

Binding of Major Histocompatibility Complex Class II to the Invariant Chain–derived Peptide, CLIP, Is Regulated by Allelic Polymorphism in Class II

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Summary

Major histocompatibility complex class II–associated invariant chain (Ii) provides several important functions that regulate class II expression and function. One of these is the ability to inhibit class II peptide loading early in biosynthesis. This allows for efficient class II folding and egress from the endoplasmic reticulum, and protects the class II peptide binding site from loading with peptides before entry into endosomal compartments. The ability of Ii to interact with class II and interfere with peptide loading has been mapped to Ii exon 3, which encodes amino acids 82–107. This same region of Ii has been described as a nested set of class II–associated Ii peptides (CLIPs) that are transiently associated with class II in normal cells and accumulate in human histocompatibility leukocyte antigen-DM–negative cell lines. Currently it is not clear how CLIP and the CLIP region of Ii blocks peptide binding. CLIP may bind directly to the class II peptide binding site, or may bind elsewhere on class II and modulate class II peptide binding allosterically. In this report, we show that CLIP can interact with many different murine and human class II molecules, but that the affinity of this interaction is controlled by polymorphic residues in the class II chains. Likewise, structural changes in CLIP also modulate class II binding in an allele-dependent manner. Finally, the specificity and kinetics of CLIP binding to class II molecule is similar to antigenic peptide binding to class II. These data indicate that CLIP binds to class II in an analogous fashion as conventional antigenic peptides, suggesting that the CLIP segment of Ii may actually occupy the class II peptide binding site.

In recent years it has become clear that invariant chain (Ii)¹ plays a central role in the functional expression of MHC class II molecules. Ii has been shown to regulate class II assembly, endosomal localization, association with antigenic peptide, cell surface expression, and functional interaction with CD4-positive T cells (1). This is dramatically illustrated in the analysis of mice lacking Ii expression (2–4), where the level of class II expression is reduced and there are significant defects in the generation and function of the CD4-positive population of T cells. One interesting feature of Ii is that different functions can often be associated with distinct segments of the Ii protein. For example, endosomal localization can be attributed to sequences in the cytosolic and transmembrane domains (5–10), class II association is mediated by amino acids 80–104 encoded by exon 3 in Ii (11–13), antigen presentation can be selectively enhanced by the p41 form of Ii (9,

14), and T cell interaction can be mediated by the chondroitin sulfate form of Ii (15).

One segment of Ii that has been the focus of much recent attention is the class II–associated Ii peptide (CLIP) region. CLIP was first identified by sequence analysis of peptides eluted from class II (16). Interestingly, in normal cells, CLIP represents only a small component of the peptide associated with class II (16–18), whereas in B cells that lack expression of HLA-DM (19, 20) or carry structural defects in class II (21), CLIP can occupy >50% of the class II molecules (22, 23). Although these results do not exclude the possibility that CLIP may simply be the predominant peptide available for class II loading in HLA-DM–negative B cells, recent studies (24) suggest that class II associated with CLIP is an intermediate in the normal processing and dissociation of Ii from class II. Interestingly, CLIP is encoded by exon 3 of Ii, the same region that is required for class II–Ii association (11–13). Taken together, these results suggest that the CLIP region of Ii forms an intimate and essential interaction with class II that persists into endosomal compartments. During pro-

¹ Abbreviations used in this paper: CLIP, class II–associated invariant chain peptide; ER, endoplasmic reticulum; Ii, invariant chain.

teolysis of Ii in endosomes, cleavage on either side of the CLIP region results in the transient association of class II with CLIP peptide in normal cells and the persistence of the class II–CLIP complex in HLA-DM defective cell lines.

One important function of Ii is the ability to interfere with class II peptide binding (25, 26). This can have profound effects on both the ability of Ii to modulate presentation of endogenous antigens (27, 28) and the ability of Ii to facilitate class II folding and transport from the endoplasmic reticulum (ER) to the Golgi apparatus (29–32). Interestingly, the ability of Ii to interfere with class II peptide loading has also been mapped to the CLIP segment (13). Thus, the CLIP segment of Ii is essential both for Ii association with class II and for the chaperone functions of Ii in the ER.

Although it is not clear where CLIP binds to class II, one possibility is that CLIP actually binds the class II peptide binding site. This possibility was originally raised when CLIP was first isolated from class II and shown to interfere with class II binding to antigenic peptides (16). In this model, CLIP would function by acting as a surrogate peptide for class II in the ER, possibly providing similar functions for class II as peptide binding in the ER does for class I (33). Alternatively, CLIP may bind outside of the peptide binding groove and modulate class II peptide binding allosterically. This possibility is supported by the inability of CLIP to generate SDS-stable class II dimers (34) (although this may be allele-dependent [17, 35], and some structural constraints on assembly of CLIP into the class II peptide binding groove (discussed in 1, 18). In this report, we show that CLIP association with class II is regulated by allelic polymorphism and is remarkably similar in biochemical parameters to class II association with known antigenic peptide. These results support the possibility that CLIP interacts with class II within the peptide binding groove.

Materials and Methods

Cell Lines and MHC Purification. The following EBV-transformed homozygous cell lines were used as sources of human HLA class II molecules (36): LG2 (DRB1*0101 [DR1]); MAT (DRB1*0301 [DR3]); PREISS (DRB1*0401 [DR4w4]); BIN40 (DRB1*0404 [DR4w14]); SWEIG (DRB1*11011 [DR5]); and PITOUT (DRB1*0701 [DR7]). In some instances, transfected fibroblasts were used: L416.3 (DRB5*0101 [DR2w2a]); TR81.19 (DRB3*0101 [DR52a]); and L257.6 (DRB4*0101 [DRw53]). For mouse class II molecules, the following cell lines were used: A20 (I-A^d, I-E^d) (37); CH12 (I-A^k, I-E^k) (37); LS102.9 (I-A^s) (38); and DB27.4 (I-A^b) (39).

MHC molecules were purified essentially as described previously (40). Briefly, human class II molecules were purified by affinity chromatography using the LB3.1 (36) monoclonal antibody. Mouse class II molecules were purified by the use of the MKD6 [I-A^d (37)]; 10.3.6 [I-A^k (37)]; 14.44 [I-E^d and I-E^k (37)]; and Y3JP [I-A^a and I-A^b (38)] monoclonal antibodies.

MHC Peptide Binding Assays. Purified mouse or human class II molecules (5–500 nM) were incubated with 5–25 nM ¹²⁵I-radiolabeled peptides for 48 h in PBS containing 5% DMSO in the presence of a protease inhibitor mixture. Purified peptides were iodinated using the chloramine-T method (41). The final concen-

trations of protease inhibitors were: 1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM *N*-ethylmaleimide, and 200 μM *N*α-*p*-tosyl-L-lysine chloromethyl ketone. Final detergent concentration in the incubation mixture was 2.6% digitonin (I-A^d and I-A^k) or 0.05% NP-40 (all other class II molecules). Class II–peptide complexes were separated from free peptide by gel filtration on Sephadex G-50 (Pharmacia, Piscataway, NJ) or TSK2000 (TOSOHAAAS, Montgomeryville, PA) columns, and the fraction of peptide bound was calculated as previously described (42). In preliminary experiments, each of the DR preparations was titered in the presence of fixed amounts of radiolabeled peptides to determine the concentration of class II molecules necessary to bind 10–20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed using this class II concentration. In the inhibition assays, inhibitory peptides were typically tested at concentrations ranging from 120 μg/ml to 1.2 ng/ml. The data were then plotted, and the dose yielding 50% inhibition was measured. Each peptide was tested in two to four independent experiments.

Peptide Synthesis. Peptides were synthesized as previously described (43). Peptides were purified to >95% homogeneity by reverse-phase HPLC. The purity of these synthetic peptides was assayed on an analytical reverse-phase column and their composition ascertained by amino acid analysis, sequencing, and/or mass spectrometry analysis.

Association and Dissociation Rates, and Scatchard Analysis. To measure association rates, 20–60 nM purified DR1 was incubated with 40,000 cpm (5–25 nM) of either radiolabeled ¹²⁵I YHA307-319 or I¹²⁵ human Ii 80–103 (M → L analogue) in the presence of protease inhibitor cocktail and determined as described in the previous section. Percent bound radioactivity was measured by gel filtration after the indicated incubation times.

To measure dissociation rates, large amounts of preformed complexes were prepared by incubating radiolabeled peptides with purified class II molecules for 2 d at room temperature. The conditions employed in this step were identical to those employed in the analytic direct binding assays, except the class II concentration was usually two- to threefold higher and radiolabeled peptide concentration 10-fold higher. After 2 d, peptide–class II complexes were isolated by gel filtration and incubated at room temperature in the presence of at least a 1,000-fold excess of unlabeled peptide (30 μg/ml) and protease inhibitors for varying amounts of time. The percent of peptide still bound to class II was then measured by repeating the gel filtration step at the indicated times.

Scatchard analysis was performed by incubating, in the conditions described above, ~100 nM of purified DR1 for 2 d at room temperature in the presence of 5–25 nM radiolabeled ¹²⁵I YHA307-319 or I¹²⁵I human Ii 80–103 (M → L analogue) and varying amounts of unlabeled HA 307–319 or human Ii 80–103.

Results and Discussion

Affinity of Class II–CLIP Interactions Is Determined by Allelic Polymorphism in Class II. To examine the ability of CLIP to interact with various class II molecules, synthetic CLIP peptides of both mouse (Ii 85–101) and human (Ii 80–103) origin were assayed for their ability to compete for binding of radiolabeled antigenic peptides. The data are summarized in Table 1. Very little difference, if any, was detected between the inhibitory capacity of mouse and human CLIP. Both peptides bound to all the class II molecules tested, indicating

Table 1. Inhibitory Capacity of CLIP and CLIP Analogues in Binding Assays Using Radiolabeled Peptide Antigens and Purified Class II

	IC ₅₀ nM values		
	Mouse Ii 85–101	Human Ii 80–103	Human Ii 80–103 (M-L analogue)
Class II mouse			
IA ^b	74	32	44
IA ^d	5.3	7.5	1,760
IA ^k	13,333	16,666	>20,000
IA ^s	32	49	1,459
IE ^d	1,186	682	ND
IE ^k	304	364	ND
Class II human			
DR1	1.2	0.89	0.63
DR2w2a	15	31	91
DR3	77	118	2,650
DR4w4	49	141	1,216
DR4w14	16	12	417
DR5	114	441	1,333
DR7	24	40	21
DR52a	27,647	16,786	ND
DRw53	13	8.2	97

Inhibitory capacity of the mouse Ii 85–101 (sequence KPVSQMRMA-TPLLMRPM), human Ii 80–103 (sequence LPKPPKPVSKMRMAT-PLLMGALPM), and human Ii 80–103, M → L analogue (sequence YLPKPPKPVSKLRLATPLLLQALPL) in various binding assays using radiolabeled peptide antigens and purified class II molecules of mouse and human origins.

that CLIP binding is remarkably degenerate in its ability to interact with different class II molecules.

Although both mouse and human CLIP bound to all class II molecules tested, the relative apparent affinity of this interaction was dramatically affected by polymorphism in class II (Table 1). For example, the inhibitory ability of CLIP varied between I-A^d and I-A^k by >1,000-fold, and between DR1 and DR52a by >10,000-fold. Interestingly, CLIP has been readily isolated from normal cells in the naturally processed form from those class II molecules that have the strongest inhibitory capacities, such as DR1, I-A^d, and I-A^b (16, 17, 44). In contrast, CLIP has not been detected, or detected at low levels, in peptides isolated from normal cell class II molecules with lower binding affinity for CLIP, such as DR3, DR7, I-A^k, and I-E^k (18, 45). Taken together, these results demonstrate that CLIP is promiscuous in its binding to class II molecules, consistent with the role of CLIP in class II folding and in class II-Ii association. However, the relative inhibiting capacity of CLIP peptides can be modulated by allelic polymorphism in class II that are mostly localized in the antigen

binding groove, consistent with the possibility that CLIP may also bind directly to the peptide binding site in class II.

Identification of CLIP Sequences that Regulate Allele-specific Class II Interaction. To measure direct binding of CLIP to class II, CLIP peptides were synthesized that contained an NH₂-terminal tyrosine. In addition, the various methionine residues in the CLIP sequence were changed to leucine to avoid oxidation during the labeling procedure. Although in most cases the analog CLIP peptides were able to compete for peptide binding at an efficiency similar to that of wild-type peptides, in some cases the analogues showed a dramatic loss in class II binding (Table 1). The most significant effect was for I-A^d binding, where the human analog peptide had <2,000-fold binding activity, as did the wild-type peptide. These data indicate that for some alleles, in particular I-A^d, I-A^s, and DR4, one or more of the four methionine residues in the CLIP peptide are critical for class II association. In addition, the strict allele association of the sensitivity of the methionine to leucine analogues further supports the idea that the ability of CLIP to interact with class II is controlled by polymorphic residues within class II.

Specificity and Kinetics of Class II Association with CLIP Are Indistinguishable from Class II Association with Antigenic Peptide. To further characterize class II-CLIP interactions, the binding of radiolabeled CLIP was compared with binding of radiolabeled antigenic peptide HA 307–319. DR1 was chosen because it bound the wild-type and analog (radiolabeled) CLIP similarly, and to avoid problems in interpretation of the results due to contaminating products of the B3 or B5 DR loci. When the kinetics of CLIP-DR1 and HA 307–319-DR1 interactions were investigated, both peptides displayed virtually indistinguishable on-rates. In both cases, the association rate appeared to follow a slow pseudo-first order kinetics and was nearly complete by 2–3 d at room temperature (Fig. 1 A). Likewise, once formed, both complexes were remarkably stable, with half-lives of 1–3 d at 37°C and 3–9 d at room temperature (Fig. 1 B). Interestingly, CLIP association rates were moderately (threefold) faster than those of HA 307–319. This may reflect the recent finding that CLIP is more readily dissociated from class II at low pH than are antigen peptides (46).

By Scatchard analysis, similar affinities were detected in two independent experiments for DR1 association with CLIP (1.1–2.1 nM) and with HA 307–319 (1.2–3.8 nM). Most importantly, the Scatchard analysis allowed estimation of the fraction of DR1 molecules accessible to binding of the HA 307–319 or the CLIP peptide. The figures obtained were remarkably similar (4.0–6.6% and 5.4–7.3% for the CLIP peptides and HA 307–319, respectively) (Fig. 2). These results suggest that: (a) only a fraction of the DR1 molecules are receptive to binding for the HA 307–319, presumably because the binding grooves of the remaining molecules are occupied by preexisting naturally processed peptides; and (b) the same fraction is accessible to DR1 binding for the CLIP peptide, suggesting that either the HA peptide and CLIP bind in the same antigen binding groove of class II, or that if the binding sites are not completely overlapping, occupancy

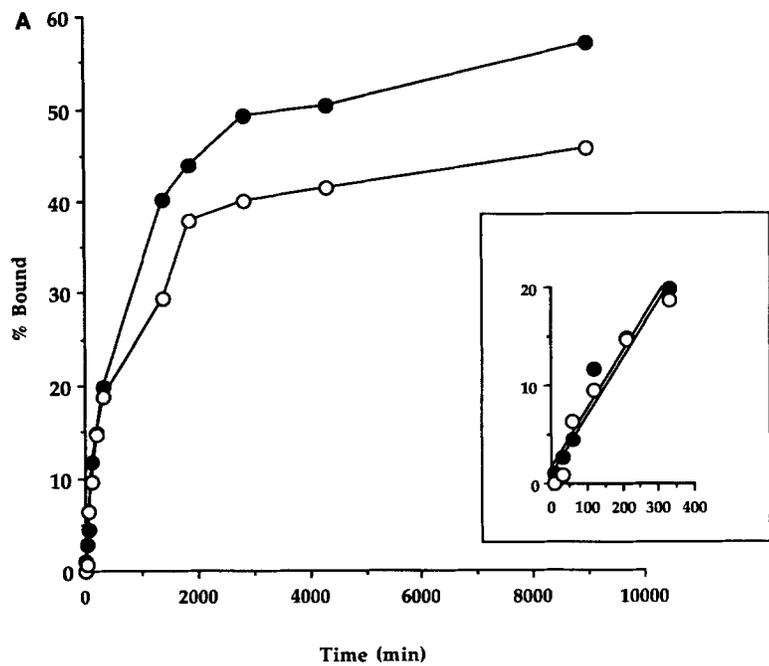


Figure 1. Kinetics of association of HA 307-319 (●) and human Ii 80-103 (M → L) to purified DR1. Association rates are shown in *A*, and the inset shows an enlargement of the early linear part of the binding isotherms. Dissociation rates are shown in *B*. Closed and open symbols refer to DR1/HA 307-319 and DR1/human Ii 80-103 (M → L) complexes, respectively. The experiments were performed both at room temperature (*squares*) or 37°C (*circles*).

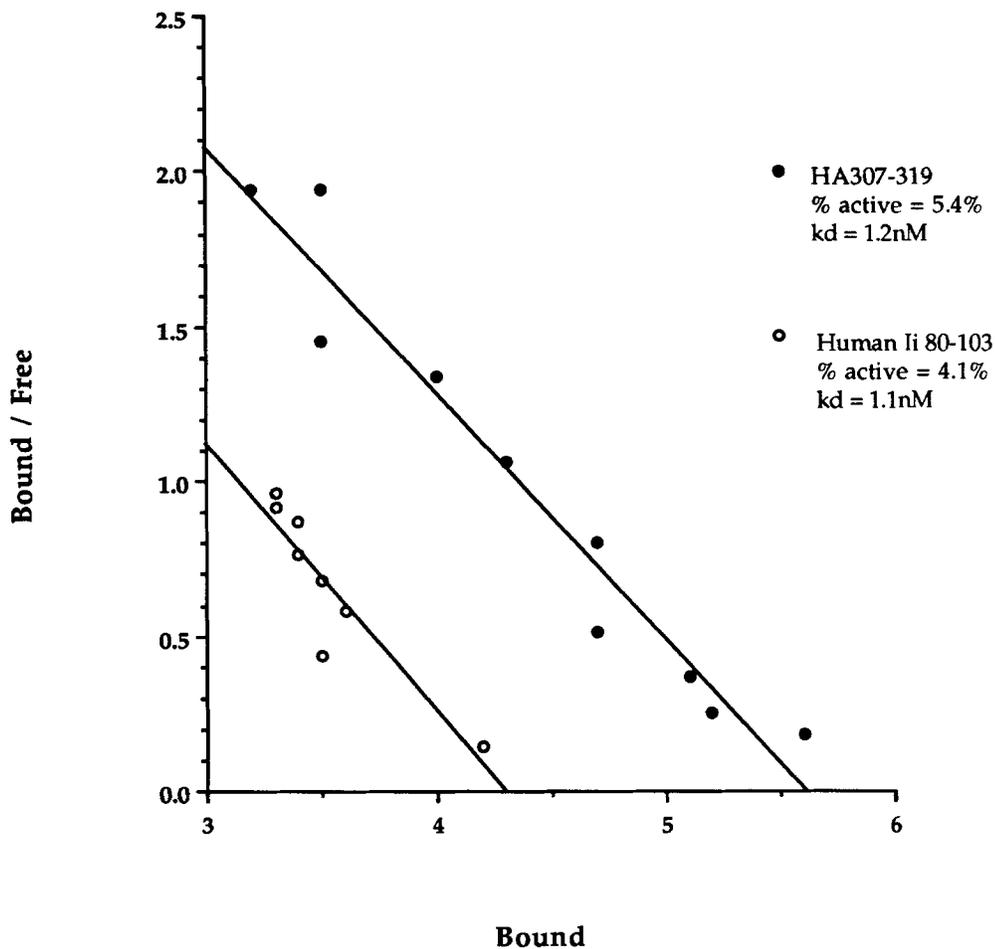
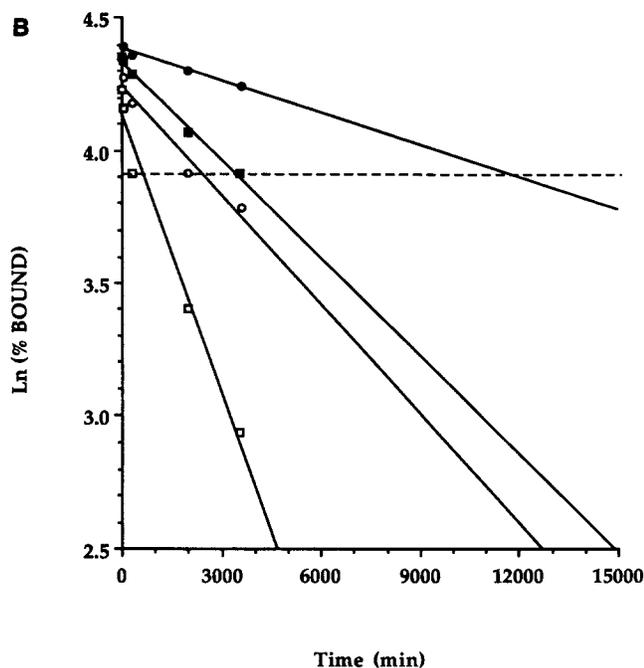


Figure 2. Scatchard plots for DR1/HA 307-319 (*closed circles*) and DR1/human Ii 80-103 (*open circles*) interactions.



of the antigen binding site would preclude occupancy of the CLIP binding site.

Finally, the specificity of the HA 307–319 and CLIP binding to DR1 was probed by the use of a panel of various peptide ligands with differing affinities for the DR1 antigen binding

groove. The same peptides were tested in parallel for their capacity to inhibit DR1-HA 307–319 and DR1-CLIP interactions. The results of this analysis are shown in Table 2. A striking similarity was observed between the two sets of data: the OVA 323 reiterative, ML65Kd 464–487, poly A .52, and the HA 307–319 and CLIP peptides were very good inhibitors of both DR1-CLIP and DR1-HA 307–319 interactions. Peptides TT 830–843, ROIV, and TT 830–843 (831Y) were intermediate inhibitors in both cases, and peptides HA 307–319 (309H), L. rep 12–26, OVA 323–336, and Lol p I 1–20 were very poor inhibitors of both interactions. In conclusion, these data illustrate the remarkable similarities in the specificity of the CLIP and HA 307–319 interactions with DR1, as probed by their capacity of being inhibited by an excess of unrelated peptide ligands.

In summary, biochemical analysis of the interaction between CLIP peptides and purified class II molecules indicates that this interaction is remarkably similar to class II binding of conventional antigenic peptides. CLIP binding is very promiscuous, yet MHC polymorphism can dramatically affect the capacity of antigenic peptides to bind class II. CLIP binding to DR1 displays the slow off-on rates characteristic of peptide binding in the antigen binding groove. Scatchard analysis suggests that the same fraction of sites is accessible for binding to CLIP and the antigenic peptide HA 307–319, and the specificity of the two interactions, in terms of their sensitivity to inhibition from unrelated ligands, is virtually indistinguishable. Although not conclusive, these data are compatible with only two explanations. According to the first

Table 2. Inhibitory Capacity of a Panel of Unrelated Peptides for the DR1/¹²⁵I HA 307–319 and DR1/¹²⁵I Human Ii 80–103, M → L Analog Interactions

Peptide	Sequence	Relative inhibitory capacity for	
		DR1/ ¹²⁵ I HA307–319	DR1/ ¹²⁵ I Human Ii 80–103 (M-L analogue)
HA 307–319	PKYVKQNTLKLAT	1.0	1.0
Human Ii 80–103	LPKPPKPVSKMRMATPLLMGALPM	5.6	12
OVA 323–339 Reiterative	YYVRAVRAYLTT	5.2	4.5
<i>M. leprae</i> 65kD Y464–487	YTLLQAAPALDKLKLGTGDEATGANI	6.0	10
Poly A .52	yAA(14)AAAKTAAAAa-NH2	17	14
TT 830–843	QYIKANSKFIGITE	0.50	0.38
ROIV	YAHAAHAAHAAHAAHAA	0.067	0.10
TT 830–843 analogue	QYQKANSKFIGITE	0.14	0.060
HA 307–319 analogue	PKHVKQNTLKLAT	0.0019	0.0013
Lambda rep. 12–26	YLEDARRLKAIYEKKK	0.0004	0.0005
OVA 323–336	ISQAVHAAHAEINE	0.0002	0.0017
Lol p1 1–20	IAKVPPGPNITAEYGDKWLD	0.0002	0.0002

To avoid discrepancies due to differences in sensitivity to inhibition of the two assays due to differential specific activity of the two labeled compounds, the data are expressed in relative inhibitory capacity. Before standardization, the IC₅₀% for the HA 307–319 was 5.0 and 30 nM in the ¹²⁵I HA 307–319 and ¹²⁵I human Ii 80–103 assays, respectively.

explanation, two different binding sites exist for CLIP and antigenic peptides. These two binding sites are so structurally similar or strictly interdependent at the structural level as to be indistinguishable at the biochemical level. It should be noted that the crystal structure of DR1 molecules has not

revealed any obvious peptide binding sites other than the antigen binding groove. Thus, according to a much simpler and perhaps more likely alternative, CLIP binds in the very same binding groove used by antigenic peptides.

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