

Self-Peptide Released from Class II HLA-DR1 Exhibits a Hydrophobic Two-Residue Contact Motif

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Summary

Peptide fragments of foreign and self-proteins are of great immunologic importance as their binding to major histocompatibility complex (MHC) class I or II molecules makes an interaction with a corresponding T cell receptor possible. Recently, allele-specific peptide sequence motifs proved to be responsible for MHC binding, no matter whether self- or non-self-antigens were involved. Up to now, all investigated human class II-associated peptides were derived from foreign antigenic proteins. Therefore, we undertook sequence and binding analyses with a 16-mer self-peptide (SP3) that has been eluted from HLA-DR1. Here we demonstrate, by synthetic polyalanine-based 13-mer analogues of SP3, that two bulky hydrophobic anchor residues with relative spacing *i, i+8* are sufficient for high affinity binding. This is consistent with the hydrophobic *i, i+8* binding pattern recently found for DR-restricted T cell epitopes. Nevertheless, highly helical alanine-based design peptides with anchor spacing *i, i+9* exhibit maximal affinity, whereas replacement of alanine by helix destabilizing proline abrogates binding. Thus, a two-residue contact motif is the common minimal requirement of self- and foreign peptides for high affinity anchoring to HLA-DR1. In contrast to class I, the anchor spacing of DR1-associated peptides seems to bear some variability due to conformational diversity.

T lymphocyte Ag receptors recognize complexes built up by class I or class II MHC molecules and peptide fragments of processed antigens on the surface of an APC (1). There is growing evidence that the discrimination of self from non-self cannot be the task of an APC since the cellular processing and MHC loading machinery is not able to distinguish self- from non-self-peptide antigens (2). Tolerance versus self seems to be due to negative selection of anti-self-T cells in the thymus and clonal anergy in the periphery (3, 4). Endogenously processed self-peptides, therefore, play a central role in several instances: (a) thymic MHC-bound self-peptides are involved in positive and negative selection of the T cell repertoire (5); (b) the stability of MHC/self-peptide complexes determines the number of MHC molecules available for foreign peptide antigens (6); and (c) a considerable portion of alloreactive T cell clones recognize MHC/self-peptide complexes (7). Sequence analyses of self-peptides from murine as well as human class I have shown that all of them are octa- or nonamers being fixed to the MHC molecule by allele-specific anchor residues (8, 9). High-resolution structural analyses revealed that HLA-B27 binds nonamer self-peptides in extended conformation (10). In contrast to these class I results, self-peptides released from murine class II proved to be 13–17

amino acids long having no sequence pattern comparable to class I self-peptides (11).

To unravel the corresponding human class II rules, we prepared a self-peptide mixture eluted from detergent-solubilized HLA-DR1. Our earlier results rationalized that DR1 molecules bear hydrophobic pockets that are filled upon peptide binding (12). This might occur by one to three apolar side chains that have been found with most of the DR-restricted foreign peptides (13–18), although the motifs defined by different groups applying different approaches are not uniform. Our findings presented here do strongly favor a hydrophobic motif consisting of two aromatic or aliphatic anchors, the spacing of which is not constant in all peptides, probably due to some folding flexibility of the peptide backbone.

Materials and Methods

Cells. EBV-transformed homozygous and consanguineous cell line WT-100 was used as a source for the isolation of HLA-DR1 and -DQw5 molecules. The cell line was maintained in vitro at 37°C, in 5% CO₂ by culture in RPMI 1640 (Gibco Laboratories, Grand Island, NY), containing 5% heat-inactivated FCS, 2 mM glutamine, and antibiotics.

Isolation of HLA-DR1 and -DQw5. Cells were lysed and the resulting homogenate prepared for affinity chromatography with mAbs L243 (anti-DR) (19) and TÚ22 (anti-DQ) (20), essentially as described elsewhere (12).

Preparation of Self-peptides. Endogenous peptides were released as previously described (12). The self-peptide pool and the three subpools SP1-SP3 were collected and dried by lyophilization.

Sequence Analyses. The self-peptide pool of 400 μ g solubilized HLA-DR1 and the subpools SP1-SP3 were lyophilized and subjected to sequencing by Edman degradation. It was performed in a pulsed-liquid sequencer (477A) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (120 A; Applied Biosystems, Inc., Foster City, CA). Glass-fiber filters were coated with 1 mg BioBrene Plus (Applied Biosystems, Inc.).

Peptide Synthesis. SP3 and IM(18-29) and all analogues were synthesized by continuous flow solid-phase peptide synthesis, using a synthesizer (9050; MilliGen, Eschborn, Germany) based on Fmoc/Bu^t strategy. The polyalanine 13-mer peptides were synthesized by a multiple automatic peptide synthesizer system (Zinsser Analytic, Frankfurt, Germany) based on Fmoc/Bu^t strategy. Lysine was incorporated at the COOH terminus for means of solubility. All peptides were purified by HPLC, and the identity was tested by ion-spray mass spectrometry.

Fluorescence Labeling. NH₂-terminal labeling was performed as previously described (12).

HPSEC Peptide-binding Assay. NH₂-terminally 7-amino-4-methyl-coumarin-3-acetic acid (AMCA) fluorescently labeled peptides (200 and 300 μ M) were coincubated with solubilized HLA-DR1 (3 μ M) or with HLA-DQw5 (3 μ M) for 48 h at 37°C in 50 mM sodium acetate, 0.1% (wt/vol) CHAPS, pH 5.3, respectively. In the competition assay with polyalanine-based 13-mer analogues of self-peptide SP3, NH₂-terminally labelled AMCA-SP3(1-16) (50 μ M) was coincubated with DR1 (3 μ M) and the competitor peptide (100 and 200 μ M), respectively, under the same conditions as cited above. 25- μ l aliquots of each sample were analyzed on a Superose 12 HR 10/30 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with the buffer cited above. High-performance size exclusion chromatography (HPSEC) was performed as previously described (21). The AMCA fluorescence signals eluting with HLA-DR or -DQ were recorded by an integrator (D-2500; Merck-Hitachi, Darmstadt, Germany) and taken for calculating the amount of bound AMCA-labeled peptide.

Results

Preparation of Self-peptides from HLA-DR1. Acid treatment of detergent-soluble HLA-DR releases endogenously bound self-peptides that can be separated by reversed-phase HPLC (8). Since we started with highly purified DR heterodimers previously freed from unspecifically or low affinity bound material by ultramembrane filtration (30-kD size exclusion), we can be sure that the acid-eluted components of the low molecular mass fraction have been bound specifically and with high affinity. Fig. 1 *a* shows the HPLC chromatogram of the self-peptide fraction (SPF) eluted from DR1. The profile is dominated by a characteristic clustering of signals between 5 and 19 min. Consistent with previous results from murine class I- and class II-derived peptide mixtures (8, 11), the SPF of HLA-DR1 elutes with 20-40% acetonitrile in the HPLC fractionation.

Sequence Analysis of a Self-peptide Mixture. The self-peptide

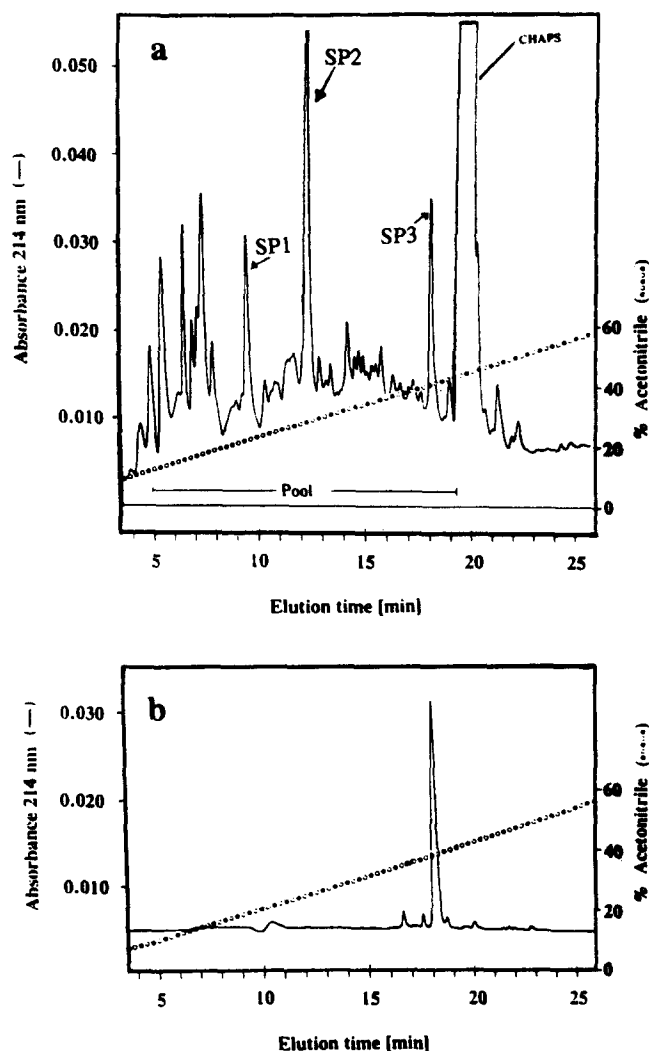


Figure 1. (a) Reverse-phase HPLC profile of low molecular mass fraction obtained after HCl-treatment of solubilized HLA-DR1 detected by absorbance at 214 nm. Fractions from 5 to 19 min (self-peptide pool) devoid of subpools SP1-SP3 were pooled and subjected to Edman sequence analysis. The dotted line gives the percentage of acetonitrile in the gradient. (b) Reverse-phase HPLC profile of synthetic SP3(1-16), AILEFRAMA-QFSRKT, detected by absorbance at 214 nm.

mixture, except SP1-SP3, was sequenced as a whole by conventional Edman degradation, as recently introduced (8). In contrast to the accumulation of certain amino acids in certain sequencing cycles, found with class I peptide mixtures (8), in our case isoleucine, leucine, and tyrosine simultaneously exhibit predominant signals in cycle 1 as well as in cycle 2. Alanine shows a weaker but prominent signal in cycle 1 (data not shown). All other cycles do not contain any other significant maxima. Since there is no increase of any residue beyond cycle 16, the apparent maximal length of the majority of analyzed self-peptides is 16 residues.

Sequence Analysis of SP3. Sequencing of the self-peptide subpools SP1-SP3 led to a definitive sequence only in the case of SP3, whereas in SP1 and SP2 most of the sequencing cycles exhibited two to three amino acids (data not shown). The

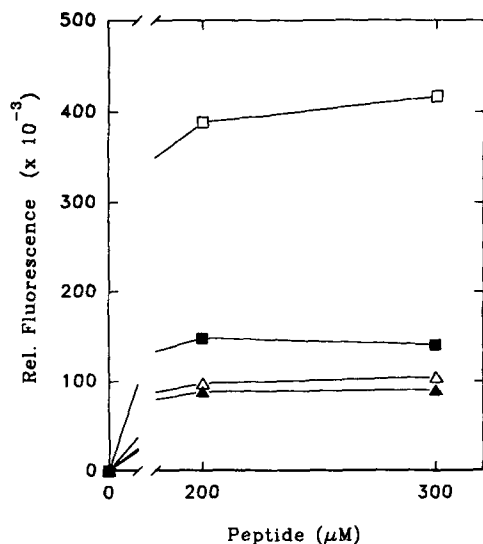


Figure 2. Affinity and selectivity of binding of self-peptide SP3 compared with IM(18-29) in an HPSEC peptide-binding assay with NH₂-terminally fluorescence labeled peptides under saturating peptide concentrations. Relative fluorescence at λ_{Em} 450 nm after coincubation of IM(18-29) and DR1 (■), SP3(1-16) and DR1 (□), SP3(1-9) and DR1 (▲), and SP3(1-16) and DQw5 (Δ).

sequence we got from SP3 is AILEFRAMAQFSRKTD. Its authenticity is confirmed by coelution of the synthetic peptide (Fig. 1 b). SP3 is characterized by a hydrophobic NH₂-terminal core and a COOH-terminal hydrophilic tail. The NH₂-terminal hydrophobic region is suited for an alignment according to the motif, recently deduced from a T cell assay with DR-restricted foreign T cell epitopes (18); thereafter, an aromatic or hydrophobic residue in position 1 (i), followed by S, T, A, V, I, L, P, C in position 6 (i+5), and another hydrophobic residue in position 9 (i+8), form a tentative DR

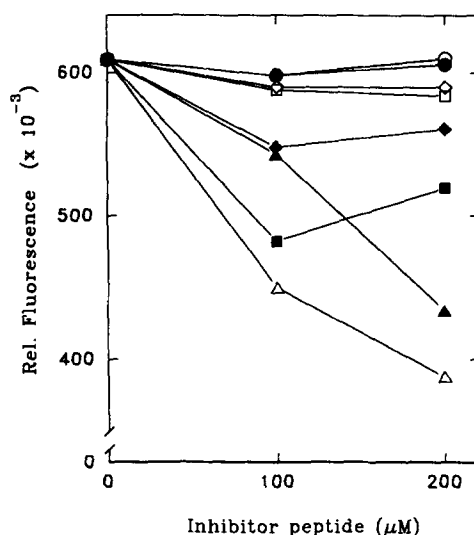


Figure 3. Competition assay with polyalanine-based 13-mer derivatives of the DR1-associated self-peptide SP3. Given is the decrease of fluorescence at λ_{Em} 450 nm, measured in an HPSEC assay after coincubation of DR1, (AMCA)-SP3(1-16) in presence or absence of the following inhibitory peptides: AL(A)₁₀K (□), AL(A)₉FK (short: F10) (■), AL(A)₈FAK (short: F9) (Δ), F8 (▲), ALA(PA)₃FAAK (◇), F7 (◆), F6 (○), and (A)₉FAAK (●). The sequences are given in one-letter code, respectively.

motif. In our SP3 sequence, very similarly, Leu-3 and Phe-11 obey the spacing rule i, i+8, and may function as anchors relevant for binding to DR1. In addition, the hydrophobic core region is comparable with the DR1-restricted T cell epitope influenza matrix peptide IM(18-29) by its high helical propensity, as previously proposed for IM(18-29) (14, 16). The binding capacities of SP3 and IM(18-29), both exhibiting the i, i+8 motif, were compared in a recently introduced HPSEC binding assay (21). The NH₂-terminally fluorescei-

Table 1. Alignment of DR1-restricted T Cell Epitopes and SP3

T cell epitope*		Protein source†
K	K I A K M E K A S S V F	Circumsporozoite protein (381-393)
	E V W R E E A Y H A A	Ragweed (51-61)
	Q Y I K A N S K F I G I T	Tetanus toxin (830-842)
E	A L V R Q G L A K V A	Ribonuclease (101-112)
	R V K R G L T V A V A G	<i>M. tuberculosis</i> (3-14)
A	K L A K L A G G V A V I K	<i>M. leprae</i> (65 kD) (412-425)
G	P L K A E I A Q R L E	Influenza matrix protein (18-29)
P	K Y V K Q N T L K L A T	Influenza hemagglutinin (307-319)
A	I L E F R A M A Q F S R K T D	Self-peptide SP3
i	i+8	Relative anchor position

The self-peptide sequence of SP3 was aligned according to the experimentally verified anchor spacing i, i+8 of influenza matrix peptide (18-29) (14, 18). * The sequences of the DR1-restricted T cell epitopes were aligned according to the NH₂-terminal bulky aromatic or aliphatic residue that is relevant for binding as recently described (12, 18).

† See references 14 and 22 for a complete list of references.

nated peptide SP3(1-16) shows high affinity binding to DR1 as evidenced by a threefold binding capacity compared with IM(18-29) (Fig. 2). An 80% smaller signal upon coincubation with HLA-DQw5 proves isotype-specific binding of SP3(1-16). The same reduction in affinity is observed with the COOH-terminally truncated derivative SP3(1-9). This result reconfirms the importance of the NH₂-terminal half, but simultaneously implicates other contact sites beyond position 9 crucial for high affinity binding. According to the postulated motif, Phe-11 could play this role.

Competition Assay with SP3-based Polyalanine Design Peptides. To answer the question whether Phe-11 is the COOH-terminal anchor of SP3, we created polyalanine-based 13-mer analogues of SP3 and tested them in an HPSEC competition assay with AMCA-labeled SP3(1-16) (Fig. 3). In alignment to IM(18-29), we chose Leu-3 as the NH₂-terminal anchor and shortened the peptide by the NH₂-terminal alanine (Table 1). The resulting peptides with Leu at position 2 and Phe, varying from position 12 to 8, proved to be powerful competitors, as already a fourfold excess was sufficient to compete efficiently with SP3(1-16) for binding. Most important, design peptides with anchor spacing *i, i+9* compete best (F9), although peptides with the pattern *i, i+8* (F8) and *i, i+10* (F10) are powerful competitors, too. The relevance of the NH₂-terminal anchor is shown by a sharper decline in competition after substitution of leucine by alanine as compared with substitution of phenylalanine. Significantly, leaving the anchor residues in their original distance (*i, i+8*) and placing three proline residues into the region between leads to a considerable loss of affinity and emphasizes the importance of folding propensities of amino acid side chains located between the putative anchor positions.

Discussion

As a whole, the sequence analysis of DR1-associated self-peptides reveals some similarities to the analyses of endogenously processed peptides of class I HLA-A2.1 (8) and HLA-B27 (9), or to murine class II molecules I-A^b and I-E^b (11). The strong accumulation of isoleucine, leucine, and tyrosine in positions 1 and 2 of the pool sequencing favors the view that these amino acids may function as anchor residues that are necessary for making contact with the DR1 peptide-binding groove. Since all three hydrophobic residues are predominant in both positions the NH₂-terminal anchor of DR1 might be out of frame, having variable distance from the NH₂ terminus. Alternatively, leucine, isoleucine, and tyrosine might form a double anchor at positions 1 and 2. Leucine was also found as a putative anchor of self-peptides eluted from class I HLA-A2.1, but exclusively in position 2 with an invariant distance from the NH₂ terminus (8). In the case of HLA-B27, nearly all of the sequenced self-peptides exhibited an arginine anchor at position 2 and, comparable

with our findings, in 7 of the 11 peptides arginine reappeared in position 1 (9). Additionally, our result is in contrast to the murine self-peptide analogues, where no invariant residues could be found (11). More important, human class II self-peptides are considerably larger than their class I counterparts. Whereas class I peptides are octa- or nonamers (8, 9), the majority of our class II peptides contains not more than 16 residues. This is in accordance with murine self-peptides that proved to be 13-17 amino acids long (11).

Common Hydrophobic Motif of DR1-associated Self and Foreign Peptides. The alignment of DR1-restricted T cell epitopes according to their putative NH₂-terminal hydrophobic anchor shows two parallels to our pool sequence analysis (Table 1): (a) similarly, bulky aliphatic residues as isoleucine, leucine, valine, and aromatic tyrosine residues appear in the NH₂-terminal anchor position, their importance being tested in a recent cellular binding assay with biotinylated derivatives (14); and (b) close to the putative first anchor most often stands another bulky hydrophobic amino acid that might function as a coanchor, fitting into one of the postulated hydrophobic pockets of DR1 (12). Two consecutive hydrophobic residues close to the NH₂-terminus reoccur in SP3 (Ile-2, Leu-3). This might explain the higher binding affinity of SP3 compared with IM(18-29), which lacks a comparable coanchor. Besides that, the alignment in Table 1 strengthens the importance of a COOH-terminal hydrophobic residue in position *i, i+8* or *i+9* relative to the NH₂-terminal anchor. The exact spacing seems to depend on the portion of helical conformation of the core-region bordered by the two anchors. Peptides with high helical propensity, as the polyalanine-based SP3 analogues, are rather compressed, their anchor residues being spaced according to *i, i+9*. The same seems to be true with the foreign T cell epitope circumsporozoite protein (381-393) (Table 1). Peptides, as influenza hemagglutinin(18-29) or IM(18-29), with lower helical propensity might adopt a slightly more extended conformation; thereby the distance of the contact sites is reduced to *i, i+8*. Our self-peptide SP3 also has a core region with considerable helical propensity and sticks to the same *i, i+8* rule. Helix-destabilizing prolines defold a previously helical peptide core, thereby reducing the affinity considerably (Fig. 3). Nevertheless, binding peptides seem to have some conformational freedom, due to a higher degree of folding flexibility in the binding groove of class II compared with class I molecules, no matter whether self- or non-self-antigens are involved. The immunological relevance of the putative hydrophobic two-residue contact motif may be that primarily hydrophobic regions, normally located in the interior of folded protein antigens and therefore "invisible" to the B cell receptor, become immunogenic, provided that a stringent selection by unfolding, processing, and proper targeting makes them "visible" for the helper T cell receptor.

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