

HLA-A2 Subtypes Are Functionally Distinct in Peptide Binding and Presentation

By Dan Barouch,* Thomas Friede,‡ Stefan Stevanović,‡
Lynda Tussey,* Kathrine Smith,§ Sarah Rowland-Jones,*
Veronique Braud,* Andrew McMichael,*
and Hans-Georg Rammensee‡

From the *Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom; ‡Abteilung Tumorstoffwechsel, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany; and §Laboratory of Molecular Biophysics, Department of Biochemistry, Oxford OX1 3QU, United Kingdom

Summary

Nearly half of HLA-A2-positive individuals in African populations have a subtype of HLA-A2 other than the A*0201 allele. We have isolated the common African HLA-A2 subtype genes from Epstein-Barr virus-transformed B cell lines and have established stable class I reduced transfectants expressing these alleles. We have studied the peptide binding and presentation properties of A*0201, A*0202, A*0205, A*0214, and A*6901 by a combination of approaches: assaying direct binding of labeled synthetic peptides, studying the ability of antigen-specific cytotoxic T lymphocytes to recognize peptide-pulsed cells, and sequencing peptide pools and individual ligands eluted from cells. We find that A*0201-restricted peptides can also bind to A*0202 but do not bind strongly to the other alleles in this study. We show that some cytotoxic T lymphocytes can recognize all subtypes capable of binding an antigenic peptide, whereas others are subtype specific. Sequencing of eluted peptides reveals that A*0202 has a similar peptide motif to A*0201, but that A*0205, A*0214, and A*6901 have different motifs. These data strongly support a model in which residue 9 (Phe or Tyr) of the A2/A68/A69 molecules is a critical factor in determining the specificity of the B pocket of the major histocompatibility complex and the position 2 anchor residue of associated peptides. We conclude that a single-amino acid difference in the major histocompatibility complex can be sufficient to cause a dramatic change in the nature of bound peptides, implying that individuals with closely related HLA subtypes may present very different repertoires of antigenic peptides to T cells in an immune response. It is likely to be a general phenomenon that very similar class I subtypes will behave as functionally distinct HLA allotypes.

HLA class I molecules are highly polymorphic cell-surface glycoproteins that present antigenic peptides to CTLs (1). HLA-A2 is the most common class I allele defined by serology and consists of at least 16 closely related subtypes that differ by 1–5 amino acids (2, 3). The A*0201 allele has been studied extensively, and its high resolution crystal structure (4, 5) and peptide binding properties (6–8) are known. Far less is known about the other A2 subtypes. No individual ligands have been previously reported, and none of their pool sequence motifs have been determined, with the exception of A*0205 (9). The polymorphisms of the A2 subtypes are concentrated in the peptide-binding groove of the molecules (10), and several reports show that antigen-specific CTLs can fail to recognize certain HLA-A2 subtypes pulsed with the antigenic peptide (11, 12). It has been unclear, however, whether these observations are

due to differences in the ability of the subtypes to bind the peptides or to conformational differences in the presentation of these peptides to CTL.

With the recent finding that the HLA-A2 subtypes are very common in non-Caucasian populations (13), the importance in determining their peptide binding and presentation properties has greatly increased. Such knowledge is crucial for studying the T cell response in diseases in Africa and Asia. Population studies have shown that, among HLA-A2-positive individuals, the non-A*0201 subtypes are relatively infrequent in most Caucasian populations (~5% of A2-positive individuals) but much more abundant in other populations (13). In Singapore Chinese, the most common HLA-A2 subtypes are reported to be A*0207 (45%), A*0201 (23%), A*0203 (23%), and A*0206 (8%) (13). In a study of Black Africans from both East and West Africa, the

HLA-A2 subtype frequencies are A*0201 (62%), A*0202 (22%), A*0205 (14%), and A*0214 (3%) (13). A study limited to Black Africans from Kenya shows slightly different frequencies, with A*0201 (55%), A*0202 (25%), A*0214 (12%), and A*0205 (8%) most common, and a geographically isolated Caucasian population from Sardinia also shows an interesting distribution, with A*0201 (59%) and A*0205 (41%) as the major HLA-A2 subtypes (13a). We have focused our study on the common African HLA-A2 subtypes and have also included A*6901 for its similarity to A*0201.

Because homozygous B cell lines were not available, we established transfectant cell lines expressing these alleles. We chose class I reduced (C1R)¹ cells as the ideal recipient cell line for all the class I genes (14, 15) since they have very low levels of HLA-A,B expression. In this paper, we report peptide binding studies, CTL killing assays, and peptide elution studies using these transfectants. The combination of these techniques has led to a detailed picture of the ability of the HLA-A2 subtypes to bind and to present peptides. We find that very small changes in the HLA-A2 molecule, especially if these differences are located in the specificity pockets of the peptide-binding groove, can dramatically alter its ability to bind exogenous peptides, to react with CTLs, and to present endogenous peptides.

Materials and Methods

Cells, Synthetic Peptides, and Antibodies. C1R cells, which were produced from the human B lymphoblastoid line Licr.Lon.Hmy2 (14–16), were used as the recipient for the transfectant class I genes. EBV-transformed B cell lines from a number of African individuals were obtained from Dr. F. Gotch. Cells were grown at 37°C with 5% carbon dioxide in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Peptides were obtained from the Medical Research Council AIDS Reagent Project (NIBSC, South Mimms, UK) or were synthesized using standard Fmoc chemistry on a peptide synthesizer (SMPS 350; Advanced Chem Tech UK, Cambridge, UK), purified by reverse-phase HPLC, and checked by mass spectrometry. The peptides used were as follows: pol HIV-1 reverse transcriptase 476–484, ILKEPVHGV (17); gag HIV-1 p17 77–85, SLYNTVATL (18); influenza matrix protein 58–66, GILGFVFTL (19, 20); a soluble, iodinated derivative of the influenza matrix peptide, YGILGKVFTL, that has been shown to have identical binding properties as the native peptide (21–23); and the hepatitis B virus (HBV) core protein 18–27, modified for iodination (F6Y), FLPSDYFPSV (24, 25). mAbs were affinity purified on protein A–Sepharose beads (Sigma Chemical Co.) from hybridoma supernatant by standard procedures and included W6/32 (anti-class I monomeric; 26), BB7.2 (anti-A2/A69; 27), MA2.1 (anti-A2/B17; 28), and ME1 (anti-B7/B22/B27; 29).

Molecular Cloning of the HLA-A2 Subtypes and Establishing Stable Transfectant Cell Lines. DNA cloning techniques were performed essentially as described (30). 5×10^6 EBV-transformed B cells from

a number of African individuals were treated with RNAzol B (Bio-genesis Ltd., Bournemouth, UK) according to the manufacturer's instructions, and total cellular messenger RNA was alcohol precipitated and resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized with reverse transcriptase using an oligo-dT primer (Collaborative Research Inc., Lexington, MA), and the complete HLA-A locus alleles were specifically amplified by the PCR using synthetic oligonucleotide primers. The forward primer (5' GTC GAA TTC GCC GCC ATG GCC GTC ATG GCG CCC CGA 3') and reverse primer (5' CGC GGA TCC TCA TCA CAC TTT ACA AGC TGT 3') included engineered restriction sites that facilitated cloning of the PCR products as EcoRI/BamHI fragments into M13 bacteriophage (Boehringer Mannheim Corp.). After total sequencing of individual clones and identification of the desired HLA-A2 subtype alleles, the genes were subcloned into pBluescript II KS(-) (Stratagene, La Jolla, CA) and then cloned as HindIII/BamHI fragments into the expression vector pKG4 (obtained from Dr. A. Townsend, Institute of Molecular Medicine, Oxford, UK). 20 µg of CsCl-purified plasmid DNA was used to transfect 10^7 C1R cells by electroporation (Gene Pulser system; Bio-Rad Laboratories, Richmond, CA). After 24 h, 1.8 mg/ml geneticin G-418 sulfate (GIBCO BRL, Gaithersburg, MD) was added to select for transfectants, and cell growth was observed after 3 wk. High-expressing lines were established by sorting the cells with BB7.2-coated sheep anti-mouse dynabeads (Dyna, Inc., Great Neck, NY) according to the manufacturer's instructions. High-expressing clones were established by limiting dilution in 96-well plates. Expression of the transfected genes was monitored regularly throughout the study by flow cytometry (Becton Dickinson & Co., Cockeysville, MD) using a panel of mAbs and an FITC-conjugated anti-mouse secondary antibody according to standard protocols.

Peptide Binding Assay. Binding of synthetic peptides to HLA molecules in cell lysates was assayed essentially as described (23). Peptides were iodinated on either histidine or tyrosine by incubating 1 µg peptide in 100 µl PBS for 60 s with 0.5 mCi of ¹²⁵I (Amersham Corp., Arlington Heights, IL) and 10 µl of 5 mg/ml chloramine T. The reaction was terminated by the addition of 30 µl of 5 mg/ml sodium metabisulfate and 2.5 ml of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% NP-40, 0.1% BSA, and 0.2% sodium azide, and the labeled peptide was purified on a gel filtration Sephadex G-25 column (Pharmacia Biotech, Inc., Piscataway, NJ). 10^7 C1R cells expressing a class I allele were washed twice in PBS, starved for 1 h in methionine-free media, metabolically labeled with 100 µCi of [³⁵S]methionine (Amersham Corp.) for 2 h, and lysed for 30 min on ice in 1 ml of 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% NP-40. The lysates were centrifuged for 15 min in a microfuge, and 100 µl of 10% insolubilized protein A (Sigma Chemical Co.) and 1×10^5 to 5×10^6 cpm of labeled peptide were added to the supernatant. After an overnight incubation at 4°C and centrifugation as before, 15 µg of purified BB7.2 (or W6/32 for the C1R-B7 control experiments) was added. After incubation for 1 h, the class I complexes were immunoprecipitated with 100 µl of 50 mg/ml protein A–Sepharose beads, washed three times in lysis buffer, and analyzed by reducing SDS-PAGE on a 16.5% gel. The gels were fixed, dried, and exposed to autoradiographic film, and the intensities of the heavy chain and peptide bands were quantitated by scanning densitometry. The quantitation of peptide binding normalized to the amount of heavy chain present was calculated by the following formula: (peptide intensity – background)/(heavy chain intensity – background). We estimate the peptide concentrations in the binding assay to be ~100 pM (10^5 cpm) to 5 nM (5×10^6 cpm).

¹Abbreviations used in this paper: C1R, class I reduced; HBV, hepatitis B virus.

CTL Recognition Assay. Standard ^{51}Cr release assays were performed (21) using two established peptide-specific CTL clones or lines, which were generated essentially as described (31, 32). Target cells were washed with PBS and labeled with 100 μCi of ^{51}Cr (Amersham Corp.) for 1 h at 37°C. After washing, the target cells were plated at 5×10^3 /well with peptide and CTL, media alone, or 5% Triton X-100 in a total reaction volume of 165 μl /well. After 5 h at 37°C, 20- μl aliquots were removed, and the radioactivity release was determined using a beta-plate counter. The percent specific lysis was calculated according to the following formula: (experimental cpm - media release)/(triton release - media release).

Elution and Sequencing of Self Peptides. HLA-binding peptides were TFA-extracted from C1R transfectant cell lines essentially as described (6). 2×10^{10} cells were lysed in PBS/1% NP-40 with protease inhibitors for 2 h stirring in an ice bath. The lysate was homogenized, sonicated, centrifuged at 3,500 rpm for 10 min, and ultracentrifuged at 100,000 g for 1 h. After filtration, the cleared lysate was passed over a 2-ml pre-column of glycine-blocked Sepharose beads and a 4-ml immunoaffinity column of CNBr-activated Sepharose beads (Pharmacia Biotech, Inc.) linked to purified BB7.2 antibody. Both columns were washed with PBS/0.5% NP-40, PBS/0.1% NP-40, and PBS, and the peptides were extracted with 0.1% TFA. The TFA extracts were passed through a Centricon-10 (Amicon Inc., Beverly, MA) apparatus to remove large proteins, and the peptides were separated by reverse-phase HPLC (SMART system; Pharmacia Biotech, Inc.) on a $\mu\text{RPC C2/C18}$ column (2.1 \times 100 mm). Pool sequences and individual peptide ligands were determined by Edman degradation sequencing using a protein sequencer (476A; Applied Biosystems, Inc., Foster City, CA).

Results

C1R Transfectants. C1R cells stably transfected with the HLA-A*0202, A*0205, and A*0214 alleles (sequence polymorphisms are detailed in Fig. 1 A) were established. Flow cytometry FACS[®] analysis was used to monitor surface expression; the expression levels of all the transfectants were approximately equal. All cell lines were stained with purified W6/32, BB7.2, MA2.1, and ME1 antibodies (26–29) with the expected results (data not shown), and expression levels were monitored regularly. Fig. 1 B shows a typical staining of one cell line.

Binding of A*0201-restricted Peptides. The abilities of the HLA-A2 subtypes and of HLA-A69 to bind three A*0201-restricted peptides (the pol, gag, and modified flu matrix peptides) were studied using the binding assay and calculations detailed in Materials and Methods. The gels in Fig. 2 clearly show the ^{35}S -labeled heavy chain and the ^{125}I -labeled peptide in each experiment, and Fig. 2 E shows the quantitated peptide binding, which is calculated relative to the amount of heavy chain present. The β_2 -microglobulin band was occasionally weak because this protein is often poorly labeled with [^{35}S]methionine. Note that the peptides (~ 1 kD) have anomalous molecular weights on these gels because of their charges and nonlinear binding of SDS. As depicted in Fig. 2, all three peptides were able to bind well to A*0201, and none could bind to the negative control B*0701, also ex-

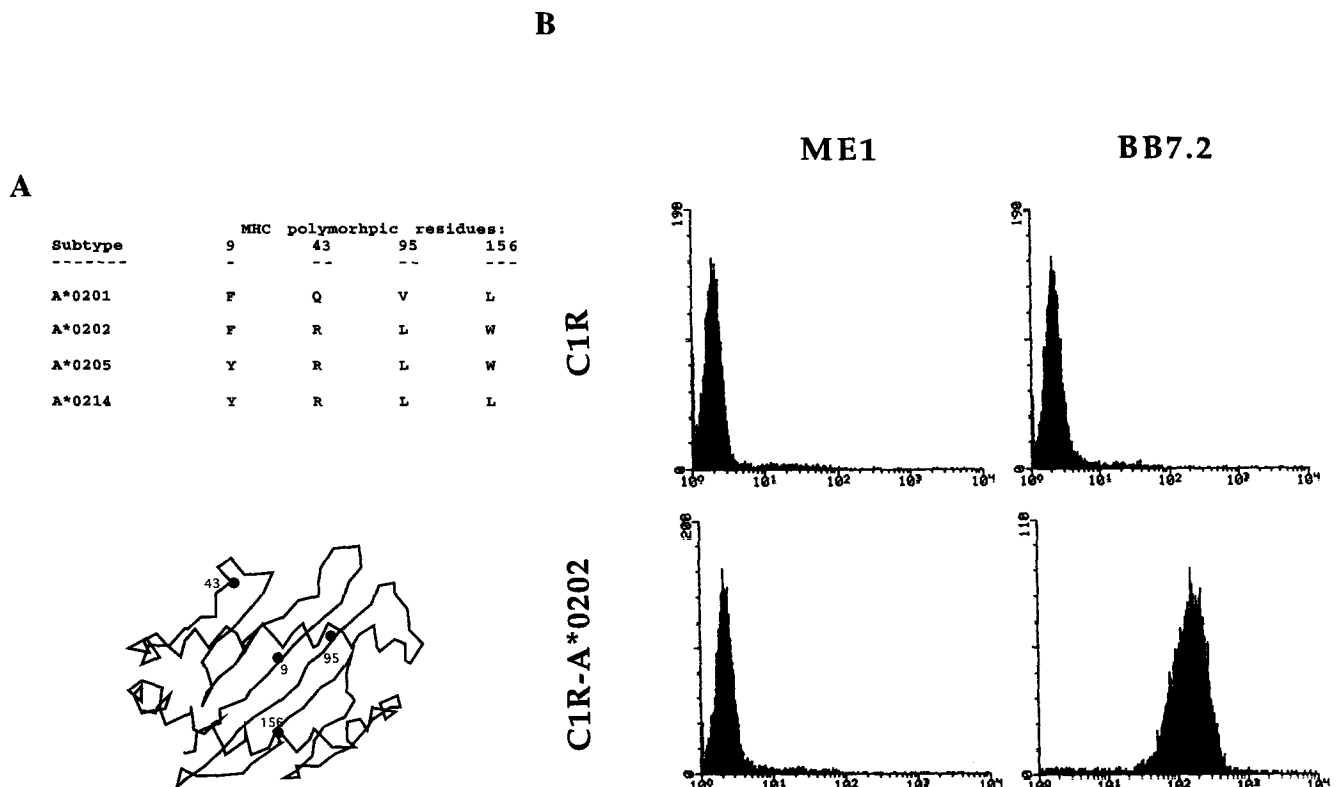


Figure 1. C1R transfectants expressing HLA-A2 subtypes. (A) Comparison of the amino acid differences of A*0201, A*0202, A*0205, and A*0214 and their positions in the peptide-binding groove of the HLA-A2 molecule (4, 5). (B) Sample FACS[®] profiles of ME1 or BB7.2 staining of untransfected C1R cells or C1R-A*0202 cells. BB7.2 staining of the A2 subtype transfectants was ~ 100 –200 times background staining.

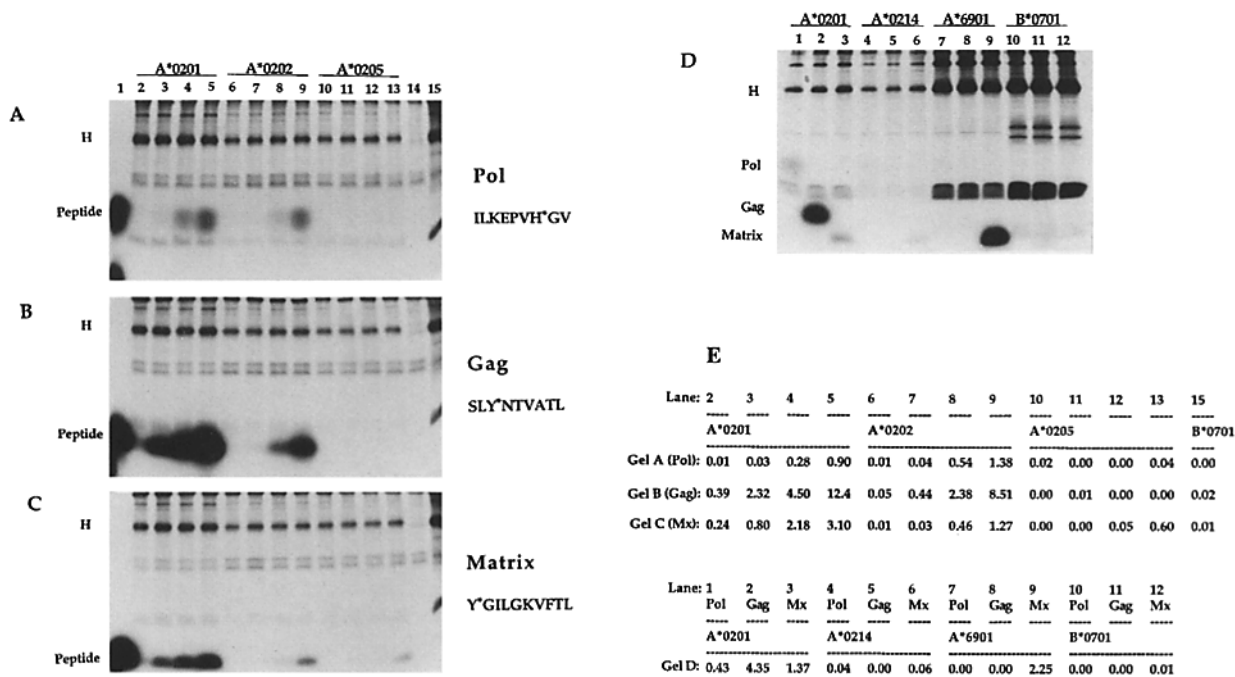


Figure 2. In vitro binding assays of labeled synthetic peptide to class I molecules in cell lysates. 16.5% SDS-PAGE analysis of immunoprecipitates from binding assays shows the presence of the ^{35}S -labeled class I heavy chain and possible associated ^{125}I -labeled peptide. The pol peptide (A, D), the gag peptide (B, D), and the modified flu matrix peptide (C, D) were assayed for binding to the A*0201, A*0202, and A*0205 molecules (A–C) as well as to the A*0214 and A*6901 molecules (D). The asterisk denotes the iodinated position in the peptide. In A–C, free peptide is shown in lane 1, and the binding assays used 10^5 cpm (lanes 2, 6, and 10), 5×10^5 cpm (lanes 3, 7, and 11), 2×10^6 cpm (lanes 4, 8, and 12), and 5×10^6 cpm (lanes 5, 9, 13–15) peptide. Cell lines were C1R–A*0201 (lanes 2–5), C1R–A*0202 (lanes 6–9), C1R–A*0205 (lanes 10–13), untransfected C1R (lane 14), and C1R–B*0701 (lane 15). In D, 2×10^6 cpm pol peptide (lanes 1, 4, 7, and 10), gag peptide (lanes 2, 5, 8, and 11), or modified flu matrix peptide (lanes 3, 6, 9, and 12) were examined for binding to lysates from C1R–A*0201 (lanes 1–3), C1R–A*0214 (lanes 4–6), C1R–A*6901 (lanes 7–9), and C1R–B*0701 (lanes 10–12) cells. In E, the quantitated peptide binding from the assays is shown (see Materials and Methods).

pressed in C1R cells. (The contribution of HLA-C to the intensity of the heavy-chain band in the C1R–B7 lanes, in which the immunoprecipitation uses W6/32, is minimal [data not shown] according to flow cytometry.) All three peptides also bound A*0202 well, but they did not in general react strongly with A*0205 and A*0214. The flu matrix peptide bound weakly to A*0205, and very long exposures of the autoradiographic films (data not shown) suggest that there is a very low level of reactivity of the flu matrix peptide for A*0214 and of the pol peptide for both A*0205 and A*0214. No reactivity whatsoever was observed for the gag peptide for A*0205 and A*0214 using this assay. The pol and gag peptides also did not bind A*6901, but the flu matrix peptide did bind A*6901 very well (Fig. 2 D).

CTL Recognition of HLA-A2 Subtypes. Two antigen-specific CTLs, one specific for the pol peptide and one for the flu matrix peptide, were tested for their ability to lyse the C1R transfectants pulsed with various concentrations of synthetic peptides. Both CTLs were totally peptide specific (data not shown). The pol-specific CTL clone recognized the pol peptide presented by C1R–A*0201 and C1R–A*0202 cells equally well, had a low level of recognition of pol-pulsed C1R–A*0205 cells, but did not recognize pol-pulsed C1R–A*0214 and C1R–B*0701 cells (Fig. 3 A). Note that this killing pattern correlates very closely with the ability of the A2 subtypes to bind this peptide (Fig. 2). The inhibition of

killing at high peptide concentrations is probably due to a slight toxicity of the peptide. The flu matrix-specific CTL line recognized only matrix-pulsed C1R–A*0201 well, and had only a very weak recognition of C1R–A*0202 at very high peptide concentrations (Fig. 3 B). Because A*0202 can bind the flu matrix peptide quite well (Fig. 2 C) but cannot present it in a manner that is recognized well by the CTL, this CTL line appears to be subtype specific.

Sequencing of Eluted Peptides. Edman sequencing of the peptides naturally presented by the C1R transfectants revealed clear peptide motifs (6) as well as several individual ligands for most subtypes. Fig. 4 depicts the sequencing results, indicating the pool sequence anchor residues (in bold), auxiliary anchors, and preferred residues, as well as the individual ligands and their presumed source proteins. In the raw data (not shown), all the peptide position 2 (P2) anchors were 100–1,500% over the corresponding signal in the first (P1) sequencing cycle and were >100 pmol in quantity. Previous work has shown that A*0201 has a Leu anchor at P2 and Val/Leu at P9 (6), and that A*6801 has a Val/Thr anchor at P2 and Arg/Lys at P9 (33). A*0202 was found to have anchors very similar to A*0201 (Leu at P2 and Leu/Val at P9), but A*0205 and A*0214 had significantly different motifs (Val/Gln/Leu at P2 and Leu at P9). A previous determination of the A*0205 motif is very similar to the one presented here (9). A*6901, which is a class

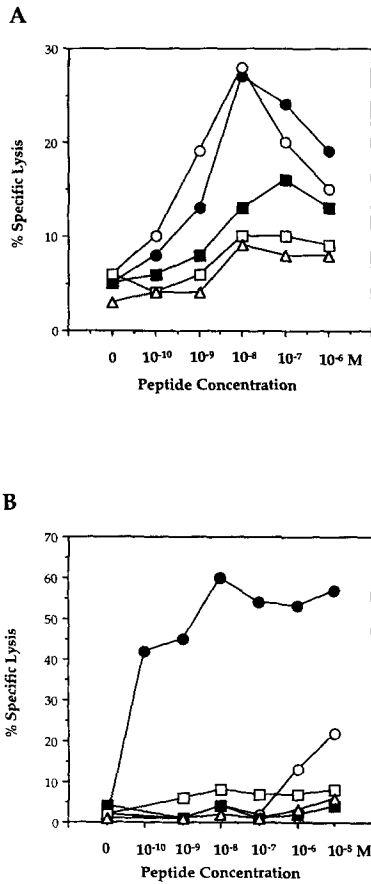


Figure 3. CTL recognition of HLA-A2 subtypes by peptide titration curves. (A) Recognition of pol peptide-pulsed C1R transfectants by a pol-specific CTL clone using an E/T ratio of 6:1. (B) Recognition of matrix-pulsed C1R transfectants by a flu matrix-specific CTL line using an E/T ratio of 15:1. —●—, A*0201; —○—, A*0202; —■—, A*0205; —□—, A*0214; —△—, B*0701.

I allele consisting of the $\alpha 1$ domain of A*6801 and the $\alpha 2$ domain of A*0201, had an interesting motif that was essentially the P2 anchor of A*6801 (Val/Thr/Ala) and the P9 anchor of A*0201 (Val/Leu). The preferred residues in other positions seemed generally to reflect the trends of A*0201, including a hydrophobic residue at P3, a polar residue at P4, and a hydrophobic residue at P6. In addition, A*6901 had a very strong Glu signal at P1, which is also seen in A*6801 but not A*0201. The sequences of several individual ligands confirm these pool sequences and clearly show the mixed Val/Gln/Leu anchor of A*0214 (Fig. 4).

Discussion

We have established a series of C1R transfectant cell lines that has facilitated the study of peptide binding and presentation of HLA-A2 subtypes. The results of the peptide binding experiments show that three A*0201-restricted peptides bind well to A*0202, but much less well (or not at all) to A*0205 and A*0214. This assay was used because of its sensitivity, only requiring peptides in the 10^{-10} to 10^{-8} M

A*0201		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors		L						V	
		M				V			L
A*0202		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors		L							L
preferred	AF	A	FA	ED	I	IL	HQ		V
	GV					VT			
	I								
A*0205		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors		V							L
		QL				I			
preferred	FV	I	IM	DE		LW	HQ	YK	
	YA		YP	KG			V		
				P					
ligands	A	V	P	D	E	I	P	P	L (hsp90)
	F	A	Y	D	G	K	D	Y	I (human MHC I alpha)
A*0214		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors		VQ							L
		L				IL			
						VF			
preferred	FY	A	IL	DE		KN		RY	VM
	VA		MD	PG					
			YP	K					
ligands	F	V	L	P	E	L	P	S	V (expressed sequence tag)
	Y	V	I	S	V	V	F	R	L (connexin 32)
	F	Q	L	E	I	N	V	R	L (unknown)
	F	Q	L	D	D	L	K	V	E L (L35 ribosomal protein)
	H	L	P	S	D	F	T	P	A V (bovine hemoglobin)
	I	L	M	E	H	I	H	K	L (60S ribosomal protein)
	F	A	Y	D	G	K	D	Y	I (MHC I alpha chain)
	F	A	Y	D	G	K	D	Y	L (HLA-E precursor)
	S	T	L	H	L	V	R	L	(ubiquitin)
A*6801		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors			VT						RK
		DE							
A*6901		<u>Position</u>							
	1	2	3	4	5	6	7	8	9
anchors		VT							VL
		A	IF			IF			
			LM			L			
preferred	E	VY	DE	P	V		YR	M	
			RP				Q		
ligands	E	T	F	N	T	P	A	H	Y V (gamma actin)
	E	T	V	A	V	G	V	I	K A V (elongation factor)

Figure 4. Sequences of peptides eluted from C1R transfectants. Both pool motifs and individual ligands are shown. The pool sequences of A*0201 and A*6801 (6, 33) are included for comparison, and a similar pool sequence of A*0205 has been previously reported (9).

range, which is much less than is required by many other MHC/peptide binding assays. The peptides studied differed in their degree of specificity, with the gag peptide totally A*0201/02 specific (according to this assay) and the flu matrix peptide also reactive to A*0205 (weakly) and A*6901 (strongly). This result coincides with the previous

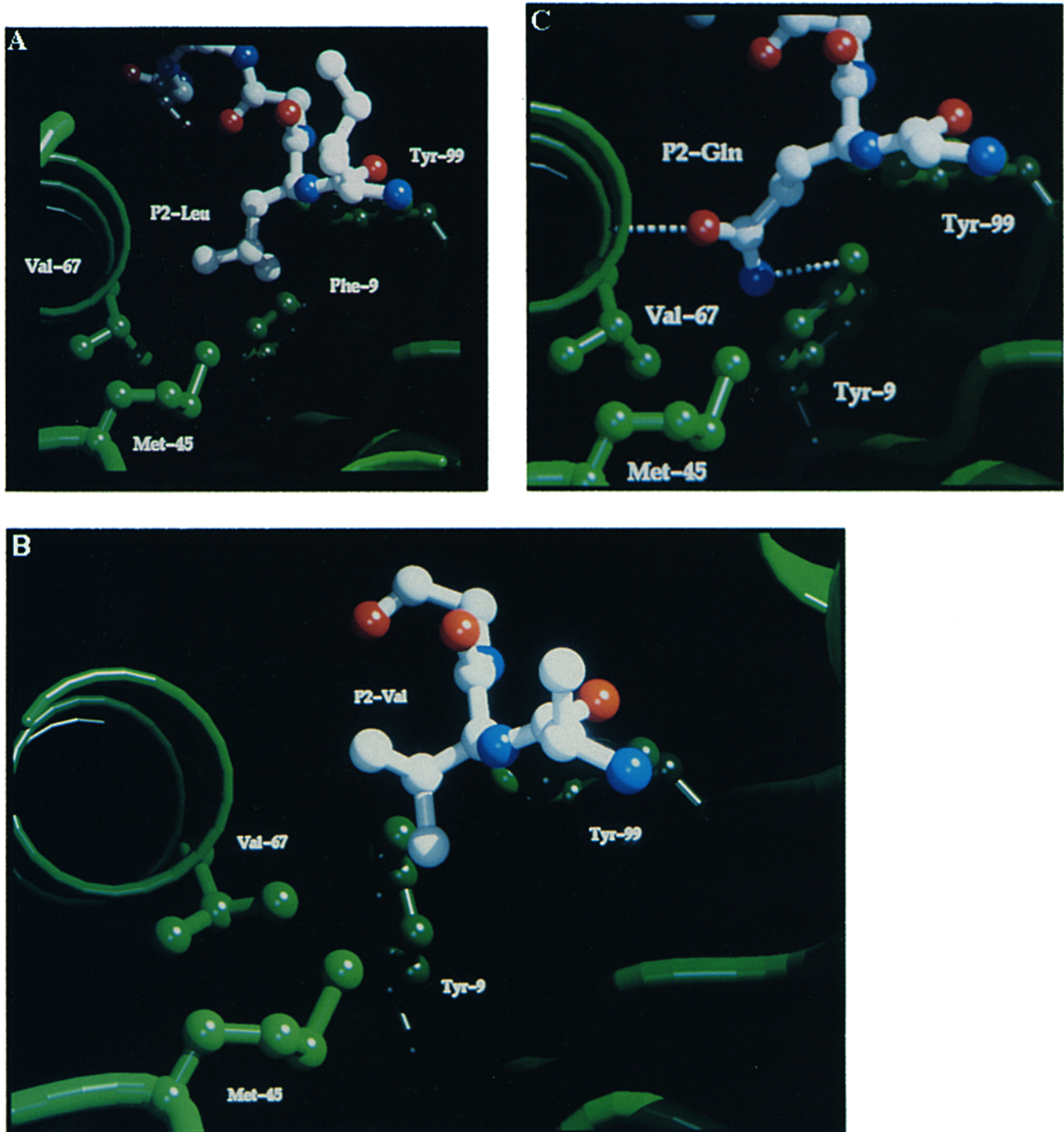


Figure 5. Views of the B pocket through the $\alpha 1$ helix of A*0201 (5), A*6801 (36), and the model of A*0205/0214. The A*0201 and A*6801 structures are taken directly from the published coordinates, and the modeled B pocket of A*0205/0214 was generated by a substituting Tyr-9 for Phe-9 in the A*0201 structure. The images were generated using Molscript (38). The HLA main-chain and key side-chain residues are depicted in green, and the bound peptide is represented in white with nitrogens in blue and oxygens in red. (A) A*0201 complexed with the HIV reverse transcriptase peptide, showing the P2-Leu peptide anchor residue interacting with a hydrophobic B pocket (5). This B pocket structure is probably very similar in A*0202. (B) A*6801 complexed with the influenza peptide, showing the P2-Val peptide anchor residue in the B pocket (36). This B pocket structure is probably very similar in A*6901. (C) Modeled structure of A*0205 or A*0214 showing the Val-67 conformation of A*0201, the Tyr-9 residue in the same orientation as the Phe-9 of A*0201, and a P2-Gln peptide anchor residue in the B pocket. Possible hydrogen-bonding contacts are shown with dotted lines. It is possible that the Tyr-9 and Val-67 residues will adopt orientations similar to those shown in B when A*0205/0214 has a P2-Val peptide anchor.

finding that some flu matrix-specific, A*0201-restricted CTL clones can also recognize HLA-A69 cells pulsed with this peptide (34). The sequences of eluted peptides coincide well with the peptide binding assays and show that A*0202 has a very similar peptide motif compared with A*0201, but that A*0205, A*0214, and A*6901 have significantly different motifs. The CTL experiments show that some CTLs are subtype specific whereas others cross-react among a number of subtypes.

As indicated in the data summary in Table 1, position 9 of the HLA-A2, A68, and A69 molecules can be either Phe or Tyr. The alleles that have Phe-9 have a Leu P2 peptide anchor, but the alleles that have Tyr-9 generally have a smaller and/or polar P2 residue (Val and either Gln, Thr, or Ala). A*0205 and A*0214 also have some leucine in the P2 anchor, suggesting that the B pocket of these alleles might be able to adopt different structures to accommodate these different residues. Position 9 of the MHC is in the bottom of the B pocket of the peptide-binding groove, and thus it is not surprising to find that this position greatly affects the P2 anchor of the bound peptides (4, 5). A comparison of the peptides that bind to A*0202 and A*0205, which differ only in this one residue (actually only one oxygen atom), demonstrates that this Phe-9 to Tyr-9 change alone is sufficient to alter the specificity of the B pocket and the nature of bound peptides.

The data implicating the relevance of position 9 of the MHC in peptide binding coincides well with the reported high resolution HLA crystal structures. The structures of A*0201 and A*6801 show that the different P2 anchors of these molecules are the result of two factors: the amino acid difference at position 9 of the MHC and the rotamer conformational shift of Val-67 (35, 36). Fig. 5, A and B, shows the B pocket of A*0201 and A*6801 with a P2-Leu and a P2-Val, respectively (using the structural coordinates provided by references 5 and 36). We predict that the B pocket of A*0202 will be very similar to A*0201 and that the B pocket of A*6901 will be similar to A*6801. A comparison of these crystal structures shows that the C^{δ2} atom of the P2-Leu in A*0201 packs very closely to Phe-9 in a space that is occupied by the hydroxyl group of Tyr-9 in the corresponding location in the A*6801 structure (35). In addition, the Val-67 orientation in A*6801 sterically blocks the innermost part of the B pocket (35). It is likely that residue 9 has a significant effect in determining the hydrophobic or hydrophilic nature of the B pocket, whereas the shift of Val-67 has a largely steric effect that determines the size of peptide side chains that fit in the B pocket.

Fig. 5 C shows a modeled B pocket structure of A*0205/0214 with a P2-Gln peptide anchor. This model was generated using the computer graphics program FRODO (37) and visualized with Molscript version 1.4 (38). It involves a Phe to Tyr substitution of the A*0201 B pocket structure, and the Val-67 and Tyr-9 orientations allow a P2-Gln to fit with possible hydrogen bonds (~2.5 Å in length) to the hydroxyl group of Tyr-9 and the main-chain nitrogen of Val-67. Such a B pocket structure might also accommodate a P2-Leu. A P2-Val, however, might require the Val-67

Table 1. Summary of Peptide Binding and Peptide Sequencing Data

Allele	Residue 9	Capable of binding			Peptide	
		pol	gag	matrix	P2	P9
A*0201	F	++	++	++	L	VL
A*0202	F	++	++	+	L	L
A*0205	Y	-	-	+/-	VQL	L
A*0214	Y	-	-	-	VQL	L
A*6901	Y	-	-	++	VT	VL
A*6801	Y	ND	ND	ND	VT	RK
B*0701		-	-	-		

Note the correlation between residue 9 of the MHC and position 2 anchor of the associated peptide. Some pool sequence data involving A*0201, A*0205, and A*6801 (6, 9, 33) have been included for comparison.

and Tyr-9 orientations seen in A*6801 (Fig. 5 B). Conformational shifts of MHC residues to accommodate different peptide anchors have been observed in the single-peptide crystal structures of HLA-A2 (5) and HLA-B53 (Smith, K., S. W. Reid, K. Harlos, A. J. McMichael, D. I. Stuart, J. Bell, and E. Y. Jones, manuscript submitted for publication).

The data in this study also coincide well with reports of functional studies involving variant A2 molecules. A previous pool sequencing of peptides eluted from A*0205 (9) shows similar results to the pool motif presented in Fig. 4. In addition, our conclusions corroborate the recent findings of Sudo et al. (38a), which determined the peptide binding motifs of other A2 subtypes and found that A*0204 (Phe-9) and A*0207 (Phe-9) have a Leu P2 anchor, whereas A*0206 (Tyr-9) has a Val/Gln P2 anchor. Furthermore, the single-amino acid substitution (Phe-9 to Tyr-9) in the A*0201 molecule was investigated in a number of reports. This mutant was shown to diminish dramatically binding of the HIV pol and influenza matrix peptides (23, 39) and also to abolish or abrogate CTL reactivity (23, 39–41). Analyses of large panels of influenza matrix-specific CTLs demonstrate that most, but not all, clones and lines fail to recognize this mutant (42, 43). The low level of peptide binding (23, 39) and the rare CTL recognition (42, 43) observed with this mutant are consistent with the data in this report showing a low cross-reactivity of A*0201-restricted peptides to A*0205 and A*0214 and a low level of leucine at the P2 position of peptides eluted from these alleles.

In contrast with these findings, two other groups have recently suggested that the peptide binding properties among A2 subtypes actually are very similar (44–46). One group has studied the binding of the HBV core peptide 18–27 on whole PHA-blast cells isolated from PBLs (44). They report cross-reactivity of this peptide with many alleles (including A*0201, A*0202, A*0205, A*0206, A*6802, and A*6901) and define this group to be an A2-like “supertype”

that all share a common peptide binding motif (hydrophobic residues at position 2 and the COOH terminus). The other group (45, 46) has also reported a broad cross-reactivity of peptide binding among the A2 subtypes and other HLA-A locus alleles using a novel heavy chain refolding assay. Perhaps the individual peptides studied by these groups possess an unusual degree of cross-reactivity, but there is no evidence of broad CTL cross-reactivities among these HLA molecules. In fact, the A*0202 and A*0205 subtypes were first identified because they failed to present the influenza matrix peptide to influenza-specific CTL (47–49). Our results support the view that different peptides have different degrees of cross-reactivity among these alleles, and in our peptide binding assay the HBV peptide has a low level of cross-reactivity. It is also possible that the various in vitro binding/folding assays have different stringencies that depend on the conditions, concentrations, and approaches used. However, considering that these alleles have different pool sequence motifs, different individual peptides presented by cells, different peptide binding properties, and different modeled crystal structure B pockets, we believe that significant functional differences exist in terms of peptide binding and presentation. Some peptides are expected to be cross-reactive, but others bind specifically to certain subtypes and not to others.

The ability of the HLA-A2 subtypes to bind different peptides has significant implications in the T cell response in disease in vivo. By presenting different antigenic peptides to CTL, these alleles are functionally distinct. Even the subtypes that seem to present the same peptides (such as A*0201

and A*0202) can be functionally distinct through differential recognition by CTL of the MHC polymorphic residues or the conformational presentation of a peptide. The fact that sequence-related HLA molecules in fact behave as distinct allotypes, together with the finding that the HLA-A2 subtypes are common in certain populations (13), suggests that they might confer relative selective advantages, possibly by efficiently presenting antigenic epitopes from pathogens in these geographical areas. This was shown to be the case for HLA-B53, which confers protection to severe malaria in West Africa (50). Differences in peptide motifs have also been reported for subtypes of HLA-B39 (51), DR4 (52; Friede, T., V. Gnau, G. Jung, W. Keilholz, S. Stevanovic, and H. G. Rammensee, manuscript submitted for publication), B27 (53), and B51 (54), but the B44 (55) subtype motifs studied so far do not show markedly different pool sequences. Recent reports show that there are similarities in peptide presentation of immunodominant EBV epitopes among the B27 subtypes (56), but that there are also important subtype-specific effects in the association of B27 with spondyloarthritis, with the putative lack of disease association of B*2703 (57, 58) and B*2706 (59) thought to be due to differential peptide presentation. It is likely that significant functional differences within a group of HLA subtypes will prove to be a general phenomenon. This should not be surprising considering that the DNA polymorphisms among subtypes have been conserved in evolution and nearly always cause an amino acid difference in the peptide-binding groove of the class I molecule.

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Address correspondence to Andrew McMichael, University of Oxford, Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, UK.

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