

# Identification of a Motif for HLA-DR1 Binding Peptides Using M13 Display Libraries

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## Summary

Oligonucleotides encoding peptides known to bind to HLA-DR1 molecules have been inserted into the gene III of filamentous M13 phages. DR1 molecules purified from human lymphoblastoid cell lines could specifically bind to these peptide sequences expressed on the phage surface. A M13 phage peptide library was next constructed and screened with DR1 molecules. After four rounds of selection, more than 80% of the phages were able to bind to DR1. Competition experiments with both isolated phages and corresponding synthetic peptides showed that the binding was specific. Sequence analysis of the peptide encoding region of 60 phages binding to DR1 molecules and comparison with phages of the original library revealed two potential anchor positions. The first was an aromatic residue (Tyr, Phe, or Trp) at the NH<sub>2</sub> terminus of the peptide sequences, and the second was located three residues downstream and consisted of Met or Leu. In addition, the negatively charged amino acids Asp and Glu were mostly excluded from the DR1 binding sequences, and the small amino acid residues Gly and Ala were enriched at position 6. As for DR1, this approach should enable one to easily determine the binding motifs of other MHC class II alleles and isotypes. Furthermore, it could have interesting applications in the design of major histocompatibility complex-specific antagonists.

The MHC class II molecules are highly polymorphic membrane glycoproteins that bind peptide fragments of proteins and display these peptides for recognition by CD4<sup>+</sup> T cells (1). Because one individual expresses only a limited set of different MHC molecules, every molecule must be able to bind a large number of diverse peptides to guarantee T cell-mediated immunity to the universe of antigens. Nevertheless, any given class II molecule shows some selectivity in its ability to bind peptides.

Different approaches have been taken to determine motifs of peptide-binding to MHC molecules. Using the Edman degradation method (2–4) and more recently, tandem mass spectroscopy (5), endogenous peptides of various MHC class I alleles were sequenced, and position-dependent enrichment of particular amino acids have been demonstrated.

For class II molecules some peptide residues involved in MHC interaction have been identified using peptide analogs with single amino acid substitution or biotinylated amino acids (for review see reference 6). In addition, naturally processed peptides of foreign proteins bound to self class II molecules have been identified (7). However, the endogenous peptides analyzed consisted of only a few different peptides and no clear binding motifs could be defined.

We have chosen a new approach to define the characteristics of peptides binding to MHC class II molecules. Here, we demonstrate that class II molecules were able to bind pep-

ptides displayed on a phage surface. This allowed us to use M13 peptide libraries (8–10) to screen for phages that bind to HLA-DR1 molecules. The comparison of the peptide pool binding to DR1 with the original peptide pool of the M13 library revealed new insights in peptide-DR1 interaction.

## Materials and Methods

**Reagents and Strains.** M13mp19, *Escherichia coli* XL1Blue and SCS1 were obtained from Stratagene Inc. (La Jolla, CA). Oligonucleotides were obtained from MedProbe (Oslo, Norway). Peptides were synthesized with a MilliGen peptide synthesizer (model 9050; Milligen/Bioresearch, Burlington, MA) using Fmoc chemistry, and were purified by preparative reverse-phase (RP) HPLC. Homogeneity of each peptide was confirmed by analytical RP-HPLC. Characterization was performed by fast atomic bombardment-mass spectroscopy (FAB-MS), and expected molecular ions were observed. Streptavidin on 4% beaded agarose and bovine albumin, fraction V, were obtained from Sigma Chemical Co. (St. Louis, MO). Biotin-XX-NHS was from Calbiochem Corp. (La Jolla, CA). MAb L243 (IgG2a) recognizing the DR heterodimer (11) was used as purified immunoglobulins. The hybridomas cells were obtained from the American Type Culture Collection (Rockville, MD).

**Purification and Biotinylation of HLA-DR1.** DR1 molecules were isolated from the human EBV-B cell line, HOM-2, as described elsewhere (12). A 10- $\mu$ M solution of affinity-purified DR1 was biotinylated with 200  $\mu$ M Biotin-XX-NHS in 0.25 M NaHCO<sub>3</sub>/0.2% NP-40 for 1 h at room temperature.

**Construction of Vectors.** The vector M13amp was constructed as follows. A  $\beta$ -lactamase gene was obtained by PCR amplification using the plasmid pUC 19 and the two oligonucleotides 5-ACG TGG ATC CGT ATA TAT GAG TAA ACT TGG-3 and GTA AGG GAA TTC GGG AAA TGT GCG CGG AAC CC-3. The PCR product was digested with EcoRI and BamHI and placed into the corresponding sites of the M13mp19 polylinker. To construct the library vector M13stufferbb, the sequence CTC CGC TGA of the protein III coding region of M13amp was converted to CTC GGC CGA by in vitro mutagenesis to create an EclXI site. The two oligonucleotides 5-GGC CGA GCT CGG TCA AGC TGA CGA AGC TGC GGT ACC-3 and 5-GGC CGG TAC CGC AGC TTC GTC AGC TTG ACC GAG CTC-3 were annealed and inserted into the EclXI site to create a stuffer with a SacI and KpnI site. For the construction of M13HA, M13TT and M13NNX, the vector M13stufferbb was digested with SacI and KpnI and the stuffer was removed. The following oligonucleotides were annealed and inserted to replace the stuffer: 5-CGG TGG TGG TCC GAA ATA CGT TAA ACA GAA CAC CCT GAA ACT GGC TAC CGG CGG CGG GGT AC-3 and 5-CCC GCC GCC GCC GGT AGC CAG TTT CAG GGT GTT CTG TTT AAC GTA TTT CGG ACC ACC ACC GAG CT-3 for M13HA; 5-CGG TGG TGG TTC CGG TCC GGA CAA AGA ACA GAT CGC TGA CGA AAT CAA CGG CGG CGG GGT AC-3 and 5-CCC GCC GCC GCC GTT GAT TTC GTC AGC GAT CTG TTC TTT GTC CGG ACC GGA ACC ACC GAG CT-3 for M13TT; and 5-CGG TGG CGG CAA CGC TCT GTA CAA ATT GAA CGC TGT TGC TGC GGC CGC AGG GGG AGG AGG GGT AC-3 and 5-CCC TCC TCC CCC TGC GGC CGC AGC AAC AGC GTT CAT TTT GTA CAG AGC GTT GCC GCC ACC GAG CT-3 for M13NNX.

**Construction of the M13 Peptide Library.** The library was in principle constructed as described (8–10), with some changes. M13stufferbb DNA was digested to completion with SacI and KpnI, and the stuffer was removed by gel electrophoresis to reduce any background. A random oligonucleotide 5-C GGT GGC GGC GGT (NNS)<sub>9</sub> GGG GGA GGA GGG GTA C-3 (S=G,C and N=G,C,T,A) encoding two flanking polyglycine spacers and a random core nonamer was annealed to two half-site oligonucleotides 5-ACC GCC GCC ACC GAG CT-3 and 5-CCC TCC TCC CCC-3 in a 1:1:1 ratio. The digested M13stufferbb DNA was ligated to the annealed oligonucleotides in an estimated 1:5 ratio. The ligated DNA was transformed by electroporation into *E. coli* SCS1 cells. Twenty transformations, each containing 40  $\mu$ l of cells and  $\sim$ 200 ng of ligated DNA, were performed to produce 24 million independent clones. The library was amplified in LB<sup>3</sup>/ampicillin media as described (10). The overall amplification factor was 10<sup>4</sup>.

**M13DR1 Binding Assay.** Up to 1 billion M13 phages displaying peptides of known or random sequences were mixed with a similar number of M13mp19 phages as a reference and incubated with 10–50 pmol biotinylated DR1 in binding buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 0.2% NP-40). After at least 24 h incubation at room temperature, BSA-blocked streptavidin on 4% beaded agarose was added and incubated for 10 min. The M13 phage/DR1 complexes were purified by washing the solid phase several times with binding buffer. In some experiments, an additional wash step with 70 mM citrate-phosphate pH 3/500 mM NaCl was performed. The phages were eluted with elution buffer (0.1 N glycine-HCl, pH 2.2, 1 mg/ml BSA) for 10 min and neutralized with 2 M Tris base as described (8–10). Up to 40 binding reactions have been performed simultaneously. The ratio of the phages displaying peptides to M13mp19 phages was determined in the initial mixture and the eluates by plating both

on X-Gal indicator plates. An enrichment of white (phages displaying peptides) vs. blue plaques (M13mp19) indicated peptide-based binding (9).

**Library Screening.** 20 billion phages of the amplified M13 peptide library were incubated with DR1 under the same conditions as described for the M13/DR1 binding assay. The phage eluate was concentrated and washed with 150 mM NaCl/50 mM Tris-Cl, pH 7.5 by ultradiation (Microsep Microconcentrator 30 k cutoff, Filtron Technology Corp., Northborough, MA). Up to 10 million eluted phages were used to infect 500  $\mu$ l of *E. coli* XL1blue plating cells (OD=1). The infected cells were transferred to 7 ml Luria-Bertoni (LB)<sup>3</sup> media in a 25-cm<sup>2</sup> tissue culture flask. After 1 h incubation (37°C/200 rpm), ampicillin was added to a final concentration of 20  $\mu$ g/ml. After overnight incubation, the amplified phages were harvested and purified twice with polyethylene glycol. The screening procedure was repeated three times. The eluate of the fourth round was used to isolate and sequence individual M13 clones.

**Peptide/DR1 Binding Assay.** The binding assay was performed as previously described (12a). Briefly, 25 pmol of affinity-purified DR1 and 30 pmol of <sup>125</sup>I-radiolabeled hemagglutinin (HA) 307–319 peptide were mixed in binding buffer. After incubation for 24 h at 37°C, the samples were applied onto columns (Bio-Spin 30; Bio-Rad Laboratories, Richmond, CA) and centrifuged at 1,100 g for 2 min. The radioactivity in the void volume was directly counted in a  $\gamma$ -scintillation counter. To measure specific binding of other peptides to DR1, indicated amounts of unlabeled peptides were added to the reaction mixture, and their ability to compete with the labeled HA peptide was determined as described above.

## Results

**Specific Interaction of DR1 Molecules with Peptides Displayed on the Phage Surface.** To investigate whether HLA-DR molecules were able to bind peptides displayed on phage surfaces, peptides known to bind to DR1 were placed on the NH<sub>2</sub> terminus of the M13 phage protein III (Table 1). The protein III is a minor coat protein of the M13 phage and the region, containing the peptide inserts, is known to be exposed on the phage surface (13). The peptide sequences were flanked by two glycine spacers to maximize the flexibility of the peptide insert.

Fig. 1 a shows that the M13 constructs bind specifically to DR1 molecules. M13 phages (M13HA and M13NNX), displaying the DR1 binding influenza HA 307–319 peptide (14) and the *Plasmodium falciparum* NNX 83–91 (Sinigaglia, F., unpublished observation), bound to DR1 molecules, as judged by the enrichment of M13HA and M13NNX phages versus the M13mp19 reference phages (90–95% specific phage binding). Conversely, phages displaying the tetanus toxoid (TT) sequence 763–775, used here as a negative control, did not bind to DR1. The ability of the DR1-binding synthetic peptides HA 307–319 and TT 830–843 (15) to compete with the phage-DR1 binding (Fig. 1 b) provided additional evidence that the binding occurred in the peptide-binding groove.

**Construction of a M13 Peptide Library and Isolation of Phages**

<sup>1</sup> Abbreviations used in this paper: HA, hemagglutinin; LB, Luria-Bertoni medium; RP, reverse phase; TT, tetanus toxoid.

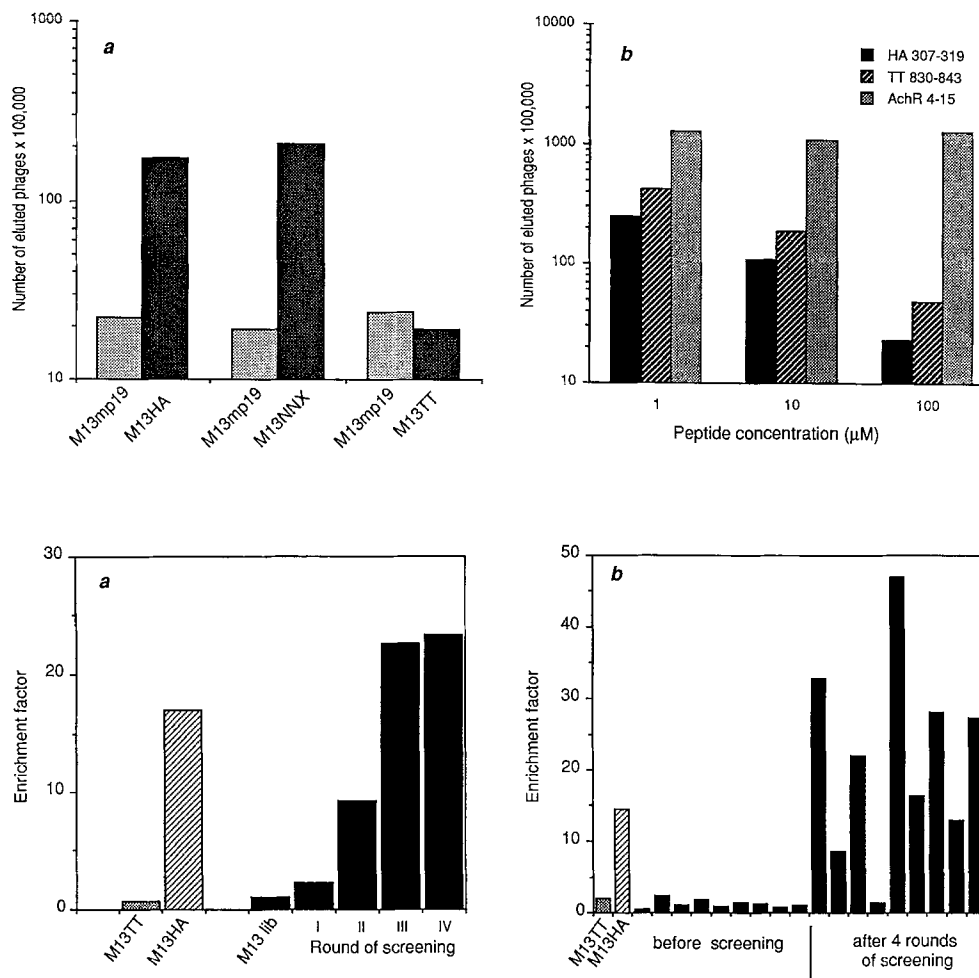
**Table 1.** Phage Constructs

Phage	NH <sub>2</sub> terminal region of M13 phage pIII	Peptide
M13HA	AEL GGG <u>PKYVKQNTLKLAT</u> GGGG VP - pIII	HA 307-319
M13NNX	AEL GGG <u>NALYKMNAVA</u> AAAA GGGG VP - pIII	NNX 83-91
M13TT	AEL GGG <u>SGPDKEQIAD</u> Ein GGGG VP - pIII	TT 763-775
M13lib	AEL GGGG <u>XXXXXXXXXX</u> GGGG VP - pIII	Random oligo

The NH<sub>2</sub> terminal region of the modified M13 protein III of each construct is shown. Amino acid sequences of peptides with known binding characteristics to DR1 (HA, NNX, and TT) and the random peptide region of the M13 peptide library are underlined. The single letter code for amino acids is: A = Ala, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr, and X = random amino acid.

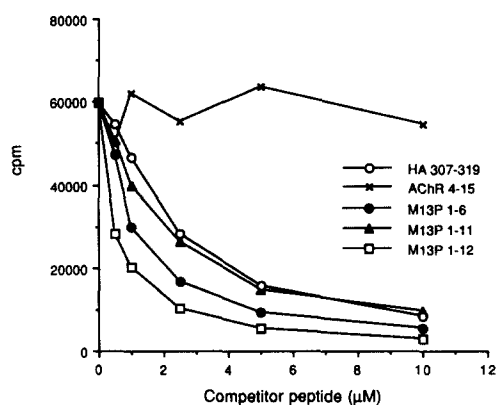
**Binding to DR1.** The oligonucleotides encoding peptides of known binding characteristics were replaced by random oligonucleotides, and a M13 peptide library with 20 million independent phages was generated (M13Lib). The random peptide inserts were nine amino acid residues in length and flanked by four glycines on each side (Table 1). Four rounds of successive selection and amplification were performed. To monitor the enrichment for DR1 binders, the binding ca-

capacity of the libraries was tested before and after each round of screening (Fig. 2 a). Whereas the original library (M13lib) did not show any binding to DR1, the DR-1 selected libraries, after three and four rounds of screening, showed binding that was of a similar magnitude to that obtained with M13HA phages (20 times M13mp19 background). The binding was specific as judged by peptide competition assays (not shown). Individual phages were then isolated and tested for DR1



**Figure 1.** DR1 molecules bind specifically to peptides displayed on the phage surface. (a) M13/DR1 binding assay. Binding of M13 constructs to DR1 was determined by their enrichment as compared with the reference phage M13mp19. For M13 constructs, the phage input was 1 billion, and for M13mp19, 2 billion. (b) Phage/peptide competition assay. Increasing amounts of peptides were added to compete for DR1/M13HA phage binding. Binding and elution conditions were the same as in a. Peptide from the human acetylcholine receptor  $\alpha$  subunit (AchR 4-15) was used as negative control. Phage input: M13HA, 2 billion.

**Figure 2.** M13/DR1 binding assay with whole libraries and isolated phages. (a) DR1 binding to the original library (M13lib) and to the amplified libraries after each round of screening was determined by the enrichment of the library phages as compared with the reference phages (M13mp19). (b) Nine phages before and after four rounds of screening were isolated, and binding to DR1 was determined for each phage as in a.



**Figure 3.** Inhibition curves for peptides based on sequences corresponding to those of three randomly selected DR1-bound phages (*M13P1-6*, *M13P1-11*, and *M13P1-12*). <sup>125</sup>I-labeled HA 307-319 and HLA-DR1 were incubated in the presence of indicated concentrations of the following peptides: *M13P1-6* (GGYRHAQAYGVGG), *M13P1-11* (GGWFGQLQAAQGG), and *M13P1-12* (GGYRQMSAPTLGG). HA 307-319 and AChR 4-15 were used as positive and negative controls, respectively. The samples were analyzed by spin column gel filtration as described in Materials and Methods. The sequences of the peptides *M13P1-6*, *M13P1-11*, and *M13P1-12* correspond to the peptide encoding regions of DR1 selected phages and are flanked by two Gly on both sites to standardize the length with that of the control peptides.

binding. As shown in Fig. 2 *b*, eight of nine phages isolated after four rounds of screening bound to DR1, whereas none of the phages isolated from the original library did. As above, the specificity of the phage-DR interaction was demonstrated by phage-peptide competition assays (data not shown). We next sequenced the peptide-encoding region of three of these phages and corresponding peptides were synthesized. The three peptides were indeed able to bind to DR1 with binding capacities within the same range of the HA 307-319 peptide (Fig. 3).

**Sequence Motifs of DR1-binding Peptide Inserts.** To investigate the structural characteristics of peptides capable of binding to DR1 molecules, the peptide-encoding regions of

60 DR1-binding phages were sequenced (Table 2). All peptides sequenced were different, which confirms the independent origin of the phages analyzed. When the overall amino acid composition of the sequences listed in Fig. 5 was compared with sequences of 60 control phages isolated from the original library, and of 35 control phages that also underwent three rounds of amplification (but without DR1 selection), several differences were noted (Fig. 4 *a*). The most striking difference was a significant enrichment of Met and Tyr, whereas the negatively charged amino acids Asp and Glu were almost absent from the DR1 binding sequences. As shown in Fig. 4, *b* and *c*, enrichment of a specific amino acid depends on the position within the peptide sequence. Although Tyr is mainly located at the NH<sub>2</sub> terminus of the peptide insert, the peak of Met is found in the middle. To determine the relative positions of these potential anchor residues and to further identify structural characteristics of the DR1-binding peptides, an unbiased alignment was performed using the "Pileup" program of the GCG (Genetics Computer Group Inc., Madison, WI) program package. The relative abundance of each amino acid at different positions of the aligned sequences is shown in Fig. 5. 48% of the aligned sequences contain a Tyr residue at position 1. The other residues whose frequencies were clearly increased at this position were Phe (25%) and Trp (13%), which also have aromatic side chains. In addition to the aromatic residues enriched at position 1, at position 4 we detected mostly Met (50%) and Leu (28%). Further constraints were found at position 6, which shows a preference for the small amino acids Ala (32%) and Gly (22%). The increased frequency of Gly at position 9 is due to the Gly of the spacer which shows up after the alignment. If we only consider the amino acid residues of the random peptide coding region, 45% of the peptides would have a Leu at position 9.

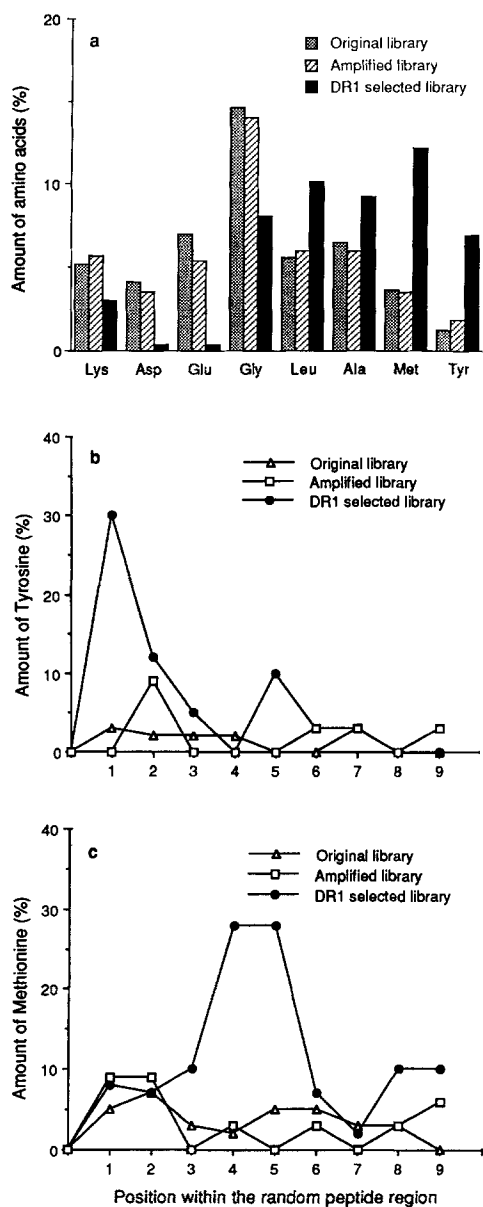
## Discussion

MHC class II molecules must bind a large number of different peptides to guarantee T cell-mediated immunity to

**Table 2.** Amino Acid Sequences of the Random Peptide Region of 60 Phages Bound to DR1

MFQRMAVDA	YRQMSAPTL	WFGQLQAAQ	YRHAQAYGV	MPRQYTQM I	FHKVYRGLL
WQMAMSRHL	S I M M M GAVR	WYTL L K S R L	YK P M L A S V G	Y A A M R G G Q I	Y K P L W G Q M T
NYQMMGTMR	FERARSRL	YLRMLQ I L K	NWRALRGAL	YRQM W V N R A	F M A M R A V R L
FTMLMNQVM	VYRAQGPQM	MMSRMLGQT	SSGRYARLG	T F Y R M G T Q M	W I G Q M Y A A K
YRMQSNHVS	DRSMEWRMM	FSA I R N R I L	YRAMQT T L S	N Y L G M K P R V	R G F R H M M A A
YQQMGARLM	SWRH I Q T R V	MYGLMLAQR	YQALRAYWQ	R F T Q M M A V A	W K G L Q G A L S
FMRLGGGHL	I W H M Q N A R V	YKLLRATQM	LWQSFMQMA	F H Q L R G G S L	Y R G M Q R R T L
YGKLRGGTL	QV G K Y L G L G	TYRGMAGFR	R V F Q N L R A S	W R Q M Y R A Q L	R S Y S L A P G R
TWNSLRGRL	QRYNAMRAA	Y I M Q M N R V V	L T L M Q A K R L	R F T Q M G A L A	Y Y G K M P A L V
PWQAMRGWL	KK I M S Y H A L	YRGM SAFRA	M F H R M S G L Q	G Q R S Y S S M Q	Y M M Q K S V R A

The phages were selected from the library M13lib by DR1 and isolated after four rounds of screening.



**Figure 4.** (a) Amino acid content of the peptide inserts before and after DR1 selection. The overall amino acid composition of the sequences listed in Table 2 was compared with sequences of 60 control phages, isolated from the original library, and of 35 control phages, that also underwent up to three rounds of amplification but without DR1 selection. Only the amino acids significantly (>2%) increased or reduced are shown. (b and c) Position-specific enrichment of Tyr and Met after DR1 selection.

the universe of foreign antigens. The binding of a peptide to a particular class II allele is nevertheless specific. From this, we can deduce that the amino acid residues of a peptide pool binding to a given class II molecule cannot be randomly distributed over the peptide sequences, but rather have a defined distribution, reflecting the physicochemical properties needed for the interaction with that particular MHC molecule. The determination of these characteristics requires a large pool

of peptides. Here we have chosen a new stochastic method to meet this requirement.

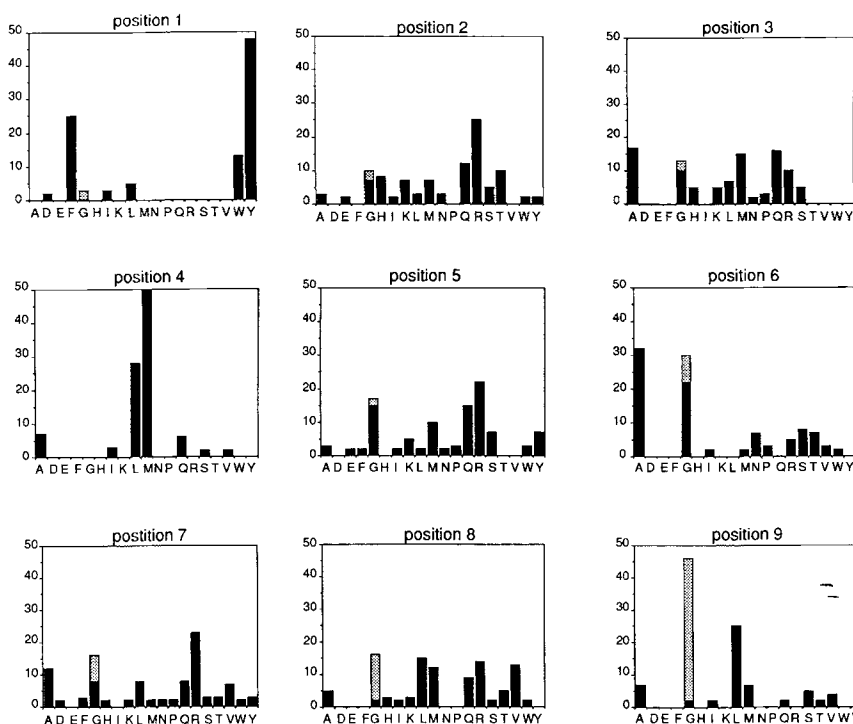
As shown in Fig. 1, HLA-DR molecules were able to bind specifically to peptides displayed on the phage surface as part of the M13 phage protein III. This binding might be facilitated by the exposure of the peptide insert on the phage surface and by its flexible conformation because of the presence of the Gly spacers. Our results also support previous observations that some T cell clones appear to recognize complete folded protein antigens or denatured antigens without further processing (16, 17). From the proposed model of class II molecules (18), it is believed that both ends of the peptide-binding groove of MHC class II may be open, in contrast to MHC class I molecules where both ends are closed (19). This may explain why class II molecules, in contrast to class I, can even bind determinants on intact proteins.

To construct our library, we chose a 9 mer as a random peptide insert, as suggested by previous findings showing that the optimal peptide length for binding is between 8 and 12 amino acids with the central 5–8 residues contributing the majority of the specific contacts (6, 20).

By sequencing the inserts of 60 phages that bound HLA-DR1 molecules, we could identify a DR1 peptide-binding motif. At the NH<sub>2</sub> terminus of the peptide-encoding region we found a striking predominance of aromatic residues. This is consistent with findings of Jardetzky et al. (21) showing the importance of Tyr<sub>309</sub> for the interaction of HA 307–319 with DR1. Three residues downstream, we identified a second restricted position with Met or Leu found in nearly 80% of the peptide sequences analyzed. Furthermore, the small amino acid residues Ala and Gly were significantly enriched at position 6. From these data we can deduce that DR1 molecules possess a pocket for the aromatic side chains Tyr, Phe, or Trp, and perhaps another one able to accommodate Met or Leu (both hydrophobic with similar size). Although the side chains of the anchor amino acid positions might interact with specific pockets in the binding cleft of DR1, the enrichment for small amino acids could instead reflect the fact that larger side chains at this position are not tolerated. Indeed in the class II model (18), a putative aromatic binding pocket has been identified in the  $\alpha_1$  domain of DR1 (Shiratori, Y., and D.M. Doran, unpublished observation). This aromatic pocket is big enough to accommodate the entire tyrosine side chain. More detailed information on the three-dimensional structure of MHC class II molecules is required, however, to clarify the nature of the DR1/peptide binding.

Our results also show that certain amino acid residues are significantly reduced in DR1 binders. In particular, we found that the negatively charged amino acids Glu and Asp were virtually excluded from the DR1 binding sequences. This indicates that in addition to specific anchor residues, the overall amino acid composition of a given peptide is important for MHC binding.

Recently, O'Sullivan et al. (22) have proposed a DR1 binding motif by determining the effect of single amino acid substitutions of peptides HA 307-319 and TT 830-843 for binding to different DR alleles. The proposed motif consisted



**Figure 5.** Position-specific composition of aligned DR1-bound peptide sequences. The 60 DR1-bound peptide sequences shown in Table 2 were aligned using the PileUp program of the GCG program package. Dark and light columns represent the amino acids of the random peptide region or the Gly of the spacer, respectively.

of an aromatic or hydrophobic residue at the NH<sub>2</sub> terminus of the peptide (position 1), a noncharged and relatively small residue at position 6, and a relatively hydrophobic residue at position 9. Similar findings were also reported by Rothbard et al. (23) using biotinylated analogs of the HA 307-319 peptide. Our data are in substantial agreement with these results, except for the Met/Leu "anchor" described above. This difference is most likely due to the large number of peptides that have to be synthesized before one is really able to identify all the important positions for MHC-binding. In this respect, the possibility to analyze an almost unlimited number of peptide sequences using the M13 peptide library is certainly a more powerful way to characterize MHC class II-binding motifs.

The fact that not all the random peptide sequences con-

tain all the anchor positions described, as the HA peptide does not have the internal Met or Leu at the predicted position, suggests that two or maybe only one critical residue may be sufficient for DR1 interaction. We are currently evaluating the relative influence of the different anchor residues for peptide binding.

Using the approach described here, we are also defining motifs for other HLA class II alleles and isotypes with the aim of studying the effect, in terms of peptide binding, of discrete mutations in MHC class II molecules that are associated with susceptibility to particular autoimmune diseases (for review see reference 24). Furthermore, the knowledge of anchor positions and other general rules of peptide-binding to MHC molecules should prove useful for the design of MHC-specific antagonists.

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