JOURNAL OF VIROLOGY, Nov. 2001, p. 10958–10968 Vol. 75, No. 22
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.22.10958–10968.2001

Molecular Determinants of Peptide Binding to Two Common Rhesus Macaque Major Histocompatibility Complex Class II Molecules

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Received 5 July 2001/Accepted 13 August 2001

Major histocompatibility complex class II molecules encoded by two common rhesus macaque alleles Mamu-DRB1*0406 and Mamu-DRB*w201 have been purified, and quantitative binding assays have been established. The structural requirements for peptide binding to each molecule were characterized by testing panels of single-substitution analogs of the two previously defined epitopes HIV Env242 (Mamu-DRB1*0406 restricted) and HIV Env482 (Mamu-DRB*w201 restricted). Anchor positions of both macaque DR molecules were spaced following a position 1 (P1), P4, P6, P7, and P9 pattern. The specific binding motif associated with each molecule was distinct, but largely overlapping, and was based on crucial roles of aromatic and/or hydrophobic residues at P1, P6, and P9. Based on these results, a tentative Mamu class II DR supermotif was defined. This pattern is remarkably similar to a previously defined human HLA-DR supermotif. Similarities in binding motifs between human HLA and macaque Mamu-DR molecules were further illustrated by testing a panel of more than 60 different single-substitution analogs of the HLA-DR-restricted HA 307–319 epitope for binding to Mamu-DRB*w201 and HLA-DRB1*0101. The Mamu-DRB1*0406 and -DRB*w201 binding capacity of a set of 311 overlapping peptides spanning the entire simian immunodeficiency virus (SIV) genome was also evaluated. Ten peptides capable of binding both molecules were identified, together with 19 DRB1*0406 and 43 DRB*w201 selective binders. The Mamu-DR supermotif was found to be present in about 75% of the good binders and in 50% of peptides binding with intermediate affinity but only in approximately 25% of the peptides which did not bind either Mamu class II molecule. Finally, using flow cytometric detection of antigen-induced intracellular gamma interferon, we identify a new CD4$^+$ T-lymphocyte epitope encoded within the Rev protein of SIV.

During the last few years, great advancements have been made in the direct quantification of immune responses, as a result of increased accuracy of epitope prediction technology and the availability and widespread use of revolutionary techniques, such as intracellular cytokine staining, enzyme-linked immunospot assay, and tetramer staining (6, 34, 44). Advances in the measurement of immune responses in both murine and human systems have involved cancer and infectious disease applications. Among the areas that have benefited most are basic studies aimed at understanding disease pathogenesis and its relation to immunity. Studies of a more-applied nature have benefited as well, providing the tools for more-accurate quantitation of immunological responses elicited by various vaccine constructs and formulations (25, 48).

Because of the overall similarity of rhesus macaque and human immune systems, rhesus macaques are utilized in disease models for transplantation, AIDS, malaria, and other important human diseases (8, 19, 27, 28, 38, 39, 45, 54). Many macaque genes that encode proteins important in the immune system are similar to those of humans. As far as major histocompatibility complex (MHC) class I molecules are concerned, genes found in rhesus macaques are homologous to the HLA-A and -B genes, and accurate techniques have been developed to type particular class I alleles in macaques (9, 29). However, alleles at either the A or B locus cluster together, not with their HLA-A or -B human counterparts, suggesting that allelic lineages are not shared evolutionarily over the 35 million years separating humans and rhesus macaques (9). Peptide binding motifs specific for several common Mamu class I types have been defined (5, 14, 50), and epitopes have been identified for a number of them (2, 3, 14, 15, 17, 20, 50). Subsequently, several different tetrameric Mamu MHC/epitope complexes have been prepared for use as reagents. As a result, a number of different studies have been performed, which start to shed light on issues such as those involved in simian immunodeficiency virus (SIV) mutation and cytotoxic T-lymphocyte (CTL) escape (4, 16) or in recognition of different epitopes in different phases of infection (4). At the applied level, this knowledge has been successfully utilized to monitor the immune responses elicited by various vaccine constructs and also to engineer macaque-specific epitope-based vaccines (15, 33).

By contrast, the Old World monkey species whose MHC class II has been studied most completely is the rhesus macaque. At the genetic level, an unprecedented number of different configurations of the DRB region have been detected, together with extensive allelic polymorphism of the DR beta chain (13). Although 116 Mamu-DRB alleles have been re-

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ported, just over half of the alleles have been allocated to a certain haplotype (13, 30, 36). It would thus appear that, although Mamu-DR molecules do share with human HLA-DR molecules the presence of a monomorphic alpha chain, they are also associated with an unparalleled degree of complexity, apparent at both the haplotype and allelic levels (13, 30, 36, 42, 52). Multiple methods to detect various different alleles and configurations are currently being developed (30, 36, 42).

At the molecular level, two human immunodeficiency virus (HIV) Env epitopes recognized by Mamu-DRB*w201- and Mamu-DRB1*0406-restricted Th cells have been described (35). The same group has recently reported the establishment of a peptide or class II tetrameric reagent and its application to the study of class II-restricted immune responses in SIV-infected macaques (32). Geluk and coworkers have also reported another Mamu-DRB-restricted epitope, derived from HSP60 of *Mycobacterium tuberculosis* (21).

Little is known at the level of the specific motifs recognized by Mamu class II molecules. Humans and rhesus macaques share several MHC-DRB loci and lineages, but most DRB alleles appear to be of postspeciation origin (42). Thus, it is speculated that some similarity to HLA-DRB alleles at the level of specific peptide binding motifs could exist. This speculation is also based on the recognition of an identical epitope by HLA-DRB1*0301-restricted T cells in humans and Mamu-DRB1*03-positive macaques (21).

Despite these early insights, the field has been hampered by the lack of knowledge regarding amino acid sequence motifs associated with Mamu class II molecules and of quantitative assays to measure the interaction between Mamu class II molecules and candidate epitopes. Our research group has utilized classical receptor-ligand assays based on purified MHC molecules and synthetic peptides to establish quantitative assays specific for more than 50 different murine and human MHC molecules. These assays have been utilized to define the structural motifs associated with peptide binding to the various alleles (31, 53). More recently, we have also applied this experimental strategy to the definition of motifs recognized by class I molecules derived from nonhuman primates, such as macaques and chimpanzees (7, 14). In the current study, we report for the first time the establishment of quantitative assays to measure the interaction between Mamu class II molecules and their peptide ligands and to define the structural requirements of such interactions.

MATERIALS AND METHODS

Cells. RM3 cell lines transfected with Mamu-DRA and either Mamu-DRB*w201 or Mamu-DRB1*0406 cDNA were utilized as the source of rhesus macaque MHC molecules (35). RM3 is a MHC class II-negative derivative of the human Epstein-Barr virus (EBV)-transformed B-LCL Raji cell line (11). Cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U (100 μg/ml) penicillin-streptomycin, and 10% heat-inactivated fetal calf serum (FCS).

Peptides and iodine-125 labeling. Peptides were obtained as lyophilized crude products from Chiron Mimotopes (San Diego, Calif.) or synthesized at Epimmune using standard *tert*-butoxycarbonyl (t-Boc) or 9-fluorenylmethoxy carbonyl (F-moc) solid-phase synthesis methods as previously described (47). Peptides subsequently used as radiolabeled probes were further purified by standard high-pressure liquid chromatography (HPLC) methods, and their composition was ascertained by mass spectrometry analysis. Peptides were stored in stock solutions at either 10 or 20 mg/ml in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, Mo.) and then diluted to required concentrations with phosphate-buffered saline (PBS). HPLC-purified peptides were radiolabeled with ^{125}I according to the chloramine-T method (23).

Individual 15-mer peptides overlapping by 5 amino acids were synthesized according to the predicted amino acid sequences of $SIV_{mac}239$ (GenBank accession no. M33262).

Affinity purification of Mamu-DR molecules. Mamu class II molecules were purified from cell lysates using affinity chromatography as previously described (22, 49). Mamu-DRB1*0406 and -DRB*w201 were captured using Sepharose CL-4B beads conjugated with the anti-HLA-DR antibody LB3.1 (ATCC 422). The LB3.1 antibody was determined to be cross-reactive with Mamu-DR by flow cytometry (data not shown). After the cell lysates were passed over the column, macaque class II DRB*w201 and DRB1*0406 molecules were eluted and concentrated. Protein purity and concentration and effectiveness of depletion steps were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Class II peptide binding assay. Quantitative assays for the binding of peptides to soluble Mamu-DRB1*0406 and Mamu-DRB*w201 molecules was based on the inhibition of binding of a radiolabeled standard probe peptide. These assays were performed using the same protocol described for the measurement of peptide binding to HLA class II molecules (49). The radiolabeled probe used in the Mamu-DRB*w201 binding assays was the Y486→F analog of the previously described Mamu-DRB*w201-restricted T-helper cell epitope HIV Env 482–497 (ELYKYKVVKIEPLGVA) (35). The radiolabeled probe used in the Mamu-DRB1*0406 binding assays was an analog of the HIV Env 242–262 (VSTVQC THGIRPVVSTQLLL; Y addition at the C terminus) Mamu-DRB1*0406-restricted T-helper cell epitope (35). In preliminary experiments, the titers of rhesus macaque MHC preparations were determined in the presence of fixed amounts of radiolabeled peptide to determine the concentration of class II molecules necessary to bind 10 to 20% of the total radioactivity. All subsequent competitive inhibition and direct binding assays were then performed using these class II concentrations.

For competitive-inhibition assays, a dose range (0.001 to 100 nM) of unlabeled competitor peptide was coincubated with 1 to 10 nM ^{125}I-radiolabeled probe and the MHC for 48 h at room temperature.

All assays were done in PBS containing 0.05% Nonidet P-40 (NP-40) and in the presence of a protease inhibitor mixture. The final concentrations of protease inhibitors were 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.3 nM 1,10-phenanthroline, 73 mM pepstatin A, 8 mM EDTA, 6 mM *N*-ethylmaleimide, and 200 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Assays were performed at pH 7.0. Class II peptide complexes were separated from free peptide by gel filtration on TSK200 columns (Tosohaas, Montgomeryville, Pa.).

Class II peptide complexes were also separated from free peptide in some assays using TopCount microplate scintillation counting technology. A 96-well Optiplate (Packard Instrument Co., Meriden, Conn.) was precoated for 24 h at room temperature with 100 μl of LB3.1 antibody per well (30 μg/ml in PBS). Plates were then blocked for 24 h at room temperature with 250 μl of 0.3% NP-40 in PBS per well. Blocking solution was removed, and class II peptide complexes were transferred to and allowed to bind to the antibody-coated Optiplate for 3 to 4 h at room temperature. Unbound material was removed, and plates were washed once with PBS (250 μl/well). To each well was then added 100 μl of Microscint 20 scintillation fluid (Packard). Radioactivity was quantified using a TopCount Scintillation detector (Packard). The fraction of MHC-bound peptide was then calculated as previously described (23).

The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was then calculated. Under the conditions used in these assays, where the concentration of label was less than the concentration of MHC and IC_{50} was greater than or equal to the concentration of MHC, the measured IC_{50}s are reasonable approximations of the true K_d values. Each peptide was tested in two or three independent experiments, and all different replicate observations were contained in a threefold range. For assays that utilized the TopCount microplate scintillation counting, IC_{50}s were calculated relative to the IC_{50} achieved by the indicator peptide tested by HPLC. For a positive control, in each experiment the unlabeled version of the radiolabeled probe was tested.

Peptide pools and antibodies for intracellular staining. Peptides, 15 amino acids in length, overlapping by 11 amino acids and which spanned the entire Rev protein sequence of SIVmac239 were synthesized (Chiron Mimitopes). Peptides were divided into three pools (each peptide at 10 mg/ml in 10% DMSO–PBS) for use in intracellular staining experiments. Individual peptides were also resuspended at 1 mg/ml in 10% DMSO–PBS. Antibodies used for staining included anti-CD4-APC (clone RPA-T4), anti-CD8-PerCP (clone RPA-T8), fluorescein isothiocyanate (FITC)-conjugated anti-gamma interferon (anti-IFN-γ) (IFN-γ

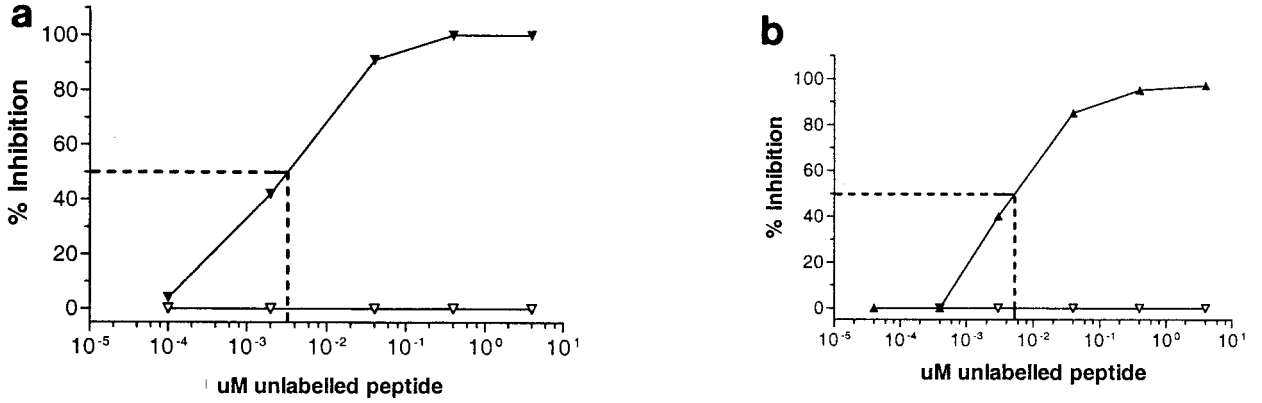

FIG. 1. Dose-dependent inhibition of binding to rhesus macaque MHC class II molecules by excess unlabeled peptide. (a) Hiv Env 242–261 (solid triangles) was used to inhibit radiolabeled Hiv Env 242–261 binding to Mamu-DRB1*0406. (b) Hiv Env482–501 (solid triangles) was used to inhibit radiolabeled Hiv Env 482–501 analog binding to Mamu-DRB*w201. The unrelated peptide (open triangles) integrin β3 (YAWASDEALPLGSPR) served as a negative control for both experiments depicted. Dotted lines indicate concentration of peptide needed to achieve 50% inhibition of binding of the radiolabelled peptide.

clone 4S.B3), and anti-CD69-PE (clone FN50) and were supplied by BD Pharmingen (San Diego, Calif.).

Intracellular staining procedure. Intracellular staining for cytokine production in peripheral blood mononuclear cells (PBMC) was performed on an animal (no. R93062) typed Mamu-DRB*w201 positive, which had been previously immunized with DNA constructs encoding the entire SIVmac239 genome using the Powderject gene gun. Briefly, PBMC were isolated from whole peripheral blood by Ficoll-diatrizoate density gradient centrifugation. Cells were resuspended at 5×10^6/ml in RPMI 1640 medium (Life Technologies, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biocell Labs Inc., Rancho Dominguez, Calif.), 2 nM glutamine, 20 nM HEPES, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Anti-CD28 and anti-CD49d antibodies (BD Pharmingen) (2.5 μg/ml each) were added to the cell suspension, and cells were divided into aliquots in 200-μl volumes into microtubes (Costar; Corning Inc., Corning, N.Y). Each Rev peptide pool (1 μg of each peptide/sample) was tested in three different samples. Flu peptide (SNEGSYFI; 1 μg/sample) was used as a negative control, and staphylococcal enterotoxin B (SEB) (10 μg/ml; Sigma) was used as a positive control. After the cells were incubated at 37°C for 1.5 h, the protein transport inhibitor brefeldin A (10 μg/ml; Sigma) was added to allow intracellular accumulation of cytokines. Samples were incubated for a further 5 h before staining. Samples were washed once with fluorescence-activated cell sorter (FACS) buffer (2% FBS–PBS), cells were pelleted by centrifugation, and pellets were resuspended in 100 μl of FACS buffer. Cells were surface stained for CD4 and CD8 for 40 min at room temperature. Cells were washed twice with FACS buffer and fixed in 2% paraformaldehyde overnight at 4°C. Cells were washed once with FACS buffer and twice with 0.1% saponin–FACS buffer in order to permeabilize cell membranes. Cells were stained intracellularly for IFN-γ and CD69 for 50 min at room temperature. Following two more washes with 0.1% saponin buffer to remove unbound antibodies, cells were resuspended in 2% paraformaldehyde and stored at 4°C until analyzed. Acquisition was performed on a FACS Caliber flow cytometer collecting 100,000 to 200,000 lymphocyte gated events per sample.

RESULTS

Establishment of Mamu-DRB*w210 and Mamu-DRB1*0406 binding assays. Two previously identified $CD4^+$ T-cell epitopes, HIV-1 Env 482–497 (ELYKYKVVKIEPLGVA; Mamu-DR*w201 restricted) and HIV-1 Env 242–261 (VSTVQCTHG IRPVVSTQLLL; Mamu-DRB1*0406 restricted) (35), were used to establish Mamu-DRB*w201 and Mamu-DRB1*0406-specific binding assays. Peptides were ^{125}I radiolabeled and tested for their capacity to bind to Mamu-DRB*w201 and -DRB1*0406 molecules purified from RM3 transfectants. More specifically, a Y486→F analog of the HIV Env 482-0497 epitope was used to probe for binding to Mamu-DRB*w201, and an analog of HIV Env 242–261 with Y added to the C terminus was used to probe for Mamu-DRB1*0406 binding. A trace amount of each radiolabeled peptide was tested over a range of MHC concentrations to determine the concentration of the MHC molecules required to bind 15% of the total radioactivity (data not shown). All subsequent inhibition assays were then performed using these MHC class II concentrations.

First, to establish binding specificity, we determined whether excess unlabeled ligand would inhibit the binding of the radiolabeled probe. Inhibition curves for the interaction of HIV Env 242–261 and HIV Env 482–497 with Mamu-DRB1*0406 and -DRB*w201, respectively, are shown in Fig. 1. The IC_{50} for the unlabeled Env 242–261 epitope and Mamu-DRB1*0406 was determined to be 3.3 nM (Fig. 1a), while the IC_{50} for Env 482–497 epitope Mamu-DRB*w201 binding was 4.9 nM (Fig. 1b). These results are in good agreement with IC_{50}s detected for other epitopes binding to their restriction element in humans (53). By contrast, unrelated control peptide derived from integrin β_3 did not inhibit either assay, when tested at concentrations up to 3 μM. In conclusion, these results illustrate the establishment of sensitive binding assays, specific for Mamu-DRB1*0406 and -DRB*w201.

Binding capacity of human and rhesus macaque class II-restricted epitopes. Next, the newly established assays were utilized to probe the binding capacity of a panel of known epitopes restricted by either macaque (Mamu) or human (HLA)-DR molecules (Table 1). We found that the Mamu-DRB1*0406-restricted epitope HIV gp120.242–261 bound its relevant restriction element with 3.3 nM affinity and with more than a 100-fold-less affinity to the other Mamu-DR molecule tested (408 nM; Mamu-DR*w201). Conversely, the two Mamu-DRB*w201 epitopes tested bound their known restricting element with good affinity (4.9 to 33 nM). Binding to Mamu-DRB1*0406 of the same Mamu-DRB*w201 epitopes was either weak (237 nM for the HIV gp120.482–497 epitope) or undetectable ($IC_{50} \geq 5{,}000$ nM for the SIV Gag 260–274 epitope). Two epitopes (MBP29–48 and HSP65.3–13) which are restricted by other Mamu-DR molecules did not bind at all

TABLE 1. Specificity of peptide binding to Mamu-DRB1*0406 and DRB*w201

Sequence	Protein	Allele	Binding capacity (IC_{50} [nM])[a]		Reference
			Mamu-DRB1*0406	Mamu-DRB*w201	
VSTVQCTHGIRPVVSTQLLL	HIV gp 120.242–261	Mamu-DRB1*0406	3.3	408	35
ELYKYKVVKIEPLGVA	HIV gp 120.482–497	Mamu-DRB*w201	237	4.9	35
GNIYRRWIQLGLQKC	SIV Gag 260–274	Mamu-DRB*w201	–	33	D.I.W.[b]
LPRHRDTGILDSIGRFFGGD	MBP 29–48	Mamu-DRB1*0306 or 0305	–	–	37
YKTIAFDEEARR	HSP65 P3–13	Mamu-DRB1*03, HLA-DR3	–	–	21
AKFVAAWTLKAAA	PADRE	Multiple HLA-DR alleles	121	9.4	1
PKYVKQNTKLAT	HA*307–319	HLA-DR1, -DR2, -DR4w4, -DR5, -DR7	–	29	10, 40
QYIKANSKFIGITE	TT830–843	HLA-DR7, -DR8w2, -DR9, -DR5w11, -DR2w2b2	–	245	40, 43
QYKVLVLNPSVAAT	HCV NS3	HLA-DR4, -DR11, -DR12, -DR13, -DR16	–	350	12
EALIHQLKINPYVLS	Unknown	HLA-DR5w12	–	–	18
YAWASDEALPLGSPR	Integrin b3	HLA-DR52a	–	–	56

[a] Values represent the concentration of test peptide required to inhibit 50% of the radiolabeled peptide ELYKFKVVKIEPLGVA (Mamu-DRB*w201) and VSTVQCTHGIRPVVSTQLLL (Mamu-DRB1*0406). –, >5,000 nM.
[b] Personal communication with David I. Watkins.

to Mamu-DRB1*0406 and Mamu-DR*w201. These data demonstrate that, as in the case of class II molecules derived from other species, Mamu class II peptide binding is allele specific and that the specificity correlates with known restrictions. They also illustrate that the two different Mamu-DR molecules exhibit some degree of cross-reactivity. This is not completely surprising because these two molecules share monomorphic alpha chains, and extensive beta-chain homologies. A similar phenomenon has also been noted for both murine and human class II molecules encoded by the same allelic locus (24, 41, 46, 51, 55).

Next, rhesus macaque DR molecules were also tested for their ability to bind known HLA-DR-restricted epitopes. The pan-HLA-DR epitope PADRE (1) bound to both Mamu-DRB1*0406 and Mamu-DRB*w201 with affinities of 121 and 9.4 nM, respectively. Detectable affinities for Mamu-DRB*w201 were also measured for the three promiscuous human HLA-DR-restricted epitopes HCV-1 NS3 1248–1261, HA*307–319, and TT830–849 (10, 12, 40, 43, 49). By contrast, two other natural ligands of human HLA class II molecules, which are not promiscuous but rather selective binders, did not bind to either one of the two Mamu-DR molecules tested. These data suggest that rhesus macaque and human HLA class II molecules can have overlapping binding specificities.

Definition of core binding regions of HIV Env 242–261 and Env 482–497. Next, we analyzed the core regions of HIV Env 242–261 and Env 482–497 crucial for binding to Mamu-DRB1*0406 and -DRB*w201, respectively. A series of N- and C-terminal truncation analogs of the HIV Env 242–261 and Env 482–497 peptides were synthesized and tested for their ability to inhibit binding of the radiolabeled indicator peptides to Mamu-DRB1*0406 and -DRB*w201, respectively (Table 2).

In the case of Mamu-DRB1*0406, removal of the first six residues from the N terminus of the HIV Env 242–261 peptide did not significantly alter the ability to bind this macaque class II molecule, while removal of the T_{248} residue resulted in a decrease in binding capacity of approximately 50-fold. No significant change was noted upon removal of H_{249} and G_{250}, but removal of I_{251} led to a complete loss of activity. Removal of the first two leucine residues at the C-terminal L_{260} and L_{261} resulted in a 20-fold or more decrease in the binding capacity. Further removal of the L_{259} residue led to complete loss of binding capacity. These results suggest that the residues crucial for Mamu-DRB1*0406 binding are contained within the core binding region IRPVVSTQL (HIV Env 251–259).

In the case of Mamu-DRB*w201, removal of the first four N-terminal residues had no appreciable effect on the binding capacity of the peptides (Table 2). Removal of the subsequent residue, Y_{486}, resulted in a greater than 200-fold drop in affinity for MHC binding. Similarly, C-terminal truncations revealed that the removal of the last three residues had no appreciable effect. Removal of the next residue, L_{494}, led to a drop in binding affinity that was greater than 200-fold. These results indicate that the residues crucial for DRB*w201 binding are contained within the core region 486–494 (YKVVKI EPL). The identification of this core binding region for Mamu-DRB*w201 correlates with a previous study identifying the same nine amino acids as the minimal epitope able to be presented to T cells in a proliferation assay (35).

Definition of HIV Env 248–261 residues involved in Mamu-DRB1*0406 binding. In order to determine which residues within the HIV Env 248–261 epitope are crucial for interaction with Mamu-DRB1*0406, single-amino-acid-substitution analogs of the residues contained within the core binding regions (and adjacent amino acids) were synthesized and tested for their binding. Four to nine different substitutions were introduced at each position, and the effects of conservative, semiconservative, and nonconservative substitutions were investigated. We defined main anchor positions as those associated with at least a 10-fold reduction in binding capacity for the majority of analogs tested. The results of this analysis are shown in Fig. 2a. Significant effects can be seen with substitutions at I_{251}, V_{254}, S_{256}, T_{257}, and L_{259}. In position 251, a negative-charged residue (E) and a positive-charged residue (K) were not tolerated, displaying 80- to 100-fold reductions in relative binding. Additionally, an N substitution resulted in a >200-fold reduction in binding capacity. Hydrophobic or aromatic residues (L, M, and F) were well tolerated at this position.

Other residues critical to the capacity of HIV Env 248–261

TABLE 2. Definition of the core binding region for rhesus macaque MHC class II Mamu-DRB1*0406 and -Mamu-DRB*w201

Peptide	Sequence	Binding capacity (IC_{50} [nM])[a]
HIV Env 242–261	VSTVQCTHG**IRPVVSTQL**LL[b]	10.5
	STVQCTHGIRPVVSTQLLL	NT
	TVQCTHGIRPVVSTQLLL	NT
	VQCTHGIRPVVSTQLLL	7
	QCTHGIRPVVSTQLLL	NT
	CTHGIRPVVSTQLLL	NT
	THGIRPVVSTQLLL	9.5
	HGIRPVVSTQLLL	455.5
	GIRPVVSTQLLL	684.5
	IRPVVSTQLLL	133
	RPVVSTQLLL	–
	PVVSTQLLL	–
	VSTVQCTHGIRPVVSTQLL	356.5
	VSTVQCTHGIRPVVSTQL	219.5
	VSTVQCTHGIRPVVSTQ	–
	VSTVQCTHGIRPVVST	NT
	VSTVQCTHGIRPVVS	NT
	VSTVQCTHGIRPVV	–
	VSTVQCTHGIRPV	NT
	VSTVQCTHGIRP	NT
	VSTVQCTHGI	–
HIV Env 482–497	ELYK**YKVVKIEPL**GVA	15
	LYKYKVVKIEPLGVA	21
	YKYKVVKIEPLGVA	10
	KYKVVKIEPLGVA	17
	YKVVKIEPLGVA	20
	KVVKIEPLGVA	–
	VVKIEPLGVA	–
	ELYKYKVVKIEPLGV	19
	ELYKYKVVKIEPLG	22
	ELYKYKVVKIEPL	73
	ELYKYKVVKIEP	–
	ELYKYKVVKIE	–

[a] Concentration of test peptide required to inhibit by 50% the binding of the radiolabeled peptides, HIV Env 482–497 ELYKYKVVKIEPLGVA (for Mamu-DRB*w201) and HIV Env 242–261 VSTVOCTHGIRPVVSTQLLL (for Mamu-DRB1*0406). Values represent the averages of two or three assays. NT, not tested; –, >1,000 nM.

[b] The core binding regions determined in this study are shown in bold type.

to bind Mamu-DRB1*0406 include V_{254} and S_{256}. All substitutions tested at the V_{254} position resulted in reduced binding capacities. Charged residues (E and K) resulted in a 20- to 40-fold reduction in binding capacity, as the binding of the L- and N-substituted peptides was reduced 10-fold. A four- to sixfold reduction in binding was noted for the remainder of the peptides with substitutions at this position. At the S_{256} position, five of the seven substitutions resulted in >10-fold reduction in binding affinity. The conserved T substitution at this position was the only substitution that was well tolerated, displaying a fourfold increase in binding capacity.

Reductions in peptide binding of greater than 10-fold can also be seen at the T_{257} and L_{259} positions. Charged residues (E and K) were not well tolerated at the T_{257} position and were associated with 30- to 100-fold reductions in binding capacity. Additionally, a C substitution at this position caused a 30-fold reduction in binding affinity for Mamu-DRB1*0406. Finally, at the L_{259} position, an N substitution resulted in an 80-fold reduction in binding, and charged residues (E and K) reduced the binding by 15- to 20-fold.

In summary, the results of the single-substitution analogs of the Mamu-DRB1*0406 epitope show that the majority of substitutions at the V_{254}, S_{256}, and L_{259} residues reduced the binding affinity greater than 10-fold, indicating these residues are crucial for Mamu-DRB*0406 binding capacity. Additionally, three of the seven substitutions at both I_{251} and T_{257} were not tolerated, suggesting that these residues may also play a critical role in Mamu-DRB1*0406 peptide binding.

Structural requirements of epitope binding to Mamu-DRB*w201. A similar analysis was performed next to characterize HIV Env 484–495 binding to Mamu-DRB*w201. The Y_{486}, V_{489}, and L_{494} residues were found to play a critical role in binding capacity (Fig. 2b). Four of the five substitutions at L_{494} resulted in 15- to 30-fold reductions in binding capacity. Aromatic residues (Y and F) appear to be preferred at this position. It was found that substituting the small residue G or the charged residues E and K at the V_{489} position resulted in 10- to 15-fold reductions in binding capacity. All of the substitutions for L_{494} resulted in decreased binding affinity, ranging from 15- to 600-fold decrease in relative binding affinity. A reduction in the relative binding affinity of analogs with substitutions of charged residues (H and K) was also observed at the I_{491} residue.

In the next series of experiments, we further studied the structural features of peptide binding to Mamu-DRB*w201, by taking advantage of the fact that the human HA 307–319 epitope also binds Mamu-DR*w201 with an affinity of 29 nM (Table 1). A panel of single-amino-acid-substituted analogs of the HA 307–319 epitope has previously been used by our laboratory for similar peptide binding motif analysis of HLA-DR molecules (40). This same panel of analogs was tested for their ability to bind Mamu-DRB*w201 (Fig. 3a). The most crucial residues in the HA 307–319 peptide in determining the Mamu-DRB*w201 binding motif were Y_{309} and L_{317}. All the semi- and nonconservative substitutions for the Y_{309} resulted in a 50- to 10,000-fold reduction in relative binding capacity. In the L_{317} position, five of the seven substitutions tested resulted in >10-fold reduction in relative binding capacity (40- to 1,400-fold range). Less striking but still significant effects were also detected at positions V_{310}, T_{314}, and L_{315}. Substitution of a negative-charged residue (E) was not tolerated at V_{310} or T_{314} with 30- to 40-fold reductions in relative binding, respectively. Additionally, substitution of a positive-charged residue (K) was not tolerated at position T_{314}, showing a similar reduction in binding. Substitution with alanine at L_{315} led to a 20-fold decrease in binding affinity.

Interestingly, the binding patterns of HA 307–319 analogs closely resembled the pattern previously noted when the same peptides when tested for binding to HLA-DR1 molecules (Fig. 3b). In the case of DR1, Y_{309}, Q_{312}, T_{314}, L_{315}, and L_{317} act as anchor residues. These results are similar to data published previously (40).

A general Mamu-DR motif. The data presented above suggest that the Mamu-DRB1*0406 binding motif is based on a preference for a hydrophobic or aromatic anchor residue in position 1 (P1), and other primary and secondary analogs located at P4, P6, P7, and P9. Mamu-DR*w201 is associated with a similar, yet clearly distinct, binding specificity also associated with P1, P4, P6, P7, and P9. These two binding motifs are very similar to the previously described HLA-DR binding motifs (40 to 41), which are also characterized by a P1-P4-P6-P7-P9

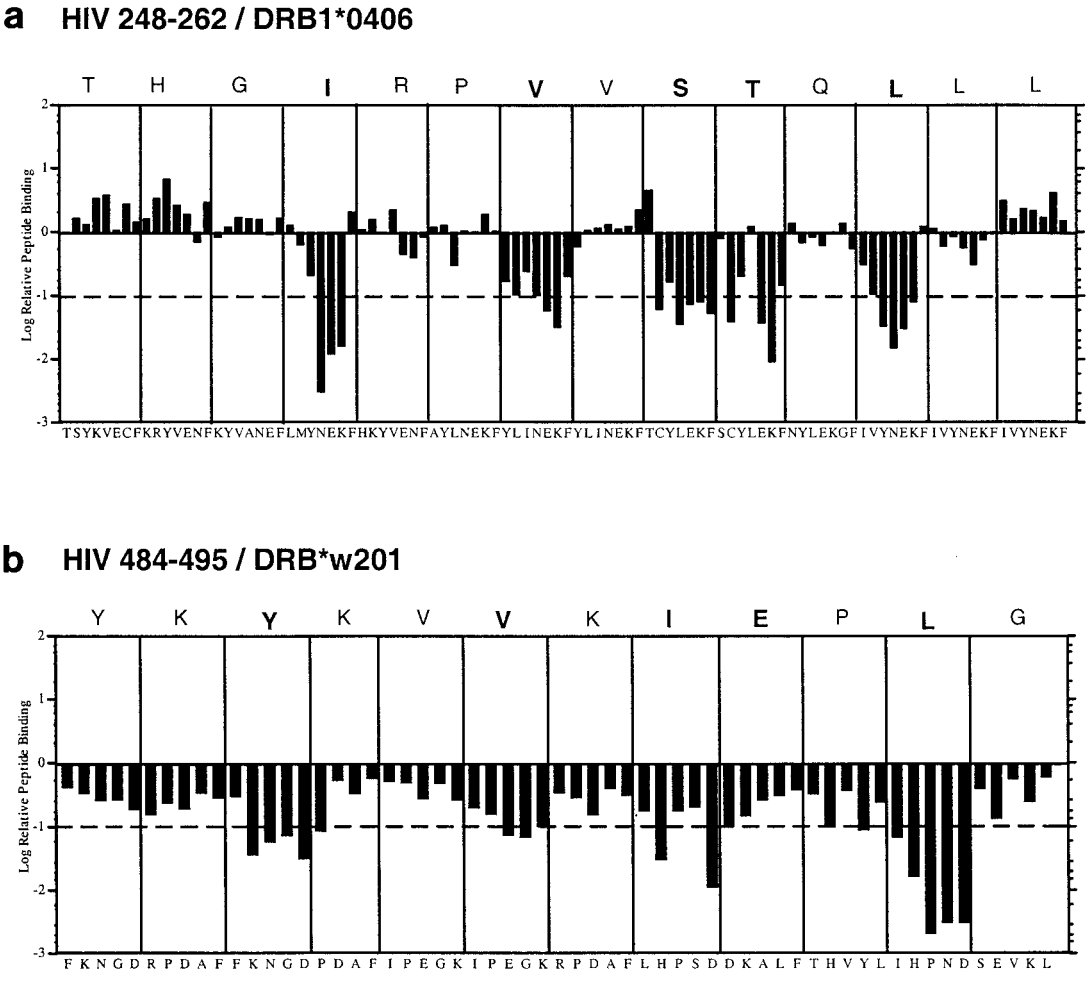

FIG. 2. Definition of epitope residues crucial for binding to rhesus macaque MHC class II molecules. (a) Relative binding of single-amino-acid-substituted analogs of Hiv Env 248–262 (THGIRPVVSTQLLL) to Mamu-DRB1*0406, normalized to the binding of the unsubstituted peptide (3.3 nM). (b) Relative binding of single-amino-acid-substituted analogs of Hiv Env 482–497 (ELYKYKVVKIEPLGVA) to Mamu-DRB*w201, normalized to the binding of the unsubstituted peptide (4.9 nM). The dashed line denotes a 10-fold reduction in binding compared with that of the unsubstituted peptide.

spacing of anchor residues. Human and macaque binding motifs are similar not only in their general anchor spacing but also in anchor specificity, as illustrated by the remarkable similarity of the binding patterns of HA 307–319 analogs to human HLA-DR*0101 and macaque Mamu-DR*w201.

Previous studies have defined a general HLA-DR supermotif based on the presence of three main anchors at P1, P6, and P9 (26, 40, 49). The data presented above suggest that a similar general motif might be extended to macaque Mamu-DR and that an overlap in peptide binding repertoire exists between rhesus macaque and human MHC class II molecules. We hypothesized that a general Mamu-DR peptide binding motif may be defined as L, I, V, M, A, F, Y, and W at P1 of the core binding region of the peptide; L, I, V, M, F, Y, S, T, Q, and A at P6; and L, I, V, M, F, and Q at P9. Differences in fine specificity at these anchors, as well as at the other P4 and P7 anchor positions would modulate allelic specificity.

Identification of SIV_{mac}239-derived Mamu-DRB1*0406 and -DRB*w201 peptides. To test this hypothesis and to identify peptide ligands derived from SIV proteins that could represent candidate Mamu-DR epitopes, we investigated the binding of a set of overlapping peptides spanning the entire predicted amino acid sequences from SIV_{mac}239. A total of 311 peptides (20-mers overlapping by 5 amino acids) were synthesized and tested for binding to Mamu-DRB1*0406 and -DRB*w201 (Table 3). At peptide concentrations of 1 to 1,000 nM, of the 311 peptides, 10 peptides bound both DR molecules and 239 peptides did not bind either Mamu-DR molecule. A total of 62 peptides (19 for peptides that did not bind to Mamu-DRB*w201 and 43 for peptides that did not bind to Mamu-DRB1*0406) bound only one of the molecules. Thus, concordant results were observed in (239 + 10)/311 = 80.1% of the cases ($P = 0.0094$). These results demonstrate that, as anticipated from the single-substitution data, a large overlap exists in peptide binding specificity between the two different Mamu-DR molecules studied. Conversely, it should also be noted that the majority of Mamu-DR binders were selective binders, in that they bound only one of the two molecules tested, thus illustrating the crucial influence of DR polymorphism on peptide binding specificity.

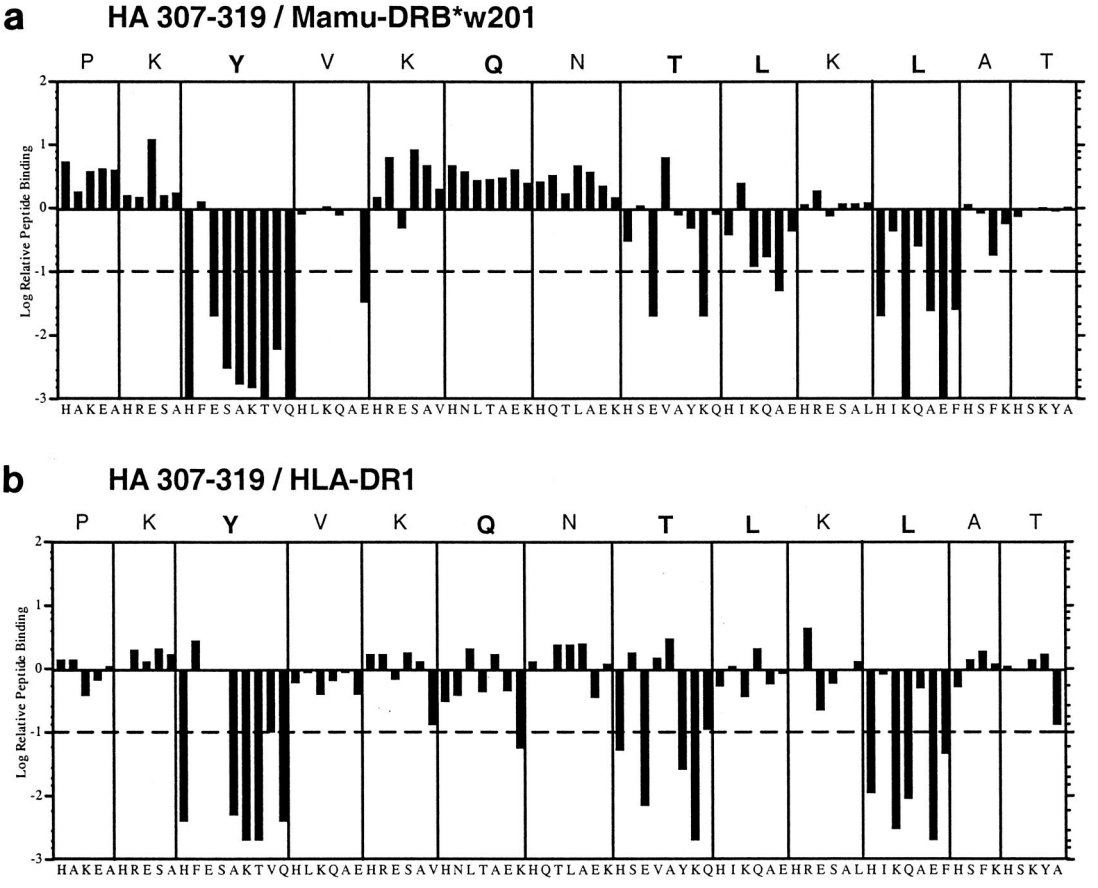

FIG. 3. (a) Relative binding of single-amino-acid-substituted analogs of HA 307–319 (PKYVKQNTLKLAT) to HLA-DR1, normalized to the binding of the unsubstituted peptide (4.3 nM). (b) Relative binding of single-amino-acid-substituted analogs of HA 307–319 to Mamu-DRB*w201, normalized to the binding of the unsubstituted peptide (29 nM). The dashed line denotes a 10-fold reduction in binding compared with that of the unsubstituted peptide.

Finally, the data were also inspected for a correlation between the presence of the putative Mamu-DR motif and Mamu-DR binding. In terms of predictability, it was noted that a total of 72 of 102 (72%) of motif-carrying peptides bound one DR molecule or both (Table 4). In general, for both Mamu-DR*w201 and -DRB1*0406, more than 75% of good binders ($IC_{50} \leq 100$ nM) carried the motif, while a little over 50% of intermediate binders and 25% of nonbinders also carried the same motif ($P = 8 \times 10^{-7}$). These results highlight the significance of the proposed motif but also support the notion that additional criteria might be defined to allow for definition of more-stringent, allele-specific motifs associated with the different Mamu-DR molecules.

Identification of a novel SIV-derived epitope. Based on the results described above, we tested three pools of peptides containing 15-mers, overlapping by five amino acids, spanning the entire Rev protein sequence of $SIV_{mac}239$ for their capacity to induce IFN-γ production from PBMC of Mamu-DRB*w201-positive macaques vaccinated with the entire $SIV_{mac}239$ genome. Fresh PBMC were incubated with brefeldin A in the presence of mitogenic or antigen-specific stimulation and stained for CD4, CD69, and IFN-γ. The expression of the CD69 lymphocyte activation molecule and the production of IFN-γ from the CD4 gated population of lymphocytes is shown in Fig. 4. PBMC from this animal responded to both Rev pools A and B (Fig. 4a). For controls, the treatment of cells with a nonspecific Flu peptide (SNEGSYFI) did not induce the production of IFN-γ, whereas the SEB positive-control mitogen induced very strong production of IFN-γ. When peptides within Rev pool A were tested individually using the same intracellular staining procedure (Fig. 4b), Rev peptides 3 and 4 did induce production of IFN-γ. These two peptides contain an overlapping peptide (RKRLRLIHLLHQT) which had been shown to bind to Mamu-DRB*w201 in the binding assays with an IC_{50} of 34 nM. These results indicate that $CD4^+$ lymphocytes in a SIV-vaccinated macaque are functionally active and capable of responding to the RKRLRLIHLLHQT peptide through the production of intracellular IFN-γ.

DISCUSSION

Herein, we report establishment of molecular binding assays for two common rhesus macaque class II molecules, Mamu-DRB*w201 and -DRB1*0406. These assays allowed us to probe the peptide binding characteristics of these two molecules and to establish prominent structural requirements for these interactions. Based on these results, a putative motif associated with Mamu-DR binding peptides was proposed.

TABLE 3. SIV-derived overlapping 15-mer peptide binding to Mamu-DRB*w201 and Mamu-DRB1*0406

Sequence	Protein	Position	Binding capacity (IC_{50} [nM])[a]		Mamu-DR motif
			Mamu-DRB1*0406	Mamu-DRB*w201	
GVPAWRNATIPLFCA	Env	31	7	242	+
LIRILQRALFMHFRG	Vpr	61	51	31	+
GFLGFLATAGSAMGA	Env	531	62	–	–
FAKIKNTHTNGVRLL	Pol	561	66	284	+
WDFISTPPLVRLVFN	Pol	621	70	19	+
MEEEKRWIAVPTWRI	Vif	1	88	21	+
RRWRRRWQQLLALAD	Rev	41	–	13	+
NIYRRWIQLGLQKCV	Gag	261	–	33	+
RKRLRLIHLLHQTNP	Rev	11	–	34	+
GTDIKVVPRRKAKII	Pol	1021	–	35	+
YLCLIQKALFMHCKK	Vpx	71	–	36	+
WHKVGKNVYLPPREG	Env	441	–	52	–
KRRRTPKKAKANTSS	Tat	81	–	59	–
EFRQYTAFTLPSVNN	Pol	331	651	61	+
LALADRIYSFPDPPT	Rev	51	–	86	+
EMIKKSEIYVAWVPA	Pol	731	–	98	–
WRRDNRRGLRMAKQN	Vif	171	104	–	–
IVAVHVASGFIEAEV	Pol	841	104	–	+
CAFRQVCHTTVPWPN	Env	611	139	483	–
QFSLWRRPVVTAHIE	Pol	111	143	239	–
WLSTYAVRITWYSKN	Vif	81	165	145	+
TIVLMAVHCMNFKRR	Pol	941	166	–	+
ITTEQEIQFQQSKNS	Pol	971	203	–	–
DCTHLEGKIIIVAVH	Pol	831	227	–	–
ANFPGLAKVLGILA	Vif	201	240	171	+
RIREQANSVETIVLM	Pol	931	259	–	+
VILLRIVIYIVQMLA	Env	701	319	–	+
ILQRLSATLQRIREV	Env	801	340	–	+
SAMGAASLTLTAQSR	Env	541	413	841	+
KGTIMTGDTPINIFG	Pol	181	623	–	–
EVRRAIRGEQLLSCC	Vif	121	629	–	–
INIFGRNLLTALGMS	Pol	191	689	–	+
AILLLSVYGIYCTLY	Env	11	732	–	+
TSRPTAPSSGRGGNY	Gag	121	740	–	–
MHFRGGCIHSRIGQP	Vpr	71	846	–	–
LDRFGLAESLLENKE	Gag	41	893	–	–
LAIQQLQNLAIESIP	Rev	71	927	–	+
FMPKCSKVVVSSCTR	Env	251	–	108	+
PVTIMSGLVFHSQPI	Env	321	–	115	+
HKYQVPSLQYLALKV	Vif	141	–	139	+
DVKRYTTGGTSRNKR	Env	511	–	143	–
CISEADASTPESANL	Tat	21	–	155	+
IVQQQQQLLDVVKRQ	Env	561	–	159	+
NQQAELEAFLMALTD	Pol	681	–	162	–
SLESSNERSSCISEA	Tat	11	–	193	–
ALKHFDPRLLTALGN	Vpr	31	–	210	+
YVPHFKVGWAWWTCS	Vif	41	–	242	–
TMSAEVAELYRLELG	Env	481	–	245	+
GVRLLAHVIQKIGKE	Pol	571	–	248	+
FLIRQLIRLLTWLFS	Env	771	–	250	+
NIIVDSQYVMGIITG	Pol	701	–	251	+
ALGMSLNFPIAKVEP	Pol	201	–	255	–
LTVWGTKNLQTRVTA	Env	581	–	266	+
PRELIFQVWQRSWEY	Vpx	41	–	277	–
RIREVLRTELTYLQY	Env	811	–	309	+
KYHSNVKELVFKFGL	Pol	781	–	381	–
LSRVYQILQPILQRL	Env	791	–	407	+
YHCQFCFLKKGLGIC	Tat	61	–	412	–
SRNKRGVFVLGFLGF	Env	521	–	504	+
HGDTLEGAGELIRIL	Vpr	51	–	571	–
TAHIEGQPVEVLLDT	Pol	121	–	727	+
VVKRQQELLRLTVWG	Env	571	–	778	+
CVKLSPLCITMRCNK	Env	101	–	821	–
RYIYKVLPQGWKGSP	Pol	351	–	825	–
KDGQLEEAPPTNPYN	Pol	251	–	830	–
KEPFQSYVDRFYKSL	Gag	291	–	856	+
ALAPVPIPFAAAQQR	Gag	371	–	953	+
GEGHGAGGWRPGPPP	Vpx	91	–	958	–
TWGTTQCLPDNGDYS	Env	51	–	969	–
ARLMAEALKEALAPV	Gag	361	–	980	+

[a] Values represent the concentration of test peptide required to inhibit 50% of the radiolabeled peptide ELYKFKVVKIEPLGVA (Mamu-DRB*w201) and VSTVQCTHGIRPVVSTQLLL (Mamu-DRB1*0406). –, IC_{50} of >1,000 nM.

TABLE 4. Presence of the putative Mamu-DRB peptide binding motif in SIV-derived overlapping 15-mer peptides

SIV-derived overlapping 15-mer peptides	Mamu-DR motif-positive peptides[a]	
	Mamu-DRB1*0406	Mamu-DRB*w201
Good binders (1–100 nM)	5/6 (83)	9/12 (75)
Intermediate binders (101–1,000 nM)	12/22 (54)	25/41 (56)
Nonbinders (>1,000 nM)	85/283 (30)	68/258 (26)
Total	102/311 (33)	102/311 (33)

[a] The number of Mamu-DR motif-positive peptides to the total number of peptides is shown, with the percentage indicated in parentheses. The values for the good binders were statistically significantly different from the values for the intermediate binders and nonbinders ($P = 0.00000008$).

The relevance of this motif was tested with a set of overlapping peptides spanning the entire SIV genome. The biological relevance of this analysis was highlighted by the demonstration of recall IFN-γ production from $CD4^+$ lymphocytes in Mamu-DRB*w201-positive macaques directed against one Mamu-DRB*w201 binder. Our report is the first to describe quantitative molecular assays to study peptide interactions with the Mamu MHC class II molecules expressed by rhesus macaques. The MHC class II complex of macaques is relatively diverse, with a large number of active duplicated genes, each associated with a discrete but limited set of polymorphisms. The availability of assays to evaluate the binding function of these class II molecules and the elucidation of their complex genetic organization should allow for an increased understanding of their biological functions.

The availability of these assays allowed the definition of structural requirements of the interactions of the two molecules Mamu-DRB*w201 and -DRB1*0406. In both cases, the residues important for peptide binding were spaced according to a P1, P4, P6, P7, and P9 pattern, with P1, P6, and P9 being the main anchors. P1 was most crucial for B1*0406, while P9 was the most important for B*w0201. The preferred side chains for the various anchor positions varied for the two molecules but were in general hydrophobic or aromatic in nature. As a result of similarities in their binding preferences, a significant overlap was also demonstrated in the peptide binding repertoire of the two Mamu-DR molecules, and a putative Mamu-DR motif was defined.

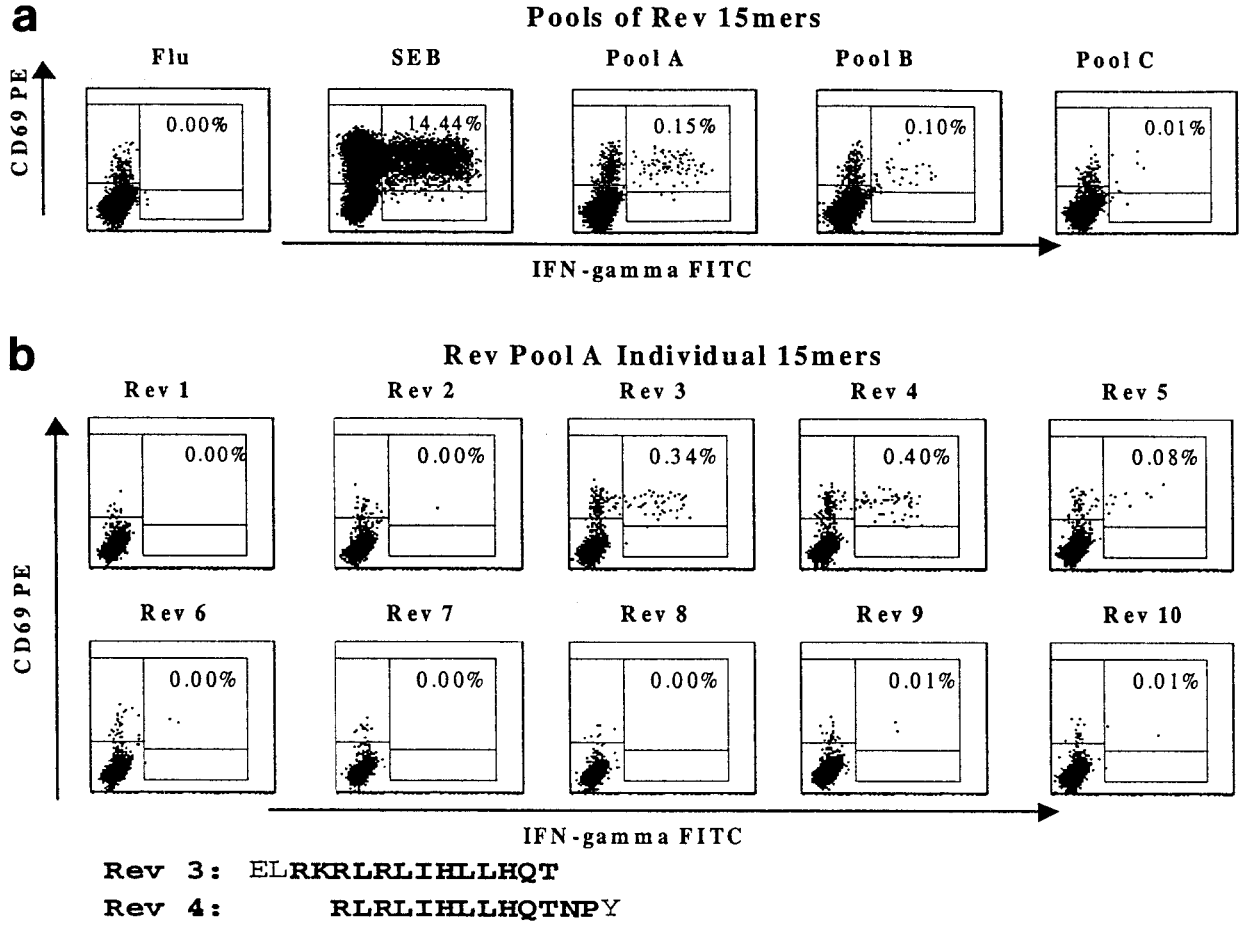

FIG. 4. Intracellular staining performed on PBMC of a Mamu-DRB*w201-positive macaque immunized with DNA constructs encoding the entire SIV genome. Plots show events gated through both $CD4^+$ and lymphocyte gates. (a) Three pools containing 15-mer peptides overlapping by 11 amino acids spanning the entire Rev protein sequence of $SIV_{mac}239$. (b) Peptides from Rev pool A tested individually. Bold type in peptide sequence indicates a peptide which had been predicted to bind Mamu-DRB*w201 in binding assays.

It was noted that the general spacing of anchor residues and peptide binding of the two Mamu-DR molecules studied are remarkably similar to those of the general motif recognized by human HLA-DR molecules, previously described in detail by other studies (12, 40, 49). This finding raises the possibility that similar epitopes might be recognized by humans and other primates, thus facilitating the design and testing of epitope-based vaccines destined for human use. The notion of some limited cross-reactivity between human- and macaque-derived DR molecules was demonstrated by the observation that certain epitopes, known to be promiscuous binders to several HLA-DR molecules, were also shown to bind Mamu-DR molecules.

As mentioned above, the current set of experiments led to the definition of a general Mamu-DR motif. Seventy-two percent of motif-carrying peptides bound either Mamu-DRB1*0406, -DRB*w201, or both. Further experiments will test whether the motif described herein is predictive of binding capacity to other common DR alleles. More-comprehensive analysis will also allow a more-precise definition of the allele-specific motifs associated with each individual Mamu-DR molecule.

Finally, our analysis has mapped a number of SIV-derived peptides which bind the two Mamu-DR molecules studied. The results of experiments with PBMC derived from SIV-vaccinated macaques have directly demonstrated that at least one SIV-derived peptide is recognized by helper T-lymphocyte responses in the course of natural infection. We anticipate that the availability of well-defined epitopes will allow exact quantitation of class II-restricted responses in disease models that utilize rhesus macaques (10–13). As in the case of class I responses, this should in turn expand our knowledge and understanding of immune functions, directly applicable to development of vaccines for human use.

ACKNOWLEDGMENTS

This work is supported in part by NIH grants 2 R44 AI38081–03 (to R.W.C.), R01 A141913, R01 AI48238–01, and R21 AI45461 (to D.I.W.), and RR00167 (to the Wisconsin Regional Primate Research Center). This work was also supported in part by NIH NIAID contract N01-AI-95362. D.I.W. is an Elizabeth Glaser Scientist.

We thank Norman L. Letvin and Marcelo J. Kuroda for the generous gift of the Mamu-DR-transfected RM3 cell lines. The expert secretarial assistance of Robin Delp is gratefully acknowledged.

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