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Identification of the Peptide Binding Motif for HLA-B44, One of the Most Common HLA-B Alleles in the Caucasian Population

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Received March 24, 1995; Revised Manuscript Received June 2, 1995[®]

ABSTRACT: Most peptides that bind to a particular MHC class I molecule share amino acid residues that are thought to physically “anchor” the peptide to polymorphic pockets within the class I binding site. Sequence analysis of endogenous peptides bound to HLA-B44 revealed two potential dominant anchor residues: Glu at P2 and Tyr, or occasionally Phe, at P9. *In vitro* assembly assays employing synthetic peptides and recombinant HLA-B44 produced by *Escherichia coli* revealed that an acidic amino acid at P2 was necessary for promoting stable peptide binding to HLA-B44. Surprisingly, although Tyr was almost exclusively found at P9 of the endogenous peptide sequences, a wide variety of amino acid residues such as Leu, Ala, Arg, Lys, His, and Phe could be tolerated at this position. Using this information, we identified antigenic peptides from the influenza virus components nonstructural protein 1 and nucleoprotein that are presented by HLA-B44 to antiinfluenza type A cytotoxic T lymphocytes. In addition, cytotoxic T lymphocytes induced by these antigenic peptides were shown to be capable of recognizing endogenously processed peptides from influenza-infected cells, indicating a potential use for these peptides in vaccine development. Finally, molecular models were created to investigate the possible ways in which the anchor residues might function to stabilize the binding of peptides to HLA-B44, and these models indicate that the acidic residue at P2 most likely interacts primarily with Lys 45 of the HLA-B44 heavy chain and makes additional contacts with Ser 67 and Tyr 9.

Class I molecules bind small peptides that are generated by proteolysis of cytoplasmic proteins (Townsend & Bodmer, 1989; Rammensee *et al.*, 1993), either self proteins (De Plaen *et al.*, 1988) or proteins encoded by infectious agents reproducing intracellularly (Townsend *et al.*, 1986). When class I molecules are complexed with a foreign or altered-self peptide, cells bearing these complexes may be destroyed by cytotoxic T lymphocytes (CTL) carrying the appropriate T cell receptor (Townsend & Bodmer, 1989). The peptides that are bound to particular class I molecules can be studied for characterization of salient features such as length and the presence of particular amino acid residues (aa)¹ that are thought to physically “anchor” the peptide to pockets within

the class I binding groove (Falk *et al.*, 1991). Knowledge of these anchor residues has proven useful in predicting which peptides from infectious agents (Pamer, 1991), transfected genes (Rotzschke *et al.*, 1991), or tumorigenic proteins (Wallny *et al.*, 1992) are likely to bind to a particular class I molecule. We report the structural characterization of endogenous peptides that bind to HLA-B44, one of the most common HLA-B alleles in the Caucasian population, having a gene frequency of 11.9%. We also report the identification of several antigenic peptides from influenza nonstructural protein 1 (NS1) and nucleoprotein (NP) based upon knowledge of dominant anchor residues for HLA-B44 endogenous peptides. Finally, we constructed molecular models to investigate how the anchor residues of the peptide might contact aa in the pockets of the peptide binding groove.

MATERIALS AND METHODS

Cells and Antisera. HLA-B44 molecules were isolated from the human plasma cell line Hmy2.C1R deficient in class I expression (Zemmour *et al.*, 1992) transfected with the HLA-B* 4403 cDNA. HLA-B44 full-length cDNA was obtained by PCR amplification (DiBrino *et al.*, 1993) of cDNA made from RNA isolated from the human lymphoblastoid B cell line W1B (HLA-A23, -A24, -B44). The cDNA was completely sequenced to confirm identity to the published sequences for HLA-B*4403 (Fleischhauer *et al.*, 1991; Zemmour, 1992, 1993) (previously called HLA-B44.1: new), cloned into the expression vector RSV.neo (Hogan, 1988), and transfected into Hmy2.C1R cells (Storkus *et al.*, 1987) as previously described (Winter *et al.*, 1991).

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[®] Abstract published in *Advance ACS Abstracts*, July 15, 1995.

¹ Abbreviations: aa, amino acid residue; CTL, cytotoxic T lymphocyte; P, position of an amino acid residue in a peptide; PΩ, carboxy-terminal position of a peptide; PΩ-1, position preceding the carboxy-terminal position of a peptide; pn, peptide number; sc, side chain; NS1, nonstructural protein 1 of influenza virus; NS2, nonstructural protein 2 of influenza virus; NP, nucleoprotein of influenza virus; PB1, basic polymerase 1 protein of influenza virus; PB2, basic polymerase 2 protein of influenza virus; M1, matrix protein of influenza virus; NS1-Vac, recombinant vaccinia virus containing the NS1 gene of influenza virus; NS2-Vac, recombinant vaccinia virus containing the NS2 gene of influenza virus.

Cell surface expression of HLA-B44 was detected with the human class I specific monoclonal antibody W6/32 (Parham *et al.*, 1979). W6/32 was isolated from ascites fluid using protein A-agarose as described (Harlow & Lane, 1988).

Isolation of Peptides from HLA-B44. A protocol for isolation of peptides has been described (DiBrino *et al.*, 1993). Briefly, HLA complexes were isolated from detergent lysates of 1.5×10^{10} HLA-B44-transfected Hmy2.C1R cells by immunoaffinity chromatography using W6/32-coupled Sepharose, prepared with CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's protocol. Ten milligrams of antibody was coupled per 2 mL of beads (wet volume). Glycine coupled to Sepharose, used as a nonspecific adsorbent prior to immunoaffinity chromatography, was prepared in a similar manner. After extensive washing of the beads, associated peptides were eluted from immunoaffinity columns at acidic pH as described (Falk *et al.*, 1991). Peptides were separated from the HLA heavy chain and β 2-microglobulin (β 2m) by centrifugation using Centriprep-10 microconcentrators (Amicon, Beverly, MA). Reversed-phase HPLC of peptides was performed on a C18 Nova-Pak column (Millipore, Bedford, MA), using Beckman (Fullerton, CA) System Gold instrumentation that included a photodiode-array detector (model 168). The gradient consisted of 0.1% trifluoroacetic acid-acetonitrile, 96:4, for 5 min followed by a linear increase to 40% acetonitrile over 45 min. The flow rate was 1 mL/min and the fraction size was 0.2 mL.

Analysis of Peptide Binding to Class I Molecules. HLA-B44 complexes were prepared using HLA class I heavy chains that are derived from *Escherichia coli* inclusion bodies, ^{125}I - β 2m, and peptides (Parker *et al.*, 1992a). Because the HLA heavy chain is refolded from inclusion bodies prepared from *E. coli*, there are no endogenous peptides that can stabilize the complex in the absence of the added synthetic peptide. To obtain the HLA-B44 heavy chain, DNA encoding aa 1–278 from an HLA-B44 cDNA clone, derived as described above, was amplified by PCR using the primers 5'-GAGACCTGGGCCGGATCCCACTC-CATGAGGTATTTC-3' and 5'-GATGGGGAAGCTTCATT-AGGAAGACGGCTCCCATCT-3', respectively. This PCR product was then inserted in place of the HLA-A2 encoding cDNA in the *E. coli* expression plasmid pHN1⁺/HLA-A2 using the sites encoded by the primers (Parker *et al.*, 1992b). The HLA-B44 coding region was sequenced to confirm that no nucleotide changes had been introduced during PCR amplification. HLA-B8 and HLA-A1 heavy chains were also prepared as described (Parker *et al.*, 1992b). HLA complexes were reconstituted using the HLA heavy chain (0.2 μg), ^{125}I - β 2m (12 000 cpm; specific activity 1×10^{17} to 1×10^{18} cpm/mol), and peptides (10 μg) in a final volume of 50 μL as described previously (Parker *et al.*, 1992a). Complex formation was analyzed by HPLC gel filtration in 25 mM 4-morpholineethanesulfonic acid buffered to pH 6.5 containing 150 mM NaCl as described (Parker *et al.*, 1992b). This assay measures peptide binding indirectly by monitoring the ability of peptides to promote incorporation of ^{125}I -labeled β 2m into HLA heavy chain/ β 2m/peptide heterotrimeric complexes. The stability of HLA complexes was assessed by measuring the rate of dissociation of ^{125}I - β 2m taken at various time points. The correlation of rate of β 2m dissociation with stability has been previously established (Parker *et al.*, 1992b). Moreover, our data on the stability of MHC class I/peptide complexes correlate well with data

on the affinity of peptides for class I molecules obtained by other laboratories (Parker *et al.*, 1995).

Peptide Sequence Analysis. N-Terminal aa sequence analyses were performed with a model 477A protein sequencer coupled to a model 120A PTH analyzer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's program, NORMAL-1. Reversed-phase HPLC analysis of the PTH aa was done using a Brownlee PTH-C-18 column (2.1 \times 220 mm). It was not possible to assign Cys residues as PTH-Cys is not recovered in sufficient yield.

Peptide Synthesis. Peptides were synthesized as described (Parker *et al.*, 1992a). Amino acid compositional, mass spectrophotometric, and analytical reversed-phase HPLC analyses were performed in order to confirm purity and sequence of the synthetic peptides.

Generation and Assay of CTL. Influenza A virus-specific CTL responses were induced *in vitro* from PBL of HLA-typed normal volunteer donors with live A/Udm virus as previously described (Biddison, 1980). HLA typing was performed by the HLA Typing Laboratory, Department of Transfusion Medicine, NIH. CTL were assayed on ^{51}Cr -labeled Hmy2.C1R cells transfected with HLA-B44 or HLA-A2 genes as described (Winter *et al.*, 1991). Target cells were either pulsed with peptides for 1 h at 37 °C at 1 $\mu\text{g}/\text{mL}$ and washed once or infected with influenza virus or recombinant vaccinia viruses as described (Winter *et al.*, 1991). Seed stocks of recombinant vaccinia viruses (Bennick & Yewdell, 1988) expressing influenza A virus genes nonstructural protein 1 or 2 (NS1-Vac, NS2-Vac), basic polymerase 1 or 2 (PB1-Vac, PB2-Vac), nucleoprotein (NP-Vac), or matrix protein 1 (M1-Vac) were gifts of Dr. Jack Bennick (Laboratory of Viral Diseases, NIAID).

Molecular Modeling. Molecular models of HLA-B44 bound to each of two different peptides, AEIPAVAAY and GEISPSPSL, were constructed starting from the coordinates of the X-ray structure of HLA-B27 residues 1–275, β 2m, and the peptide ARYAASSTEL (Madden *et al.*, 1991). Using the Protein Design module within QUANTA 3.3 (Molecular Simulations, Waltham, MA), the appropriate substitutions for HLA-B44 were placed on the HLA-B27 backbone by superimposition of the C α atoms and retention of the C α –C β bond for each substitution. Following 100 steps of energy minimization by the adopted basis Newton–Raphson (ABNR) algorithm, the structure was subjected to simulated annealing using X-PLOR (Brunger, 1990) using heating to 1000 K and slow cooling to 300 K, as described (Corr *et al.*, 1992). Final structures were again subjected to 100 steps of ABNR minimization. In previous control experiments, we have found that when a model of HLA-A2 was built starting from the crystal structure of HLA-B27, the rms deviation of all C α atoms was <1.5 Å (Corr *et al.*, 1993).

RESULTS

Isolation and Sequences of HLA-B44-Associated Peptides. HLA-B44 complexes were isolated from detergent lysates of HLA-B44-transfected Hmy2.C1R cells as described in Materials and Methods. After separation of the endogenously bound peptides from the HLA-B44 heavy chain and β 2m, the peptides were fractionated by reversed-phase HPLC (Figure 1A). An enlarged view of the region in which most of the peptides eluted is shown in Figure 1B.

The results for sequence analyses for 27 selected HPLC fractions or combination of fractions are shown in Table 1.

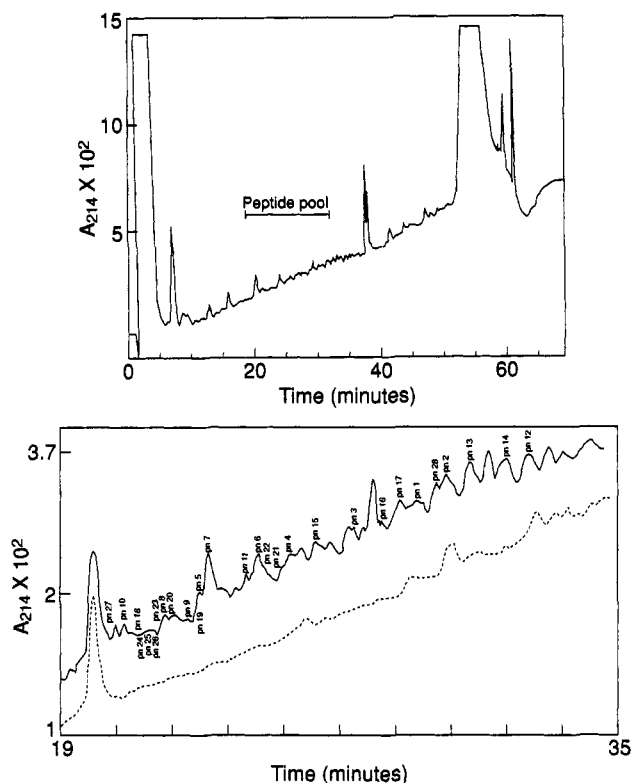


FIGURE 1: (A) Reversed-phase HPLC of peptides eluted following immunoaffinity purification using the mAb W6/32. (B) Enlarged view of the elution profile for the region between 20 and 35 min for HLA-B44-associated peptides. Superimposed on the profile for the W6/32 eluate (—) is the corresponding region of the chromatogram for material obtained upon elution from a nonspecific adsorbent consisting of glycine coupled to Sepharose (---). The number assigned to a peptide sequence in Table 1 is denoted "pn".

The majority of the sequences are nine amino acid residues (aa) in length, while four are decamers and four others are probably not full-length sequences presumably due to low yield. In many instances, several peptides were present in the HPLC fractions because, in addition to the primary sequence, other aa were present at certain cycles in lower yield. The sequence of peptide number 19 (pn19) corresponds to aa 37–46 of human ribosomal protein S21 (Bhat & Morrison, 1993).²

Certain types of aa are over-represented at particular positions (P) in the peptide sequences. Glu is present at P2 in all of the peptide sequences. Tyr is present in approximately 85% of the full-length sequences at the carboxy-terminal position of the peptide (P Ω), while Phe is present in the remaining 15%. Also over-represented, but not to as great an extent, are Asp at P1, Ile at P3, Pro at P4, and Val at P6.

Synthetic Peptides Corresponding to the Sequences of the Endogenous Peptides Form Complexes with HLA-B44 *in Vitro*. In order to confirm that the sequences represent peptides that can bind to HLA-B44, peptides with sequences corresponding to five of the endogenous peptide sequences were synthesized. All of these peptides formed complexes

with HLA-B44 (Table 2) but, as expected, not with HLA-A1 or HLA-B8 (data not shown). One of the peptides (pn7B44) was synthesized in two forms, as a decamer and as a nonamer, because of the tentative assignment of Tyr at P10 and the secondary assignment of Tyr at P9 (see Table 1). Both peptides DEVGIVTKY and DEVGIVTKMY bound to HLA-B44 *in vitro*. However, the half-time for dissociation of β 2m ($t_{1/2}$) (240 min) of the nonamer peptide was similar to that of the other four nonamer peptides (240–360 min) (Table 2), whereas the $t_{1/2}$ of the decamer was significantly shorter (60 min) (Table 2).

Anchor Residue Requirements for Peptide Binding to HLA-B44. In order to determine the importance of anchor residues for peptides that bind to HLA-B44, peptides were synthesized that contained Ala except at selected positions of interest (Table 3). First, we tested the requirement for Glu at P2 because Glu was observed in all of the endogenous peptide sequences at P2 (Table 1). The data show that an acidic aa at P2 strongly favors interaction with HLA-B44; complexes formed with AAIAAVAKY are over ten times less stable than complexes formed with AEIAAVAKY (18 vs 240 min). Glu appeared to be slightly better than Asp at P2 since AEIAAVAKY formed complexes that were twice as stable as those formed with ADIAAVAKY (240 vs 120 min, respectively).

Second, Tyr was observed at P Ω in the majority of the full-length endogenous peptide sequences, while Phe was present at P Ω in the remainder. The P Ω side chain (sc) of the peptide has been shown to bind in the F pocket in the crystal structures that have been solved for human class I molecules (Bjorkman *et al.*, 1987; Madden *et al.*, 1991; Saper *et al.*, 1991; Silver *et al.*, 1992a). We reasoned that Lys, Arg, His, and Leu might, in addition, be accommodated at P Ω because peptides that bind to some other class I molecules that have F pocket aa similar to that of HLA-B44 have basic and hydrophobic P Ω aa (see Tables 5 and 6 and Discussion). Indeed, we found that poly(Ala) peptides having these P Ω anchor residues bound to HLA-B44, forming complexes that differed in their degree of stability (Table 3). The relative hierarchy for P Ω aa in terms of binding stability is Leu (900 min) > His (600 min) > Phe (480 min) > Tyr (240) > Ala = Arg = Lys (Table 3). AEIAAAAAAE binds very poorly ($t_{1/2}$ of 24 min, Table 3), indicating that Glu is much less well tolerated than the other aa that we tested at P Ω .

Third, since Ile, Pro, and Val were observed in about 40% of the endogenous peptide sequences at P3, P4, and P6, respectively, these aa were tested to determine whether they might act as auxiliary anchor residues. Our data indicate that Ile at P3 or Pro at P4 can act to stabilize binding, as AEIAAAAKY and AEAPAAAAY, but not AEIAAAAAAY, bind to HLA-B44 and form stable complexes (Table 3). Ile appears to stabilize binding better than Pro since the $t_{1/2}$ for AEIAAAAAAY is 600 min as compared to that of AEAPAAAAY which is 360 min (Table 3). The ability of Val at P6 to function as an auxiliary anchor residue appears to be dependent upon the sequence of the remainder of the peptide. The presence of Val appears to be stabilizing when the P Ω aa is Leu and destabilizing when the P Ω aa is Tyr (compare AEIAAVAKL, AEIAAVAKY, and AEIAAAAKY, $t_{1/2}$ of 900, 240, and 780 min, respectively). Finally, since there is not a high degree of over-representation of a single aa at the position preceding the carboxy-terminal position (P Ω -1), we assumed that the aa at P Ω -1 does not

² The databases used were GenBank (release 74.0), GenPept (Release 74.0), EMBL (Modified, Release 31.0), HRA-Protein (Release 8.0), HRA-Nucleic (Release 8.0), PIR-Protein (Release 33.0), PIR-Nucleic (Release 36.0), NRL-3D (Release 8.0), SwissProt (Release 23.0), Vecbase (Release 3.0), PROSITE (Release 9.0), TFDAA (Release 5.1) (Devereux *et al.*, 1984). Numerals in brackets indicate the aa residue location within the protein.

Table 1: HLA-B44 Endogenous Peptide Sequences^a

position no.										peptide no.
1	2	3	4	5	6	7	8	9	10	
S	E	I	D	L	I	L	X	(Y)		pn1
T/K/N		L/F/V	P/K	I/F/V	V	K		(Q)		
A	E	I	P	R	T	F	K	Y		pn2
S/K/T		D/L/F	G	D/S/Q	V					
A	E	I	A	I	V	P				pn3
K/Q/T		L/V/N	P							
A	E	I	V							pn4
A	E	M	G	K	F	K	F	S	Y	pn5
		L/A	K	I/N		T	A	Y		
S	E	I	D	T	V	A	K	Y		pn6
W/K	P	F/L	E/Y/T	P/V/G	L/N/K	H/K	N/Q/(L)			
D	E	V	G	I	V	T	K	M	(Y)	pn7
S/E/Q		M/A/L/I	K/N/Q	A	(I/G/F)	S	A/F	Y		
A	E	I	P	T	R	V	N	Y		pn8
R/D/S		A/T/F/L	(G/K)		T	K	E/(H/F)	(F)		
A	E	V	D	K	V	T	G	(Y)		pn9
S/D/T		A/L/I	G/P							
F	E	P	T	V	V	K	K	Y		pn10
D/R	R	V/(N)		L	I					
D	E	F	I	G	V	A	(L)	Y		pn11
Q/S/R		I/M/H	P/Q	T/(A)		T/I	(k)			
D	E	I	P	L	V					pn12
N/V/I		L/A/V								
N	E	M	P	I	L	N	X	Y		pn13
D/K/M		L/T/I				Y				
D	E	I	P	F	V	X	X	Y		pn14
S/V/N		F/L/Y						D		
D	E	(I)	(L)	(I)	(F)	(Q)	X	F		pn15
(S/T)		(V/L/A)	(P)	(V)	(T/Q/K)					
D	E	I	G	V	I	D	L	Y		pn16
(S/M/T)		A/L/V	K/P	I/F/T/Q	P	F/K/M	Y/Q			
D	E	(L)	(P)	(I)	(I)	(L)	(L)	F		pn17
(T/G/V)		(I/V/F)	(L/K/V)	(V/F)	(V/Y)	(K/A/Q)	(K)			
D	E	N	P	L	V	K	Q	Y		pn18
S/T/Q		Y/F/I/A	K/L/T	A	G	I	R	F		
A	E	V	D	K	V	T	G	R	F	pn19
T/S/D			P/G					Y		
T	E	A	I	L	(R)	(M)	E	Y		pn20
D/F/K		V/N/L	Q	V/M/K			K/Q/N			
S	E	I	R	V	N					pn21
T/M/V		F/N/V/A								
S	E	I	P	V	N	K	X	Y		pn22
T/M/A		A/V/F	Y/K	I	A	F		L/T/G/F/S		
T	E	A	P	K	T	K	X	Y		pn23
K/S/D			L/E/K		A	Q		F		
D	E	A	P	A	V	I	(S)	Y		pn24
A/G/S		N/V/M	E/K/G	I		F	(D)	F		
S	E	A	I	H	T	F	Q	Y		pn25
D/Q/K		F/N/D	P/R/K	K/L			T/H			
A	E	G	I	V	T	G	Q	Y		pn26
D/S/K		I/A/F	L/N/E	A		D/K/VN		F/D		
D	E	V	P	D	L	E	R	K	Y	pn27
S/T/N		A/I/H	Q/S/R	T/A	K	N/V		Y/A/F		
P1	P2	P3	P4	P5	P6	P7	P8	P9		
	E	I	P		V			Y		

^a Primary aa assignments are given in rows with a corresponding peptide number that refers to a peak in Figure 1B. Letters positioned beneath the primary assignments indicate the presence of secondary aa assignments at these positions. Letters in parentheses denote tentative assignments; X denotes unknown aa. Dominant anchor residues are bolded.

contribute significantly to binding and is probably solvent exposed; therefore, Lys was added at this position to some of the poly(Ala) peptides to help increase peptide solubility. Our data indicate that this assumption is reasonable (compare AEIAAAAY, $t_{1/2} = 600$ min, to AEIAAAKY, $t_{1/2} = 780$ min, Table 3). We conclude that P2 and P9 act as typical dominant anchor residues, while P3, P4, and P6 sometimes act as auxiliary anchor residues, and that P8 is, for some peptides at least, unimportant for binding.

T Cell Epitopes Derived from Influenza Virus NS1 and

NP Predicted from the HLA-B44 Peptide Binding Motif. In order to determine if knowledge of aa critical for peptide binding could be used to identify antigenic peptides restricted to HLA-B44, we performed the following experiments. First, we determined which proteins from an infectious agent, influenza A virus, could serve as the target(s) for an immune response. Bulk culture CTL from an HLA-B44⁺ donor generated against A/Udorn influenza virus were shown to recognize predominantly HLA-B44-transfected Hmy2.C1R target cells that were infected with recombinant vaccinia

Table 2: HLA-B44 Endogenous Peptides Bind to HLA-B44

peptide sequence	peptide no.	% ¹²⁵ I-β2m	
		incorporation ^a (min)	t _{1/2} ^b (min)
SEIDLILGY	pn1B44	86	240
SEIDTVAKY	pn6B44	85	240
DEVGIVTKMY	pn7B44	46	60
DEVGIVTKY ^c		48	240
AEIPTRVNY	pn8B44	88	360
AEIPRTFKY	pn2B44	77	300
no peptide		1	

^a HLA complexes were detected by gel filtration as described in Materials and Methods. Incorporation of ¹²⁵I-β2m at levels greater than 5% indicates specific binding. Data are the average of two experiments.

^b t_{1/2}, half-time for dissociation in minutes at 37 °C. ^c Nonamer variant of pn7B44; see Results.

Table 3: Poly(Ala) Peptides with Anchor Residues Bind to HLA-B44

peptide sequence	% ¹²⁵ I-β2m incorporation ^a	t _{1/2} ^b (min)	peptide sequence	% ¹²⁵ I-β2m incorporation ^a	t _{1/2} ^b (min)
AEIAAAAAAY	1		AEIAAVAKH	43	600
AEIAAAAAAY	18	600	AEIAAVAKF	12	480
AEAPAAAAAY	61	360	AEIAAVAKK	21	180
AAIAAVAKY	18	18	AEIAAVAKR	36	180
AEIAAVAKY	63	240	AEIAAAAKY	39	780
AEIAAVAKA	26	180	AEIAAAAAE	12	24
ADIAAVAKY	26	120	no peptide	1	
AEIAAVAKL	8	900			

^a HLA complexes were detected by gel filtration as described in Materials and Methods. ^b t_{1/2}, half-time for dissociation in minutes at 37 °C.

Table 4: Predicted HLA-B44 Antigenic Peptides from Influenza Type A Nonstructural Protein 1 and Nucleoprotein

peptide sequence	source ^a	% ¹²⁵ I-β2m incorporation ^b	t _{1/2} ^c (min)	SD ₅₀ ^d (nM)
IETATRVGK	NS1 55–63	5		
GEISPLPSL	NS1 158–166	69	180	10–100
KEESDEALK	NS1 70–78	1		
TEIRASVGK	NP 23–31	2		
LEEHPGAGK	NP 79–87	1		
MELIRMIKR	NP 191–199	0		
FDMSNEGSY	NP 480–488	2		
LELRSRYWA	NP 379–387	21	120	100–1000
GERQNATEI	NP 17–28	12	60	
RESRNPNGA	NP 293–301	0		
FEDLRVLSF	NP 338–346	17	180	10–100
LELRSRYWAI	NP 379–388	14	240	1–10
NO PEPTIDE		4		

^a Source, protein from which the peptide was derived and the aa numbers identifying its location in the native protein; NS1, nonstructural protein 1; NP, nucleoprotein. ^b HLA complexes were detected by gel filtration as described in Materials and Methods. ^c t_{1/2}, half-time for dissociation in minutes at 37 °C. ^d SD₅₀, half-maximal sensitization dose as determined in Winter (1991), no. 73.

viruses that expressed the type A influenza virus nonstructural protein 1 (NS1-Vac) or nucleoprotein (NP-Vac) genes (Figure 2A). Then, in order to identify potentially antigenic peptides, the NS1 and the NP protein sequences were scanned for nonamer subsequences that have a P2 Glu, as Glu had been observed in all of the endogenous peptide sequences at P2 and was shown to promote stable binding. Of 14 such peptides that were identified from NS1, three were chosen for synthesis (Table 4). Eight of 40 peptides that were identified from NP were synthesized. We biased our decision to synthesize a particular peptide according to the hierarchy for aa binding at PΩ discussed above. One of

the three NS1 peptides tested, GEISPLPSL (aa 158–166), bound to HLA-B44 and had a t_{1/2} within the range observed for the endogenous peptides (t_{1/2} of 180 min for GEISPLPSL, 240–360 min range for the endogenous peptides). Antiinfluenza A/Udm CTL recognized HLA-B44⁺ target cells pulsed with this peptide (half-maximal sensitization dose, SD₅₀, 10–100 nM). Three of the NP peptides bound to HLA-B44 *in vitro* (Table 4), and two sensitized target cells for lysis (FEDLRVLSF, aa 338–346, and LELRSRYWA, aa 379–387) (Table 4 and Figure 2B,C) in a peptide-specific and HLA-B44-restricted manner. The SD₅₀ for nonamer peptide LELRSRYWA (NP 379–387) is 100–1000 nM. We reasoned that the extended decamer peptide LELRSRYWAI (NP 379–388) might be a more optimal antigenic peptide since the aa at P10, Ile, is very similar to Leu, and PΩ Leu was shown to be superior to PΩ Ala in the poly(Ala) peptides (Table 3). The decamer formed complexes that were slightly more stable than complexes formed with the nonamer (240 vs 120 min, Table 4), but the SD₅₀ of the decamer (1–10 nM) was much lower than that of the nonamer. FEDLRVLSF also sensitized target cells for lysis, with an SD₅₀ of 10–100 nM.

Next, one peptide from each of the source proteins, GEISPLPSL (NS1 158–166) or FEDLRVLSF (NP 338–346), plus rIL2 was used to stimulate HLA-B44⁺ donor PBL to determine if the peptide-specific CTL could be induced, and if so, would the CTL recognize an endogenously processed epitope from the relevant protein. The donor was able to generate CTL that recognized NS1 158–166- or NP 338–346-pulsed Hmy2.C1R cells that were transfected with HLA-B44 but not the same cells transfected with HLA-A2, demonstrating that these CTL were HLA-B44 restricted (Figure 3). HLA-B44-transfected target cells pulsed with one of the endogenous peptides, pn1, were not recognized, demonstrating the peptide specificity of these CTL (Figure 3). Anti-NS1 158–166 CTL and anti-NP 338–346 CTL lysed HLA-B44 transfected Hmy2.C1R target cells infected with NS1-Vac or NP-Vac, respectively (Figure 4). The irrelevant Vac-protein constructs were not recognized, indicating that these peptide-specific CTL could specifically recognize endogenously processed forms of the NS1 and NP proteins.

DISCUSSION

The establishment of one important arm of cell-mediated immunity to infectious agents and tumors requires the development of cytotoxic T cells that recognize specific antigenic peptides in association with class I MHC molecules (Townsend & Bodmer, 1989). Random screening for peptides that sensitize target cells for lysis is costly and labor intensive, and identification of the biologically relevant natural antigenic peptide from several overlapping peptides is often not possible. An alternative approach involves the characterization of the salient features of peptides that are bound to particular class I molecules (Table 5). In the majority of cases, the identification of two dominant anchor residues has facilitated the identification of antigenic peptides (Pamer, 1991; Rotzschke *et al.*, 1991). In other instances, the situation is more complex, as for HLA-B14, which uses combinations of several dominant anchor residues to achieve binding (DiBrino *et al.*, 1994a).

We have performed aa sequence analyses on 27 HLA-B44-associated peptide fractions and have observed the

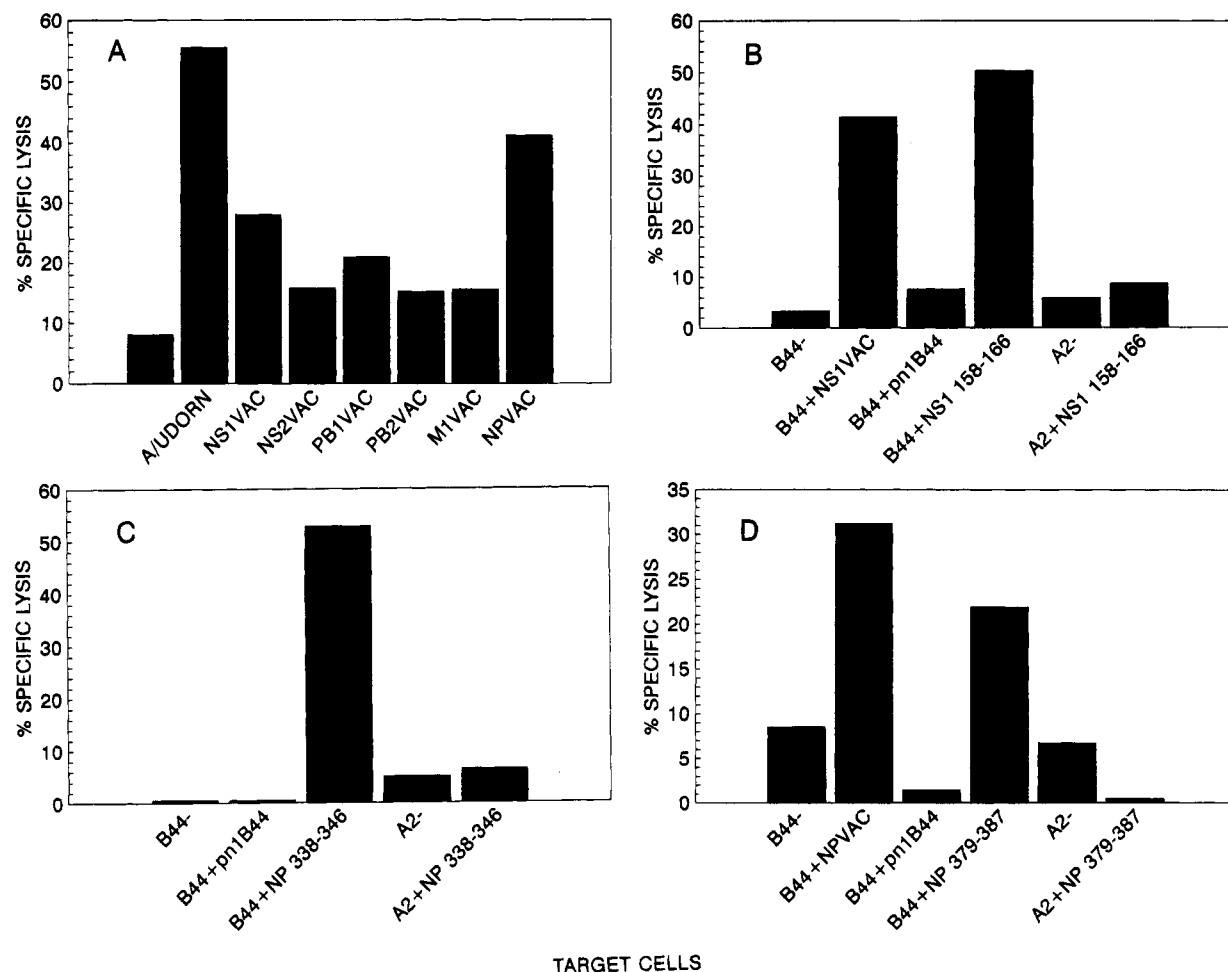


FIGURE 2: Influenza A virus-immune CTL recognition of epitopes presented by HLA-B44. (A) PBL from donor Q196 (HLA-A30, A34, B8, B44, Cw4, Cw7, DR2, 3, DQw1, DRw52) were stimulated three times *in vitro* with A/Udorn virus and assayed against Hmy2.C1R target cells transfected with HLA-B44 and either uninfected (–) or infected with A/Udorn or the indicated vaccinia virus recombinants. E:T = 2:1. (B) PBL from donor Q196 were stimulated six times *in vitro* with A/Udorn virus and assayed on Hmy2.C1R target cells transfected with either HLA-B44 or HLA-A2 and were incubated with nothing (–), infected with NS1VAC, or incubated for 1 h at 37 °C with 1 µg/mL pn1B44 or NS1 158–166 peptides. E:T = 10:1. (C) PBL from donor Q226 (HLA-A2, 24, B15, 44) were stimulated twice *in vitro* with A/Udorn and assayed on target cells as described in (B). E:T = 5:1. (D) PBL from donor Q227 (HLA-A26, 33, B38, 44) were stimulated four times *in vitro* with A/Udorn and assayed as described in (B). E:T = 10:1.

presence of Glu at P2 in all of these sequences (Table 1). An acidic aa at P2 was shown to be important for stable binding of poly(Ala) peptides containing the additional necessary anchor residues at P3 or P4 and PΩ. The $t_{1/2}$ of complexes formed with AEIAAVAKY is 10–30-fold lower than that of complexes formed with peptides having the aforementioned combinations of anchor residues, for example, AEIAAAAAAY, AEAPAAAAAY, or AEIAAVAKY (Table 3). Tyr was observed at PΩ in most of the sequences (Table 1), indicating that the PΩ aa is a dominant anchor residue, and our binding data support this observation (Table 3, $t_{1/2}$ of complexes formed with AEIAAAAAE = 18 min vs 600 min for AEIAAAAAAY). In addition, we have demonstrated that several other aa can be tolerated at PΩ in a hierarchical fashion: Leu > His > Phe > Tyr > Ala = Arg = Lys (Table 3). The peptide sequences were slightly enriched for Asp at P1, for Ile at P3, for Pro at P4, and for Val at P6 (see Table 1), and Ile at P3 and Pro at P4 were required for the formation of stable HLA-B44 complexes (for example, AEIAAAAAAY and AEAPAAAAAY bind to HLA-B44 while AEIAAAAAAY does not; Table 3). Thus, HLA-B44 is similar to the other class I molecules that have been studied previously in the apparent requirement for auxiliary anchor residues for stable complex formation (Table 5). Curiously,

the combination of Val at P6 and Tyr at P9 appeared to be destabilizing since AEIAAVAKL and AEIAAAAAAY formed more stable complexes with HLA-B44 than did AEIAAVAKY (Table 3, 900 and 600 min, respectively, vs 240 min). Perhaps the bulky Tyr residue is not accommodated as well at PΩ as is Leu when Val is present at P6. Or it may be the case that Val at P6 in combination with Tyr at PΩ and Ile at P3 is nonfavorable. In support of these possibilities, we note that there is only one endogenous peptide out of 27 that has all three of these aa (SEIDTVAKY, pn6) and it has a $t_{1/2}$ of 240 min (Table 1).

Crystallographic studies and modeling of peptide structures in the peptide binding sites of other class I molecules help us to explain binding specificity in terms of the aa composition and geometry of the pockets that bind the sc of anchor residues of peptides (Saper *et al.*, 1991; Fremont *et al.*, 1992; Guo *et al.*, 1992; Madden *et al.*, 1992, 1993; Silver *et al.*, 1992). In crystal structures for class I molecules that bind peptides with P2 and PΩ anchor residues, the P2 sc extends into the B pocket and interacts primarily with the aa at position 45 of the HLA molecule, while the PΩ sc faces down into the F pocket. HLA-B44, HLA-B40, and HLA-B37 each bind peptides having a negatively charged P2 anchor residue (Table 5). In the case of HLA-B44 and HLA-

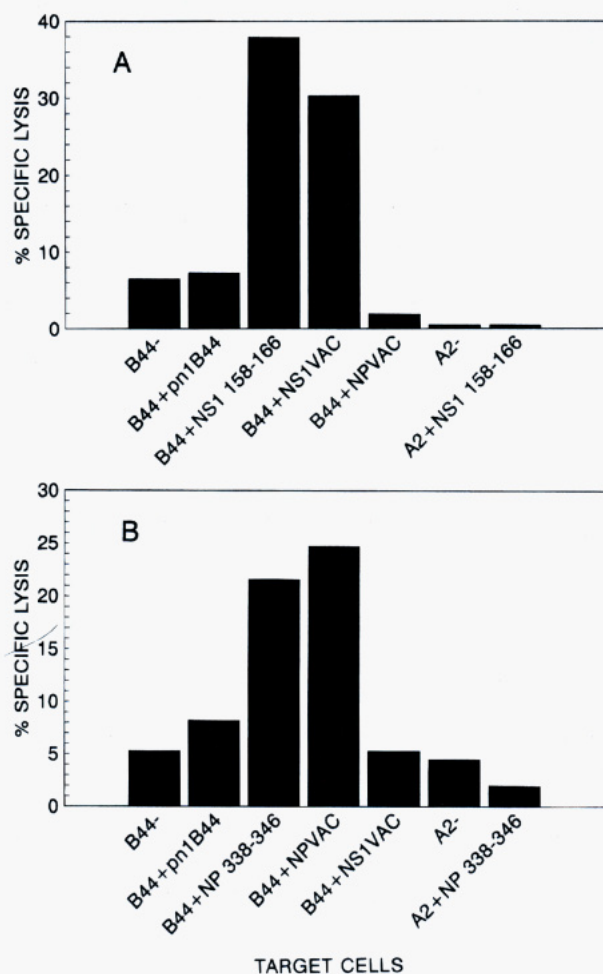


FIGURE 3: Generation of CTL with influenza virus epitope peptides presented by HLA-B44. (A) PBL from donor Q227 were stimulated four times *in vitro* with NS1 158–166 and assayed on the indicated target cells as described in the legend to Figure 2. E:T = 10:1. (B) PBL from donor Q227 were stimulated *in vitro* with NP 338–346 and assayed on the indicated target cells as described in the legend to Figure 2. E:T = 10:1.

Table 5: Dominant and Auxiliary Anchor Residues for Peptides That Bind to Selected Class I Molecules^a

position	1	2	3	4	5	6	7	8	9	ref
HLA-A1	T/I	E/D	P			L/M/I		Y		DiBrino <i>et al.</i> , 1994b
HLA-A3	L	F/Y				NC	L/F/Y	Y/K		DiBrino <i>et al.</i> , 1993
HLA-A11		Hy						K		Zhang <i>et al.</i> , 1993
HLA-Aw68		V/T						R/K		Guo <i>et al.</i> , 1992
HLA-A31	Hy	Ar/L					L/F	R		Falk <i>et al.</i> , 1994
HLA-A33	Hy							R		Falk <i>et al.</i> , 1994
HLA-B14	R/K	L/Y/F		R/H	I/L			L		DiBrino <i>et al.</i> , 1994a
HLA-B27	R	Y/F/I						R/K		Jardetzky <i>et al.</i> , 1991
HLA-B37	D/E				V/Hy			Hy		Falk <i>et al.</i> , 1993
HLA-B40	E	F/I						L		Harris <i>et al.</i> , 1993
HLA-B44	E	I	P			V		Y		DiBrino <i>et al.</i> , 1995

^a Dominant anchor residues are bolded; NC, noncharged aa; Hy, hydrophobic aa.

B40 (Harris *et al.*, 1993), Glu is exclusively observed at P2 of the endogenous peptide sequences (Table 5). This is remarkable especially in light of the observation that Glu is only 2-fold better than Asp at P2 in peptides that bind to HLA-B44 (compare AEIAAVAKY with ADIAAVAKY, $t_{1/2}$ of 240 vs 120 min, respectively) (Table 3). In contrast, the pooled sequence data of Falk *et al.* for HLA-B37 reveals a strong enrichment for Asp and a weaker enrichment for Glu at P2 (Falk *et al.*, 1993) (Table 5). Notably, HLA-B40 and HLA-B44 have Lys 45 in the B pocket, while HLA-B37 has Thr 45 (Table 6). It is possible that HLA-B37 pocket geometry and composition may render His 9 (also present in HLA-B40) exceptionally important in determining binding specificity for HLA-B37 and so may explain the observation that both Glu and Asp are found at P2. We constructed a

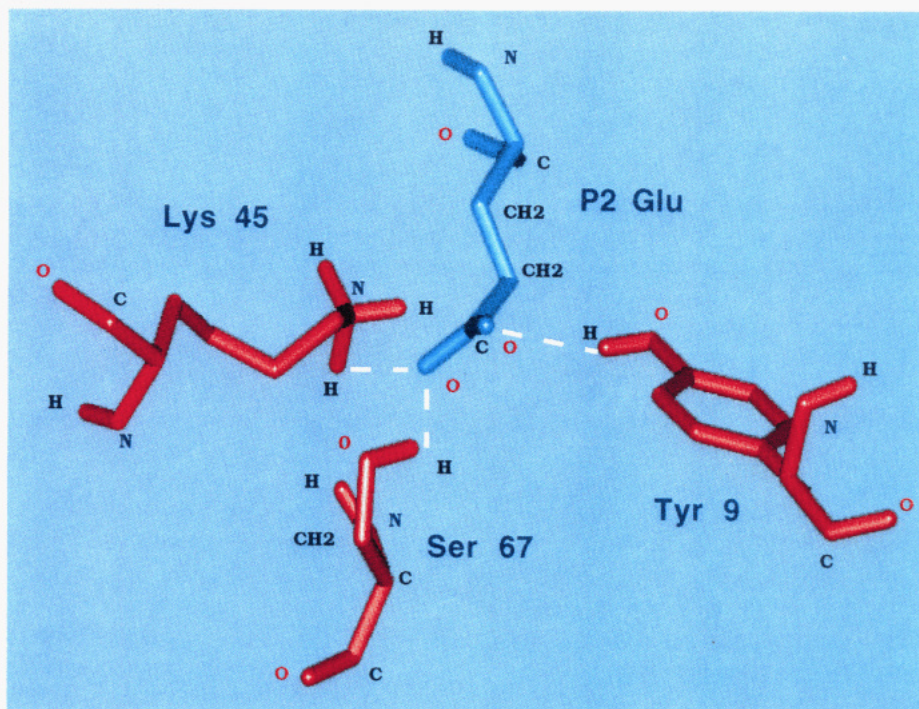


FIGURE 4: Representation of orientation of binding of the P2 Glu in the HLA-B44 binding groove. Peptide AEIAAVAKY (blue) is modeled in the HLA-B44 binding site to demonstrate the interactions of the P2 Glu sc with Lys-45, Ser-67, and Tyr-9 (red). Hydrogen bonds (white-dotted lines) are indicated.

Table 6: Comparison of Polymorphic Pocket Residues for Selected Class I Molecules^a

pocket B aa ^b	7	9	24	25	34	45	63	66	67	70	99
HLA-A1	Y	F	A	V	V	M	E	N	M	H	Y
HLA-A3	Y	F	A	V	V	M	E	N	V	Q	Y
HLA-Aw68	Y	Y	A	V	V	M	N	N	V	Q	Y
HLA-A11	Y	Y	A	V	V	M	E	N	V	Q	Y
HLA-A31	Y	T	A	V	V	M	E	N	V	Q	Y
HLA-A33	T	Y	A	V	V	M	N	N	V	H	Y
HLA-B14	Y	Y	S	V	V	E	N	Q	C	N	Y
HLA-B27	Y	H	T	V	V	E	E	Q	C	K	Y
HLA-B37	Y	H	S	V	V	T	E	I	S	N	S
HLA-B40	Y	H	T	V	V	K	E	Q	S	N	Y
HLA-B44	Y	Y	T	V	V	K	E	Q	S	N	Y

pocket F aa	77	80	84	116	123	143	146	147
HLA-A1	N	T	Y	D	Y	T	K	W
HLA-A2	D	T	Y	Y	Y	T	K	W
HLA-A3	D	T	Y	D	Y	T	K	W
HLA-Aw68	D	T	Y	D	Y	T	K	W
HLA-A11	D	T	Y	D	Y	T	K	W
HLA-A31	D	T	Y	D	Y	T	K	W
HLA-A33	D	T	Y	D	Y	T	K	W
HLA-B14	S	N	Y	F	Y	T	K	W
HLA-B27	D	T	Y	D	Y	T	K	W
HLA-B37	S	L	Y	D	Y	T	K	W
HLA-B40	S	N	Y	Y	Y	T	K	W
HLA-B44	N	T	Y	D	Y	T	K	W

^a The pockets within the peptide binding site of the class I molecule are defined according to Saper *et al.* (1991). Amino acid sequences are from Zemmour and Parham (1992). ^b Numerals represent amino acid position number of the HLA molecule.

model of HLA-B44 in order to demonstrate that it is sterically possible for the P2 Glu to contact B pocket aa Lys 45, presumably by means of a salt bridge, and also to make hydrogen bonds with Ser 67 and Tyr 9 (Figure 4). Our models are similar to models of Thorpe and Travers (1994), who concluded that peptides having a P2 Asp would interact with lower affinity than those having a P2 Glu due to a reduction in the number of hydrogen bonds formed in the P2 pocket as a consequence of the shorter Asp residue. Specifically, they postulated that potential contact with Ser 67 and Tyr 9 would be obviated if the salt bridge between P2 Asp and Lys 45 was conserved as the major interaction within the pocket. In contrast to HLA-B44, molecules such as HLA-B14 and HLA-B27 bind peptides that have a P2 basic anchor residue and the compensating acidic charge is Glu 45.³

In order to understand why a variety of aa can be accommodated at PΩ of peptides that bind to HLA-B44—aromatic aa, basic aa, and hydrophobic aliphatic aa (Table 3)—it is helpful to review the binding specificities of other peptide/class I systems. Crystallographic data have demonstrated that the PΩ sc protrudes into the F pocket of the HLA binding site (Fremont *et al.*, 1992; Guo *et al.*, 1992; Madden *et al.*, 1992, 1993; Silver *et al.*, 1992) and that the aa at position 116 appears to make an especially important contribution to binding. HLA-A3, HLA-Aw68, HLA-B27, and some other class I molecules as well bind to endogenous peptides that have basic PΩ aa (Table 5) and share most F pocket aa, including Asp 116, with HLA-B44 and HLA-A1. The latter two class I molecules, in contrast, bind

exclusively to endogenous peptides having aromatic PΩ aa (Tables 5 and 6). A key residue in determining the relative abundance of basic aa vs aromatic aa at PΩ appears to be aa 77, which is Asn in both HLA-A1 and HLA-B44 but is Asp in the other molecules. Asp 77 is in van der Waals contact with the Arg at PΩ in the crystal structure of HLA-Aw68 complexed with a nonapeptide (Silver *et al.*, 1992). In spite of the difference in the dominant anchor residue at PΩ, HLA-B44 is very similar to HLA-B27 with respect to its ability to tolerate the same wide range of PΩ aa. It has been postulated for HLA-B27 binding peptides that different PΩ sc of varying lengths can contact different F pocket aa (Jardetzky *et al.*, 1991); presumably this is also true for peptides that bind to HLA-B44. Thus there appears to be a correlation between a preference for Tyr and Phe at PΩ, as in the HLA-B44 and HLA-A1 endogenous peptide sequences, and the residues at both positions 77 and 116, as noted previously (Kubo *et al.*, 1994) that is important. HLA-B37 shares Asp 116 with all of the aforementioned HLA molecules, but in contrast has Ser 77. Like HLA-B44, it can bind peptides enriched for hydrophobic aa at PΩ (Falk *et al.*, 1993). Since two of the antigenic peptides that we identified contained aliphatic rather than aromatic aa at PΩ, it is clear that endogenous peptides containing aa at PΩ other than Tyr or Phe can be generated *in vivo* and represented on the cell surface. It is evident from the endogenous peptide sequences shown in Table 1 that HLA-B44 binds peptides with aa other than Tyr at PΩ. Our binding data (Tables 3 and 4) indicate that other aa can function about as well as Tyr at PΩ and that peptides with such PΩ aa can be recognized by CTL (Figures 2 and 3). Our models also indicate that the HLA-B44 F pocket can readily accommodate non-Tyr residues (data not shown). Thus, it is a bit perplexing that most of the endogenous peptide sequences that we obtained had Tyr at PΩ. Possible explanations for the apparent discrepancy are (1) Tyr at PΩ is favored for *in vivo* folding of the HLA-B44 complex and/or (2) the antigen processing machinery may selectively produce peptides with PΩ Tyr in consort with HLA-B44 expression. The peptide binding motif for HLA-B44 enabled us to identify three different antigenic peptides from influenza virus. Using vaccinia virus recombinants, we established that antigenic peptides could be processed from both the influenza NS1 and NP proteins (Figure 2). There are 14 peptides from influenza A NS1 and 40 peptides from NP that contain Glu at P2. Of these, we chose to synthesize three NS1 peptides and eight NP peptides, a subset of those peptides with PΩ aa known to be tolerated by HLA molecules with similar F pockets (Jardetzky *et al.*, 1991; DiBrino *et al.*, 1994a). Of these 11 peptides, four bound (Table 4), and three of these, one NS1 peptide, GEISPLPSL (aa 158–166), and two NP peptides, FEDLRVLSF (aa 338–346) and LELRSRYWA (aa 379–387), were recognized in the context of HLA-B44 by antiinfluenza CTL (Figure 2 and Table 4). In general, on the basis of our analysis of synthetic peptides (Table 3), the peptides that did not bind detectably to HLA-B44 in our assay have less optimal aa at PΩ (Ala or basic aa, Tables 3 and 4) while GEISPLPSL has the most optimal aa at PΩ.⁴ These results emphasize the importance of doing binding studies to supplement the observations made from sequence

³ HLA-B8 also has Glu 45, but does not have a P2 basic anchor residue; this has been explained by the presence of a large, bulky aa, Phe, at position 67 which probably influences the depth of the pocket (Sutton *et al.*, 1993).

⁴ Note that when Val at P6 was substituted with Ala, Tyr was almost as stabilizing as Leu at PΩ.

analysis of endogenous peptides in order to more accurately predict antigenic peptides.

CTL generated against GEISPLPSL or FEDLRVLSF were able to recognize endogenously processed peptides from the respective proteins (Figure 3). GEISPLPSL was the only NS1-derived peptide able to bind to HLA-B44 (Table 4) and, similar to LELRSRYWAI, has an aliphatic aa at P Ω . Notably, a longer peptide containing FEDLRVLSF has previously been found to be the predominant target of antiinfluenza CTL in HLA-B37-positive people (McMichael *et al.*, 1986), and LELRSRYWAI overlaps two previously identified antigenic peptides: SRYWAIRTR, which is restricted to HLA-B27 (Huet *et al.*, 1990; Kvist & Hamann, 1990), and ELRSRYWAI, which is restricted to HLA-B8 (Sutton *et al.*, 1993). Thus, evidence continues to accumulate that particular subregions of proteins are for some reason unusually antigenic. It is not yet clear whether this is due to efficiency of processing or some other selection process.

In conclusion, anchor residues were identified for peptides that bind to HLA-B44, one of the most common HLA-B alleles in the Caucasian population. Sequence analysis of endogenous peptides revealed two dominant anchor residues at P2 and P Ω of the peptides. An expanded peptide binding motif was created using the results of *in vitro* assembly assays that employed synthetic poly(Ala) peptides with anchor residues. We constructed computer models of HLA-B44 complexed with different peptides in order to explore the possible ways in which the anchor residues might interact with HLA-B44 pocket aa. Antigenic nonamer peptides from influenza NS1 and NP proteins were identified using the P2 and P Ω anchor residues, and we demonstrated that peptide-specific CTL were able to recognize endogenously processed epitopes from influenza proteins. The cumulative data provide a rationale for identifying peptides that can be presented to T cells by HLA-B44.

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