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BRIEF COMMUNICATION

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Predominant role of N-terminal residue of nonamer peptides in their binding to HLA-B* 5101 molecules

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Previous studies on the binding of HLA class I molecules to chemically synthesized peptides carrying primary anchor residues (Ruppert et al. 1993; Parker et al. 1994; Schönbach et al. 1995, 1996) have shown that not only are primary anchor residues critical for major histocompatibility complex (MHC) class I peptide binding, but also that secondary residues play an important role in MHC class I peptide binding. Our recent studies (Schönbach et al. 1995, 1996) have demonstrated that in addition to two primary anchor residues at position 2 (P2) and the C-terminus, secondary anchor residues can be identified by statistical analysis. However, there are other methods to analyze the effect of secondary anchors on MHC class I peptide binding, as shown by Udaka and co-workers (1995).

In a previous study using the HLA-B*5101 stabilization assay (Kikuchi et al. 1996), we failed to clarify the role of secondary anchor residues of HLA-B*5101 binding peptides by statistical analysis because the ability of HLA-B*5101 binding peptides to stabilize HLA-B*5101 molecules is very weak. We therefore improved the stabilization assay for HLA-B*5101 binding peptides by extending the incubation time as follows: RMA-S-B*5101 cells cultured at 26 °C for 18–24 h were incubated at 26 °C for 3 h with various concentrations of peptides followed by a 3 h incubation at 37 °C. The cells were then stained with TP25.99 HLA class I α3 domain specific monoclonal antibody (mAb) (D'Urso et al. 1991: Tanabe et al. 1992) and FITC-conjugated IgG of sheep mouse-specific Ig antibodies. The mean linear fluorescence intensity (MFI) of the cells was measured by using a FACScan. The relative MFI was obtained by subtracting the MFI of cells not loaded with peptide and stained with TP25.99 mAb from the MFI of peptide-loaded cells stained with TP25.99 mAb. The affinity of a peptide was represented by the half-maximal binding level (BL_{50}) which is the peptide concentration yielding the half-maximal mean fluorescence intensity. Binding peptides were classified according to the BL_{50} into three categories: high binder ($BL_{50} \leq 10^{-4}$ M), medium binder ($10^{-4} < BL_{50} \leq 10^{-3}$ M), and low binder (10^{-3} M $< BL_{50}$). High, medium, low, and nonbinders were given ranks 3, 2, 1, and 0, respectively, and the mean binding rank (MBR) was calculated.

We tested 127 nonamer peptides (Sakaguchi et al. 1997) carrying the anchor residues at P2 (Pro, Ala, and Gly) and P9 (Ile, Val, Leu and Met) which were selected from the sequence of the SF2 strain of human immunodeficiency virus-1 (Sanchez-Pescador et al. 1985) and the JT strain of hepatitis C virus protein (Tanaka et al. 1992). The MBR of these peptides increased from 0.22 to 0.42 by the improved stabilization assay (Sakaguchi et al. 1997). Subsequently we conducted an analysis of these nonamer peptides to determine secondary anchor residues which contribute to the HLA-B*5101-peptide interaction. The frequency of binding peptides and the MBR was calculated for each amino acid or groups of amino acids at each non-primary anchor position (positions 1, 3, 4, 5, 6, 7, and 8) in a Mann-Whitney U-test (Table 1). Positive effects on the peptide binding to HLA-B*5101 molecules were found for aromatic (Tyr, Phe, Trp, and His) and aliphatic (Leu, Val, Ile, and Met) hydrophobic residues at P1 ($P < 0.01$). Likewise, small amino acids, Ala and Gly at P6, significantly enhanced the binding to HLA-B*5101 molecules ($P < 0.05$). In contrast, negative effects on the peptide binding to HLA-B*5101 molecules were observed for Gly and Ala at P1 ($p < 0.05$).

In order to confirm the effect of the secondary anchor residues, the binding of peptides mutated at P1 and P6 was tested. The substitution of hydrophobic residues Tyr and Leu for Asn at P1 of NPPIPVGEI increased the binding to HLA-B*5101 molecules. Moreover, two mutations, Tyr and Val for Leu at P1 of LPCRIKQII did not affect the binding

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Table 1 Effect on nonanchor residues in peptide binding to HLA-B*5101 molecules

Amino acid	Position of amino acid in peptides						
	1	3	4	5	6	7	8
Y,F,W,H	6/10[1] (60.0%[2]) 1.60 *[3]	2/10 (20.0%) 0.30	1/11 (9.1%) 0.09	3/15 (20.0%) 0.40	2/7 (28.6%) 0.43	2/12 (16.7%) 0.33	1/4 (25.0%) 0.50
L,V,I,M	12/23 (52.3%) 1.09*	8/26 (30.8%) 0.73	8/27 (29.6%) 0.56	7/23 (30.4%) 0.78	5/36 (13,9%) 0.31	7/20 (35.0%) 0.65	7/24 (29.2%) 0.67
S,TÇ	2/19 (10.5%) 0.21	5/18 (27.7%) 0.61	5/18 (27.8%) 0.72	5/19 (26.3%) 0.63	4/12 (33.3%) 0.58	1/17 (5.9%) 0.18	7/21 (33.3%) 0.71
P	0/7 (0%) 0	5/11 (45.5%) 1.18	2/11 (18.2%) 0.45	3/10 (30.0%) 0.80	1/ 7 (14.3%) 0.43	4/ 9 (44.4%) 0.78	3/11 (27.3%) 0.73
G,A	1/26 (3.8%) 0.04**	3/23 (13.0%) 0.22	4/15 (26.7%) 0.67	4/24 (16.7%) 0.25	9/18 (50.0%) 1.00**	3/17 (17.6%) 0.47	4/22 (18.2%) 0.27
Q,N	4/12 (33.3%) 0.50	2/ 8 (25.0%) 0.38	0/10 (0%) 0	0/5 (0%) 0	3/17 (17.6%) 0.41	2/11 (18.2%) 0.55	1/16 (6.3%) 0.19
R,K	2/20 (10.0%) 0.50	3/20 (15.0%) 0.30	8/24 (33.3%) 0.58	2/21 (9.5%) 0.10	3/17 (17.6%) 0.41	8/22 (36.4%) 0.68	2/15 (13.3%) 0.13
D,E	2/10 (20.0%) 0.60	1/11 (9.1%) 0.09	1/11 (9.1%) 0.27	5/10 (50.0%) 0.90	2/13 (15.4%) 0.38	2/19 (10.5%) 0.26	4/14 (28.6%) 0.64

[1] No. of binding peptides / Total No. of peptides
[2] Percentage of binding peptides
[3] MBR (Mean Binding Rank): MBR of the peptides carrying the corresponding amino acid(s) at each position was calculated
* $P < 0.01$ ** $P < 0.05$

Table 2 Effect of amino acid substitution at P1 on peptide binding to HLA-B*5101 molecules

Peptide	Peptide concentration (μM)						
	1000	333	100	33	10	1	BL_{50}
NPPIPVGEI	22.3*	19.3	9.0	3.7	0.8	0	1.9×10^{-4}
YPPIPVGEI	37.3	33.0	29.3	22.4	14.9	4.6	1.1×10^{-5}
LPPIPVGEI	33.7	30.8	24.8	18.5	10.8	3.2	2.3×10^{-5}
DARAYDTEV	22.9	21.5	15.9	9.5	3.8	0.6	7.6×10^{-5}
YARAYDTEV	25.8	23.7	15.3	9.9	4.3	0.9	6.9×10^{-5}
LARAYDTEV	26.0	22.8	13.1	7.9	3.4	0.4	1.0×10^{-4}
YAPPIGGQI	35.2	34.4	23.1	14.1	8.7	4.1	3.1×10^{-5}
DAPPIGGQI	36.0	34.5	27.7	22.0	16.1	5.0	1.2×10^{-5}
NAPPIGGQI	29.9	27.0	20.8	10.3	8.8	1.9	4.8×10^{-5}
LPCRIKQII	27.9	21.5	13.1	9.0	4.5	0.5	1.2×10^{-4}
YPCRIKQII	30.4	26.9	18.9	13.8	8.5	1.6	4.1×10^{-5}
VPCRIKQII	27.7	22.3	13.0	9.6	4.4	0.6	1.0×10^{-4}
DPCRIKQII	25.0	17.9	10.3	6.4	2.8	0.2	2.2×10^{-4}
PPCRIKQII	0	0	0	0	0	0	–
GPCRIKQII	0	0	0	0	0	0	–
APCRIKQII	0	0	0	0	0	0	–

* Relative MFI = MFI value of peptide loaded RMA-S-B*5101 cells - MFI value of peptide unloaded RMA-S-B*5101 cells

of the peptide to HLA-B*5101 molecules (Table 2). These data support the result of the statistical analysis which predicts that hydrophobic residues at P1 increase the binding of the peptide to HLA-B*5101 molecules.

A pool sequence analysis of HLA-B51 (Falk et al. 1995) showed that in addition to hydrophobic residues, Asp was detected as a preferred residue at P1 of self-peptides eluted from HLA-B*5101 molecules. Therefore, we examined the role of Asp at P1 in the peptide binding to HLA-B*5101 molecules. The affinity of the mutated peptide DPCRIKQII to HLA-B*5101 molecules was almost identical to that of LPCRIKQII (Table 2). A similar finding was observed between DARAYDTEV and its mutants (YARAYDTEV and LARAYDTEV) as well as between YAPPIGGQI and DAPPIGGQI (Table 2). These results indicate that Asp at P1 exerts a positive effect on the peptide binding to HLA-B*5101 molecules, although our analysis of 127 peptides did not show a statistically significant positive effect on the peptide binding to HLA-B*5101 molecules (Table 1).

On the other hand, the substitution of Ala or Gly for Leu at P1 of LPCRIKQII critically affected HLA-B*5101-peptide binding (Table 2), supporting the finding that the small

residues Ala and Gly at P1 have a negative effect on HLA-B*5101 peptide binding. Pro also seems to have a negative effect on HLA-B*5101 peptide binding because none of the seven peptides carrying Pro at P1 bound to HLA-B*5101 molecules (Table 1). Indeed, the substitution of Pro for Leu at P1 of LPCRIKQII critically affected the peptide binding to HLA-B*5101 molecules (Table 2). Taken together, these results indicate that the small residues Ala and Gly as well as Pro at P1 have a negative effect on HLA-B*5101 peptide binding.

The role of Ala and Gly at P6 in peptide binding was investigated by using mutated peptides. The binding of LPCRIKQII and NANPDCKTI to HLA-B*5101 molecules was compared with its mutant peptides at P6. The substitution of Ala for Lys at P6 of LPCRIKQII slightly enhanced the affinity of the peptide to HLA-B*5101 molecules (LPCRIKQII : $BL_{50} = 1.2 \times 10^{-4}$, LPCRIAQII : $BL_{50} = 5.7 \times 10^{-5}$). Conversely, the substitution of Ala and Gly for Cys at P6 of NANPDCKTI slightly decreased the affinity of the peptide for HLA-B*5101 molecules [(NANPDCKTI : $BL_{50} = 8.2 \times 10^{-5}$, NANPDAKTI : $BL_{50} = 1.0 \times 10^{-4}$ and NANPDGKTI : $BL_{50} = 2.0 \times 10^{-4}$) (data not shown)]. Thus, the experiment using the mutated peptides indicated that only occasionally do Ala and Gly at P6 have a positive effect on the peptide binding to HLA-B*5101 molecules. These findings imply that P6 is not a strong anchor and might have little effect on HLA-B*5101 peptide binding. Another possibility is that the efficiency of Ala and Gly at P6 is modified by neighboring amino acids.

Here we demonstrated that hydrophobic residues at P1 enhanced the peptide binding to HLA-B*5101 molecules, whereas Pro and the non-bulky residues Gly and Ala reduced the peptide binding to HLA-B*5101 molecules. P1 interacts with A-pocket residues and is crucial in the hydrogen bonding of amino acid side-chains at the N-terminus (Madden et al. 1991). Therefore, hydrophobic residues, especially bulky ones, seem to favor binding to HLA-B*5101 molecules. The non-polar side-chains of Gly and Ala appear too small to interact with Asn 63 and Tyr 7, while Pro seems to interfere with the hydrogen-bonding due to the restrained, secondary NH_2^+-group. Recent studies of HLA-B*3501 binding peptides also showed that hydrophobic residues at P1 have a positive effect on the binding of 9-mer to 11-mer peptides to HLA-B*3501 molecules (Schönbach et al. 1995, 1996). Since there is only one amino acid substitution (Tyr to His) at residue 171 in the A-pocket between HLA-B*3501 and B*5101, it is assumed that the A-pocket of both HLA class I molecules have similar structures. Therefore, the substitution of P1 may not influence peptide binding. Indeed, our previous study showed that a single amino acid substitution at residue 171 has no effect on the peptide binding to HLA-B*5101 (Kikuchi et al. 1996). The residues forming the A-pocket are conserved in MHC class I molecules, especially in the HLA-B5, B35 cross-reacting group (HLA-B51, B52, B53, B78, and B35), suggesting that hydrophobic residues and Asp at P1 may play an important role in peptide binding to these HLA class I molecules.

In the present study, we analyzed secondary anchor residues of nonamer peptides bound to HLA-B*5101 which is associated with Behçet's disease (Ohno et al. 1973, 1978; Mizuki et al. 1993). Statistical analysis of secondary anchor residues in nonamer peptides showed that P1 plays an import role in the peptide binding to HLA-B*5101 molecules, which was further confirmed by using mutant peptides. However, the statistical analysis failed to identify Asp at P1 as secondary anchor residues, whereas pool sequencing of HLA-B*5101 binding self-peptides suggested a predominant role of Asp at P1 on HLA-B*5101 peptide binding. We analyzed only each of five peptides carrying Asp and Glu at P1. This number was not sufficient to observe a statistically significant effect on HLA-B*5101 peptide binding. An increase in the number of test peptides carrying Asp at P1 should clarify the effect of Asp at P1 by statistical analysis. Thus, the present study suggests that both statistical analysis of the binding of the chemically synthesized peptides to HLA class I molecules and pool sequencing of HLA class I binding self-peptides are required to identify and characterize the primary and secondary anchor residues.

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