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Binding of an invariant-chain peptide, CLIP, to I-A major histocompatibility complex class II molecules

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ABSTRACT **Invariant chain (Ii) associates with major histocompatibility complex (MHC) class II molecules and is crucial for antigen presentation by class II molecules. The exact nature of Ii interaction with MHC class II molecules remains undefined. A nested set of Ii peptides, CLIPs (class II-associated Ii peptides), have been eluted from various MHC class II molecules, suggesting that CLIPs correspond, at least in part, to the Ii motif which blocks the conventional peptide binding site in MHC class II molecules. Here we report how CLIPs interact with class II MHC molecules, I-A. We have identified regions critical for binding of CLIPs and I-A class II molecules. In most cases, the binding of CLIPs to a number of I-A molecules is modulated by the steric bulk of methionine residues at positions 93 and 99. In addition, the binding of CLIPs to an I-A molecule, I-A^u, is sensitive to substitutions at aspartic acid-59 in the α chain and threonine-86 in the β chain, whereas the binding of an antigen-derived peptide is not. Taken together, these results provide an insight as to how CLIPs bind to MHC class II heterodimers.**

Invariant chain (Ii), a monomorphic glycoprotein, plays a crucial role in the assembly of major histocompatibility complex (MHC) class II molecules (1–3). The Ii gene encodes two polypeptide chains, one called p31 (M_r 31,000), and another, less abundant, called p41 (M_r 41,000) (4). Ii functions as a surrogate peptide in the endoplasmic reticulum, interferes with peptide loading, and enhances folding of MHC class II molecules (5–8) for their exit from the endoplasmic reticulum to endocytic compartments (6, 7, 9–11). Ii and MHC class II molecules make a nine-chain complex composed of three Ii chains and three $\alpha\beta$ heterodimers before their exit from the endoplasmic reticulum (12). This complex is then transported into acidic endosomal compartments via the Golgi complex, where Ii is removed. The removal of Ii allows the binding of exogenously derived peptides to MHC class II molecules. These molecules are then transported to the cell surface for presentation to T cells of the helper subtype. MHC class II molecules which fail to bind the antigenic peptides within an antigen-presenting cell (APC) may form large aggregates and may eventually be degraded (13). Because Ii protects MHC class II molecules in the endoplasmic reticulum and directs the complex into a compartment for antigenic peptide interaction with MHC class II molecules, Ii has been recognized as an important molecule in antigen processing for MHC class II molecules (see ref. 3 for review). However, the exact nature of Ii binding to MHC class II molecules remains undetermined.

Recently, a nested set of peptides, CLIPs (class II-associated Ii peptides) derived from amino acids 80–104 of Ii has been isolated and sequenced from various MHC class II molecules (14–19). The segment of Ii (corresponding to exon 3) that is critical for association with MHC class II molecules also contains the CLIP sequence (20). The binding of CLIPs appears to be different from that of normal antigenic peptides. Unlike the latter, CLIPs fail to confer SDS stability on class II dimers (18).

To understand the biological function and the mechanisms of CLIP binding to MHC class II molecules, we have synthesized a set of peptides with single amino acid substitutions along the length of the CLIP sequence to determine residues crucial for its binding to MHC class II molecules. Subsequently, multiple substitutions were generated at certain key positions in CLIP to determine whether CLIP binding to MHC class II molecules is modulated sterically. Finally, site-specific mutants in I-A^u were utilized to determine the interaction of CLIPs with MHC class II molecules. Overall, these results show that the binding of CLIPs to MHC class II molecules is regulated by the steric bulk of methionine residues. In addition, we have identified one conserved residue and one nonconserved residue in I-A^u that are required for CLIP interaction.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Peptides. B-cell lines A20 (I-A^d), CH27 (I-A^k), and M12.C3 transfected with cDNAs encoding I-A^u α and β chains (21) were used as APCs. An ovalbumin (Ova) 323–339 peptide-specific, I-A^d-restricted T-cell hybridoma, 3DO 5.4.8 (22), a myelin basic protein (MBP) *N*-acetyl 1–11 (Ac1–11) peptide-specific, I-A^u-restricted T-cell hybridoma, 1934.4 (21), and a hen egg lysozyme (HEL) 46–61 peptide-specific, I-A^k-restricted T-cell hybridoma, 3A9 (23), were used in this study. Normally cells were maintained in RPMI 1640 with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), 2 mM glutamine, and 0.05 mM 2-mercaptoethanol. Cells transfected with cDNA encoding I-A^u or site-specifically mutated I-A^u were cultured in medium with G418 (200 μg/ml). The drug was removed from the cells prior to assays. Peptides were synthesized by standard *tert*-butoxycarbonyl chemistry and by purified HPLC, amino acid composition was determined, and molecular weight was confirmed by mass spectroscopy.

T-Cell Hybridoma Assays. T-cell hybridoma cells (2.5×10^4) were incubated with an equal number of APCs and the indicated concentrations of antigenic peptides in the presence or absence of competitor peptides in flat-bottom 96-well plates. After 24 hr, 50 μl of supernatant was harvested from each well and tested for interleukin 2 (IL-2) activity on the IL-2-dependent cell line HT-2 (21). Data are presented as means of duplicate and triplicate wells. In experiments where

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Abbreviations: Ii, invariant chain; CLIP, class II-associated Ii peptide; MHC, major histocompatibility complex; APC, antigen-presenting cell; IL-2, interleukin 2; Ova, ovalbumin; MBP, myelin basic protein; HEL, hen egg lysozyme.

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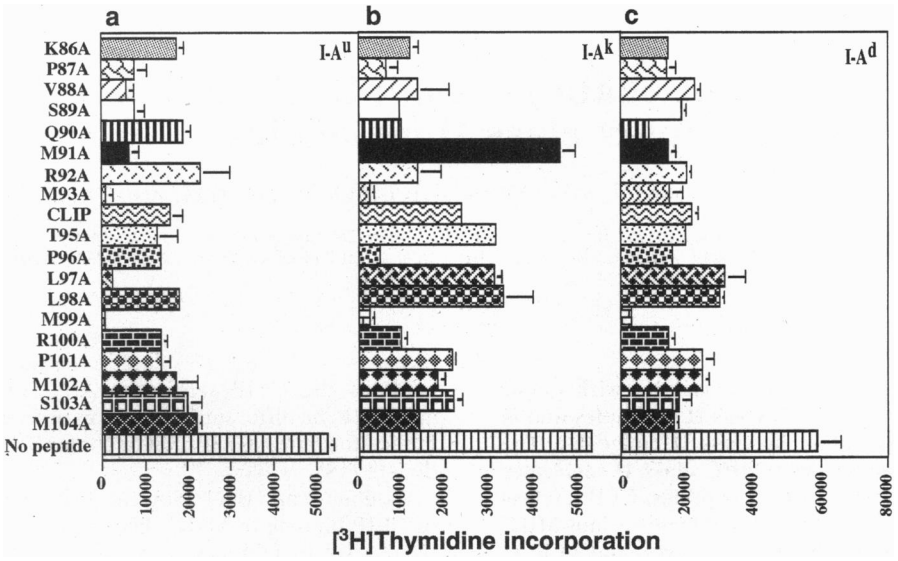

FIG. 1. Competition for presentation by alanine-substituted CLIP-(86–104). Peptides singly substituted with alanine (e.g., lysine to alanine at 86, or K86A) were tested as competitors with MBP-(Ac1–11), HEL-(46–61), and Ova-(323–339) peptides for presentation to a T-cell hybridoma to determine binding to I-A^u, I-A^k, and I-A^d, respectively (see *Materials and Methods*). Multiple concentrations of CLIP-(86–104) were used in competition assays. A single concentration of CLIP-(86–104) (50 μM for I-A^u and 25 μM for I-A^k and I-A^d) which inhibited 50% and 75% of the antigenic T-cell stimulation is shown for brevity. Stimulation was measured as supernatant IL-2 activity causing [^{3}H]thymidine incorporation in the IL-2-dependent cell line HT-2.

standard deviations are not shown, the values ranged from 1% to 15% about the mean.

Cell Surface Peptide-Binding Assay. Cells (10^5) expressing I-A^u or I-A^d were incubated in normal culture medium with biotinylated Ova-(322–339) peptide for 18–20 hr. Cells were washed three times with ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin and 0.05% sodium azide. Fluorescein isothiocyanate-conjugated streptavidin (Pierce) was then added and incubated for 30–40 min on ice. After three washes, cells were analyzed by flow cytometry (21). For inhibition of binding by competitor peptides, competitor peptides and biotinylated Ova-(322–339) were coincubated for the entire duration of the incubation. Specific binding is expressed as percentage of inhibition of control binding of biotinylated Ova-(322–339) in the absence of competitor (22).

Biochemical Analysis of Purified I-A^u Molecules. I-A^u were affinity purified from 10^{10} cells as described (22). Samples (10 μl) of I-A^u (pH 6.35–6.5) were acidified with 3.25 μl of a pH 3 solution of glycine. Immediately after acidification, samples were exposed to 5 μl of 5 mM peptide solutions (pH 7). This treatment resulted in a final pH of 4.5–5. Reaction mixtures were incubated at 37°C for 2 hr and neutralized with 1.5 μl of 2 M Tris·HCl (pH 8.5). Samples were run under nonreducing conditions in SDS/polyacrylamide gels at 4°C, blotted onto nitrocellulose membranes for 1–2 hr, blocked with 10% milk powder, and hybridized with I-A^u-specific antibody 10.3.6 (provided by H.O.M.) overnight. Membranes were washed with 10% milk powder and incubated with horseradish peroxidase-linked anti-mouse IgG (Amersham) for 45 min. Membranes were developed with an enhanced chemiluminescence (ECL) kit (catalogue no. RPN 2109; Amersham) according to the standard protocol. Membranes were exposed to x-ray films for up to 1 hr.

RESULTS AND DISCUSSION

We have used CLIP-(86–104) as a competitor peptide with antigenic peptides to determine its binding to various I-A MHC class II molecules. We first established that CLIP-(86–104) competed with antigenic peptides for presentation by I-A^k, I-A^d, and I-A^u MHC class II molecules to antigen-specific T-cell hybridomas (Fig. 1).

To determine the requirements for binding of CLIP-(86–104) to I-A, peptides with single amino acid substitutions with alanine at each position were synthesized and their ability to compete for antigen presentation with antigenic peptides in T-cell hybridoma assays was determined. Strikingly, peptides with substitutions of alanine for methionine in CLIP-(86–104) at positions 93 and 99 (M93A and M99A) displayed enhanced binding as measured by their ability to compete for antigen presentation with MBP-(Ac1–11), HEL-(46–61), and Ova-(323–339) peptides on cells expressing I-A^u (Fig. 1*a*), I-A^k (Fig. 1*b*), and I-A^d (Fig. 1*c*), respectively. Despite certain haplotypic differences there are clearly general similarities in the way these peptides competed for presentation by the various I-A molecules.

Table 1. Inhibition of biotinylated Ova-(322–339) binding by alanine-substituted CLIP-(86–104)

Peptide	% inhibition I-A^u	% inhibition I-A^d
CLIP-(86–104)	38.6	64.0
M91A	39.1	58.3
R92A	13.6	62.0
M93A	46.0	72.7
P96A	60.8	70.1
L97A	37.5	50.6
M99A	72.7	77.9
Ova-(323–339)	52.8	58.3

Percent inhibition of cell surface class II (I-A^u or I-A^d) binding of biotinylated Ova-(322–339) is shown. B cells expressing I-A^u or I-A^d were incubated with biotinylated Ova-(322–339) (15 μM) either alone or with competitor peptides (100 μM). Peptides with critical substitutions in CLIP-(86–104) are shown. Control binding (as measured by mean fluorescence) of biotinylated Ova-(322–339) to I-A^u and I-A^d was 106 with a background of 5.98 and 53.5 with a background of 13.2, respectively. All peptides have been tested in this assay at least five times. Competition with only key CLIP analogues is shown because all other alanine-substituted peptides competed essentially like wild-type CLIP-(86–104).

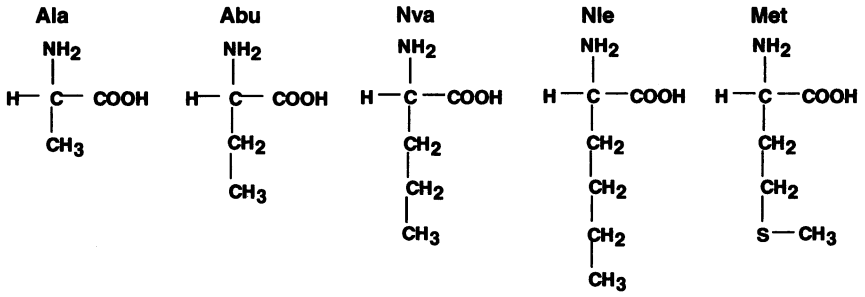

FIG. 2. Structures of alanine, α-aminobutyric acid (Abu), norvaline (Nva), norleucine (Nle), and methionine.

A similar pattern was observed when alanine-substituted CLIP peptides were tested for binding to I-A^u and I-A^d in a cell-surface flow cytometry-based assay (Table 1) (21). An I-A$^{u/d}$-binding peptide, Ova-(323–339), competed at least as well as CLIP-(86–104). Surprisingly, the majority of the other alanine-substituted peptides bound essentially like CLIP-(86–104) in both assays (Fig. 1 and Table 1). This implies that there are only a few residue-specific binding determinants in CLIP and that binding may occur via more general hydrophobic mechanisms that are little affected by alanine substitutions.

To investigate the enhanced binding to I-A observed when methionine-93 and methionine-99 were replaced by alanine and to determine its implications for CLIP binding, sets of CLIP-(86–104) peptides with multiple substitutions of hydrophobic residues at these positions were synthesized. If the presence of methionine at 93 and 99 modulates binding due to steric occlusion, and higher binding affinity results when this repulsion is obviated by alanine substitutions, then incorporation of residues with intermediate steric bulk might result in intermediate binding properties. In this case, one can argue that the CLIPs of Ii have evolved to have an "intermediate" affinity, so that they can be more easily displaced by peptides generated in endosomes. Thus, peptides with norleucine (Nle), norvaline (Nva), and α-aminobutyric acid (Abu) at positions 93 and 99 in the CLIP sequence were synthesized (Fig. 2). These substitutions provide residues approximating to the side-chain length of methionine decreasing progressively by a single methylene group to that one greater than alanine. Fig. 3 shows competition for presentation by three different cell lines expressing I-A^u, I-A^k, or I-A^d. In each case, there appeared to be reduced binding as residues with longer side chains were used. For example, peptides with Nle at 93 and 99 in place of methionine (M93Nle and M99Nle) competed like the wild type CLIP-(86–104) for all I-A molecules used in presentation assays. On the other hand, peptides with Abu residues at 93 and 99 (M93Abu and M99Abu) competed for presentation essentially like M93A and M99A. These results indicate that steric hindrance by bulky residues, such as methionines, at positions 93 and 99 may modulate the interaction with I-A molecules. Moreover, these data provide

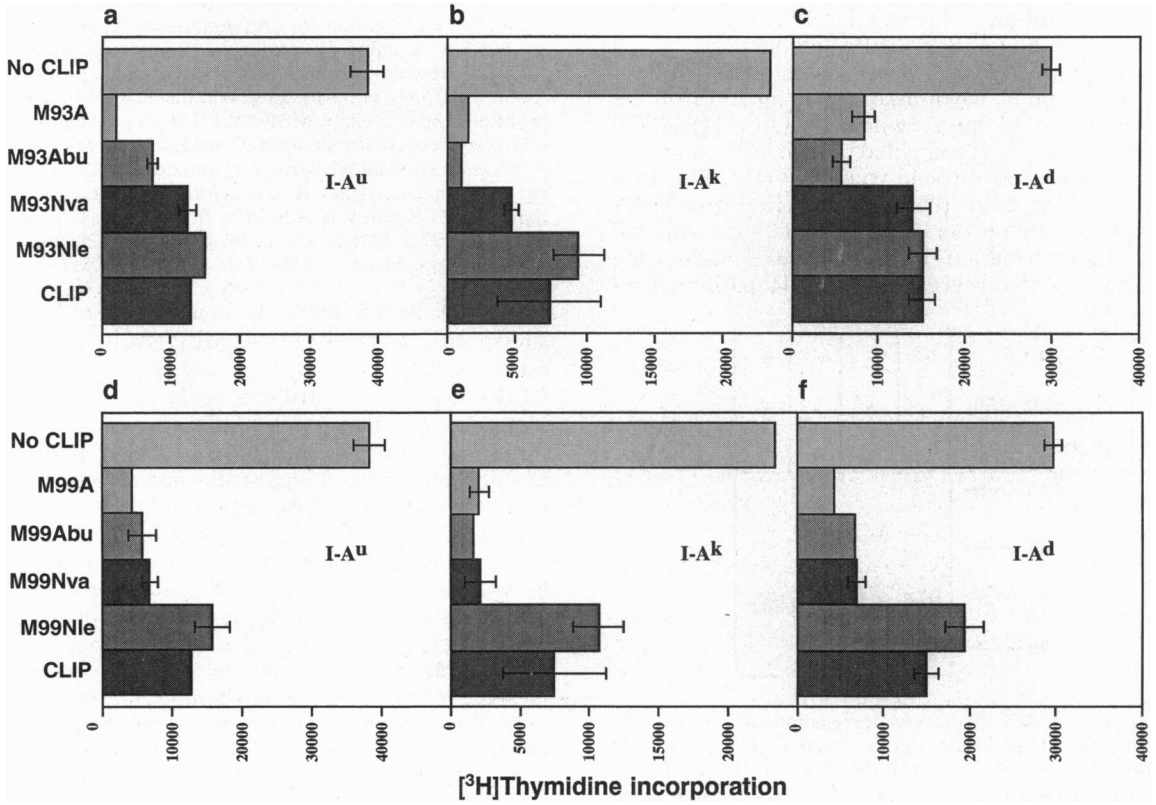

FIG. 3. Competition by peptides substituted at position 93 (*a–c*) or 99 (*d–f*) in CLIP-(86–104) for presentation on cells expressing I-A^u (*a* and *d*), I-A^k (*b* and *e*), or I-A^d (*c* and *f*). Cell numbers and antigen peptide concentrations were as in Fig. 1. Peptides were synthesized and experiments were carried out as in Fig. 1.

evidence that CLIPs with methionine residues at 93 and 99 bind with an "intermediate," or submaximal affinity. Three other methionine residues in the CLIP-(86–104) sequence have no general effect on I-A binding. Whether or not these methionines are utilized in binding to I-E class II molecules is an interesting question.

The peculiar biochemical properties of CLIPs as observed by Riberdy *et al.* (18), together with the striking pattern of these same residues (methionine-93 and -99) conferring parallel properties of CLIP binding to various MHC class II molecules (Figs. 2 and 3), argue that CLIPs bind in a distinct manner from antigen-derived peptides. This is further substantiated by our biochemical analysis on purified I-A^u class II molecules, which showed that Ova-(323–339) prevents SDS-stable high molecular mass (110–120 kDa) class II aggregation whereas CLIP-(86–104) does not (Fig. 4). Clearly, more rigorous studies utilizing wide range of antigen and Ii-derived peptides are needed to demonstrate convincingly that CLIPs bind in a different manner.

We next asked whether CLIP-(86–104) interacts at distinct locations or residues in I-A molecules. The binding region in class II molecules is not known for the Ii or CLIP association. To address this, we have utilized several sets of site-specific mutants in I-A^u class II molecules (C.P., A.M.G., H.O.M., unpublished work). Aspartic acid, a conserved residue at position 59 in the α chain, was mutated to either lysine (D59K) or alanine (D59A). Threonine, a nonconserved residue at position 86 in the β chain, was mutated to either serine (T86S) or leucine (T86L) (Fig. 5*a*). All the mutants present MBP-(Ac1–11) peptide essentially like wild-type I-A^u. Therefore, we asked whether CLIP-(86–104) would compete similarly for binding to these mutants. If CLIP-(86–104) failed to compete for binding to any of the mutants, this would suggest that the mutated residue in I-A^u was involved in CLIP-(86–104) binding. The results obtained employing the dose of CLIP-(86–104) that competed most effectively with MBP-(Ac1–11) in a T-cell hybridoma assay are shown in Fig. 5. CLIP-(86–104) inhibited the T-cell hybridoma response by up to 90%. The same dose of CLIP-(86–104) peptide, when used as a competitor on the D59K mutant, failed to compete with MBP-(Ac1–11). At the same time, CLIP-(86–104) competed well on D59A mutants, although not as well as on wild-type I-A^u (Fig. 5*b*). In a separate experiment, CLIP-(86–104) competed with MBP-(Ac1–11) very poorly on the T86L mutants but competed normally on the T86S mutant (Fig. 5*c*). These results indicate that CLIP-(86–104) interacts in proximity to aspartic acid-59

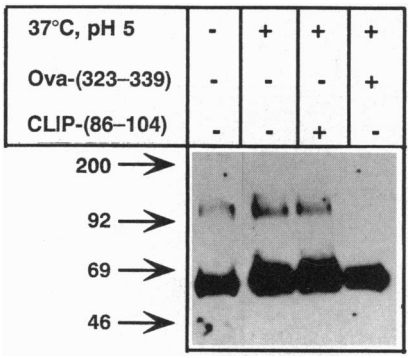

FIG. 4. Ii peptides fail to prevent aggregation of I-A^u class II molecules. Purified I-A^u molecules were exposed to pH 4.5–5 at 37°C in the presence or absence of CLIP-(86–104) or Ova-(323–339) peptide for 1 hr. Western blot analysis was carried out with I-A^u-specific antibody 10.3.6. Aggregated (≈110 kDa) and compact (≈65 kDa) forms of I-A^u are shown with appropriate molecular mass (kDa) markers. The high molecular weight aggregates in MHC class II preparation have been reported previously (22).

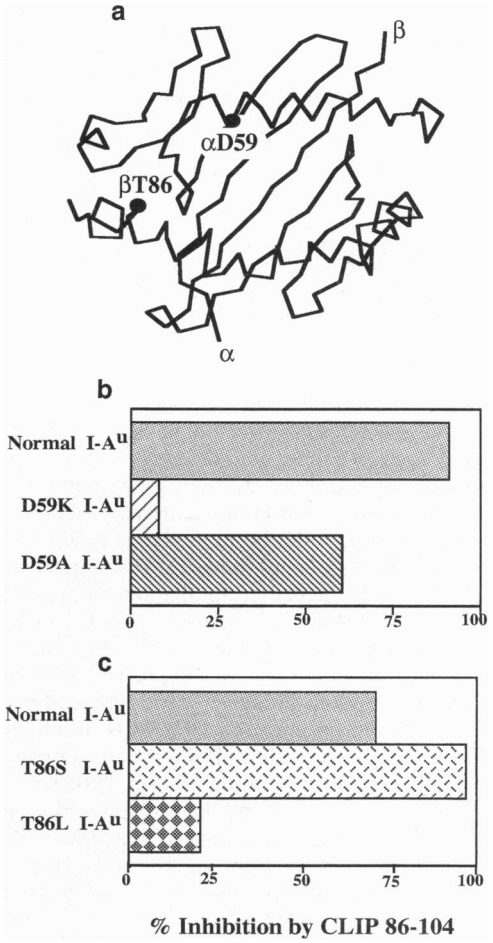

FIG. 5. Competition for presentation by CLIP-(86–104) on site-specific I-A^u mutants. (*a*) Amino acids mutated in I-A^u. (*b*) Competition for presentation on site-specific mutants at position 59 in the α chain. (*c*) Competition for presentation on site-specific mutants at position 86 in the β chain. MBP-(Ac1–11) (25 μM) and CLIP-(86–104) (400 μM) were coincubated with T hybridoma cells in the presence of APCs expressing either normal or mutated I-A^u. Supernatants were harvested and tested for IL-2 activity. [^{3}H]Thymidine incorporation (cpm) by HT-2 cells was as follows. In *b*: normal I-A^u, 10,941 ± 304; D59K, 28,089 ± 547; D59A, 22,590 ± 673. In *c*: normal I-A^u, 59,714 ± 9512; T86S, 44,629 ± 6946; T86L, 35,221 ± 2657.

in the α chain and threonine-86 in β chain of I-A^u. Since the site-specific mutants present MBP-(Ac1–11) normally, different amino acids in I–A^u are involved in binding to CLIP-(86–104) and MBP-(Ac1–11). Mutations at two other conserved positions in I-A^u (leucine to glutamic acid at position 55 in the α chain and tyrosine to phenylalanine at position 60 in the β chain) did not affect the binding of CLIP-(86–104) or MBP-(Ac1–11) (data not shown).

Interestingly, crystal structures of MHC class II molecules (24, 25) predict aspartic acid at 59 in the α chain to be at the back of the α-helix and not in the binding groove. Therefore, it is not surprising that the presentation of MBP-(Ac1–11) peptide is not affected by mutation at this position. However, it is possible that substitutions at 59 in the α chain perturb the local conformation in the class II molecules such that it affects only the CLIP binding. On the other hand, threonine at position 86 in the β chain is predicted to be on the penultimate turn of the α-helix and may form the outermost region of the peptide-binding groove. Since T86L mutation does not affect the presentation of MBP-(Ac1–11) but affects the ability of CLIP-(86–104) to compete, it is conceivable that CLIPs bind in proximity to this threonine residue in the β chain, thereby

blocking premature entry of antigenic peptide into the binding groove of MHC class II molecules. Perhaps it is also significant that both aspartic acid at 59 in the α chain and threonine at 86 in the β chain are located on the opposite sides of the binding groove. A crystal structure of a class II with Ii or CLIPs is required to confirm the nature of this interaction unequivocally.

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