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Immunology

Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding

(HLA antigens/antigen presentation/kinetics)

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ABSTRACT **The invariant chain (Ii) prevents binding of ligands to major histocompatibility complex (MHC) class II molecules in the endoplasmic reticulum and during intracellular transport. Stepwise removal of the Ii in a trans-Golgi compartment renders MHC class II molecules accessible for peptide loading, with CLIP (class II-associated Ii peptides) as the final fragment to be released. Here we show that CLIP can be subdivided into distinct functional regions. The C-terminal segment (residues 92–105) of the CLIP-(81–105) fragment mediates inhibition of self- and antigenic peptide binding to HLA-DR2 molecules. In contrast, the N-terminal segment CLIP-(81–98) binds to the *Staphylococcus aureus* enterotoxin B contact site outside the peptide-binding groove on the α1 domain and does not interfere with peptide binding. Its functional significance appears to lie in the contribution to CLIP removal: the dissociation of CLIP-(81–105) is characterized by a fast off-rate, which is accelerated at endosomal pH, whereas in the absence of the N-terminal CLIP-(81–91), the off-rate of C-terminal CLIP-(92–105) is slow and remains unaltered at low pH. Mechanistically, the N-terminal segment of CLIP seems to prevent tight interactions of CLIP side chains with specificity pockets in the peptide-binding groove that normally occurs during maturation of long-lived class II–peptide complexes.**

Major histocompatibility complex (MHC) class II molecules are heterodimeric peptide receptors that bind antigenic peptides and present them to $CD4^+$ T cells (1). Class II α and β subunits associate with an invariant chain (Ii) trimer shortly after biosynthesis in the endoplasmic reticulum (ER), generating a stable nine-subunit $(\alpha\beta Ii)_3$ complex (2, 3). The nonameric complex leaves the ER and is targeted by a sorting signal in the cytoplasmic tail of Ii into endocytic post-Golgi compartments (4, 5). In these acidic endosome/lysosome-like vesicles, termed MHC class II compartments (MIIC; ref. 6), proteolysis of Ii by cathepsin B and cathepsin D is thought to release MHC class II αβ dimers (7, 8), with subsequent loading by endocytically generated self- or antigenic peptides (9, 10). In the ER and during transport, Ii prevents endogenous ligands from binding to class II molecules (11–13), although a small percentage of peptides may already bind in the ER (14, 15).

Degradation of Ii is a regulated process with stepwise removal of Ii segments from class II MHC molecules (16). There is evidence that the final Ii fragment to be removed constitutes the membrane-proximal region (17, 18), which is designated CLIP (class II MHC-associated Ii peptides; ref. 19), and encompasses residues 81–105 of the p33 form of human Ii (20). In agreement with the hypothesis that αβCLIP is a common intermediate of class II maturation, CLIP remains associated with the majority of HLA-DR (DR) molecules in HLA-DM-negative mutant cells that are deficient in peptide loading (18, 19, 21), whereas class II molecules from HLA-DM-positive wild-type cells contain smaller amounts of CLIP (22–28). The CLIP region has been shown to be sufficient for stable binding of truncated Ii constructs to class II molecules (29–31) and for inhibition of antigenic peptide binding (19, 21–23, 28). Thus, removal of CLIP is a prerequisite for efficient loading of class II molecules in the MIIC, but it is not understood which structural features of CLIP facilitate its specific release and why replacement by antigenic peptides is favored.

To address this question, we functionally dissected CLIP and show here that the C-terminal region is sufficient for inhibition of peptide binding, whereas the N-terminal segment is crucial for rapid dissociation from MHC class II molecules.

MATERIALS AND METHODS

Cells. The Epstein–Barr virus-transformed homozygous cell line LD2B was maintained at 37°C under 5% CO_2/95% air in RPMI 1640 medium (GIBCO) containing heat-inactivated fetal calf serum (GIBCO) and antibiotics and was used for isolation of HLA-DR2 (DR2).

Purification of DR2. Cells were lysed, and the resulting homogenate was prepared for affinity chromatography with the anti-DR monoclonal antibody L243 (32) essentially as described (33). In the washing and elution buffer of the affinity chromatography, 0.1% Zwittergent-12 (Calbiochem) was used as a detergent to solubilize DR2 molecules.

Peptide Synthesis and Labeling. Peptides were synthesized by continuous-flow solid-phase peptide synthesis with standard 9-fluorenylmethoxycarbonyl chemistry. N-terminal 7-amino-4-methylcoumarin-3-acetic acid (AMCA; Molecular Probes)-labeling was performed as described (33). Labeling did not influence the affinity of the peptides, as equal amounts of unlabeled peptide inhibited ≈50% of the labeled peptide. Peptides were purified by HPLC, and their identity was tested by laser-desorption mass spectrometry. Final concentrations were determined by weight.

Peptide-Binding Assay. Solubilized DR2 (0.5 μM) was coincubated with the respective AMCA-labeled peptide (1 μM) for 48 h at 37°C in 150 mM sodium phosphate, pH 6.0/0.1% Zwittergent-12. For inhibition studies, indicated

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Abbreviations: MHC, major histocompatibility complex; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; CLIP, class II MHC-associated invariant chain peptides; CLIP-(81–105), CLIP-(92–105), synthetic peptides containing residues 81–105 or 92–105 of CLIP; DR and DR1–DR3, HLA-DR and HLA-DR1–HLA-DR3; HA-(307–319), synthetic peptide representing amino acids 307–319 of influenza virus hemagglutinin; HLA, human leukocyte antigen; HPSEC, high-performance site-exclusion chromatography; Ii, invariant chain; MIIC, MHC class II-containing compartment; SEB, *Staphylococcus aureus* enterotoxin B.

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amounts of inhibitor were included. All samples were analyzed on a Pharmacia Superdex 75 HR 5/20 high-performance size-exclusion chromatography (HPSEC) column equilibrated with 150 mM sodium phosphate, pH 6.0/0.1% Zwittergent-12/15% (vol/vol) acetonitrile. The flow rate was 0.5 ml/min. The column run-through went through a Merck fluorescence spectrophotometer and a Merck UV detector set up in series. Fluorescence signals eluted with DR2 $\alpha\beta$ dimers were recorded by a model D-2500 integrator (Merck-Hitachi). For quantitation, fluorescence intensity was measured as the height of the peak associated with the respective $\alpha\beta$ dimer; $\alpha\beta$ dimers were eluted after 3 min.

RESULTS

N- and C-Terminal "Halves" of CLIP-(81–105) Contribute Cooperatively to High-Affinity Binding. For identification of segments of the 25-mer CLIP-(81–105) fragment that mediate binding and prevent association of antigenic peptides to DR2 molecules, four truncation variants of CLIP-(81–105) were synthesized and tested in an *in vitro* binding assay based on high-performance size-exclusion chromatography. To determine the equilibrium constant K_d, peptides were N-terminally labeled with the fluorophor AMCA and coincubated with DR2 for 48 h. The fluorescence associated with elution of $\alpha\beta$ dimers after gel-filtration was used for quantitation. CLIP-(81–105), the most prominent CLIP variant on products of all *HLA-DR* alleles investigated (18, 21–24), was of high affinity (K_d = 23 nM), whereas the N-terminally truncated version, CLIP-(90–105), which so far has been found associated only with HLA-DR1 (DR1) and DR2 molecules (23), displayed reduced affinity (K_d = 230 nM). Peptide CLIP-(92–105), which is shortened further by two *N*-terminal residues, retains only moderate affinity (K_d = 1.2 μM). Thus, the N-terminal segment is important for high-affinity binding of CLIP-(81–105) to DR2 molecules (Table 1). However, the region 92–104 also contributes to the affinity of CLIP-(81–105), as the complementary N-terminal proline-rich 12-mer CLIP-(81–92) does not bind stably (K_d > 100 μM). C-terminal elongation resulting in CLIP-(81–98) restores moderate binding (K_d = 3.0 μM).

The importance of the C-terminal CLIP-(92–104) segment is also reflected by its strong inhibitory effect on the binding of AMCA-labeled influenza hemagglutinin (HA) peptide AMCA-HA-(307–319) (Table 1): the IC_{50} value, where half-maximal inhibition is attained, is 1.8 μM for CLIP-(92–105) and 1.4 μM for the longer fragment CLIP-(90–105). CLIP-(81–105) is only 5 to 6 times better (IC_{50} = 290 nM), whereas CLIP-(81–92) and CLIP-(81–98) failed to block peptide binding ($IC_{50} \gg 100$ μM). High-affinity binding of CLIP-(81–105) accompanied by strong interference with peptide binding was also observed for DR1, DR3, and murine H-2E^k (unpublished data), implying a promiscuous binding mode that involves monomorphic determinants on class II molecules.

Table 1. Dissociation equilibrium constant K_d and inhibitory potential IC_{50} of CLIP truncation variants on DR2

	CLIP variant	K_d, nM	IC_{50}, nM
CLIP-(81–105)	LPKPPKPVSKMRMATPLLMQALPMG	23	290
CLIP-(81–98)	LPKPPKPVSKMRMATPLL	3000	NI
CLIP-(81–92)	LPKPPKPVSKMR	ND	NI
CLIP-(92–105)	RMATPLLMQALPMG	1200	1800
CLIP-(90–105)	KMRMATPLLMQALPMG	230	1400

K_d values were determined by an *in vitro* peptide-binding assay using Scatchard analysis. For this purpose, the indicated CLIP variants were AMCA-labeled and measured for binding to DR2 in the concentration range 1 nM–10 μM. Fluorescence intensity divided by the respective peptide concentration (bound/free) was plotted versus peptide concentration, and the slope of the linear least-squares regression fit was used for K_d calculation. For calculation of IC_{50} values, the indicated peptides were used as inhibitors (10 nM–100 μM) against 1 μM AMCA-HA-(307–319) (K_d = 140 nM) binding to DR2 under standard conditions. NI, no inhibition with ≤100 μM of the respective inhibitor; ND, no detection of stable binding under the assay conditions (K_d > 100 μM).

Inhibitory Motif of CLIP on DR2. Conventional antigenic peptides bind to class II molecules by using some of their side chains at defined anchor positions for specific interaction with pockets within the peptide-binding groove (34). The structural requirements for antigenic peptide binding have been identified for products of several MHC class II alleles including *HLA-DR2* (24, 35). To elucidate the inhibitory motif of CLIP, we tested truncated variants of CLIP-(81–105) and single-alanine mutants of CLIP-(92–105) for inhibition of AMCA-HA-(307–319) binding on HLA-DR2 (Fig. 1). N-terminal truncations of CLIP-(81–105) revealed that most of the inhibitory potential resides in the C-terminal segment, CLIP-(92–105). The mutational analysis of this segment showed that the C-terminal residues Met-99, Leu-102, Pro-103, and Met-104 are crucial for inhibition by CLIP-(92–105), as their replacement by alanine strongly reduced inhibition (Fig. 1 *Upper*). This is consistent with the finding that CLIP-(81–98), in which the above residues are absent, does not block HA-(307–319) binding (Fig. 1; compare Table 1). This hydrophobic cluster of residues at the CLIP C-terminal is clearly different from the type and position of anchor residues essential for binding of self- or antigenic peptides to the DR2 groove (Fig. 1 *Lower*): conventional high-affinity groove binding peptides of DR2a are characterized by a large hydrophobic anchor residue at relative position *i*, followed by a further hydrophobic residue at position $i + 3$ and a positively charged residue at position $i + 6$ or $i + 7$ (24). These differences in the ligand motifs suggest that the CLIP-(92–105) segment does not occupy the same specificity pockets as do conventional groove-binding peptides.

Kinetics of CLIP-(81–105) and CLIP-(92–105). In the case of HLA-DR3 (DR3), CLIP has been found to be a powerful inhibitor of antigenic peptide binding; but in contrast to conventional DR3–peptide complexes, the DR3–CLIP complexes readily dissociated during SDS gel electrophoresis (19, 21). To investigate formation and disassembly of DR2–CLIP complexes under milder conditions, CLIP or antigenic peptide HA-(307–319) was incubated with DR2 in 0.1% Zwittergent-12 detergent solution at pH 6.0 and assayed by gel-filtration. Under these conditions, 1 μM HA-(307–319) required 9.2 h for half-maximal formation of stable complexes ($t_{1/2,on} \approx 9.2$ h; Table 2) that is comparable to $t_{1/2,on}$ obtained for the same HA peptide binding to DR1 under similar conditions with or without detergent (36). In contrast, 1 μM CLIP-(81–105) bound very fast ($t_{1/2,on} \approx 6$ min), but elimination of the N-terminal amino acids resulting in CLIP-(92–105) significantly decreased the association or on-rate to $t_{1/2,on} \approx 5.3$ h. The N-terminal CLIP segment also determined the fast dissociation or off-rate of CLIP at pH 6.0 (Table 2): CLIP-(81–105) dissociated fast ($t_{1/2,off} \approx 25$ min), whereas CLIP-(92–105) displayed higher stability ($t_{1/2,off} \approx 2.8$ h), comparable to that of the antigenic peptide HA-(307–319) ($t_{1/2,off} \approx 5.7$ h), although the dissociation curve of CLIP-(92–105) was biphasic after 48 h of incubation, indicating a residual portion of rapidly dissociating complexes (see Fig. 2 *Right*). At pH 4.5—the most extreme pH described for endosomes—the presence of the N-terminal region mediates a further 4-fold increase of the DR2–CLIP-(81–105) disociation rate ($t_{1/2,off} \approx 6$ min), whereas CLIP-(92–105) dissociation was not influenced by low pH ($t_{1/2,off} \approx 2.9$ h). The off-rate of HA-(307–319) was also increased at pH 4.5 ($t_{1/2,off} \approx 1.2$ h). We suggest that the N-terminal region prevents CLIP-(81–105) from stable binding to DR2, at the same time enabling CLIP-(81–105) to associate and dissociate at high frequency.

Short-Lived DR2–CLIP-(81–105) Complexes Do Not Mature. *In vitro* studies have shown that a rapidly formed short-

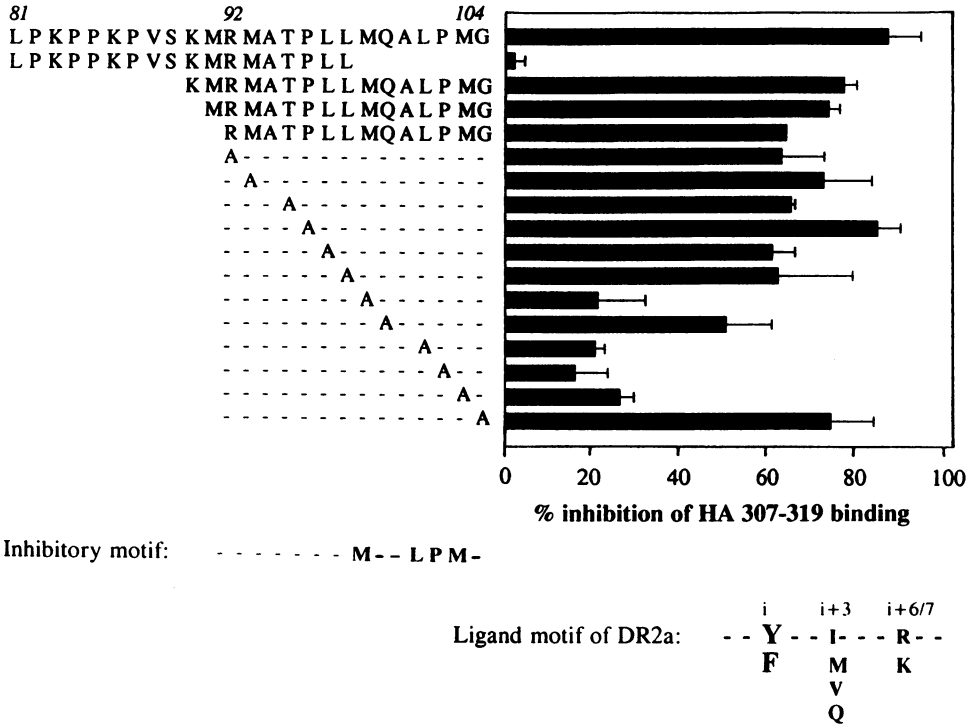

FIG. 1. Inhibitory motif of CLIP on HLA-DR2. (*Upper*) Truncation variants of CLIP-(81–105) and single-alanine substitutions of CLIP-(92–105) were used to inhibit AMCA-HA-(307–319) binding to DR2. Binding of 1 μM AMCA-HA-(307–319) was assayed at 37°C and at pH 6.0 for 48 h in the absence and presence of a 20-fold molar excess (20 μM) of the indicated CLIP variants. Error bars indicate the errors from three independent experiments. (*Lower*) For comparison, the antigenic peptide motif for DR2a is also given, which was derived from alanine-scanning-mutants of the high-affinity DR2-binding myelin basic protein peptide MBP-(87–99) competing against AMCA-HA-(307–319) (ref. 24).

lived complex precedes stable binding of antigenic peptides into the peptide-binding groove of class II molecules (37–39). As low-affinity peptide occupancy was found to preserve class II from inactivation at physiological temperature, it was speculated that Ii–class II complexes may behave similarly (38). Therefore, we compared the off-rates of CLIP-(81–105) and CLIP-(92–105) complexed to DR2 after different incubation times *in vitro* (Fig. 2). As expected, with the antigenic peptide HA-(307–319), a time-dependent maturation of the DR2–HA-(307–319) complex was observed (Fig. 2 *Left*): when binding was allowed for only 2 h, the resulting class II–HA peptide complexes dissociated rapidly ($t_{1/2,off} \approx 25$ min). However, complexes formed during 48 h of incubation were stable with a $t_{1/2,off} \approx 5.7$ h, which is typical for long-lived class II–peptide complexes (Fig. 2 *Left* and Table 2). In contrast, 48 h of incubation of DR2 with CLIP-(81–105) did not yield stable complexes ($t_{1/2,off} \approx 25$ min), and similar off-rates were obtained irrespective of the incubation time (Fig. 2 *Center*). CLIP-(92–105) behaved more like HA-(307–319) (Fig. 2 *Left* and *Right*): complexes formed after 2 h were short-lived ($t_{1/2,off} \approx 10$ min), whereas dissociation after 16 or 48 h was biphasic (Fig. 2 *Right*): $t_{1/2,off}$ values of ≈25 min and 2.8 h were obtained. However, unlike DR2–CLIP-(81–105) complexes, maturation of DR2–CLIP-(92–105) complexes occurred because the portion of long-lived complexes increased with time.

Table 2. Kinetics of CLIP and antigenic peptides binding to DR2

	$t_{1/2}$, h		
	HA-(307–319)	CLIP-(81–105)	CLIP-(92–105)
On-rate, $t_{1/2,on}$*	9.2	0.10	5.3
Off-rate, $t_{1/2,off}$†			
pH 6.0	5.7	0.41	2.8‡
pH 4.5	1.2	0.10	2.9

*Half-times of association ($t_{1/2,on}$) were determined from the apparent first order-rate constants of association with 0.5 μM DR2 and the following concentrations of AMCA-labeled peptides: 10 μM HA, 10 μM CLIP-(81–105), and 10 μM CLIP-(92–105).

†Half-times of dissociation ($t_{1/2,off}$) were determined from the first-order rate constants of the dissociation. For this purpose, 0.5 μM DR2 and the respective AMCA-labeled peptides at 1 μM were coincubated for 48 h, and the DR2–peptide complexes were separated from unbound AMCA-labeled peptides by HPSEC. Then the pH was adjusted to 6.0 or 4.5 by adding acetic acid, and 20-μl aliquots were reinjected at 10- to 60-min intervals.

‡In the case of CLIP-(92–105), the dissociation rate of the long-lived complex is given by using values at $t > 30$ min, where the plot of ln bound fluorescence versus time is linear (see Fig. 2 *Right*).

***Staphylococcus aureus* enterotoxin B (SEB) Interferes with CLIP Binding.** The observed 50-fold higher affinity of CLIP-(81–105) compared with CLIP-(92–105) (see Table 1) argues in favor of additional contact sites for the N-terminal region on class II molecules. For investigation of these binding sites, we used the inhibition by superantigen SEB. X-ray crystallography has shown that SEB binds exclusively to the α1 domain outside the groove of DR1 molecules and does not interfere with self-peptide binding into the groove (40). In agreement with this, SEB had no effect on binding of AMCA-labeled HA-(307–319) or MBP-(85–105) to DR2 (Fig. 3 *Left*). Similar results have been obtained with AMCA-HA-(307–319) on DR1 (H.K., unpublished data). In contrast, SEB was a potent

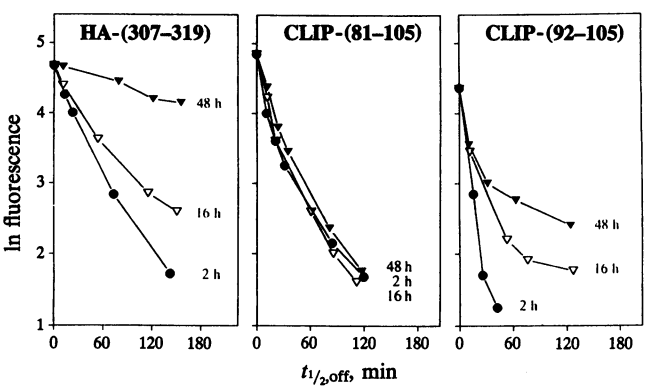

FIG. 2. Dissociation rates of HA-(307–319), CLIP-(81–105), and CLIP-(92–105) complexed with HLA-DR2 depend on the incubation time. Complexes of DR2 (0.5 μM) and the indicated AMCA-labeled peptides (10 μM) were formed during 2, 16, or 48 h of incubation and separated from unbound peptides by HPSEC, and the off-rates were determined at pH 6.0 and 37°C as described in the footnotes to Table 2.

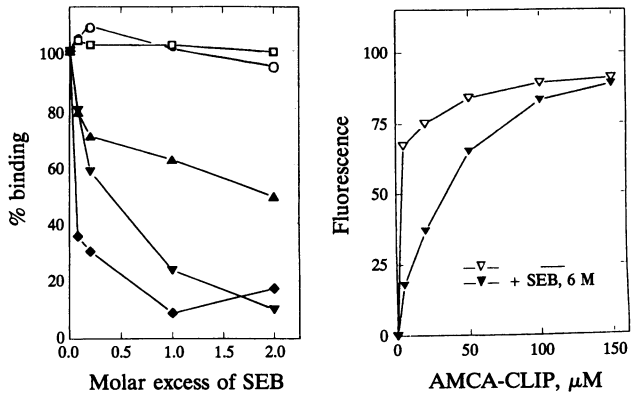

FIG. 3. SEB competes for binding of CLIP. (*Left*) SEB (0.1–2 μM) was used to inhibit binding to DR2 of 1 μM AMCA-CLIP-(81–105) (▼), 1 μM AMCA-CLIP-(92–105) (▲), 1 μM AMCA-CLIP-(81–98) (◆), and the following antigenic peptides: 1 μM AMCA-MBP-(85–105) (○) and 1 μM AMCA-HA-(307–319) (□). The results are expressed as a percentage of the binding obtained in the absence of SEB. (*Right*) Increasing amounts of 5–150 μM AMCA-CLIP-(81–105) were bound to 0.5 μM DR2 in the presence (▼) or absence (▽) of 6 μM SEB, revealing a competitive mode of inhibition.

inhibitor of AMCA-CLIP-(81–105) binding to DR2 (Fig. 3 *Left*). This inhibition followed competitive kinetics, as it could be overcome by increasing amounts of AMCA-CLIP-(81–105) (Fig. 3 *Right*). AMCA-CLIP-(81–98) was even more strongly inhibited by SEB, whereas AMCA-CLIP-(92–105) was significantly more weakly inhibited than AMCA-CLIP-(81–105) (Fig. 3 *Left*), suggesting that it is predominantly the N-terminal region of CLIP-(81–105) that shares an overlapping binding site with SEB. Similar observations were made with soluble DR1 and DR3 molecules (data not shown).

DISCUSSION

There is accumulating evidence that the CLIP region of Ii, which is encoded by exon 3, is predominantly responsible for tight association with class II MHC molecules and inhibition of premature loading (29–31). Moreover, CLIP seems to be the final proteolytic fragment of Ii that remains bound to class II molecules (17, 18). Since CLIP and antigenic peptide binding are mutually exclusive (14), CLIP release is essential for efficient antigen presentation. The respective mechanism is not clear, but it appears reasonable to assume that this mechanism functions in a CLIP-specific manner.

In this study we demonstrate that CLIP-(81–105) has intrinsic properties facilitating its own removal. The experiments indicate a dissection of CLIP-(81–105) into two functionally distinct halves that complement each other. The highly and positively charged N-terminal segment mediates rapid on/off kinetics but does not directly interfere with antigenic peptide binding. In contrast, the C-terminal half [CLIP-(92–105)] displays slow on/off kinetics and is sufficient for inhibition of peptide binding (Table 2 and Fig. 2). Both halves act cooperatively conferring high affinity to CLIP-(81–105) for binding to DR2 (Table 1) and also for DR1 and DR3 (data not shown). The fact that the N-terminal half of CLIP-(81–105) is able to interact with DR molecules but is irrelevant for physical inhibition of antigenic peptide binding probably explains the partial lack of correlation between K_d and IC_{50} values: for example, Lys-90 and Met-91 at the N terminus of CLIP-(90–105) increase the K_d value 5-fold relative to CLIP-(92–105), whereas the IC_{50} values of both peptides are comparable.

The observed fast on-rate of CLIP-(81–105), caused by its N-terminal portion, is consistent with the reported fast assembly of Ii with αβ dimers *in vivo* (2), which prevents premature ligand binding in the ER. It should be noted that under equilibrium conditions, CLIP-(92–105) is found to have 1/50th of the affinity of CLIP-(81–105) (see Table 1), whereas on- and off-rates for each peptide are in the same order of magnitude, respectively (see Table 2). Thus, equilibrium constants cannot be directly derived from kinetic measurements in this case, most probably because binding reflects a multistep mechanism.

At pH 4.5, which is found in endocytic compartments where removal of CLIP is thought to occur, we found accelerated dissociation of CLIP-(81–105) but not of CLIP-(92–105) (Table 2), again emphasizing the important role of the N-terminal region for rapid release of CLIP. CLIP-(81–105)-specific release at low pH has also been described for purified *ex vivo* DR1–CLIP (41) as well as for DR3–CLIP complexes *in vivo* and *in vitro* (18, 42). Thus, the interaction of CLIP with DR molecules is weakened at low pH irrespective of the *HLA-DR* allele. This may be accomplished by protonation of monomorphic glutamic or aspartic acid side chains on DR molecules that are negatively charged at neutral pH, allowing them to interact with positively charged side chains of Lys-83 and/or Lys-86 of the CLIP N-terminal region, which are highly conserved. Since binding of this region to MHC class II molecules is strongly blocked by SEB (Fig. 3), the respective contact sites most likely are located in the SEB contact area on the α1 domain of DR molecules. Thus, Asp-17, Glu-21, and Asp-35 lying in close proximity on the α1 domain of DR are candidate contact residues. Interactions between positively and negatively charged residues may also account for the observed accelerated dissociation of DR2–HA-(307–319) complexes at low pH (see Table 2), as the positively charged Lys-317 of HA-(307–319) is part of the anchor motif relevant for binding to DR2a molecules (see Fig. 1 *Lower*; ref. 24). Accordingly, pH 4.0 treatment releases CLIP-(81–105) but not other DR1-associated self-peptides because binding of the latter does not involve positively charged peptide anchor residues (34). The postulated interactions between the positively charged N-terminal half and negatively charged side chains on the α1 domain of MHC class II molecules is also consistent with recent findings that positively charged primary amines and fatty acids promote CLIP dissociation *in vitro* (18).

Recent cellular binding studies involving mutation of position Glu-11 inside the α1 DR1 groove revealed reduced binding of SEB and prompted the authors to hypothesize that SEB interferes with a subset of groove-binding peptides, but direct evidence has not been presented (43). For our own studies, we can exclude interference by SEB with CLIP-(81–98) via a binding site inside the groove because CLIP-(81–98) does not inhibit binding of the groove binding peptide HA-(307–319) (Table 1 and Fig. 1 *Upper*). Interaction of the CLIP N-terminal segment with the α1 domain of class II molecules outside the groove is probably the first and rate-determining step during CLIP-(81–105) binding. Since this is a low-affinity step (see Table 1), SEB with its moderate affinity (K_d = 1 μM; ref. 44) easily competitively blocks CLIP-(81–105) (see Fig. 3), although the final DR2–CLIP-(81–105) complex appears to be of high affinity (see Table 1).

Previous studies using cellular binding assays suggested distinct binding sites on HLA-DR for Ii and staphylococcal enterotoxins (45). These authors looked at inhibition of SEB binding by Ii, whereas we have investigated inhibition of CLIP by SEB, which might explain the apparent discrepancy. It is possible that CLIP may not block SEB binding because the SEB contact area is fairly large, involving at least 20 residues of the DR molecule. In contrast, CLIP is likely to cover only a small part of this area.

The CLIP-(81–92) region seems to influence also the second step of CLIP-(81–105) binding: DR2–CLIP-(81–105) complexes dissociate with a relatively constant fast off-rate irrespective of the incubation time with which they were generated (Fig. 2 *Center*), whereas DR2–CLIP-(92–105) complexes mature with increasing time, showing an initial fast off-rate but a

considerable portion of slowly dissociating complexes after 48 h of incubation (Fig. 2 *Right*). This is reminiscent of the maturation of groove-binding peptide–DR complexes (Fig. 2 *Left*). Maturation in terms of increasing stability is thought to involve at least two conformational states (38, 39). In the short-lived initial complex weak interactions between the peptide and the class II molecule dominate. Subsequent conformational changes allow the formation of stable complexes via tight contacts between peptide anchors and several specificity pockets (34, 38). Accordingly, the inability of DR2–CLIP-(81–105) complexes to mature seems to imply that binding of the N-terminal CLIP segment prevents the formation of tight side-chain/pocket contacts of the C-terminal CLIP region that might occur in absence of the N-terminal CLIP region. Whatever these contact sites are, they do not appear to be the same ones used by conventional groove-binding peptides, because the clustering of anchor residues Met-99, Leu-102, and Pro-103 at the CLIP C terminus is clearly different from the established DR2a peptide-binding motif (Fig. 1; refs. 24 and 35). In this context it is of interest that the myelin basic protein peptide Ac(1–14)A4C15 behaves in a similar manner to CLIP-(81–105), as it forms exclusively short-lived complexes (with H-2A^k), but is fully functional in T-cell proliferation assays (46). In contrast to CLIP-(81–105), this peptide has a low affinity reminiscent of kinetic intermediates (34). The special feature of CLIP-(81–105) displaying high affinity under steady-state conditions but fast off-rates, is probably due to its very fast on-rate that compensates for the low stability (see Table 1).

If CLIP-(81–105) were to reflect the kinetic behavior of the whole Ii, the fast off-rate of CLIP-(81–105) at 37°C would be disadvantageous for the task of Ii to prevent premature loading of class II in the ER. However, this difficulty seems to be overcome by trimerization of Ii. In a trimeric Ii scaffold containing three $\alpha\beta$ class II dimers, not only simultaneous breakage of three CLIP–$\alpha\beta$ interactions would have to occur for complete dissociation of the complex but also disassembly of the Ii trimer. Since there is additional stabilization by membrane anchoring, such an event is highly unlikely; therefore, $(\alpha\beta\text{Ii})_3$ is stable unless proteolysis generates single $\alpha\beta$–CLIP complexes.

The fast dissociation of CLIP-(81–105) at pH 6.0 and 37°C *in vitro* explains the observation that DR1 molecules isolated from endosomal vesicles containing large amounts of DR1–CLIP-(81–105) complexes displayed 5- to 10-fold higher binding capacity for antigenic peptides than did conventional DR1 preparations isolated from whole-cell lysates (ref. 47; H.K., unpublished observations).

However, there is evidence that acidic pH and the intrinsic property to dissociate are not always sufficient for quantitative and rapid removal of CLIP *in vivo*: in contrast to wild-type cells, HLA-DM-negative mutants accumulate $\alpha\beta$–CLIP complexes (18, 19, 42, 48). Therefore HLA-DM appears to be involved in the removal of CLIP-(81–105), but the requirement for HLA-DM does not seem to be absolute because products of some class II alleles, such as *H-2A^k*, are loaded with peptides in the absence of HLA-DM (49). It has been speculated that HLA-DM directly removes CLIP, but so far no HLA-DM–CLIP complexes could be found (50). Alternatively, HLA-DM or an unknown molecule that is dependent on HLA-DM might be transiently engaged in a complex with $\alpha\beta$CLIP to labilize the CLIP–$\alpha\beta$ interaction and/or to prevent rebinding of CLIP.

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