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Residue 116 determines the C-terminal anchor residue of HLA-B*3501 and -B*5101 binding peptides but does not explain the general affinity difference

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Abstract HLA-B*3501 and -B*5101 molecules, which belong to the HLA-B5 cross-reactive group, bind peptides carrying similar anchor residues at P2 and the C-terminus, but differences are observed in the preference for a Tyr residue at the C-terminus and the affinity of peptides. A recent study of HLA-B*3501 crystal structure suggested that residue 116 on the floor of the F-pocket determines a preference for anchor residues at the C-terminus. In order to evaluate the role of the residue 116 in the peptide binding to both HLA-B*3501 and HLA-B*5101 molecules, we generated HLA-B*3501 mutant molecules carrying Tyr at residue 116 (B*3501–116Y) and tested the binding of a panel of nonamer peptides to the B*3501–116Y molecules by a stabilization assay with RMA-S transfectants expressing the mutant molecules. The substitution of Tyr for Ser at residue 116 markedly reduced the affinity of nonamer peptides carrying Tyr at P9, while it enhanced that of nonamer peptides carrying Ile and Leu at P9. On the other hand, the affinity of peptides carrying aliphatic hydrophobic residues at P9 to B*3501–116Y molecules was much higher than that to HLA-B*3501 and HLA-B*5101 molecules. These results indicate that residue 116 is critical for the structural difference of the F-pocket between HLA-B*3501 and HLA-B*5101 which determines the C-terminal anchor residues, while leaving other residues which differ between HLA-B*3501 and HLA-B*5101 may be responsible for the low peptide binding property of the latter.

Key words HLA-class I · Peptide · F-pocket · HLA-B35 · HLA-B51

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Introduction

Recent sequencing studies of self-peptides isolated from purified major histocompatibility complex (MHC) class I molecules have shown that the peptides bound to the MHC class I molecules have allele-specific motifs and characteristic anchor residues (Falk et al. 1991, 1993, 1994; Jardetzky et al. 1991). The critical role of anchor residues in MHC class I-peptide binding was confirmed by a peptide binding assay using synthetic peptides carrying anchor residues (Parker et al. 1992; Takamiya et al. 1994). HLA-B51 and HLA-B35 molecules form a part of a serologically cross-reactive group (CREG) called HLA-B5, B35 CREG including HLA-B52, HLA-B53 and HLA-B78 (Tait et al. 1992). Since HLA-B5, B35 CREG molecules are also cross-recognized by some alloreactive T cells (Matsumoto et al. 1990), it is suspected that these molecules bind peptides carrying similar anchor residues. In fact, previous pool sequencing studies of self-peptides eluted from HLA-B*3501 and HLA-B*5101 molecules showed that they share the same anchor residues, Pro and Ala, at position 2 (P2) and Ile, Leu, and Met at position 9 (P9), while HLA-B*3501 but not HLA-B*5101 favors peptides carrying Tyr at the C-terminus (Falk et al. 1993, 1995). These findings were further confirmed by peptide binding studies using chemically synthesized peptides (Kikuchi et al. 1996; Sakaguchi et al. 1997; Schönbach et al. 1995).

The functional differences between HLA-B*3501 and HLA-B*5101 molecules are known to some extent. The former assembles very rapidly, while the assembly rate of the latter is slow (Hill et al. 1993). The difference in the assembly rate may result from the affinity of peptides to these molecules because the affinity of peptides to HLA-B*5101 molecules is much weaker than that to HLA-B*3501 molecules (Sakaguchi et al. 1997). Since the affinity of peptides to HLA class I molecules is critically dependent on the anchor residues and the B-pocket of HLA-B*3501 is identical to that of HLA-B*5101, it is assumed that the structural difference of the F-pocket formed by the substitution (Ser in HLA-B*3501 and Tyr in HLA-B*5101) at residue 116 determines the affinity of peptides to these HLA class I molecules and the C-terminus of binding peptides. X-ray crystallographic analysis of HLA-B*3501 molecules suggested that the prevalence of Tyr at the C-terminus of HLA-B*3501 binding peptides arises from the ability of the Tyr hydroxyl group to form hydrogen bonds to both Ser at residue 116 and Tyr at residue 74 (Smith et al. 1996). In the present study, we investigated the binding of peptides to HLA-B*3501 mutant molecules carrying Tyr at residue 116 (HLA-B*3501–116Y) and compared them to that of HLA-B*3501 molecules in order to clarify the role of the substitution at residue 116 between HLA-B*3501 and HLA-B*5101 in peptide binding to these HLA class I molecules.

Materials and methods

Cells

RMA-S cells expressing human β_2-m (RMA-S-h β_2m) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). RMA-S-hβ2m cells expressing HLA-B*3501 (RMA-S-B*3501) were previously generated (Takamiya et al. 1994) and maintained in RPMI 1640 medium containing 10% FCS and 0.15 mg/ml hygromycin B.

*Generation of a single amino acid mutant of HLA-B*3501 gene*

The single-strand DNA from pHLA-B*3501 was used as template to produce the *HLA-B*3501* mutant gene. Site-directed mutagenesis was carried out by using a one-base mismatched 20-mer synthetic oligonucleotides according to the method of Kunkel (1985). Ser at residue 116 of HLA-B*3501 was substituted by Tyr (HLA-B*3501–116Y).

*Generation of RMA-S transfectant expressing B*3501–116Y*

The *HLA-B*3501–116Y* gene was transfected by electroporation together with the hygromycin resistant gene into RMA-S-hβ_2m cells. After selection with hygromycin B, hygromycin-resistant cells were isolated from separate wells. Surface expression of HLA-B*3501–116Y molecules on transfected cells was detected by flow cytometry with the combination of anti-HLA-Bw6 mAb, SFR8.B6 (Radka et al. 1982) and anti-HLA class I α3 domain monoclonal antibody (mAb) TP25.99 (Tanabe et al. 1992). RMA-S-hβ_2m cells expressing HLA-B*3501–116Y (RMA-S-B*3501–116Y) were maintained in RPMI 1640 medium containing 10% FCS and 0.15 mg/ml hygromycin B.

Peptides

The nonamer peptides carrying the HLA-B*3501 and HLA-B*5101 binding motifs were previously generated and used for the binding to HLA-B*3501 or HLA-B*5101 molecules (Kikuchi et al. 1996; Sakaguchi et al. 1997; Schönbach et al. 1995, 1996). The peptides were prepared with an automated multiple peptide synthesizer, Shimadzu Model PSSM-8, using the Fmoc strategy followed by cleavage (Nokihara et al. 1992). The peptides were shown to be homogeneous by liquid secondary ion mass spectrometry and reverse-phase HPLC.

Peptide binding assay by flow cytometry

The binding of peptides to HLA-B*3501 and HLA-B*3501–116Y molecules was tested as previously described (Takamiya et al. 1994). Briefly, RMA-S-B*3501 and RMA-S-B*3501–116Y were cultured at 26 °C for 18–24 h. Cells (2×10^5) in 50 µl of phosphate buffered saline (PBS) supplemented with 20% NCS (PBS-NCS) were incubated at 26 °C for 1 h with 50 µl of a solution of peptides at concentration of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M and then at 37 °C for 3 h. After washing with PBS-NCS, cells (2×10^5) were incubated for 30 min on ice with an appropriate dilution of a mixture of TP25.99 mAb and SFR.B6 mAb. After two washes with PBS-NCS, cells were incubated for 30 min on ice with an appropriate dilution of fluoroscein isiothiocyanate-conjugated IgG of sheep mouse Ig specific antibodies (Silenus Laboratories, Hawthorn, Australia). Cells were then washed three times with PBS-NCS and the fluorescence intensity was measured using a FACScan. RMA-S-B*3501 and RMA-S-B*3501–116Y cultured at 26 °C or at 37 °C and stained with mAb under the same experimental conditions were used as controls.

Analysis of peptide binding

Peptides at a concentration of 10^{-4} M giving more than 25% of the mean fluorescence intensity (MFI) of RMA-S-B*3501 and B*3501–116Y were evaluated as binding peptides. MFI of the RMA-S transfectants was obtained by subtracting the MFI value of the RMA-S transfectants incubated at 37 °C for 3 h and stained with a mixture of TP25.99 and SFR8.B6 mAb from the MFI value of the RMA-S transfectants cultured at 26 °C stained with TP25.99 mAb and SFR8-B6 mAb. The half-maximal binding level (BL_{50}) which is the peptide concentration yielding the half-maximal MFI was calculated using the LEAST program (Biomedical Research Center, Olympus Optical Corp., Hachioji, Tokyo, Japan). Binding peptides were classified according to the BL_{50} into three categories: high binder ($BL_{50} < 10^{-5}$ M), medium binder ($10^{-5} > BL_{50} < 10^{-4}$ M) and low binder ($BL_{50} < 10^{-4}$ M). High, medium, low, and non-binders were then assigned the ranks 3, 2, 1, and 0, respectively. Mean binding rank (MBR) of a group of peptides was calculated. For example, two high binders, one medium binder, and one non-binder yield the rank 8. The MBR of these peptides is 8/4 = 2.00.

Results

*Stabilization assay of HLA-B*3501–116Y molecules using RMA-S transfectants*

RMA-S-hβ2m cells expressing HLA-B*3501–116Y (RMA-S-B*3501–116Y) were generated by the transfection of the genes encoding HLA-B*3501–116Y mutant into RMA-S-hβ_2m cells.The surface expression of HLA-B*3501–116Y molecules on the transfectants was examined using TP25.99 anti-HLA class I α3 domain mAb after the cells were cultured at 37 °C or at 26 °C overnight. The expression of HLA-B*3501–116Y molecules on the transfectants

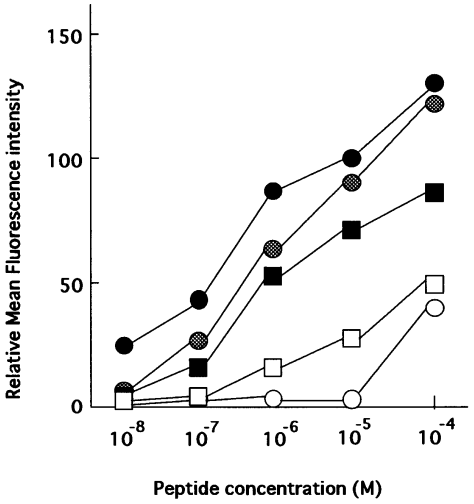

Fig. 1 The binding of nonamer peptides carrying two anchor residues, Pro at P2 and Tyr, Ile or Leu at P9 to HLA-B*3501–116Y molecules. Binding of nonamer peptides, EPILRSLAY (○), IPIKSQWTI (●), WPLYQNEGL (▒), YPLTFGWCF (■), and RPQVPLRPM (□) to HLA-B*3501–116Y molecules was tested using RMA-S-B*3501–116Y

cultured at 26 °C was higher than that on the transfectants cultured at 37 °C and it was almost identical to that of HLA-B*3501 on RMA-S-B*3501 cells cultured at 26 °C (data not shown). Similar to HLA-B*3501 surface expression on RMA-S-B*3501 cells, HLA-B*3501–116Y molecules which are induced at 26 °C disappeared rapidly after the transfectants were incubated at 37 °C (data not shown). We concluded from these results that the RMA-S-B*3501–116Y transfectants can be used for a stabilization assay under the same condition as previously described for RMA-S-B*3501 cells. Indeed, some nonamer peptides carrying Pro at P2 and Ile, Leu, Met, Tyr or Phe at P9 stabilized HLA-B*3501–116Y molecules in a peptide-dose dependent fashion (Fig. 1).

Table 1 Binding of nonamer peptides carrying Tyr at position 9 to HLA-B*3501 and B*3501-116Y molecules

Peptide	Binding rank (BL_{50})	
	B*3501	B*3501-116Y
NPDIVIYQY	H (1.9×10^{-7})	N
NPEIVIYQY	H (2.8×10^{-7})	N
SPSSTPLLY	H (9.9×10^{-8})	N
VPKVSGLQY	H (1.2×10^{-6})	N
VPGAAYALY	H (1.4×10^{-7})	N
VPTSTIVQY	H (3.2×10^{-7})	N
LPIWARPDY	H (3.7×10^{-7})	L ($>10^{-3}$)
DPCSLQCPY	H (6.4×10^{-6})	L ($>10^{-3}$)
EPILRSLAY	H (2.3×10^{-6})	L ($>10^{-3}$)
SPPSTPLLY	H (3.1×10^{-8})	L ($>10^{-3}$)
TPITIPMEY	H (4.1×10^{-7})	M (1.2×10^{-5})
MPLANNSEY	H (2.1×10^{-7})	M (1.0×10^{-6})
NPAQEDDQY	L (1.6×10^{-4})	N
TPGQEITQY	L ($>10^{-3}$)	N
QPGYPWPLY	L ($>10^{-3}$)	N

H: High binder, M: Medium binder, L: Low binder, N: Non-binder

Reduction of the binding of nonamer peptides carrying Tyr at the C-terminus by the substitution of Tyr for Ser at residue 116

Twenty-one nonamer peptides carrying Tyr at the C-terminus were tested for their binding to both HLA-B*3501 and HLA-B*3501–116Y. Sixteen of these peptides were previously shown as HLA-B*3501 binding peptides (Schönbach et al. 1995). Twelve and three peptides belonged to high and low binders, respectively (Table 1). Of the twelve high binders for HLA-B*3501, ten peptides showed low or no binding to HLA-B*3501–116Y molecules, while two peptides revealed reduced affinity to the mutant molecules

Fig. 2A–C The effect of the substitution of Tyr for Ser at residue 116 on the binding of nonamer peptides carrying Tyr, Ile, or Leu at P9. Binding of nonamer peptides **A** EPILRSLAY, **B** EPGFGQSLL, and **C** LPPTTGPPI to HLA-B*3501 molecules (●) was compared with that of these peptides to HLA-B*3501–116Y (○). % expression = (MFI of the RMA-S tranfectants pulsed with each peptide/MFI of the RMA-S transfectants cultured at 26 °C) × 100

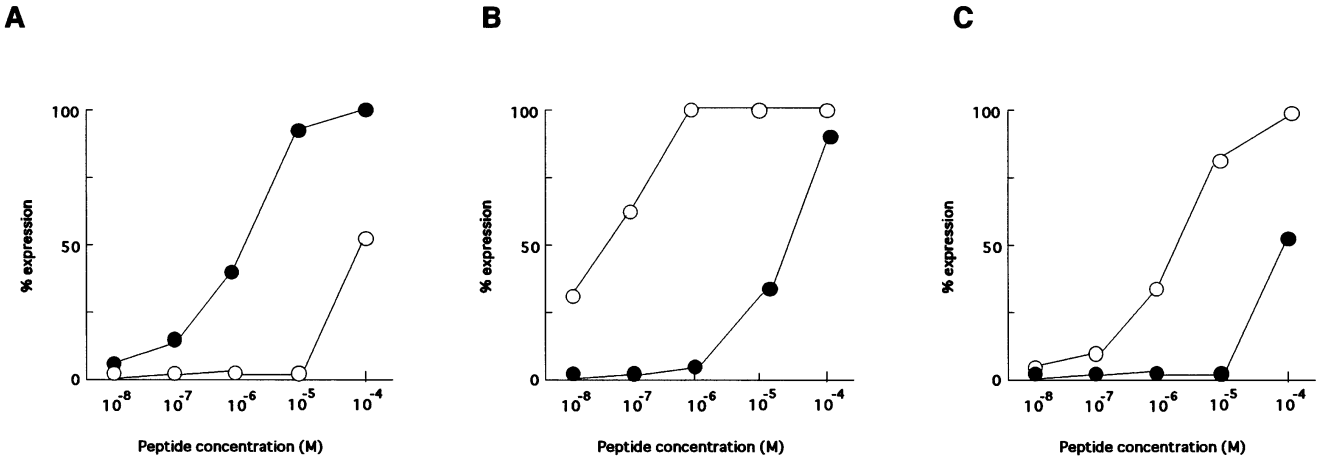

Table 2 Binding of nonamer peptides carrying Leu at position 9 to HLA-B*3501 and B*3501-116Y molecules

Peptide	Binding rank (BL_{50})	
	B*3501	B*3501-116Y
QPDKSESEL	N	H (4.1×10^{-6})
KPCVKLTPL	N	H (7.4×10^{-6})
SPGQRVEFL	N	H (2.2×10^{-6})
FPGFGQSLL	N	H (2.3×10^{-8})
QPSGPWKTL	N	H (7.3×10^{-6})
PPQEEGEPL	N	M (1.4×10^{-5})
TPKDKTKWL	N	M (4.0×10^{-5})
DPPQPEYPL	N	L (3.4×10^{-4})
TPWPTSQGL	L (1.9×10^{-4})	H (6.5×10^{-8})
SPPITWPLL	L ($>10^{-3}$)	H (1.6×10^{-6})
VPLKRLEEL	L ($>10^{-3}$)	H (8.3×10^{-6})
RPIVSTQLL	L ($<10^{-3}$)	H (3.9×10^{-7})
TPQDLNTML	M (6.0×10^{-5})	H (4.3×10^{-7})
IPLTEEAEL	M (2.2×10^{-5})	H (6.1×10^{-7})
SPLTTQNTL	H (3.1×10^{-6})	H (2.7×10^{-7})
WPLYGNEGL	H (4.8×10^{-6})	H (1.8×10^{-7})
HPNIEEVAL	H (2.5×10^{-7})	H (4.5×10^{-8})
EPEPDVAVL	H (2.6×10^{-7})	H (3.3×10^{-10})
TPIPAASQL	H (4.9×10^{-7})	H (3.3×10^{-8})
TPLKNTSVL	H (2.4×10^{-7})	H (1.7×10^{-8})
RPDYNPPLL	H (9.5×10^{-6})	H (2.7×10^{-7})
DPNPQEVVL	H (2.8×10^{-8})	H (5.3×10^{-7})
YPLASLKSL	H (1.3×10^{-6})	H (1.6×10^{-6})
TPAETTVRL	H (2.2×10^{-8})	H (5.4×10^{-7})
IPAASQLDL	H (3.0×10^{-7})	H (2.6×10^{-7})
YPLTSLRSL	H (9.2×10^{-7})	H (2.3×10^{-6})

H: High binder, M: Medium binder, L: Low binder, N: Non-binder

(Table 1, Fig. 2A). Three low binders for HLA-B*3501 did not bind to HLA-B*3501–116Y molecules (Table 1). These results suggest that the substitution at residue 116 of HLA-B*3501 affects the binding of the peptides carrying Tyr at the C-terminus.

Enhancement of the binding of nonamer peptides carrying aliphatic hydrophobic residues Ile and Leu by the substitution of Tyr for Ser at residue 116

Thirty-six nonamer peptides carrying Leu at the C-terminus were tested for the binding to HLA-B*3501–116Y molecules. Eighteen peptides bound to HLA-B*3501 molecules (Table 2), while 18 peptides did not bind to these molecules (Table 3). Eight nonbinders for HLA-B*3501 bound to HLA-B*3501–116Y molecules. Four low binders and two medium binders for HLA-B*3501 showed high affinity for HLA-B*3501–116Y, while seven high binders for HLA-B*3501 displayed increased affinity for HLA-B*3501–116Y (Table 2, Fig. 2B). On the other hand, two high binders for HLA-B*3501 revealed the same affinity to HLA-B*3501–116Y and three high binders for HLA-B*3501 showed slightly reduced affinities to the mutant HLA-B*3501 molecules (Table 2).

Similarly, sixteen nonamer peptides carrying Ile at the C-terminus were tested for the binding to HLA-B*3501–116Y molecules. Only five peptides bound to HLA-B*3501 molecules (Table 4). Four of these HLA-B*3501 binding peptides bound to HLA-B*3501–116Y molecules with the same affinity. On the other hand, seven peptides that were not capable of binding to HLA-B*3501 bound to HLA-B*3501–116Y (Table 4, Fig. 2C). These results suggest that the substitution of Tyr for Ser at residue 116 enhances the binding of nonamer peptides carrying Leu and Ile.

Minimum effect of the substitution of Tyr for Ser at residue 116 on the binding of nonamer peptides carrying Met or Phe

We tested eight nonamer peptides carrying Phe and ten nonamer peptides carrying Met at the C-terminus. Six peptides carrying Phe and four peptides carrying Met at the C-terminus bound to HLA-B*3501 molecules (Table 3). These HLA-B*3501 binding peptides also bound to HLA-B*3501–116Y molecules, but the effect of the substitution at residue 116 on the binding of each peptide varied

Table 3 Binding of nonamer peptides carrying anchor residues at positions 2 and 9 to HLA-B*3501 and B*3501-116Y molecules

Amino acids at position 9	Binding molecules	Binding rank				Total	MBR*
		High	Medium	Low	No		
Y	B*3501	12**	0	3	5	20	1.95
	B*3501-116Y	1	1	4	14	20	0.45
F	B*3501	3	1	2	2	8	1.63
	B*3501-116Y	2	1	3	2	8	1.38
L	B*3501	12	2	4	18	36	1.22
	B*3501-116Y	23	2	1	10	36	2.06
I	B*3501	5	0	0	14	19	0.79
	B*3501-116Y	8	3	1	7	19	1.63
M	B*3501	2	1	1	6	10	0.90
	B*3501-116Y	1	1	4	4	10	0.90
Total	B*3501	34	4	10	45	93	1.29
	B*3501-116Y	35	8	13	37	93	1.44

* Mean Binding Rank
** Number of peptides

Table 4 Binding of nonamer peptides carrying Ile at position 9 to HLA-B*3501 and B*3501-116Y molecules

Peptide	Binding rank (BL_{50})	
	B*3501	B*3501-116Y
LPPVVAKEI	N	H (4.5×10^{-6})
QPRGRRQPI	N	H (2.2×10^{-6})
LPPTTGPPI	N	H (2.7×10^{-6})
NPPIPVGEI	N	H (1.4×10^{-6})
TPSQKQEPI	N	M (3.4×10^{-5})
CPKVSFEPI	N	M (2.4×10^{-5})
DPGLADQLI	N	L ($>10^{-3}$)
YPCTVNFTI	H (3.8×10^{-7})	H (6.1×10^{-7})
IPTSGDVVI	H (4.7×10^{-7})	H (2.0×10^{-7})
LPCRIKQII	H (2.3×10^{-6})	H (1.4×10^{-6})
IPIKSQWTI	H (3.9×10^{-7})	H (2.1×10^{-7})
LPALSTGLI	H (1.1×10^{-6})	M (1.4×10^{-5})

H: High binder, M: Medium binder, L: Low binder, N: Non-binder

Table 5 Binding of nonamer peptides carrying Phe and Met at position 9 to HLA-B*3501 and B*3501-116Y molecules

Peptide	Binding rank (BL_{50})	
	B*3501	B*3501-116Y
APPEESFRF	H (5.8×10^{-6})	M (5.2×10^{-5})
LPGCSFSIF	M (6.5×10^{-5})	H (3.3×10^{-6})
YPLTFGWCF	H (2.8×10^{-7})	H (6.7×10^{-7})
LPKLPGVPF	H (3.4×10^{-7})	L (2.5×10^{-4})
DPEIVMHSF	L ($>10^{-3}$)	L ($>10^{-3}$)
SPPAVPQTF	L ($>10^{-3}$)	L ($>10^{-3}$)
APPPSWDQM	N	L ($>10^{-3}$)
SPAIFQSSM	H (4.1×10^{-6})	L (1.6×10^{-4})
GPAATLEEM	H (7.2×10^{-7})	M (1.0×10^{-5})
VPVKLKPGM	N	L ($>10^{-3}$)
RPQVPLRPM	L ($>10^{-3}$)	L ($>10^{-3}$)
TPEGIIPSM	M (1.7×10^{-5})	H (4.9×10^{-8})

H: High binder, M: Medium binder, L: Low binder, N: Non-binder

(Table 5). These results suggest that the substitution of Tyr for Ser at residue 116 has a minimum effect on the binding of peptides carrying Phe and Met at the C-terminus.

Confirmation of the effect of the substitution at residue 116 on the binding of nonamer peptides carrying five hydrophobic residues by using a set of nonamer peptides mutated at P9

In order to confirm the effect of the substitution of Tyr for Ser at the residue 116 on the binding of peptides carrying the hydrophobic residue at P9, we tested the binding of four sets of nonamer peptides mutated at P9 to HLA-B*3501 and HLA-B*3501–116Y molecules (Fig. 3). NPDIVIYQY effectively bound to HLA-B*3501, while the binding of this peptide to HLA-B*3501–116Y was critically reduced. The mutated peptides NPDIVIYQL and NPDIVIYQI showed weak binding to HLA-B*3501 (BL_{50}: 1.8×10^{-4}, $>10^{-3}$, respectively), while the affinity of these peptides to HLA-B*3501–116Y was very high (BL_{50}: 5.3×10^{-8}, 8.9×10^{-7}, respectively) (Fig. 3A). The mutated peptides NPDIVIYQF and NPDIVIYQM revealed about 10-fold higher affinity to HLA-B*3501–116Y than to HLA-B*3501. Similar results were observed in three other peptides. The peptides carrying Tyr at P9 showed high affinity for HLA-B*3501 but very low affinity for HLA-B*3501–116Y (Fig. 3B–D). The binding of the peptides carrying Leu or Ile at P9 to HLA-B*3501–116Y is much higher than that to HLA-B*3501 (Fig. 3B–D). On the other hand, the effect of the substitution of Tyr for Ser at residue 116 on the peptides carrying Phe or Met varied (Fig. 3B–D). Thus, these results confirmed that the substitution of Tyr for Ser at residue 116 affects the binding of nonamer peptides carrying Tyr at P9, while it enhances that of nonamer peptides carrying Leu or Ile at P9.

*The substitution of Tyr for Ser at residue 116 is not responsible for low-affinity binding of peptides to HLA-B*5101 molecules*

The substitution of Tyr for Ser at residue 116 of HLA-B*3501 drastically reduced the binding of nonamer peptides carrying Tyr at the C-terminus and enhanced the binding of nonamer peptides carrying Ile and Leu at the C-terminus (Table 3, Fig. 3). These results suggest that the structure of the F-pocket is critical for C-terminal anchor residues of HLA-B*3501 and HLA-B*5101 binding peptides. Previous studies showed that the affinity of HLA-B*5101 binding peptides is much weaker than that of HLA-B*3501 (Sakaguchi et al. 1997). In fact, the HLA-B*3501–116Y binding peptides carrying Leu or Ile at the C-terminus shown in Tables 2 and 4 were previously tested for the binding to HLA-B*5101 molecules (Kikuchi et al. 1996). Only one (YPLASLKSL) of 26 peptides carrying Leu at the C-terminus and seven of twelve peptides carrying Ile at the C-terminus weakly bound to HLA-B*5101. Since both HLA-B*3501 and HLA-B*5101 binding peptides carry the same anchor residues at position 2, it is thought that the structural difference of the F-pocket between HLA-B*3501 and HLA-B*5101 determines the general affinity of peptides to these HLA class I molecules. However, there was no difference between MBR of 93 peptides for HLA-B*3501 and that for HLA-B*3501–116Y (Table 3). Thus, the results of the present study strongly suggest that the substitution of Tyr for Ser at residue 116 is not solely responsible for the general affinity difference seen between HLA-B*3501 and HLA-B*5101.

Discussion

HLA-B*3501 and HLA-B*5101 binding peptides have similar anchor residues at position 2 and the C-terminus, but at the C-terminus Tyr is a stronger anchor for HLA-B*3501 than aliphatic hydrophobic residues (Schönbach et al. 1995, 1996). HLA-B*5101 molecules failed to bind

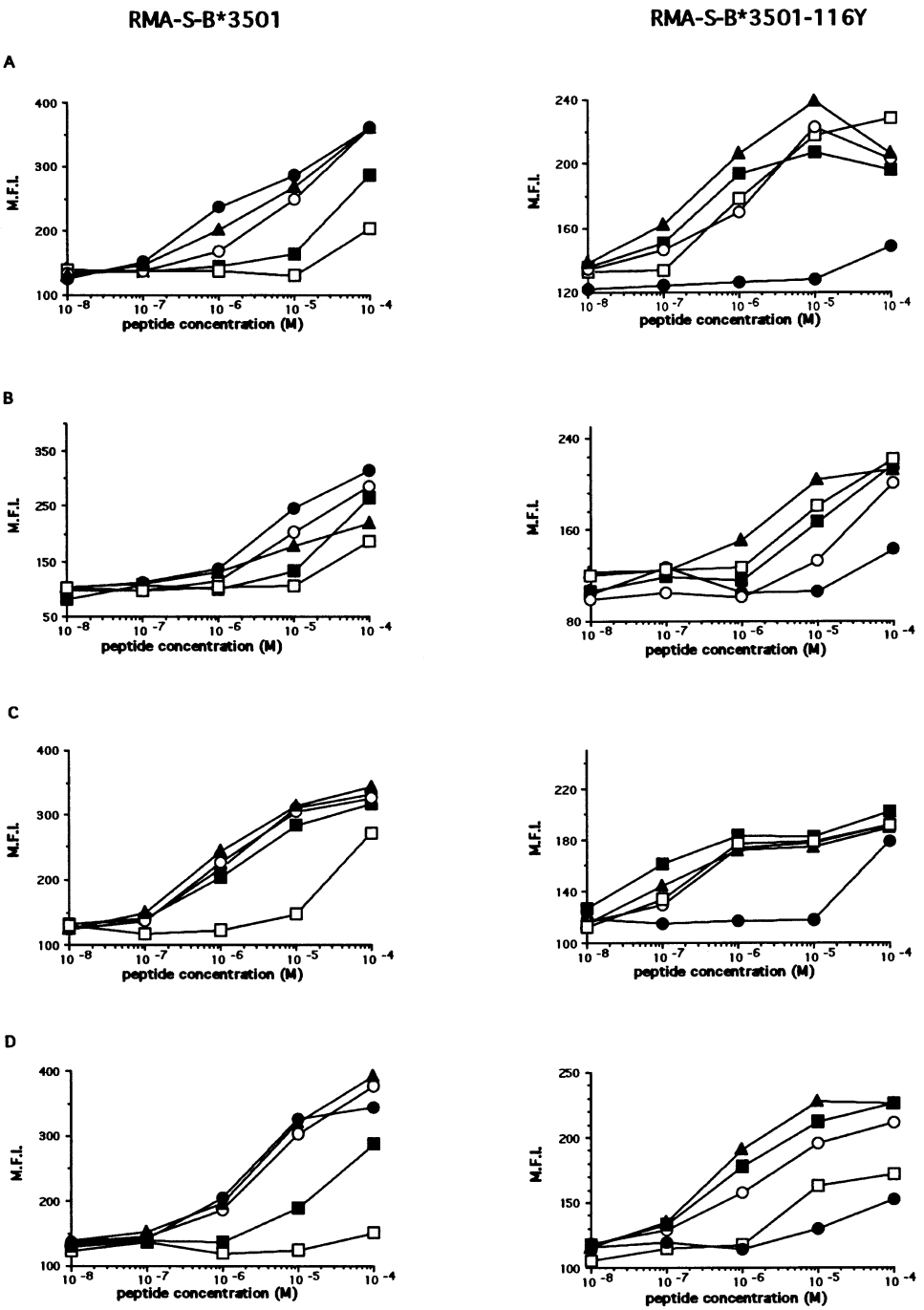

Fig. 3A–D The effect of substitution of Tyr for Ser at residue 116 on the binding of nonamer peptides mutated at P9. Binding of four sets of nonamer peptides **A** NPDIVIYQY, **B** RPIVSTQLL, **C** IPLTEEAEL, **D** LPPVVAKEI carrying mutations at the C-terminus to Tyr (●), Phe (○), Leu (■), Ile (□) or Met (▲) to HLA-B*3501 molecules was compared to that of these peptides to HLA-B*3501–116Y molecules. BL_{50} of each peptide for HLA-B*3501 (S) and HLA-B*3501–116Y (Y) is as follows. (A) NPDIVIYQY (S:6.0 × 10^{-7}, Y:> 10^{-3}), NPDIVIYQF (S:3.2 × 10^{-6}, Y:3.8 × 10^{-7}), NPDIVIYQL (S:1.8 × 10^{-4}, Y:5.3 × 10^{-8}), NPDIVIYQI (S: > 10^{-3}, Y:8.9 × 10^{-7}) and NPDIVIYQM (S:1.3 × 10^{-6}, Y:6.8 × 10^{-8}), (B) RPIVSTQLY (S:1.6 × 10^{-6}, Y:> 10^{-3}), RPIVSTQLF (S:4.7 × 10^{-6}, Y:1.3 × 10^{-4}), RPIVSTQLL (S:8.8 × 10^{-4}, Y:6.4 × 10^{-6}), RPIVSTQLI (S:> 10^{-3}, Y:5.5 × 10^{-6}) and RPIVSTQLM (S:5.1 × 10^{-5}, Y:1.6 × 10^{-6}). (C) IPLTEEAEY (S:8.6 × 10^{-7}, Y:> 10^{-3}), IPLTEEAEF (S:7.4 × 10^{-7}, Y:7.1 × 10^{-7}), IPLTEEAEL (S:1.1 × 10^{-6}, Y:1.7 × 10^{-8}), IPLTEEAEI (S:> 10^{-3}, Y:4.3 × 10^{-7}) and IPLTEEAEM (S:4.6 × 10^{-7}, Y:3.1 × 10^{-7}), (D) LPPVVAKEY (S:7.5 × 10^{-7}, Y:> 10^{-3}), LPPVVAKEF (S:1.3 × 10^{-6}, Y:8.8 × 10^{-6}), LPPVVAKEL (S:3.6 × 10^{-5}, Y:4.2 × 10^{-7}), LPPVVAKEI (S:> 10^{-3}, Y:3.2 × 10^{-6}) and LPPVVAKEM (S:8.5 × 10^{-7}, Y:2.8 × 10^{-7})

peptides carrying Tyr at the C-terminus but are capable of binding peptides carrying aliphatic hydrophobic residues Ile, Leu, and Val at the C-terminus (Kikuchi et al. 1996; Sakaguchi et al. 1997). These findings suggest that the structural difference of the F-pocket between HLA-B*3501 and HLA-B*5101 determines the C-terminal residues of peptides bound to these HLA class I molecules. The three-dimensional structure of the HLA-B*3501 molecule suggests that the specificity of HLA-B*3501 F-pocket for Tyr of the C-terminus arises from the ability of the Tyr hydroxyl group to form hydrogen bonds to both Ser at residue 116 and Tyr at residue 74 (Smith et al. 1996), implying that the substitution at residue 116 between HLA-B*3501 and HLA-B*5101 determines the specificity of the C-terminus binding peptides, since Tyr at residue 74 is conserved between these HLA class I molecules. Moreover, previous pool sequencing studies of peptides eluted from HLA-B*3503 molecules showed that the peptide motif of HLA-B*3503 differs from that of HLA-B*3501 in the predominant occurrence of Tyr as the C-terminal anchor (Steinle et al. 1995), although both HLA-B35 alleles differ by a single amino acid substitution (Ser and Phe for HLA-B*3501 and HLA-B*3503, respectively) at residue 116. These studies strongly suggest that residue 116 determines

the C-terminal anchor residue of HLA-B35 and B51 binding peptides. The present study confirmed this concept by showing that the specificity of the C-terminus of binding peptides is altered by the substitution of Tyr for Ser at residue 116 of HLA-B*3501.

Sakaguchi and co-workers (1997) showed that the affinity of HLA-B*5101 binding peptides is much weaker than that of HLA-B*3501 binding peptides. HLA-B*5101 and HLA-B*3501 differ by four substitutions on the α_1 domain, which are associated with Bw4/Bw6 epitopes, and eight substitutions on the α_2 domain (Hayashi et al. 1989; Ooba et al. 1989). Since anchor residues at position 2 and the C-terminus are the most critical sites for the peptide binding to HLA-B*3501 and HLA-B*5101 molecules and the B-pocket of HLA-B*3501 is identical to that of HLA-B*5101, it is assumed that the interaction of the C-terminal anchor residues with the F-pocket determines the affinity of peptides to both HLA class I molecules. Consequently, both the residues associated with Bw4/Bw6 epitopes and residue 116 on the floor of the peptide binding groove forming the F-pocket are candidates which determine the affinity of peptides. The present study showed that the substitution of Tyr for Ser at residue 116 does not influence the general affinity of peptides to HLA-B*3501 molecules, suggesting that the substitutions associated with the HLA-Bw4/Bw6 epitopes in the α_1 helix may influence the affinity of peptides. Since HLA-B*3501 and B*5301 differ by the HLA-Bw4/Bw6 epitopes in the a_1 domain but are identical in the a_2 domain, the comparison of B*3501 and B*5301 peptide binding should clarify the role of the HLA-Bw4/Bw6 epitope in the affinity determination of peptides. Another possibility is that the A-, D-, and E-pocket-forming residues (residues 94, 95, 97, 104, 114, 152, and 171) of the a_2 helix affect the general affinity of peptide binding. Substitutions of the pocket forming residues might alter the specificity of secondary anchor residues and the affinity of binding.

It is well known that some HLA class I alleles are associated with several diseases. HLA-B27 is strongly associated with ankylosing spondylitis (Brewerton et al. 1973). A recent study demonstrated that one HLA-B27 subtype, *HLA-B*2709*, is not associated with this disease (D'Amato et al. 1995). *HLA-B*2709* differs from the most frequent and disease-associated *HLA-B*2705* allele by a single amino acid substitution of His for Asp at residue 116 which controls the specificity of the C-terminal anchor of the binding peptides. *HLA-B*2705* bound nonamer peptides carrying Arg and Tyr at position 9, whereas *HLA-B*2709* failed to bind these peptides. On the other hand, the *HLA-B*2702* allele which is associated with ankylosing spondylitis does not bind peptides carrying Arg at the C-terminus (Rötzschke et al. 1994). Taken together, these studies suggest that Tyr is a good candidate for the C-terminal residue of the disease-triggering peptide(s). HLA-B51 (B*5101 and B*5102) is associated with Behçet disease whereas HLA-B35 and B53 are not associated with this disease (Ohno et al. 1973, 1978; Mizuki et al. 1993, 1994). Both HLA-B*5101 and HLA-B*3501 bind the peptides carrying similar anchor residues at position 2 and the C-terminus. The HLA-B*3501 but not the HLA-B*5101 molecule accepts peptides carrying Tyr at the C-terminus. These findings imply that the Behçet disease-triggering peptide(s) might carry aliphatic hydrophobic residues at the C-terminus.

In the present study we demonstrated that the residue 116 critically influence the C-terminus of binding peptides to HLA-B*3501 and HLA-B*5101, while it does not determine the general affinity of binding peptides. Further studies of peptide binding to HLA-B*5301 and HLA-B*3501 mutants carrying mutations at residues facing the A-, D-, and E-pockets as well as those investing differences in stability of the HLA class I molecules and the association with β_2m are expected to clarify the reasons why the affinity of HLA-B*5101 binding peptides tends to be much lower than that of HLA-B*3501 binding peptides.

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