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EFFECT OF ANCHOR RESIDUE MODIFICATIONS ON THE STABILITY OF HLA-A11/PEPTIDE COMPLEXES

R. Gavioli*[1], Q-J. Zhang°, M. Marastoni§, R. Guerrini§, E. Reali*, R. Tomatis§, M. G. Masucci° and S. Traniello*

*Department of Biochemistry and Molecular Biology, University of Ferrara, Italy
°Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden
§Department of Pharmaceutical Chemistry, University of Ferrara, Italy

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Summary. MHC class I antigens bind peptides derived from endogenous proteins and present them to cytotoxic T lymphocytes. This binding is selective and shows high allele specificity. Peptides binding to HLA-A11 contain a hydrophobic or a small polar amino acid at position 2 and a lysine at the carboxy terminus. Peptide analogues, derived from previously identified high affinity peptides and carrying amino acid substitutions in position 2, were used to determine the requirements for formation of stable HLA-A11/peptide complexes. By kinetic analysis we were able to discriminate among apparent and true binders. Only analogues carrying in position 2 the amino acids valine, threonine and isoleucine formed stable complexes with HLA-A11 with a half life ≥ 72 hours.

MHC class I molecules bind short peptides, 8-10 amino acids long, derived from proteins expressed inside the cells and present them at the cell surface for surveillance by cytotoxic T lymphocytes (CTL). Bound peptides form an integral part of MHC molecules and, together with β2microglobulin, induce stable trimolecular complexes (1).

Sequencing of naturally presented peptides eluted from class I molecules has revealed some of the rules governing the interaction between peptides and MHC (2). These rules are different for each class I allele, leading to the definition of peptide binding motif characterized by allele-specific length and the presence of anchor positions always occupied by residues with closely similar side chains.

Molecular details of MHC/peptide association reveal that the peptide binding site is localised in a groove formed by the two α-helices lying across an eight-stranded β pleated sheet (3, 4). The groove accommodates peptides in an extended conformation, allowing the interaction of the amino acid side chains with six defined pockets, designated A to F, which are characteristic for each MHC allele. The main anchor residues, usually positions 2 and 9, interact with pockets B and F respectively. The selectivity of pockets B and F has been studied in crystallised class I molecules (3, 4), by site-directed mutagenesis (5, 6) and peptide binding (7). The presence of correct anchor residues is in many cases not sufficient to ensure high affinity MHC/peptide interactions suggesting that other residues contribute to a stable association with MHC molecules (7).

[1]To whom correspondence should be addressed. Fax:+39-532-202723.

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We have previously defined a motif for HLA-A11 binding peptides which predicts the presence of a hydrophobic amino acid in position 2 and Lysine in position 9 (8). Here, we assess the interaction between pocket B of HLA-A11 and the peptide anchor position 2 by using synthetic analogues derived from two peptides, previously identified as high affinity ligands for HLA-A11. The ultimate purpose of this study was to define conditions for the establishment of stable HLA-A11/peptide complexes, since stability may be regarded as a critical feature in ensuring efficient monitoring of the cell's interior by the immune system.

Materials and Method

Cell lines. The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T cell line CEM (9). An HLA-A11 positive subline (T2/A11) was obtained by transfection of a genomic HindIII fragment containing the HLA-A11 coding sequence (10).

Synthetic peptides. Peptides were synthesized by solid phase method as previously described (11). Crude deprotected peptides were purified by HPLC; amino acid analysis and NMR properties were consistent with peptide sequence and purity was > 98%. The amino acid sequences of the peptides in single letter code were: IVTDFSVIK (IVT), derived from EBV nuclear antigen-4 aa 416-424 (12), and AIFQSSMTK (AIF), derived from the HIV pol antigen aa 325-333 (8). Analogues carrying amino acid substitutions in position 2 (p2) are referred to as (x)2.

Generation of CTL cultures and clones. HLA-A11 restricted EBV-specific CTLs were obtained from EBV seropositive donors as described (13). Single cell cloning was done by limiting dilution as described (12).

Detection of peptide binding to HLA-A11 class I molecules. i). by immunoprecipitation and one-dimension isoelectric focusing (1D-IEF): $3x10^6$ cells were metabolically labelled overnight at 26° C in RPMI containing 100 μCi ^{35}S-methionine and 10^{-4}M peptides. The labelling was stopped by the addition of 1 mM cold methionine and the cells were further incubated at 37°C for 5 h, washed and treated for 1 h at 4°C with the W6/32 mouse monoclonal antibody, directed against a monomorphic determinant on the HLA class I/ß2m complexes, and extensively washed before lysis. The surface HLA class I-W6/32 complexes were precipitated from the cell lysates as described (8). The HLA-A11 molecules were resolved by 1D-IEF and detected by autoradiography (14). The intensities of HLA class I heavy chain polypeptides were measured from suitably exposed autoradiograms with a LKB laser spectrophotometer equipped with a scanning attachment.
ii). by immunofluorescence: aliquots of $1x10^6$ T2/A11 cells were cultured overnight in 1 ml serum free medium containing 10^{-4}M of the indicated peptides. Surface expression of HLA-A11 molecules was detected by indirect immunofluorescence using the mouse mAb AUF 5.13 (15), which is specific for a polymorphic determinant expressed on HLA-A3 and -A11. The mean fluorescence intensity was measured with a FACS analyser (8).

Cytotoxicity tests. Four hr ^{51}Cr-release assays were performed as previously described (13). For the peptide sensitization assays, $4x10^3$ ^{51}Cr-labelled cells were placed in triplicate wells of 96 V-shaped well plates. Peptides were added to each well, and the plates were incubated for 1 hr at 37°C before addition of $4x10^4$ effector cells (12).

Stability of HLA/peptide complexes. T2/A11 cells were labelled with ^{51}Chromium for 1 h at 37°C, washed twice and treated with 50 μg/ml mytomycin C for 1h at 37°C to block cell proliferation. Cells were washed twice and aliquots of $3x10^6$ cells were incubated for 1 h at 37° in 3 ml RPMI, and treated with 10^{-7}M peptide. Then cells were extensively washed and resuspended in RMPI + 20% FCS and maintained at 37°C for kinetic experiments. Cells viability was detected before cytotoxicity experiments and was always >90%; spontaneous ^{51}Chromium release was always <20%.

Results

Effect of p2 analogues on HLA-A11 expression. Two previously identified HLA-A11 binding peptides were chosen for these studies. The two peptides, IVTDFSVIK (IVT), aa 416-424, derived from the Epstein-Barr virus nuclear antigen 4 (EBNA4) and AIFQSSMTK (AIF), aa 325-333, derived from the HIV pol antigen, were

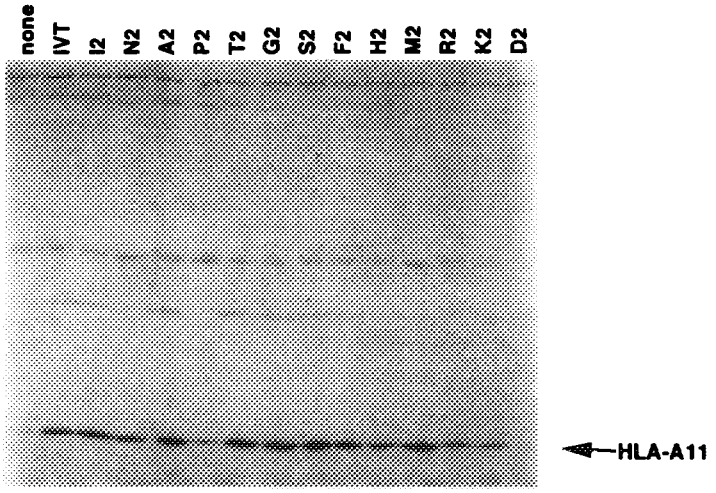


Figure 1. Expression of HLA A11 detected by 1D-IEF. HLA class I antigens were immunoprecipitated from total lysate of metabolically labelled and peptide pulsed T2/A11 using the W6/32 mAb. The W6/32 precipitates were treated with neuroaminidase for 3 hr at 37°C. The HLA class I heavy chains and b2m polypeptides were resolved by 1D-IEF. One representative experiment out of 3 is shown.

previously shown to induce high levels of surface HLA-A11 expression in the transfected T2/A11 mutant cell line (8). The IVT peptide and a series of synthetic analogues, differing from the original sequence by the single amino acid substitutions in position 2, were tested for their ability to associate with HLA-A11 molecules. HLA-A11/peptide association was assessed by the induction of surface HLA-A11 expression in the T2/A11 mutant cell line (16), as detected by immunofluorescence with the HLA-A11 specific mAb and immunoprecipitation followed by 1D-IEF. Results obtained with both detecting methods showed that the I2, A2, M2, G2, S2 and T2 analogues, in which the hydrophobic V2 was replaced with hydrophobic or polar amino acids, were still able to induce HLA-A11 expression at levels comparable to those obtained with the original IVT peptide (Figure 1 and Table 1). An apparent discrepancy between the two assays was observed with S2 and T2 analogues, which yielded HLA-A11/ peptide complexes with poor reactivity with the AUF5.13 mAb. This is likely to be due to the restricted specificity of this monoclonal for a particular subset of HLA-A11/peptide complexes (Q-J. Z., unpublished results). A net decrease in binding was observed by both detecting procedures with the N2, F2 and H2 analogues, while P2, K2, D2 and R2 analogues did not stabilize the expression of HLA-A11 molecules at the cell surface.

A parallel series of experiments carried out with a panel of AIF analogues indicated that this peptide has a more stringent requirements for p2 occupancy in that only analogues carrying hydrophobic aliphatic residues were active (Figure 2). In contrast to the IVT-G2 and the IVT-S2 analogues the AIF-G2 and the AIF-S2 peptides were not able to induce HLA-A11 expression.

Effect of p2 analogues on sensitization of target cells to lysis. IVT peptide, corresponding to the immunodominant EBNA4 epitope (12), was shown to induce HLA-A11-restricted lysis of T2/A11 cells at subpicomolar concentrations (8). The IVT peptide and corresponding p2 analogues were used in titration experiment in order to define the molar concentration required for half-maximal lysis. The I2, M2, A2 and G2 analogues were able to sensitize target cells to lysis at concentrations as low as 10^{-13}-10^{-14}M. T2 and S2 were active at 10^{-11}M; in contrast, a net decrease in activity occurred with N2, H2, R2, K2, D2, F2 and P2 analogues, which showed biological activity only at molar concentrations higher than 10^{-9} (Table I). As shown

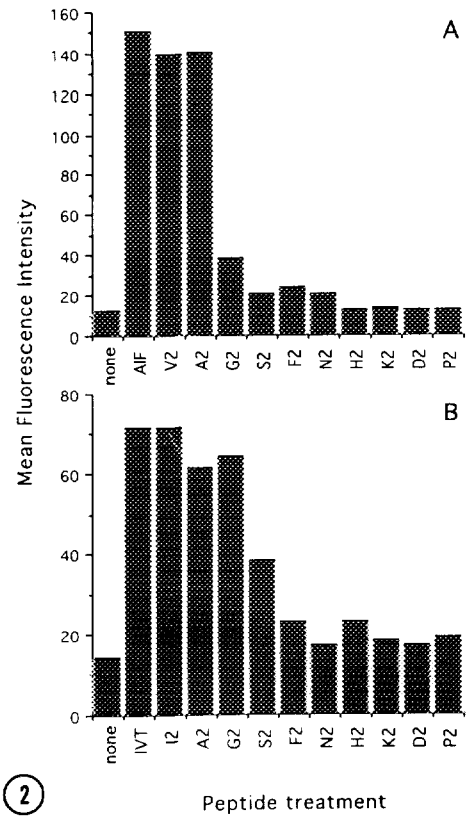
Table 1. Induction of surface HLA-A11 expression and CTL sensitization by IVT analogue peptides

Peptide	HLA A11 expression[a]		CTL Lysis[c]
	Fluorescence[a]	1D-IEF[b]	
none	14	-	--
I V T D F S V I K	71	10.7	$5x10^{-14}$
- I - - - - - - -	71	13	$5x10^{-14}$
- M - - - - - - -	71	13.9	$5x10^{-14}$
- A - - - - - - -	61	14.3	10^{-13}
- G - - - - - - -	64	11.6	$5x10^{-14}$
- T - - - - - - -	24	12.9	10^{-11}
- S - - - - - - -	38	12.5	$5x10^{-11}$
- N - - - - - - -	17	7.3	$5x10^{-9}$
- H - - - - - - -	23	6.2	10^{-8}
- R - - - - - - -	23	4.3	$5x10^{-6}$
- K - - - - - - -	18	2.9	$5x10^{-7}$
- D - - - - - - -	17	0.7	10^{-7}
- F - - - - - - -	23	7.8	$5x10^{-9}$
- P - - - - - - -	19	2.1	10^{-7}

a. Mean fluorescence intensity. T2/A11 cells were incubated overnight in medium alone or medium containing 100 mM of the indicated peptides. HLA-A11 expression was measured by FACS analysis after staining with the AUF 5.13.mAb. Mean of 4 experiments.
b. The intensities of HLA-A11 molecules, resolved by 1D-IEF, were quantitated with a laser spectrophotometer and data are expressed as increase of intensity using a value of 1 for HLA-A11 molecules detected in untreated T2/A11. Mean of 3 experiments.
c. Molar concentration of peptide required for half maximal lysis of 1 h preincubated T2/A11 cells. Mean of 4 experiments.

in Fig.3, plotting of the peptide activity in relation to the hydropathy index of the various p2 aliphatic substitutions (17) reveals a direct correlation between the two parameters.

Stability of HLA/peptide complexes. IVT analogues that bound with high efficiency to HLA-A11 molecules were compared for their capacity to induce stable HLA-A11/peptide complexes. Excess molar concentrations of the G2, M2, I2, A2, S2 and T2 analogues able to induce target cell lysis at concentrations lower than 10^{-10}M were used to pulse T2/A11 cells. After peptide pulsing the cells were extensively washed, mytomycin C treated to avoid proliferation, and used in kinetic experiments as targets of IVT-specific CTL clones (Figure 4). Equal levels of lysis were observed at time 0 with targets pulsed with the different peptides. T2/A11 cells that were pulsed with the IVT peptide were efficiently lysed by IVT-specific clones over a 3 day period. The levels of specific lysis did not change with time when the same clone was used as effector (not shown). The I2 and T2 analogues showed a similar kinetic. In contrast a net decrease of lysis was observed already after one day



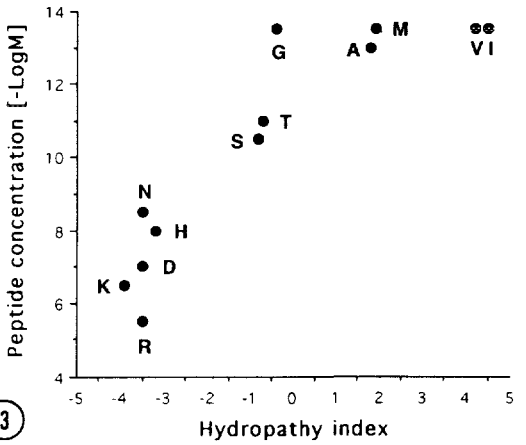


Figure 2. Expression of HLA-A11 detected by immunofluorescence. T2/A11 cells were preincubated overnight at 37°C with 10^{-4}M of the indicated peptides before detection of surface HLA-A11 expression by using the AUF 5.13 mAb. Panel A: AIF analogues; Panel B: IVT analogues. The results are expressed as mean fluorescence intensity. Mean ± S.E. of 4 different experiments.

Figure 3. Relation between peptide activity and hydropathy index of the various p2 substitutions. Peptide activity is expressed as molar concentrations of peptide required for half-maximal lysis of T2/A11 cells preincubated for 1h with the indicated peptides and used as target of IVT-specific CTL clones. Mean of 4 separate experiments performed with different IVT-specific clones.

with target cells pulsed with the G2, A2, M2 and S2 analogues; the decrease was considerably marked after two days, and lysis reached basal levels after 3 days.

Discussion

Peptides presented by HLA class I molecules are uniform in length and have specific motifs that are unique for the presenting allele (2). Identification of these allele-specific motifs may allow the exact prediction of natural T-cell epitopes. We have shown that the HLA-A11 binding motif is defined by the presence of hydrophobic residues in position 2 and a lysine in position 9 (8); this prediction has been confirmed by other investigators that observed a similar preference after sequencing the naturally processed peptides eluted from HLA-A11 molecules (18). In contrast to the strict requirement for lysine in position 9, a large number of amino acids seems to be accepted in the second position of the peptide ligand (18 and this paper). Amino acid in position 2 interacts with pocket B of the peptide binding groove (19). Comparison of HLA-A11 and HLA-A2 sequences suggests that the B pocket of HLA-A11 is constituted by hydrophobic amino acids at the bottom, and by polar and charged amino acids at the top.

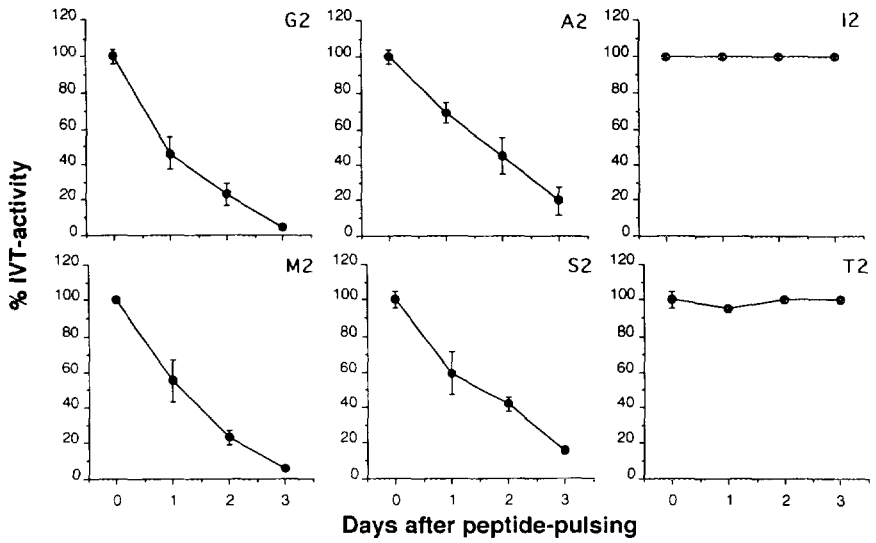


Figure 4. Stability of HLA-A11/peptide complexes. T2/A11 cells were pulsed with 10^{-7}M of the IVT peptide or the indicated p2 analogues, mytomycin C treated and used as a target of IVT-specific CTL clones. The results are expressed as % IVT activity. The % specific lysis of T2/A11 treated with IVT were 45, 47, 44 and 35 at time 0, after 1, 2 and 3 days,respectively. Mean ± S.E. of 5 different experiments.

In the present report, a detailed study of the interaction between the anchor position 2 of the peptide ligand and HLA-A11 class I molecules was performed by analysing different parameters. These included the capability of exogenous peptides to: i) stabilise empty molecules at the cell surface in processing mutant cell lines; ii) induce target cell lysis by specific CTL clones; iii) form stable HLA-A11/peptide complexes at the cell surface.

We have shown that all IVT-p2 analogues with hydrophobic residues or with G, T and S are able to associate with HLA-A11. The capability to bind HLA molecules seems to be directly related to the hydropathy index of the side chains. Surprisingly, the G2 analogue still binds to HLA-A11 in spite of loss of the p2 anchor position. This indicates that secondary anchors mediated by other amino acid residues contribute to the interaction of this peptide with HLA-A11. The influence of such secondary anchors appears to be weaker in the interaction of HLA-A11 with the AIF peptide since the AIF-G2 analogue failed to stabilize HLA-A11 expression.

IVT-p2 analogues exhibited a different behaviour when their ability to produce stable HLA-A11/peptide complexes was tested. Kinetic analysis, aimed at assessing the stability of complexes for up to three days, discriminated the peptides into "apparent" and "true" binders. The G2, A2, M2 and S2 analogues lost 30-50% of activity within one day, and drastically dropped to almost no activity by day 3. On the contrary, the I2 and T2 analogues yielded stable complexes, with kinetics comparable to that of IVT. It is noteworthy that all peptides capable to form stable complexes with HLA-A11 molecules carry in position 2 an amino acid with a branched side chain and methyl groups on C_β atom. This residue may be necessary to contact the hydrophobic bottom of the B pocket.

The L2 analogue that was not included in this series of experiments, was previously shown to bind HLA-A11 and to sensitize target cells to lysis as efficiently as IVT (20). This mutated IVT epitope was originally described in EBV strains isolated from Chinese donors, lympho blastoid cell lines carrying the mutated virus failed to present this epitope at the cell surface (20), suggesting that this peptide forms unstable

complexes with HLA-A11. It is noteworthy that although V and L have a similar hydrophobicity the L side chain lacks the methyl group on C_β atom.

In conclusion, we have characterized the requirements for p2 occupancy of HLA-A11 binding peptides, and we have set a new approach for the study of HLA-peptide stability. This parameter, in contrast to peptide binding experiments performed with isolated HLA molecules, is relevant for evaluating functionality of HLA complexes. If a peptide dissociates rapidly after initial interaction with class I antigens, this would reduce effective peptide display, owing to loss during intracellular transit or upon arrival at the cell membrane. It is reasonable to postulate that stable association with the HLA molecules is a pre-requisite for efficient presentation of antigenic T cell epitope.

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