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The peptide binding specificity of the MHC class II I-A molecule of the Lewis rat, RT1.B^{l}

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Abstract

The specificity of peptide binding to MHC molecules is defined by binding motifs composed of several relatively conserved anchor positions. The peptide binding motifs of murine MHC class II I-A molecules are functionally important but poorly characterized. Here we use peptide binding studies and isolation of naturally presented peptides to characterize the peptide binding motif of the MHC class II I-A molecule, RT1.B^{l}, a molecule that is involved in experimental autoimmunity in the Lewis rat. We now report that, similar to other class II motifs, the RT1.B^{l} motif consists of a nonamer sequence with four major anchor positions (P1, P4, P6 and P9). Residues at P4 and P9, rather than at P1, appeared to be particularly important for binding. Negatively charged residues were favored at P9, consistent with the presence of a serine at position 57 of the RT1.B^{l} β chain. This RT1.B^{l} motif could be observed in the dominant autoantigenic T cell epitopes mapped previously in the Lewis rat. These results highlight a general similarity and some important differences in the organization of MHC class II peptide binding motifs. The reported RT1.B^{l} motif should facilitate the prediction and design of T cell epitopes for the induction and control of experimental autoimmune diseases in Lewis rat models.

Introduction

MHC class I and II molecules are highly polymorphic membrane glycoproteins that bind processed peptides and present them for recognition by T cells. The binding of peptides to MHC molecules is a unique interaction in which, on the one hand, a high affinity is required, and, on the other hand, each MHC allele should be able to bind many diverse peptides. Recent progress in solving the structures of MHC molecules has explained how this delicate balance in peptide binding is achieved at the structural level (reviewed in 1). Sequence-independent interactions between the peptide binding groove of the MHC and the peptide backbone stabilize peptide binding and confer a fixed N- to C-terminal orientation to the bound peptides. In addition, polymorphic pockets in the groove interact with only a few amino acid side chains at so-called anchor positions in a peptide. It is the several anchor positions, each utilizing a particular set of structurally similar amino acid residues, which comprise the allele-specific peptide binding motif. The identification of such motifs is important for the understanding of MHC–peptide interactions and their biological consequences, including the genetic influence of the MHC on autoimmunity (2,3). In addition, the characterization of specific motifs can facilitate the prediction and design of T cell epitopes.

The identification of peptide motifs for MHC class II molecules is generally hampered by size heterogeneity and relatively degenerate amino acid usage at anchor positions in MHC class II peptide ligands (4). Recently, several approaches have been used successfully for the analysis of MHC class II motifs, including screening of random peptide phage libraries (5) or designer peptide libraries (6,7), pool (8,9) and individual (10) sequencing of natural MHC class II ligands, and extensive substitution analysis of known antigenic peptides (11). Together with the determination of the crystal structure of a human MHC class II HLA-DR molecule (12) and of its complex with a peptide (13), these studies have led to the identification of detailed, structurally supported peptide motifs for some MHC class II molecules, mainly for alleles of HLA-DR and of the homologous murine I-E proteins

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(reviewed in 14). These motifs generally consist of a nonamer sequence with anchor residues at positions (P) 1, 4, 6 and 9. Nevertheless, the peptide motifs of many other important MHC class II products such as murine I-A molecules are still poorly characterized. In particular, it is not clear whether these motifs are organized along the lines of the DR/I-E motifs.

The Lewis rat is an inbred strain that is particularly suitable for the induction of various experimental autoimmune diseases (15). In these experimental models, the injected autoantigen is presented by rat MHC class II molecules to populations of self-reactive CD4$^+$ T cells that mediate the disease. The Lewis rat homolog of the mouse I-A molecule, RT1.B^l, appears to be a restriction element for the majority of autoantigenic epitopes. Therefore, the analysis of the RT1.B^l–peptide interaction could be helpful both for the dissection of peptide binding and for the experimental study of autoimmunity. To this end, we examined the peptide binding specificity of RT1.B^l using peptide binding studies and the isolation of natural MHC ligands.

Methods

Peptides

Peptides were synthesized on an automated multiple peptide synthesizer (AMS 422; Abimed, Langenfeld, Germany) using standard Fmoc chemistry. The quality of each peptide was confirmed by analytical reverse-phase HPLC. For use in the binding assay, the peptides were dissolved at 20 mg/ml in DMSO, which ensured complete solubilization.

Competition assay of peptide binding to RT1.B^l

It has been observed previously in our laboratory that *in vivo* activated macrophages, even after chemical fixation, can induce antigen-specific proliferation of CD4$^+$ T cell clones (16). We exploited this observation to develop a competition assay of peptide binding to RT1.B^l based on peptide presentation by fixed activated macrophages to an RT1.B^l-restricted T cell clone. Activated peritoneal macrophages were obtained from 2-month-old female Lewis rats (Harlan Olac, Bicester, UK) that had been injected i.p. 3 days before with 0.1 mg concanavalin A in PBS. The Lewis rat CD4$^+$, TCR V$_\beta$8.2$^+$, RT1.B^l-restricted T cell clone A1 specific for amino acids 71–90 of guinea pig myelin basic protein (GBP) was obtained by limiting dilution cloning of a GBP-specific T cell line. Macrophages were resuspended in DMEM with 10% FCS, seeded at 2×10^5/well into 96-well flat-bottom plastic plates and incubated for 2 h at 37°C in a 7.5% CO_2 atmosphere. Confluent monolayers of adherent macrophages were isolated in the wells after washing out the non-adherent cells. The cells were washed with PBS, fixed with 0.1% glutaraldehyde for 1 min, and washed with 1 M lysine/PBS and then with PBS. All peptide dilutions were made in DMEM supplemented with antibiotics only. A single concentration (4 μM) of indicator peptide 71–90 of GBP and a 0.2–200 μg/ml concentration range of the test peptide were added to duplicate wells and the plates were incubated for 2 h at 37°C. The wells were washed with PBS, resting A1 T cells (5×10^4/well in DMEM with 10% FCS) were added and [^{3}H]thymidine incorporation was determined 3 days later using the Matrix 96 direct β-counter (Packard Instrument, Meriden, CT). The concentration of test peptide, in μM, required to inhibit the control T cell proliferative response to the indicator peptide by 50% (ID_{50}) was calculated for each test peptide. Note that these values are intended only for estimating relative binding capacities of diffrent peptides. Similar relative binding of reference peptides was obtained when the assay was performed at pH 5. All results of binding experiments presented in this paper are specific for RT1.B^l since different results were obtained testing the peptides for binding to two other murine I-A haplotypes (data not shown). Each result is representative of two to five separate experiments.

RT1.B purification and peptide analysis

As a source of RT1.B^l, we used the Lewis rat CD4$^+$ T cell clone A2b specific for amino acids 180–88 of *Mycobacterium tuberculosis* heat shock protein (HSP) 65 (17). During prolonged culture *in vitro*, a subclone of A2b acquired the ability to continuously grow in the presence of IL-2 and to express MHC class II molecules at a high level (data not shown). The A2b cells were grown in DMEM supplemented with 5% FCS and 40 U/ml rIL-2 (Cetus, Emeryville, CA) to $1.5–2\times10^6$ cells/ml, collected and frozen at –70°C. For MHC purification, 30 ml of pelleted A2b cells was lysed on ice in PBS containing 1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin and pepstatin (2 μg/ml each), and the lysate was ultracentrifuged for 1 h at 100 000 *g*. The supernatant was passed with recirculation over a Sepharose–glycine column followed by a column of Sepharose-coupled anti-RT1.B mAb OX6. The material bound to the columns was released with 0.1% TFA and passed through Centricon-10 ultrafiltration membranes (Amicon, Witten, Germany). The eluate from the Sepharose–OX6 column contained the RT1.B α and β chains as confirmed by SDS–PAGE. The filtrates were separated on a reverse-phase HPLC column (μRPC C2/C18, 2.1×100 mm) using a SMART system (Pharmacia, Freiburg, Germany). The HPLC conditions were as described (8). The eluate from the Sepharose–glycine column was used as a negative control. Dominant single peaks were sequenced individually, whereby 10% of the volume of fractions containing dominant peaks and the remaining fractions were pooled and sequenced as such. The fractions were sequenced by Edman degradation on a pulsed-liquid protein sequencer 476A with on-line PTH-amino acid analysis (Applied Biosystems, Weiterstadt, Germany).

Results

Identification and alignment of the peptide ligands of RT1.B^l

In order to characterize a possible RT1.B^l peptide binding motif, we wished to analyze many different peptides binding to RT1.B^l with high affinity. Such peptides were identified using two experimental approaches. First, we screened a large batch of synthetic peptides for their capacity to bind to RT1.B^l. The screening was performed using a peptide binding assay based on the inhibition of antigen presentation by fixed antigen-presenting cells (18) and adapted for use with T cell clones. This assay, measuring relative binding capacities of peptides, was suitable since a quantitative analysis of

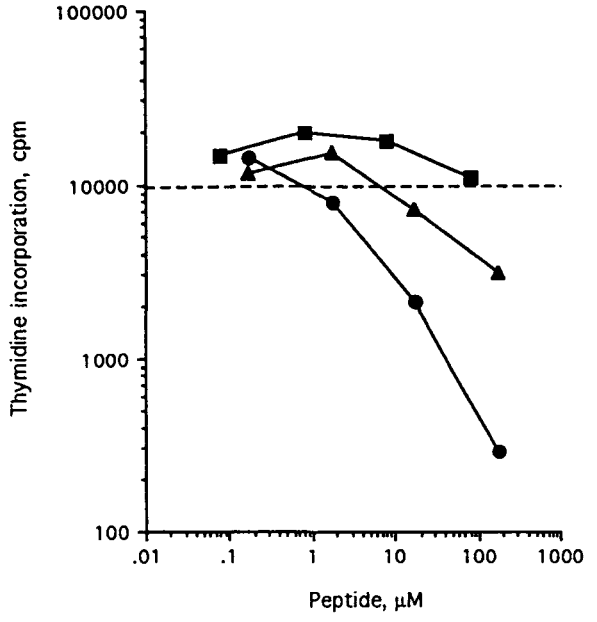

Fig. 1. The competition assay of peptide binding to RT1.B^{l}. Fixed activated macrophages were incubated with a single concentration of the reference RT1.B^{l}-binding peptide (GBP 71–90) and a dose range of test peptides. The binding of test peptides to RT1.B^{l} was revealed by inhibition of the proliferative response of a GBP-specific T cell line, measured as [^{3}H]thymidine incorporation. The results represent mean c.p.m. of duplicate cultures. The peptides include *M. tuberculosis* HSP65 178–86A$_{183}$ (circles), *M. tuberculosis* HSP65 180–88 (triangles) and GBP 91–110 (squares). The dashed line shows 50% of the response in the absence of a competitor peptide.

peptide binding was not necessary. To calibrate the assay, we examined the binding of three peptides (*M. tuberculosis* HSP65 178–86A$_{183}$, *M. tuberculsosis* HSP65 180–88 and GBP 91–110) that were reported previously to possess high, intermediate and low affinities respectively for purified RT1.B^{l} molecules (19). As shown in Fig. 1, similar results were consistently obtained for these peptides using the inhibition of antigen presentation assay (ID_{50} ~ 1, 10 and >100 μM, respectively). The screened peptides included two complete sets of 20-mers, overlapping by five amino acids, spanning the whole sequence of the mouse 60 kDa heat-shock protein (mHSP60) and the homologous *Escherichia coli* protein GroEL, as well as other peptides; >100 peptides were thus studied. Table 1(A) summarizes the peptides that were found to bind well (ID_{50} < 10 μM) to RT1.B^{l}. The core epitopes could be identified for some of the peptides by the alignment of homologous peptides.

In addition, we identified natural peptide ligands of RT1.B^{l}. This was necessary since peptide binding *in vitro* might not reflect possible constraints on antigen processing and MHC–peptide interactions *in vivo*. Therefore, we isolated and sequenced the prominent peptide fractions bound to RT1.B^{l} molecules purified from an MHC class II-positive Lewis rat T cell clone A2b. Four independent peptide sequences were obtained (Table 1B). The capacity of the corresponding synthetic peptides to bind RT1.B^{l} was confirmed using the competition binding assay. Three of the peptides showed a high RT1.B^{l} binding capacity (ID_{50} < 10 μM), while the fourth peptide (HSP70 420–37) showed moderate binding.

Next, the identified high-affinity binders and natural ligands of RT1.B^{l} (Table 1), as well as several previously reported peptides with strong RT1.B^{l} binding (19), were inspected for the presence of conserved sequence features. Indeed, it became apparent that the majority of the peptides contained a negatively charged residue (mainly Glu) near the C-terminus, which was preceded by a small residue, mainly Ala, two amino acid residues away. Since small residues are often found at position 6 (P6) in the HLA-DR motifs and negatively charged residues were previously found at P9 in the HLA-DRB1*0405 motif (14), we reasoned that these positions might represent P6 and P9 of the RT1.B^{l} motif, respectively. Therefore, we aligned the peptides or their known core epitopes with respect to these residues (Table 2) and searched for additional conserved features, particularly at P1 and P4. As shown in Table 2, P4 was predominantly occupied by residues with large hydrophobic, polar and positively charged side chains. Surprisingly, P1 was absent in some cases; nevertheless, a preference for polar and aliphatic residues was noted. An additional conserved position, P3, became apparent, which was occupied by similar medium-size residues, Val, Ser and Thr. A general preference for large polar residues was also observed at P7. In contrast, this motif was not observed in the negative binders (data not shown).

In addition, pool sequencing of the peptides eluted from RT1.B^{l} molecules of A2b cells was performed (data not shown). As observed with pool sequencing of other MHC class II ligands, a strong Pro signal was found at cycle 2, presumably representing the MHC class II processing motif (8). Most importantly, clear signals for Glu and Asp residues were observed at sequencing cycles 11–14, indicating the presence of negatively charged amino acids near the C-terminus of less abundant natural RT1.B^{l} ligands.

Therefore, the peptides binding to RT1.B^{l} appared to share a typical MHC class II binding motif consisting of four anchor positions P1, P4, P6 and P9, as well as an additional position P3. Some positions (P3, P6 and P9) were more restricted, while others (P1 and P4) were more degenerate. Notably, P1 differed from the previously described DR/I-E motifs in that it was (i) not necessarily hydrophobic, (ii) relatively unrestricted and (iii) missing in some cases, suggesting that it may not be a primary anchor position.

Design of peptides binding to RT1.B^{l}

Since the motif described above was based on the arbitrary alignment of peptides, it was necessary to prove that the assignment of the anchor positions was correct. To address this point, we chose a peptide with a precisely known MHC binding core. Recent evidence suggests that the peptide 85–101 of the invariant chain, also termed CLIP, contains a nonameric 'supermotif' sequence (amino acids 90–98: MRMATPLLM) which allows it to bind promiscuously to different MHC class II proteins (21–24). Positions 1, 3 and 9 in this sequence are occupied by the unusually flexible Met residue which can fit into pockets of different size, while P4 and P6 contain small residues avoiding any possible negative effects of polymorphic amino acid in the corresponding pockets. Indeed, as shown in Table 3(A), the mouse CLIP peptide

Table 1. Peptide ligands of RT1.B^{l}

Protein	Epitope	Sequence	ID_{50} (μM)
(A) High affinity binders			
mHSP60	255-75	QSIVPALEIANAHRKPLVIIA	<0.1
mHSP60	166-85	EEIAQVATISANGDKDIGNI[a]	0.7
GroEL	136-55	PCSDSKAIAQVGTISANSDE[a]	0.7
A2b TCRβ	71-90	PNLTFSTLTVNNARPEDSSV[b]	2.2
mHSP60	421-40	VTDALNATRAAVEEGIVLGG[a]	3.5
GroEL	391-410	KKARVEDALHATRAAVEEGV[a]	2.8
mHSP60	106-25	NEEAGDGTTTATVLARSIAK	4
mHSP60	316-35	MAIATGGAVFGEEGLNLNLE	6
mHSP60	226-45	SPYFINTSKGQKCEFQDAYV	6.5
GroEL	121-40	KAVTAAVEELKALSVPCSDS	7
(B) Naturally bound peptides			
Ribophorin I	203-	DIPAYSQDTFKVHYEN	3
Actin β/γ	195-	ERGYSFTTTAEREIVR	5
Nucleobindin	140-53	LDPQNQHTFEARDL	8
HSP70 family	420-	TIPTKQTQTFTTYSDNQP	50

Over 100 peptides were tested for binding to RT.1B^{l} using a competition binding assay and those found to bind with high affinity (ID_{50} < 10 μM) are shown in Section (A).

[a]Homologous peptides of mHSP60 and GroEL having the same core sequence (underlined) are aligned.

[b]The immunogenic core sequence (20) is underlined.

Section (B) shows the sequences of major individual peptide fractions eluted from purified RT.1B^{l} molecules. All sequences show a full match to the corresponding rat proteins. The C-terminal position is not indicated for the peptides that may contain additional amino acids at the C-terminus. Three truncation variants of the HSP70 peptide with the same core sequence (underlined) were identified. ID_{50} represents the RT.1B^{l} binding capacity of synthetic peptides measured in the competition binding assay.

Table 2. Alignment of the RT1.B^{l} peptide ligands

		1 34 6 9
Ovalbumin	323-39	ISQAVHAAHAEINEAGR[a]
GBP	72-85A_{79}	QKSQRAQDENPV[a]
M. tuberculosis HSP65	180-86A_{183}	TFGAQLE[a]
mHSP60	255-75	QSIVPALEIANAHRKPLVIIA
mHSP60	168-79	IAQVATISANGDK
A2b TCRβ	77-86	TLTVNNARPE
mHSP60	423-36	DALNATRAAVEEGI
mHSP60	106-25	NEEAGDGTTTATVLARSIAK
mHSP60	316-35	MAIATGAVFGEEGLNLNLE[b]
		G
mHSP60	226-45	SPYFINTSKGQKCEFQDAYV
GroEL	121-40	KAVTAAVEELKALSVPCSDS
Ribophorin I	203-18	DIPAYSQDTFKVHYEN
Actin β/γ	195-210	ERGYSFTTTAEREIVR
		or ERGYSFTTTAEREIVR
Nucleobindin	140-53	LDPQNQHTFEARDL
HSP70 family	421-34	IPTKQTQTFTTYSD

[a]The binding cores of RT.1B^{l} peptide ligands shown in Table 1 as well as of the peptides described previously are aligned according to the proposed anchor positions (highlighted in bold).

[a]Data from (19).

[b]The two Gly residues may form a kink resembling a single amino acid side chain as suggested by molecular modeling (not shown).

binds well to RT1.B^{l}. The truncated CLIP peptide 89–99 also showed strong binding (data not shown), confirming the binding in the correct frame. The substitution of P9 for Ala did not change the binding significantly, although it was shown previously to increase the binding of CLIP to mouse I-$A^{u,k,d}$ molecules (25). In contrast, the introduction of a Glu residue at P9 increased the binding of CLIP to RT1.B^{l} 10-fold. These results confirm our proposed assignment of anchor positions in the RT1.B^{l} motif.

Since P9 of the RT1.B^{l} motif appeared to have an unusual preference for negatively charged residues, we wished to examine the accomodation of different amino acid at P9 in more detail. For this purpose, we tested a panel of short 9-mer peptides with several non-conserved amino acid substitutions at P9. At P7 and P8, these peptides contain Arg residues which are unlikely to be accomodated at either P6 or P9 of RT1.B^{l} and, therefore, should ensure binding in the correct frame. Table 3(B) shows that, indeed, the Glu residue con-

Table 3. Binding of substituted peptides to RT1.B^{l}

Peptide	ID_{50} (μM)
(A) Binding of the invariant chain 85-101 (CLIP) peptide[a]	
1 9	
KPVSQMRMATPLLMRPM	2
KPVSQMRMATPLLARPM	3
KPVSQMRMATPLLERPM	0.2
(B) Binding of peptides substituted at P9	
AAALAARRE	5
AAALAARRA	30
AAALAARRT	45
AAALAARRL	>200
AAALAARRQ	>200
AAALAARRK	>200
AAALAARRY	>200
(C) Binding of designed polyalanine peptides	
AAAAAAAAAAAA	>170
AAAAAAAAAEAA	40
AAAAHAAAAAAA	30
AAAAHAAAAEAA	0.7
AAAALAAAAEAA	50
AQAAHAAAAEAA	0.7

The binding of variants of the CLIP peptide and of the substituted polyalanine peptides was measured in a competition binding assay and the ID_{50} values are shown.

[a]The rat CLIP peptide differs from the mouse peptide at one position (89: Q to P). The binding core (underlined) and anchor positions are according to (22).

ferred the highest binding capacity. Smaller residues Ala and Thr were less preferred, while larger residues decreased the binding significantly. These data confirm the strong preference for negatively charged residues at P9 in the RT1.B^{l} motif.

To further analyze the proposed RT1.B^{l} motif, we synthesized 12-mer polyAla peptides with successive additions of the anchor residues and tested their binding to RT1.B^{l}. As shown in Table 3(C), the polyAla backbone alone did not bind detectably to RT1.B^{l}. The addition of single anchor residues such as a Glu residue near the C-terminus or of an aromatic His residue in the middle conferred intermediate binding capacity (ID_{50} = 30–40 μM). However, the addition of both His and Glu residues together at P4 and P9, respectively, yielded a peptide with a very high binding capacity for RT1.B^{l} (ID_{50} = 0.7 μM). Note that the consensus Ala residue was present at P6 in this case. Interestingly, the addition of a P1 anchor residue (Table 3B) and further addition of preferred residues at P3 and P8 (not shown) did not improve binding. The preference for large residues at P4 was confirmed by introducing an aliphatic Leu residue instead of His: in this case, only intermediate binding was observed. Again, these results are allele-specific, since very different relative binding was observed for these peptides using two other mouse I-A molecules (not shown). Thus, similar to previous studies using HLA-DR molecules (26), simplified polyAla peptides could bind well to RT1.B^{l} provided that key anchor residues were introduced. These data show the importance of the P4 and P9 anchor residues for peptide binding to RT1.B^{l}.

The proposed RT1.B^{l} binding motif is summarized in Table 4. Note that at least three and typically four to five anchor positions in the peptides binding to RT1.B^{l} (Table 2) must be occupied by consensus residues. Consequently, in spite of the possible accomodation of multiple amino acid at some positions, the overall motif appears quite stringent, a condition that should restrict the potential epitopes within a protein.

Occurrence of the motif in T cell epitopes in the Lewis rat

To test the relevance of the proposed sequence motif to actual immune responses, we sought the occurrence of the motif in peptides that were shown previously to stimulate T cells in the Lewis rat. These peptides are mainly derived from self-antigens and serve as targets of various actively induced or adoptively transferred autoimmune diseases. For this purpose, we examined the immunodominant epitopes, which are likely to be among the best MHC binders within a given protein (6); in addition, RT1.B^{l} restriction was confirmed for at least some of them. Table 5 summarizes the disease-inducing autoantigens for which a single immunodominant epitope was identified in the Lewis rat. As shown in Table 5, the immunodominant peptide of a protein invariably contained a RT1.B^{l} motif. In some of the proteins, such as GBP, P2 and S100β, the single known dominant peptides were the only peptides in the protein sequence that conformed to the motif. In addition, the motif was present in many epitopes (notably, the most potent ones) that were shown to be immunogenic or pathogenic in the Lewis rat after immunization with single peptides (not shown). Thus, the proposed RT1.B^{l} peptide motif is present in the majority of previously mapped immunogenic epitopes in the Lewis rat.

Discussion

In this study, we analyzed the peptide binding specificity of the Lewis rat MHC class II I-A molecule, RT1.B^{l}. Previously, the affinities of several disease-associated peptides and their substituted analogs for RT1.B^{l} were reported (20) without any attempt to define a binding motif. In another study, a two-position motif (SxxxxE) for RT1.B^{l} was suggested from the sequences of several autoantigenic T cell epitopes; using this motif, the authors were able to predict disease-inducing peptides in cardiac myosin (34) and proteolipid protein (35). However, this simplified amino acid combination is unlikely to represent a complete RT1.B^{l} peptide motif, since MHC class II motifs usually contain several anchor positions with many possible residues. Indeed, the majority of known T cell epitopes or peptides with good RT1.B^{l} binding capacity lack this two-position combination. Furthermore, no structural or biochemical verification of this motif was made.

Here we used isolation of natural peptide ligands and peptide binding studies to delineate a peptide binding motif of RT1.B^{l}. In addition, we have undertaken molecular modeling of the RT1.B^{l} structure and interactions with peptides by homology to the solved crystal structures of HLA-DR1 (12) and of HLA-DR1–peptide complex (13) (data not shown). The modeled binding groove of RT1.B^{l} seems to contain four prominent pockets 1, 4, 6 and 9, with several key residues apparently affecting specificities of the pockets. Thus, residues β86Ser, β89Arg and α31Glu appear to create a polar environment in pocket 1 so that it could accomodate a broad range of polar, charged and hydrophobic side chains. A small indent in the binding groove corresponding to P3

Table 4. The putative RT1.B^{l} peptide binding motif

Positions	1	2	3	4	5	6	7	8	9
Amino acids	polar, aliphatic		**T,S,V** A,M	**F,H** Q,K,R N,T,L,I		**A,S,V,T** P,L	large		**E,D** aliphatic

The major anchor positions and the putative consensus amino acid are in bold type.

Table 5. Occurrence of the RT1.B^{l} peptide motif in immunodominant T cell epitopes in the Lewis rat

Protein	Epitope	Sequence	Disease[a]	Restriction	Reference
GBP	72-85	**Q**K**S**Q**R**S**Q**D**E**NPV	EAE	RT1.B^{l}	27
Bovine P2	60-70	TEI**S**F**K**L**G**Q**E**F	EAN	ND	28
Rat S100β	76-91	F**V**S**M**V**T**T**A**C**H**E**F**F**E**HE	EAP	RT1.B^{l}	29
Bovine rhodopsin	331-42	DEAS**T**T**V**S**K**T**E**T	EAU	ND	30
Rat PO	180-99	SSKRGRQT**P**V**L**Y**A**M**L**D**H**SRS	EAN	ND	31
M. tuberculosis HSP65[b]	180-88	**T**F**G**L**Q**L**E**L**T	AA	RT1.B^{l}	32
Torpedo californica AchR	α100-116	YAIVH**M**T**K**L**L**L**D**YTGKI	EAMG	ND[c]	33

Peptides reported to be immunodominant in the Lewis rat are aligned according to the proposed RT1.B^{l} peptide motif (highlighted in bold).
[a]AA, adjuvant arthritis; EAE, experimental autoimmune encephalomyelitis; EAN, experimental autoimmune neuritis; EAMG, experimental autoimmune myasthenia gravis; EAP, experimental autoimmune panencephalitis; EAU, experimental autoimmune uveoretinitis.
[b]Obtained after immunization with *M. tuberculosis* microorganisms.
[c]The peptide was shown to bind weakly to RT1.B^{l} (19).

favors medium-size residues such as Val and Thr. Pocket 4 seems to be enlarged by the small residues β13Gly, β78Val and α9Ala, while residues β26Asp and β74Glu confer a negative charge to the pocket. Consequently, large hydrophobic, polar and positive but not negative residues would be favored at P4. P6, similar to HLA-DR1, appears small. In P9, a serine at β57 seems to provide a hydroxile group to the pocket and to expose the positive charge of α76Arg, making negatively charged residues most suitable at P9. Since the crucial residues (α62Asn, α69Asn and β82Asn) involved in hydrogen bond formation with the peptide backbone are conserved in RT1.B^{l}, the peptides are likely to bind in the same extended conformation as shown in HLA-DR (13,22). Finally, energy calculations suggest a major contribution of residues at P4 and P9 rather than at P1 for peptide binding. Thus, our experimental data are consistent with the modeled structure of RT1.B^{l}.

The RT1.B^{l} peptide motif described here appears to fit well into the emerging general picture of MHC class II motifs (4). Thus, the core sequence contains nine amino acids with anchor residues at positions P1, P4, P6 and P9. The majority of the binding energy is apparently contributed by large hydrophobic, polar or charged residues at P4, P9 and in some cases P1. However, it is likely that additional preferences at apparently non-anchor positions might influence peptide binding to RT1.B^{l}. For example, a general preference for large and polar residues was observed at P7, which might contribute energy to the interaction (Table 2). In addition, two anchor positions (P3 and P6) can accept side chains of a limited length. The amino acid usage seems to be relatively relaxed at some positions such as at P1 and P4 but more restricted at other positions (e.g. Glu at P9).

Nevertheless, distinct features were revealed in the RT1.B^{l} motif. All HLA-DR and I-E peptide motifs described so far contain a hydrophobic (aromatic or aliphatic) residue at P1 (14) and, at least for HLA-DR molecules, this is the most important anchor position providing the majority of the binding energy. In contrast, in the RT1.B^{l} motif, the amino acid usage at P1 was quite degenerate, with polar residues such as Gln preferred. In addition, P1 in general appeared to be a rather secondary anchor position, the majority of the binding energy being provided by P4 and P9.

Another particular feature of the RT1.B^{l} motif is the presence of negatively charged residues at P9. Molecular modeling suggests a major role for a Ser residue at position 57 of the RT1.B^{l} β chain in determining the specificity of P9. The importance of β57 for the specificity of peptide binding by HLA-DR molecules has been demonstrated previously (36). Negatively charged residues have been observed near the C-terminus of the peptides naturally bound to HLA-DRB1*0405 (14) and I-A^{g7} (37) molecules, both of which have a Ser instead of the usual Asp at β57. Thus, the presence of Ser instead of Asp at β57 appears to confer a preference for negatively charged residues at P9 and, therefore, to change the overall peptide binding specificity of a particular MHC class II allele. These data are consistent with an important role of the polymorphism at β57 in susceptibility to autoimmune diseases (38).

The proposed RT1.B^{l} motif is qualitative and possibly incomplete, since it does not not cover all residues at all positions. Additional studies such as global amino acid replacement (6,7,11) might be performed to reveal all possible amino acid preferences, and so render the motif more quantitative and predictive. Nevertheless, the presence of the proposed motif in the majority of known T cell epitopes and the successfull design of high-affinity RT1.B^{l} binders

support its utility in the prediction of T cell epitopes, which is a commonly encountered problem in studies of experimental autoimmunity in the Lewis rat. Indeed, using the described motif, we have been able to predict peptides binding to RT1.B^{l} and capable of inducing an RT1.B^{l}-restricted T cell response in the Lewis rat (F. Mor *et al.*, submitted). In addition, our analysis of the RT1.B^{l}–peptide interactions should be useful for the design of MHC blockers, TCR antagonists and other specific agents for experimental therapy of autoimmune diseases.

Note added in proof

Since submission of the manuscript, two articles have been published which describe peptide binding motifs of HLA-DQ molecules (39,40). Similar to our data, these studies show the relaxed nature of P1 (39,40) and the role of β57 in determining the specificity of P9 (40).

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Abbreviations

GBP	guinea pig myelin basic protein
HSP60/65/70	heat shock protein of 60/65/70 kDa
mHSP60	mouse HSP60 protein

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