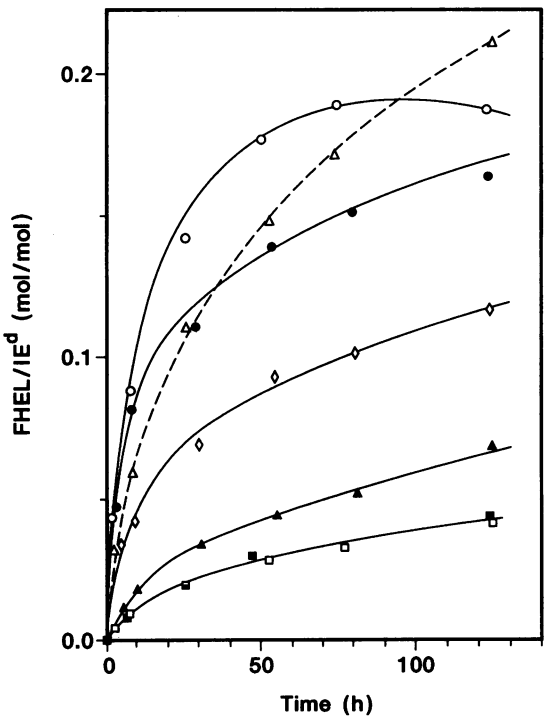

FIG. 3. Effect of increasing concentrations of poly(L-lysine) on kinetics of binding of 5 μM FHEL to 0.3 μM IE^d at pH 7.0: 0 μM (□), 5 μM (▲), 50 μM (◇), 200 μM (○), and 500 μM (●). Effect of 3.3 mM L-lysine is also shown (■); for comparison, the binding of FHEL to IE^d in 150 mM NaCl/McIlvaine's citrate phosphate buffer, pH 5.0/1 mM DM is shown (△; dashed line).

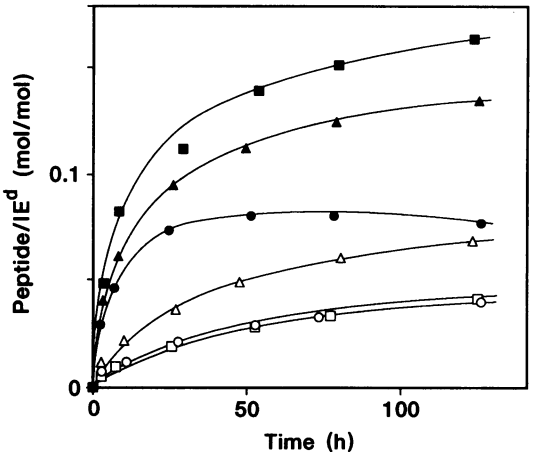

FIG. 4. Stimulatory effect of 200 μM poly(L-lysine) (solid symbols) on binding of FDyn (△), FHEL (□), and FCysHEL (○) to IE^d. Open symbols depict binding at pH 7 in the absence of poly(L-lysine). Experimental conditions are as in Fig. 3.

by poly(L-lysine) is less and appears to be reversed after longer incubation times (Fig. 4). In separate work, we have shown that this is due to the greater susceptibility of the IE^d–FCysHEL complex to poly(L-lysine)-induced dissociation. This result points to the dual effect poly(L-lysine) has on peptide binding to IE^d, which was also observed for Dyn (Figs. 1*B* and 2). Poly(L-lysine) also stimulates the binding of FDyn to IE^d but to a lesser extent than that of FHEL (Fig. 4).

The enhancing effect of poly(L-lysine) on the binding of HEL to IE^d is also apparent from the T-cell stimulation experiments in Fig. 5. A comparison of the dose–response curves in the presence and absence of 50 μM poly(L-lysine) (Fig. 5*B*) shows that the homopolymer does not shift the level of maximum IL-2 production to a lower peptide concentration, as the net increase in IL-2 production induced by poly(L-lysine) decreases with increasing concentrations of HEL. This points to a mechanism in which the molar ratio of poly(L-lysine)/HEL rather than the absolute poly(L-lysine) concentration is decisive.

Class II Specificity. To address the question of the specificity of the effect of poly(L-lysine) on peptide binding to class II molecules, HPSEC experiments were carried out with detergent-solubilized IE^k and IA^d. The peptide HEL has also been shown to bind to IE^k and to activate specifically an IE^k-restricted T-cell hybridoma (18). Fig. 6 shows that poly(L-lysine) exerts a stimulatory effect on the binding of FHEL to detergent-solubilized IE^k, albeit less pronounced than in the case of IE^d (cf. Fig. 3). No effect of poly(L-lysine) was detectable on the binding of the IA^d-restricted peptide FOva to IA^d (Fig. 6).

DISCUSSION

Two peptides, Dyn and poly(L-lysine), that have the ability to accelerate the dissociation of IE^d–peptide complexes have been shown to enhance the binding of a second peptide to detergent-solubilized IE^d. The balance between stimulation of binding on the one hand and of dissociation on the other depends on the molar ratio of the peptides present. The enhanced peptide binding to the class II protein is peptide and class II specific (Figs. 4 and 6), indicating that the site of the interaction between the second peptide and the class II molecule is the antigen binding site. The Dyn/poly(L-lysine)-enhanced binding results in the case of HEL in functional MHC–peptide complexes as shown by the T-cell stimulation experiments.

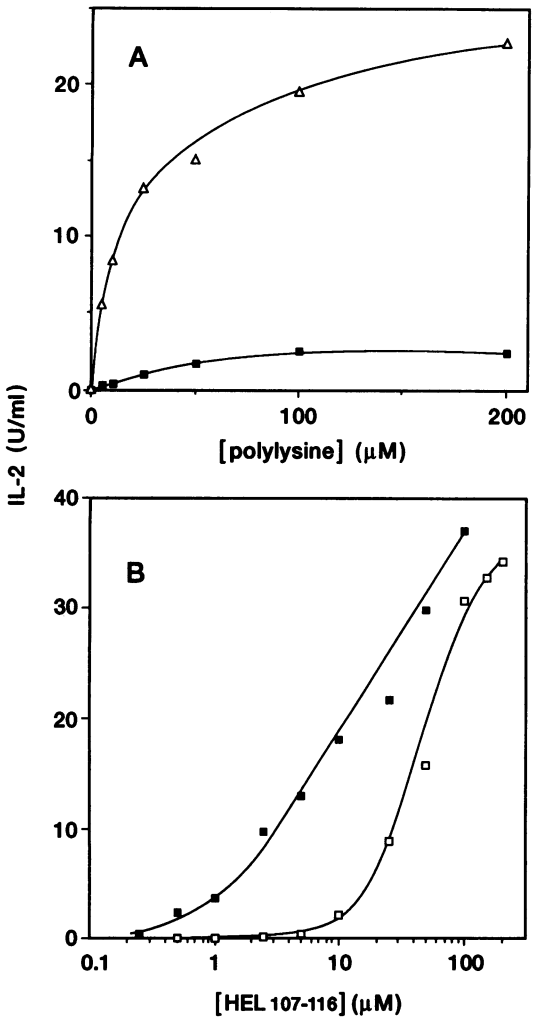

FIG. 5. (*A*) Enhancement of IL-2 production of the T-cell hybridoma D2-1E5 in response to 1 μM (■) and 5 μM (△) HEL by increasing concentrations of poly(L-lysine). (*B*) Dose–response of D2-1E5 cells to HEL in the absence (□) and presence (■) of 50 μM poly(L-lysine). A20 cells were used as antigen presenting cells; for experimental details, see *Materials and Methods*. Poly(L-lysine) in the concentration range used does not affect the viability of HT2 cells.

One might argue that the stimulatory effect of poly(L-lysine) on T-cell stimulation is due to enhanced cell–cell adhesion induced by this polycation. However, in view of the consistency of the binding and stimulation data obtained for both Dyn and poly(L-lysine), we are confident that the poly(L-lysine)-enhanced T-cell stimulation reflects enhanced HEL binding to IE^d.

The data are consistent with a molecular mechanism in which the association of an exogenous peptide with a preexisting class II–peptide complex yields an intermediate two-peptide complex that catalyzes the displacement of the first (endogenous) peptide from the binding groove, thus creating the opportunity for itself or a third peptide to bind in the groove. Whether or not displacement or replacement will occur depends critically on the properties and concentrations of the peptides and on the MHC–(endogenous) peptide complex involved.

As mentioned, positively charged amino acid residues facilitate peptide interaction with IE^d. This selectivity is most likely based on electrostatic interaction with negatively charged residues in the IE^d binding groove. A structural model for class II proteins based on the crystal structure of a class I molecule (19) applied to IE^d reveals a large number

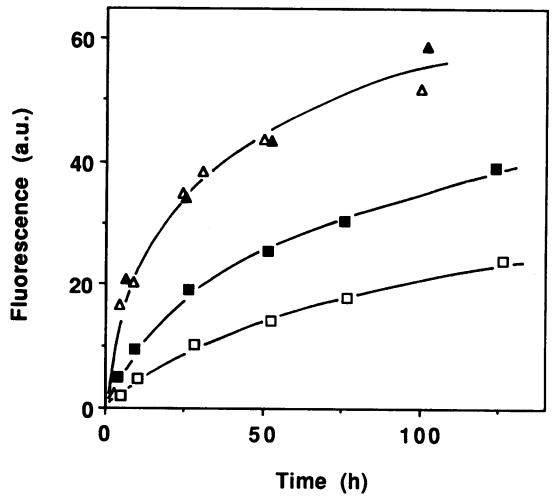

FIG. 6. Time course of binding of FOva to IA^d (△) and of FHEL to IE^k (□) at pH 7 and 37°C in the absence (open symbols) and presence (solid symbols) of 200 μM poly(L-lysine). Concentration of fluoresceinated peptide is 5 μM and that of class II molecules is 0.3 μM. Binding is expressed as fluorescence signal associated with the αβ heterodimer. For comparison, the fluorescence signal of FHEL bound to IE^d after incubation for 125 h under the same conditions amounts to 50 arbitrary units.

of negative charges lining the binding groove (20). If the (endogenous) peptide present in the binding groove does not shield all the negative charges all the time, exogenous positively charged peptides like HEL, Dyn, and poly(L-lysine), present in excess, are likely to adsorb to these. Depending on the properties of the endogenous and the exogenous peptide adsorbed to the second "binding" site, this may result in a destabilization of the complex leading to the release or replacement of the endogenous peptide. In separate work, we have found that the efficiency of the displacement of different fluorescein-labeled peptides from IE^d by a panel of unlabeled peptides is determined by both peptides—i.e., it is different for each combination of peptides.

If the peptide-induced destabilization of the complex proceeds to the point where the endogenous peptide is forced out, the exogenous peptide may take its place provided that it has the ability to form a stable complex with the αβ heterodimer. The data indicate that this situation applies to Dyn, which can form a stable complex with IE^d (Fig. 1*A*). However, with Dyn and HEL both present in equal excess, there is a clear preference for HEL to occupy the binding groove following destabilization by Dyn (Figs. 1*B* and 2), probably because an αβ–HEL complex is thermodynamically more favorable than an αβ–Dyn complex. In the presence of higher Dyn/HEL molar ratios, the IE^d–HEL complex is destabilized.

Poly(L-lysine) can adsorb to and destabilize a large subpopulation of IE^d–endogenous peptide complexes. Whether poly(L-lysine) can also replace the endogenous peptide and form a stable αβ–poly(L-lysine) complex has not directly been determined but is considered unlikely as even a 100-fold molar excess of poly(L-lysine) over FHEL does not inhibit FHEL binding to IE^d (Fig. 3). By destabilizing the class II–peptide complex, poly(L-lysine) opens the door for the binding of other peptides that do have the ability to replace the endogenous peptide and form stable complexes (HEL/Dyn), thus giving rise to the observed enhancements (Figs. 3–5).

The enhancement of HEL binding by poly(L-lysine) is more efficient than that by Dyn because (*i*) Dyn can compete with HEL for binding in the groove (Fig. 1); (*ii*) HEL–IE^d complexes, once formed, are more susceptible to dissocia-

tion by Dyn than by poly(L-lysine); and (*iii*) *a priori* poly(L-lysine) may be able to destabilize a broader subpopulation of the IE^d–endogenous peptide complexes than Dyn. Although HEL is not very effective in destabilizing preformed peptide–IE^d complexes, the data in Fig. 5*B* indicate that it does compete with poly(L-lysine) in binding, since the enhancement of T-cell stimulation by 50 μM poly(L-lysine) becomes less pronounced at higher concentrations of HEL.

The stimulatory effect of poly(L-lysine) on binding of FHEL to IE^k (Fig. 6) indicates that the proposed mechanism applies in general to mouse class II molecules of the E isotype and suggests that it accounts for the results reported previously (2). IE molecules share the same α chain, which contains a large number of acidic amino acid residues in the proposed binding groove (20). In retrospect, the two-peptide–MHC complex intermediate proposed here may be related to the previously identified kinetic intermediate in the binding reaction of pCytc88–104 to IE^k (21). No effect of poly(L-lysine) was detected on the binding of FOva to detergent-solubilized IA^d (Fig. 6).

One important implication of the results reported here and in previous studies (2, 3) is that relative binding affinities of peptides for MHC molecules derived from comparative competition experiments with only one labeled peptide may yield a distorted picture. The use of $\alpha\beta$ heterodimers devoid of endogenous peptides (22, 23) in this type of study is to be preferred.

All the biological implications of this work are not clear. MHC class II molecules bind peptides in endosomal compartments in the cell (24). Conceivably, the (antigenic) peptides produced in these compartments use a mechanism similar to that proposed here in competing to replace the invariant chain or derived peptides (25, 26) and possibly chaperone proteins that are associated with the newly synthesized $\alpha\beta$ heterodimers. This opens the intriguing possibility that nonantigenic portions of a processed antigen can affect the binding of antigenic peptides.

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