

An Insulin Peptide That Binds an Alternative Site in Class II Major Histocompatibility Complex

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Summary

We report that a peptide from the B chain of insulin, B(10-30), binds with high affinity to multiple class II proteins, including IA^{b,d,k}, IE^{d,k}, and DR1. The ability of B(10-30) to inhibit the binding of other peptide antigens to class II does not correlate with its affinity for class II. B(10-30) only weakly inhibits the binding of antigenic peptides. Conversely, peptides with high affinity for the peptide-binding groove of various class II proteins do not inhibit B(10-30) binding. The rate of association of B(10-30) with class II is unusually rapid, approaching saturation in 1–2 h compared with 1–2 d for classical peptide antigens in the same conditions. The dissociation rate is also relatively rapid. The B(10-30) peptide inhibits the binding of the superantigen staphylococcal enterotoxin B (SEB) to IA^k. It also inhibits SEB-mediated T cell activation. These observations support the conclusion that B(10-30) binds to a site outside the peptide-binding groove. Our findings indicate that short-lived peptide–class II complexes can be formed through interactions involving the SEB-binding site and raise the possibility that alternative complexes may serve as T cell receptor ligands.

The TCR on CD4⁺ T cells recognizes peptide antigens associated with class II MHC glycoproteins expressed on the surface of APC (1). It is now clear that peptides associate with class II molecules through a single common binding site, the peptide-binding “groove.” The first evidence for a single binding site came from the studies of Guillet et al. (2, 3), who demonstrated that the stimulation of antigen-specific T cell hybridomas by peptide antigen was inhibited by unrelated peptides that shared a common capacity to be recognized by T cells in association with the same class II molecules. The demonstration of specific binding of peptides to purified class II proteins provided a basis for the molecular analysis of class II–peptide interactions (4, 5). Buus et al. (6) demonstrated that the ability of a peptide to inhibit binding of a specific peptide to purified class II proteins directly correlated with the capacity of the peptide to bind to that particular class II protein. Thus all peptides with high affinity for a class II protein compete for a single common binding site (6). This observation was universally confirmed in subsequent class II–peptide-binding studies. The high-resolution crystal structure of HLA-DR1 has recently provided a detailed image of this common peptide-binding site (7–9).

Another class of antigens, the bacterial and retroviral superantigens, bind to class II molecules to form complexes that stimulate T cells sharing selected T cell receptor V β domains (10, 11). The best characterized of these proteins, the staphylococcal enterotoxins and toxic shock syndrome toxin (TSST)¹ 1, each bind with high affinity to a wide spectrum

of different class II molecules (12–16). Experiments with mutant class II proteins demonstrated that amino acid substitutions differentially affect the binding of superantigens and antigenic peptides (17–20). The conclusion that superantigens bind to a site outside of the peptide-binding groove is further supported by the observation that TSST-1 does not prevent the binding of antigenic peptide to HLA-DR (21). The structures of staphylococcal enterotoxin B (SEB) and TSST-1 bound to HLA-DR1, determined by x-ray crystallography, demonstrate that these superantigens interact with overlapping sites in the $\alpha 1$ and $\alpha 1/\beta 1$ domains, respectively, that are outside of the peptide-binding groove (9, 22).

Here we report that a peptide from the B chain of insulin binds to a variety of class II proteins with high affinity. Like staphylococcal superantigens, this peptide does not efficiently inhibit the binding of antigenic peptides known to bind in the peptide-binding groove. Conversely, antigenic peptides do not inhibit the binding of the B chain peptide. This violates the general rule that unstructured peptides bind to a common site in class II molecules. Further evidence for an alternative peptide-binding site(s) and the potential implications of this finding are discussed.

Materials and Methods

Class II Purification. IE^d and IA^d were purified from detergent-solubilized A20 B lymphoma (23) membrane preparations using 14-4-4 (anti-IE^d and -IE^k) and MKD6 (anti-IA^d) mAb immunoaffinity columns (24), and IE^k and IA^k were purified from detergent-solubilized CH27 (25) B lymphoma membrane preparations using 14-4-4 and 10-2-16 (anti-IA^k) mAb immunoaffinity columns. DR1

¹Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; TSST, toxic shock syndrome toxin.

was purified from detergent-solubilized membrane preparations of the EBV-transformed homozygous LG2 cell line (DRB1*0101) using an LB3.1 (26) mAb immunoaffinity column, as previously described (24).

Protein and Peptide Antigens. Beef insulin was purchased from Elanco Products Co. (Indianapolis, IN). Peptides were synthesized in the Emory University Microchemical Facility as described previously (24). SEB was purchased from Toxin Technology, Inc. (Sarasota, FL), reconstituted, and stored as directed. Peptide sequences were MAT(17-31), SGPLKAEIAQRLEDV; HEL(46-61), NTDGSTDYGLQINSR; mCc(82-103), FAGLKKANERADLIAYLKQATK; pCc(91-104), RADLIAYLKQATAK; rabies NS(101-120), GVQIVRQIRSGERFLKIWSQ; p190(211-224) (malaria A1), YKLNIFYDLLRAKL; HIV gp41(577-595), GIKQLQARILAVERYLKDQ; NP7TPO(535-551), LDPLIRGLL-ARPAKLQV; HA(255-271), FESTGNLIAPEYGFKIS; HA(306-318), PKYVKQNTLKLAT; I(1-18), MDDQRDLISNHEQLP-ILG; B(1-30), FVNQHLGSHLVEALYLVCGERGFFYTPKA; B(1-22), FVNQHLGSHLVEALYLVCGER; B(10-30), HLV-EALYLVCGERGFFYTPKA; B(17-30), LVCGERGFFYTPKA; B(23-30), GFFYTPKA. MAT(17-31), B(10-30), mCc(82-103), HEL(46-61), and SEB were biotinylated by reaction with excess *N*-hydroxysuccinimide-biotin in dimethyl formamide (24). The peptides were precipitated in acetone and purified using HPLC, and SEB was dialyzed against PBS. Rabies NS(101-120), malaria A1 peptide, NP7TPO(535-551), HA(306-318), and HIV gp41(577-595) were biotinylated through the α -amino group before deprotection and cleavage.

Class II–Peptide-binding Assay

Solutions. The solutions used in this assay include the following: PBS = 150 mM sodium chloride, 7.5 mM sodium phosphate, dibasic, 2.5 mM sodium phosphate, monobasic, pH 7.2; BBS = 0.125 M borate-buffered saline, pH 8.2; binding buffer = 100 mM citrate/phosphate, 1 mM *N*-ethylmaleimide, 5 mM EDTA, 1 mM iodoacetamide, 1 mM benzamide, 1 mM PMSF, and 0.2% NP-40; TTBS = 500 mM Tris, pH 7.5, and 0.1% Tween-20; MTB = 5% skim milk, 1% BSA, 500 mM Tris, pH 7.5, and 0.1% Tween-20; MTBN = MTB + 0.5% NP-40; neutralization buffer = 3.5% skim milk, 0.7% BSA, 335 mM Tris, pH 7.5, 0.07% sodium azide, 0.07% Tween-20, and 0.35% NP-40; europium assay buffer = 100 mM Tris, 0.15 M NaCl, 1% sodium azide, 2 μ M diethylenetriaminepentaacetic acid (Sigma Chemical Co., St. Louis, MO), 0.5% BSA, and 0.01% Tween-40; enhancement solution A = 1 M acetate, pH 3.1, 60 mM benzoyl trifluoroacetone (Sigma Chemical Co.), 850 μ M yttrium oxide (Y_2O_3 ; Sigma Chemical Co.), and 5% Triton X-100; and enhancement solution B = 2 M Tris, pH 7.0, and 200 mM 1,10-phenanthroline (Sigma Chemical Co.) (27, 28).

Microtiter Assay Plate Preparation. 96-well microtiter immunassay plates (Immulon II; Dynatech, Chantilly, VA) were prepared by initial coating with 20 μ g/ml affinity-purified goat anti-mouse Ig in PBS for 18 h at 4°C. The plates were washed with PBS and incubated with 20 μ g/ml of class II-specific mAb in BBS for 18 h at 4°C or 2 h at 37°C. The microtiter assay plates were washed with TTBS and blocked with MTB for 30 min at 24°C. The assay plates were washed with TTBS, and 50 μ l MTBN was added to each well before sample addition.

Binding Reactions. Purified class II (20–100 nM) was incubated with various concentrations of biotin-antigen in 30 μ l binding buffer at 37°C. After incubation, reactions were neutralized with an equal volume of neutralization buffer, and 50 μ l was

transferred to prepared microtiter assay plates and incubated for 2 h at 4°C for capture of antigen–class II complexes.

Detection of Antigen–Class II Complexes. Bound biotin-antigen was detected using two different methods. Detection with avidin-alkaline phosphatase is as follows. Plates were washed with TTBS and incubated with 100 μ l of 5 μ g/ml avidin-alkaline phosphatase for 90 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 (24). OD was read at 405 nm with a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA). Detection with europium-streptavidin was as follows. Plates were washed with TTBS and incubated with 100 μ l of 100 ng/ml europium-labeled streptavidin (Wallac Oy, Turku, Finland) in assay buffer for 1 h at 4°C (27). Plates were washed with TTBS, and 100 μ l of enhancement solution A was added per well. After plates were shaken gently at room temperature for 10 min, 20 μ l of enhancement solution B was added per well and the assay plates shaken at room temperature for 10–15 min (28, 29). Fluorescence of each sample well was measured at 615 nm using a time-resolved fluorimeter (model 1230 ARCUS; LKB Wallac, Finland). The data points represent the OD or the mean fluorescent counts per second/1,000 (cps $\times 10^{-3}$) of duplicate or triplicate samples. These procedures allow comparison of relative OD or fluorescence within individual experiments but not between experiments. In saturation experiments, free peptide concentrations were assumed to equal total peptide for Scatchard plot analysis. The results shown are representative of a minimum of two separate experiments.

Culture Conditions and Lymphokine Assay. Cultures were performed in triplicate 96-well tissue culture plates in a final volume of 200 μ l/well of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 μ M 2-ME, 10 μ g/ml gentamicin, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μ g/ml streptomycin. The OVA-specific, IA^d-restricted T cell hybridoma 3DO-54.8 (5×10^4 /well), which recognizes SEB presented by class II MHC (30), was incubated with CH27 B lymphoma cells (5×10^4 /well), SEB, and peptides for 18 h. Flat-bottom, 96-well tissue culture plates were coated with various concentrations of the anti-CD3 mAb 145-2C11 in PBS for 1.5 h at 37°C. The plates were washed and 3DO-54.8 T cells (5×10^4 /well) were added and cultured for 18 h in the presence or absence of peptide. Lymphokine production, reflecting T cell activation, was quantified by using the IL-2-dependent T cell line CTLL. Culture supernatants (100 μ l) were transferred to flat-bottom 96-well tissue culture plates, freeze-thawed once, and cultured with 10^4 CTLL/well for 24 h. Each well was pulsed with 1 μ Ci of [³H]thymidine during the final 10 h of culture. IL-2 (units per milliliter) was calculated from a standard curve using recombinant human IL-2.

Results

Promiscuous Binding of an Insulin B Chain Peptide to Class II Proteins. Individual antigenic peptides generally bind to a limited number of different class II proteins, and specificity is determined by the presence of critical amino acid side chains appropriately spaced in the peptide sequence so that they can properly interact with allele-specific polymorphic pockets in the class II peptide-binding site (6, 8, 31). We observed that a peptide from the B chain of insulin, B(10-30), was unusual because it bound to all class II proteins that we tested. Biotinylated B(10-30) was incubated with purified class II proteins followed by capture with class II–

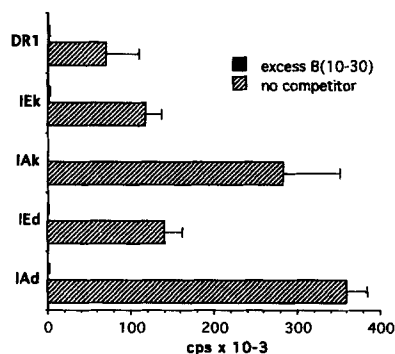


Figure 1. B(10-30) binds specifically to a broad spectrum of class II molecules. Triplicate samples of IA^d (25 nM), IE^d (100 nM), IA^k (50 nM), IE^k (100 nM), and DR1 (50 nM) were each incubated with 1 μ M biotin-B(10-30) or 1 μ M biotin-B(10-30) and 100 μ M B(10-30) at pH 5 for 18 h at 37°C. Peptide-class II complexes were captured with class II-specific mAbs, and biotin-B(10-30) binding was assayed as described in Materials and Methods.

specific mAb and peptide detection with europium-streptavidin (27). B(10-30) binds the human class II protein, DR1 as well as IE^k, IA^k, IE^d, IA^d (Fig. 1), and IA^b (data not shown). The extent of binding during an 18-h incubation differs among class II proteins with greater binding to IA than IE or DR molecules. Binding is saturable (see below) and inhibited by unlabeled B(10-30) (Fig. 1). No signal is obtained with the use of an irrelevant mAb. Thus B(10-30) binds specifically to various class II proteins.

The same core region of insulin B chain is required for binding to different class II proteins. Intact insulin does not inhibit the binding of biotin-B(10-30) to IA^k (Fig. 2 *a*), IE^k (Fig. 2 *b*), or IA^d (Fig. 2 *c*), indicating that the protein disulfide bonds must be cleaved to generate a free, unstructured B chain for class II association. Full-length B chain, B(1-30), and B(10-30), but not B(17-30), inhibit the binding of biotin-B(10-30) to IE^k, IA^k, and IA^d. The COOH-terminal residues of the B chain are not required in the core epitope since B(1-22) inhibits the binding of biotin-B(10-30). These results suggest that the minimal epitope required

for binding to different class II molecules is located within B(10-22).

Evidence for an Alternative Binding Site. The relative affinity of antigenic peptides for a given class II protein can be determined by comparing their inhibitory capacity in competitive binding assays with labeled peptide. As illustrated in Fig. 3, *a-d*, the relative ability of a panel of peptides to inhibit binding of labeled peptides to DR1 does not vary with the different labeled peptides used. This is consistent with the general observation that peptides compete for a single common binding site in class II molecules and that their ability to compete is determined only by their relative affinities. A different result, however, is obtained with B(10-30). The affinity of this peptide for DR1 is ~ 3 logs lower than HA(306-318) as measured in competition assays using four different biotin-labeled peptides, including HA(306-318) and MAT(17-31), which were defined by specific T cells (32) (Fig. 3, *a-e*). By contrast, these peptides are unable to compete with biotin-B(10-30) for binding to DR1 (Fig. 3 *e*). Low concentrations of HA(306-318) and MAT(17-31) consistently inhibit the binding of biotin-B(10-30) by 25–50%. However, the degree of inhibition is not further increased as the concentration of competitor is increased, whereas binding is completely inhibited with increasing concentrations of unlabeled B(10-30).

Similar results are obtained with IE^k and IA^k. Low concentrations of the high affinity peptide pCc(91-104) inhibit the binding of 3 different biotin-peptides to IE^k (Fig. 4, *a-c*). HEL(46-61), an immunodominant IA^k-specific peptide (4), and B(10-30) bind to the groove of IE^k with at least 3 logs lower affinity than pCc(91-104). The apparent order of affinity is reversed in competition experiments using biotin-B(10-30) (Fig. 4 *d*). HEL(46-61) inhibits the binding of biotin-HEL(46-61) to IA^k at 2–3 logs lower concentration than either pCc(91-104) or B(10-30) (Fig. 5 *a*). By contrast, unlabeled B(10-30) is much more potent than HEL(46-61) in inhibiting the binding of biotin-B(10-30) to this class II protein (Fig. 5 *b*). These results suggested that B(10-30) may bind to a site in multiple class II proteins that is outside the peptide-binding groove.

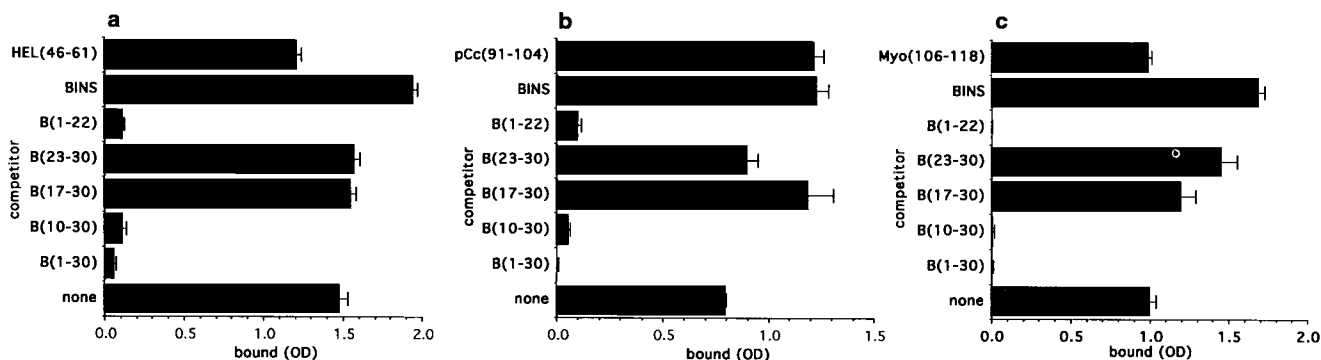


Figure 2. A common core sequence of insulin B chain determines binding to multiple class II proteins. Purified IA^k (*a*), IE^k (*b*), or IA^d (*c*) (100 nM each) were incubated in triplicate with 0.5 μ M biotin-B(10-30) for 78 h at 37°C at pH 5 with a panel of competitors (100 μ M). Biotin-peptide binding was measured as described in Materials and Methods.

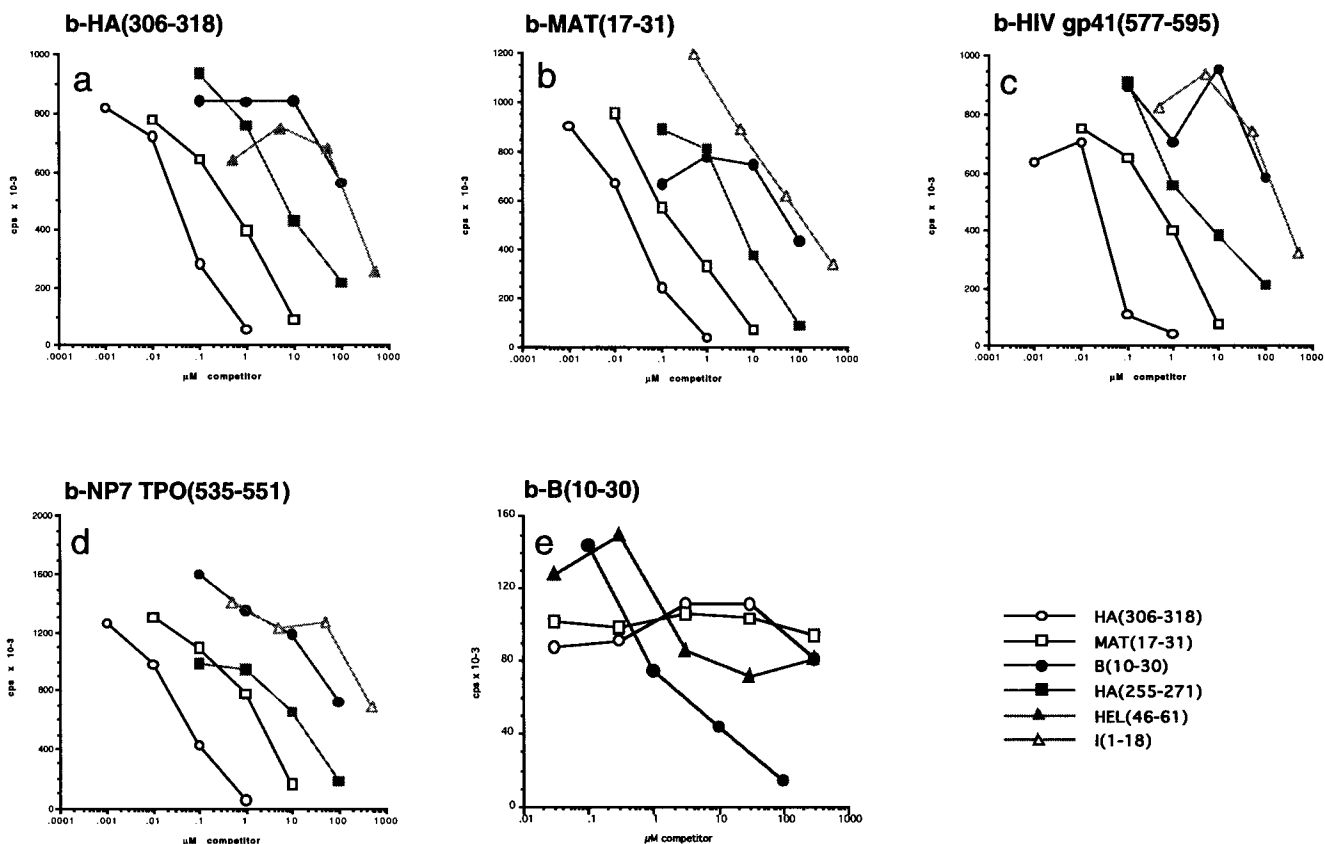


Figure 3. B(10-30) binds an alternative site in DR1. Purified DR1 (75 nM) was incubated with (a) 0.05 μM biotin-HA(306-318), (b) 0.2 μM biotin-MAT(17-31), (c) 0.02 μM biotin-HIV gp41(577-595), (d) 0.1 μM biotin-NP7 TPO(535-551), or (e) 0.1 μM biotin-B(10-30) in the presence of a panel of competitors at a range of concentrations. All incubations were carried out in duplicate at pH 7 for 18 h at 37°C. Peptide-class II complexes were captured with the mAb L-243, and biotin-peptide binding was measured as described in Materials and Methods. Fluorescence signals in the absence of competitor were (a) 819.5, (b) 1163.8, (c) 710.6, (d) 932.6, and (e) 207.6 cps $\times 10^{-3}$.

B(10-30) Binds Rapidly and with High Affinity to Class II Proteins. Antigenic peptides bind with remarkably slow association rates to purified class II proteins, reaching saturation only after 24–48 h (5, 24, 33). Binding appears to involve a rate-limiting peptide exchange reaction that is associated with a conformational change in class II molecules (24, 34, 35). Once formed, the complexes are very stable, resulting in an overall apparent equilibrium dissociation constant, K_d , in the range of 0.01–4 μM (4, 5, 24, 33). By contrast, superantigens bind with relatively fast kinetics with half-times in minutes or seconds rather than hours (15, 36, 37). The dissociation rates are also relatively rapid (36), and reported K_d values are in the range of 0.02–3 μM (15, 37–39).

The kinetics of binding of B(10-30) to class II was measured by incubating biotin-B(10-30) with purified class II proteins for various time periods. After incubations, excess unlabeled B(10-30) was added to prevent further biotin-peptide binding during the capture and biotin-peptide detection steps. Biotin-B(10-30) saturates the alternative binding site of IA^k within 1 h (Fig. 6 a). A second, slower phase of binding is often observed consistent with additional binding through the peptide-binding groove. Similar kinetics are observed with IA^d , IE^d , IE^k , and DR1 (data not shown).

Scatchard analysis of 18-h binding data gave an apparent K_d of 0.25 μM for IA^k (Fig. 6 b). A similar value, 0.62 μM , was obtained for DR1 (Fig. 6 c). These values probably underestimate the binding affinity because it is likely that a fraction of bound peptides dissociates during the immunoassay, which requires 2–3 h. The apparent K_d values are in the same range as those observed for binding of peptides to the peptide-binding groove, whereas the association rate is much faster. Thus, it is inferred that the dissociation rate must also be faster than for antigens bound to the conventional peptide-binding site, and preliminary experimental results support this conclusion (data not shown). The pH dependence of binding of B(10-30) is also different from other peptides, which generally bind to murine class II proteins much better at pH 5 than at pH 7 (24). Binding of B(10-30) to murine class II proteins is not enhanced at low pH (data not shown).

B(10-30) Inhibits the Binding of SEB Superantigen to Purified IA^k . The pattern of competitive inhibition experiments obtained with B(10-30) suggests two possible mechanisms. B(10-30) may bind to multiple different class II proteins through a common site that is distinct from the peptide-binding groove. An alternative explanation is that this pep-

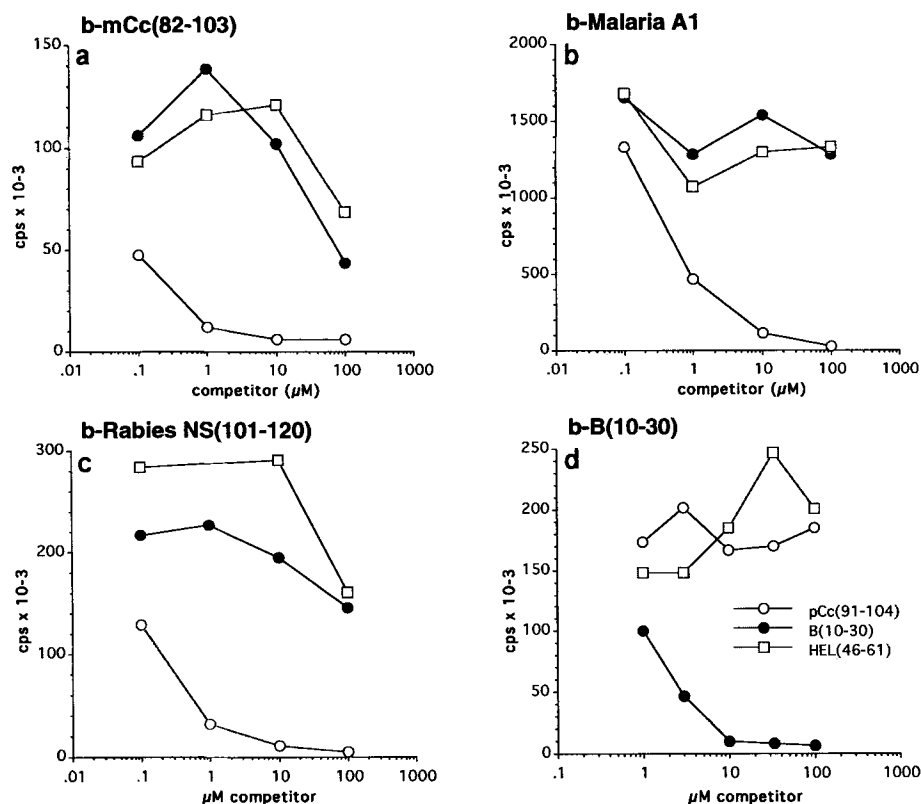


Figure 4. B(10-30) binds an alternative site in IE^k. Purified IE^k (25 nM) was incubated with (a) 0.5 μM biotin-mCc (82-103), (b) 1.0 μM biotin-malaria A1 peptide, (c) 1.0 μM biotin-rabies NS (101-120), or (d) 0.5 μM biotin-B(10-30), alone or in the presence of a panel of competitors at a range of concentrations. All incubations were carried out in duplicate at pH 5 for 18 h at 37°C. Peptide-class II complexes were captured with 14-4-4 mAb, and biotin-peptide binding was measured as described in Materials and Methods. Fluorescence signals in the absence of competitor were (a) 108.7, (b) 704.0, (c) 187.9, and (d) 196.0 cps × 10⁻³.

tide preferentially binds to a different subpopulation of class II molecules. Staphylococcal enterotoxin superantigens bind to various relatively nonpolymorphic sites in the α1 and β1 domains of class II molecules outside of the peptide-binding groove (9, 17–22). The high resolution crystal structures of SEB bound to DR1 provide a detailed image of a superantigen binding site (9). We performed experiments to determine if B(10-30) binds to a site in IA^k overlapping with the SEB binding site determined by the crystal structure. The binding of biotin-SEB to IA^k was measured by immunoassay using the europium detection system. We took advantage of the fact that the IA^k-specific mAb 10-2-16 does not interfere with SEB binding. The specificity of the SEB-binding assay is illustrated in Fig. 7 a. A strong signal was obtained when IA^k was incubated for 2 h with 25 nM

biotin-SEB, followed by capture with 10-2-16 and detection with europium-streptavidin. No signal was obtained in controls containing either protein alone, or after capture with an inappropriate mAb, MKD6, that does not bind IA^k. SEB binding was saturable, and Scatchard analysis gave an apparent K_d of 62 nM (Fig. 7 b).

B(10-30) inhibited the binding of biotin-SEB to IA^k in a dose-dependent fashion (Fig. 7 c). No inhibition was observed with HEL(46-61), which binds to the peptide-binding groove of IA^k with high affinity (4, 40). Competitive binding experiments with B chain peptides demonstrated that the same core region, B(10-22), that was required to inhibit the binding of biotin-B(10-30) (Fig. 2) also was required to inhibit the binding of biotin-SEB to IA^k (Fig. 7 d). We conclude that B(10-30) binds to a site that overlaps

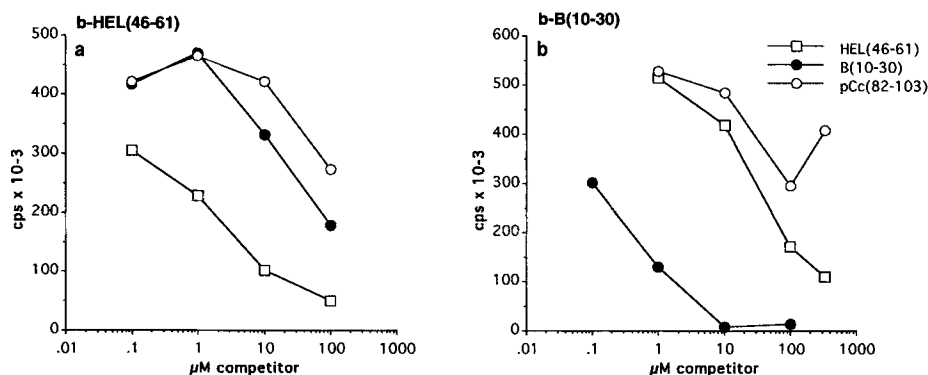


Figure 5. B(10-30) binds an alternative site in IA^k. Purified IA^k (50 nM) was incubated with (a) 0.5 μM biotin-HEL(46-61) or (b) 1.0 μM biotin-B(10-30) at pH 5 for 18 h at 37°C alone or in the presence of competitors at a range of concentrations. In each experiment, peptide-class II complexes were captured with 10-2-16 mAb, and biotin-peptide binding was measured as described in Materials and Methods. Fluorescence signals in the absence of competitor were (a) 397.5 and (b) 312.2 cps × 10⁻³.

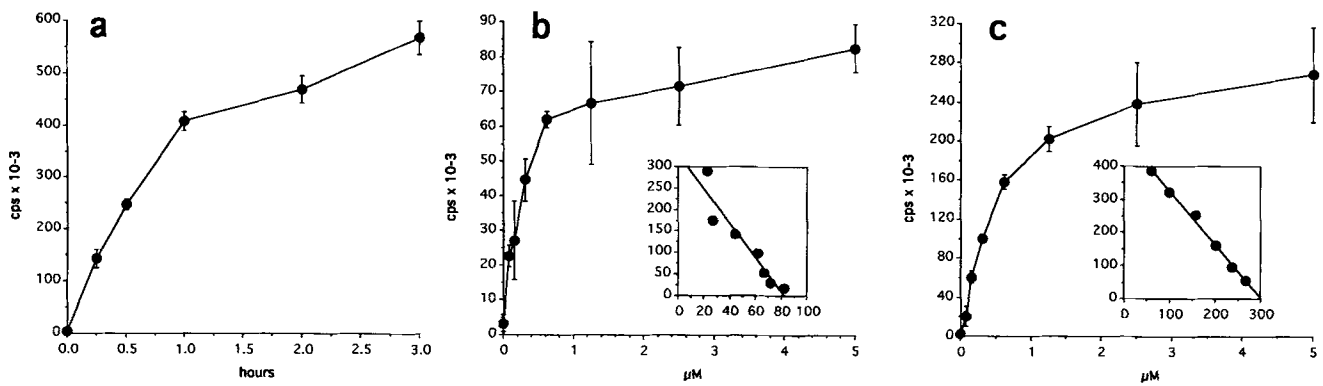


Figure 6. Rapid, high-affinity binding of B(10-30) to class II proteins. (a) Purified IA^k (50 nM) was incubated in triplicate with 5 μM biotin-B(10-30) at pH 5 for various times at 37°C. At time 0, excess unlabeled B(10-30) was added to all tubes to block additional binding of biotin-peptide. Peptide-class II complexes were captured with 10-2-16, and biotin-peptide binding was measured as described in Materials and Methods. (b) Purified IA^k (50 nM) was incubated in triplicate for 18 h at 37°C at pH 5 with a range of concentrations of biotin-B(10-30). Peptide-class II complexes were captured with 10-2-16, and biotin-peptide binding was assayed as described. $K_d = 0.25 \mu\text{M}$ by Scatchard analysis. (c) Purified DR1 (75 nM) was incubated in triplicate for 18 h at 37°C at pH 7 with a range of concentrations of biotin-B(10-30). $K_d = 0.62 \mu\text{M}$ by Scatchard analysis.

with the SEB-binding site in class II molecules and is outside the peptide-binding groove.

B(10-30) Inhibits SEB-mediated T Cell Activation. SEB stimulates V β8.3-expressing T cells such as the OVA-specific, IA^d-restricted hybridoma 3DO-54.8 (30). Activation is dose dependent and requires class II-positive APC. B(10-30) inhibited the response of 3DO-54.8 to SEB in the presence of CH27 (H-2^k) APC, whereas the control peptide, HEL(46-61), did not (Fig. 8 a). This effect was only evident with limiting concentrations of SEB. Inhibition was not a result of T cell toxicity since stimulation by the anti-CD3 mAb 154-2C11 was not altered in the presence of B(10-30) or control peptide (Fig. 8 b).

Discussion

In this report we demonstrate that a peptide from the B chain of insulin binds with high affinity to class II molecules through a site that is distinct from the peptide-binding groove. Evidence for an alternative binding site includes (a) promiscuous binding to multiple different class II molecules, (b) lack of inhibition in competitive peptide-binding experiments, (c) unusually fast binding kinetics, and (d) the ability of B chain peptides to inhibit binding of the superantigen SEB to IA^k and to inhibit SEB-mediated T cell activation.

B(10-30) was observed to bind to at least six different class II proteins. The suggestion that this peptide binds to different class II molecules in a similar manner was supported by the observation that the same core sequence, B(10-22), was required for binding to each protein. Promiscuous class II-binding peptides have previously been described (31, 41-45). However, it is very unusual for peptides to bind to both DR as well as multiple murine IA and IE molecules (6, 31, 46-48). Data from the HA(306-318)/DR1 cocrystal structure suggest that a core sequence of ~12 amino acid residues of the peptide intimately interact

with the peptide-binding groove. Given the high degree of polymorphism in the peptide-binding pockets of class II molecules, it seems unlikely that the 13-amino acid core sequence, B(10-22), would bind to multiple different class II molecules through the peptide-binding groove.

The results of competitive peptide-binding experiments provided striking contrast to the findings of previous studies. The ability of B(10-30) to inhibit the binding of known antigenic peptides did not correlate with its binding affinity. B(10-30) only weakly inhibited the binding of allele-specific antigenic peptides to DR1, IE^k, and IA^k. Conversely, high affinity groove-binding peptides did not inhibit the binding of biotin-B(10-30). Two general mechanisms could account for these results. The B chain peptide may bind to a site distinct from the peptide-binding groove, or it may bind to a different subpopulation of molecules. The hypothesis that B(10-30) binds to an alternative site is strengthened by the binding kinetics data. Peptide binding to class II molecules is generally very slow, reaching saturation only after 24-48 h (5, 24, 33). By contrast, B(10-30) binding reaches a plateau in 1-2 h, and the dissociation rate is also much higher than that observed for other peptides. These kinetics are similar to those observed for superantigen binding to class II molecules (15, 37). The pH dependence of binding also differs from other peptides, which generally bind to mouse class II molecules much better at pH 5 than pH 7. We observe very little effect of pH in this range on B(10-30) binding. In addition, B(10-30) differs from other high affinity peptides because it does not form SDS-stable complexes with class II molecules (data not shown).

Convincing evidence for an alternative binding site came from studies with the superantigen SEB, which binds outside of the peptide-binding groove (9, 17, 36). B(10-30), but not HEL(46-61), inhibited the binding of SEB to IA^k. The same core sequence of B chain that was required to inhibit the binding of biotin-B(10-30) to various class II proteins was also required to inhibit the binding of SEB to IA^k.

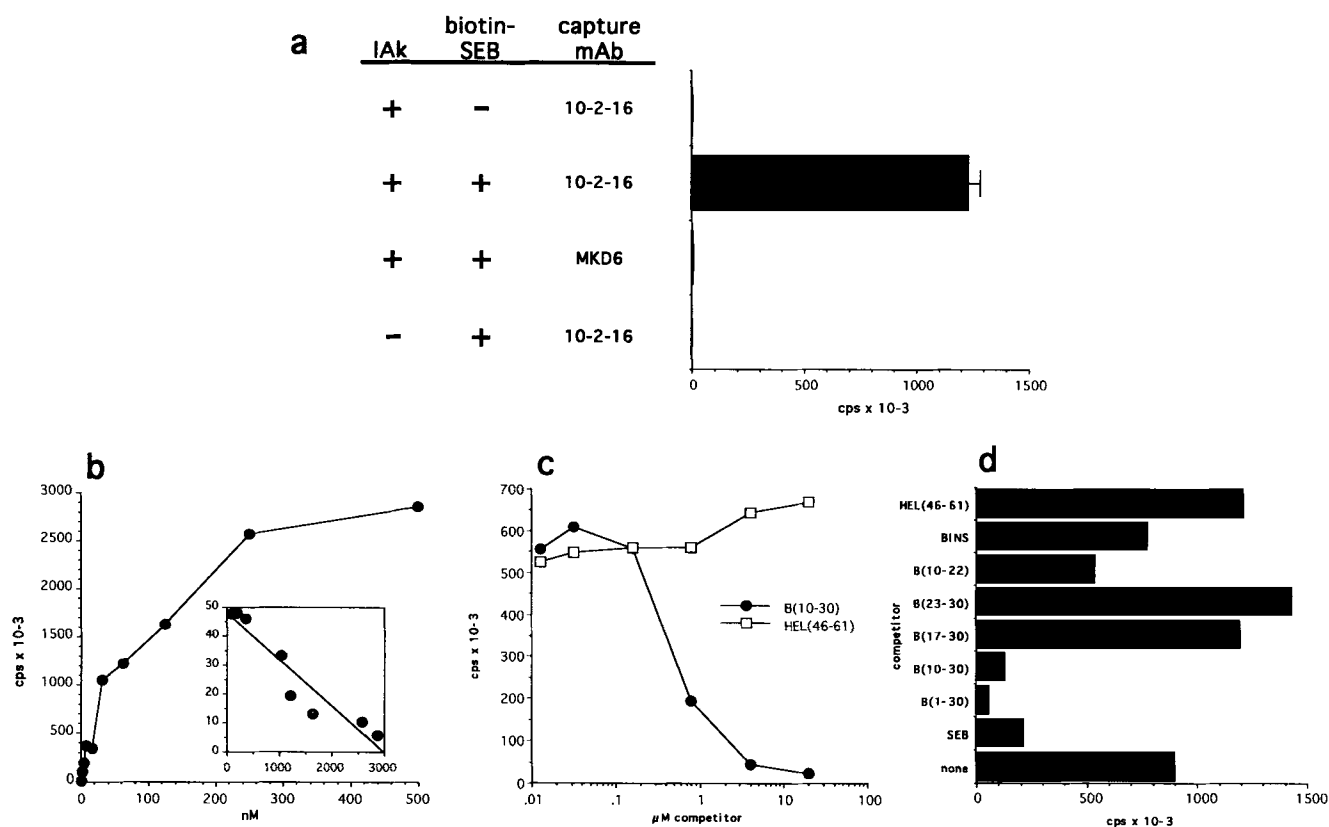


Figure 7. B(10-30) inhibits the binding of SEB to IA^k. (a) Biotin-SEB (25 nM) was incubated in triplicate alone or with 25 nM purified IA^k at 37°C for 2 h at pH 7. Biotin-SEB-class II complexes were captured with a mAb specific for IA^k (10-2-16) versus a control mAb (MKD6). IA^k was incubated alone as a negative control. (b) Purified IA^k (25 nM) was incubated in triplicate for 2 h at 37°C at pH 7 with a range of concentrations of biotin-SEB or alone. Antigen-class II complexes were captured with 10-2-16, and biotin-SEB binding was assayed as described. $K_d = 62$ nM by Scatchard analysis. (c) Purified IA^k (25 nM) was preincubated in triplicate at pH 7 for 1 h with a range of concentrations of B(10-30) or HEL(46-61). Biotin-SEB was added to each sample (25 nM final concentration), and the incubation was continued for 2 h at 37°C. Biotin-SEB-class II complexes were captured with 10-2-16 and measured with the europium fluorescence assay. (d) Purified IA^k (25 nM) was preincubated in duplicate at pH 7 for 1 h with a panel of competitors at 5 μ M concentration (SEB was at 24 μ M). Biotin-SEB was added to each sample (20 nM final concentration), and incubation continued for 2 h at 37°C. Biotin-SEB-class II complexes were captured with 10-2-16 and measured with the europium fluorescence assay.

B(10-30) also inhibited SEB-mediated activation of V β 8.3-bearing T cells. SEB exclusively interacts with the α 1 domain of DR1, contacting amino acid residues from the first and third turns of the β sheet and from the external face of the α helix (9). It is reasonable to assume that SEB binds to a similar site in IA^k (9). The assumption that SEB binds outside of the peptide-binding groove of IA^k is directly supported by the results of mutational studies with A α ^k (17). Therefore, we can conclude that B(10-30) binds to a site distinct from the peptide-binding groove that overlaps with the SEB-binding site. High affinity binding of an unstructured peptide to a site in class II molecules other than the peptide-binding groove has not previously been described.

The demonstration of an alternative binding site for B(10-30) does not rule out the possibility that this peptide may bind to a subpopulation of class II molecules. Although it is difficult to quantify the precise fraction of molecules that bind B(10-30), it is evident that all molecules do not bind the peptide under the assay conditions used. The relatively low degree of binding may be in part a result of peptide dissociation during the incubation periods required to separate bound

from free peptide. It is likely that B(10-30) can bind to a major fraction of the molecules because this peptide completely inhibits SEB binding in a dose-dependent manner. It is well established that SEB binds to class II molecules containing previously bound peptides (9, 17, 36). However, Thibodeau et al. (49) have recently demonstrated that superantigens SEB and TSST-1 bind to an overlapping site on different subsets of DR1 molecules. These data suggest that the identity of the peptides in the peptide-binding groove may influence superantigen binding (49). It is possible that groove-bound peptides may also influence the binding of B(10-30).

It is unlikely that B(10-30) inhibits SEB binding because of homology with the class II-binding region of SEB. Pontzer et al. (50) were able to inhibit the binding of SEA with a peptide from the NH₂ terminus of SEA. However, <50% inhibition was observed with millimolar peptide concentrations (50). In our study, complete inhibition of SEB binding to IA^k was observed with 4 μ M B(10-30). We have not been able to identify any significant homology between B(10-30) and sequences in SEB.

Tampe et al. (51) have demonstrated that two peptide mol-

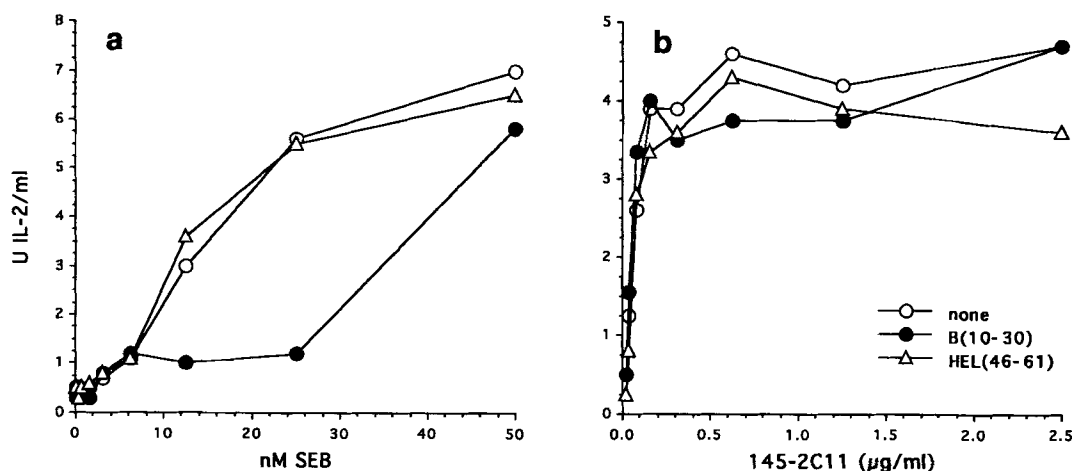


Figure 8. B(10-30) inhibits SEB-mediated T cell activation. (a) 3DO-54.8 T cells (5×10^4 cells/well) were cultured with CH27 cells (5×10^4 cells/well) for 1.5 h alone, with $50 \mu\text{M}$ B(10-30), or with $50 \mu\text{M}$ HEL(46-61), followed by addition of various concentrations of SEB and further culture for 18 h. Culture supernatants were collected and assayed for IL-2 production as described in Materials and Methods. (b) 96-well flat-bottom tissue culture plates were coated with a range of concentrations of the anti-CD3 mAb 145-2C11 (0.01–2.5 $\mu\text{g/ml}$). The T hybridoma 3DO-54.8 was added to the plates (5×10^4 cells/well) and cultured for 18 h alone or with $50 \mu\text{M}$ peptide as indicated. Culture supernatants were collected and assayed for IL-2 production as described in Materials and Methods.

ecules can bind simultaneously to a single class II molecule. It has been shown that certain peptides can enhance the dissociation (52) or association (53) of unrelated peptides. This mechanism presumably occurs through a two-peptide intermediate. The site in the class II protein where the second peptides may bind has not been defined. It is possible that they bind to the same site as B(10-30). Thus, although peptides bound in the groove may influence the binding of superantigens to external sites (49), the binding of peptides to an external site may also influence the binding of peptides to the groove. The fast kinetics of binding of B(10-30) to class II are reminiscent of the kinetics of formation of short-lived peptide–class II complexes that may represent intermediates in the formation of long-lived complexes (34, 54–56). Although these short-lived complexes have been shown to stabilize empty class II molecules at 37°C (56), there is no unequivocal evidence for their role as intermediates in the formation of stable complexes. Indeed, Mason and McConnell (57) have demonstrated short-lived complexes between IA^k and a peptide from myelin basic protein that do not lead to the formation of long-lived complexes. Our results with insulin B(10-30) raise the possibility that peptides may interact with a site outside the peptide-binding groove to form the short-lived complexes described in previous studies (34, 54–57). The concept that peptides other than B(10-30) can bind to a common site outside the peptide-binding groove is supported by the observation that a tryptic digest of OVA inhibits biotin–B(10-30) binding to class II (data not shown). In addition, we have found that the IA^d -binding peptide Myo(106–118) inhibits the binding of biotin–B(10-30) to multiple class II proteins. The overlapping peptide Myo(110–121) was shown by Beeson and

McConnell (54) to form both short-lived and long-lived complexes with IE^d .

The ability of peptides to bind an alternative site in class II molecules introduces the possibility that such peptide–class II complexes may be recognized by T cells. Although the interaction of B(10-30) with class II shares some features with superantigen binding, it is clear that this peptide does not stimulate large populations of T cells like superantigens. Superantigens interact with class II molecules and T cell receptor $\text{V}\beta$ domains through separate domains whose function depends on the integrity of the tertiary structure. Unlike superantigens, B(10-30) is a relatively short unstructured peptide with a spatial orientation that is probably determined by its interaction with class II. In this way it is similar to classical groove-binding peptide antigens. The suggestion that short-lived peptide complexes can be recognized by T cells is supported by the observation that an antigenic peptide from myelin basic protein binds to IA^k with a rapid rate of association and a 30-min dissociation half-time (57). Despite its antigenicity, this peptide does not form long-lived complexes with IA^k (57). In preliminary studies we have isolated insulin B chain-reactive, IE^k -restricted T cell hybridomas. It seems likely that these T cells may recognize B chain bound to the alternative site since B chain has very low affinity for the peptide-binding groove of IE^k as measured by competitive binding experiments with pCc(91–104). In addition, we have previously characterized a group of T cells that recognize a B chain epitope in an MHC-unrestricted but class II-dependent manner (58). We are presently exploring the possibility that these cells recognize complexes formed by insulin B chain associated with an alternative binding site in class II molecules.

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