Enhanced Binding of Peptide Antigen to Purified Class II Major Histocompatibility Glycoproteins at Acidic pH

By Peter E. Jensen

From the Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322

Summary

Helper T lymphocytes recognize peptide antigens stably associated with class II major histocompatibility complex (MHC) glycoproteins on the surface of antigen-presenting cells and serve to regulate a wide variety of immune responses. A previous study from our laboratory had demonstrated that the functional association of various peptide antigens with the antigen-presenting cell membrane was increased at pH 5 as compared to pH 7, consistent with the potential role of acidic endosomal compartments in antigen processing. The mechanism for this effect was not determined. In the present study, assays using purified class II glycoprotein were used to further define this mechanism. The potential requirement for pH-dependent interactions involving non-MHC membrane components was excluded in functional assays with purified class II reconstituted in artificial membranes containing only neutral phospholipids and cholesterol. The association of HEL(104-120) with I-E^d, and OVA(323-339) with I-A^d, was increased at pH 5, as measured by activation of specific T cell hybridomas. An enzyme immunoassay was developed to measure the binding of biotin-labeled peptides to purified class II in detergent micelles. The pH dependence of binding paralleled our previous functional results. Optimum binding of biotin-HEL(104-120) to I-E^d was observed at pH ~4.5, whereas maximum binding of biotin-Myo(106-118) to I-A^d occurred at pH ~5.5. The latter peptide also bound weakly to I-E^d, but with a pH dependence similar to that observed using HEL(104-120). Further experiments with biotin-HEL(104-120)/I-E^d indicated that both the apparent affinity and the apparent concentration of peptide-binding sites are increased as hydrogen ion concentration is increased from pH 7 to pH 5. The effect of pH in this range was largely reversible and was not associated with a change in peptide dissociation that could be measured with our assay system. Binding was not inhibited in the presence of 1.5 M NaCl, suggesting that electrostatic interactions between HEL(104-120) and I-E^d are not essential for binding. It is proposed that protonation of a critical group(s) in the class II molecule regulates its capacity to form stable complexes with peptide. However, this effect alone does not fully account for the rapid kinetics of peptide binding observed in experiments with intact antigen-presenting cells.

Recent studies have greatly clarified the general features of the antigen processing pathway that prepares antigen for recognition by class II–restricted helper T cells (1, 2). Antigen is taken up through endocytosis or phagocytosis into an intracellular pathway in APC, where it is modified, possibly through limited proteolytic cleavage, and re-expressed on the surface of APC. At some point in this pathway, antigen encounters class II glycoproteins (Ia), forming stable complexes. A common feature among many of the organelles in the endocytic pathway of eucaryotic cells, including APC, is the presence of H^+ ATPases responsible for generating an internal acidic environment (3). Low pH is an important factor in the regulation of receptor-ligand traffic during endocytosis and recycling. In addition, the activity of enzymes such as lysosomal acid hydrolases are profoundly influenced by pH. Drugs that increase the pH of intracellular compartments reversibly inhibit antigen processing (4). Vacuolar acidification may regulate antigen processing by affecting proteolytic cleavage of antigen (5), subcellular trafficking, dissociation of invariant chain from newly synthesized class II (6), or by regulating the association of antigen with class II.

We have recently reported evidence supporting the latter possibility (7). The functional association of artificially processed peptide antigen with aldehyde-fixed B cells or B cell membranes was assessed by measuring specific activation of class II–restricted T cell hybridomas after exposing the B cells to peptide under various conditions. A marked increase in the rate and extent of functional peptide binding

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was observed after incubation with peptide at pH 5, as compared to neutral pH. It was concluded that antigen/class II complex formation is favored in the acidic environment provided by compartments in the endocytic pathway. In the present report we describe experiments that extend our previous observations and indicate that acidic pH directly facilitates the binding of peptide to purified class II glycoproteins. Hydrogen ion concentrations in the physiological range were observed to modulate both the apparent affinity and the apparent number of available peptide-binding sites in preparations of purified I-E^d, but not the extent of peptide dissociation. These effects did not fully account for the relatively rapid rate of antigen/class II complex formation that occurs under physiological conditions.

Materials and Methods

Class II Purification and Reconstitution. I-E^d and I-A^d were purified from detergent-solubilized A20 B lymphoma membrane preparations using a modification of the procedure of Gorga et al. (8). Nuclei were removed after hypotonic lysis by centrifugation at 2,000 *g* for 10 min. Membranes were pelleted by centrifugation at 100,000 *g* for 60 min and solubilized at 5 × 10^8 cell equivalents/ml in 0.5% NP-40, 0.15 M NaCl, 50 mM TRIS, pH 8.0, containing 5 mM PMSF, 2 mM EDTA, and 1% aprotinin. After clearing by centrifugation at 100,000 *g* for 60 min, the lysate was passed sequentially through Sepharose-4B, mouse IgG Sepharose, MKD6-Sepharose (9), and 14-4-4-Sepharose (10) immunoaffinity columns. After loading, the mAb columns were individually washed with 50-column volumes of 50 mM Tris, 0.5% NP-40, 0.5 M NaCl, pH 8.0, followed by 20 vol 50 mM Tris, pH 8.0, and 10 vol of 50 mM Tris, 0.15 M NaCl, pH 8.0, containing 1% *N*-octylglucoside. Purified class II was eluted in 50 mM glycine, pH 11.0, containing 1% *N*-octylglucoside. The eluate was immediately neutralized, dialyzed, concentrated, and stored at 4°C. Class II preparations, analyzed by SDS-PAGE and coomassie blue staining, were essentially homogeneous. Yields routinely ranged from 100 to 200 μg class II/10^{10} A20 cells.

Purified class II was reconstituted into liposomes from solutions containing 1% deoxycholate, 275 μM phospholipid, 75 μM cholesterol, and 1 μM class II by extensive dialysis against PBS. The phospholipids used were L-α-dipalmitoyl phosphatidylcholine and L-α-dilinoleoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) in a 9:1 molar ratio (11). Liposomes were supported by incubation with 2.5 × 10^6 washed 10-μm silica gel particles (Sigma Chemical Co.) for 2 h at 24°C. Liposome-coated particles were washed and resuspended in buffer immediately before use.

Peptides and T Cell Cultures. Peptide antigens were synthesized as previously described (12). Sequences of peptides were HEL(104-120), GMNAWVAWRNRCKGTDVY; OVA(323-339), ISQAYHAAHAEINEAGRY; Myo(106-118), FISEAIIHVLHSR; HEL(46-61), NTDGSTDYGILQINSR; pCC(91-104), RADLIAYLKQATAK. The cloned I-E^d-restricted T cell hybridoma line, Hd-1.AC5, was derived from BALBc mice immunized with hen egg lysozyme (12). DO-11.10 T cell hybridomas recognize processed OVA in association with I-A^d (5). Aliquots of class II liposome-coated particles were incubated with 30 μM peptide in 0.15 M citrate/phosphate buffer, pH 5.0 or 7.2, for various time periods. Washed particles were cultured (10^5 particles/well) with T cell hybridomas (10^5/well) in duplicate wells of 96-well tissue culture plates for 24 h in RPMI containing 10% FCS. In some experiments, fixed APC were pulsed with peptide and cultured with T cells as previously described (7). Lymphokine production, reflecting T cell activation, was measured by culturing serial dilutions of culture supernatant with the IL-2-dependent HT-2 cell line (10^4/well) (13). Results are expressed in U/ml lymphokine, where I U is defined by stimulation of 50% of the maximum response determined by a standard curve. Concentrations <10 U/ml could not be measured in the assay.

Peptide Binding Assay. HEL(104-120) and Myo(106-118) were biotinylated by reaction with a 2.5-mol excess of biotinamidocaproate *N*-hydroxysuccinimide ester in *N,N*-dimethyl formamide, followed by precipitation with acetone and HPLC purification. Biotin was attached through the free α amino group in Myo(106-118) and through both the α and ε amino groups in HEL(104-120). The modified peptides retained biological activity, although potency was slightly reduced. Biotinylated peptides were incubated with 40–80 nM I-A^d or I-E^d at 37°C in a final volume of 30 μl in the presence of 0.2% NP-40, 30–100 mM citrate/phosphate, 1 mM PMSF, 2 mM EDTA, and 5 mM *N*-ethyl maleimide. Sodium azide (0.01%) was included in some experiments. Samples were diluted to 200 μl in 200 mM Tris-HCl buffer, pH 7.5, containing 5% skim milk, 0.1% BSA, 0.1% Tween 20, and 0.5% NP-40. Class II/peptide complexes were separated from free peptide by incubation on microtiter assay plates coated with MKD6 (I-A^d) or 14-4-4 (I-E^d) mAb for 60 min at 37°C. Equivalent results were obtained in experiments using 25-9-17 (14) instead of MKD6 to capture I-A^d. Assay plates were prepared by incubation with 50 μg/ml affinity-purified goat anti-mouse Ig followed by incubation with 50–200 μg/ml purified mAb. Plates were blocked with 50 mM Tris, pH 7.5, containing 5% skim milk, 0.1% BSA, and 0.1% Tween 20, and thoroughly washed before sample addition. Bound biotinylated peptide was detected by incubation with 5 μg/ml avidin-alkaline phosphatase for 60 min at 24°C followed by incubation with 1 mg/ml *p*-nitrophenyl phosphate in 4 mM $MgCl_2$, 0.5 M Tris, pH 10.0. Each data point represents the mean specific absorbance at 405 nm of duplicates or triplicates. Absorbance, measured in absence of class II glycoproteins or in the absence of biotinylated peptide, was found to be similar to that observed in the presence of excess unlabeled peptide, and was generally <10% of the maximum value in the assay. Nonspecific absorbance, subtracted from the results, could be attributed largely to a small degree of nonspecific binding of avidin-alkaline phosphatase in the immunoassay. In saturation experiments, free peptide concentrations were assumed to equal total peptide for Scatchard plots. This is a reasonable approximation because the concentration of class II was <10% of the apparent K_d (15). Apparent K_d and B_{max} were calculated from Scatchard plots as described (16, 17).

Results

Accessory Molecules Are Not Required for Enhanced Peptide/Class II Complex Formation at Low pH. A recent study from our laboratory demonstrated that the functional association of peptide antigen with APC membranes, as measured by specific T cell activation, was strikingly increased at acidic pH (7). Class II–restricted T cell hybridomas were used to assess the binding of various peptide antigens to aldehyde-fixed APC. The rate and extent of functional peptide binding was markedly increased at pH 5 as compared to pH 7.3. The pH dependence of binding was preserved after pretreatment of fixed APC with pH 5 buffer, suggesting that pH had a direct effect on the interaction of peptide with APC mem-

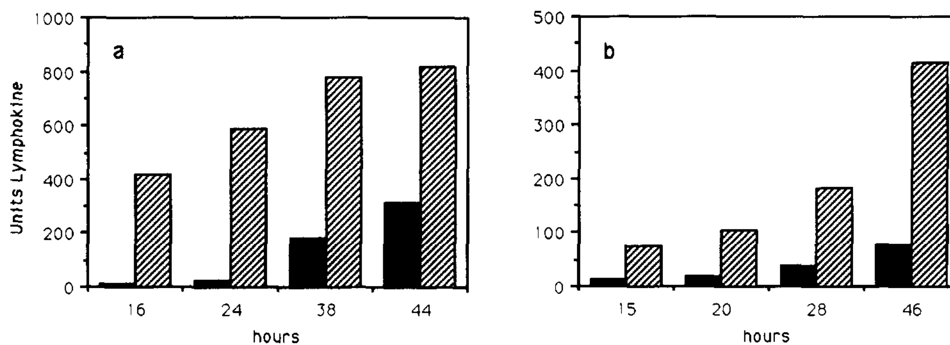

Figure 1. Effect of pH on the functional association of peptide with purified class II in supported liposomes. (*a*) Response of Hd-1.AC5 T cells to supported I-E^d liposomes exposed to HEL (104-120). (*b*) Response of DO-11.10 T cells to I-A^d liposomes exposed to OVA(323-339). I-E^d and I-A^d liposome-coated particles were suspended in 0.15 M citrate/phosphate buffer, pH 7.2 (*filled bars*) or pH 5.0 (*hatched bars*). Aliquots were incubated with 30 μM peptide for various time periods, washed, and cultured (10^5 particles/well) with T cell hybridomas (10^5/well) in duplicate wells of 96-well tissue cultures plates for 24 h in RPMI containing 10% FCS. Lymphokine production was determined as described in Materials and Methods.

branes. Potential pH-dependent interactions involving membrane components, other than class II, could not be excluded in these experiments. To explore the possibility that accessory molecules may be involved in the pH dependence of peptide/class II complex formation, further experiments were performed with artificial membranes reconstituted with purified class II. I-A^d and I-E^d were purified from detergent-solubilized A20 B lymphoma membranes by immunoaffinity chromatography and reconstituted into liposomes by detergent dialysis. Silica gel particles, coated with liposomes, were capable of stimulating class II–restricted T cell hybridomas in cultures containing appropriate peptide antigens. T cell responses were completely dependent upon the presence of the appropriate class II glycoproteins (data not shown). The effect of pH on peptide binding was evaluated in this system. I-E^d liposomes were exposed to 30 μM HEL(104-120) for various periods of time, washed, and cultured with Hd-1.AC5 T cells. The extent of peptide/class II complex formation was greater at pH 5 as compared to pH 7 for all time points (Fig. 1 *a*). Similar results were obtained with I-A^d liposomes and OVA(323-339) using DO-11.10 T cells (Fig. 1 *b*). These results indicated that accessory membrane proteins are not required for pH-dependent peptide binding, supporting the hypothesis that pH directly regulates the binding of antigen to Ia.

No lymphokine was detected in cultures containing liposomes that had been exposed to peptide for 9 h or less. Incubation periods >24 h were required to saturate functional binding sites at pH 5 in these experiments (Fig. 1). By contrast, apparent saturation was approached in <2 h at the optimum pH in our previous studies (7) using aldehyde-fixed APC. It was possible that the rapid apparent saturation observed in our previous experiments resulted from the inability of the T cells to distinguish between very high peptide/class II densities. To address this possibility, fixed APC were exposed to peptide for 4 and 18 h, washed, and titered into cultures with specific T cell hybridomas. Alternatively, anti–class II antibody was titered into cultures containing a uniform number of peptide-pulsed APC to modulate the effective density of Ia on individual cells. No difference was detected in the density of peptide/class II complexes on 4-h vs. 18-h pulsed APC (Fig. 2). Thus, there appears to be a real difference in the time required to saturate binding sights on class II liposomes as compared to fixed APC.

Measuring Peptide/Class II Complexes with an Immunoassay. An immunoassay was developed to measure direct binding of peptide antigen to purified class II in detergent solutions. Biotinylated derivatives of HEL(104-120) and Myo(106-118) were synthesized and found to retain activity in T cell assays, with a slight reduction in potency, indicating that the biotin group does not prevent peptide interaction with Ia or the TCR. Biotin peptide was incubated with purified class II at 37°C to allow binding followed by dilution and neutralization in buffer containing milk protein. Bound and free peptide were separated by binding to microtiter wells coated with anti–class II mAb. After washing, bound biotinylated peptide was measured by incubation with excess avidin-alkaline phosphatase followed by substrate. Binding was dose dependent, saturable, and inhibited by unlabeled peptide. No signal was obtained by using microtiter plates coated with antibody specific for irrelevant class II glycoproteins. Control experiments demonstrated a linear relationship between absorbance at 405 nm and soluble avidin-enzyme concentration. It is therefore assumed that absorbance is proportional to the quantity of bound peptide.

The specificity of the assay was evaluated by measuring the capacity of several peptides, representing known T cell determinants, to inhibit the binding of biotin-HEL(104-120) to I-E^d and biotin-Myo(106-118) to I-A^d (Fig. 3). The observed specificity of inhibition corresponded to the known specificity of the peptides tested, as determined from functional and binding experiments. OVA(323-339) (18, 19) and Myo(106-118) (20, 21) are known to bind to I-A^d, whereas HEL(104-120) binds I-E^d (7, 22). HEL(46-61) (23, 24) and pCC(91-104) (11, 25) have very low affinity for these class II molecules. Myo(106-118) partially inhibited the binding of biotin/peptide to I-E^d at higher concentrations, although

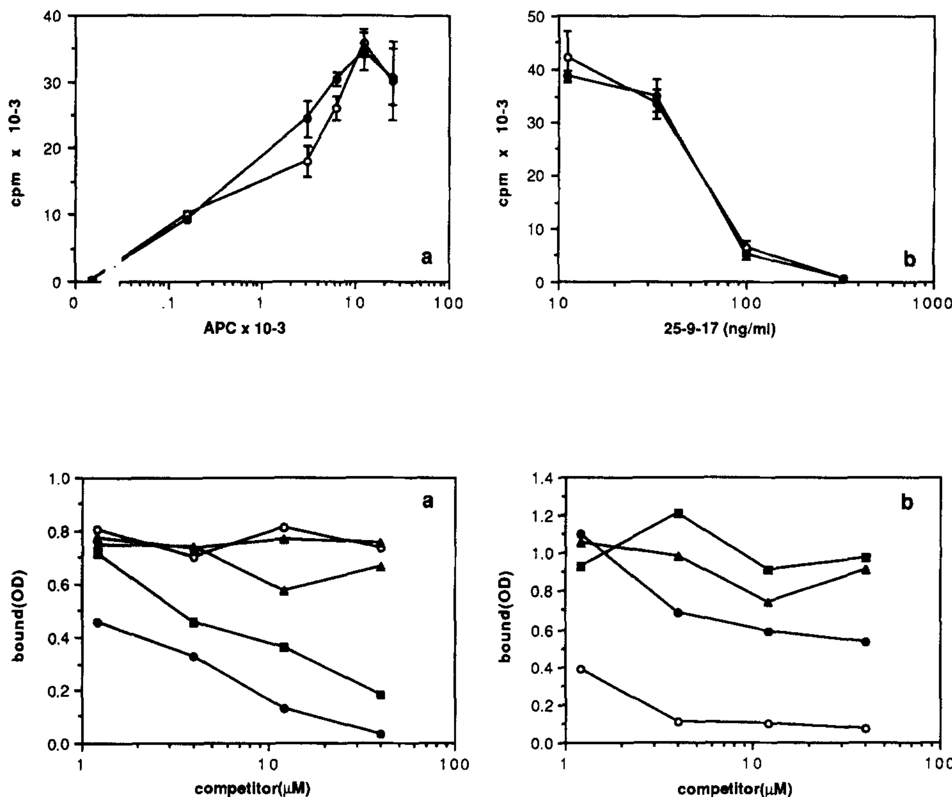

Figure 2. Rapid saturation of functional binding sites on fixed APC. Fixed TH2.2 B cells (7) were incubated for 4 h (*filled symbols*) or 18 h (*open symbols*) in 0.15 M citrate/phosphate, pH 5.0, containing 20 μM OVA(323-339). After washing, treated APC were titered into culture with 10^5 DO-11.10 T cells (*a*). Alternatively, 4 × 10^4 APC were cultured with DO-11.10 in the presence of various concentrations of the anti-I-A^d mAb, 25-9-17 (*b*). The amount of lymphokine secreted into cultures was assayed by [^{3}H]thymidine uptake of IL-2-dependent HT-2 cells cultured with a 1:2 dilution of T cell supernatant. Results represent mean ± SD cpm of triplicate cultures.

Figure 3. Inhibition of biotin-peptide binding with unlabeled peptides. (*a*) Purified I-A^d was incubated with 2 μM biotin-Myo(106-118) in the presence of various concentrations of unlabeled peptides for 48 h in duplicate, and binding was measured by immunoassay as described in Materials and Methods. The control signal, measured in the absence of unlabeled peptide, was 0.90 U. (*b*) Purified I-E^d was incubated with 2 μM biotin-HEL(104-120) for 24 h. Control binding was 1.08 absorbance units. Unlabeled peptides: Myo(106-118) (●), HEL(104-120) (○), pCC(91-104) (△), OVA(323-339) (■), HEL(46-61) (▲).

it is not known as a T cell determinant recognized in association with I-A^d. A similar result has been reported by Sette et al. (26). The affinity of I-E^d for biotin-HEL(104-120) is somewhat less than that for unmodified HEL(104-120). We cannot formally exclude the possibility that the anti–class II antibodies differentially recognize class II with a given peptide bound as opposed to other forms of the molecule. However, the antibodies used do block activation of T cells with a wide range of antigen specificities and quantitatively detect class II in immunofluorescence and immunoprecipitation studies. In addition, comparable results are obtained in immunoassays using two different anti-IA^d antibodies, MKD6 (9) and 25-9-17 (14).

Effect of pH on Binding of Biotin Peptides to Purified Class II. The binding of biotin-HEL(104-120) to I-E^d and biotin-Myo(106-118) to I-A^d was clearly pH dependent as measured by immunoassay (Fig. 4). The pH dependence of binding was in close agreement with our previously reported results from functional assays using unmodified peptides and fixed APC. Optimum binding was observed at pH 5.4–6.0 for biotin-Myo(106-118)/I-A^d. By contrast, a more acidic optimum pH of binding was observed with biotin-HEL(104-120)/I-E^d. The binding of biotin-HEL(104-120) to I-E^d was more sensitive to pH values in the range of 5–7 than was the binding of biotin-Myo(106-118) to I-A^d. We were interested in determining the relative influence of peptide sequence vs. class II structure on the pH optimum. Biotin-Myo(106-118) binds well to I-A^d and weakly to I-E^d. Optimum binding of this peptide to I-E^d occurred at a more acidic pH as compared to that to I-A^d (Fig. 4). This result suggests that class II structure has a major influence on the optimum pH of Myo(106-118)/class II binding and is consistent with the hypothesis that pH regulates peptide binding by altering the ionization state of groups within class II.

The characteristics of binding of biotin-HEL(104-120) to IE^d were further evaluated. The rate of association was markedly increased at pH 5 as compared to pH 7 (Fig. 5). The time period required to approach saturation was comparable to that observed in the functional experiments with class II liposomes. Saturation data from 66 h incubation periods consistently demonstrated pH dependence in both the apparent dissociation constant (K_d) and the apparent concentration of binding sites (B_{max}) (Fig. 6). The calculated K_d decreased approximately threefold as hydrogen ion concentration was varied from pH 6.0 (2.33 μM) to pH 5.0 (0.76 μM), reflecting an increase in affinity. The measured K_d at pH 5 ranged from 0.4 to 2 μM with different IE^d preparations and similar experimental conditions. The pH dependence within individual experiments was quite consistent. The apparent B_{max} increased approximately threefold as hydrogen ion concentra-

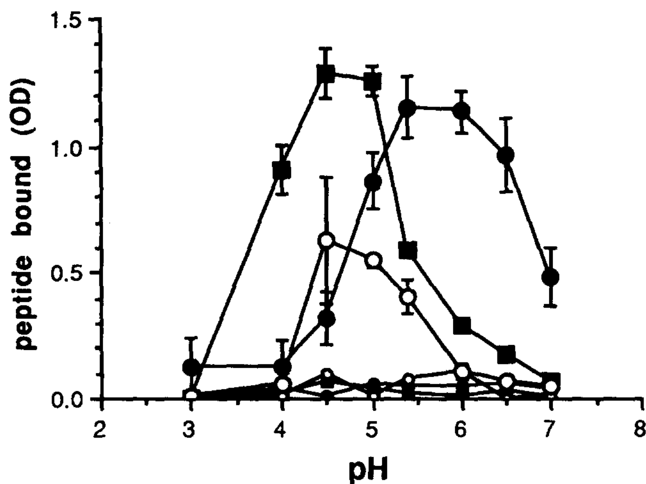

Figure 4. Optimum pH for binding of peptides to purified class II glycoproteins. Biotinylated peptides were incubated with purified I-A^d or I-E^d for 40 h at various pHs, and binding was measured by immunoassay as described in Materials and Methods. Data represent mean ± SD specific OD of triplicates (*large symbols*): 2 μM biotin-Myo(106-118)/I-A^d (●); 2 μM biotin-HEL(104-120)/I-E^d (■); or 10 μM biotin-Myo(106-118)/I-E^d (○). Nonspecific signal (*small symbols*), determined by addition of 200 μM unlabeled peptide, was subtracted from the data. Measurements reported for HEL(104-120)/I-E^d and Myo(106-118)/I-E^d were taken at 40 and 60 min, respectively, after substrate addition to facilitate comparison. Data for I-A^d binding are from a separate experiment. Results are representative of at least six experiments each.

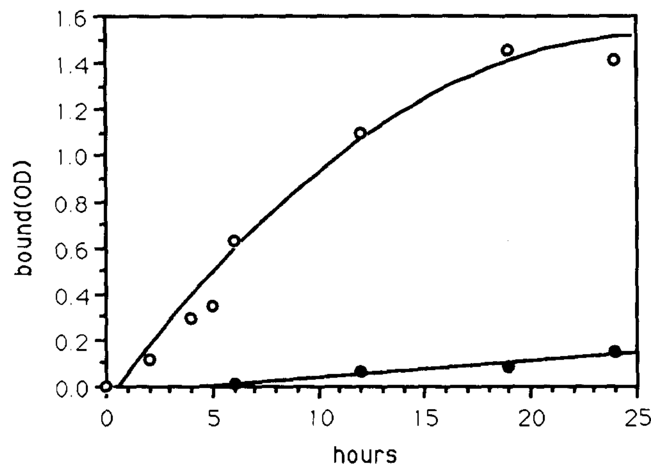

Figure 5. Increased rate of association of peptide to I-E^d at pH 5. Purified I-E^d was incubated with 20 μM biotin-HEL(104-120) at pH 5.0 (*open symbols*) or pH 7.2 (*filled symbols*) for various time periods in duplicate tubes. Binding was measured by immunoassay as described in Materials and Methods.

tion was increased from pH 6.0 (0.6 U) to pH 5.0 (1.8 U). The relative difference in apparent B_{max} observed at optimal vs. suboptimal hydrogen ion concentrations was maintained in experiments with incubation periods up to 140 h (data not shown). Very similar results were obtained in preliminary experiments measuring the binding of biotin-Myo(106-118) to IA^d (data not shown).

Since the maximal extent of peptide binding was affected by pH, we were particularly concerned about the possibility that exposure to pH 5 may: (*a*) increase the number of available peptide-binding sights by inducing dissociation of endogenous peptides already bound to Ia before purification; or (*b*) induce an irreversible conformational change in class II to increase the fraction capable of binding peptide. Pretreatment in pH 5 buffer had little effect on the pH dependent binding of peptide to fixed APC in our previous experiments (7). To further evaluate these possibilities, purified I-E^d was pre-treated for 24 h at pH 5 or 7.2, and aliquots of each were then incubated for 18 h with biotin-HEL(104-120) at each pH. Pretreatment at pH 5 had a very slight effect on the extent of peptide binding, as measured by immunoassay (Fig. 7 *a*). This effect increased with longer incubation periods in pH 5 buffer (Fig. 7 *b*). However, the pH dependence of peptide binding was retained after extensive I-E^d pretreatment in low pH buffer, indicating that a major component of the effect of pH on peptide binding is reversible.

The effect of pH on peptide dissociation was evaluated in

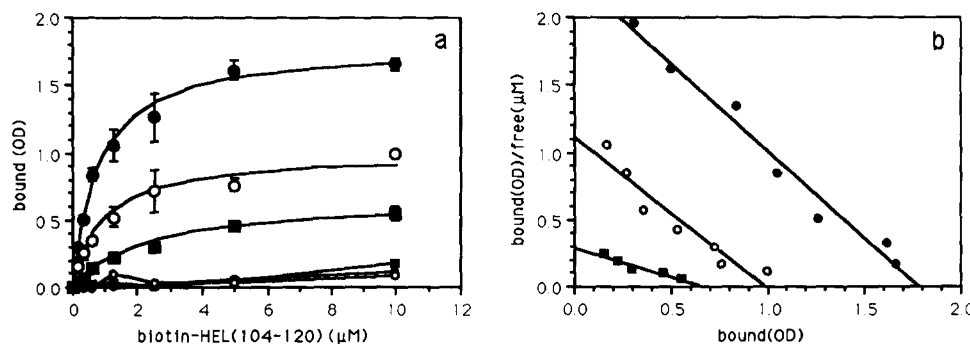

Figure 6. Effect of pH on K_d and B_{max}. Various concentrations of biotin-HEL(104-120) were incubated for 66 h with 40 nM I-E^d in citrate/phosphate buffer at pH 6.0 (■), pH 5.5 (○), or pH 5.0 (●) in triplicate tubes. (*a*) Specific binding (*large symbols*) was determined by subtraction of signal measured in the presence of 400 μM unlabeled peptide (*small symbols*). (*b*) Scatchard plots of the saturation data. The correlation coefficients for linear fit were >0.9 in each case. Data were derived from absorbance after 20-min (pH 5.0), 30-min (pH 5.5), or 45-min (pH 6.0) incubations with substrate to increase data points within the linear range of the assay and were corrected for comparison. Points with absorbance <0.2 U were excluded from the plots.

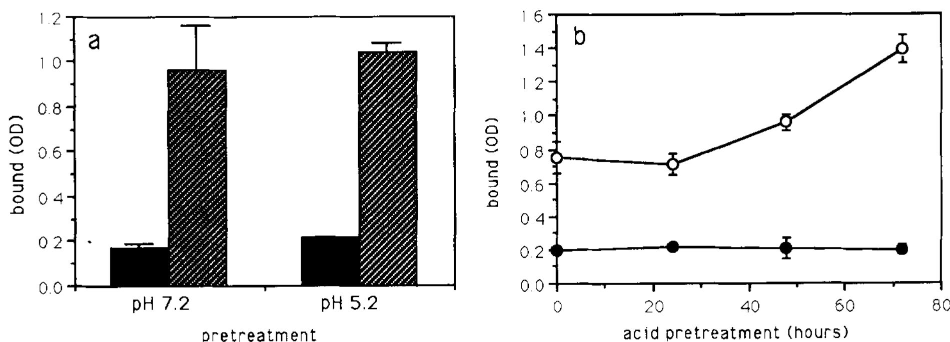

Figure 7. The effect of pH on peptide binding is largely reversible. (*a*) I-E[d] (160 nM) was pre-treated in 0.2% NP-40, 30 mM citrate/phosphate, pH 5.0 or pH 7.2, containing protease inhibitors for 24 h at 37°C. Aliquots (10 μl) were then incubated in triplicate with 5 μM biotin-HEL(104-120) for 18 h after dilution and adjustment to pH 7.2 (*filled bars*) or pH 5.0 (*hatched bars*) in 30 μl 200 mM citrate/phosphate buffer containing 0.2% NP-40. Binding was measured by immunoassay and results represent mean absorbance ± SD. (*b*) I-E[d] (160 nM) was pretreated for various time periods at 37°C in pH 5.0 citrate/phosphate buffer. Aliquots were incubated in triplicate for 18 h with 5 μM biotin-HEL(104-120) after dilution and adjustment to pH 5.0 (*open symbols*) or pH 7.2 (*filled symbols*). Nonspecific signal, determined by addition of 200 μM unlabeled peptide, was subtracted from the results.

functional and direct binding assays. No difference in peptide dissociation at pH 7 vs. pH 5 could be detected using several experimental protocols that involved extended incubation of peptide-pulsed fixed APC in the presence of excess competing peptide at each pH and indirect measurement of dissociation with T cell hybridomas (data not shown). To further evaluate dissociation, biotin-HEL(104-120) was allowed to bind to I-E[d] and free peptide was removed by dialysis. Aliquots were diluted in buffer containing 250 μM unlabeled HEL(104-120) and incubated for 66 h at pH 5 or 7.2. Remaining biotin-peptide/class II complexes were measured by immunoassay (Fig. 8 *a*). Alternatively, mixtures containing biotin peptide/class II complexes were simply diluted in buffer containing excess unlabeled peptide, without dialysis, and incubated for 66 h at pH 5 or 7 before immunoassay (Fig. 8 *b*). No pH-dependent difference in the extent of peptide dissociation was detected in these experiments. Potential heterogeneity in the susceptibility of different peptides to pH 5–induced dissociation cannot be excluded. Indeed, dissociation of a subset of low affinity peptides bound to the purified class II could account for the effect of pretreatment in low pH buffer described above (Fig. 7 *b*).

It is possible that pH regulates binding by altering the charge of amino acid side chains in peptide or class II with resultant effects on electrostatic interactions between peptide and class II that are important for binding. Bogen and Lambris (27) have proposed that electrostatic interactions between basic amino acids in peptide and acidic residues in I-E[d] may play a critical role in determining the affinity of peptides for this MHC molecule. The potential role of electrostatic interaction in peptide binding was assessed by evaluating the effect of high concentrations of NaCl on binding of biotin-HEL (104-120) to purified I-E[d]. We were surprised to observe that concentrations up to 1.5 M did not inhibit peptide binding (Fig. 9).

Discussion

The major conclusion from this study is that pH directly regulates the interaction of peptide with class II glycopro-

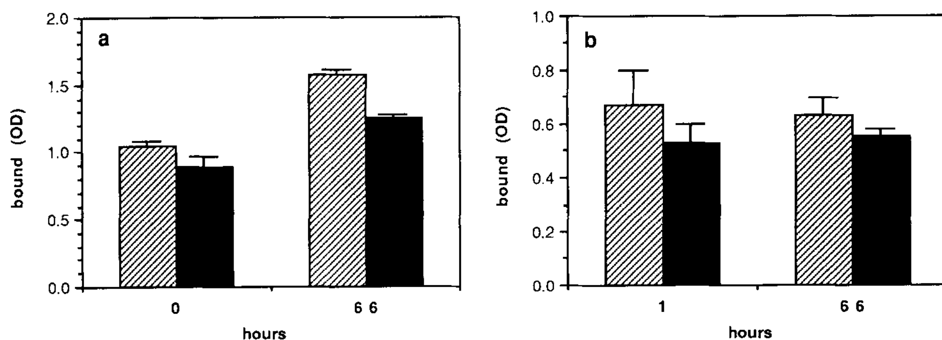

Figure 8. Effect of pH on peptide dissociation. (*a*) I-E[d] (60 nM) was incubated with 10 μM biotin-HEL(104-120) for 60 h in 0.2% NP-40, 0.15 M NaCl, 50 mM citrate/phosphate, pH 5.35, containing protease inhibitors. Free peptide was removed by extensive dialysis, and aliquots were incubated in 100 mM citrate/phosphate, pH 7.2 (*filled bars*) or pH 5.0 (*hatched bars*) containing 250 μM unlabeled HEL(104-120). Bound peptide was measured immediately or after a 66-h incubation at 37°C by immunoassay as described in Materials and Methods. Results are comparable within an immunoassay, but not between assays, and represent mean ± SD of triplicate determinations. (*b*) I-E[d] was incubated with 3 μM biotin-HEL(104-120) for 60 h at pH 5.0. Samples were then diluted in 5 vol 0.2% NP-40, 200 mM citrate/phosphate, pH 7.2 (*filled*) or pH 5.0 (*hatched*), containing 300 μM HEL(104-120) and protease inhibitors. Remaining biotin-HEL(104-120)/I-E[d] complexes were compared by immunoassay after 1- and 66-h incubations.

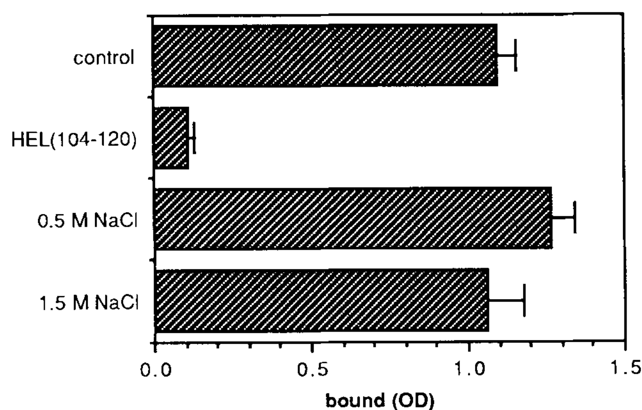

Figure 9. Binding is not inhibited by high concentrations of NaCl. I-E^d was incubated with 3 μM biotin-HEL(104-120) for 42 h in the presence of 60 μM HEL(104-120), 0.5 M NaCl, or 1.5 M NaCl as indicated. Bound biotin-peptide was measured by immunoassay as described in Materials and Methods.

tein without the obligate participation of accessory molecules. We have demonstrated that peptide binding is facilitated by acidic pH regardless of whether class II is in the environment of the plasma membrane, in artificial lipid bilayers, or isolated in detergent micelles. Our results do not exclude the possibility that interactions involving membrane components, other than class II, may play an important role in peptide/class II complex formation under physiological conditions. The discrepancy in the time periods required to approach saturation with peptide in experiments using purified class II and our previous results with fixed APC is consistent with this possibility. Apparent saturation was approached within 1–2 h at pH 5 in experiments with fixed APC, closely corresponding with the observed kinetics of antigen processing. By contrast, saturation required >20 h at optimal pH in experiments using purified class II. Qualitatively similar results were obtained in functional experiments using class II liposomes and in direct binding immunoassays. We were concerned that the maximum T cell responses observed in our previous study may have actually reflected threshold effects on T cell activation, rather than true saturation of functional peptide-binding sites. However, the results of titration experiments suggest otherwise. Thus, there appears to be a real difference in the time period required to saturate purified class II as opposed to class II present in the natural membrane environment at a given peptide concentration. It is possible that the effective local concentration of peptide is increased by interaction of peptide with non–class II membrane components, such as the HSP-70-like peptide receptor described by Lakey et al. (28) or through nonspecific hydrophobic (29) or electrostatic (7) interactions.

It is clear, however, that pH does have a marked direct effect on peptide binding by class II. We have had qualitatively similar results in functional assays using four different peptide antigens recognized in association with three different class II glycoproteins (OVA[323-339]/I-A^d, Myo[106-118]/I-A^d, HEL[104-120]/I-E^d and pCC[88-104]/I-E^k). These results are confirmed and extended in experiments using purified class II. To further characterize this phenomenon, an immunoassay was used to measure binding of biotin-labeled peptides to purified class II in detergent micelles. The specificity of binding, stability of formed complexes, and pH dependence of binding measured with the immunoassay accurately corresponded to results from functional experiments, indicating that the assay measures physiologically relevant binding interactions. Binding experiments with biotin-HEL(104-120)/I-E^d demonstrated that both the apparent K^d and B_{max} are affected by hydrogen ion concentrations in the range of pH 5.0–7.2, with no detectable change in the rate of peptide dissociation. Similar results were obtained in preliminary experiments with biotin-Myo(106-118)/I-A^d (data not shown).

Binding interactions could be altered by ionization of critical groups in peptide or in class II. The net charge, solubility, or conformation of peptide may be altered as a function of hydrogen ion concentration with potential effects on binding. HEL(104-120) contains no histidine, the amino acid residue most likely to ionize in the relevant pH range. We had previously reported that alkylation of carboxyl groups in HEL(104-120) had no effect on the pH dependence of binding as measured by T cell activation (7). This result suggested that ionization of groups in the peptide were not of major importance in the pH dependence of class II binding. Further support for this conclusion comes from the observation that 1.5 M NaCl does not inhibit peptide binding. Thus, electrostatic interactions between HEL(104-120) and I-E^d are not essential for binding. We cannot exclude the possibility that electrostatic repulsion may inhibit binding in certain situations. Indeed, it seems reasonable to expect that the ionization state of groups in peptide will influence binding for some peptide/class II combinations. The pH dependence of peptide binding was not altered by the presence of detergent, which may have effects on the solubility or conformation of peptides, providing further evidence in support of a mechanism involving ionizations in class II. Our results do not distinguish between potential effects on the ionization of groups in class II that directly interact with peptide as opposed to indirect effects on groups that may affect conformational transitions in class II. The latter possibility is appealing, however, because of the results indicating that electrostatic interactions are not essential for peptide binding, because of the slow kinetics of binding, and because a similar pH dependence was observed using several different peptide/class II combinations.

The effect of pH on the peptide binding is largely reversible. We previously reported that pretreatment of fixed APC for extended time periods with pH 5 buffer had little effect on the pH dependence of peptide binding. Similar results were obtained with purified class II, although some increase in peptide binding at pH 5 was observed after extended pretreatment in low pH buffer. It is possible that this reflects an increase in available binding sites after dissociation of a subpopulation of low-affinity peptide complexes present in the purified I-E^d. It is evident, however, that the major effect requires coincident exposure of class II to low pH and peptide. Thus, the effect of pH cannot simply be attributed to an increase in available peptide-binding sites by acid-induced

dissociation of endogenous peptide bound to class II before purification, or to an irreversible effect on class II conformation. We have been unable to find any difference in the rate of peptide dissociation at pH 5 vs. pH 7 in functional assays or experiments with purified class II. We cannot exclude the possibility that peptide antigens are heterogeneous with respect to their sensitivity to dissociation at pH 5. The peptides used in our experiments may be resistent because they were selected by virtue of immunodominance. By contrast to dissociation, both the apparent affinity (as reflected by decreased K_d) and the apparent B_{max} are increased as hydrogen ion concentration is increased from pH 7 to pH 5. The ratio of apparent B_{max} at optimal vs. that at suboptimal pH remains relatively unchanged using incubation periods from 66 to 140 h in length.

Our findings can be accounted for by an extension of the kinetic intermediate model for peptide binding proposed by Sadegh-Nasseri and McConnell (30). There investigators observed the rapid formation of unstable pCC(88-104)/I-E^k complexes (C_i),[1] and it was proposed that they represent intermediates in the formation of terminal peptide/class II complexes (C_t) that are very stable. To account for the pH dependence of apparent B_{max}, we envision a simplified kinetic model in which there is a pH-dependent equilibrium between protonated and unprotonated forms of C_i (Fig. 10). Also included in this model is the formal possibility of ionization of the free class II glycoprotein. Hydrogen ions can be regarded as modulators of stable complex formation under the experimental conditions used. In this scheme, α is a constant that describes the degree of interaction between the ligand binding and the proton binding steps, and is assigned a value greater than one. Intermediate complexes would not be detected in our experiments because of rapid peptide dissociation during the course of the immunoassay. It is assumed that protonation of a critical group(s) in C_i is required for transformation of C_i to C_t. We speculate that the latter step may involve a ligand-induced conformational change in class II or peptide displacement event. Once formed, C_t appears to be insensitive to hydrogen ion concentrations in the range of pH 5 to pH 7. This suggests that critical groups in class II do not ionize in this pH range due to alteration in pK after stable peptide binding, or that the state of protonation is not critical after stable complex formation. Using the rate constants reported by Sadegh-Nasseri and McConnell (30), and binding constants in the micrometer range, this minimal scheme predicts that both K_d and B_{max} are pH dependent (data not shown). Interpretation of the effects of pH on available binding sites is complicated by the likelihood that new sites continue to become available as a heterogeneous population of endogenous peptides slowly dissociates (31). Final conclusions will require kinetic experiments using homogeneous class II preparations, lacking peptide or containing a defined peptide.

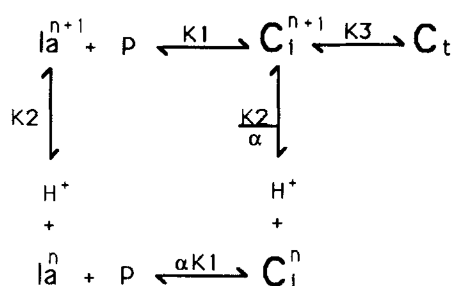

Figure 10. Model for regulation of stable peptide/class II complex formation by pH. An extension of the kinetic intermediate model of Sadegh-Nasseri and McConnell (30) is proposed, with description of pH-dependent equilibria between protonated (Ia^{n+1}) and unprotonated forms (Ia^n) of Ia and the unstable intermediate complex (C_i). It is envisioned that only the protonated form of the intermediate complex (C_i^{n+1}) is capable of forming a stable terminal complex (C_t) with peptide (P) through a step with slow on and off rates.

It is possible that different class II glycoproteins have a characteristic optimum pH for peptide binding that is determined by the pK of a group(s) whose ionization state regulates conformation or conformational flexibility. The pH profile of binding of biotin-Myo(106-118) to I-E^d was very similar to that of biotin-HEL(106-118) to I-E^d, and very different from that observed with biotin-Myo(106-118) and I-A^d (Fig. 4). I-A^d may bind peptides more readily than I-E^d in early endosomes with internal pH between 6.0 and 6.5 (32, 33). I-A^d and I-E^d may have similar capacity to bind peptides in more acidic endosomal compartments with pH between 5.0 and 5.5. A characteristic pH sensitivity might influence the repertoire of antigenic peptides available for recognition by T cells in association with products of different class II genes as a function of peptide availability in different intercellular compartments in APC. The composition of peptides available in distinct endosomal organelles may be influenced both by route of antigen uptake (34) and by potential differences in protease activity between organelles (35). This possibility can be tested by analysis of the pH dependence of binding using a large number of peptide/class II combinations.

The conclusion that antigen/class II complex formation is markedly facilitated at hydrogen ion concentrations in the range of pH 5.0–6.5 is consistent with the widely accepted idea that complex formation occurs in acidic subcellular compartments in APC. The observation that apparent B_{max} is increased at pH 5 may contribute toward the relatively low percent saturation observed by others in peptide binding studies done at neutral pH (24). Further studies will be required to determine the effect of pH on the stoichiometry of binding. This was not feasible with the experimental procedures used in the current study. The effect of pH on peptide binding may also contribute towards the inhibition of antigen processing induced by agents that raise the pH of acidic organelles (4). However, endosomal acidification does not appear to provide a mechanism for dissociation of peptide/class II complexes and re-utilization of recycling class II for presentation of new antigens (19). This is consistent with our previous results demonstrating a requirement for de novo protein syn-

[1] *Abbreviations used in this paper:* C_i, unstable peptide/class II complexes; C_t, terminal peptide/class II complexes.

thesis in antigen processing (36). As a final note of caution, we must consider the possibility that the observed increase in peptide binding at acidic pH may actually reflect the use of immunodominant determinants that were selected in vivo for their capacity to bind class II in the acidic environment of organelles in the endosomal pathway of APC. This possibility can be tested by analysis of the binding characteristics of peptides initially selected for their capacity to stably associate with class II in vitro at neutral pH.

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Address correspondence to Peter E. Jensen, Department of Pathology, Room 775 WMB, Emory University School of Medicine, Atlanta, GA 30322.

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