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Role of anchor residues in peptide binding to three HLA-A26 molecules

Key words:
HLA-A26; natural ligand; peptide; anchor

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Abstract: To investigate the role of anchor residues in HLA-A26 binding peptides, we analyzed the binding of various peptides to three HLA-A26 molecules using the HLA class I stabilization assay. Of twenty nonamer peptides carrying anchors at P2 and P9, 3, 6 and 3 peptides bound to HLA-A*2601, HLA-A*2602 and HLA-A*2603, respectively. The peptide EV-IPMFSAL bound most strongly to these three HLA-A26 molecules. Analysis using mutants of this peptide at P1, P2 or P9 showed that acidic amino acids at P1 and five hydrophobic residues (Val, Thr, Ile, Leu and Phe) at P2 are anchor residues for the three HLA-A26 molecules while with exception of positively charged amino acids, a broad range of amino acids function as P9 anchor residues. These anchors were further evaluated using 38 nonamer peptides carrying anchor residues at P1, P2 and P9. Nineteen of these peptides bound to at least one HLA-A26 molecule. The frequency of HLA-A26 binding peptides was higher for peptides carrying all three anchor residues than for peptides carrying only P2 and P9 anchor residues. These results indicate that in addition to P2 and P9 anchors, the P1 anchor plays an important role in peptide binding to three HLA-A26 molecules.

HLA-A26 is one of the most common HLA-A alleles in Asia. In Japan, only three subtypes, A*2601, A*2602 and A*2603, have been identified with allele frequencies of 6.2%, 1.3% and 1.3%, respectively (1). These three HLA-A26 molecules differ at residues 74, 76, 77 and 116 (A*2601: D74, A76, N77 and D116; A*2602: D74, A76, N77 and N116; A*2603: H74, V76, D77 and D116) (2). Peptide motifs for HLA-A*2601, HLA-A*2602, and HLA-A*2603 have been shown by pool sequencing of natural ligands (2). HLA-A26 binding peptides carry anchor residues at P2 and the C-terminus with two auxiliary anchors at P1 and P6. Glu was identified as the strongest and Asp as a weaker P1 anchor. Val , Thr, Ile, Leu and Phe were P2 anchor residues. Small differences in P9 anchor preference were identified between the HLA-A26 subtypes: Tyr and Phe for HLA-A*2601 and Tyr, Phe, Met and Leu for HLA-A*2602 and HLA-A*2603.

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Pool sequencing studies do not provide precise information concerning the interaction between HLA class I molecules and binding peptides. Peptide binding assays are required to confirm pool sequencing data and to precisely analyze HLA class I molecule-binding peptide interactions. Indeed, recent studies of peptide binding to HLA class I molecule using the HLA class I stabilization assay with RMA-S transfectants expressing HLA-B*3501 (4, 5), HLA-B*5101 (6, 7), HLA-A*2402 (8) and HLA-A*1101 (9) clarified the details of HLA class I molecule-binding peptide interactions. In the present study, we investigated the role of the anchor residues in peptide binding to HLA-A26 molecules using an HLA-A26 stabilization assay with RMA-S transfectants expressing the three HLA-A26 subtypes and the human β_2 molecule.

Material and methods

Cells

Three HLA-A26 genes (A*2601, A*2602 and A*2603) were transfected by electroporation into human β_2 microglobulin (β_2-m) gene transfected RMA-S(RMA-S-hβ_2m) cells along with the hygromycin-resistant genes. After selection by hygromycin B, hygromycin-resistant cells were isolated from separate wells. The surface expression of HLA-A*26 molecules on transfected cells was confirmed by flow cytometry using anti-HLA class I α_3 domain monoclonal antibody (mAb) TP25.99 (10). RMA-S-hβ_2m cells expressing HLA-A*26 (RMA-S-A*26) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.2 mg/ml hygromycin B.

Peptides

Sequences derived from the human immunodeficiency virus type-I SF2 strain (HIV-1: Env, Gag, Pol, Nef) were screened for HLA-A26 binding peptide motifs. Peptides were synthesized using an automated multiple peptide synthesizer, Shimadzu Model PSSM-8, with the Fmoc strategy followed by cleavage as previously described (11). The peptides were shown to be homogeneous by liquid secondary ionmass spectrometry and reverse-phase high-performance liquid chromatography (HPLC).

Peptide binding assay by flow cytometry

Peptides binding to HLA-A*26 molecules was determined as described previously (12). Briefly, RMA-S-A*26 cells were cultured at 26°C for 14~18 h. Cells (2×10^5) in 50 µl phosphate buffered saline (PBS) supplemented with 20% FCS (PBS-FCS) were incubated with a 50 µl solution of peptides at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M at 26°C for 1 h and then at 37°C for 3 h. After washing with PBS-FCS, the cells were incubated for 30 min on ice with an appropriate dilution of mAb TP25.99. After two washes with PBS-FCS, the cells were incubated for 30 min on ice with an appropriate dilution of fluorescein isothiocyanate conjugated sheep IgG anti-mouse Ig antibody (Silenus Laboratories, Hawthorn, Australia). The cells were washed three times with PBS-FCS and the fluorescence intensity was measured using a FACSCalibur (Becton Dickinson, Mountain View, CA, USA). RMA-S-A*26 cells cultured at 26°C and 37°C were stained with mAb TP25.99 under the same experimental conditions and used as controls.

Analysis of peptide binding to HLA-A*26 molecules

Peptides which at a concentration of 10^{-3} M gave more than 25% of the mean fluorescein intensity (MFI) of RMA-S-A*26 cells were defined as binding peptides. This criterion was previously used for HLA-B*3501- (4) and HLA-A*2402-binding peptides (8). The relative MFI of RMA-S-A*26 cells was calculated by subtracting the MFI value of peptide-unloaded RMA-S-A*26 cells stained with mAb TP25.99 from the MFI value of peptide-loaded RMA-S-A*26 cells stained with mAb TP25.99. The half-maximal binding level (BL_{50}) was calculated as the peptide concentration yielding the half-maximal MFI. Binding peptides were classified into three categories according to the BL_{50}: high binding ($BL_{50}<10^{-5}$ M), medium binding ($10^{-5}\leq BL_{50}<10^{-4}$) and low binding ($BL_{50}\geq10^{-4}$). High-, medium-, low- and non-binding peptides were ranked as 3, 2, 1 and 0, respectively. The mean binding rank (MBR) of a group of peptides was then calculated. For example, if in a group of 10 peptides, three were high binding, one was medium binding, one was low binding and five non-binding, then the MBR is 12/10=1.20. The MBR of each amino acid group and each peptide length were analyzed by the Mann-Whitney U-test (Stat View 4.02; Abacus Concepts, Berkeley, CA, USA).

Results

Generation of characterization of RMA-S transfectants expressing HLA-A26 subtype molecules

Three RMA-S transfectants expressing HLA-A26 subtype genes were generated following separate transfection of HLA-A*2601, HLA-A*2602 and HLA-A*2603 genes into RMA-S-hβ_2m cells. Transfectant cells cultured at 26°C had a much higher HLA-A26 surface expression level than the transfectants cultured at 37°C (Fig.

1). There was little difference in HLA-A26 surface expression between the three transfectants (Fig. 1). HLA-A26 surface expression on the transfectants cultured at 26°C rapidly decreased following incubation at 37°C. There was no significant difference in the decreasing HLA-A26 expression rate between the HLA-A26 subtypes (Fig. 1). Taken together, these results indicate that these transfectants are suitable for use in peptide binding assay using the same conditions as described previously (12). Further, these results suggest that the affinity of peptides to the three HLA-A26 subtypes should be comparable. We therefore employed this method to evaluate peptide binding to the three HLA-A26 molecules.

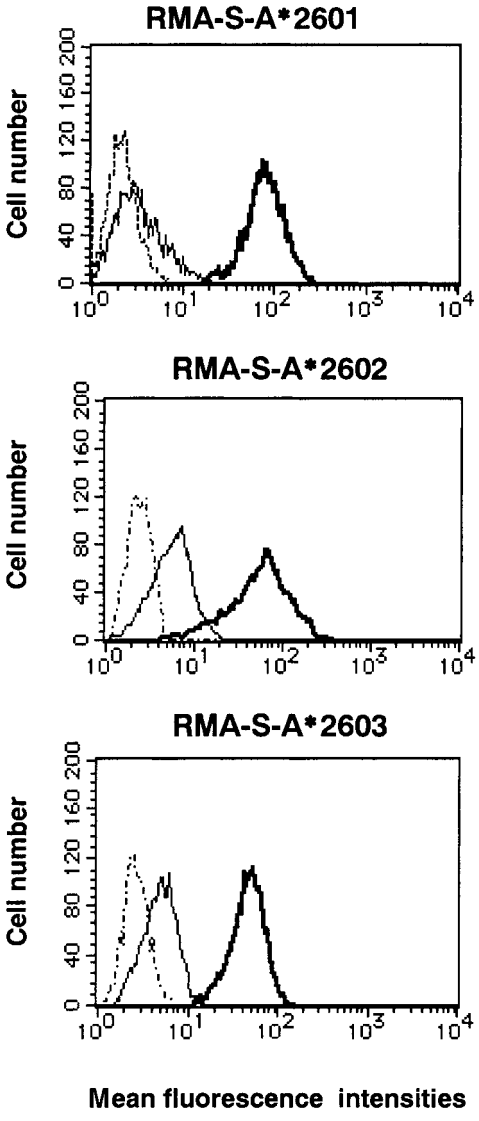

Fig. 1. **Surface expression of HLA-A26 on RMA-S-A26 transfectants.** Transfectant cells were incubated at 26°C overnight and then the cells were stained with TP25.99 mAb and FITC-labeled anti-mouse Ig before (bold line) and after (straight line) 3 h incubation at 37°C. Cells stained with only FITC-labeled anti-mouse Ig (dotted line) were used as a negative control.

Binding of peptides carrying anchor residues at P2 and P9 to three HLA-A26 molecules

We screened the protein sequences derived from the human immunodeficiency virus type-I (HIV-1) SF2 strain for 9-mer peptides with HLA-A26 anchor residues at P2 (Val, Thr, Ile, Leu and Phe) and P9 (Tyr, Phe, Met and Leu). Twenty such peptides were synthesized and analyzed by the stabilization assay. RMA-S-A*26 cells cultured overnight at 26°C were incubated for 1 h with the peptides, and then incubated at 37°C for 3 h. Surface expression of HLA-A26 was examined by a flow cytometry using the anti-HLA class I α_3 domain mAb TP25.99. Of twenty peptides, six bound to HLA-A*2602 while only three bound to HLA-A*2601 and HLA-A*2603 (Table 1). The highest binding peptide EVIPMFSAL has previously been identified as an HLA-A26-restricted HIV-1 epitope (13).

Binding of nonamer peptides to three HLA-A26 molecules

Peptide sequence	Peptide binding affinity (BL_{50})		
	A*2601	A*2602	A*2603
A F S P E V I P M	NB	$\geqq 10^{-4}$	NB
E V I P M F S A L	2.9×10^{-7}	1.8×10^{-7}	5.1×10^{-7}
A T P Q D L N T M	NB	$\geqq 10^{-4}$	NB
D I A G T T S T L	NB	$\geqq 10^{-4}$	NB
T L Q E Q I G W M	$\geqq 10^{-4}$	1.2×10^{-6}	$\geqq 10^{-4}$
E T P G I R Y Q Y	$\geqq 10^{-4}$	$\geqq 10^{-4}$	$\geqq 10^{-4}$
D V K D T K E A L	NB	NB	NB
I V Q N L Q G Q M	NB	NB	NB
V T N P A N I M M	NB	NB	NB
G L N K I V R M Y	NB	NB	NB
N I M M Q R G N F	NB	NB	NB
F L G K I W P S Y	NB	NB	NB
P T A P P E E S F	NB	NB	NB
P L T S L R S L F	NB	NB	NB
D T G A D D T V L	NB	NB	NB
P V N I I G R N L	NB	NB	NB
E L A E N R E I L	NB	NB	NB
I I R D Y G K Q M	NB	NB	NB
T V L D V G D A Y	NB	NB	NB
K I Q N F R V Y Y	NB	NB	NB

NB: not binding

Table 1

Analysis of anchor residues using mutant peptides

In order to investigate the role of the anchor residues in peptide binding to HLA-A26, a panel of peptides based on the sequence EVIPMFSAL with substitutions at P2 or P9 were synthesized and then analyzed by the stabilization assay.

Pool sequencing of HLA-A26 binding self-peptides revealed that in addition to Val, Thr, Ile and Leu, Phe at position 2 is also an anchor residue for HLA-A26. However, analysis of peptides with various mutations at P2 showed that the substitution of Phe for Val remarkably reduced its binding to HLA-A*2601 molecules (Fig. 2). However, one peptide carrying Phe at P2 (AFSPEVIPM) bound weakly to HLA-A*2602 (Table 1). These results suggest that Phe may be a P2 anchor but it is much weaker than other hydrophobic residues. Peptides carrying aromatic hydrophobic residues had much lower affinity to HLA-A*2601 than peptides carrying aliphatic hydrophobic residues (Fig. 2). The same findings were observed in peptides binding to HLA-A*2602 and HLA-A*2603 (data not shown). These results suggest that HLA-A26 molecules have relatively small B-pockets. Peptides carrying Leu or Ile at P2 bound to the three HLA-A26 molecules more effectively than those carrying Ala or Met at P2. These findings are consistent with the pool sequencing analysis of HLA-A26-binding peptides.

Previous pool sequencing analysis showed that HLA-A*2601 preferentially binds to peptides with Tyr or Phe at P9, and that HLA-A*2602 and HLA-A*2603 preferentially bind to peptides with Tyr, Phe, Met or Leu at P9 (3). We investigated the role of these P9 residues in the peptide binding using variations of the EVIPMFSAL peptide. With the exception of peptides carrying positively charged residues at P9, all peptides tested effectively bound to HLA-A*2601 (Fig. 3). Similar findings were observed with the binding of these peptides to HLA-A*2602 and HLA-A*2603 (data not shown). These results demonstrate that hydrophobic residues, small residues, and negatively charged residues at P9 function as anchors for these HLA-A26 molecules. The peptide carrying the positively charged residue Arg at P9 had much lower affinity for HLA-A*2601 and HLA-A*2602 than the peptide carrying the negatively charged residue Asp. However there was little difference between these two peptides in affinity for HLA-A*2603 (Fig. 4). This suggests that HLA-A*2603 had a less positively charged F-pocket than HLA-A*2601 and HLA-A*2602.

A previous pool sequencing study showed that in addition to P2 and P9 anchor residues, negatively charged residues at P1 are auxiliary anchors (3). To evaluate the role of P1 residues, we synthesized a panel of peptides based on EVIPMFSAL with substitutions at P1. Peptides carrying negatively charged amino acids at P1 effectively bound to HLA-A*2601 molecules while the binding of those carrying positively charged residues was markedly reduced (Fig. 5). Peptides carrying hydrophobic residues at P1 revealed weak binding to HLA-A*2601. Similar findings were observed in peptide binding to HLA-A*2602 and HLA-A*2603 (data not shown). Thus, acidic amino acids at P1 function as anchors for these HLA-A26 molecules.

Binding of peptides having anchor residues at P1, P2 and P9 to three HLA-A26 subtype molecules

The analyses using mutant peptides suggested that residues at P1, P2 and P9 play an important role in HLA-A26 peptide binding. Therefore, we re-evaluated peptide binding to HLA-A26 using pep-

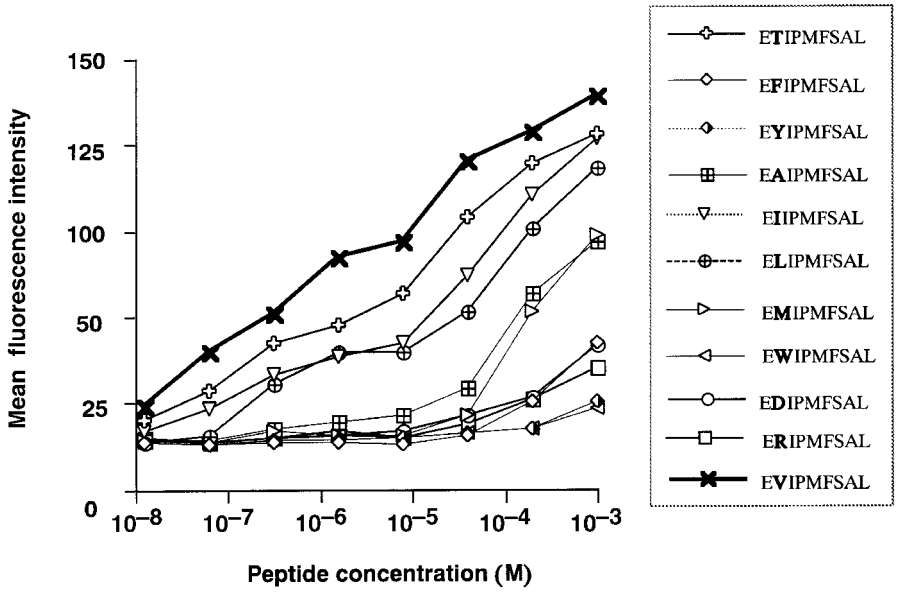

Fig. 2. **Binding of EVIPMFSAL and mutant peptides at P2 to HLA-A*2601 molecules.**

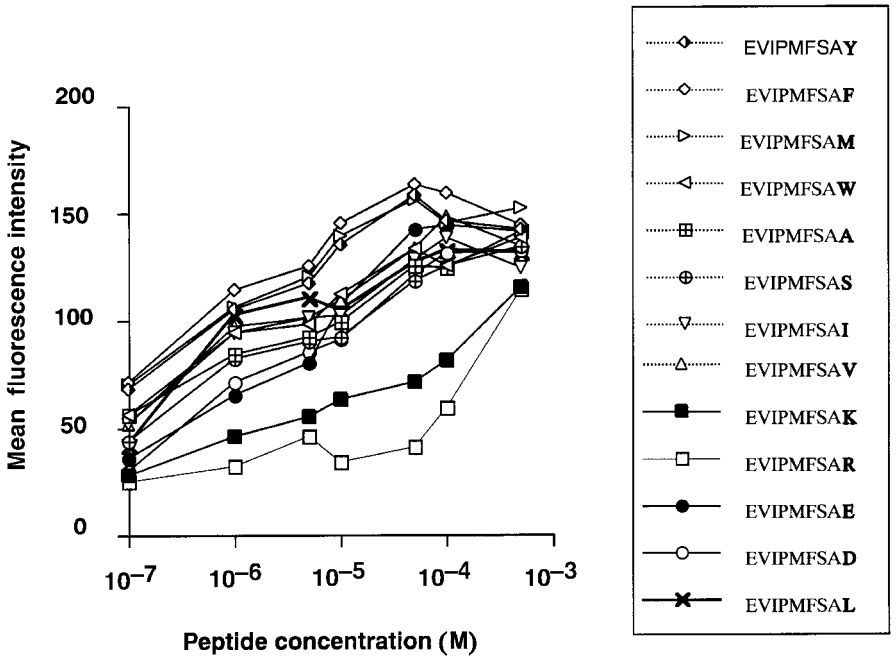

Fig. 3. **Binding of EVIPMFSAL and mutant peptides at P9 to HLA-A*2601 molecules.**

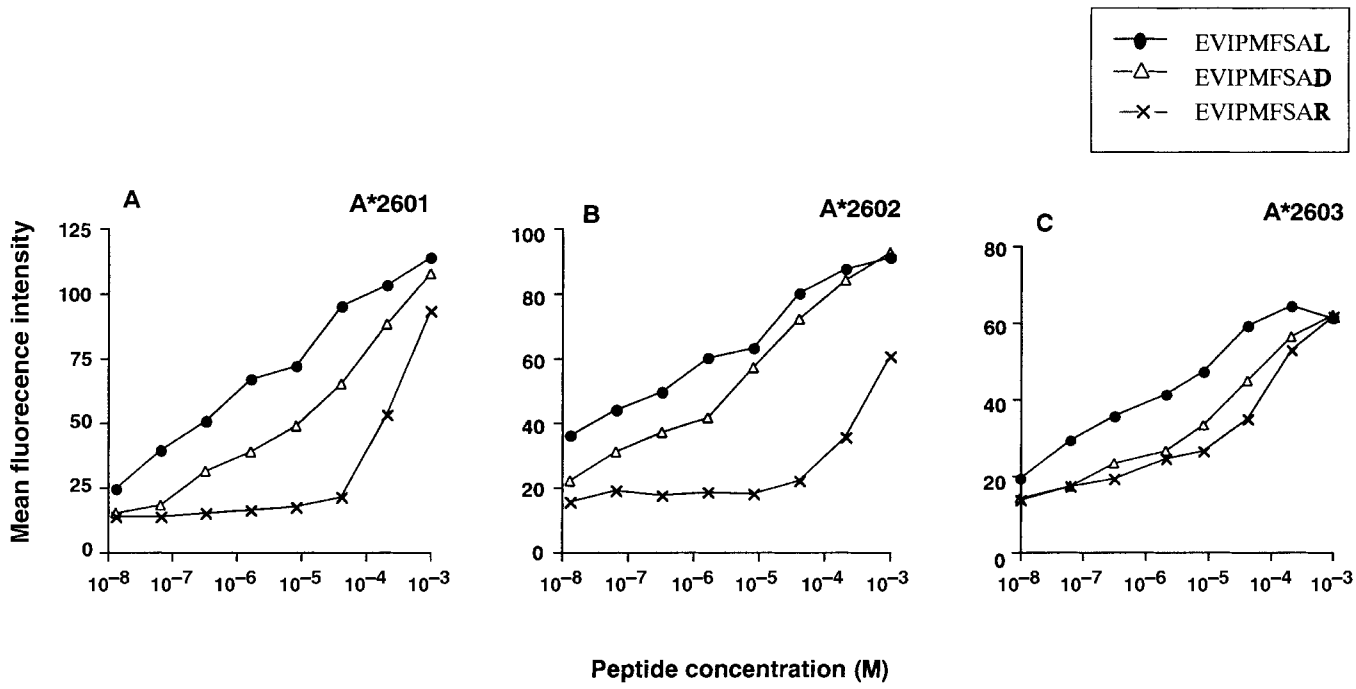

Fig. 4. **Binding of EVIPMFSAL and mutant peptides carrying Asp or Arg at P9 to three HLA-A26 molecules.**

tides with anchor residues at these three positions. The sequences of HIV-1 SF2 Pol, Nef, Gag and Env proteins were screened for 9-mer peptides containing all three anchor residues. Thirty-eight peptides were synthesized and analyzed by the stabilization assay. Nineteen of the 38 peptides bound to at least one of the three HLA-A26 molecules; 13, 18 and 10 peptides bound to HLA-A*2601, HLA-A*2602 and HLA-A*2603, respectively (Table 2). The percentage of HLA-A26 binding peptides was much higher for peptides carrying

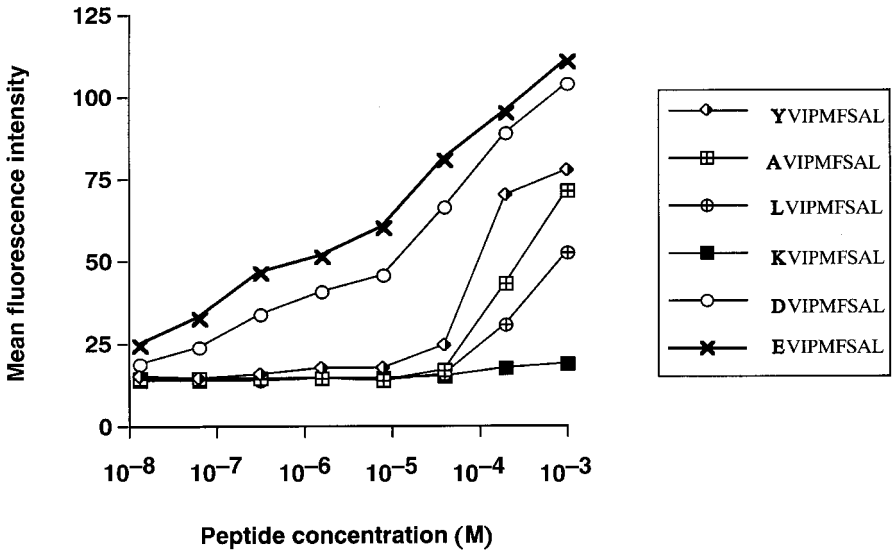

Fig. 5. **Binding of EVIPMFSAL and mutant peptides at P1 to HLA-A*2601.**

all three anchor residues than for peptides carrying P2 and P9 anchor residues (A*2601: 15.0%; A*2602: 30.0%; A*2603: 15.0%) (Table 1). In addition, the MBR of the former peptides (Table 2) was higher than that of the latter peptides (A*2601: 0.25; A*2602: 0.50; A*2603: 0.25) (Table 1). These results indicate that together with P2 and P9 anchor residues, P1 anchor residues play an important role in peptide binding to HLA-A26.

The MBR of peptides carrying Glu at P1 seems to be higher than that of peptides carrying Asp (Table 3). However there is statistically no difference in MBR between these peptides (data not shown). Analysis using mutant peptides also showed that the affinity of EVIPMFSAL was higher than that of DVIPMFSAL not only for HLA-A*2601 (Fig. 5) but also for HLA-A*2602 and HLA-A*2603 (data not shown). These results indicate that Glu is a stronger anchor than Asp. On the other hand, there is no significant difference in MBR between peptides carrying the P2 anchor residues (data not shown).

Pool sequencing of HLA-A26 binding self-peptides indicated that the hydrophobic residues Ile, Leu, Val, Met, Tyr and Phe at P6 are auxiliary anchor residues for HLA-A26 with a little difference between the three subtypes. We attempted to evaluate the effect of these residues at P6. Although the frequency of binding peptides and the MBR are higher for peptides carrying these residues than those which do not (Table 4), there was no statistical difference between the two groups (data not shown). This suggests that these residues at P6 may only be weak auxiliary anchors.

Binding of 9-mer peptides carrying three anchor residues to HLA-A26 molecules

	Number of binding peptides		
Binding affinity	A*2601	A*2602	A*2603
High	2	3	1
Medium	2	4	1
Low	9	11	8
None	25	20	28
Percentage of binding peptides	34.2	47.4	26.3
Mean binding rank	0.50	0.74	0.34

Table 2

Discussion

Pool sequencing of HLA-A26-binding peptides identified five P2 anchor residues and four P9 anchor residues. The present study confirmed these anchor residues and further revealed that other hydrophobic residues such as Val, Ile and Trp, as well as small or negatively charged residues at P9 function as anchors for HLA-A26. Pool sequencing study showed that Met and Ile are P9 anchor residues for HLA-A*2602 and HLA-A*2603 but not for HLA-A*2601 whereas the present study could not demonstrate this difference between HLA-A26 subtypes. The discrepancy between the two studies might be explained by the fact that constrains of processing may bias the use of P9 residues in naturally processed peptides. Identification of CTL epitopes presented by HLA-A26 molecules will clarify whether peptides having the P9 anchor residues identified by the present study can be processed and presented to HLA-A26 molecules.

In addition to P2 and P9 anchors, we confirmed that two acidic acid residues at P1 are anchors for three HLA-A26 molecules. More-

Effect of positively charged residues at P1 on the peptide binding

	A*2601		A*2602		A*2603	
Amino acid at position 1	NBP*/NTP**	MBR	NBP/NTP	MBR	NBP/NTP	MBR
E	10/23 (43.5%)	0.70	12/23 (52.1%)	0.91	8/23 (34.8%)	0.48
D	3/15 (20.0%)	0.20	6/15 (40.0%)	0.47	2/15 (13.8%)	0.13
Total	13/38 (34.2%)	0.50	18/38 (47.3%)	0.74	10/38 (26.3%)	0.34

NBP: number of binding peptides
NTP: number of total peptides tested

Table 3

over, comparison between peptides carrying two anchors at P2 and P9 and those carrying three anchors showed that a P1 anchor increases the affinity of peptides to HLA-A26 molecules. Although the pool sequencing study showed that Asp is more frequent than Glu at P1, the present study demonstrated that Glu is a stronger anchor than Asp. This discrepancy might also be explained by processing bias. Acidic amino acids have also been observed as P1 auxiliary anchors in natural ligands of other HLA class I molecules such as A*6801, A*6901, A*3303, B*1401 and B*0801 (14–17). These HLA molecules carry the positively charged amino acid Arg at residue 62. In contrast, HLA-A*0201, HLA-B*2705 and HLA-B*5801, which bind peptides carrying basic amino acids at P1, have the negatively charged amino acid Glu at residue 63 (10). This supports the idea that the presence of a positively charged amino acid Arg at residue 62 and the absence of a negatively charged amino acid at residue 63 are responsible for preference of acidic residues at P1.

Effect of hydrophobic residues at P6 of nonamer peptides on peptide binding to HLA-A26

	A*2601	
Amino acid at P6	NBP/NTP	MBR
I,L,V,M	4/7 (57.1%)*	0.86
others	9/31 (29.0%)	0.42
total	13/38 (34.2%)	0.50
	A*2602	
Amino acid at P6	NBP/NTP	MBR
I,L,V,M,Y,F	7/9 (77.7%)	1.00
others	11/29 (37.9%)	0.66
total	18/38 (47.3%)	0.74
	A*2603	
Amino acid at P6	NBP/NTP	MBR
I,L.V,Y,F	4/8 (50.0%)	0.63
others	6/30 (20.0%)	0.26
total	10/38 (26.3%)	0.34

* percentage of binding peptides

Table 4

Although previous pool sequencing study only identified minimum differences between natural ligand of the three HLA-A26 subtypes, the present study did identify different binding affinity of peptides between these HLA-A26 subtypes. The affinity of EVIPMFSAR was much lower than that of EVIPMFSAD for HLA-A*2601 and HLA-A*2602, while HLA-A*2603 effectively bound both peptides. These HLA-A26 subtypes differ at residues 74, 76, 77 and 116. HLA-A*2601 and HLA-A*2602 are identical at D74, A76 and N77 but differ at 116 (D at HLA-A*2601 and N at HLA-A*2602), while HLA-A*2603 has the residues H74, V76, D77 and D116. Thus, HLA-A*2603 differs from the other subtypes by a negatively charged residue 77 (N77 for A*2601 and A*2602, D77 for A*2603). This suggests that D77 might generate a less positively charged environment in the F-pocket of HLA-A*2603.

TLQEQIGWM effectively bound to HLA-A*2602 but bound only weakly to HLA-A*2601 and HLA-A*2603 (Table 1). Of the 58 peptides analyzed, 24 bound to HLA-A*2602 while 16 and 13 bound to HLA-A*2601 and HLA-A*2603, respectively (Tables 1 and 2). Thus, HLA-A*2602 is the best binder for nonamer peptides carrying HLA-A26 anchors. As HLA-A*2602 differs from other two subtypes by an Asp at residue 116, this residue at the bottom of the F-pocket might significantly influence peptide binding.

In the present study, we confirmed the identity of P2 and P9 anchor residues in nonamer peptides which bind to three HLA-A26 subtypes and further demonstrated the important role of acidic amino acid residues at P1 in peptide binding. In addition, we showed differences in peptide binding between the three HLA-A26 subtypes. The present study is expected to contribute to not only immunological studies of T-cell epitopes presented by HLA-A26 but also to studies concerning the molecular recognition of HLA-peptide-T-cell receptor (TCR) interaction.

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