Supermotifs Enable Natural Invariant Chain-derived Peptides to Interact with Many Major Histocompatibility Complex–Class II Molecules

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Summary

Class II–associated invariant chain peptides (CLIPs) compete with natural allele-specific ligands for binding to several purified HLA-DR molecules. Truncation and substitution analysis showed that a minimal sequence of 13 amino acids is sufficient for excellent binding to DR17 and DR1. Hydrophobic residues at relative positions 1 and 9 (P1 and P9) which are shared among these DR-ligands, and are found to be anchored in complementary pockets by x-ray crystallography allow specific binding. Two flanking residues at either end next to the specific contact sites Met_{107} and Met_{115} contribute to binding irrespective of their side chains, suggesting H-bonds to the major histocompatibility complex (MHC) molecule. Thus, CLIPs behave like conventional ligands, however, lack their allele-specific contact sites. Introduction of the DR17-specific contact site aspartate at P4 dramatically improves invariant chain–peptide binding to DR17, but reduces DR1 binding. By contrast, binding to DR1, but not DR17 is strongly improved after introduction of the DR1-specific contact site alanine at P6. In addition, analyzing the fine specificity of the hydrophobic contact sites at P1 and P9, CLIP variants reflected the allele-specific preferences of DR17- or DR1-ligands, respectively, for aliphatic or aromatic residues. Alignment studies suggest that CLIPs are designed for promiscuous binding in the groove of many MHC class II molecules by taking advantage of one or more supermotifs. One such supermotif, for example, does not include the DR17-specific contact site aspartate at P4, which in conventional natural ligands like Apolipoprotein (2877-94) is necessary to confer a stable conformation. Introduction of aspartate at P4 generates a CLIP variant that is stable in the presence of sodium dodecyl sulfate, such as allele-specific ligands. Studying the stability of class II–CLIP complexes at pH 5, we found that CLIPs, similar to anchor-amputated ligands, can be released from class II molecules, in contrast to conventional natural ligands, which were irreversibly bound. Taken together, our data provide compelling evidence that CLIP peptides bind into the class II groove.

MHC class II molecules are heterodimeric transmembrane glycoproteins that present immunogenic peptides to T lymphocytes. Such peptides are normally derived from the endocytic pathway (1, 2), whereas class I molecules predominantly bind peptides generated from proteins synthesized in the cell. These functional dichotomies between class I and II MHC molecules are preserved by the invariant chain (Ii)[1], a nonpolymorphic, non-MHC–encoded transmembrane protein (3, 4). After assembly of α and β polypeptides with Ii in the endoplasmic reticulum, an NH_2-terminal signal sequence of Ii drives the stoichiometric complex into the endosomal system (5, 6). During this transport Ii prevents interaction of class II molecules with peptides or segments of intact proteins (3, 4). Proteolytic cleavage of the Ii in a post-Golgi compartment releases α/β dimers, making the class II binding site available (7). The precise mechanisms involved in forming class II–peptide complexes are unclear, although the existence of antigen processing–deficient mutants argues that at least one additional gene product, HLA-DM, encoded in the MHC region, is involved. In such mutants, a limited variety of self peptides was found on MHC class II molecules (8–10). The dominant sequences were derived from a distinct region of the Ii chain and were presented as nested sets on either ends, with a common core (8, 9). Similar class II–associated invariant chain peptides (CLIPs) have been found in wild-type cells of humans and mice (11–14), implying that

[1] *Abbreviations used in the paper:* ApoB, Apolipoprotein B; CLIP, class II–associated invariant chain peptide; Ii, invariant chain.

these complexes may be biosynthetic intermediates that accumulate in these mutants (10, 15). From several studies it was clear that CLIPS can compete in vitro with natural ligands for class II binding (8, 9, 16). However, the question remained how they bind to class II molecules. It is especially unclear whether CLIPs bind in the MHC class II binding groove, or at another site of the MHC molecule. Such a question became more interesting, since it was recently found that the sequence of CLIP and the segment indispensable for Ii–class II interaction are related (17, 18). We reproduced the competitive effect of CLIP-binding with natural ligands and addressed the problem of the interaction between class II and CLIP by studying the binding characteristics of CLIP peptides and variants of CLIP peptides to different class II molecules. Our recent study of allele-specific contact sites of natural DR17 ligands (19, 20) prompted us to choose HLA-DR17 and HLA-DR1 alleles that have been found to be associated with CLIPs in vivo. The latter were intensively studied for their binding motifs (21–24) and crystallographic data provided evidence how contact sites of natural ligands are accommodated in the DR1 peptide binding groove (25, 26). Comparative studies of CLIP-binding motifs with motifs of conventional natural class II ligands and modulation at allele-specific contact sites revealed a high degree of compatibility, suggesting CLIP-binding in the peptide binding groove.

Materials and Methods

Cell Lines and Antibodies. EBV-transformed human B cells from a local homozygous donor (RUP, DR17, and DR52a, confirmed by oligonucleotide typing) and LG2 cells (DR1) were grown in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% FCS (Boehringer-Mannheim, Mannheim, Germany), 20 mM Hepes, 2 mM glutamine, and antibiotics and expanded in roller bottles to 0.5×10^{10}–1×10^{10} cells.

mAb L243 obtained from the American Type Culture Collection (Rockville, MD) was used for immunoprecipitation of MHC molecules and analysis of native HLA-DR17 or HLA-DR1 molecules in ELISA. mAb L243 binds to a nonpolymorphic determinant present on DR molecules.

HLA-DR Purification. The purification of HLA-DR17 and HLA-DR1 molecules from homozygous EBV-transformed B cell line RUP or LG2 using L243-coupled, CNBr-activated Sepharose (Pharmacia, Freiburg, Germany) was performed as described (20). DR1 or a mixture of HLA-DR17/52a molecules was eluted with 25 mM Na_2CO_3, 0.15 M NaCl, 0.1% NP40, and 0.1 mM PMSF, pH 11.0, neutralized immediately with 50 mM Tris/HCl at pH 8.4 an stored at 4°C until use.

Peptides. Peptides were synthesized on a multiple synthesizer (model SMPS 350; Zinsser Analytik, Frankfurt/M, Germany) using Fmoc/tBu strategy and purified by reversed phase HPLC (model 600; Waters, Eschborn, Germany) to achieve a purity of >90%. Structure and purity were confirmed by electrospray mass spectrometry (model API III; Sciex, Toronto, Canada). Apolipoprotein (Apo) B(2877-2894) and HLA-A2(103-117) were NH_2-terminally biotinylated on line by five coupling steps forming Biotin-ε-Lys-β-Ala-Ahx-β-Ala-[ApoB100 2877-2894] or in solution by a 3.5 molar excess of biotinyl-ε-aminocaproyl-*N*-hydroxysuccinimide ester BACNHS; Sigma Chemical Co., St. Louis, MO) at 20°C for 2 h.

Peptide Binding Assay. The peptide binding assay was performed as described by Jensen (27) and elsewhere (20). Briefly, purified HLA-DR molecules (100 nM) were incubated at 37°C with 2 μM biotinylated agonist with or without competitor peptide in 96-well microtiter plates (Greiner, Nürtingen, Germany) in binding buffer (50 μl) containing 25 mM Na-carbonate, 50 mM Tris, 2 mM EDTA, 0.01% azide, 0.1 mM PMSF, and 0.1% NP40 adjusted to pH 5 by 1 M citrate. After 72 h, MHC–peptide complexes were separated from free peptides by immunoprecipitation with immobilized L243 and detected by successive incubation at 20°C with streptavidin (10 μg/ml) and biotinylated peroxidase (1 ng/ml; both from Dianova, Hamburg, Germany) for 45 min, respectively, followed by incubation with ABTS (1 mg/ml; Boehringer-Mannheim). The absorbance at 405 nM was measured by an ELISA reader (Multiskan Plus; Titertek, Meckenheim, Germany) after about 45 min, and nonspecific signals (quadruplicates, typically 15% of maximal absorbance) were subtracted from the data.

Stability of Class II–Ligand Complexes. 100 nM DR17 molecules was preloaded with 2 μM biotinylated peptides for 4 d (37°C, pH 5) and separated from excess peptides by 10-K ultrafiltration (Amicon Corp., Beverly, MA). The time course of dissociation of DR17 complexes in the presence of 100 μM unlabeled ApoB(2877-2892) was determined by incubation at 37°C, pH 5 and stable complexes were detected as described in the previous paragraph.

SDS-PAGE and Western Blot Analysis. HLA-DR17 molecules were isolated from the homozygous EBV-transformed B cell line RUP or LG2 as described above (20). 1 μM class II molecules was incubated for 3 d in binding buffer at pH 5 in the presence of 25 μM peptide and neutralized by addition of 50 mM Tris, pH 8.8. An equal volume of 2× nonreducing Laemmli sample buffer was added and samples were run on 10% SDS-PAGE. After transfer (Multiphor; Pharmacia) the Nylon membrane was blocked with 6% Casein/0.05% Na-azide/10 mM EDTA/PBS, pH 7.4, subsequently treated with streptavidin (2.5 μg/ml), and complexes were detected with biotinylated alkaline phosphatase (100 ng/ml) and chemiluminiscence.

Results and Discussion

Overlapping peptides corresponding to the Ii sequence Ii (97-120) (numbering from the NH_2 terminus of the longer p35 form) (28) were synthesized. Peptides were initially tested for their ability to compete with the natural DR17 ligand Apolipoprotein B100 [ApoB(2877-2894)] for binding to purified HLA-DR17 (20). MHC–peptide association was performed in solution at pH 5, shown to be optimal for natural ligands including CLIP(97-120) to bind to DR17 (data not shown). Fig. 1 *a* shows the inhibiting effect of six Ii 12-mer peptides versus 2 μM ApoB(2877-2894). Ii(106-117) competed in a dose-dependent manner, whereas the other peptides showed no effect. Thus, only Ii(106-117), a sequence contained in all Ii ligands found on DR molecules, blocked the binding of ApoB(2877-94) to DR17.

To study the binding of Ii(106-117) in detail, we analyzed contact residues of the 12-mer by single Ala substitutions. At most positions we observed only slight shifts in the binding capacity (Fig. 1 *b*). However, Ala substitutes for Met_{107} or Met_{115} did not or did only marginally compete, even at high concentrations, corresponding to a decrease of the binding capacity of at least 100- and 10–20-fold, respectively (Fig. 1 *c*). This notion is consistent with the failure of Ii(97-113) (data not shown), Ii(103-114), and Ii(109-120) (Fig. 1 *a*),

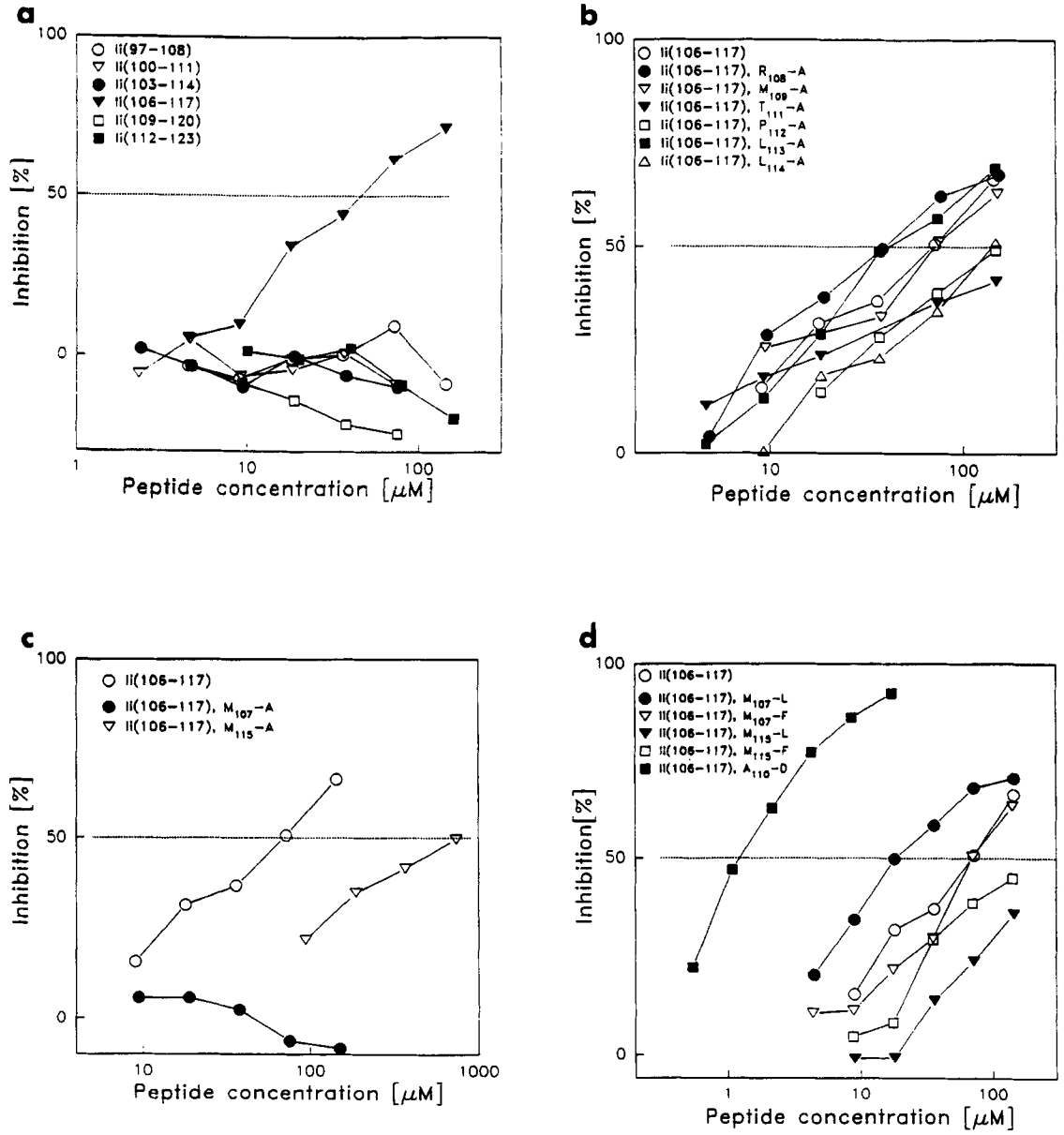

Figure 1. Ability of increasing amounts of Ii 12-mers derived from the CLIP sequence and variants of Ii(106-117) to compete with 2 μM biotinylated ApoB(2877-2894) for binding to purified DR17 molecules. Binding of biotinylated Apo(2877-2894) in the absence of competitor was 0.599 ± 0.021 (*a*), 0.602 ± 0.032 (*b* and *d*), and 0.507 ± 0.017 (*c*) OD_{405} U (quadruplicates). Nonspecific signal in the absence of MHC molecules was 0.063 ± 0.003 (*a*), 0.084 ± 0.002 (*b* and *d*), and 0.065 (*c*) (quadruplicates). Percent inhibition of agonist-binding, performed in duplicates, was expressed using the formula: 1 − [(signal with competitor − background/signal without competitor − background)] × 100%.

lacking either Met_{107} or Met_{115}, to compete with ApoB(2877-2894) for binding to DR17. Moreover both methionines are contained in the nested set of CLIPs. In conclusion, we have identified two hydrophobic DR17 contact sites of CLIPs, Met_{107} and Met_{115}.

Phe or Leu substitution for Met_{115} resulted in a lesser decrease of the binding capacity as compared with the Ala substitution, whereas the same substitutions of Met_{107} maintained the parental binding capacity of Ii(106-117) or even improved it (Fig. 1 *d*). Thus, a conservative exchange for hydrophobic residues like leucine or phenylalanine can replace Met_{107} and Met_{115} better than the small alanine. Aspartate at these positions completely disrupt binding (data not shown). We could reconfirm these findings with CLIP(97-120) and therefore rule out a different mechanism in binding of the longer CLIPs compared with the truncated forms (data not shown).

Met_{107} and Met_{115} are spaced by seven amino acids. This is the same spacing of hydrophobic/aromatic contact residues, at relative position P1 and P9, as found in natural DR17 ligands interacting with the peptide binding groove (19, 20, and Table 1). In addition to this partial homology between Ii(106-117)

Table 1. *Contact Sites in HLA-Class II peptide Motifs Suggest Supermotifs Used by CLIPs*

	Relative Position									Reference
	1	2	3	4	5	6	7	8	9	
HLA-DR17 motif	**L I F M**			**D**		**K R**			**Y L F**	19
CLIP(97-120)	M	R	M	A	T	P	L	L	M	8, 9
CLIP(97-119)	M	R	M	A	T	P	L	L	M	8, 9, 11
CLIP(98-119)	M	R	M	A	T	P	L	L	M	9, 11
CLIP(99-120)	M	R	M	A	T	P	L	L	M	8, 9
CLIP(99-119)	M	R	M	A	T	P	L	L	M	11
HLA-DR1 motif	**Y F V L**			**M L A**		**A G S**			**L A M**	14, 21-24
CLIP(97-121)	M	R	M	A	T	P	L	L	M	11
CLIP(97-120)	M	R	M	A	T	P	L	L	M	11
CLIP(97-119)	M	R	M	A	T	P	L	L	M	11
CLIP(98-121)	M	R	M	A	T	P	L	L	M	11
CLIP(98-120)	M	R	M	A	T	P	L	L	M	11
CLIP(98-119)	M	R	M	A	T	P	L	L	M	11
CLIP(99-120)	M	R	M	A	T	P	L	L	M	11
CLIP(99-119)	M	R	M	A	T	P	L	L	M	11
CLIP(100-119)	M	R	M	A	T	P	L	L	M	11
CLIP(106-120)	M	R	M	A	T	P	L	L	M	11
CLIP(106-119)	M	R	M	A	T	P	L	L	M	11
CLIP(106-120)*	M	A	T	P	L	L	M	Q	A	11
CLIP(106-120)*	A	T	P	L	L	M	Q	A	L	11
HLA-DR2a motif	**F Y**	**K**		**I A V**						45
CLIP(97-120)	M	R	M	A	T	P	L	L	M	11
CLIP(98-120)	M	R	M	A	T	P	L	L	M	11
CLIP(98-119)	M	R	M	A	T	P	L	L	M	11
CLIP(99-120)	M	R	M	A	T	P	L	L	M	11
CLIP(99-119)	M	R	M	A	T	P	L	L	M	11
CLIP(100-119)	M	R	M	A	T	P	L	L	M	11
CLIP(106-124)	M	R	M	A	T	P	L	L	M	11
CLIP(106-119)	M	R	M	A	T	P	L	L	M	11
HLA-DQ7 motif	**F Y I L M**				**V L I M**		**Y F M L**			14
CLIP(97-115)	M	A	T	P	L	L	M			14
CLIP(99-117)	M	A	T	P	L	L	M	Q	A	14
CLIP(111-126)	L	M	Q	A	L	P	M	G	A	14
CLIP(111-125)	L	M	Q	A	L	P	M	G	A	14

Specific contact sites demonstrated in this study are indicated in bold, noncore contact sites are double underlined; and contact residues deduced from ligand and/or binding motifs are underlined. Chemically related residues found on the anchor positions of the postulated ligand or binding motif are also accepted.

* An alternative use of contact sites shown with CLIP(106-120) may be possible.

and natural DR17 ligands, however, there is a major difference. The natural DR17 ligand motif indicates the four specific contact sites P1, P4, P6, and P9. P4 is the allele-specific anchor dominantly occupied by aspartate. Introduction of aspartate instead of Ala_{110} at P4 into Ii(106-117) (numbered from Met_{107} designated as P1) generated a 20-fold more potent competitor as compared with the parental Ii-peptide (Fig. 1 *d*). This striking improvement of the binding capacity upon a single amino acid substitution strongly indicates the CLIPs have the basic structural features for binding into the peptide binding groove, although CLIPs are suboptimally endowed with allele-specific contact residues (Table 1).

The relative binding capacity of the Ii(106-117) was ~50 μM, whereas the longer peptide Ii(97-120) required just 1 μM to achieve the same effect (Fig. 2 *a*). Similar binding capacities (0.7–3.8 μM) were found for the majority of natural ligands isolated from DR17 molecules (Fig. 2 *b*). The great difference in the binding capacity of truncated CLIPs versus

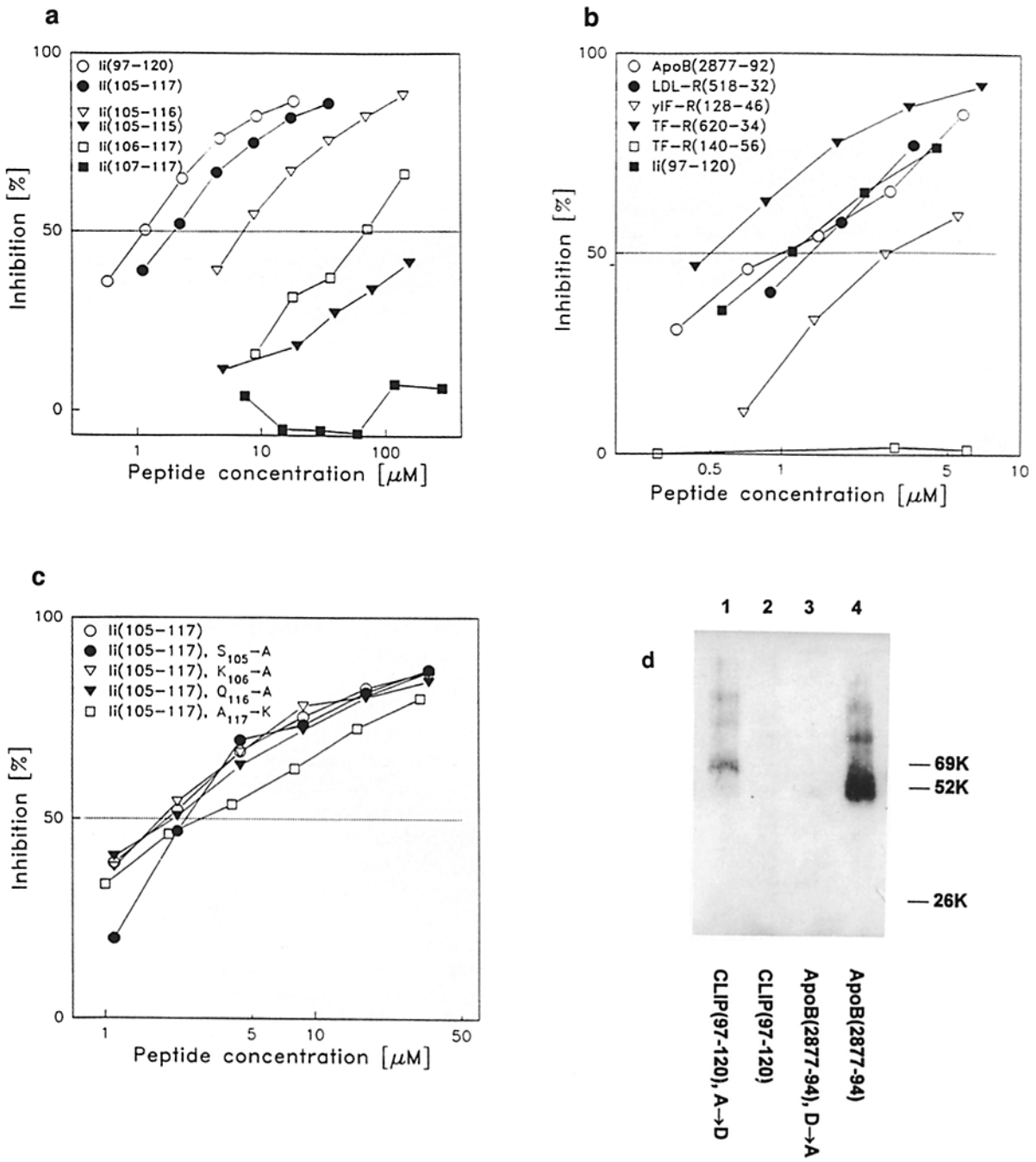

Figure 2. Binding of Ii-peptides, variants, and specific DR17 ligands to DR17 molecules. In competition assays the binding capacity of CLIP(97-120) was compared with truncated variants (*a*) and to the specific DR17 ligands (9) (Low density lipid receptor [*LDLR*], IFN-γ receptor [*γIF-R*], transferrin receptor [*TF-R*]) TF-R (140-56), a natural DR1 ligand was included as a control (*b*). The contribution of flanking residues on binding was investigated by removal (*a*) or substitution of single amino acids next to the hydrophobic contact sites Met_{107} or Met_{115} (*c*). Binding of biotinylated ApoB(2877-2894) in the absence of competitor was 0.602 ± 0.034 (*a* and *c*) and 0.735 ± 0.034 (*b*) OD_{405} U (quadruplicates). Nonspecific signal in the absence of MHC-molecules was 0.084 ± 0.002 (*a* and *c*) and 0.057 ± 0.002 (*b*) (quadruplicates). SDS stabilization of HLA-DR17 dimers under nonreducing conditions analyzed in Western blot in the presence of natural DR17 ligands and their variants, altered at the allele-specific contact site (*d*). In the presence of DTT no signal was detectable (not shown).

CLIPs of natural length is likely due to the flanking residues. Removal of the 8 NH_2-terminal and of the 3 COOH-terminal residues [Ii(105-117)] had little effect on binding (Fig. 2 *a*). However, further removal reduced the binding capacity dramatically (Fig. 2 *a*). To determine the specificity of the four residues Ser_{105}, K_{106}, Q_{116}, and A_{117}, which were obviously contact sites outside the core region, these amino acid residues were substituted for a variety of amino acids. It did

not matter whether such flanking residues were substituted for Ala (Fig. 2 *c*) or even for charged residues (data not shown), since the parental binding capacity of Ii(106-117) was always retained. These data indicate that residues at positions 105, 106 and 116, 117 are necessary for binding, but irrespective of their side chains. A similar contribution on binding was previously shown for cytochrome c peptides and demonstrated for the flanking regions of I-A^k- and DR17-specific ligands (20, 29, 30) and it is likely that such flanking residues are forming H-bonds to the MHC molecule as illustrated for HA(307-319) bound to DR1 (26). Thus, our results support the view that CLIPs bind in the DR17-peptide binding groove, like allele-specific ligands.

X-ray crystallography has clearly demonstrated how a ligand is accommodated by pockets along the peptide binding groove of the DR1 molecule, thereby explaining postulated ligand and binding motifs (14, 21–26). We therefore extended on CLIP-binding studies to DR1. Purified DR1 molecules were loaded with biotinylated natural DR1 ligand HLA-A2(103-117) and competition by Ii-peptides was measured. Among the six 12-mers tested, Ii(106-117), the peptide which contained contact sites for DR17 binding, was the best inhibitor (Fig. 3 *a*). This finding is not unexpected, since this sequence is contained in each CLIP found on DR1. Studying the contact sites within the 12-mer Ii(106-117) we again found that Met_{107} and Met_{115} efficiently contribute to binding (Fig. 3 *b*). Such results were reproduced with the long CLIP(97-120) and confirmed that the dominant contact sites are Met_{107} and Met_{115} (data not shown). The effects upon Ala substitution of these methionines on DR1 binding are significant although less impressive than those observed on DR17 binding, but became striking upon substitution for any charged residue (Figs. 1 *c* and 3 *b*, data not shown). Furthermore, substitution of M_{107} for phenylalanine strongly improved the capacity of Ii(106-117) to bind to DR1 (Fig. 3 *c*) but not to DR17 (Fig. 1 *d*). Such results are consistent with the fine specificity of DR1 and DR17 ligands at P1. Aromatic residues dominate over aliphatic residues at P1 among DR1 ligands (21–24), whereas the opposite preferences were found for DR17 ligands (19, 20). In the same vein, the effects on binding upon substitution of Met_{115} can be interpreted. Substitution for alanine showed a less impressive reduction than that for phenylalanine, whereas substitution for leucine even improved binding to DR1 (Fig. 3 *c*). These results are in perfect agreement with the fine specificity of DR1 ligands at P9 (24). Leucine dominates over methionine and an equivalent frequency was observed for alanine, whereas phenylalanine is not preferred. Thus, we have shown that CLIPs share M_{107} and M_{115} as contact sites P1 and P9 for DR1 and DR17 molecules. The side chain at P4 can be accommodated by the second pocket of the DR1 binding groove (26). P4 of DR1 ligands is predominantly occupied by residues with aliphatic side chains (14, 24). Leucine occupies P4 of the natural DR1 ligand HLA-A2(103-117) and alanine occupies P4 of CLIPs. The importance of aliphatic residues in these ligands was demonstrated upon Asp substitutions. In any case, the binding capacity dramatically dropped (Fig. 3, *b* and *d*). Thus, the same substitution shows strikingly different effects on CLIP-binding to DR1 and DR17, which is consistent with the allele-specific binding requirements of class II ligands at P4, aliphatic residues in DR1, aspartate in DR17 ligands. Moreover, introduction of the DR1-specific contact site alanine at P6 (14, 24) improved the binding capacity of the Ii-peptide dramatically (Fig. 3 *c*). In summary, we have shown how CLIPs adapt to different allele-specific requirements. Side chain–dependent as well as –independent (data not shown for DR1) contact sites of CLIPs fit perfectly with natural allele-specific ligands of DR1 and DR17. The use of hydrophobic contact sites common among DR ligands (Table 1) enable CLIPs to bind well to different class II molecules including DR17, which has an unusual and highly specific ligand motif (19). The lack of allele-specific contact sites in CLIPs is useful to keep potential negative effects at a minimum, particularly avoiding charged residues (20, 31) which could jeopardize binding to other class II molecules. Thus, CLIPs appear to be designed to be promiscuous MHC binders. This is illustrated in the excellent binding capacity of CLIPs found to be associated with DR17 and DR1 (9, 16 and Figs. 2 *b* and 3 *e*).

In addition to DR17 and DR1, CLIPs were found to be associated with a variety of class II molecules (11–14). Alignment studies suggest that CLIPs possess a sequence compatible with motifs of different class II alleles and isotypes (Table 1). CLIPs, for example, found to be associated with DR4Dw4 on T2 transfectants (32) use the same minimal sequence as DR1 and DR17 for binding with Met_{107} as main anchor (Malcherek, G., and A. Melms, unpublished results). Met_{115} does not serve as anchor at P9, the position, which is not conserved by hydrophobic residues within DR4Dw4 ligands (24). These allele-specific effects should have consequences on the capacity to bind to DR4Dw4. Indeed, the capacity of CLIP(97–120) to compete with natural DR4Dw4 ligands is reduced in vitro (Malcherek, G., and A. Melms, unpublished results) and probably in vivo, explaining the absence of CLIPs on DR4Dw4 wild-type cells. In consequence, CLIPs have the ability to bind to a series of HLA-DR allele products by taking advantage of supermotifs, which enable variable binding affinities depending on the respective allele (32). These supermotifs are not limited to the arrangement of contact sites of HLA-DR ligands, but are also compatible with motifs of isotypes like HLA-DQ (Table 1). Common to all these motifs is the use of a core sequence, which completely lacks a third of the NH_2-terminal part of the long CLIP-form Ii(97–120) for binding (Table 1).

From our data it becomes clear that CLIPs cannot fully meet the optimal allele-specific requirements of all class II motifs. CLIPs appear to have found a balance by avoiding residues resulting in high affinity for some alleles but adverse effects for others.

Figs. 1 and 3 show, that the lack of allele-specific contact sites does not preclude the excellent binding capacity of CLIPs. However, this lack becomes apparent if the MHC dimer stability in the presence of SDS is studied. CLIP(97-120) is not able to generate SDS-stable complexes with DR17 (8, and

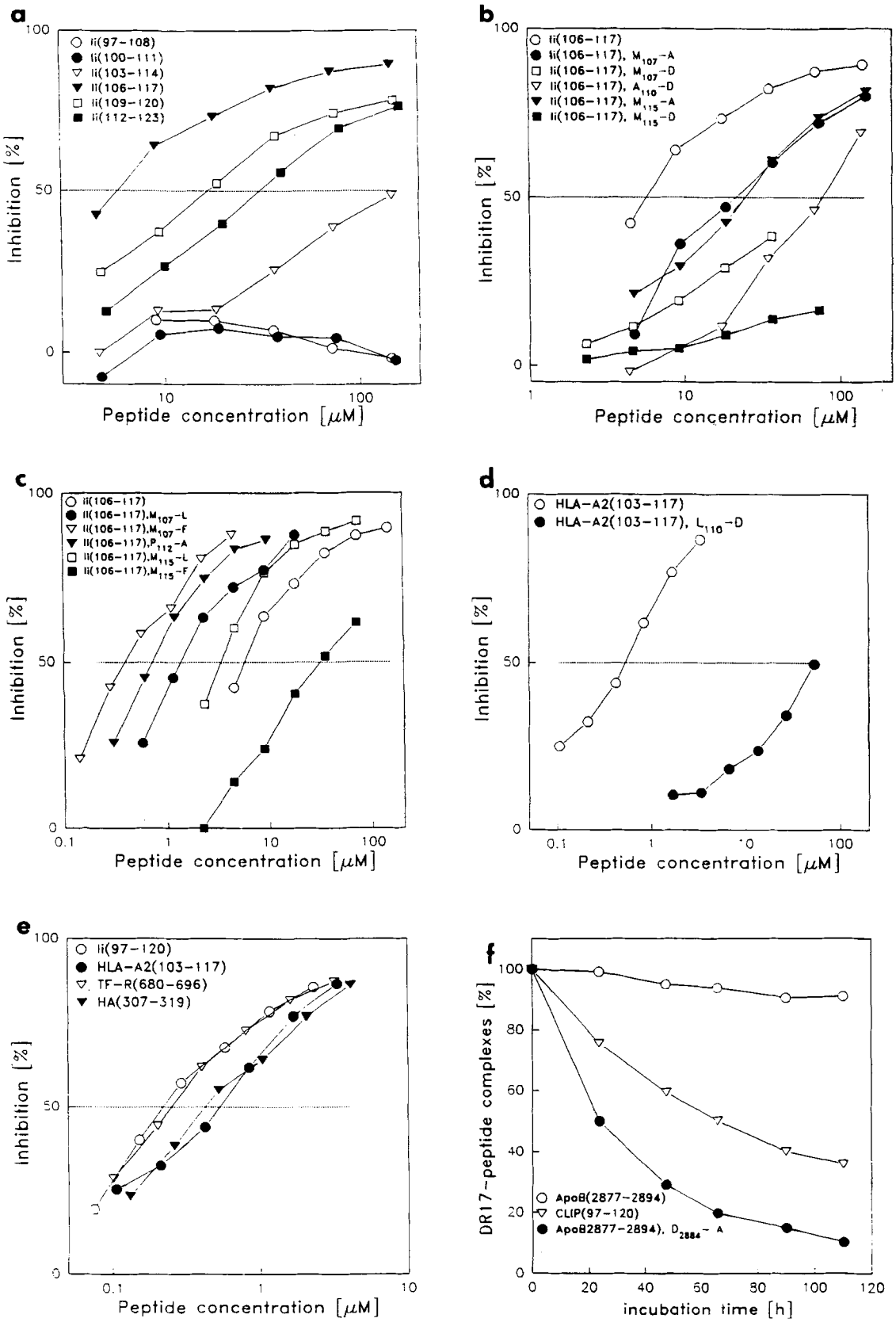

Figure 3. Ability of Ii-peptides, variants, and natural DR1 ligands to compete with 2 μM biotinylated HLA-A2(103-117) for binding to purified DR1 molecules (*a*–e). Binding of biotinylated HLA-A2(103-117) in the absence of competitor was 0.516 ± 0.014 OD_{405} U (quadruplicates). Nonspecific signal in the absence of class II molecules was 0.091 ± 0.002 (quadruplicates). Stability of MHC class II–peptide complexes in the presence of 100 μM natural ligands (*f*).

Fig. 2 *d*), whereas ApoB(2877-2894) does (Fig. 2 *d*). SDS stability of the latter is lost if we substitute Asp_{2884} for Ala at P4 (Fig. 2 *d*). Nevertheless, the variant retains a high capacity for binding to DR17 (data not shown). Thus, excellent binding in the MHC peptide binding groove does not necessarily coincide with SDS-stable association (30). Furthermore, the stability of HLA-DR17 α/β dimers appears to be dependent on the presence of the DR17-specific contact site aspartate. Aspartate introduced in CLIP(97-120) at P4 confers to SDS resistance (Fig. 2 *d*). In the same vein, complexes of HLA-DR4, DR11 with CLIPs are unstable in the presence of SDS (32), in contrast to complexes of DR1 with CLIPs (data not shown; 16). CLIPS are able to provide more specific contact sites for interaction with DR1 than with DR17, DR4, or DR11 (24, and Table 1). Thus, our findings clearly show that the different behavior in SDS is a direct consequence of CLIP binding after allele-specific rules and argues in favor rather than against groove binding (17).

High affinity, antigenic peptides are irreversibly associated with class II molecules in living cells (33), inducing a conformation required for surface expression. Once formed, this association is irreversible in the presence of SDS (34, and Fig. 2 *d*) and even in the presence of high amounts of conventional natural ligands (Fig. 3 *e*). In contrast, CLIPs behave like anchor-amputated variants of allele-specific ligands. These do not usually induce SDS stability and can be released from the binding groove by competing natural ligands at low pH (35, and Fig. 3 *e*). The intact Ii, however, blocks peptide binding until reaching the class II loading compartment (36–39). The physiological effects on antigen presentation are evident in Ii-negative cells, which express a different array of antigenic peptides. Some new peptides not seen on normal, Ii-positive cells occur, whereas other peptides are missing (40). Thus, Ii apparently determines presentation of antigenic peptides, probably also in the form of CLIPs succeeding the Ii. Our data indicate that CLIPs are fitted to bind into class II grooves, and that they can be replaced by high affinity, allele-specific ligands. Thus the role of CLIPs as anchor-amputated substitutes of allele-specific ligands could be, because of their nature as peptides, to provide a mechanism for effectively loading of class II molecules with antigenic peptides. Thereby CLIPs might also select binding of antigenic peptides in a way that binding of low affinity peptides present in the class II loading compartment is avoided, and the access to the groove is limited for high affinity ligands only.

Under this hypothesis, removal of displaced CLIPs by HLA-DM molecules, possibly serving as a sink for CLIPs, appears to be a prerequisite for efficient loading of allele-specific ligands, since the class II molecules of HLA-DM–defective cells are predominantly occupied by CLIPs (10, 41). In summary, our data provide compelling evidence that CLIPs as free peptides bind in the class II binding groove. This is the direct conclusion from studies focused on CLIPs performed at the peptide level. Our studies do not exclude, however, a second mode of CLIP-binding to class II molecules at a site other than the groove, as has been postulated by Kropshofer et al. (42). In our present studies we have addressed the question of how CLIPs bind to class II molecules, but we cannot determine when this interaction occurs. Recent findings that CLIPs are almost identical to a region of Ii indispensable for binding to class II molecules (17, 18) argued for a single binding site of CLIP as a free peptide and CLIP as a part of the Ii. The possibility that the intact Ii already occupies and prevents access to the binding groove cannot be ruled out. Polypeptides, in general, have the ability to bind to class II molecules and are able to compete with natural ligands (43, and Malcherek, G., and A. Melms, unpublished observations). Ii as a whole molecule exhibits an extending conformation (44), which should facilitate groove binding via the CLIP segment. Moreover, the instability in the presence of SDS coincides between class II–Ii and class II–CLIP complexes (4, 8, 32, 34, and Fig. 2 *d*), a feature shown not to be in contradiction with groove binding. An alternative explanation for the generation of class II–CLIP complexes is that CLIPs happen to be class II binding peptides which are released upon Ii proteolysis and subsequently compete with endocytically generated peptides for groove binding. A mutagenesis-based study focused on specific residues and shown in our study to be important for binding should help to determine whether the CLIP region interacts with the class II binding groove in the intact $\alpha\beta$-Ii complex as well.

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