Immunology 1996 **88** 482–486

Analysis of peptide-binding motifs for two disease associated HLA-DR13 alleles using an M13 phage display library

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SUMMARY

Major histocompatibility complex (MHC) molecules bind peptides bearing an appropriate 'sequence motif' for MHC binding. The use of phage display libraries exploits the ability of MHC class II molecules to exchange peptides in solution and thus select out peptide sequences with high-affinity binding from a large array of random peptides. We have analysed the peptide binding motifs of HLA-DRB1*1301 and *1302 using affinity purified HLA-DR13 molecules to purify sequentially HLA-DR13-binding peptides from a large random library of M13 phage containing nonamer inserts in the pIII coat protein. These DR13 alleles differ only at position 86 of the HLA-DRβ chain, where they contain valine and glycine residues respectively. These alleles were chosen because of their association with protection from severe malaria and chronic hepatitis B virus infection in West Africa. Analysis of the phage bound to these DR molecules suggests binding motifs. We compare the results derived from the use of the phage display library with results obtained from analysis of eluted peptides and peptide-binding studies. This analysis shows that although there is a common theme to motifs derived using different methods, there are also subtle variations between them.

INTRODUCTION

Major histocompatibility complex (MHC) class II molecules are important in presenting peptides derived from exogenous proteins on the surface of the cell for recognition by $CD4^+$ T cells. MHC molecules exhibit allele specificity in their ability to bind different peptide ligands. Analysis of this specificity has resulted in the concept of 'peptide-binding motifs' for different MHC alleles. The peptide-binding motifs of MHC class II molecules have been studied by a large number of methods including analysis of naturally occurring peptide ligands by both individual peptide sequencing[1,2] and pool sequencing of eluted peptides.[3,4] In addition, the ability of MHC class II to exchange peptides and bind new ligands in solution has allowed extensive use of peptide-binding assays for analysis of the effects of peptide sequence on binding to HLA-DR.[5,6] The ability of MHC class II molecules to exchange peptides in solution has also allowed the use of random nonamer peptide libraries encoded in the pIII coat protein of the M13 phage to investigate the specificity of binding of several MHC class II alleles.[7,8] This method allows the analysis of the ability of MHC class II molecules to select for high-affinity peptides in the presence of a large number of potential ligands.

Received 26 February 1996; revised 17 April 1996; accepted 22 April 1996.

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In this report we have used affinity-purified HLA-DR proteins and the M13 phage display library to analyse the peptide-binding specificity of two HLA-DR13 variants, HLA-DRB1*1301 (DRB1*1301) and HLA-DRB1*1302 (DRB1 *1302). DRB1*1302 has been associated with protection from severe malarial anaemia in West Africa,[9] and both DRB1*1301 and *1302 have been associated with protection from chronic hepatitis B virus infection in the same region.[10] From our analysis we derive a peptide-binding motif for these alleles. Analysis of the results derived by this method and comparison with motifs derived by other methods allows identification of a motif common to these different methods, as well as identification of the variations observed using different methods of analysis.

MATERIALS AND METHODS

The Epstein–Barr virus-transformed B-cell lines HHKB (DRB1*1301, DRB3*0101)[11] and WT-47 (DRB1*1302, DRB3*0301)[12] (obtained from S. Marsh and J. Bodmer) were used as sources of HLA-DR protein. Ten grams of cell pellet were lysed in buffer containing CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate) detergent, and then the HLA-DR protein was purified as previously described using an L-243 affinity column.[13] The DRB1*1301 and *1302 gene products were then purified from the DRB3 products using the TAL15·1 monoclonal antibody[14] (obtained

from S. Marsh and J. Bodmer). The purified DR13 molecules were then biotinylated.[7]

Selection of DRB1*1301 and *1302 binding phage was performed using the M13 phage display library and methodology previously described.[7] Briefly, random peptide inserts of nine amino acids flanked at each end by four glycine residues were incorporated into the pIII phage coat protein of M13. Pools of 10^{10} phage were mixed with 50 pmol biotinylated DR13 molecules at pH 7 at room temperature for two days. The phage–DR complexes were purified using bovine serum albumin (BSA)-blocked streptavidin on agarose beads and washed extensively. The bound phage were eluted using 0·1 M glycine pH 2·2 and amplified overnight in *Escherichia coli* XL1blue. The amplified phage were harvested by two rounds of polyethylene glycol/NaCl precipitation. Two or three additional rounds of binding were performed. Specific selection of HLA-DR13-binding phage was demonstrated by showing enrichment of phage library isolates over non-insert-containing

M13 phage eluted from DRB1*1301

W Y T A Q R S L Y	W G S R A E S R V	S T R G W P W A R	F P A R S I S H K
W T S V M V S A F	Y G P G L R W K S	W P I V D V S A P	F I R K H L N K L
F W Y W H T T Q R	S W T V V H I S S	G W A I E S I R W	G I G M G H S E Q
D K M G R T S R W	W V L R S I R A Q	A V L W W F N E R	M K A V I G R T M
W A G R S I S Y K	A T W A E Q W R T	L E V P L R Q H Y	D K M G R T S R W
W T V R T L S S R	W G E D P E A R R	R R G E V L G A R	G R Q G R G D M W
W A G R S I S Y K	W K M S S W R W D	N L G R M G A R H	E A R G E V W G R
W T V R T L S S R	Y K M G R T S R W	E I R Q A L A R E	C A R S G M S G W
F W E V V T I T N	G Y K P R W H N C	D R N L E G R G K	N E M T T M I E W
L I S W E N F A Q	I G E Q G W R H R	E L R R F L E L L	G Q Q E R V W P S
W E I K S L S G I	A Q V W G R R R M	E A L E Q L G A L	P D S Q Q I N L E
W V V V T L N L W	W A G P T P S D E	L R S T N A R S A	N T H K D R V H L
W M A M S V S E K	L Q H R W H K L E	V I T P D P S E V	H Q K R V E E S A
Q V G P Y E W R R	V R A L W E A K S	R S Q I P V R E L	
S R H W E G R G K	F M T M W A W E T	Q I R M N T N E F	**Total = 58**

M13 phage eluted from DRB1*1302

Y W W T W S R A G	M E F R A G S H A	S Y V S Y N E G E	T Q G K R G Y N S
W T S R T L S A R	K S V A F M K G R	P I S S T R I E G	R Q T V E G H R L
W A R H R T G S E	R S F S R V L E E	A R Q P R G H M W	P V N D H P L E K
S V W L R W R G C	P M F Q L W E G Q	H T R R K W G G E	S M A A T V G A R
A A K W Q K R V E	T Q L R G R R L N	R G G V H D W A R	G G A G V A K H E
Q A G W Y W W V R	A V D N Q L H E R	D R R P H N W G E	K S G K E V E R R
I G E Q R W R H R	L H A R Q L P R G	G A P P R A F A G	S D T C T C H T R
V V I D R W E I R	I K S L L R K E L	M A Q E R F L E M	T S R H E Q A R N
L G G V H Y W R R	L I S P E Q P P Q	S H G P K N F G E	S R G G S N M R E
K G W L G G R A S	P M S Q Y S V G Q	A A G A S A A Q L	R S T S S C S E R
W R S L R T L L E	M E T Y L R I S S	A G R A C A L A G	
R K E P W G E M S	A I Y S R R V L R	P S A T S P L E K	**total = 46**

Unselected M13 phage library

S R V T F L H E T	R V R L G E C G T	K A L T T H K E P	E A Q E Q Q P N A
R M Y E T A G P V	G G Q G Y A R R E	T R I I L P G R V	E G R A E N A R E
G D V E G I P Q V	E S L G E R M R A	L S R H L M K I V	S E S T Q E V R R
V A W S V W G L W	S W P A A G F A W	G H S E T P G R F	G G S E N D H E A
E S G R R W D G G	G V Y S M R G T V	R L A A K S Q N P	E D V T G E A H R
S L G R I E A Y K	Y C A E Y G G S G	F W W M E M A S G	S E T L T G P R A
G K G Q N L W M G	Q L G R P A G L P	G V Y S M R G T V	K V T R M P V K P
A S L L S R R M E	K K L V S Q V K T	R A V R A Q L Q R	V I V Y P K E M T
V S G I K T R E N	A K D Y E D S T G	E T Q D E F Q H R	A V E P S D S G E
E R L E C E T K N	L R F P T N F G E	R G D K L V R N D	V T K G N V D R R
D E W E V K N G A	F S A P A H L R E	I Q Q L R S W Q M	A R S P D M R G G
E S Q G A R E L W	F H G Y Q T S G E	V R W V R A C A S	Q S D R E D D R S
K G T T A R D Y S	S H G P K N F G E	F K W I R A E A Q	D P T S A R G E K
K A P P P W V E A	E T G S K F I G E	G Y C R R R T A A	E G S G Q W R T A
P G E E L V M G T	S M R G W D N R E	E D N L Q T R G P	H E M R P A K R T
T W E Y R F G G G	S S V P F S R E A	F E I G Q T R Y R	T V E G N P E E S
F L A M E G V E Y	P G G S A V S L S	V K L P P R E S Q	E Q M V R G G A S
W S H S G A T W R	G T S Q D Y S G R	G H K E E G N A L	K G A N D E R S A
P G F G G T E S S	P E W E L G Q R R	D L H E P R D R A	V S G A A S R A R
K Q D G M F S W K	K S Q S G V F Q S	R L W Y E H V A V	S R E T Q G R A E
S W P T E K K E A	R V V V N G A W G	T Y T G R Q K R M	V S S N E M C G R
C S D W Q E R D R	E R G R K Q S S I	L T K G N V D R R	M G S N N V E G Q
K S P D L Q P V P	A F S W E D V S K	E S V N V S E Q T	H K Q T E R P P Q
A E R A L L R R M	S T T I G P R G A	V K A N D S G A P	G K V D R T T Q G
T D G R L M N L R	T A S F R L R G L	V K G K M M K T R	**total = 99**

Figure 1. Sequences of all peptides: sequences of peptides eluted from DRB1*1301, DRB1*1302 and of the unselected phage pool are listed.

wild-type M13 phage. Finally, phage selected on each of the HLA-DR alleles and also unselected phage from the original library were sequenced and compared. The frequency of each amino acid at each position in the different peptide pools was calculated when the peptides were aligned either by their N-termini or by the closest hydrophobic residue to the N-terminus.

RESULTS

The sequences of phage clones obtained from both the HLA-DR13 selected and unselected phage are shown in Fig. 1. Comparison of the binding of HLA-DR selected phage over wild-type phage showed that after three rounds of selection approximately 95% of phage bound specifically to HLA-DR. The frequency of different amino acids at different positions in the peptides was analysed. For the unselected phage pool the mean frequency of each amino acid (averaging the frequency at each position) and the standard deviation from this mean were calculated. The same analysis was then performed on the phage pools which had bound to DRB1*1301 and *1302. As both the unselected phage pool and the DR-associated pools showed variations in the frequency of different amino acids over their length, we considered that an increase in the frequency of an amino acid above mean plus three standard deviations of the frequency in the unselected pool was a significant enrichment. Thus analysis of the variation in frequency of the DRB1*1301- and *1302-associated pools indicates regions where there has been selection for particular amino acids (Fig. 2). It should be noted that it is quite likely that the first amino acid of the random peptide does not always lie in the first position of the binding groove. As a result of this amino acids tend to be enriched over a number of neighbouring residues rather than simply at one position. Analyses which seek to align the hydrophobic anchors of the peptides have sought to avoid this problem, although they may introduce their own biases. Here we have also compared the peptide pools by aligning peptides by the closest hydrophobic amino acid (F/I/L/V/W/Y) to the N-terminus (Fig. 2).

DRB1*1301

Position	1	2	3	4	5	6	7	8	9
aligned by N-terminal	F W	I T	M	M		I L T	I S T	H	W
aligned by hydrophobic anchor	F I L W Y	G T	I M	R	H T	I L	S		K

DRB1*1302

Position	1	2	3	4	5	6	7	8	9
aligned by N-terminal		I		P	H R	C W	H L W	H	E
aligned by hydrophobic anchor	F I L V W Y		S	H M W	R	L H	E	C H I R	E

Figure 2. Amino acid enrichments seen in phage pools: the mean and standard deviation of the proportion of each amino acid over the length of the peptide was calculated from the unselected pool. This was then compared with the frequency of each amino acid at each position in the HLA-DR13-selected pools. Positions where the frequency of an amino acid is greater than the mean plus three standard deviations of the unselected pool are shown. This is performed for the pools when aligned by their N-termini and when aligned by the hydrophobic residue closest to the N-terminus; Hø, hydrophobic; +ve, positively charged.

Comparison between the results obtained for each allele indicates common trends, most importantly for a large hydrophobic or aromatic N-terminal residue. Surprisingly, tryptophan and other aromatic residues are highly enriched in the first position of DRB1*1301, although these residues were not seen in analysis of eluted peptides.[13] Aromatic residues were also common in this position amongst the DRB1*1302 eluted phage, and have been seen in the other HLA-DR alleles which have been studied.[7,8] Thus this methodology seems to select for aromatic residues at this position much more than is seen in naturally processed amino acids. However, whereas DRB1*1302 and the other HLA-DR alleles which have been analysed using the phage display library all have glycine at position β86 and are able to utilize an aromatic primary anchorresidue at this position, DRB1*1301 (β86 valine) does not. Difficulties using this system to analyse HLA-DR subtypes which have valine at position β86 have been observed previously in studies of HLA-DR4 subtypes.[15] For this reason the data obtained for DRB1*1301 must be interpreted with caution, since our previous results suggest that aromatic amino acids at relative position 1 of the peptide are observed less frequently in naturally occurring peptides than the results obtained using the phage display library would suggest.

Additional positions of enrichment are found in positions 5 to 9, where there appears to be a preference for charged or polar residues and at position 7 where hydrophobic residues seem increased in frequency. The enrichment for charged and polar residues has been previously noted in analysis of eluted peptides.[13] Enrichment for hydrophobic amino acids around position 7 is also present but less prominent in the data obtained from pool sequencing.[16]

DISCUSSION

Previous work by ourselves and others has attempted to identify peptide-binding motifs for DRB1*1302 using analysis of eluted peptides[13,16] or peptide binding studies with multiply substituted peptide analogues.[17] Comparison of these results with those obtained using the phage display library indicate agreement on the major anchors involved in peptide binding, but some variation in the apparent role of minor anchors (Fig. 3). In particular it is interesting to note that where multiple substitutions of an individual peptide have been used to analyse peptide-binding motifs,[17] direct binding studies have not agreed with T-cell recognition studies. Thus whereas Boitel *et al.*[17] found that position 4 of the peptide was crucial for peptide binding, and position 6 irrelevant, T cells recognized all variants at position 4 equally (indicating that it was unimportant for binding or T-cell recognition) and their pattern of recognition at position 6 was in agreement with the binding motif identified by analysis of individual peptides (i.e. lysine and arginine were the only substitutions tolerated).

Where other HLA-DR alleles have been studied using multiple methods of analysis, similar differences have been observed between the minor anchor residues (Fig. 3). Possible

explanations for these differences are that this variation in 'minor anchors' reflects the fact that the current methods of analysis are insufficiently sensitive to identify these minor preferences, or that the use of different experimental techniques has effects on apparent peptide-binding specificity. One apparent example of this latter effect is the observation by Boitel and colleagues[17] of clear differences in binding properties using the same peptides in either *in vitro* peptide-binding assays with purified HLA-DR, or peptide binding to antigen-presenting cells and T-cell recognition. As the latter is in agreement with the observations made with eluted peptides, it may be that differences in peptide-binding specificity arise as a result of the conditions used for *in vitro* binding.

Analysis of the peptide-binding specificity of two HLA-DR13 alleles using a phage display library suggests a motif for peptide binding to these alleles. Results obtained by this method are in broad agreement with those obtained through analysis of eluted peptides from these alleles, and suggest an additional anchor may be present. One discrepancy between data obtained using the phage display library and those obtained from analysis of eluted peptides is the frequency of aromatic amino acids at primary hydrophobic anchor position of the DR*1301 peptides. It appears that although aromatic amino acids are able to be accommodated in this position of the peptide under the conditions used in the phage display library, they are rarely seen in naturally processed peptides.[13]

HLA-DRB1*1302 has been associated with protection from severe malaria in West Africa,[18] and both DR*1301 and *1302 have been associated with protection from chronic hepatitis B virus infection.[10] The data we have obtained using the phage display library provide information about the peptide-binding specificities of these molecules. Using these data in conjunction with data on the sequences of naturally processed peptides[13] provides a powerful tool for prediction of likely T-cell epitopes from *Plasmodium falciparum* and hepatitis B virus-derived proteins.

HLA-DR13 motifs

*(HLA-DRB1*1302)* position	1	2	3	4	5	6	7	8	9
Pool sequencing and individual peptide sequencing of eluted peptides (13)	F I L Y (V)					+ve or polar			Y
In vitro peptide binding (17)	Hø (I/V)			N					G A
T-cell recognition in vitro (17)	Hø (I/V)	K	A		S	K R	F Y		G A
Phage display library (this study)	Hø				+'ve or polar	+'ve or polar	Hø	+'ve or polar	+'ve or polar

HLA-DR1 motifs

*(HLA-DRB1*0101)* position	1	2	3	4	5	6	7	8	9
Sequencing of individual eluted peptides (2)	+ve					H-bond			Hø
Pool sequencing of eluted peptides (4)	Hø			L A I V M N		+/- or polar	+/- or polar		Hø
Peptide binding (19,20)	Hø			+ve or polar		small			Hø
Phage display (7)	Hø			M L		G A			(L)

HLA-DR4 motifs

*(HLA-DRB1*0401)* position	1	2	3	4	5	6	7	8	9
Sequencing of individual eluted peptides (21)	F/L V								N/Q S/T
Peptide binding with many unrelated peptides, truncation analysis and single amino acid substitutions (22)	F/W Y/L I/V M			(no R or K)		S/T L/I V/M	(no R or K)		(no R or K, no D or E)
Phage display library (8)	W Y			M (A/V/ L)		T/S	L/Q (M/ N)		
Individual peptide sequencing and binding studies with single amino acid substituted analogs (23)	Y/W I/V			D/A (no K or R)		S/T (no I or L)	I/L (no K or R)		(no R or K, no D or E)

Figure 3. Comparison of HLA-DR motifs derived by different methods. (a) HLA-DRB1*1302; (b) HLA-DRB1*0101 and (c) HLA-DRB1*0401.

ACKNOWLEDGMENTS

We would like to thank J. Bodmer and S. Marsh for generously providing antibodies and cell lines used in these experiments. MPD is supported by the Lionel Murphy Foundation and the Wellcome Trust. AVSH is a Wellcome Trust Principal Research Fellow.

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