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Analysis of three HLA-A*3303 binding peptide anchors using an HLA-A*3303 stabilization assay

Key words:
HLA-A*3303; HLA class I molecules; peptide

Acknowledgments:
We thank Dr. Ferrone for the gift of mAb TP25.99 and Sachiko Sakai for secretarial assistance. This work was supported by a Grant-in-Aid for Scientific Research (10557034 and 10167217) from the Ministry of Education, Science, Sport and Culture, the government of Japan, and a grant from Japan Health Science Foundation.

Abstract: The affinity of 232 8- to 11-mer peptides carrying HLA-A*3303 anchor residues at position 2 (P2) (Ala, Ile, Leu, Val, Phe or Tyr) and the C-terminus (Arg) was analysed by a stabilization assay using RMA-S transfectants expressing HLA-A*3303 and human β_2-microglobulin. One hundred and nineteen of these peptides (51.3%) bound to HLA-A*3303, confirming that these residues are anchors for HLA-A*3303. Evaluation of P2 residues demonstrated that binding of peptides with Phe or Tyr at P2 is stronger than that of peptides with aliphatic hydrophobic residues at P2. This was confirmed by analysis of a panel of peptides mutated at P2. Analysis of the C-terminal mutant peptides showed that substitution of Lys for Arg had minimal influence on binding to HLA-A*3303. This implies that peptides carrying HLA-A*1101 anchor residues (Val, Ile, Phe or Tyr at P2 and Lys at the C-terminus) can bind to HLA-A*3303. However, such peptides showed lower binding for HLA-A*3303 than for HLA-A*1101. Thus, Arg at the C-terminus is much stronger anchor for HLA-A*3303 than Lys. The preference for Arg and Lys at the C-terminus by HLA-A*1101 and HLA-A*3303 respectively may be due to sequences of three residues (70, 97 and 114) forming the F-pocket of these HLA class I molecules. Statistical analysis of 232 peptides further showed a positive effect of negatively charged residues at P1 for peptide binding to HLA-A*3303. Thus, residues at P1, P2 and the C-terminus play an important role in peptide binding to HLA-A*3303.

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HLA-A33 is one of the most common HLA-A alleles in Asia (1). Three HLA-A33 subtypes, HLA-A*3301, -A*3303 and -A*3304, have been reported (2). HLA-A*3303 allele has been identified in Japan with frequency of 12.8% (3). Pool sequencing of natural ligands demonstrated that HLA-A*3303 binding peptides have an anchor at P9 and an auxiliary anchor at P2 (4). Arg was identified as the C-terminal anchor while Ala, Ile, Leu, Phe, Tyr and Val were evaluated as P2 auxiliary anchor residues. All six ligands identified in this study contained Arg at the C-terminus. Although most HLA class I binding peptides carry hydrophobic C-terminal anchor residues (5), positively charged C-terminal anchor residues have also been identified in HLA-A*3101, HLA-A*1101, HLA-A*0301 and HLA-A*6801 binding peptides (4–8).

Received 26 October and
accepted for publication 28 December 1999

Tissue Antigens . ISSN 0001-2815

Tissue Antigens 2000: **55**: 296–302
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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. We have clarified the details of various HLA class I molecule-binding peptide interactions using the HLA class I stabilization assay with RMA-S transfectants expressing HLA-B*3501 (9, 10), HLA-B*51 (11, 12), HLA-A*2402 (13), HLA-A*1101 (8) and HLA-A*26 (14). In the present study, we investigated the role of anchor residues in peptide binding to HLA-A*3303 using an HLA-A*3303 stabilization assay with RMA-S transfectants expressing HLA-A*3303 and human β_2-microglobulin.

Material and methods

Cells

The HLA-A*3303 gene was transfected by electroporation into human β_2 microglobulin transfected RMA-S(RMA-S-hβ_2m) cells. Following selection by hygromycin B, resistant cells were isolated from separate wells. Surface expression of HLA-A*3303 molecules on transfected cells was confirmed by flow cytometry using the anti-HLA class I α3 domain monoclonal antibody (mAb) TP25.99 (15). RMA-S-A*1101 cells were previously generated (8). RMA-S-hβ2m cells expressing HLA-A*3303 (RMA-S-A*3303) and RMA-S-A*1101 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.2 mg/ml hygromycin B.

Peptides

The amino acid sequences of Env, Gag, Pol and Nef from the human immunodeficiency virus type-I (HIV-1) SF2 strain were screened for HLA-A*3303 binding peptide motifs. Candidate peptides were synthesized using an automated multiple peptide synthesizer, Shimadzu Model PSSM-8, with the Fmoc strategy followed by cleavage as previously described (16). The peptides were shown to be homogenous by liquid secondary ionmass spectrometry and reverse-phase high-performance liquid chromatography (HPLC). Peptides containing HLA-A*1101 anchor residues were previously synthesized (8).

Stabilization assay

Peptides binding to HLA-A*3303 was determined using the stabilization assay as described previously (17). Briefly, RMA-S-A*3303 cells were cultured at 26°C for 14~18 h. Cells (2×10^5) in 50-µl phosphate-buffered saline supplemented with 20% FCS (PBS-FCS) were incubated with a 50 µl solution of peptides (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, or 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 then the fluorescence intensity was measured using a FACS Calibur (Becton Dickinson, Mountain View, CA, USA). RMA-S-A*3303 cells cultured at 26°C and 37°C were stained with mAb TP25.99 under the same experimental conditions and used as controls. Similarly, peptide binding to HLA-A*1101 was determined as described previously (8).

Analysis of peptide binding to HLA-A*3303 and HLA-A*1101 molecules

Peptides which at a concentration of 10^{-3} M gave more than 25% of the mean fluorescence intensity (MFI) of RMA-S-A*3303 cells cultured at 26°C were defined as binding peptides. This criterion was previously used for HLA-B*3501 (9, 10) and HLA-A*2402 (13) binding peptides. The relative MFI of RMA-S-A*3303 cells was calculated by subtracting the MFI value of peptide-unloaded RMA-S-A*3303 cells stained with mAb TP25.99 from the MFI value of peptide loaded RMA-S-A*3303 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 assigned values of 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 were non-binding, then the MBR would be 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

Analysis of a panel of peptides containing hydrophobic residues at P2 and Arg at the C-terminus using RMA-S transfectants expressing HLA-A*3303

RMA-S transfectants expressing HLA-A*3303 were generated following transfection of the HLA-A*3303 gene into RMA-S-hβ_2m

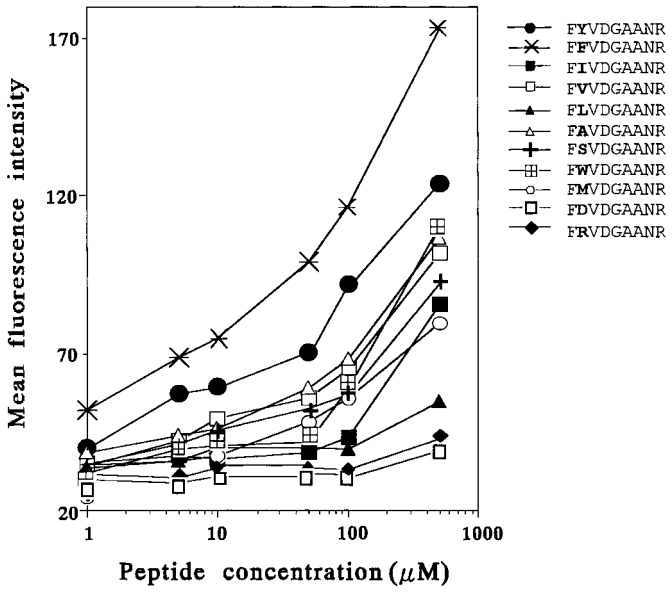

Fig. 1. **The effect of substitution at P2 on binding of the 9-mer peptide FYVDGAANR to HLA-A*3303 molecules.** The BL_{50} (M) of these peptides is: F**Y**VDGAANR 1.7×10^{-5}, F**F**VDGAANR 4.0×10^{-6}, F**I**VDGAANR 8.5×10^{-3}, F**V**VDGAANR 1.2×10^{-4}, F**L**VDGAANR $>10^{-3}$, F**A**VDGAANR 8.8×10^{-5}, F**S**VDGAANR 3.5×10^{-4}, F**W**VDGAANR 6.5×10^{-4}, F**M**VDGAANR 2.6×10^{-4}, F**D**VDGAANR: no binding, and F**R**VDGAANR: no binding.

cells. Transfectant cells cultured at 26°C had a much higher HLA-A*3303 surface expression level than those cultured at 37°C. HLA-A*3303 surface expression on the cells cultured at 26°C rapidly decreased following incubation at 37°C (data not shown). These transfectants are therefore suitable for use in stabilization assays using the same conditions as described previously (9, 13, 14). We therefore employed this method to evaluate peptide binding to HLA-A*3303.

We screened the protein sequences derived from HIV-1 SF2 strain for 8- to 11-mer peptides containing HLA-A*3303 anchor residues at P2 (Ala, Ile, Leu, Val, Phe and Tyr) and the C-terminus (Arg). 232 peptides (45 8-mer, 79 9-mer, 55 10-mer and 53 11-mer peptides) were chemically synthesized and analyzed by the stabilization assay. One hundred and nineteen of these peptides (51.3%) bound to HLA-A*3303 molecules (Table 1). These results strongly suggest that these P2 and C-terminal residues function as anchors for HLA-A*3303 binding.

A previous sequencing study of HLA-A*3303 ligands showed a strong Arg signal at P9 but no further C-terminal signal, suggesting that the majority of HLA-A*3303 ligands are 9-mer peptides (2). However, this study also identified six independent ligands with length of 9- to 12-mer, implying that longer peptides can be ligands for this allele. To clarify the effect of peptide length on peptide binding to HLA-A*3303, we analysed the affinity of each 8- to 11-mer peptides for HLA-A*3303. Approximately half of the 9- to 11-mer peptides analysed (49.1–64.6%) bound to HLA-A*3303 while only 26.7% of the 8-mer peptides bound to HLA-A*3303 (Table 1). The MBR of 8-mer peptides was significantly lower than those of 9-mer ($p<0.0001$), 10-mer ($p<0.005$) and 11-mer peptides ($p<0.05$). These results support the above mentioned pool sequencing study. Similar findings have been observed for peptides binding to HLA-B*3501 (10), HLA-A*2402 (13) and HLA-A*1101 (8).

Role of hydrophobic P2 anchor residues

Previous pool sequencing analysis of HLA-A*3303 ligands identified six auxiliary anchor residues (Ala, Ile, Leu, Phe, Tyr and Val) at P2 (4). We analyzed the effect of P2 residues on peptide binding to HLA-A*3303. Effective binding of 9-mer peptides containing Phe or Tyr at P2 was observed while only 20% of 9-mer peptides containing Ile at P2 bound to HLA-A*3303 (Table 2). The same findings were found following analysis of all 232 8- to 11-mer peptides (Table 3). These results together indicate that aromatic hydrophobic residues are stronger P2 anchors than aliphatic hydrophobic residues.

To directly examine the effect of P2 anchor residues on peptide binding to HLA-A*3303, we synthesized P2 mutants of a peptide FYVDGAANY which showed high binding to HLA-A*3303. Ten P2

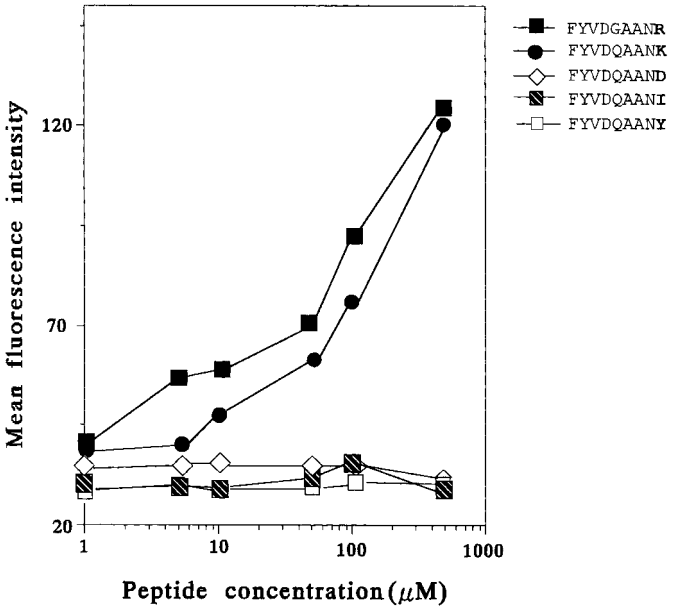

Fig. 2. **The effect of substitution at the C-terminus on binding of the 9-mer peptide FYVDGAANR to HLA-A*3303.** The BL_{50} (M) of these peptides is: FYVDGAAN**R** 1.7×10^{-5}, FYVDGAAN**K** 5.5×10^{-5}, FYVDGAAN**D**: no binding, FYVDGAAN**I**: no binding, and FYVDGAAN**Y**: no binding.

Effect of peptide length on binding to HLA-A*3303

Peptide length	NBP/NTP		MBR
8-mer	12/45	(26.7%)	0.33
9-mer	51/79	(64.6%)	1.14
10-mer	30/55	(54.5%)	0.82
11-mer	26/53	(49.1%)	0.75
Total	119/232	(51.3%)	0.82

NBP=number of binding peptide, NTP=number of peptides tested, MBR=mean binding rank

Table 1

Effect of P2 residues on the binding of 9-mer peptides to HLA-A*3303

P2 residues	NBP/NTP		MBR
A	8/12	(66.7%)	1.08
I	1/5	(20.0%)	0.20
L	17/27	(63.0%)	1.00
V	14/22	(63.7%)	1.18
F	2/2	(100.0%)	1.50
Y	9/11	(81.2%)	1.82
Total	51/79	(64.6%)	1.14

Table 2

mutants were tested for binding to HLA-A*3303 (Fig. 1). A mutant peptide with Phe at P2 showed stronger binding than the original peptide, while mutant peptides with the aliphatic hydrophobic residues Ala, Val, Leu or Ile at P2 were more weakly than original peptide. These results are mostly consistent with the peptide panel analysis shown in Table 2. Since the binding of peptides containing Trp, Ser or Met at P2 is similar to that of peptides containing other aliphatic hydrophobic residues, peptides containing these P2 residues might be able to bind to HLA-A*3303. Peptides with Arg or Asp at P2 failed to bind to HLA-A*3303. These results confirmed that the aromatic hydrophobic residues Tyr and Phe are stronger anchors than the aliphatic hydrophobic residues.

Role of the C-terminal anchor residue Arg

Binding of four mutant peptides with Lys, Asp, Ile or Tyr at P9 was compared to that of original peptide FYVDGAANR. Only the peptide with the positively charged residue Lys at P9 bound to HLA-A*3303 (Fig. 2). These results indicate that peptides with the P2 anchor residue and Lys at the C-terminus can bind to HLA-A*3303. Since ligands for HLA-A*1101 contain Val, Ile, Phe, Tyr or

Effect of P2 residues on the binding of 8- to 11-mer peptides to HLA-A*3303

P2 residue	NBP/NTP		MBR
A	20/37	(54.1%)	0.70
I	10/36	(27.8%)	0.42
L	31/66	(47.0%)	0.74
V	27/54	(50.0%)	0.83
F	12/17	(70.6%)	1.06
Y	19/22	(86.4%)	1.68
Total	119/232	(51.3%)	0.82

Table 3

Thr at P2 and Lys at the C-terminus (4, 8), HLA-A*1101 binding peptides may also be able to bind to HLA-A*3303. To clarify this, we examined the binding of peptides containing HLA-A*1101 anchors to HLA-A*3303. Three high-binding, six medium-binding and

Cross-binding to peptides containing anchor residues for HLA-A*1101

Peptide	BL_{50}	
	HLA-A*1101	HLA-A*3303
S I A D T T N Q K	4.1×10^{-6}	NB
G I I Q A Q P D K	4.1×10^{-5}	NB
I I A T D I Q T K	7.0×10^{-5}	NB
T I I L P C R I K	1.7×10^{-4}	NB
D I Q T K E L Q K	NB	NB
S I L D I R Q G P K	2.2×10^{-6}	NB
I V I W G K I P K	2.8×10^{-5}	6.8×10^{-4}
C V H Q R I D V K	$>10^{-3}$	NB
L V D F R E L N K	$>10^{-3}$	NB
S V L S G G E L D K	5.4×10^{-6}	NB
K V L F L N G I D K	1.2×10^{-5}	NB
L V Q N A N P D C K	5.9×10^{-5}	NB
V V I Q D N S D I K	8.4×10^{-5}	NB
V V E S M N N E L K	1.2×10^{-4}	NB
V F I H N F K R K	NB	NB
D F N L P P V V A K	$>10^{-3}$	$>10^{-3}$
G F A I L K C N N K	$>10^{-3}$	NB
A Y F S V P L D K	$>10^{-3}$	$>10^{-3}$
T Y Q I Y Q E P F K	2.1×10^{-4}	NB
Q Y N V L P Q G W K	NB	$>10^{-3}$

NB: no binding

Table 4

Effect of P1 residues on binding to HLA-A*3303

Amid acids	Number of peptides	MBR	Significance
Y F W H	17	1.18	
L V I M	51	0.98	
S T C	38	0.87	
P	17	0.24	$P<0.01$
G A	40	0.48	
Q N	15	1.07	
R K	30	0.40	
D E	24	1.46	$P<0.005$

Table 5

eight low-binding peptides for HLA-A*1101 were examined, as well as three non-binding peptides. The MBR of 9- and 10-mer peptides with Ile, Val, Phe or Tyr at P2 for HLA-A*3303 was 0.00, 0.13, 0.33 and 0.66, respectively (Table 4). This was much lower than the MBR of 9- and 10-mer peptides containing the corresponding residues at P2 and Arg at the C-terminus (Ile:0.47, Val:0.97, Phe:1.86, Tyr:1.75). Thus, peptides with HLA-A*1101 anchor residues showed cross-binding to HLA-A*3303, but the affinity of these peptides for HLA-A*3303 was much weaker than those having Arg at the C-terminus.

Role of the P1 residue

It is well known that in addition to P2 and the C-terminus other positions influence peptide binding to HLA class I molecules. To define the influence of such residues on peptide binding to HLA-A*3303, we performed a statistical residue-position analysis as previously described for HLA-B*3501 binding peptides (9, 10). The MBR and frequency of amino acid or groups of amino acids at each position were tested for their statistical significance in a Mann-Whitney U-test. The number of peptides of each length was not enough for a statistical analysis. Therefore, since peptide length is thought to have a little effect on the role of P1 in peptide binding, we only analysed the role of P1 using all 232 8- to 11-mer peptides. A positive effect was identified in negatively charged residues at P1, while a negative effect was observed for the small residue Pro (Table 5). These results suggest that HLA-A*3303 has an A-pocket with a positively charged environment, as also observed in HLA-A*26 (14).

Discussion

Pool sequencing of HLA-A*3303 ligands revealed that the hydrophobic residues Ala, Ile, Leu, Phe, Tyr and Val are P2 anchor residues for this allele (4). The present study defined differences in binding affinity between these residues and showed that the aromatic hydrophobic residues Phe and Tyr were much stronger anchors than the aliphatic hydrophobic residues. This is in contrast to P2 anchors of HLA-A*1101 in which aliphatic hydrophobic residues are stronger P2 anchors than aromatic hydrophobic residues (8). These observation suggest that HLA-A*3303 contains a deep B-pocket.

Previous pool sequencing studies identified the C-terminal anchor for HLA-A*3303 and HLA-A*1101 binding peptides as the positively charged residues Arg and Lys respectively (4). These C-terminal anchors were confirmed by a previous study (8) and the present study using a peptide binding assay. HLA-A*3303 and HLA-A*1101 have similar F-pockets (Table 6) which differ at only three positions, residues 70, 97 and 114 (Gln, Ile, Arg for HLA-A*1101 and His, Met and Gln for HLA-A*3303). These residues may therefore determine preference for either Arg or Lys at the C-terminus of binding peptides. This is supported by analysis of the F-pocket sequence of various HLA molecules (Table 6). Residues 70, 97 and 114 are identical between HLA-A*3303 and HLA-A*3101, and both bind to ligands with Arg at the C-terminus. Similarly, residues 70, 97 and 114 are identical between HLA-A*1101 and HLA-A*0301, and both bind to ligands with Lys at the C-terminus. Furthermore, HLA-A*6801 binds to ligands with either Arg or Lys

Residues forming the F-pocket of HLA class I alleles whose ligands carry hydrophilic C-terminal residues *Table 6*

Allele	Residue 70	74	76	77	80	81	95	97	114	116	142	143	147	C-terminal anchor residue of ligands
A*3303	**H**	D	V	D	T	L	I	**M**	**Q**	D	I	T	W	R
A*3101	**H**	D	V	D	T	L	I	**M**	**Q**	D	I	T	W	R
A*1101	**Q**	D	V	D	T	L	I	**I**	**R**	D	I	T	W	K
A*0301	**Q**	D	V	D	T	L	I	**I**	**R**	D	I	T	W	K
A*6801	**Q**	D	V	D	T	L	I	**M**	**R**	D	T	T	W	R, K

at C-terminus (6, 7). Residues 70 and 114 of this allele (Gln and Arg, respectively) are the same as HLA-A*1101, while residue 97 (Met) is the same as HLA-A*3303. These observations suggest that Met at residue 97 accepts Arg in the F-pocket while the other two residues form an F-pocket suitable for binding to Lys.

The present study demonstrated that peptides containing HLA-A*1101 anchor residues can bind to HLA-A*3303 with much lower affinity than peptides with HLA-A*3303 anchors. These findings support a previous pool sequencing study where only Arg was detected at the C-terminal anchor of natural HLA-A*3303 ligands. However, substitution of Lys at the C-terminus of the peptide FYVDGAANK did not affect binding to HLA-A*3303. This suggests that the C-terminal substitution, which is often seen in HIV-1, might have little influence on HLA-A*3303-restricted T-cell recognition.

Previous pool sequencing analysis showed that Asp and Glu are the preferred P1 residues (4). In the present study, statistical analysis of 232 peptides with respect to P1 demonstrated that the negatively charged residues Asp and Glu at P1 have a positive effect on peptide binding to HLA-A*3303, suggesting that negatively charged residues at P1 are also anchor residues for HLA-A*3303. Negatively charged anchor residues at P1 have also been observed in natural ligands of other HLA class I molecules such as HLA-A*6801, -A*6901, -A*1401, -A*2601, -A*2602, -A*2603, and -B*0801 (7, 18, 19). Since all these HLA molecules have Arg at residue 62, this residue may be critical for generation of an A-pocket suitable for binding to peptide with negatively charged residues at P1.

Only one epitope presented by HLA-A33 has been reported (20). The epitope LLGPGRPYR derived from tyrosinase-related protein 2, which contains P2 and the C-terminal anchors for HLA-A*3303, is recognized by melanoma-specific HLA-A33-restricted cytotoxic T-lymphocytes (CTL). Several HIV-1 peptides containing these two anchor residues are recognized by HLA-A*3303-restricted CTL (unpublished observation). Thus, CTL epitopes are effectively identified by using anchor residues. In the present study, we clarified the role of anchor residues at P1, P2 and the C-terminus using a stabilization assay with RMA-S-A*3303 cells. Our findings are expected to contribute to studies of HLA-A*3303-restricted T-cell recognition.

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