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Binding of 8-mer to 11-mer peptides carrying the anchor residues to slow assembling HLA class I molecules (HLA-B*5101)

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Abstract The binding of 303 8-mer to 11-mer peptides carrying the anchor residues at P2 and the C-terminus to HLA-B*5101 molecules was examined by a stabilization assay in which peptides were incubated with RMA-S-B*5101 cells at 26 °C for 3 h. Analysis of the binding of these peptides to HLA-B*5101 molecules showed that Pro and Ala at P2, and Ile, Val, and Leu at the C-terminus functioned as anchor residues, while Gly at P2 and Met at the C-terminus were weak anchors. Pro was a stronger anchor residue than Ala at P2, while Ile was the strongest anchor at the C-terminus. Among 8-mer to 11-mer peptides, the 9-mer peptides showed the strongest binding to HLA-B*5101 molecules. This is in contrast to our recent findings that 10-mer and 11-mer peptides bind to HLA-B*3501 molecules as effectively as 9-mer peptides. Since both HLA class I molecules have the same B-pocket and the binding peptides carry the same anchor residues, it is assumed that the structure of the F-pocket may restrict the length of binding peptides. The ability of HLA-B*5101 binding peptides to stabilize the HLA-B*5101 molecules was markedly lower than that of HLA-B*3501 binding peptides to stabilize the HLA-B*3501 molecules. It is known that HLA-B*5101 is a slow assembling molecule, while HLA-B*3501 assembles rapidly. The results imply that the slow assembling of HLA-B*5101 molecules results from the low affinity of peptides to HLA-B*5101 molecules.

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Introduction

Recent studies of the sequencing 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 an allele-specific motif 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). Most peptides bound to MHC class I molecules are 9-mer, while 8-mer, 10-mer, and 11-mer peptides are also capable of binding to the MHC class I molecules (Parker et al. 1992; Guo et al. 1992; Takamiya et al. 1994). Studies of the binding of HLA-A2 molecules (Ruppert et al. 1993; Parker et al. 1994) and HLA-B35 molecules (Schönbach et al. 1995) with chemically synthesized peptides carrying anchor residues have shown that not only the anchor residues are critical for MHC class I-peptide binding, but nonanchor residues also have an important role in MHC class I-peptide binding.

HLA-B51 and HLA-B35 molecules form part of a serologically cross-reactive group called HLA-B5, B35 CREG including HLA-B52, HLA-B53, and HLA-B78 (Tait et al. 1992). These HLA-B5, B35 CREG molecules are also cross-recognized by some alloreactive T cells (Matsumoto et al. 1990). HLA-B51 and B35 molecules differ by five amino acids associated with HLA-Bw4/Bw6 public epitopes and eight amino acids in the α2 domain (Hayashi et al. 1989; Ooba et al. 1989). Since the B- and F-pockets of the HLA-B51 molecules are similar to those of the HLA-B35 molecules, it is suspected that self-peptides bound to both HLA class I molecules have similar anchor residues. In fact, they share the same anchor residues, Pro and Ala, at position 2 (P2), and Ile, Leu, and Met at position 9 (P9) (Falk et al. 1993, 1995). In contrast, the functional differences between these two molecules are known to some extent. The former assembles very rapidly, while the assembly rate of the latter is slow (Hill et al. 1993).

Behçet's disease is strongly associated with HLA-B51 but not with HLA-B35 and B52 (Ohno et al. 1973, 1978; Baricordi et al. 1986), though the pathogenesis of this disease remains unknown. It is speculated that the presentation of a particular peptide by HLA-B51 molecules to T cells might initiate the onset of Behçet's disease. Therefore, it is expected that analyses of HLA-B51 binding peptides might contribute to studies clarifying the pathogenesis of the disease.

We have recently established the HLA-B51 stabilization assay using the RMA-S-transfectant expressing HLA-B*5101 molecules and have shown that P2 and P9 of 9-mer peptides are the anchor residues and that the affinity of 9-mer peptides to HLA-B*5101 molecules is markedly weaker than that of HLA-B*3501 binding peptides (Kikuchi et al. 1996). In the present study, we attempted to improve the binding assay for HLA-B*5101 molecules in order to accurately evaluate the affinity of peptides to HLA-B*5101 molecules. Furthermore, we investigated the binding of 8-mer to 11-mer peptides to HLA-B*5101 molecules by the stabilization assay modified for HLA-B*5101 molecules.

Materials and methods

Cells

RMA-S cells expressing human β_2-microglobulin (β_2 m; RMA-S-h β_2m) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). RMA-S cells expressing HLA-B*5101 (RMA-S-B*5101) were previously generated (Nakayama et al. 1994). These cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml hygromycin B.

Peptides

Sequences derived from human immunodeficiency virus type-1 (HIV-1: Env, Gag, Pol, Nef, and Vif), and hepatitis C virus (HCV: C, E1, E2/NS1, NS2, NS3, NS4, and NS5), were screened for Pro, Ala, and Gly at P2, and Leu, Ile, Val, Met, and Phe at the C-terminus of 8-mer to 11-mer peptides. The peptides were prepared using an automated multiple peptide synthesizer, Shimadzu Model PSSM-8, with the Fmoc strategy followed by cleavage as previously described (Nokihara et al. 1992). Peptides were shown to be homogeneous by liquid secondary ion mass spectrometry and reverse-phase high pressure liquid chromatography.

Peptide binding assay by flow cytometry

The binding of peptides to HLA-B51 molecules was tested as previously described (Kikuchi et al. 1996). Briefly, RMA-S-B*5101 cells were cultured at 26 °C for 18–24 h. Cells (2×10^5) in 50 µl of phosphate buffered saline (PBS) supplemented with 20% FCS (PBS-FCS) were incubated at 26 °C for 3 h with 50 µl of a solution of peptides at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M and then at 37 °C for 3 h. In this condition, protease activity of FCS was ruled out (Kikuchi et al. 1996). After washing with PBS-FCS, cells (2×10^5) were incubated for 30 min on ice with an appropriate dilution of TP25.99 anti-HLA class I α3 domain monoclonal antibody (mAb; D'Urso et al. 1991; Tanabe et al. 1992). After two washes with PBS-FCS, cells were incubated for 30 min on ice with an appropriate dilution of fluorescein isothiocyanate-conjugated IgG of sheep mouse-specific Ig antibodies (Silenus Laboratories, Hawthorn, Australia). Cells were then washed three times with PBS-FCS and the fluorescence intensity was measured using a FACScan. RMA-S-B*5101 cultured at 26 °C and at 37 °C, and stained with mAb TP25.99 under the same experimental conditions were used as controls.

*Analysis of peptide binding to HLA-B*5101 molecules*

Peptides at a concentration of 10^{-3} M giving more than 25% of the mean fluorescence intensity (M. F. I.) of RMA-S-B*5101 cells cultured at 26 °C were evaluated as binding peptides. The relative M. F. I. of RMA-S-B*5101 cells was obtained by subtracting the M. F. I. value of peptide-unloaded RMA-S-B*5101 cells stained with TP25.99 mAb from the M. F. I. value of peptide loaded RMA-S-B*5101 cells stained with TP25.99 mAb. The half-maximal binding level (BL_{50}) which is the peptide concentration yielding the half-maximal M. F. I. was calculated. Binding peptides were classified according to the BL_{50} into three categories: high binder ($BL_{50} \leqq 10^{-4}$M), medium binder ($10^{-4} < BL_{50} \leqq 10^{-3}$ M) and low binder ($BL_{50} > 10^{-3}$ M). High, medium, low, and nonbinders were then given the ranks 3, 2, 1, and 0, respectively. The mean binding rank (MBR) of these peptides was calculated.

Nonparametric two group unpaired t-test

The mean binding rank of peptides carrying Pro and Ala at P2, or Ile, Leu, and Val at the C-terminus for each length was judged by the *t*-test (StatView 4.02; Abacus Concepts, Berkeley, CA). The test compares

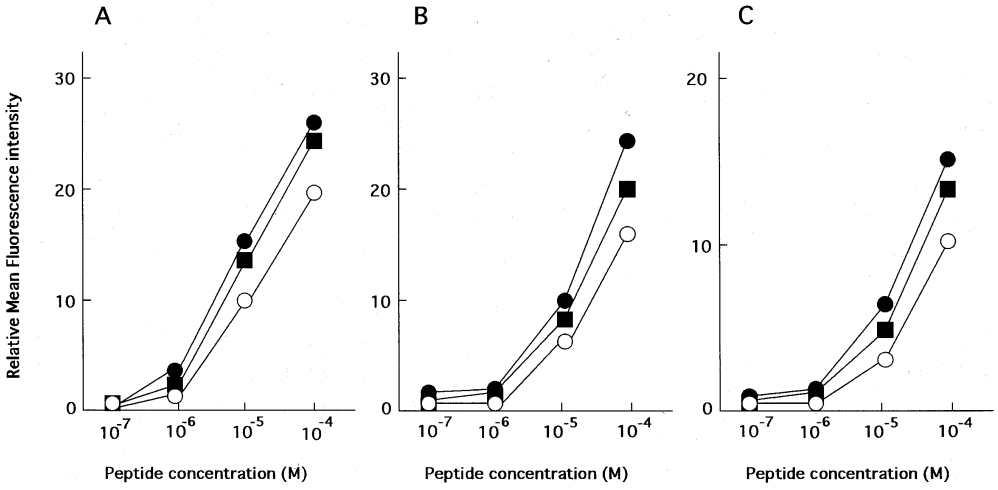

Fig. 1A–C Peptide binding to HLA-B*5101 molecules with different incubation times at 26 °C. Binding of three 9-mer peptides. **A** FPISPIETV, **B** LPPVVAKEI, and **C** DARAYDTEV to HLA-B*5101 molecules was tested with 1 h (○), 2 h (■), or 3 h (●) incubation at 26 °C. BL_{50} of each peptide is as follows: EPISPIETV: 5.5×10^{-5} (1 h), 1.3×10^{-5} (2 h), 9.4×10^{-6} (3 h), LPPVVAKEI: 1.4×10^{-4} (1 h), 2.9×10^{-4} (2 h), 1.9×10^{-5} (3 h), DARAYDTEV: $>10^{-3}$ (1 h), $>10^{-3}$ (2 h), 2.1×10^{-4} (3 h)

Table 1 Effect of an amino acid at position 2 of 8-mer, 9-mer, 10-mer, and 11-mer peptides on their binding to HLA-B*5101 molecules

	8-mer		9-mer		10-mer		11-mer		Total	
Amino acid at position 2	N.B.P.*1/ N.T.P.*2	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR
P	–	–	21/78 (26.9%)	0.60	4/25 (16.0%)	0.32	5/32 (15.6%)	0.22	30/135 (22.2%)	0.46
A	7/44 (16.0%)*3	0.34	6/21 (28.6%)	0.48	2/32 (6.3%)	0.06	3/28 (10.7%)	0.18	18/125 (14.4%)	0.27
G	–	–	2/43 (4.7%)	0.05	–	–	–	–	2/43 (4.7%)	0.05
Total	7/44 (16.0%)	0.34	29/142 (20.4%)	0.42	6/57 (10.5%)	0.12	8/60 (13.3%)	0.20	50/303 (16.5%)	0.32

*1 No. binding peptides
*2 Total No. of peptides
*3 Percentage of binding peptides

the ranks of peptides carrying anchor residues at P2 or the C-terminus among 8-mer to 11-mer peptides.

Results

*Enhancement of the peptide binding to HLA-B*5101 molecules with a long incubation time at 26 °C*

Our previous HLA class I stabilization assay for HLA-B*3501 and B*5101 molecules using RMA-S transfectants expressing HLA-B*3501 and B*5101 molecules was carried out under the following experimental conditions (Takamiya et al. 1994; Schönbach et al. 1995; Kikuchi et al. 1996): RMA-S transfectants cultured at 26 °C overnight were incubated with peptides at 26 °C for 1 h and then at 37 °C for 3 h before the cells were stained with antibodies. These studies showed that the affinity of peptides to HLA-B*5101 is markedly weaker than that to HLA-B*3501 molecules (Kikuchi et al. 1996). If the association of peptides with HLA-B*5101 molecules is dependent on the incubation time, the stabilization of HLA-B*5101 molecules may be restored in a longer incubation time at 26 °C. In order to clarify the relationship between the stabilization of HLA-B*5101 molecules and the incubation time at 26 °C, we tested the stabilization of HLA-B*5101 molecules by three 9-mer peptides in a 1 h to 3 h incubation time. As shown in Figure 1, the stabilization of HLA-B*5101 molecules was enhanced depending on the incubation time at 26 °C. BL_{50} was increased 6–10-fold at 3 h incubation compared with BL_{50} at 1 h incubation. On the other hand, incubation for longer than 3 h markedly decreased the viability of RMA-S-B*5101. Therefore, we re-estimated the binding of peptides to HLA-B*5101 molecules by the stabilization assay using the experimental condition of 3 h incubation at 26 °C.

*Effect of length of peptides on the peptide binding to HLA-B*5101 molecules*

We tested the binding of 303 peptides (44 8-mer, 142 9-mer, 57 10-mer, and 60 11-mer peptides) which carry Pro, Ala, or Gly at P2, and Leu, Ile, Val, Met, or Phe at the C-terminus to HLA-B*5101 molecules using the HLA-B*5101 stabilization assay. Twenty-nine of 142 9-mer peptides (20.4%) bound to HLA-B*5101 molecules (Table 1). The frequency of binding peptides was almost the same as that (18.4%) in a previous study (Kikuchi et al. 1996) using the 1 h incubation time at 26 °C. In contrast, MBR (0.42) of these peptides was much higher than that (0.22) measured in the previous study using the 1 h incubation time at 26 °C (Kikuchi et al. 1996). These results indicate that the affinity of peptides was evaluated to be higher, but the frequency of binding peptides to HLA-B*5101 molecules was not increased in the stabilization assay using the longer incubation time.

The sequence analysis of eluted self-peptides from HLA-B*3501 molecules revealed that HLA-B*3501 molecules are able to bind 10-mer peptides carrying Tyr at the C-terminus (Falk et al. 1993). Furthermore, a recent study (Schönbach et al. 1996) has shown that 10-mer and 11-mer peptides carrying anchor residues effectively bind to HLA-B*3501 molecules. In contrast, the study of eluted self-peptides from HLA-B*5101 molecules demonstrated that HLA-B*5101 binding peptides are mainly 9-mer peptides (Falk et al. 1995), suggesting that the binding of 10-mer and 11-mer peptides to HLA-B*5101 molecules is much weaker than that of 9-mer peptides. In order to clarify the binding of longer peptides to HLA-B*5101 molecules, we tested whether 10-mer and 11-mer peptides carrying anchor residues can effectively bind to HLA-B*5101 molecules. Of 57 10-mer peptides and 60 11-mer peptides carrying Pro or Ala at P2, and Ile, Leu, Val, Met, or Phe at the C-terminus, six and eight peptides (10.5% and 13.3%) bound to HLA-B*5101 molecules, respectively (Table 1). The MBR of 10-mer and 11-mer peptides was 0.12 and 0.20, respectively. These values were lower than those (20.4% and 0.42) of 9-mer peptides.

Furthermore, we tested the binding of 44 8-mer peptides carrying Ala at P2 to HLA-B*5101 molecules and compared it with that of longer peptides. Seven of 44 peptides bound to HLA-B*5101 molecules (Table 1). The frequency of binding peptides (16.0%) and the MBR of 8-mer peptides (0.34) was higher than those of 10-mer (6.3% and 0.06) and 11-mer peptides carrying Ala at P2 (10.7% and 0.18), but lower than 9-mer peptides carrying Ala at P2 (28.6% and 0.48). These results show that 8-mer peptides carrying anchor residues can effectively bind to HLA-B*5101 molecules though their affinity is slightly weaker than that of 9-mer peptides.

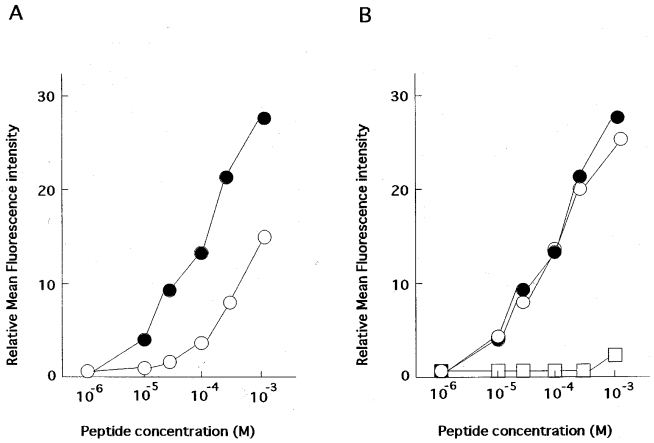

Fig. 2 A, B Binding of mutated peptides at anchor residues to HLA-B*5101 molecules. **A** Binding of mutated peptide LACRIKQII (○) to HLA-B*5101 molecules was compared with that of LPCRIKQII (●). **B** Binding of mutated peptides LPCRIKQIV (○) and LPCRIKQIL (□) to HLA-B*5101 molecules was compared with that of LPCRIKQII (●)

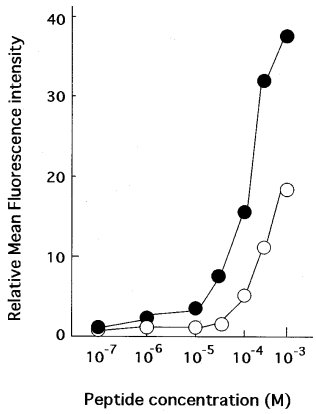

Fig. 3 Leu at P9 but no Phe at P10 of 10-mer peptide YPLTSLRSLF functions as an anchor residue. Binding of the two peptides YPLTSLRSLF (○) and YPLTSLRSL (●) to HLA-B*5101 molecules was examined at different peptide concentrations

Role of anchor residues at P2

The binding frequency and MBR of 9-mer peptides carrying Gly was much lower than that of peptides carrying Pro and Ala (Table 1), indicating that Gly at P2 is a weak anchor residue, while Pro and Ala are strong anchor residues. Since Gly at P2 is a weak anchor residue for HLA-B*5101 molecules, we attempted to re-evaluate the effect of the length of peptides on the peptide binding to HLA-B*5101 molecules by 9-mer to 11-mer peptides carrying Pro and Ala at P2. Statistical analysis showed that the affinity of 10-mer and 11-mer peptides is significantly weaker than that of 9-mer peptides ($P < 0.05$; data not shown).

Analyses of the binding data of 9-mer to 11-mer peptides showed that Pro at P2 is a stronger anchor residue than Ala at P2 (Table 1). In order to confirm this, we generated mutated peptides of LPCRIKQII. The substitution of Ala for Pro at P2 significantly decreased their binding to HLA-B*5101 molecules (Fig. 2 A). Taken together, these results reveal that at P2, Pro is a stronger anchor residue than Ala.

Role of anchor residues at the C-terminus

The effect of five amino acids at P9 on the binding of 9-mer peptides to HLA-B*5101 molecules was evaluated (Table 2). Ile at P9 is the most favored residue for binding to HLA-B*5101 molecules. This was observed in both 9-mer peptides carrying Pro and Ala at P2 (Table 3). Similarly, 9-mer peptides carrying Val at P9 effectively bound to HLA-B*5101 molecules. In contrast, the affinity of 9-mer peptides carrying Leu or Met at P9 was weak. These findings were consistent with a previous study (Kikuchi et al. 1996) in which the binding of 9-mer peptides to HLA-B*5101 molecules was measured using the 1 h incubation time at 26 °C. Furthermore, we investigated the role of three aliphatic hydrophobic residues, Ile, Val, and Leu, at P9 using mutated 9-mer peptides of LPCRIKQII. The substitution of Leu for Ile at P9 significantly decreased their binding to HLA-B*5101 molecules, while the substitution of Val did not affect the binding to HLA-B*5101 molecules (Fig. 2 B). The results confirmed that Ile and Val are stronger anchor residues for HLA-B*5101 than Leu at P9 of 9-mer peptides.

Similarly, 9-mer peptides, 10-mer, and 11-mer peptides carrying Ile at the C-terminus most effectively bound to HLA-B*5101 molecules (Table 2). In particular, 10-mer

Table 2 Effect of an amino acid at the C-terminus of 8-mer, 9-mer, 10-mer, and 11-mer peptides on their binding to HLA-B*5101 molecules

	8-mer		9-mer		10-mer		11-mer		Total	
Amino acid at C-terminus 3	N.B.P.*1/ N.T.P.*2	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR	N.B.P./ N.T.P.	MBR
I	9/9 (33.3%)*3	0.78	18/42 (42.8%)	0.90	3/19 (15.8%)	0.26	6/22 (27.3%)	0.36	30/92 (32.6%)	0.63
V	3/10 (30.0%)	0.50	6/35 (17.1%)	0.34	1/15 (6.7%)	0.07	1/8 (12.5%)	0.13	11/68 (16.2%)	0.31
L	1/15 (6.9%)	0.20	4/32 (12.5%)	0.28	1/12 (8.3%)	0.25	1/16 (6.3%)	0.19	7/75 (9.3%)	0.24
M	0/3 (0%)	0	1/18 (5.6%)	0.11	0/2 (0%)	0	0/4 (0%)	0	1/27 (3.7%)	0.07
F	0/7 (0%)	0	0/15 (0%)	0	1/9 (11.1%)	0.11	0/10 (0%)	0	1/41 (2.4%)	0.02
Total	7/44 (16.0%)	0.34	29/142 (20.4%)	0.42	6/57 (10.5%)	0.12	8/60 (13.3%)	0.20	50/303 (16.5%)	0.32

*1 No. binding peptides
*2 Total No. of peptides
*3 Percentage of binding peptides

Table 3 Effect of amino acids at both position 2 and the C-terminus of 8-mer, 9-mer, 10-mer, and 11-mer peptides on their binding to HLA-B*5101 molecules

Amino acid at position 2/ C-terminus	8-mer N.B.P.*1/ N.T.P.*2	MBR	9-mer N.B.P./ N.T.P.	MBR	10-mer N.B.P./ N.T.P.	MBR	11-mer N.B.P./ N.T.P.	MBR	Total N.B.P./ N.T.P.	MBR
P/I	–	–	12/18 (66.7%)	1.50	2/8 (25.0%)	0.50	4/8 (50.0%)	0.50	18/34 (52.9%)	1.06
P/V	–	–	4/10 (40.0%)	0.80	–	–	–	–	4/10 (40.0%)	0.80
P/L	–	–	4/32 (12.5%)	0.28	1/12 (8.3%)	0.25	1/16 (6.3%)	0.19	6/60 (10.0%)	0.25
P/M	–	–	1/9 (11.1%)	0.22	0/2 (0%)	0	0/4 (0%)	0	1/15 (6.7%)	0.13
P/F	–	–	0/9 (0%)	0	1/3 (33.3%)	0.30	0/4 (0%)	0	1/16 (6.3%)	0.06
A/I	3/9 (33.3%)*3	0.78	4/8 (50.0%)	1.00	1/11 (9.1%)	0.09	2/14 (11.8%)	0.29	10/42 (23.8%)	0.57
A/V	3/10 (30.0%)	0.50	2/7 (28.6%)	0.57	1/15 (6.7%)	0.08	1/8 (12.5%)	0.13	7/40 (17.5%)	0.28
A/L	1/15 (6.7%)	0.20	–	–	–	–	–	–	1/15 (6.7%)	0.20
A/M	0/3 (0%)	0	0/3 (0%)	0	–	–	–	–	0/6 (0%)	0
A/F	0/7 (0%)	0	0/3 (0%)	0	0/6 (0%)	0	0/6 (0%)	0	0/22 (0%)	0
G/I	–	–	2/16 (12.5%)	0.13	–	–	–	–	2/16 (12.5%)	0.13
G/V	–	–	0/18 (0%)	0	–	–	–	–	0/18 (0%)	0
G/M	–	–	0/6 (0%)	0	–	–	–	–	0/6 (0%)	0
G/F	–	–	0/3 (0%)	0	–	–	–	–	0/3 (0%)	0
Total	7/44 (16.0%)	0.34	29/142 (20.4%)	0.42	6/57 (10.5%)	0.12	8/60 (13.3%)	0.20	50/303 (16.5%)	0.32

*1 No. binding peptides
*2 Total No. of peptides
*3 Percentage of binding peptides

Table 4 Binding of 8-mer to 11-mer peptides carrying Tyr at the C-terminus to HLA-B*5101 molecules

Peptides	Peptide concentration (μM) 1000	333	100	33.3	10	1
LPCRIKQII	36.9*	29.6	18.8	12.3	5.9	0.9
YPYRLWHY	0	0	0	0	0.2	0.1
TPGPGIRY	0	0	0.8	0	1.0	1.0
LPIWARPDY	0	0	0.4	0	0.8	0.5
TPPLVKLWY	0	0	0	0	0.2	0.2
LPQAVMGSSY	0	0	0.3	0	0.9	0.6
VPLDEDFRKY	0	0	0	0	0	0
TPCTCGSSDLY	0	0	0.5	0	1.0	0.7
IPAETGQETAY	0	0	0	0	0	0

* Relative M.F.I. = M.F.I. value of peptide loaded RMA-S-B*5101 cells – M.F.I. value of peptide unloaded RMA-S-B*5101 cells

and 11-mer peptides carrying Pro at P2 and Ile at the C-terminus effectively bound to HLA-B*5101 molecules (Table 3). Similarly, 8-mer peptides carrying Ile and Val at the C-terminus could effectively bind to HLA-B*5101 molecules, while the affinity of 8-mer peptides carrying other residues was weak (Tables 2 and 3). Taken together, the results demonstrated that the aliphatic hydrophobic residues Ile, Val, and Leu at the C-terminus of 8-mer to 11-mer peptides function as anchor residues for HLA-B*5101 molecules.

Of 40 peptides carrying Phe at the C-terminus, only one (YPLTSLRSLF) bound to HLA-B*5101 molecules (Table 2). Since the binding of 9-mer peptide YPLTSLRSL to HLA-B*5101 molecules is much stronger than that of 10-mer peptide YPLTSLRSLF (Fig. 3), it is suggested that Leu at P9 has a positive effect on the peptide binding to HLA-B*5101 molecules while Phe at P10 of YPLTSLRSLF has a negative effect on the peptide binding. Previous study of X-ray crystallography of HLA-A*0201 carrying 10-mer peptide MLLSVPLLLG which contains the C-terminal anchor residue Leu at P9 showed that the C-terminal residue Gly positions out the peptide binding site and Leu at P9 occupies the F-pocket (Collins et al. 1994). It is speculated that YPLTSLRSLF also binds to HLA-B*5101, with the C-terminal residue pointing out the peptide binding site. Another possibility is that nonanchor residues of this peptide contribute to the binding to HLA-B*5101 molecules more than the C-terminal anchor residue. Similarly, only one (VPVKLKPGM) of 27 peptides carrying Met at the C-terminus bound to HLA-B*5101 molecules (Table 3). These results strongly suggest that Phe at the C-terminus is not an anchor for HLA-B*5101, while Met at the C-terminus is a very weak anchor.

Tyr at the C-terminus is a strong anchor for HLA-B*3501 molecules (Takamiya et al. 1994; Schönbach et al. 1995, 1996). On the other hand, it is assumed that Tyr is not an anchor residue for HLA-B*5101 molecules because Tyr is not detected at the C-terminus of self-peptides eluted from HLA-B*5101 molecules. We examined whether Tyr in the C-terminus is an anchor for HLA-B*5101 molecules. As shown in Table 4, 8-mer to 11-mer peptides carrying Tyr at the C-terminus did not bind to HLA-B*5101 molecules although these peptides are HLA-B*3501 binding peptides (Schönbach et al. 1995). These results indicate that the F-pocket of HLA-B*5101 molecules does not favor Tyr at the C-terminus of peptides.

Statistical analysis showed that the binding of 10-mer and 11-mer peptides carrying Ile at the C-terminus was significantly lower than that of 9-mer peptides carrying Ile ($P < 0.005$), while there is no significant difference in the binding between 8-mer and 9-mer peptides carrying Ile at the C-terminus, and between 9-mer and shorter or longer

peptides carrying Val or Leu at the C-terminus (data not shown). Thus, HLA-B*5101 molecules favor most 8-mer and 9-mer peptides carrying Ile at the C-terminus.

Discussion

A previous study demonstrated that HLA-B51 molecules assemble very slowly while the assembling of HLA-B35 molecules is rapid (Hill et al. 1993). Although the molecular mechanism of fast and slow assembling remains unknown, it is speculated that the affinity of peptides to HLA class I molecules in the ER is related to the speed of assembling. Namely, the weak binding of peptides to HLA-B51 molecules results in slow assembling, while the fast assembling of HLA-B35 molecules results from the high affinity of peptides to B35 molecules in the ER. In a previous study using the HLA-B35 stabilization assay with RMA-S-B*3501 cells (Schönbach et al. 1995), a 10-fold higher threshold was employed for the classification of HLA-B*3501 binding peptides and, moreover, a short incubation time (1 h) at 26 °C was used. Therefore, the MBR for HLA-B*3501 was calculated to be about 50 times lower than that for HLA-B*5101 in the present study. The MBR of 9-mer peptides for HLA-B*3501 is 1.42 (Schönbach et al. 1995), while that for HLA-B*5101 is 0.42. This means that the affinity of HLA-B*5101 binding peptides is about 500 times lower than that of HLA-B*3501 molecules. However, further studies to investigate the relationship between the affinity of peptides and the speed of assembling of other HLA class I molecules may be necessary to determine whether the affinity of peptides to HLA class I molecules in the ER determines the assembling speed of HLA class I molecules.

Previous studies (Falk et al. 1993, 1995) of the sequencing of self-peptides eluted from HLA-B*3501 and B*5101 molecules have shown that the small residues, Pro, Ala, and Gly are predominantly observed at P2 of self-peptides bound to B*5101 molecules, while only Pro is predominantly found at P2 of self-peptides bound to HLA-B*3501 molecules, and Ala is weakly detected at P2. These studies suggested that Pro, Ala, and Gly at P2 are strong anchor residues for HLA-B*5101, while Pro at P2 is a stronger anchor than Ala for HLA-B*3501. In the present study we revealed that 22.2% of 135 peptides carrying Pro at P2 bound to HLA-B*5101 molecules, while only 14.4% of 125 peptides carrying Ala at P2 and 4.7% of 43 peptides carrying Gly at P2 were HLA-B*5101 binding peptides. Similarly, our recent study has shown that 60.6% of 249 9-mer to 11-mer peptides carrying Pro at P2 and 27.5% of 69 peptides of 9-mer to 11-mer carrying Ala at P2 were evaluated as HLA-B*3501 binding peptides (Schönbach et al. 1996). Thus, the peptide binding assay showed that Pro is a stronger anchor than Ala at P2 for both HLA-B*3501 and HLA-B*5101 molecules, while Gly is a weak anchor residue. The discrepancy of the results between the sequencing of self-peptides and the direct analysis of peptide binding remains unknown. However, since there is no amino acid substitution at the residues forming the B-pocket between HLA-B*3501 and HLA-B*5101, it is suspected that both HLA class I molecules have the same B-pocket and favor the same anchor residues at P2.

At the C.-terminus, aromatic hydrophobic residues Tyr an Phe were stronger anchor residues for HLA-B*3501 than aliphatic hydrophobic residues (Falk et al. 1993; Schönbach et al. 1995), while only aliphatic hydrophobic residues are strong anchors for HLA-B*5101. These results imply that the F-pocket of HLA-B*5101 molecules is relatively shallow, while HLA-B*3501 has a deep F-pocket. In the residues forming the F-pocket, only one substitution is observed at residue 116 between HLA-B*3501 and HLA-B*5101 (Ooba et al. 1989). Ser in HLA-B*3501 is substituted to Tyr in HLA-B*5101. These findings strongly suggest that a shallow F-pocket of HLA-B*5101 is generated by the substitution of Tyr for Ser.

Our new recent studies showed that 10-mer and 11-mer peptides bind to HLA-B*3501 molecules as effectively as 9-mer peptides (Schönbach et al. 1996). In contrast, the affinity of 10-mer and 11-mer peptides to HLA-B*5101 molecules was significantly lower than that of 9-mer peptides. Previous analyses of self-peptides bound to HLA-B*3501 and B*5101 molecules demonstrated that Tyr was observed at P10 of B*3501 binding self-peptides, while no signal was detected at P10 of B*5101 binding self-peptides, suggesting that HLA-B*3501 is able to bind effectively peptides longer than 9-mer, while HLA-B*5101 molecules favor 9-mer peptides. The present study supported this idea. These studies together suggest that the shallow F-pocket of HLA-B*5101 molecules favors aliphatic hydrophobic residues at the C-terminus rather than aromatic hydrophobic residues, while HLA-B*3501 carrying a deep F-pocket selects aromatic hydrophobic residues at the C-terminus. The strong binding of the C-terminus of peptides into the F-pocket of HLA-B*3501 molecules may permit longer peptides to bind in a form that bulges out. A decrease of the affinity of longer peptides to HLA-B*5101 may result from weaker binding of the C-terminus of peptides into the F-pocket of HLA-B*5101 molecules.

Since the affinity of peptides to HLA-B51 molecules is very weak and the assembling of HLA-B51 molecules is very slow, it is hypothesized that HLA-B51 molecules rarely present various antigens to T cells. In fact, there is only one report of CTL epitopes presented by HLA-B51 molecules (Koziel et al. 1993). However, this may result from the fact that HLA-B51-restricted CTL epitopes have not been intensively studied. Further attempts to identify and characterize HLA-B51-restricted CTL epitopes should be very important in determining the role of slow and fast assembling of HLA class I molecules in antigen presentation.

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