Interference of Distinct Invariant Chain Regions with Superantigen Contact Area and Antigenic Peptide Binding Groove of HLA-DR[1]

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In the endoplasmic reticulum, MHC class II $\alpha\beta$ dimers associate with the trimeric invariant chain (Ii), generating a nine-subunit $(\alpha\beta Ii)_3$ complex. In the presence of Ii, the peptide binding groove is blocked, so that loading with self or antigenic peptides can only occur after proteolytic removal of Ii in specialized post-Golgi compartments. The class II-associated invariant chain peptide region of Ii (about residues 81–104) is known to mediate binding to class II molecules and blockade of the groove, but this does not exclude additional contact sites for Ii. Using a set of overlapping Ii peptides and recombinant soluble Ii, we demonstrate here that a large segment of Ii encompassing approximately residues 71 to 128 interacts with HLA-DR molecules. The N- and C-terminal regions of this Ii segment appear to bind outside the peptide groove to the contact area for the staphylococcal superantigen *Staphylococcus aureus* enterotoxin B on the α1 domain. The core region of this segment (residues 95–108) prevents binding of antigenic peptides, probably by interaction with the peptide groove. Occupation of the groove with antigenic peptides abolishes binding not only of the core region, but also that of those Ii peptides that bind outside the groove. These findings suggest the existence of distinct conformational states of class II molecules, with Ii binding preferentially to one form. *The Journal of Immunology,* **1995, 155: 4757–4765.**

The MHC class II-associated Ii[3] is a monomorphic type II transmembrane glycoprotein (1) that decisively influences the quality of Ag presentation by MHC class II molecules (2). In the human system, the Ii gene encodes four polypeptide chains originating from differential splicing of the corresponding mRNA (3): the p33 form as well as the less abundant p41 form are both found without and with N-terminal extensions resulting in the p35 and the p43 forms (4, 5). Ii forms trimers in the ER and chaperones proper assembly of MHC α- and β-subunits, finally generating a nonameric complex $(\alpha\beta Ii)_3$ (6). In the ER and during transport to endocytic compartments, $\alpha\beta$Ii complexes are not accessible for peptides (7, 8). Furthermore, the assembly with Ii prevents $\alpha\beta$ dimers from association with polypeptides (9) or self-aggregation and inactivation (10, 11). A sorting signal in the cytoplasmic tail of Ii is responsible for efficient targeting of the nine-chain complex to post-Golgi compartments of the endocytic pathway (12, 13), where loading with self or antigenic peptides is thought to occur (14–17). Stepwise proteolytic destruction of Ii, probably by members of the cathepsin family in vesicles of the endosomal-lysosomal system (18, 19), renders $\alpha\beta$ dimers accessible for peptides derived from exogenous (20) and endogenous source proteins (21). $\alpha\beta$:peptide complexes finally travel to the cell surface for subsequent presentation of peptides to T cells (20).

Biochemical studies on several naturally occurring fragments of Ii provided insight into the interaction of Ii with class II molecules; a p25 Ii fragment was found to be associated with $\alpha\beta$ dimers. p25 is a soluble C-terminal Ii fragment (22) starting at position 98 of the p31 form of murine Ii (23) or at positions 91, 93, and 99 of the human p33 form (24). In contrast, treatment of cells with the cysteine protease inhibitor leupeptin leads to accumulation of a 22-kDa membrane-anchored N-terminal leupeptin-induced polypeptide (25) that trimerizes and is still engaged in a nonameric complex with MHC $\alpha\beta$ dimers (26). Both findings are in agreement with the results of systematic exon deletions and N- or C-terminal truncations of recombinant Ii, arguing that the membrane-proximal segment formed by amino acids 81 to 105 of exon 3 is crucial for interaction of Ii with class II molecules (27–29), whereas the region between amino acids 163 and 183 seems to be essential for Ii trimerization (28). Further evidence underlining the capability of Ii region 81 to 105 to bind promiscuously to $\alpha\beta$ dimers comes from a nested set of Ii peptides spanning the above region. These CLIP (30) have been eluted from several murine (31, 32) and human class II molecules extracted from cells (21, 33, 34). They vary in length from 14 to 28 residues (21, 35) and are potent inhibitors of antigenic peptide binding to class II molecules (30, 36).

It is still a matter of discussion, whether CLIP is a residual fragment of Ii proteolysis that remains bound to class II $\alpha\beta$ dimers or whether it rebinds after proteolysis of Ii. However, the finding that the number of $\alpha\beta$:CLIP complexes is drastically reduced during transport from endosomes to the plasma membrane in B cells

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[3] Abbreviations used in this paper: Ii, invariant chain; ER, endoplasmic reticulum; CLIP, class II-associated invariant chain peptides; SEB, *Staphylococcus aureus* enterotoxin B; sDR1, soluble DR1; HA, influenza virus hemagglutinin; MBP, myelin basic protein; IM, influenza virus matrix protein; AMCA, 7-amino-4-methyl-coumarin-3-acetic acid; HPSEC, high performance size-exclusion chromatography; rIi, recombinant Ii.

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(37) and the fact that purified αβ:CLIP complexes cannot be distinguished from those generated by limited in vitro proteolysis of αβ:Ii complexes (34) favor the hypothesis that the CLIP segment of Ii associates with αβ dimers during formation of the nonameric complex early after biosynthesis in the ER. In human wild-type B cells, a small but significant amount of αβ dimers of various HLA-DR and -DQ isotypes is associated with CLIP under steady state conditions (21, 38), whereas in HLA-DM-negative Ag-processing mutants, such as T2 transfected with HLA-DR3, -DR4, or -DR11, CLIP was revealed to be the predominant self-peptide bound to DR molecules (30, 34). The presence of HLA-DM appears to prevent the accumulation of αβ:CLIP complexes and leads to proper peptide loading of class II molecules (39, 40). Thus, DM may be directly involved in the removal of CLIP, at least in the HLA-DR-associated Ag presentation pathway (41, 42).

There are findings arguing that αβ:CLIP as well as αβIi complexes behave differently from complexes with Ag-derived peptides. Ii fails to form SDS-stable complexes with αβ dimers (43), and the same has been found for certain αβ:CLIP complexes (30, 34). CLIP is selectively released at endosomal pH (34, 44–46), αβ:CLIP complexes do not undergo time-dependent maturation (46), and single mutations outside the peptide binding groove of class II molecules prevent CLIP, but not antigenic peptide, binding (36). Conversely, other studies have shown that polymorphic residues of αβ dimers are involved in CLIP binding comparable to their involvement in antigenic peptide binding (34, 47, 48). However, it should be kept in mind that at present we can exclude neither different binding modes of CLIP for different class II alleles nor the existence of more than one binding mode for CLIP for certain class II molecules.

There exists evidence that class II molecules are flexible entities that can occur in different conformations (49, 50). For example, the recently described short-lived kinetic intermediates of αβ:peptide complexes (51–53) seem to bear alternative conformations compared with the long-lived complexes known from the crystal structure of HLA-DR1 (54). Therefore, the questions arise whether αβ dimers complexed to Ii or the CLIP region are structurally different from mature class II molecules loaded with antigenic peptides and whether CLIP cooperates with other Ii stretches for binding to αβ dimers.

In the present study we addressed the question of whether, besides CLIP, other regions of Ii would interact with class II molecules. Using a set of overlapping Ii-derived peptides and recombinant Ii we demonstrate that regions flanking CLIP contribute to the interaction of DR αβ dimers with Ii. These regions appear to contact the α1 domain outside the peptide binding groove, as superantigen SEB interferes with their binding. Unexpectedly, antigenic and, therefore, bona fide groove binding peptides were also found to inhibit those Ii peptides that bound to the SEB contact area outside the groove, suggesting the existence of two different class II conformations: one stabilized by Ii binding and another that is gradually formed in the presence of antigenic peptides according to principles of the induced fit model.

Materials and Methods

Cells

EBV-transformed B-LCL LD2B (HLA workshop no. 9083) was used as a source for isolation of DR2. Cells were maintained in RPMI with HEPES (Life Technologies, Grand Island, NY) with 5% calf serum (Life Technologies) at 37°C using roller bottles. *Spodoptera frugiperda* (Sf9) were maintained at 27°C in Grace medium (Life Technologies) in the presence of 10% FCS. For protein production, cells were grown in serum-free SF900 medium (Life Technologies). Virus infection was performed as previously described (55).

Table I. *Dissociation equilibrium constant K_d of Ii peptides for binding to HLA-DR2*

Peptide	Residues	Sequence	K_d [nM]
Ii61	61–78	RLDKLTVTSQNLQLENLR	$>10^6$
Ii71	71–88	NLQLENLRMKLPKPPKPV	1,600
Ii81	81–98	LPKPPKPVSKMRMATPLL	3,000
Ii90	90–108	KMRMATPLLMQALPMGALP	35
Ii95	95–108	TPLLMQALPMGALP	280
Ii101	101–118	ALPMGALPQGPMQNATKY	4,300
Ii111	111–128	PMQNATKYGNMTEDHVMH	6,800
Ii121	121–138	MTEDHVMHLLQNADPLKV	80/10,800
Ii131	131–148	QNADPLKVYPPLKGSFPE	$>10^{6a}$
Ii141	141–158	PLKGSFPENLTHLKNTME	$>10^6$
CLIP	81–105	LPKPPKPVSKMRMATPLLMQALPMG	23

[a] The Scatchard plot of Ii121 gave a biphasic curve, indicating a low and a high affinity binding site.

Purification of DR2

LD2B cells (5×10^9) were homogenized in hypotonic lysis buffer (20 mM Tris (pH 7.8) containing protease inhibitors (0.2 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μg/ml chymostatin)). Cytosolic proteins were separated by differential centrifugation (500 × *g*, 4°C, 10 min; 12,000 × *g*, 4°C, 10 min; 140,000 × *g*, 4°C, 1 h). Pellets containing the membrane fractions were pooled and lysed with lysis buffer containing 1% Nonidet P-40 for 1 h on ice, and nuclear material was removed by centrifugation at 500 × *g* for 10 min at 4°C. Further purification of the cell extract was performed by centrifugation for 10 min at 12,000 × *g* at 4°C and ultracentrifugation at 140,000 × *g* for 1 h at 4°C. DRαβ molecules were purified from the cell extract using a column of mAb L243 (56) coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. Glycine coupled to Sepharose was used as a precolumn. The cell extract was passed over the glycine-Sepharose and L243 Sepharose columns for 16 h. The L243 column was extensively washed with 100 mM sodium phosphate, pH 8.0, and 0.5% Zwittergent-12, followed by 100 mM sodium phosphate (pH 8.0) and 0.1% Zwittergent-12, and eluted with 100 mM sodium phosphate (pH 11.0) and 0.1% Zwittergent-12. The eluted material was neutralized with 1 M HCl and concentrated by ultrafiltration with a 20-kDa cut-off membrane (Sartorius, Göttingen, Germany). Solubilized DR molecules were kept at 4°C and a concentration of about 0.5 mg/ml.

sDR1 was obtained from Sf9 cells infected with baculovirus as previously described (55).

Purification of p25

The p25 fragment of Ii was isolated from EBV-transformed B-LCL LD2B as described previously (24).

Peptides

The following peptides spanning the region between the transmembrane domain and the putative trimerization domain of Ii were used (sequences are given in Table I): Ii-(61–78) (Ii61), Ii-(71–88) (Ii71), Ii-(81–98) (Ii81), Ii-(90–108) (Ii90), Ii-(95–108) (Ii95), Ii-(101–118) (Ii101), Ii-(111–128) (Ii111), Ii-(121–138) (Ii121), Ii-(131–148) (Ii131), and Ii-(141–158) (Ii141). Numbering is according to the p33 form of human Ii (5).

In addition, the following antigenic peptides were used: PKYVKQ NTLKLAT, HA-(307–319); PVVHFFKNIVTPRTPPPSQGK, MBP-(85–105); HLVEALYLVCGERGFFYTPKA, INS-(10–30), bovine insulin β-chain; and PLKAEIAQRLEDV IM-(19–31).

Peptides were synthesized on an AMS422 Multiple Peptide Syntheziser (Abimed, Langenfeld, Germany) using F-moc chemistry and purified by reverse phase HPLC. Purified peptides were analyzed by matrix-assisted laser desorption mass spectrometry and shown to be single species. N-terminal labeling with AMCA (Lambda, Graz, Austria) was performed as previously described (57).

Peptide binding assay

Peptide binding to DR2 and sDR1 was measured by HPSEC, as previously described (58). Briefly, purified DR2 (200 nM) or sDR1 (50 nM) molecules were coincubated with AMCA-labeled peptide (10 nM to 50 μM) in the absence or the presence of inhibitor molecules in 100 mM sodium phosphate buffer (pH 6.0) and 0.1% Zwittergent-12 (Calbiochem, La Jolla, CA).

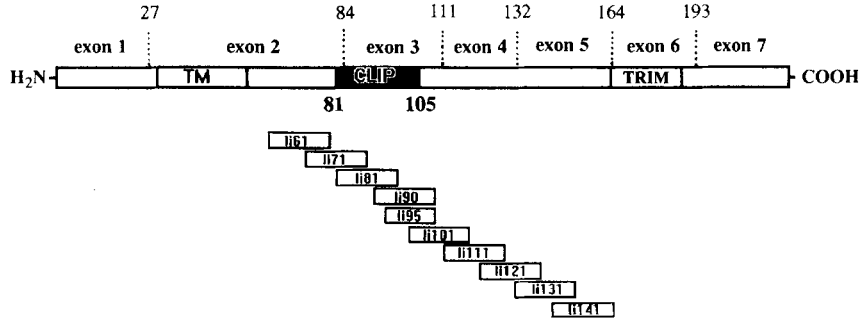

FIGURE 1. The following peptides spanning the region between the transmembrane domain (TM) and the putative trimerization domain (TRIM) of Ii were used: Ii-(61–78) (Ii61), Ii-(71–88) (Ii71), Ii-(81–98) (Ii81), Ii-(90–108) (Ii90), Ii-(95–108) (Ii95), Ii-(101–118) (Ii101), Ii-(111–128) (Ii111), Ii-(121–138) (Ii121), Ii-(131–148) (Ii131), and Ii-(141–158) (Ii141). Numbering is according to the p33 form of human Ii (5). The exon borders of the Ii gene are indicated (65).

After incubation for 40 h at 37°C, HPSEC was performed on a Superdex 75 HR 5/20 column (Pharmacia) equilibrated with 150 mM sodium phosphate buffer (pH 6.0), 0.1% Zwittergent-12, and 15% acetonitrile. The column run-through passed through a F1080 fluorescence detector (Merck-Hitachi; excitation, 350 nm; emission, 450 nm) and a L4001 UV detector (Merck-Hitachi, Darmstadt, Germany) set up in series. Signals were recorded by a model D2500 integrator (Merck-Hitachi, Darmstadt, Germany). Peptide:DR complexes eluted after 2.5 min with an elution volume of 1.5 ml. K_d values were calculated from the slope of the Scatchard plots of bound/free peptide vs bound peptide. Inhibition was calculated from fluorescence signals in the absence (F_o) and the presence (F_i) of inhibitor: % inhibition = $(F_o - F_i)/F_o \times 100$.

Recombinant Ii

The cDNA encoding the human Ii (p33 form) was obtained from the Ii expression vector IipSV51L (12) using the 820-bp fragment, which was flanked by the *Nar*I site at the 5′ end and the *Sac*I site at the 3′ end. This fragment and a double stranded synthetic oligonucleotide (TAT GGA TGA CCA GCG CGA CCT TAT CTC CAA CAA TGA GCA ACT GCC CAT GCT GGG CCG G) were introduced into the pET3a vector using the *Nde*I and *Sac*I site. The transmembrane region was deleted by removing the fragment from the *Sac*II site to the *Pst*I site, thereby restoring the 3′ C-terminal coding 40 bp with a double stranded oligonucleotide (GGC CGG CTG GAC AAA CTG ACA GTC ACC TCC CAG AAC CTG CA). At the 3′ end of the Ii coding region, a 6-His tail was introduced at the *Bst*YI site using the oligonucleotide (GAT CTG GGC CCA GTC CCC ATG CAC CAC CAC CAC CAC CAC TGA GCT). The resulting plasmid pET3a.Ii.DTMR-His was used to express rIi in *Escherichia coli* (BL21).

Expression of rIi was induced by treatment with 0.4 mM IPTG for 1 h at 37°C. Bacterial pellets were obtained by centrifugation (5000 rpm, 5 min, 4°C), resuspended in 50 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM PMSF and disrupted by freezing/thawing in liquid nitrogen. After the addition of 200 μg/ml lysozyme, the bacterial suspension was incubated for 15 min on ice. One-tenth volume of 5 M NaCl was added and incubated for an additional 15 min on ice. Bacterial extract was clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. After the addition of 2 mM $MgCl_2$ and 20 mM imidazole, the extract was loaded onto a Ni^{2+}/NTA column (Quiagen, Chatsworth, MA) and washed extensively with the following buffers: 10 column volumes of 50 mM Tris (pH 7.5), 20 mM imidazole, and 500 mM NaCl; 1 column volume of 1 M NaCl; and 20 mM HEPES (pH 7.5), 90 mM KCl, and 2 mM $MgCl_2$. Elution was performed with 250 mM imidazole. The eluted material was concentrated by ultrafiltration with a 10 kDa cut-off filter (Centriprep, Amicon, Danvers, MA) and further purified by ion exchange chromatography on a Mono S Sepharose column (Pharmacia). Elution was performed with a gradient from 90 mM to 1 M KCl. rIi eluted with 200 to 300 mM KCl and was stored at 4°C.

Results

Flanking regions of CLIP bind to DR molecules

Previous studies using Ii deletion and truncation mutants have shown that the CLIP region encoded by exon 3 is essential for binding of Ii to class II molecules (27–29). As this does not rule out additional contact sites, a series of overlapping peptides (mainly 18-mers) was synthesized spanning the region between the transmembrane and the putative trimerization domain of Ii, residues 61 to 158 (Fig. 1). Each Ii peptide was tagged at the N terminus with the fluorescence label AMCA and tested for binding to purified HLA-DR2 molecules using HPSEC and fluorescence detection. The use of peptides to mimic the behavior of defined stretches of Ii seems to be a valid approach, as the Stokes' radius of Ii trimers suggests an extended conformation without globular folding of Ii (3).

As shown in Table I, all Ii peptides except Ii61, Ii131, and Ii141 were found to bind to DR2, with peptides Ii90 and Ii95 from the CLIP region displaying the highest affinities (K_d = ~35 and 280 nM, respectively). Ii71 and Ii101 that overlap with the N- and C-terminals of the CLIP sequence, respectively, bind with lower, but significant, affinity (K_d = ~1.6 and 4.3 μM). Binding of Ii111 and Ii121 together with the lack of binding of Ii131 suggest that residues 111 to about 128 contribute to Ii binding. Likewise, the twofold higher affinity of Ii71 compared with Ii81 implies that at least some of amino acids 71 to 80 are also involved in binding. The Scatchard plot of Ii121 was biphasic, indicating two binding sites: one with low affinity (K_d = ~10.8 μM) and the other with high affinity (K_d = ~80 nM). Binding of the Ii-derived peptides was specific, as it was competed out by the respective unlabeled peptide, and no binding to purified HLA class I molecules was observed (data not shown). These data suggest that not only the CLIP region (residues 81–105), but also a larger segment of Ii ranging from approximately positions 71 to 128, can interact with class II molecules.

As class II molecules are peptide receptors, one might argue that all the Ii-derived peptides bind in the antigenic peptide binding groove like conventional peptides. To address this possibility, DR2 was simultaneously incubated with different AMCA-labeled Ii peptides. Examples are presented in Figure 2*a*. Using AMCA-Ii71 under saturating concentrations, addition of the AMCA-labeled peptides Ii90, Ii101, and Ii111 led to respective additive binding signals. Likewise, unlabeled Ii90, Ii101, and Ii111 did not inhibit AMCA-Ii71 binding (data not shown), and coincubation of the three nonoverlapping labeled peptides Ii71, Ii90, and Ii111 resulted in additive fluorescence signals, but there was no additivity when overlapping peptides such as Ii71 and Ii81 or Ii71 and Ii61 were coincubated (Fig. 2*a*). Ii121 was an exception, as it displayed only partial additive binding with Ii71. Ii121 appears to bind to an additional site on MHC class II molecules, which may be the peptide binding groove or part of it. This view is consistent with the observation that the Scatchard analysis resulted in two distinct K_d values for Ii121 (see Table I). Corresponding results for Ii peptide binding were obtained with sDR1 molecules produced in insect cells (Fig. 2*b*). sDR1 is free of detectable peptide (55) and, therefore, represents a homogeneous population. Accordingly, saturating amounts of labeled Ii71 led to 95% occupancy of sDR1 (data not shown). Simultaneous binding of Ii71 with Ii90, Ii101, and Ii101, but not with Ii61, Ii81, or Ii121 confirms the results obtained with DR2 and excludes the possibility that Ii peptides bind to distinct DR subpopulations. Together, these data indicate that the

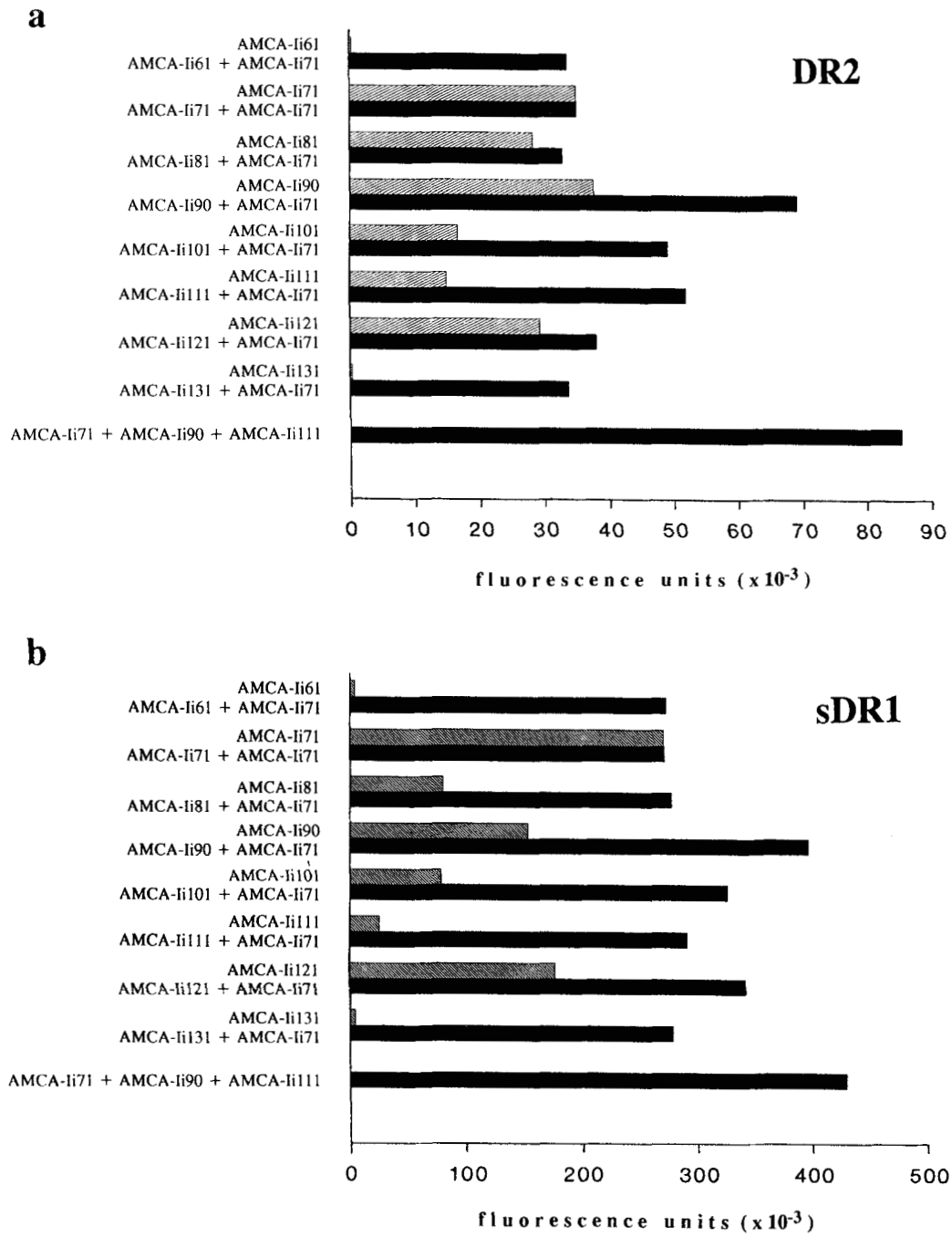

FIGURE 2. Cobinding of Ii-derived peptides. *a*, DR2 (200 nM) was coincubated with different AMCA-Ii peptides (5 μM) in the absence (hatched bars) and the presence (black bars) of saturating concentrations of AMCA-Ii71 (50 μM) for 40 h in HPSEC binding assay. *b*, sDR1 (50 nM) was coincubated with different AMCA-Ii peptides (5 μM) in the absence (hatched bars) and presence (black bars) of saturating concentrations of AMCA-Ii71 (50 μM) for 40 h in HPSEC binding assay.

nonoverlapping Ii peptides bind to distinct sites on $\alpha\beta$ dimers and rule out that all peptides bind in the groove.

Ii-derived peptides reflect binding of intact Ii

To determine whether the Ii-derived peptides mirror the behavior of the corresponding regions in the Ii polypeptide chain and bind to the respective contact sites, inhibition studies with human rIi lacking the transmembrane domain were performed. As depicted in Figure 3, coincubation of rIi with the respective AMCA-labeled Ii peptides in a 2:1 molar ratio moderately reduced binding of Ii90 and Ii95 and very efficiently reduced binding of the other Ii peptides, reflecting the different affinities of the respective Ii peptides for DR2 (Table I). At higher concentrations of rIi, binding of Ii90 and Ii95 and also that of Ii121 were completely blocked (data not shown). rIi was also able to prevent binding of AMCA-labeled antigenic peptides MBP-(85–105) from MBP and HA-(307–319) from HA (Fig. 3), but failed to block the control peptide INS-(10–30) from the bovine insulin B chain that has been suggested to occupy an alternative binding site on class II molecules (P. Jensen, personal communication). Additional evidence for correct binding

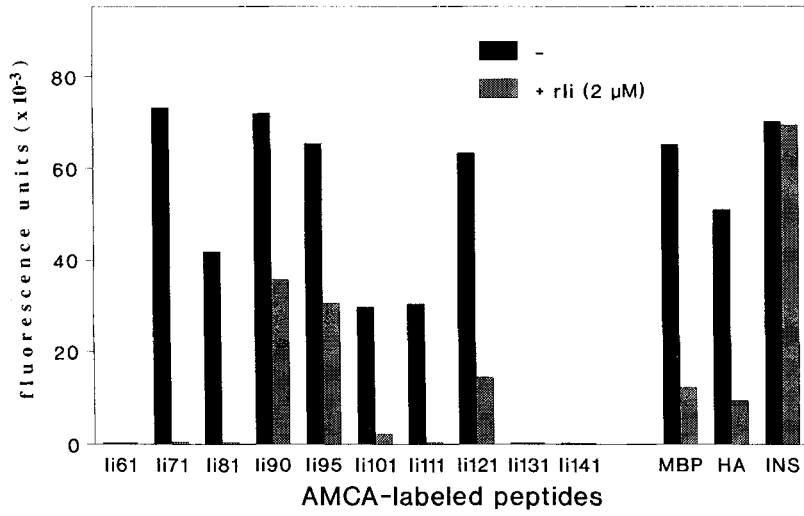

FIGURE 3. Inhibition of Ii peptide binding by rIi. DR2 (200 nM) was incubated together with the indicated peptides in its AMCA-labeled form (1 μM) in the absence (black bars) or the presence (cross-hatched bars) of rIi (2 μM) and analyzed after 40 h in the HPSEC binding assay.

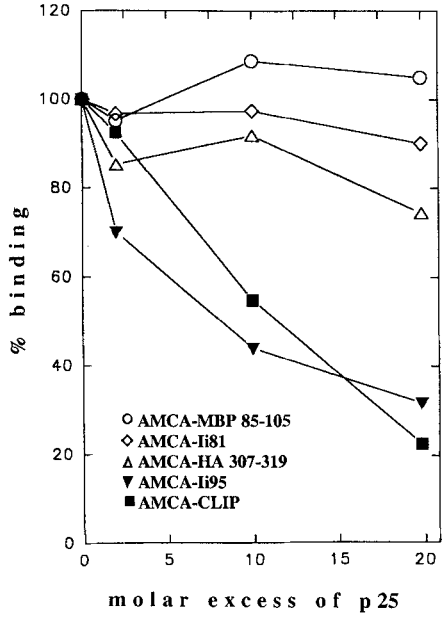

FIGURE 4. Selective inhibition of Ii peptide binding by Ii fragment p25. Titration of the inhibition by p25 (2–20 μM) of AMCA-labeled peptides Ii81, Ii95, CLIP-(81–105), MBP-(85–105), and HA-(307–319), 1 μM each, on DR2 (200 nM). p25 starts with Ala^{101}, as shown by Edman sequencing, and contains the complete C terminus of Ii.

of the Ii peptides was derived from the C-terminal Ii fragment p25, which is found associated with class II molecules ex vivo and in vitro (22, 24). p25 was isolated from the EBV-transformed B cell line LD2B. Sequencing of p25 by Edman degradation showed that its N-terminal amino acid uniformly was Ala^{101}. Thus, it shares the same N terminus with peptide Ii101. p25 selectively blocked binding of Ii peptides Ii95 and CLIP-(81–105), which overlap with the p25 sequence, but did not inhibit binding of nonoverlapping Ii81 or antigenic peptides (Fig. 4). These data for p25 are in good agreement with the binding of Ii101, Ii111, and Ii121, in qualitative and in quantitative terms. Taken together, the additive binding and the selective inhibition by rIi and p25 strongly indicate that the Ii-derived peptides are oriented on sDR1 and DR2 molecules in the same way as the corresponding regions in intact Ii.

Interaction of Ii segments with the SEB contact area

For identification of the contact region of class II molecules for Ii-derived peptides we employed SEB. The x-ray structural analysis of the co-crystal of SEB with HLA-DR1 showed that SEB binds to the α1 domain of αβ dimers exclusively outside the groove without interference with peptide binding in the groove (59). The results shown in Figure 5 confirm that SEB does not prevent groove binding of antigenic peptides; neither AMCA-MBP-(85–105) nor AMCA-HA-(307–319) binding to DR2 (Fig. 5*a*) or sDR1 (Fig. 5*b*) was influenced by SEB, even at a 20-fold molar excess of SEB (data not shown). In contrast, a twofold molar excess of SEB strongly or moderately blocked binding of all Ii peptides except Ii95 (Fig. 5, *a* and *b*). Ii95 was not inhibited even at high SEB concentrations (see *inset*, Fig. 5*a*). Only in the case of Ii101 and Ii111 were allelic differences in SEB inhibition observed; both peptides were efficiently inhibited by SEB on sDR1 (Fig. 5*b*), but only moderately on DR2 molecules (Fig. 5*a*). Inhibition of CLIP-(81—105) binding by SEB followed competitive kinetics, suggesting that both molecules competed for the same site (46). Together, these data imply that regions covering about amino acids 71 to 90 and 109 to 128 interact with sites outside the peptide binding groove on the SEB contact area on the α1 domain of DR molecules. The exact boundaries of these segments are not clear from these studies.

Interference with antigenic peptide binding

It is known that CLIP and antigenic peptides interfere with each other's binding. We addressed the question of which of the Ii-derived peptides would block antigenic peptides and vice versa. When AMCA-labeled Ii peptides were incubated for 48 h in the presence of antigenic peptide, it was observed on both sDR1 and DR2 that all Ii peptides were inhibited by antigenic peptide (Fig. 6, *a* and *c*). The inhibition was dose dependent, as 50 μM MBP-(85–105) (50-fold excess) was more efficient than 2 μM (twofold molar excess) of the same peptide. In addition, the inhibition was time dependent, as very little blockade was observed after 2 h of incubation (data not shown). The interference of groove binding peptides with those Ii peptides binding to the SEB contact area outside the groove (Ii71 and Ii111; Fig. 5) suggests different conformational states of DR molecules. Further evidence for at least two alternative conformations was obtained when the reverse experiment was performed, namely inhibition of labeled antigenic peptides by unlabeled Ii peptides. After 48-h coincubation, only Ii90 and Ii121 (the peptide with the exceptional behavior; Table I)

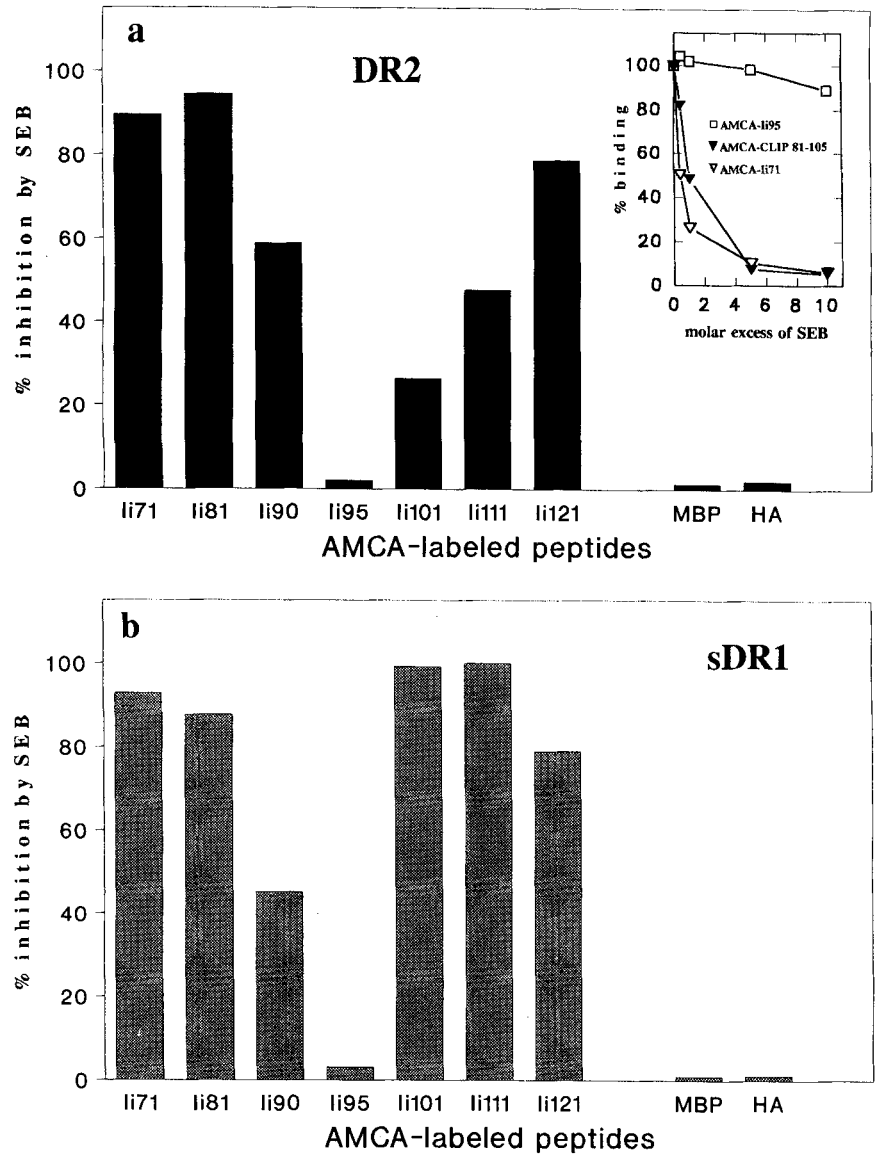

FIGURE 5. Selective inhibition of Ii peptide binding by SEB. *a*, Binding of the indicated AMCA-labeled Ii peptides (1 μM) to DR2 (200 nM) in the absence or the presence of SEB (2 μM) was analyzed by HPSEC. *Inset*, Titration of the inhibition by SEB (0.4–10 μM) of peptides AMCA-Ii71, AMCA-Ii95, and AMCA-CLIP-(81–105) on DR2 (K_d values; cf Table 1). *b*, Binding of the indicated AMCA-labeled Ii peptides (1 μM) to sDR1 (50 nM) in the absence or the presence of SEB (2 μM) was analyzed by HPSEC.

were able to decrease AMCA-MBP-(85–105) binding to DR2 and AMCA-HA-(307–319) binding to sDR1, but not the other Ii peptides despite a 100-fold molar excess (Fig. 6, *b* and *d*). The same amount of unlabeled MBP-(85–105) or HA-(307–319) led to quantitative inhibition on DR2 and sDR1, respectively. This result demonstrates that the region Ii90–108 of CLIP is essential for efficient inhibition of antigenic peptide binding to DR1 and DR2 molecules.

However, when the kinetics of inhibition were studied in more detail, we found that after short time incubation, antigenic peptide binding was also inhibited by Ii71, which binds outside the groove (Fig. 7). In the absence of Ii71, AMCA-HA-(307–319) attained half-maximal binding to sDR1 after a $t_{1/2}$ of approximately 4 h, reaching maximal binding after approximately 30 h (Fig. 7*a*). Under steady state conditions, about 85% of sDR1 molecules were occupied by AMCA-HA-(307–319). However, in the presence of Ii71, the strong inhibition observed after 1 h (75%) gradually decreased to 15% after 48 h. The time dependency of inhibition, as calculated from the data in Figure 7*a*, is depicted in Figure 7*b*. This effect is not due to different kinetics of AMCA-HA and AMCA-Ii71 binding, as the two association curves are almost superimposable (Fig. 7*a*). For ligands and inhibitors binding to the same site and stabilizing the same conformation of $\alpha\beta$ dimers, the inhibitory potential is expected to be independent of the incubation time. Indeed, when the groove binding influenza matrix peptide IM-(19–31) was used as an inhibitor for AMCA-HA-(307–319), no time dependency of the inhibitory potential was observed. Together, these data indicate distinct binding sites for Ii71 and antigenic peptides and the existence of at least two distinct conformational states of $\alpha\beta$ dimers.

Discussion

Previous studies using recombinant Ii truncation and deletion variants provided indirect evidence that the CLIP region (residues 81–104) is essential for promiscuous binding to class II molecules. Especially residues 96 to 104 were found to mediate binding to DR1 (28), and residues 90 to 104 were sufficient for inhibition of loading of DR1, DR2, and DR3 with antigenic peptides (33, 46, 48). The present study shows that Ii peptide Ii90 (residues 90–108) binds not only to DR1 and DR2, but also to peptides from the flanking regions (amino acids 71–89 and 109–128), albeit with lower affinity (Table I and Fig. 8). The moderate or low affinity of these flanking regions might explain why they were not found in studies involving coprecipitation of certain Ii truncation constructs

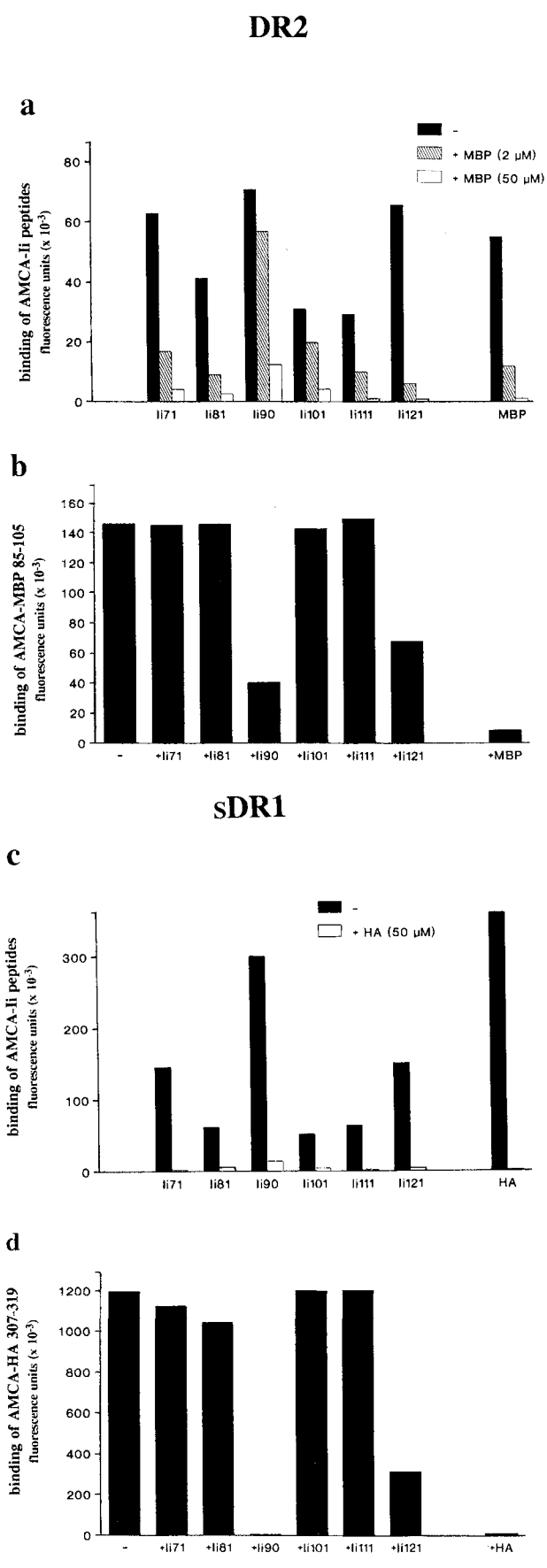

FIGURE 6. Interference of Ii peptides with antigenic peptides on DR molecules. *a*, Inhibition of Ii peptide binding by MBP-(85–105) on DR2. DR2 (200 nM) was incubated with various AMCA-labeled Ii peptides (1 μM) in the absence (black bars) or the presence of 2 μM MBP-(85–105) (hatched bars) or 50 μM MBP-(85–105) (white bars) for 48 h in HPSEC binding assay. *b*, Inhibition of AMCA-MBP 85–105 binding by Ii peptides on DR2. DR2 (200 nM) was coincubated with AMCA-MBP-(85–105) (100 nM) in the absence and the presence of the indicated Ii peptides (10 μM) or MBP-(85–105) (10 μM) for 48 h in the HPSEC binding assay. *c*, Inhibition of Ii peptide binding by HA-(307–319) on sDR1. sDR1 (50 nM) was incubated with various AMCA-labeled Ii peptides (1 μM) in the absence (black bars) or the presence of 50 μM HA-(307–319) (white bars) for 48 h in the HPSEC binding assay. *d*, Inhibition of AMCA-HA-(307–319) binding by Ii peptides on sDR1. sDR1 (50 nM) was coincubated with AMCA-HA-(307–319) (100 nM) in the absence and the presence of the indicated Ii peptides (10 μM) or HA-(307–319) (10 μM) for 48 h in the HPSEC binding assay.

with αβ dimers. Several complementary findings strongly suggest that the Ii peptides spanning the region 71 to 128 bound to the DR molecule in the same manner as in the context of the intact Ii. 1) Nonoverlapping Ii peptides displayed additive binding and did not compete with each other, whereas overlapping peptides did not bind simultaneously (Fig. 2). 2) Soluble rIi prevented binding of all Ii peptides (Fig. 3). 3) The C-terminal Ii fragment p25 interfered only with those Ii peptides that overlap with the p25 sequence (Fig. 4). Thus, it is likely that in the context of whole Ii, not only CLIP but also the flanking regions contribute to the assembly of αβ:Ii complexes.

Inhibition by the superantigen SEB provided information on the respective contact sites of Ii peptides on the DR1 and DR2 αβ dimers and suggested that the flanking regions covering residues 71 to 94 and 109 to 128 interact with the SEB contact area on the α1 domain. These results are schematically compiled in Figure 8. It should be kept in mind that determination of the precise boundaries of the respective segments awaits further studies. For example, it is not clear whether all residues of the segment 71 to 81 interact with the DR2 molecules, but the slightly higher affinity of Ii71 compared with that of Ii81 (Table I) indicates a contribution of at least some residues. Our SEB data seem to be in contrast to the earlier finding that Ii expression on the cell surface does not inhibit SEB binding to $DR1^+$ cells (60), but in agreement with our data, inhibition of SEB binding to cells by rIi was recently reported (61).

The only peptide not affected by SEB was Ii95 (Fig. 5). However, the Ii95 sequence is contained in Ii90, which is a powerful competitor of antigenic peptide binding on both DR alleles (Fig. 6, *b* and *d*). The most straightforward explanation for both findings is that peptide Ii90 binds directly to the peptide binding groove. This would be consistent with the recent suggestion that CLIP binds to class II molecules like conventional antigenic peptides, with Met^{91} of CLIP being part of a supermotif and an important anchor residue for pocket P1 of the DR groove (47, 48). Indeed, the x-ray structure of a DR3:CLIP complex visualizes residues Pro^{87} to Ala^{101} in the binding groove, with Met^{91} being one of the anchor residues (P., Gosh, M. Amaya, E. Mellins, and D. C. Wiley, personal communication). With Ii90 being located in the groove, it has to be explained how inhibition of Ii90 by SEB from outside the groove is accomplished (Fig. 5). Two possibilities come to mind. Assuming that the crystal data describing binding of CLIP to DR3 can also be extended to DR1 and DR2 molecules with Lys^{90} and Met^{91} lying in the groove, then SEB binding outside the groove could be accompanied by small structural changes inside the peptide groove that would prevent binding of Ii90, but not binding of Ii95 or the antigenic peptides HA and MBP. This model would be

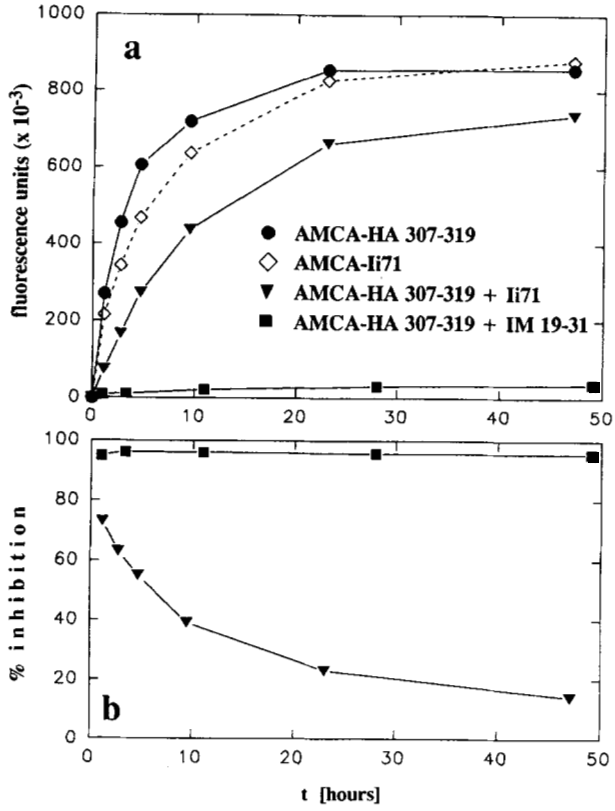

FIGURE 7. Kinetics of the inhibitory effect of Ii71 on AMCA-HA-(307–319) binding to sDR1. *a*, sDR1 (50 nM) was coincubated with 50 nM AMCA-HA-(307–319) (●), 10 μM AMCA-Ii71 (◇), 50 nM AMCA-HA-(307–319) in the presence of 10 μM Ii71 (▼) or with 50 nM AMCA-HA-(307–319) in the presence of 10 μM IM-(19–31) (■) and analyzed by HPSEC after the indicated incubation times. *b*, Inhibitory capacity of Ii71 (▼) or IM 19–31 (■) on AMCA-HA-(307–319) (50 nM) binding to sDR1 at different time points, calculated from values in Figure 7*a*.

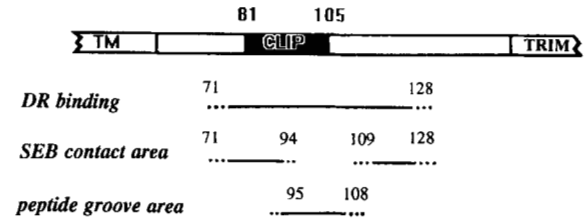

FIGURE 8. Functional domains of invariant chain.

consistent with the hypothesis that some, but not all, groove binding peptides might interfere with SEB binding (62). Alternatively, our data raise the possibility that Ii90 binds differently to DR1 and DR2 than described for DR3:crystal. On DR1 and DR2 molecules residues 90 to 94 of Ii90 might reach out of the groove, thereby directly competing with SEB for an overlapping binding area on top or outside of the binding cleft. The latter model is consistent with distinct binding modes for antigenic peptides and Ii90, as suggested by the observation that antigenic peptides preclude the binding of Ii71 and Ii111 (Fig. 6), whereas Ii90 displayed simultaneous binding to both Ii peptides (Fig. 2).

The observation that antigenic peptides were able to prevent not only binding of Ii90, but, after long-term incubation, also binding of those Ii peptides whose contact sites are outside the groove on the SEB contact area, such as Ii71 and Ii111 (Fig. 6), suggests the existence of at least two conformational states of class II molecules. One state (designated here as S2) is described by the conformation we know from the crystallographic studies with HLA-DR1 (63); it is stabilized by groove binding peptides that bind with slow on and off rates and excludes simultaneous stable binding of Ii peptides under steady state conditions (Fig. 7). The other conformational state (S1) is compatible with Ii binding and characterized by fast on and off rates of its ligands explaining the fast on rate of Ii in vivo (64) and the fast off-rate of CLIP on DR1 (44), DR3, DR4, and DR11 (34). Since antigenic peptides can compete with Ii peptides binding to the SEB contact area (e.g., Ii71; Fig. 7), they appear to be able to bind to the S1 state (or a similar intermediate conformation). With time, however, antigenic peptides (but not Ii peptides) induce a gradual shift toward the stable S2 state. Such a model would explain that inhibition of AMCA-HA by Ii71 decreases with time (Fig. 7), and inhibition of AMCA-Ii71 by antigenic peptides increases with time (data not shown). This model is consistent with previous kinetic studies also suggesting distinct conformational states of class II molecules (51–53). According to this, initial binding of antigenic peptide by a fast on rate and low affinity is followed by a conformational change resulting in a slow on rate and high affinity. Such a maturation of DR2 molecules could not be observed when CLIP was used as a ligand (46).

In conclusion, the present study adds further evidence that class II molecules are dynamic entities that can occur in distinct conformational states. These conformational modulations could be subtle, but sufficient to influence the respective binding sites. It would be advantageous if a conformation of $\alpha\beta$ favoring Ii binding were to be generated during biosynthesis, as this would facilitate rapid association with the trimeric Ii scaffold in the ER. Since CLIP has a fast off rate, it seems reasonable to assume that stabilization of the $\alpha\beta$Ii complex could be mediated by additional Ii contact sites recruiting flanking regions of the CLIP segment, as described here. These additional contact sites may also compensate for the poor binding of the CLIP peptide to certain class II alleles, such as DR4 (34) (H. Kropshofer, A. Vogt, and G. J. Hämmerling, unpublished observations) or A^k (47).

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