

# Specificity and Promiscuity among Naturally Processed Peptides Bound to HLA-DR Alleles

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## Summary

Naturally processed peptides were acid extracted from immunoaffinity-purified HLA-DR2, DR3, DR4, DR7, and DR8. Using the complementary techniques of mass spectrometry and Edman microsequencing, >200 unique peptide masses were identified from each allele, ranging from 1,200 to 4,000 daltons (10–34 residues in length), and a total of 201 peptide sequences were obtained. These peptides were derived from 66 different source proteins and represented sets nested at both the amino- and carboxy-terminal ends with an average length of 15–18 amino acids. Strikingly, most of the peptides (>85%) were derived from endogenous proteins that intersect the endocytic/class II pathway, even though class II molecules are thought to function mainly in the presentation of exogenous foreign peptide antigens. The predominant endogenous peptides were derived from major histocompatibility complex-related molecules. A few peptides derived from exogenous bovine serum proteins were also bound to every allele. Four prominent promiscuous self-peptide sets (capable of binding to multiple HLA-DR alleles) as well as 84 allele-specific peptide sets were identified. Binding experiments confirmed that the promiscuous peptides have high affinity for the binding groove of all HLA-DR alleles examined. A potential physiologic role for these endogenous self-peptides as immunomodulators of the cellular immune response is discussed.

MHC class I and II molecules are membrane-bound glycoproteins that present processed antigen to T cells and initiate an immune response (1). Crystallographic analysis of several class I molecules identified a groove composed of two  $\alpha$  helices supported by an eight-strand  $\beta$ -pleated sheet containing electron-dense material that represents bound antigenic peptide (2–4). Several groups have characterized the complex mixtures of acid-extracted class I-bound peptides by HPLC fractionation and sequencing (5–9). The majority of these peptides were 8–11 amino acids long and possessed a binding motif characteristic of peptides that bind to a given class I allele.

The characterization of naturally processed peptides bound to class II molecules provides an approach towards understanding both antigen processing and peptide binding events *in vivo*. The stability of class II molecules requires peptide binding (10, 11); however, the precise class II molecule-peptide contacts that provide this energy are not yet well defined. Identification of naturally processed peptides extracted and sequenced from class II molecules revealed that the bound peptides were longer (13–25 residues) than those bound to class I (12–15) and nested at the amino- and/or carboxy-terminal ends, suggesting that the peptide binding groove on class II molecules is open at both ends (13, 15). Although

only a limited number of source proteins were reported, peptides derived from both endogenous proteins and exogenous serum proteins were identified. The association constants (measured by competitive inhibition) for several of these peptides were in the nanomolar range, confirming the high affinity of these peptides for class II molecules.

To further our understanding of class II antigen processing and peptide binding, several different allelic forms of HLA-DR molecules were purified and the bound peptides were identified using the complementary techniques of mass spectrometry and Edman microsequencing. Each allele has a complex, yet readily distinguishable and reproducible peptide profile. The range of peptide masses is comparable among the alleles examined. To establish a representative collection of bound peptides and to identify their respective source proteins, individual fractions corresponding to both major and minor chromatographic peaks from the extracted peptide pool separations were chosen for sequence analysis. Various features relating to the allelic specificity and promiscuity of naturally processed peptides bound to different DR alleles will be described.

## Materials and Methods

*Reagents and Materials.* HLA-DR molecules were purified from homozygous EBV-transformed human B lymphocyte lines:

DR1 (DRB1\*0101) from LG-2 and HOM-2 cells, DR2b/DR2a (DRB1\*1501/DRB5\*0101) from MST cells, DR3/DRw52 (DRB1\*0301/DRB3\*0201) from WT20 cells, DR4/DRw53 (DRB1\*0401/DRB4\*0101) from Priess cells, DR7/DRw53 (DRB1\*0701/DRB4\*0101) from Mann cells and DR8 (DRB1\*0801) from 23.1 cells. DR1 was also purified from the MHC antigen-loss mutant cell lines 721.45 (kindly provided by Robert DeMars, University of Wisconsin, Madison, WI) and 721.221 (CRL 1855; American Type Culture Collection, Rockville, MD). The cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin G, and 50 µg/ml streptomycin in spinner flasks or roller bottles and stored as pellets at -80°C.

The anti-HLA-DR hybridoma LB3.1 (IgG2b) was produced in this laboratory (16). The anti-HLA-DRw53 mAb 109d6 (17) was kindly provided by R. Winchester (Columbia University, New York, NY).

Peptides representing residues 97–120 of the invariant chain (Ii) (LPKPPKPVSKMRMATPLLMQALPM), residues 182–198 of HLA-DR  $\alpha$  chain (APSPLPETTENVVICALG), residues 188–202 of the Ig  $\kappa$  chain C region (KHKVYACEVTHQGLS), residues 103–117 of the HLA-A2-like sequence (VGSDWRFLRGYHQYA), and residues 1273–1291 of bovine apolipoprotein B-100 (IPDNLF-LKSDGRIKYTLNK) were synthesized on a peptide synthesizer (430; Applied Biosystems, Inc., Foster City, CA) using solid-phase Fmoc/HBTU chemistry. Biotinylations were done on resin-bound free amino-terminal peptides. Briefly, a fivefold molar excess of *N*-hydroxysuccinimidyl-crosslinked biotin (Pierce Chemical Co., Rockford, IL) was added to a 5-mg/ml suspension of resin-bound peptide in imidazole-buffered DMSO and rocked at room temperature for 2 h. The slurry was then washed four times with 25 ml of DMSO followed by four washes with 50 ml HPLC-grade water, air dried, and stored at 4°C until deprotection/cleavage. All peptides were purified by HPLC (HPXL solvent delivery system with UV-M detector; Rainin Instrument Co., Inc., Woburn, MA) on a preparative C<sub>18</sub> reversed-phase chromatography (RPC)<sup>1</sup> column (25 × 2.2 cm, 300 Å, 10–15 µm; Vydac, Hesperia, CA) in 0.06% TFA/water with a 20–80% 50-min linear 0.055% TFA/acetonitrile gradient. The integrity of the purified peptides was confirmed by amino acid analysis (420A/130A derivatizer/HPLC after hydrolysis with 6 N HCl for 24 h in vacuo; Applied Biosystems, Inc.) and mass spectrometry on a triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI-MS) (TSQ 700; Finnigan MAT, San Jose, CA).

**Protein Purification.** Immunoaffinity purification of HLA-DR alleles was performed as reported (18) with minor modifications. The detergent-soluble lysates from Priess and Mann cells were loaded onto an immunoaffinity column prepared with the mAb 109d6 to remove the DRw53 (DRB4\*0101) allele-linked molecules before passage through the LB3.1 immunoaffinity column. DRw52 (DRB3\*0201) was not removed from the WT-20 lysates and is a minor contaminant (<10%) of the DR3 preparations. Similarly, the DR2 preparations contain both the major and minor isotypes: DR2a (DRB5\*0101) and DR2b (DRB1\*1501).

Water-soluble HLA-DR was produced by limited papain digestion, as prepared for the crystallization of HLA-DR molecules, then purified using gel filtration, concentrated by vacuum dialysis to ~10 mg/ml, and stored at 4°C (19). SDS-PAGE was performed by the method of Laemmli (20) with the single modification that samples were not boiled before analysis. Protein concentrations were determined by bicinchoninic acid assay (Pierce Chemical Co.) and/or quantitative amino acid analysis (Applied Biosystems, Inc.). Im-

mediately before peptide extraction protein samples were further purified by HPLC (6000A solvent delivery system; Waters, Milford, MA) on a size-exclusion column (300 × 7.5 mm, 250 Å, 10 µm, TSK-3000SW; Toyo Soda, Philadelphia, PA) in 25 mM 2-[*N*-morpholino] ethanesulfonic acid, 150 mM NaCl, pH 6.5, at a flow rate of 1 ml/min and spin-concentrated using Centricon 10 ultrafiltration devices (Amicon, Danvers, MA) to remove any residual small molecular weight contaminants.

**Peptide Extraction and Separation.** All protein samples (1 mg) were spin-concentrated to a final volume between 50 and 100 µl using Centricon 10 ultrafiltration devices. Bound peptides were eluted from HLA-DR by addition of acetic acid (10%, 1 ml) and incubation at 70°C for 15 min. Isolation of peptide pools from HLA-DR was accomplished by ultrafiltration. The acid-denatured protein was retained in the Centricon 10 microconcentrators, while extracted peptides were collected from the flow-through. The peptide pools were vacuum concentrated to 50 µl in a Speed-Vac (Savant Instrs., Inc., Farmingdale, NY) before HPLC separation.

Peptides were separated by HPLC (Rainin Instrument Co. Inc.) on a microbore C<sub>18</sub> RPC column (250 × 2.1 mm, 300 Å, 5 µm; Vydac) as previously described (15). Chromatographic analysis was monitored at multiple UV wavelengths simultaneously, permitting spectrophotometric evaluation of purified species before mass spectral and sequence analyses. Fractions were collected and stored at -20°C until subsequent analyses. A sample of 25 mM MES was processed in parallel from the extraction procedure through the HPLC analysis for each allele to control for possible contamination from reagents or materials.

**Mass Spectrometry and Edman Sequence Analyses.** RPC fractions were chosen for mass spectrometry analyses based on UV absorption profiles. Briefly, optimum fractions from the RPC separation were chosen based on differential UV absorbance at 210, 254, 277, and 292 nm, peak symmetry, and resolution (21). Selected fractions were further analyzed by matrix-assisted laser-desorption mass spectrometry (MALD-MS) to determine the individual mass values for the predominant peptides. 4% (4 µl) of the collected fractions was mixed with 1 µl of matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid, 10 mg/ml in 50% acetonitrile; Sigma Chemical Co., St. Louis, MO), applied to a gold plated sample disc, and dried. Mass determinations were made using a LASERMAT mass spectrometer (Finnigan MAT). Selected fractions were analyzed by sample infusion using a TSQ 700 triple quadrupole mass spectrometer.

Amino-terminal sequence analysis was accomplished by automated Edman microsequencing using a pulsed-liquid protein sequencer (477A; Applied Biosystems, Inc.). The resultant phenylthiohydantoin amino acid derivatives were identified manually using an on-line HPLC (120A; Applied Biosystems, Inc.). Initial sequence yields measured in picomoles were used to determine the total yield of peptides derived from single source proteins. Combining the mass spectral data with the sequencing analyses enables crucial verification of both the amino- and carboxy-terminal amino acids of peptides within a single sample. For example, given a low picomolar mixture of two theoretical peptides, SMILE and SMIL, the presence of the carboxy-terminally truncated peptide SMIL would not be apparent by conventional Edman microsequencing alone. The observed sequence data would be SMILE and any reduction in yield of the carboxy-terminal residue could be attributed to normal losses during procession of the chemistry. All identified peptides were aligned to regions of proteins stored in the database using the BLAST network at the National Center for Biotechnology Information (22).

**Peptide Binding.** The assay was performed as described previously (23, 24). Briefly, 5 × 10<sup>6</sup> cells were resuspended in 3%

<sup>1</sup> Abbreviation used in this paper: RPC, reversed-phase chromatography.

paraformaldehyde and incubated at room temperature for 10 min followed by a wash with 50 ml PBS. Residual paraformaldehyde was quenched by addition of 5 mM glycylglycine (incubated at room temperature for 5 min) and washed with another 50 ml PBS.  $2 \times 10^5$  cells were next resuspended in 100  $\mu$ l of complete medium, added to 100  $\mu$ l PBS containing biotinylated peptide, and incubated at 37°C for 10 h. Cells were then washed and stained with FITC-streptavidin (4.22  $\mu$ g/ml PBS, 0.1% BSA) at 4°C for 30 min. Each incubation was followed by two washes with 250  $\mu$ l PBS, 0.1% BSA at 4°C. Stained cells were subjected to flow cytometry on a FACScan® analyzer (Becton Dickinson & Co., Mountain View, CA). To measure the relative amount of fluoresceinated streptavidin bound, the mean fluorescence of 10,000 stained cells was determined.

Comparison of different homozygous B lymphoblastoid cells for their relative peptide binding capacity was done by normalizing the class II expression levels using biotinylated LB3.1 mAb. Specificity was demonstrated by blocking using the L243 anti-HLA-DR mAb and/or by competition with nonbiotinylated peptide.

## Results

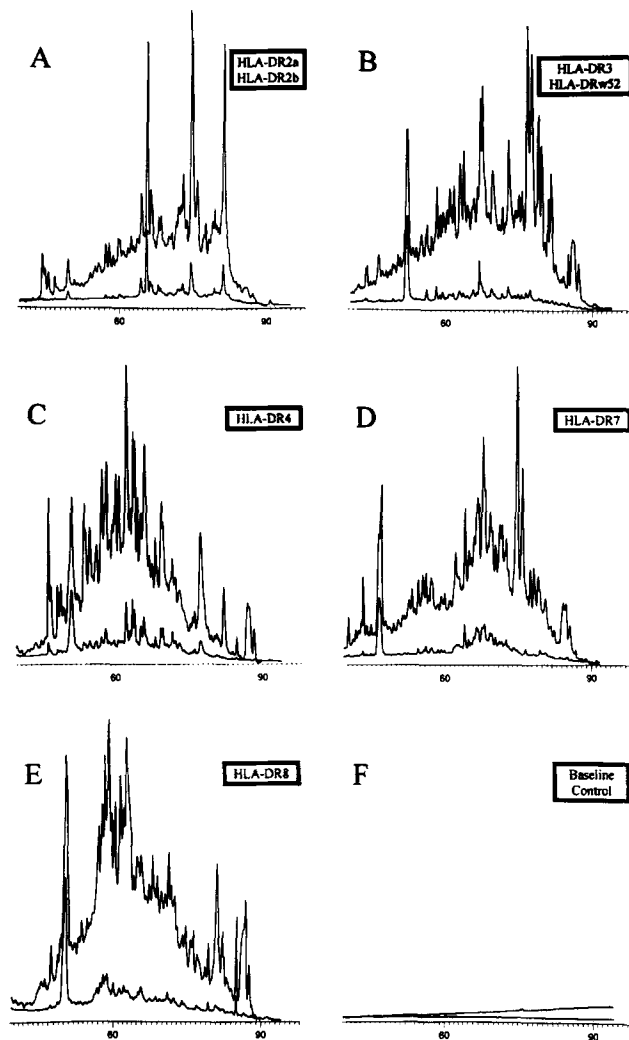
### Peptide Isolation from Immunoaffinity-purified HLA-DR Alleles

The bound peptide pools were released from 1 mg of DR2, DR3, DR4, DR7, and DR8 (~16 nmol) by acid elution with the total extracted peptide yields (~11–13 nmol, 70–80% recovery) for each DR allele similar to those previously described for DR1 (15). Moreover, purified DR molecules were stable  $\alpha/\beta$  complexes (>95% in each case), as measured by SDS-PAGE, indicating that the starting material was fully loaded with peptide (10, 25, 26); no separate  $\alpha$  and  $\beta$  chains were detectable in any preparation. Only single free  $\alpha$  and  $\beta$  chains were observed after acid elution of peptides, suggesting the complete extraction of peptides (data not shown).

Separations of the respective peptide pools were achieved using RPC and the peptide profiles from multiple extraction/separations for each of the five alleles were highly reproducible. These profiles exhibit the complexity of the bound peptide pools and the differences highlight the selectivity of individual peptides for specific alleles (Fig. 1). The bound peptides from papain-digested and detergent-solubilized DR had identical HPLC profiles. To confirm that papain solubilization of HLA-DR did not generate or alter the bound peptide repertoire, the predominant peaks from both the detergent-soluble and papain-digested DR1 profiles were analyzed and found to contain peptides identical in mass and sequence (15).

### Size Distribution of Allele-specific Peptide Pools

Aliquots from the individual RPC separation fractions (20–200 fmol) were analyzed by MALD-MS and 1,101 unique masses were identified from the peptide pools extracted from the five DR alleles (an average of 220/allele). Many individual fractions contained multiple species of varying mass, indicating coelution of peptides in single chromatographic peaks, further corroborating the complexity of the separated mixtures. The molecular mass distribution and estimated peptide length (based on an amino acid average mass value of

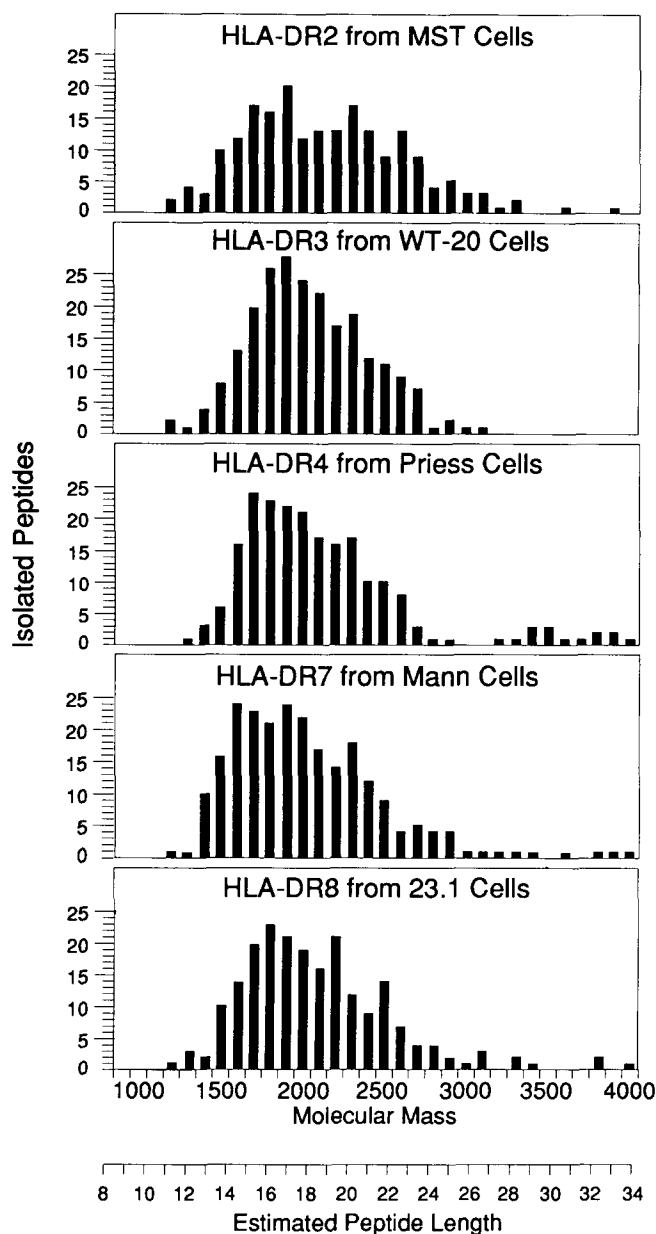


**Figure 1.** Reversed-phase HPLC separation profiles of peptide pools extracted from papain-digested HLA-DR. Each chromatogram represents the peptide repertoire as detected by UV absorbance for both 210 (sensitivity) and 277 nm (tryptophan/tyrosine content) at a full scale absorbance of 200 mAU. The profile patterns differ substantially, thus representing the various peptide fingerprints associated with each DR allele. (A) Peptides bound to DR2, including two prominent peaks at 75 and 81 min that were identified by sequence and mass analyses as amino-terminal fragments (papain overdigestion products) of the DR2a and DR2b  $\beta$  chain, respectively; (B) peptides bound to DR3; (C) peptides bound to DR4; (D) peptides bound to DR7; (E) peptides bound to DR8; (F) mock extraction buffers. The peak in B–E at ~50 min with a high 277-nm absorbance was identified as an organic contaminant from the Centricon ultrafiltration devices.

118 daltons) varied from 1,239 to 4,091 daltons (10–34 residues in length) with the mode lying between 1,700 and 2,100 daltons (15–18 residues) for each allele (Fig. 2).

### Identification and Sequence Characterization of Naturally Processed Bound Peptides

Complete sequences for 201 peptides isolated from five DR alleles were obtained by automated Edman microsequencing



**Figure 2.** Size distribution of peptides bound to different HLA-DR alleles. A minimum aliquot (see text) was removed from each RPC fraction containing peptidic material and analyzed by MALD-MS. The molecular masses are plotted against the number of isolated peptides for each allele. Peptide length was estimated by dividing the experimental mass by an average amino acid mass of 118 daltons. The mass range varied between 1,200 and 4,100 daltons with the mode lying between 1,700 and 2,100 daltons of each allele.

in combination with mass spectrometry. Source proteins of the sequenced peptides were identified by homology search of the current database (22, 27). Over 29 peptides were characterized from each DR allele. Partial sequences for an additional 94–110 peptides from unknown source proteins were also obtained.

The predominant source proteins of the naturally processed peptides sequenced from the class II molecules of human B cells were MHC-related molecules. Self-peptides derived from

MHC class I and/or II molecules were isolated from every class II allele, including peptides derived from the three class I isotypes (HLA-A, HLA-B, and HLA-C) as well as from the three class II isotypes (HLA-DR, HLA-DQ, and HLA-DP). From 201 sequenced peptides, 78 were derived from class I molecules, class II molecules, or the invariant chain (Ii). In addition, the relative abundance of these MHC-related self-peptides, as measured by initial yields from sequencing analyses, was the highest observed.

**Peptides Identified in a Single Allele.** Peptides derived from 44 integral membrane proteins (including two EBV-encoded proteins), four proteinase inhibitors, three cathepsins, six cytosolic proteins, and six bovine serum proteins representing 84 different core sequences were only detected in one allele. Peptides homologous with tubulin, an extracellular matrix-related protein not normally expressed in human lymphocytes but described as appearing on the cell surface of EBV transformed B cells (28), were also identified. Self-proteins thus contributed the large majority of bound peptides and were derived from both membrane proteins (which could have reached the endocytic pathway by endocytosis) and from cytosolic proteins (which must have intersected the endocytic/class II pathway in some other manner). Sets of peptides with common core sequences and nested at either the amino- and/or carboxy-terminal ends were observed for 38 of these peptides. Detailed descriptions of the identified peptides are listed by allele in Tables 1–5.

Additional peptides were derived from a limited set of secreted but not surface-expressed proteins, including endocytic processing enzymes and proteinase inhibitors that are present in endocytic or lysosomal vesicles. Five peptides were identified from the human cathepsins E and S and from a sequence homologous to rat cathepsin C (human cathepsin C has not been described). Peptides derived from two cytosol resident proteins implicated in intracellular transport and trafficking were also identified. A single peptide from the IFN-induced guanylate-binding protein (GBP-2), distinct from the Rab family of canonical regulatory heterotrimeric GTP-binding proteins (29), was identified, as was a nested set of peptides derived from Hsp 70. Guanylate-binding proteins can direct vesicular traffic by stimulating endosomal fusion, transduce hormonal and sensory signals across the plasma membrane, and act as initiation/elongation factors during protein synthesis (30, 31). The Hsp 70 source protein may be prp72/74 (a member of the Hsp 70 family with no sequence available from any databank), which has been implicated in the intracellular assembly of processed antigen–class II complexes (32). The other four peptides from cytosolic proteins were from cytochrome-b<sub>5</sub> reductase, EBV capsid protein, *c-myc*, and *K-ras*.

Peptides from exogenous bovine serum proteins were also isolated from each allele. 25 peptides representing 11 different core sequences that were homologous to human apolipoprotein B-100 were identified. The core sequences varied among alleles indicative of allelic specificity. Although the sequence for bovine apolipoprotein B-100 has not been described, this protein is the principal apolipoprotein in chylomicrons, very low density lipoprotein and low density lipoprotein in humans

and mice, all abundant in serum-supplemented culture media. Peptides derived from other proteins in bovine serum include fetuin (15), factor VIII, hemoglobin, complement C9, von Willebrand factor, and transferrin. With the exception of apolipoprotein B-100, the relative abundance of the serum-derived peptides was significantly lower than that observed for MHC-related self-peptides.

Sequence analyses of the peptides extracted from these five DR alleles confirm that class II antigen processing produces heterogeneous-sized peptides nested at both the amino- and carboxy-terminal ends. The characterization of potential enzymatic cleavage points was attempted by alignment of the flanking regions surrounding the identified peptide sequences. No common pattern was recognized, probably due to the exoproteolytic removal of amino acids from each end, and

the shifting nature of the binding core sequences. However, a marked preference for heterogeneity, both in number of amino acids and total number of peptides, was observed at the carboxy-terminal end as compared with the amino-terminal end.

*Promiscuous Self-peptides.* The ability of individual peptides to bind to multiple DR alleles is defined as promiscuity (also referred to in the literature as degeneracy) and has been previously described for antigenic peptides presented to DR-restricted T cells (23, 33–35) as well as in *in vitro* binding experiments (36–40). 37 peptides derived from four promiscuous self-peptide families were identified (Table 6). These peptides were derived from Ii, an HLA-A2-like sequence (described below), HLA-DR  $\alpha$  chain, and Ig  $\kappa$  chain. Each was represented by a nested set of peptides similar to those de-

**Table 1.** Naturally Processed Peptides Bound to HLA-DR2

Source protein	Protein category	Residues	Sequence	Length RT [M + H] <sup>+</sup>		Observed m/z	Yield*	
				min	m/z			
HLA-DQ $\alpha$ chain	Membrane	97–119	NIVIKRSNSTAATNEVPEVTVFS	23	52	2,477	2,478	–†
		97–112	NIVIKRSNSTAATNEV	16	49	1,717	1,717	–†
HLA-DQ $\beta$ chain	Membrane	42–59	SDVGVYRAVTPQGRPDAE	18	49	1,917	1,920	14.0
		43–59	DVGVYRAVTPQGRPDAE	17	49	1,830	1,833	–§
		43–57	DVGVYRAVTPQGRPD	15	49	1,630	1,633	–§
HLA-DR2b $\beta$ chain	Membrane	94–111	RVQPKVTVPYPSKTQPLQH	18	48	2,107	2,114	12.5
		94–108	RVQPKVTVPYPSKTQP	15	48	1,728	1,731	–§
FnR $\alpha$ chain	Membrane	586–616	LSPIHIALNFSLDQPVD SHGLR PALHYQ	30	71	3,308	3,313	11.1
K <sup>+</sup> channel protein	Membrane	173–190	DGILYYYQSGGRLRRPVN	18	71	2,127	2,133	–†
		173–189	DGILYYYQSGGRLRRPV	17	71	2,013	2,018	–†
Mannose binding protein	Membrane	174–193	IQNLIKEEAFLGITDEKTEG	20	80	2,248	2,248	–†
MET	Membrane	59–81	EHHIFLGATNYIYVLNEEDLQKV	23	75	2,746	2,747	–†
GBP-2	Cytosolic	434–450	QELKNKYYQVPRKGIQA	17	81	2,063	2,074	–†
Apolipoprotein B-100 <sup>  </sup>	Exogenous	1200–1220	FPKSLHTYANILLDRRVPQTD	21	71	2,485	2,491	22.6
		1200–1218	FPKSLHTYANILLDRRVPQ	19	71	2,269	2,277	–§
Factor VIII <sup>  </sup>	Exogenous	1775–1790	LWDYGMSSSPHVLNR	16	53	1,918	1,922	–†

Amino acid sequences (single-letter code) and mass determinations are shown for peptides isolated from HLA-DR2. All sequences were determined by automated Edman degradation using a protein sequencer (ABI 477A). RT, retention time from RPC separation; FnR, fibronectin receptor; MET, MET protooncogene member of the tyrosine kinase family of growth factor receptors; GBP-2, IFN-induced guanylate-binding protein 2. Protein category refers to the type of source protein; membrane refers to endogenous membrane associated/secretory proteins; cytosolic refers to endogenous resident cytosolic proteins; and exogenous refers to bovine serum proteins. [M + H]<sup>+</sup> refers to the calculated mass of the peptide in daltons, while the observed m/z refers to the mass in daltons determined by either a Finnigan TSQ 700 or LASERMAT mass spectrometer.

\* Yield refers to initial sequence levels of individual peptides. Total yield of peptides from a single source protein is summed.

† A yield <8 pmol.

§ Yields for coeluting peptides with identical amino termini could not be distinguished.

|| Proposed bovine serum sources with sequence homology to known human proteins.

**Table 2.** Naturally Processed Peptides Bound to HLA-DR3

Source protein	Protein category	Residues	Sequence	Length	RT [M + H] <sup>+</sup>		Obs.	Yield*
					min	m/z		
HLA-A30 <sup>†</sup>	Membrane	28-?	VDDTQFVRFSDAASQ . . .	ND	58	ND	ND	- <sup>†</sup>
HLA-DR α chain	Membrane	111-129	PPEVTVLNTPVELREPNV	19	58	2,090	2,093	- <sup>†</sup>
		111-128	PPEVTVLNTPVELREPN	18	58	1,991	1,990	- <sup>†</sup>
Invariant chain	Membrane	131-149	ATKYGNMTEHDVHMLLQNA	19	73	2,173	2,179	41.5
Acetylcholine receptor	Membrane	289-304	VFLLLLADKVPETSLS	16	69	1,745	1,750	18.5
Glucose transporter	Membrane	459-474	TFDEIASGFRQGGASQ	16	58	1,671	1,673	- <sup>†</sup>
Na <sup>+</sup> channel protein	Membrane	384-397	YGYTSYDTFSWAFL	14	43	1,721	1,721	- <sup>†</sup>
CD45	Membrane	1071-1084	GQVKKNNHQEDKIE	14	43	1,667	1,667	- <sup>†</sup>
ICAM-2	Membrane	64-76	LNKILLDEQAQWK	13	54	1,599	1,602	12.2
IFN-γ receptor	Membrane	128-148	GPPKLDIRKEEKQIMIDIFHP	21	82	2,505	2,510	25.4
		128-147	GPPKLDIRKEEKQIMIDIFH	20	82	2,408	2,412	- <sup>§</sup>
EBV gp220	Membrane	592-606	TGHGARTSTEPTTDY	15	43	1,593	1,593	- <sup>†</sup>
EBV tegument p140	Membrane	1395-1407	KELKRQYEKKLRQ	13	54	1,747	1,750	- <sup>†</sup>
IP-30	Membrane	38-59	SPLQALDFFGNGPPVNYKTGNL	22	82	2,350	2,353	- <sup>†</sup>
		38-57	SPLQALDFFGNGPPVNYKTG	20	82	2,122	2,124	- <sup>†</sup>
Cyt-b5	Cytosolic	155-172	GKFAIRPDKKSNPIRTV	18	54	2,040	2,043	- <sup>†</sup>
Apolipoprotein B-100 <sup>  </sup>	Exogenous	1276-1295	NLFLKSDGRIKYTLNKNSLK	20	67	2,353	2,360	43.1
		1273-1292	IPDNFLKSDGRIKYTLNKN	20	69	2,350	2,355	27.5
		1273-1291	IPDNFLKSDGRIKYTLNK	19	69	2,236	2,245	- <sup>§</sup>
		1273-1290	IPDNFLKSDGRIKYTLN	18	69	2,107	2,097	- <sup>§</sup>
		1273-1289	IPDNFLKSDGRIKYTL	17	69	1,993	2,001	- <sup>§</sup>
		1276-1291	NLFLKSDGRIKYTLNK	16	64	1,910	1,911	43.8
		1276-1290	NLFLKSDGRIKYTLN	15	64	1,782	1,786	- <sup>§</sup>
		1207-1224	YANILLDRRVPQTDMTF	17	67	2,053	2,059	- <sup>†</sup>
		1794-1810	VTTLNSDLKYNALDLTN	17	73	1,895	1,896	- <sup>†</sup>
								114.4

Sequence and mass determinations for peptides bound to HLA-DR3 are shown. Experimental conditions, abbreviations, and notes are as listed in Table 1 with the following additions: IP-30, IFN-γ-induced protein; Cyt-b5, NADH-cytochrome b<sub>5</sub> reductase.

<sup>†</sup> Partial sequence not verified by mass spectrometry.

scribed earlier for the allele-specific peptides. Mass analysis of fractions collected adjacent to those sequenced confirmed the presence of additional peptides corresponding precisely in mass to each of the promiscuous peptides with extensions and/or truncations at either terminal end.

The second most abundant set of characterized promiscuous self-peptides was derived from an HLA-A2-like sequence with residues 105-117 as the core. However, only two of the five cell lines studied express HLA-A2 (Priess A2/DR4 and 23.1 A2/DR8). Nevertheless, HLA-A2-like peptides were isolated from DR2 (MST cells, A3), DR3 (WT-20 cells, A30), and DR7 (Mann cells, A29), none of which expresses HLA-A2 (reconfirmed by staining with specific mAb; data not shown). These same peptides have also been identified from peptide

pools extracted from DR1 expressed in the HLA-A2-positive cell line, LG-2 (15). This sequence, in which tryptophan 107 is a unique residue and facilitates spectrophotometric detection, has been found only in HLA-A2 and HLA-A69 (which appears to have arisen by an exon exchange involving the third exon of an HLA-A2 gene encoding its α2 domain) (41). DR1 expressed in LG-2 (HLA-A2 homozygous), HOM-2 (HLA-A3 homozygous), and the mutant cell lines 721.45 (HLA-A2-positive hemizygous deletion mutant) and 721.221 (a class I negative variant of 721.45, reference 42) was purified and the bound peptide pools characterized. The HLA-A2-like peptides were found in each of the cell lines, but in varying amounts (Fig. 3). Identification of these peptides was accomplished by sequence and mass identity of the HLA-A2-like

**Table 3.** Naturally Processed Peptides Bound to HLA-DR4

Source protein	Protein category	Residues	Sequence	Length	RT [M + H] <sup>+</sup>		Obs.	Yield
					min	m/z		
HLA-A2	Membrane	28-50	VDDTQFVRFSDAASQRMEPRAP	23	61	2,639	2,641	-†
		28-48	VDDTQFVRFSDAASQRMEPR	21	59	2,471	2,473	- <sup>s</sup>
		28-47	VDDTQFVRFSDAASQRMEP	20	62	2,315	2,319	13.5
		28-46	VDDTQFVRFSDAASQRME	19	57	2,217	2,219	28.9
		30-48	DTQFVRFSDAASQRMEPR	19	58	2,256	2,263	51.7
		31-49	TQFVRFSDAASQRMEPRA	19	59	2,212	2,212	12.5
		28-44	VDDTQFVRFSDAASQR	17	58	1,957	1,963	- <sup>s</sup>
		31-47	TQFVRFSDAASQRMEP	17	59	1,985	1,987	- <sup>s</sup>
		31-45	TQFVRFSDAASQRM	15	57	1,759	1,761	- <sup>s</sup>
		31-42	TQFVRFSDAAS	12	57	1,343	1,343	- <sup>s</sup>
								106.6
HLA-Cw9	Membrane	28-50	VDDTQFVRFSDAASPRGEPRAP	23	59	2,534	2,537	- <sup>s</sup>
		31-52	TQFVRFSDAASPRGEPRAPWV	22	57	2,490	2,491	- <sup>s</sup>
		28-48	VDDTQFVRFSDAASPRGEPR	21	57	2,366	2,368	- <sup>s</sup>
		28-47	VDDTQFVRFSDAASPRGEP	20	59	2,209	2,211	- <sup>s</sup>
		28-46	VDDTQFVRFSDAASPRGE	19	59	2,112	2,114	- <sup>s</sup>
		28-45	VDDTQFVRFSDAASPRG	18	59	1,983	1,987	- <sup>s</sup>
		31-48	TQFVRFSDAASPRGEPR	18	55	2,036	2,041	11.0
		28-44	VDDTQFVRFSDAASPR	17	58	1,926	1,932	- <sup>s</sup>
		30-46	DTQFVRFSDAASPRGE	17	55	1,898	1,902	- <sup>s</sup>
		31-44	TQFVRFSDAASPR	14	55	1,597	1,604	- <sup>s</sup>
		130-150	LRSWTAADTAAQ I TQRKWEAA	21	59	2,375	2,376	-†
		129-147	DLRSWTAADTAAQ I TQRKW	19	61	2,218	2,220	147.2
		130-147	LRSWTAADTAAQ I TQRKW	18	61	2,103	2,105	- <sup>s</sup>
		129-145	DLRSWTAADTAAQ I TQR	17	62	1,904	1,909	- <sup>s</sup>
129-144	DLRSWTAADTAAQ I TQ	16	62	1,748	1,752	- <sup>s</sup>		
129-143	DLRSWTAADTAAQ I T	15	62	1,620	1,622	16.8		
								175.0
HLA-Bw62	Membrane	129-150	DLSSWTAADTAAQ I TQRKWEAA	22	69	2,421	2,423	-†
		129-148	DLSSWTAADTAAQ I TQRKWE	20	70	2,278	2,285	13.6
		129-146	DLSSWTAADTAAQ I TQRK	18	69	1,963	1,966	- <sup>s</sup>
		129-145	DLSSWTAADTAAQ I TQR	17	63	1,835	1,838	84.8
								98.4
VLA-4	Membrane	229-248	GSLFVYN I TTNKYKAF LDKQ	20	69	2,351	2,353	- <sup>s</sup>
		229-244	GSLFVYN I TTNKYKAF	16	69	1,866	1,868	20.1
HLA-DQ3.2 β chain	Membrane	24-38	SPEDFVYQFKGMCYF	15	83	1,861	1,862	-†
PAI-1	Membrane	261-281	AAPYEKEVPLSALTN I LSAQL	21	69	2,228	2,229	-†
		261-278	AAPYEKEVPLSALTN I LS	18	69	1,916	1,917	-†
Cathepsin C <sup>†</sup>	Membrane	151-167	YDHNFKVA I NADQKSWT	17	74	2,037	2,039	-†
		151-166	YDHNFKVA I NADQKSW	16	74	1,936	1,938	-†
Ig Heavy chain <sup>**</sup>	Membrane	121-?	GVYFYLQWGRSTLVSVS . . .	ND	70	ND	ND	-†
Bovine hemoglobin	Exogenous	26-41	AEALERMFLSFPTTKT	16	83	1,842	1,836	-†

Sequence and mass determinations for peptides bound to HLA-DR4 are shown. Experimental conditions, abbreviations, and notes are as listed in Table 1 with the following additions. VLA-4, a cell surface heterodimer in the integrin superfamily of adhesion receptors; PAI-1, plasminogen activator inhibitor 1.

† Proposed human protein source with sequence homology to the reported rat cathepsin C sequence.

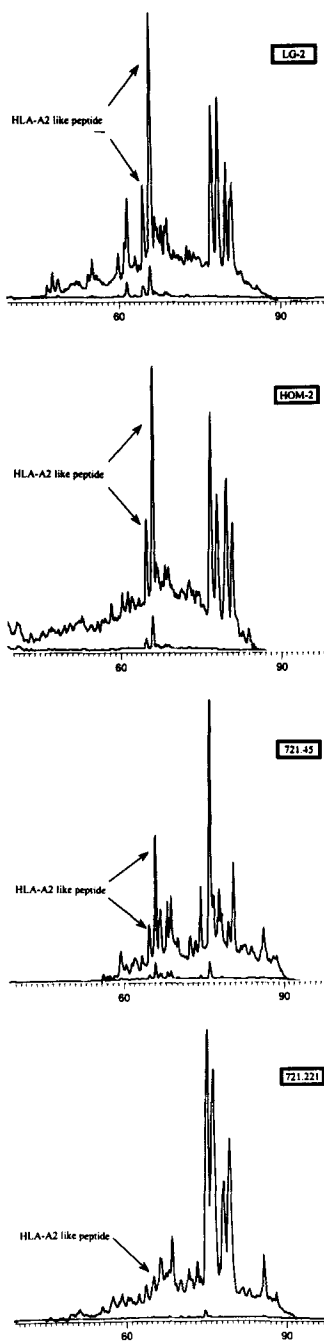
\*\* Partial sequence not verified by mass spectrometry.

Table 4. Naturally Processed Peptides Bound to HLA-DR7

Source protein	Protein category	Residues	Sequence	Length	RT	[M + H] <sup>+</sup>	Obs.	Yield*
					min	m/z	m/z	pmol
HLA-A29	Membrane	234-253	RPAGDGTQKASVWVPSGQ	20	68	2,087	2,092	~5
		234-249	RPAGDGTQKASVWV	16	65	1,718	1,718	17.8
		237-258	GDGTFQKASVWVPSGQEQRYT	22	68	2,441	2,440	~5
		237-254	GDGTFQKASVWVPSGQE	18	68	1,892	1,892	~5
		239-252	GTFQKASVWVPSG	14	68	1,463	1,465	~5
		239-253	GTFQKASVWVPSGQ	15	68	1,720	1,721	~5
		239-261	GTFQKASVWVPSGQEQRYTCHV	23	68	2,606	2,606	116.5 134.3
HLA-B44	Membrane	83-99	RETQISKINTQTYRENL	17	35	2,082	2,086	~†
		83-98	RETQISKINTQTYREN	16	35	1,969	1,971	~†
		83-97	RETQISKINTQTYRE	15	35	1,855	1,857	~†
HLA-DR $\alpha$ chain	Membrane	101-126	RSNYTPITNPPEVTVLTNSPVELREP	26	35	2,924	2,927	16.6
		58-78	GALANIAVDKANLEIMTKRSN	21	68	2,229	2,221	~†
HLA-DQ $\alpha$ chain <sup>†</sup>	Membrane	179-?	SLQSPITVEWRAQSESAQSKMLSGIGGFVL	ND	35	ND	ND	~†
		318-338	VTQYLNATGNRWCSWSLSQAR	21	74	2,442	2,445	~†
4F2	Membrane	318-334	VTQYLNATGNRWCSWSL	17	74	1,999	2,002	~†
LIF receptor	Membrane	854-866	TSILCYRKREWIK	13	35	1,696	1,701	~†
Thromboxane-A synthase	Membrane	406-420	PAFRFTREAAQDCEV	15	74	1,740	1,743	~†
K <sup>+</sup> channel protein	Membrane	492-516	GDMYPKTWSGMLVGALCALAGVLT I	25	74	2,567	2,567	~†
Hsp 70	Cytosolic	38-54	TPSYVAFTDTERLIGDA	17	71	1,856	1,857	~†
		38-52	TPSYVAFTDTERLIG	15	71	1,670	1,672	~†
EBV MCP	Cytosolic	1,264-1,282	VPGLYSPCRAFFNKEELL	18	56	2,082	2,081	19.3
		1,264-1,277	VPGLYSPCRAFFNK	14	56	1,598	1,599	~5
Apolipoprotein B-100 <sup>††</sup>	Exogenous	1,586-1,608	KVDLTFKQHALLCSDYQADYES	23	56	2,661	2,662	18.5
		1,586-1,600	KVDLTFKQHALLCS	15	56	1,689	1,688	~5
		1,942-1,954	FSDHYRGSTSHRL	13	43	1,563	1,567	16.6
		2,077-2,089	LPKYFEKRRNT I I	13	63	1,650	1,654	39.2 74.3
Complement C9 <sup>††</sup>	Exogenous	465-483	APVLSQKLSP IYNLVPVK	19	63	2,080	2,084	~†

Sequence and mass determinations for peptides bound to HLA-DR7 are shown. Experimental conditions, abbreviations, and notes are as listed in Table 1 with the following additions. 4F2, human cell surface antigen involved in normal and neoplastic cell growth; LIF receptor, leukemia inhibitory factor receptor; Hsp 70, heat-shock protein 70; EBV MCP, Epstein-Barr virus major capsid protein. <sup>†</sup>Partial sequence not verified by mass spectrometry.





**Figure 3.** Reversed-phase HPLC separation profiles of peptides bound to HLA-DR1 purified from both HLA-A2-positive and -negative cell lines. Chromatographic peaks containing the HLA-A2-like peptides are easily discerned by their prominent 277-nm UV absorbance due to tryptophan. HLA-A alleles of the homozygous cell lines are: LG-2 (HLA-A2), HOM-2 (HLA-A3), 721.45 (HLA-A2 hemizygous), and 721.221 (class I-negative deletion mutant).

labeled peaks (data not shown). Experiments to characterize a putative locus encoding the HLA-A2-like sequence in HLA-A2-negative cells are underway.

The peptides derived from Ii varied in length from 15 to 25 residues with a common core sequence at position 107–120, and were found in three of the five alleles studied here. They were prominent peptides in each of the alleles in which they were found. An interesting physical characteristic of the Ii peptides is their lack of aromatic amino acids, especially since this feature is prominent in several putative DR-binding motifs (39, 43–46).

The two remaining sets of promiscuous peptides both de-

rived from nonpolymorphic regions of their respective source proteins. A set of MHC-related peptides was derived from the HLA-DR  $\alpha$  chain (positions 182–194), common to all DR alleles. Three DR  $\alpha$  peptides varying in length from truncations at the carboxy terminus have been sequenced from four of the five DR isotypes. These peptides are similar in composition to those derived from Ii in that both groups have a relatively high proline content and no aromatic residues, but neither of these two properties are shared by the other two promiscuous self-peptides. The only peptides isolated from multiple DR isotypes not associated with the MHC were derived from the Ig  $\kappa$  chain (present in varying quantities on the surface of most of the cell lines from which the DR molecules were purified; data not shown).

#### Peptide Binding to Surface-expressed HLA-DR

Synthetic peptide analogues representing promiscuous and allele-specific peptides were used in surface-binding assays. Before biotinylation each synthetic peptide was observed to have the same RPC retention time as the naturally processed extracted self-peptide (data not shown). Each peptide contained a single biotin attached via a 22-Å spacer to the amino terminus. The cell lines used for the binding assay (23, 24) were those from which the respective DR isotypes were purified. As a negative control, the class II deletion mutant T2 was used to monitor nonspecific binding of peptide to the cell surface.

Direct binding of biotinylated peptide to class II MHC molecules on the surface of fixed cells was detected by FITC-streptavidin using flow cytometry. Peptide concentrations between 6.25 and 100  $\mu$ M were used to measure relative binding (Table 7). Specific peptide binding could be blocked by mAb L243 before addition of peptide (Fig. 4). The biotinylation of the HLA-A2-like peptide 103–117 interfered with the normal mode of binding for this peptide; however, the nonbiotinylated synthetic peptide did competitively inhibit the specific binding of the other peptides. Similarly, competitive inhibition using an excess of nonbiotinylated peptide was observed for each peptide (data not shown). Thus, it appears that the promiscuous peptides were capable of binding to multiple DR alleles. In contrast, strict allelic specificity was demonstrated by the apolipoprotein B-100 peptide 1273–1291; it bound only to DR3. No specific binding of any of the peptides to the class II-negative cell line, T2, was observed.

#### Discussion

Four properties illustrate the functional dichotomy between class I and II proteins with respect to peptide binding: (a) although both class I and II proteins assemble in the endoplasmic reticulum, only class I binds peptides before reaching the golgi; (b) cellular trafficking ensures that class II molecules travel a pathway to the cell surface separate from that of class I and encounter different types of source proteins in the endocytic structures where peptides are loaded; (c) structural differences in the binding groove/pocket geometry define the sizes of peptides from a large random pool that can bind

**Table 5. Naturally Processed Peptides Bound to HLA-DR8**

Source protein	Protein category	Residues	Sequence	Length	RT	[M + H] <sup>+</sup>	Obs.	Yield
					min	m/z	m/z	pmol
HLA-DR $\alpha$ chain	Membrane	158-180	SETVFLPREDHLFRKFFHYLPFLP	23	63	2,889	2,889	-†
HLA-DP $\beta$ chain	Membrane	80-92	RHNYELDEAVTLQ	13	81	1,588	1,591	-†
LAM Blast-1	Membrane	88-108	DPQSGALYISKVQKEDNSTYI#	21	57	2,544	2,549	32.6
		92-108	GALYISKVQKEDNSTYI#	17	55	2,116	2,118	29.0
		129-146	DPVPKPVIKIEKIEDMDD	18	61	2,081	2,086	-‡
		129-143	DPVPKPVIKIEKIED	15	61	1,720	1,725	42.6
								104.2
Ig $\kappa$ chain	Membrane	63-80	FTFTISRLEPEFAVYYC	18	61	2,202	2,204	-†
		63-77	FTFTISRLEPEFAV	15	61	1,772	1,777	-†
LAR	Membrane	1,302-1,316	DPVEMRRLLNYQTPG	14	81	1,676	1,680	-†
LIF receptor	Membrane	709-726	YQLLRSMIGYIEELAPIV	18	70	2,109	2,112	-†
IFN- $\alpha$ receptor	Membrane	271-287	GNHLYKWKQIPDCENVK	17	70	2,073	2,075	-†
IL-8 receptor	Membrane	169-188	LPFFLFRQAYHPNNSPVCY	20	63	2,401	2,403	-†
Ca <sup>2+</sup> release channel	Membrane	2,614-2,623	RPSMLQHLLR	10	73	1,251	1,255	-†
CD35	Membrane	359-380	DDFMGQLLNGRVLFPVNLQLGA	22	78	2,418	2,421	-†
CD75	Membrane	106-122	IPRLQKIKWKNYLSMNKY	17	70	2,196	2,202	-†
Calcitonin receptor <sup>55</sup>	Membrane	38-53	EPFLYILGKSRVLEAQ	16	83	1,863	1,848	-†
TIMP-1	Membrane	101-118	NRSEEFLLIAGKLQDGLLH	18	70	2,040	2,043	-†
		102-117	RSEEFLLIAGKLQDGLL	16	75	1,789	1,800	-†
		103-117	SEEFLLIAGKLQDGLL	15	77	1,633	1,646	-†
		101-112	NRSEEFLLIAGKL	12	70	1,377	1,382	-†
TIMP-2	Membrane	187-214	QAKFFACIKRSDGSCAWYRGAAPPKQEF	28	67	3,162	3,165	-†
		187-205	QAKFFACIKRSDGSCAWYR	19	67	2,235	2,234	-†
PAI-1	Membrane	378-396	DRPFLFVVRHNPTGTVLFM	19	63	2,247	2,247	-†
		133-148	MPHFFRLFRSTVKQVD	16	75	2,008	2,116	-†

Cathepsin E	Membrane	89-112	QNFTVIFDTGSSNLWVPSVYCTSP	24	63	2,663	2,664	-†
		89-104	QNFTVIFDTGSSNLWV	15	63	1,828	1,830	-†
Cathepsin S	Membrane	189-205	TAFQYIDNKGIDSDAS	17	68	1,858	1,857	-†
Cystatin SN	Membrane	41-58	DEYYRLLRVL RAREQIV	18	68	2,349	2,348	-†
Tubulin $\alpha$ -1 chain	Membrane	207-223	EAIYDICRRNLDIERPT	17	68	2,077	2,078	-†
		207-219	EAIYDICRRNLDI	13	68	1,594	1,595	-†
Myosin $\beta$ heavy chain	Membrane	1,027-1,047	HELEKIKKOVEQEKEIQAAL	21	63	2,494	2,494	-†
$\alpha$ -enolase <sup>  </sup>	Membrane	23-?	AEVYHDVAASEFF . . .	nd	58	ND	ND	-†
<i>c-myc</i>	Cytosolic	371-385	KRSFFALRDQIPDL	14	73	1,706	1,709	-†
<i>K-ras</i>	Cytosolic	164-180	RQYRLKKISKEEKTGCG	17	58	2,064	2,066	-†
Apolipoprotein B-100 <sup>  </sup>	Exogenous	1,724-1,743	KNIFHFVKVQEGKLKSNDDMM	20	66	2,394	2,399	16.1
		1,724-1,739	KNIFHFVKVQEGKLKLS	16	61	1,902	1,904	19.8
		1,780-1,799	YKQTVSLDIQYSLVTTLNS	20	58	2,272	2,274	12.3
		2,646-2,662	STPEFTILNTHIPSFT	17	85	1,918	1,929	-†
		2,647-2,664	TPEFTILNTHIPSFTID	18	85	2,059	2,073	-†
		2,647-2,662	TPEFTILNTHIPSFT	16	85	1,831	1,842	18.4
		2,885-2,900	SNTKYFHKLNIPQLDF	16	73	1,965	1,970	-†
		2,072-2,088	LPIFKFLPKYFEKRRNT	17	80	2,204	2,207	-†
		2,072-2,086	LPIFKFLPKYFEKRR	15	81	1,988	1,992	10.1
		4,022-4,036	WNFYSPQSSPDKKL	15	63	1,860	1,863	-†
								76.7
Bovine transferrin	Exogenous	261-281	DVIWELLNHAQEHFGKDKSKE	21	81	2,524	2,525	-†
		261-275	DVIWELLNHAQEHFG	15	83	1,808	1,818	16.1
		261-273	DVIWELLNHAQEH	13	78	1,604	1,609	20.5
								36.6
von Willebrand factor <sup>  </sup>	Exogenous	617-636	IALLMASQEPQRMSRNFVR	20	63	2,360	2,360	-†
		617-630	IALLMASQEPQRM	14	63	1,601	1,601	-†

Sequence and mass determinations for peptides bound to HLA-DR8 are shown. Experimental conditions, abbreviations, and notes are as listed in Table 1 with the following additions: LAM, lymphocyte activation marker; LAR, leukocyte antigen-related protein; TIMP, tissue inhibitor of metalloproteinases; *c-myc*, protooncogene member of DNA binding proteins; *K-ras*, protooncogene member of the GTP binding proteins.

† The combination of sequence analysis and mass spectrometry was able to confirm the posttranslational addition of *N*-acetylglucosamine on asparagine 104 of these peptides. Presumably, the rest of the carbohydrate attached at this site was hydrolyzed either during antigen processing or peptide extraction/separation.

‡ Proposed human source with sequence homology to known porcine calcitonin receptor.

|| Complete sequence of  $\alpha$ -enolase is unknown therefore this peptide sequence cannot be verified by mass spectrometry.

**Table 6.** Promiscuous Self-Peptides Bound to HLA-DR Alleles

HLA-DR Allele(s)	Source protein	Protein category	Residues	Sequence	Length	RT min	[M + H] <sup>+</sup> m/z	Obs. m/z	Yield* pmol/			
DR7	HLA-A2 like	Membrane	105-124	SDWRFLRGYHQYAYDGKDYI	20	68	2,554	2,556	39.2			
DR1 <sup>1</sup> , DR2, DR7			103-120	VGSDWRFLRGYHQYAYDG	18	67	2,190	2,190	2,190	28.3		
DR2			103-119	VGSDWRFLRGYHQYAYD	17	67	2,133	2,132	2,132	-s		
DR2			104-119	GSDWRFLRGYHQYAYD	16	66	2,034	2,040	2,040	-s		
DR1, DR2, DR3, DR7			103-117	VGSDWRFLRGYHQYA	15	66	1,855	1,854	1,854	52.6		
DR1, DR2			103-116	VGSDWRFLRGYHQY	14	65	1,784	1,784	1,784	-s		
DR1, DR2, DR7			104-117	GSDWRFLRGYHQYA	14	63	1,755	1,755	1,755	25.6		
DR7			104-116	GSDWRFLRGYHQY	13	63	1,685	1,688	1,688	-s		
DR1, DR2, DR7			105-117	SDWRFLRGYHQYA	13	62	1,698	1,699	1,699	15.3		
											161.0	
DR1			Invariant chain	Membrane	97-121	LPKPPKPVSKMRMATPLLMLQALPMG	25	81	2,734	2,734	nr	
DR1, DR2					97-120	LPKPPKPVSKMRMATPLLMLQALPM	24	80	2,676	2,676	2,676	11.3
DR1					98-121	PKPPKPVSKMRMATPLLMLQALPMG	24	79	2,620	2,620	2,620	nr
DR1, DR3					97-119	LPKPPKPVSKMRMATPLLMLQALP	23	78	2,545	2,544	2,544	36.5
DR1, DR2	98-120	PKPPKPVSKMRMATPLLMLQALPM			23	80	2,563	2,562	2,562	13.2		
DR1, DR2	99-120	KPPKPVSKMRMATPLLMLQALPM			22	80	2,466	2,466	2,466	-s		
DR1, DR2, DR3, DR7	98-119	PKPPKPVSKMRMATPLLMLQALP			22	76	2,432	2,432	2,432	50.7		
DR1, DR2, DR3, DR7	99-119	KPPKPVSKMRMATPLLMLQALP			21	78	2,335	2,334	2,334	-s		
DR1, DR2	100-119	PKPPKPVSKMRMATPLLMLQALP			20	80	2,207	2,207	2,207	-s		

DR2						106-124	KMRMATPLLLMQALPMGALP	19	81	2,071	2,074	-†
DR1, DR2						106-120	KMRMATPLLLMQALPM	15	80	1,732	1,732	18.9
DR1						106-119	KMRMATPLLLMQALP	14	81	1,601	1,600	nr
												130.6
DR7	HLA-DR α chain	Membrane				182-200	APSPLPETTENVCALGLTV	20	43	1,912	1,918	18.4
DR2, DR4, DR8						182-198	APSPLPETTENVCALG	17	45	1,698	1,704	13.4
DR2						182-194	APSPLPETTENVV	13	44	1,354	1,362	80.0
												111.8
DR4	Ig κ chain	Membrane				188-208	KHKVYACEVTHQGLSSPVTKS	21	47	2,300	2,304	44.0
DR4						188-207	KHKVYACEVTHQGLSSPVTK	20	50	2,212	2,213	32.1
DR4						189-206	HKVVYACEVTHQGLSSPVT	18	45	1,955	1,952	23.4
DR4						188-204	KHKVYACEVTHQGLSSP	17	47	1,883	1,883	-‡
DR4						187-203	EHKVYACEVTHQGLSS	17	47	1,915	1,922	-‡
DR4						188-203	KHKVYACEVTHQGLSS	16	57	1,787	1,787	14.8
DR4						189-204	HKVVYACEVTHQGLSSP	16	50	1,755	1,768	-‡
DR4						187-202	EHKVYACEVTHQGLS	16	45	1,828	1,823	-‡
DR4						188-202	KHKVYACEVTHQGLS	15	54	1,700	1,708	58.1
DR4						189-203	HKVVYACEVTHQGLSS	15	47	1,658	1,667	-‡
DR4						187-200	EHKVYACEVTHQG	14	54	1,629	1,633	-‡
DR7						188-201	KHKVYACEVTHQGL	14	53	1,613	1,616	-‡
DR7						188-200	KHKVYACEVTHQG	13	53	1,499	1,501	10.9
												183.3

Sequence and mass determinations for naturally processed promiscuous peptides bound to HLA-DR alleles are shown. Experimental conditions, abbreviations, and notes are as listed in Table 1 with the following additions. nr, yields from DR1 are not reported.

† DR1 refers to previously published work (15).

‡ For peptides observed binding to multiple alleles, the yields are reported for HLA-DR2-isolated peptides (similar yields were observed for the same peptides isolated from multiple DR alleles).

**Table 7.** Surface Staining of EBV-transformed Lymphoblastoid Cell Lines

Cell lines	Ii (97–120)	Ig $\kappa$ (188–202)	HLA-DR $\alpha$ (182–198)	Apo B (1273–1291)
LG-2 (DR1)	123	191	345	50
MST (DR2)	288	178	148	18
WT-20 (DR3)	170	174	176	450
Priess (DR4)	215	189	189	57
Mann (DR7)	288	171	nd	50
23.1 (DR8)	213	221	172	19
T2 (no DR)	35	42	39	40

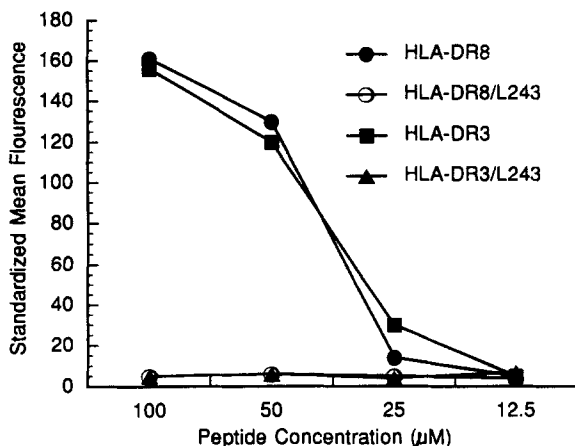
Peptide binding to cell surface expressed class II MHC molecules. The cell lines used to produce the HLA-DR molecules in this study were incubated with biotinylated synthetic peptide and specific binding was measured by flow cytometry. The data are presented as the relative mean log fluorescence. Promiscuous peptides were determined to bind to all six surface expressed DR alleles, while the apolipoprotein B-100 peptide 1273–1291 was found only to bind DR3-positive cells.

class II (10–34 residues) rather than class I molecules (8–11 residues); and (d) since the size distributions of bound peptides are different, the antigen processing machinery used to supply peptide for each is likely to be different. Peptides from antigen produced endogenously are primarily presented by class I, while peptides from exogenously administered antigens are presented by class II MHC molecules (47). However, class II MHC molecules also present peptides derived from proteins synthesized by the APC itself, although primarily derived from source proteins translated with signal peptides (membrane associated or secretory proteins) (48–55). Class II molecules can in addition bind endogenous peptides derived from cytosolic source proteins, but only after such proteins enter the class II processing compartment by either

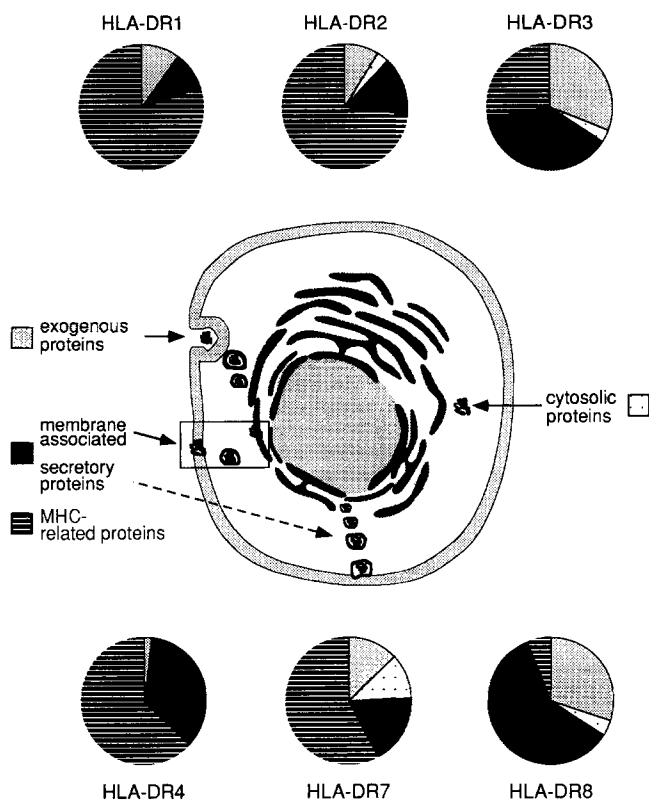
translocation or autophagy (56, 57). The relative proportion of class II-bound peptides derived from these sources has not been clear.

The peptides bound to class II MHC molecules purified from APC grown in culture without exposure to “antigen” are described here. Peptides derived from endogenous proteins (both membrane associated/secretory and cytosolic proteins) and exogenous serum sources were identified. If the peptides presented by EBV-transformed B cells grown in vitro are truly representative, then >85% of the peptides bound to class II MHC molecules in vivo are derived from endogenous self-proteins. The frequency of the different types of peptides bound to the DR alleles examined is represented in Fig. 5. The predominant endogenous peptides were derived from MHC-related source proteins, supporting earlier observations on sequenced peptides bound to DR1 and H-2A<sup>d</sup> (13, 15) and functional work describing the presentation of both class I- and class II-derived peptides to CD4<sup>+</sup> T cells (53, 58, 59). Interestingly, only a single MHC-related peptide was identified as bound to either DR11/DRw52 or H2-A<sup>k</sup>, while none at all were reported among the peptides sequenced from H-2A<sup>s</sup> or H-2E<sup>b</sup>, even though these MHC molecules were purified from similar APC. However, only a limited number of peptides were analyzed in these studies (12, 14, 46, 60). Cell-specific differences in class II antigen processing or protein immunoaffinity purification could also have introduced peptide bias.

These peptide sequences may also further our understanding of class II antigen processing in vivo. Only 27 of the 201 sequenced peptides were <15 residues in length, even though in vitro experiments have shown minimal required peptide lengths of 9–10 residues (1, 34, 38, 45, 61), thus supporting the notion that class II molecules protect bound peptides from proteolytic degradation in vivo (15) as well as in vitro (62, 63). The identification of peptides containing a single *N*-acetylglucosamine (see Table 5) confirmed an earlier in vitro functional study, but more importantly further illustrates the role of lysosomal degradation in antigen processing (64, 65). Since



**Figure 4.** Ii peptide 97–120-specific binding to surface-expressed HLA-DR was determined by direct measurement of added biotinylated peptide using flow cytometry. APC were preincubated with either the anti-DR mAb L243 to sterically obstruct the peptide binding site or a mock buffer before the addition of biotinylated peptide. Ii peptide was blocked at all concentrations measured, representative of the L243 antibody-induced blocking of peptide binding to surface-expressed DR alleles.



**Figure 5.** Relative frequency and source of peptides bound to six HLA-DR alleles. Source proteins were divided into three groups; exogenous serum proteins, endogenous membrane associated/secretory proteins, and endogenous cytosolic proteins, and the number of sequenced peptides (not the molar abundance) for each allele, was plotted. The DR1 peptides were as previously described (15). Although this representation is only based on the 223 peptides sequenced from six DR alleles (between 1.6 and 5% of the estimated total number of peptides bound to any single class II allele), it appears to show a consistent preference for certain types of source proteins. The predominance of MHC-related peptides is highlighted as a subset of the membrane-associated/secretory source protein group.

neither the immunoaffinity purification nor the mild acid extraction procedure altered the glycosylation of the class II  $\alpha$  or  $\beta$  chains (data not shown), the complex carbohydrate originally attached to the peptide must have been enzymatically cleaved in a lysosomal carbohydrase-containing compartment. Further corroboration of lysosomal processing was demonstrated by the identification of cathepsin C-, E-, and S-derived peptides and the observation of 17 cysteine-containing peptides, presumably generated after lysosomal disulfide reduction (66). Finally, the marked preference for heterogeneity at the carboxy-terminal end is consistent with the concerted action of the endosomal proteases cathepsin D and B (67). However, because no fixed cleavage sites were determined, additional exo- and endoproteases such as aminopeptidase N should also continue to be considered as part of the processing machinery.

**Allelic Specificity in Peptide Binding.** Allelic specificity was suggested by distinct HPLC profiles of peptide isolated for

each allele and by the identification for single DR alleles of 84 peptides with different core sequences. In addition to the promiscuous class I- and class II-derived peptides, 41 other peptides from six different class I molecules and 15 peptides from eight different class II molecules were processed and efficiently bound by the DR alleles. Although the predominant peptides were derived from MHC-related molecules, peptides in sufficient quantity for sequence analysis were identified from 52 source proteins not associated with the MHC. The majority of these were from integral membrane proteins expressed on the cell surface or serum-derived proteins. If non-MHC-related membrane-bound proteins were internalized and degraded, the peptide products would travel through the endocytic pathway and encounter DR molecules en route to the cell surface in endocytic vesicles (68-70). Similar source proteins were described for the peptides isolated from murine H-2A alleles (12, 13, 60). Of the few cytosolic peptides identified, a partial sequence overlap was found with the Hsp 70 self-peptide bound to H-2A (14), while a different Hsp 70 peptide sequence was reported from DR11/DRw52 (46). These peptides may be allele specific since no common sequences from these peptides were observed in more than one DR isotype.

Apolipoprotein B-100 from serum in the medium was the most abundant exogenous peptide source protein. This probably occurred as a result of the efficient internalization of apolipoprotein B-100 by the LDL receptor in clathrin-coated pits (71) and subsequent delivery of the protein to the endosomes. The binding specificity for apolipoprotein B-100 1273-1291 was determined to be limited to DR3, but additional synthetic peptides must be made to confirm the specificity of the remaining 10 apolipoprotein B-100 core sequences. This is especially significant in light of two partial sequence overlaps from apolipoprotein B-100 observed in DR2/DR3 and DR7/DR8, respectively. These sequences cannot be eliminated as candidates for promiscuity until their binding specificity is tested with synthetic analogues. Interestingly, an apolipoprotein B-100 peptide sequence not isolated from any of the five alleles reported here was identified in DR11/DRw52 (46), further suggestive of allele specificity. Additional sequence analyses and/or binding studies with synthetic peptides should determine whether all of the allele-specific observations represent truly specific binding to different DR alleles.

**Promiscuity in Peptide Binding to Class II MHC Molecules.** The identification of peptides bound to different DR alleles and confirmation of their binding capacity using synthetic peptides demonstrated the promiscuity of four of the self-peptides. The remarkable efficiency of both processing and binding of promiscuous self-peptides may suggest a physiologic role for these peptides in modulation of the immune response. Perhaps they ensure that only antigenic peptides with sufficient affinity are presented to CD4<sup>+</sup> T cells. Recognition of antigen has been demonstrated to be extremely sensitive, with 80-500 immunogenic class II/peptide complexes being sufficient for T cell stimulation (72, 73). Thus, a few immunologically active peptides could overshadow a plethora of peptides (estimated to be between 650 and 2,000;

references 13 and 15) bound to surface-expressed MHC molecules. Since overstimulation by antigen can induce peripheral tolerance (74–78), these self-peptides may serve not only as competitors to ensure that only antigenic peptides capable of long half-lives bind, but also to prevent overpresentation of a foreign peptide epitope *in vivo*. Generation of self-peptides as immunomodulators could be an efficient mechanism within the cell since the source proteins are available in sufficient concentration and the immune system in general would have developed tolerance to these sequences during maturation.

Perhaps the most interesting set of promiscuous self-peptides are those derived from Ii. Ii forms a complex with class II MHC molecules early during biosynthesis and has several roles in antigen processing/presentation. Ii functions as a chaperone, blocks premature peptide binding, and affects class II intracellular trafficking (79–86). Could Ii have evolved within its primary structure an immunomodulatory peptide sequence capable of high affinity binding to the class II binding groove? Is that sequence the site at which intact Ii binds to class II MHC molecules initially? Ii is a type II transmembrane glycoprotein with its amino terminus as the cytoplasmic tail. There are 34 residues between the ER lumenal side of its transmembrane domain (residue 72) and the beginning of the core sequence of the Ii-nested set of peptides bound to class II MHC molecules (residue 106). This length would be adequate for the Ii polypeptide to reach the cleft at the top of the class II MHC molecule, estimated to be at least 45 Å from the membrane (J. Brown, personal communication) as an  $\alpha$ -helix (51 Å at 1.5 Å/amino acid), or more than adequate as an extended polypeptide (119 Å at 3.5 Å/amino acid for a  $\beta$ -pleated sheet). Alternatively, the Ii peptides may be formed efficiently by proteolysis of Ii in the endosomes and then bind to  $\alpha/\beta$  dimers. In either case, since proteolytic cleavage efficiently dissociates Ii from class II in the antigen processing compartment, the final role of Ii may be to provide high affinity peptides to serve as competitors during the binding of antigenic peptides. At present, the additional possibility that these Ii peptides act as allosteric effectors and bind to the  $\alpha/\beta$  dimers at some site other than the binding cleft is still imaginable. Regardless of how peptide binding occurs, both the high affinity (15, 87) and promiscuous binding of the Ii peptides to DR molecules are clearly evident. Furthermore, the processing machinery for the proteolysis of Ii does not differ significantly among cell lines, since the same nested set of peptides was isolated from multiple cell lines, as well as DR1 (15), and in antigen-processing mutants expressing DR3 (87, 88). Interestingly, homologous Ii peptides from two different H-2A alleles have also been identified (12, 13), suggesting a universal function for the relatively well-conserved high affinity peptides derived from this molecule and bound to class II proteins.

Peptides derived directly from class II MHC molecules by proteolysis are also among the naturally processed bound peptides. A set of peptides from the conserved DR  $\alpha$  chain exhibited promiscuous binding like the Ii peptides. Similarly, a peptide set from the nonpolymorphic murine homologue,

H-2E  $\alpha$  chain, was identified as bound to H-2A molecules that were immunoaffinity purified from either cultured B cells (12, 13) or spleen cells, thus demonstrating that peptides from MHC-related proteins also bind to class II molecules *in vivo* (60).

The set of class I MHC-related promiscuous peptides is by far the most enigmatic. Although this peptide set is clearly related to HLA-A2 (or HLA-A69, which contains the same  $\alpha 2$  domain as HLA-A2), it was also found in cell lines that were HLA-A2 negative. This set of peptides was also present in the class II molecules purified from the deletion mutant 721.221, which lacks expression of HLA-A, -B, and -C molecules, although some class I loci are still present (42). Thus, a putative locus encoding the HLA-A2-like protein could be located within the class I subregion of the MHC. Exon shuffling *in vivo* can generate novel HLA class I molecules (89), and one possibility for the source of the HLA-A2-like self-peptides could be a nonfunctional pseudogene encoded within this region of the MHC. The reduced yield of the A2-like peptide in 721.221 could be due to a deletion in a regulatory element or promoter region located in the class I MHC loci, since large gene deletions exist in this mutant cell line. The amount of identified HLA-A2-like peptides varied among the DR alleles, suggesting two possibilities: (a) the number of fractions sequenced was too small to permit statistical evaluation; or (b) the concentration-dependent equilibrium essential for peptide binding was perturbed by either poor processing of the HLA-A2-like molecule, resulting in less peptide generated or more efficient processing of other protein sources.

The only set of promiscuous binding peptides derived from a non-MHC-related protein was from the Ig  $\kappa$  chain. Relatively high concentrations of this protein would be expected to enter the endocytic pathway since its role is to capture intact antigen on the surface of APC and traffic it to endosomes. Presentation of endogenously processed Ig light chain by class II MHC molecules has been demonstrated in B lymphoma cells with T cell stimulation assays (49, 54) and by sequence analysis of bound peptides (60). The high affinity binding of the Ig  $\kappa$  synthetic analogue to six DR alleles (including DR1 from LG-2, which is  $\lambda$  positive) supports the binding promiscuity even though Ig  $\kappa$  peptides were only isolated from two alleles. The recent sequence analysis of DR11/DRw52-bound peptides also reported this same peptide (46), further supporting its promiscuity.

The ability of peptides to bind multiple DR alleles must be dependent on the composition and location of several key amino acids within the primary structure. Peptide binding to class I MHC molecules has been demonstrated to primarily involve hydrogen bond networks between the MHC molecule and peptide backbone atoms. If a similar mechanism can be extended to peptide binding in class II molecules, then side chain exclusion in the binding cleft region could account for peptide selectivity. Hence, steric hindrance could be avoided by promiscuous peptides possessing a minimum number of bulky side chain residues leading to high affinity



binding to a maximum number of DR alleles. A similar proposal based on synthetic peptide analogue studies has been made (1).

*Motifs for Binding of Peptides to HLA-DR Alleles.* Strict allele-specific binding motifs, as defined for class I MHC molecules, may not exist for class II-bound peptides. Identification of class I motifs was aided: (a) by the relatively small size of the bound peptides (nonamers); (b) the fact that the majority of these peptides have their amino and carboxy termini fixed within the A and F pockets of the class I MHC molecules; and (c) the uniform length of the peptides bound to the alleles initially studied. The problem of deducing motifs from sequence information is more difficult for class II molecules. Bound peptides vary in size (10–34 residues) and are represented by nested sets with varying numbers of flanking residues added at either the amino or carboxy termini, making interpretation of pooled sequencing data nearly impossible. Thus, the longer peptides do not appear to have an end anchored within the binding cleft of class II MHC molecules and may extend outside the cleft at either or both ends. The minimal-length peptide could contain a motif within its core region (9–10 residues), but the core and its anchor residues may be located at various positions within different peptides. Thus, the identification of motifs from sequence information alone is difficult and some additional data are required, such as binding studies varying the length and composition of the peptides and even more definitively (and with correspondingly greater difficulty) refined crystallographic information using single peptide complexes (90–92).

Prior attempts at proposing DR binding motifs using various experimental methods have generated conflicting conclusions (15, 24, 39, 43, 45, 93–95). For example, aside from the probable presence of a hydrophobic residue important in anchoring the amino-terminal region of the peptide, neither of the motifs recently proposed for DR3 (94, 95) fits all of the naturally processed peptides found in this molecule (Table 2), illustrating the difficulty of assigning motifs based on limited sequence information.

With these limitations, can any information relating to preferred amino acids be obtained from the sequences of the naturally processed peptides bound to the different DR alleles? The peptide is likely to be oriented with its amino terminus at the left hand end of the binding cleft, as it is usually represented, and its carboxy terminus at the right hand end (44), as in class I MHC molecules. The left hand end of the cleft is composed mainly of residues contributed by the non-polymorphic DR  $\alpha$  chain. A hydrophobic pocket may exist in this region, for example, in the region of DR  $\alpha$  F26, F54, and F51 (equivalent to Y59 in the A pocket of class I molecules) and DR  $\beta$  G86 (equivalent to Y171) (96). The diallelic polymorphism of residue 86 (G or V in different alleles) in the  $\beta$  chain may affect this pocket, and could well contribute to the size of the pocket, which might influence whether a large hydrophobic amino acid (I, L, M, or V) or an aromatic residue (F, Y, or W) could be accommodated in this region (see Fig. 3 C in reference 44). Analysis of the minimum core sequences from Tables 1–6 reveals that there is a preference for hydrophobic residues near the amino-terminal end

**Table 8.** Sequence Alignment of Allele-specific Preferred Amino Acids

Allele	Sequence alignment	Conformity
		%
DR2a/DR2b	ixxxxxxxx↓ (i + 10) i = I, L, or V ↓ = H, K, or R	70 (7/10 core sequences)
DR3/DRw52	ixx↓ (i + 3) i = F, I, L, V, or Y ↓ = D, N, Q, or T	81 (13/16 core sequences)
DR4	ixxxxxxxx↓ (i + 8) i = F, L, or V ↓ = N, Q, S, or T	88 (7/8 core sequences)
DR7	ixxxx↓ (i + 5) i = F, I, L, V, or Y ↓ = N, S, or T	87 (13/15 core sequences)
DR8	ixxx↓ (i + 4) i = F, I, L, V, or Y ↓ = H, K, or R	73 (24/33 core sequences)

The minimum core sequences from the allele-specific bound peptides (Tables 1–5) were aligned to determine the existence of any prominent amino acids/positions. Conformity refers to the percentage of identified sequences in exact alignment. The spacing between key residues is listed under the alignment, with *i* referring to the index position for the listed hydrophobic residues, and ↓ referring to the preferred residues at the designated position.

for most of the bound peptides, supporting the proposal orientation of bound peptides. In fact, <1% of the bound peptides are without some type of hydrophobic residue in the amino-terminal third of any given minimum core sequence.

Allele-specific preferences for peptide residue content and location are not obvious. Six class II allele-specific binding motifs have been proposed by correlating sequence analyses from identified bound peptides with those of synthetic analogue studies (13, 15, 46, 60). However, in each instance there are peptides that bind to the particular class II molecules without an absolute compliance to the proposed preferences. To investigate if preferred residues exist in the peptides bound to individual alleles, sequence alignments were completed on the minimum core sequences for the peptides in Tables 1–5 (10, 16, 8, 15, and 33 sequences, respectively). The conformity varied from a 70% alignment in DR2 (7/10 peptides, perhaps due in part to the minor contamination of DR2a-bound peptides) to an 88% alignment in DR4 (7/8 peptides), with no set of allele-specific peptides completely conforming to a strict amino acid position preference (Table 8). It is noteworthy that the distance between the index position (*i*) and the preferred residue (*h*) varied between alleles, implying that this spacing may in part contribute to allele-specific peptide binding. The promiscuous self-peptides, demonstrated to bind to all six DR alleles, also varied in alignment conformity from 60 to 100% (the worst alignment was again observed with DR2). Thus, it appears that class II molecules govern peptide binding by subtle differences in peptide composition rather than by strictly defined side chain requirements. This con-

clusion is supported by the identification of antigenic peptides capable of binding to multiple DR alleles (23, 33–39) as well as the promiscuous self-peptides identified in this report.

As an alternative to using synthetic peptides and inferring that these findings are reflective of peptide binding *in vivo*, we have chosen to analyze those peptides that have been processed and bound to MHC molecules within cells. Thus, in addition to the identification of the bound peptides, much can be learned about the repertoire of presented antigen and the machinery involved in antigen processing. In contrast to the peptides bound to class I MHC proteins, class II-bound peptides do not have specific side chain requirements at precise distances from the termini, suggesting that the overall binding energy is provided by the sum of cooperative interactions between the peptide backbone and the cleft and does not involve the peptide termini *per se*. Although allele specificity surely exists, the identification of promiscuous self-peptides (capable of binding to multiple alleles) suggests that rigid allele-specific motifs for class II molecules do not exist, thus permitting a broad binding specificity. Perhaps this promiscuous binding both modulates the presentation of foreign antigens during an immune response and also broadens T cell tolerance to self-peptides during thymic development. Finally, the substantial amount of MHC-related bound peptide (both in terms of relative abundance and the presentation of peptides derived from the three major isotypes found among class I and II molecules) must be considered when interpreting alleloreactivity and may have significant implications for transplantation immunology.

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