Flexibility of Amino Acid Residues at Position Four of Nonapeptides Enhances Their Binding to Human Leucocyte Antigen (HLA) Molecules

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The binding affinity of synthetic nonapeptides to human leucocyte antigens (HLA) molecules of the A0201 allotype, the most common in Caucasian, is enhanced or reduced by suitable amino acid substitutions at position 4, as a result of increased or decreased chain flexibility. A higher flexibility of the bond at this position correlates with an easier accomodation of the fragment into the HLA groove, while rigidity of the peptide chain appears to interfere. These data are based on two lines of evidence: a) most natural high affnity ligands for HLA-A0201 possess, at position 4, flexible residues b) substitutions of such residues by rigid amino acids results in a decrease of binding affinity.

Introduction

Human Leucocyte Antigen (HLA) class I molecules are dimers of an α chain (44 kDa), and the non-covalently bound β2-microglobulin. In a small "groove" of its structure, the α chain binds small peptides selectively and display high allele specificity, that is dictated by complementary interactions of side chains of several amino acid residues of the bound peptide with residues of the HLA molecule. The binding of peptides to HLA is the first, essential step in the complex mechanism of antigen presentation to T lymphocytes, and as a rule, peptides displaying high affinity for the HLA molecule, are those that elicit a strong T cell cytotoxic response.

Two positions of the ligand appear to be involved predominantly in the binding with the HLA molecule, i.e. position 2 (sometimes 3) and 9, that function as anchor residues (Rammensee, 1995). Other amino acids in the structure however may play an important role in providing complementary interactions that increase the binding affinity (Di Modugno *et al.,* 1996).

HLA molecules of the A0201 allotype, the most common in Caucasians, bind natural as well as synthetic nonapeptides that possess almost invariantly Leu at position 2 and Val at position 9. The other residues (1 and 3–8) are either involved in providing additional interactions that stabilize the binding of the peptide in the groove, and/or committed to the signalling of a message to T cell receptors (Lim *et al.*, 1996).

A comparative study of ligands and peptides eluted from cells expressing HLA class I molecules (Falk *et al.*, 1991) indicates that the number of amino acid residues occupying certain positions is quite limited, and that they often share common chemical or physical properties. As a typical example, only three hydrophobic residues (Ile, Val, Leu) occur at position 6 in the large majority of high affinity binders for HLA-A0201, and a substitution can be detrimental for binding (Parker *et al*, 1994). On the contrary, some other positions display an heterogeneous variety of amino acids, and it is likely that their nature is relatively unrelevant for the binding affinity of the nonapeptide, as residues completely differing in charge, size, and hydrophilicity are very unlikely involved in providing the same type of interaction with the amino acid residues in the binding cleft.

In this investigation, we attempted to define whether in high affinity ligands for HLA-A0201, the most commonly-occurring amino acids in position 4 could be related by any chemical of physical property. Then we selected several peptides with the canonical binding motif for this allotype, and tested in binding assays with soluble HLA class I molecules whether the binding affinity of such peptides could be modified by suitable substitutions of the amino acid residue at position 4. The "*a priori*" identification of peptides with higher af-

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finity for the HLA class I might have practical importance for the selection of peptides to be tested for the *in vitro* and *in vivo* activation of cytotoxic T lymphocytes.

Materials and Methods

Nonapeptides to be tested in binding assays with HLA-A0201 molecules were selected from different proteins, as reported in Table I. All peptides but one possessed, at position 2 and 9, the "canonical" anchor motifs Leu or Ile, and Val or Leu.

Peptides were synthesized by the solid-phase method, using the Boc approach (Merrifield, 1963). At the end of the synthesis, peptides were deprotected and cleaved from the resin particles, and then purified by gel filtration on high-resolution resins Aca202 (Spectrum, Los Angeles, USA) or Trisacryl GF05 (IBF Soc. Chim., Villeneuve, France). An additional purification on HPLC, using the reverse-phase technique, was performed when necessary to achieve high purity (95% or more). A small aliquot of samples was then hydrolyzed with 6 N HCl at 108 °C, for 18 to 72 h, and then controlled for exact amino acid composition.

α-chains were obtained from JES human homozygous B-lymphoid cells (A*0201; B*2705, C1), lysed and denatured at pH 11.5, and then purified by gel filtration (Tanigaki *et al.*, 1993).

The binding of peptides to HLA-A2 molecules was performed by measuring the conformational change of unfolded HLA α chain induced by the binding of the peptide in the HLA groove, as previously reported (Tanigaki, 1992; Fruci *et al.*, 1993). Briefly, two serological activities, α and α', unique to HLA class I proteins, were defined. The a activity is specific to native, folded α chains. This activity is not found on denatured class I proteins, and is reexpressed upon renaturation. The α' activity is characteristic of unfolded HLA class I α chains.

These activities were measured by a specific radio immune assay, based on the specific inhibition of binding of radiolabeled antigens and the corresponding polyclonal antibodies. In the tables, the values reported indicate the net gain of a activity, i.e. the refolding of the chain in presence of the peptide (Tanigaki *et al.*, 1993). Details of the method are reported in a footnote of Table I.

Results and Discussion

Early data reported by the literature suggest that the majority of peptides eluted from cells bearing the HLA-A0201 specificity had, at position 4, either one of the following amino acids: Glu, Lys, Gly, Pro, Asp (Falk *et al.*, 1991). This statement is confirmed by the analysis of sequences of ligands and T cell epitopes reported by Rammensee (Rammensee *et al.*, 1995) and by binding experiments of synthetic peptides to T2 cells: most of high-affinity peptides possess, at this position, the above five reported residues, with the addition of serine and glutamine (Fisk *et al.* , 1995).

Thus, it might be postulated that the seven amino acids:
Glu, Lys, Gly, Pro, Asp, Ser, Gln
placed at position 4, favour the binding of nonapeptides to HLA-A0201.

There is apparently little chemical correlation among the seven amino acids of this group, apart the fact that they are not hydrophobic (Hopp and Woods, 1981). It is noteworthy, however, that other non-hydrophobic residues, as arginine and asparagine, are scarcely represented in the many high-affinity ligands for HLA-A0201 and T-cell epitopes described until today. Thus, the chemical or physical feature correlating the seven amino acids might not be lack of hydrophobicity:

Many attempts have been made to correlate the presence of certain amino acids at a non-anchor position with an increase, or decrease, of binding affinity for a certain allotype (Reay *et al.*, 1994), and by suitably replacing residues in synthetic nonapeptides, it has been possible to construct the best ligand for an HLA class I molecule (Rovero *et al.*, 1995). Although this approach has little practical relevance for studying T-cell activation, as some residues within the peptide might be essential for the recognition by T-cell receptors, and thus not replaceable, it nevertheless provides an insight into interactions between peptide and residues in the HLA groove.

Besides positions 2 and 9, which function as main anchor residues, high affinity ligands to HLA-A0201 have a third position that correlates with high binding. Most ligands possess, at position 6, almost exclusively hydrophobic residues.

Thus we attempted to predict the binding affinity of nonapeptides to HLA A0201 on the basis

of the following criteria: besides two valid anchor residues at positions 2 and 9, an high affinity ligand was likely to possess:

a) an hydrophobic residue at position 6,

b) any of the seven residues listed above, at position 4.

These peptides were assigned the highest binding activity (+++). Peptides with an hydrophilic residue at position 6, or lacking a "favourable" residue at position 4, were assigned a medium binding ability (++). A low binding (+) was predicted for peptides with both unfavourable combinations. Control peptides without valid anchor residues at position 2 and 9, were assigned a zero value.

Eight synthetic peptides, selected from different proteins, were tested. As reported in Table I, five peptides were expected to exhibit high binding values, i.e.

369, 402, 689, 92, HIV

Middle values were expected for 3 peptides, i.e.

435, 94, IMP

and low values for 2 peptides, i.e.

845, 96.

Peptide X had no binding motifs for HLA-A0201, and was assigned a binding value of 0.

Binding tests to soluble HLA-A0201 molecules indicate that high binding was exhibited indeed by four peptides of the first group, i.e. 369, 689, 92 and HIV. Four peptides (402, 435, 94 and IMP),

Table I. Binding affinity of peptides as determined by the α chain refolding assay.

The binding tests were performed in duplicates. The values reported are the mean of the two determinations. For duplicates, values are within + or − 12%.

Name	Sequence	**Binding affinity for HLA molecules** Predicted	found
369	Lys Ile Phe Gly Ser Leu Ala Phe Leu	+++	656
402	Thr Leu Glu Glu Ile Thr Gly Tyr Leu	+++	405
435	Ile Leu His Asn Gly Ala Tyr Ser Leu	++	410
689	Arg Leu Leu Gln Glu Thr Glu Leu Val	+++	680
845	Asp Leu Ala Ala Arg Asn Val Leu Val	+	130
HIV	Ile Leu Lys Glu Pro Val His Gly Val	+++	670
92	Asp Ile Met Pro Pro Leu Leu Phe Val	+++	500
94	Glu Val Ala Pro Pro Glu Leu Phe Val	++	350
96	Glu Phe Gly Thr Ser Ser Ser Arg Leu	+	110
IMP	Leu Leu Thr Glu Val Glu Thr Tyr Val	++	380
X	Glu Pro Arg Gly Ser Asp Leu Ala Gly		20

Briefly, 10 μl of test peptide, at known concentration, are added to 20 μl of alkaline-denatured, unfolded HLA α chains for 16 hours. A high-affinity peptide will induce partial to complete refolding of the α chain. Samples are then incubated with a known amount of specific rabbit anti-α-chain antiserum, (which will bind exclusively the newly-refolded α chain), and one hour later with ^{125}I-labeled HLA-A2 molecules. Only a fraction of the original antibodies will be now available for binding the radioactive HLA-A2 preparation. The ^{125}I-labeled antigen-antibody complex is then precipitated and counted in a γ-rays counter. Peptides with low binding affinity for HLA α chains, as peptide X, will not induce the refolding: the α chain remains thus unfolded, and will not bind anti-α-chain antibodies, which in turn will bind ^{125}I-labeled HLA molecules. Thus, there is little or no inhibition of the reaction, and radioactivity will be high. One unit is defined as the amount, in μg, of test peptides that induces a 50% inhibition of the binding. The results are presented as total α activity / ml of test peptide. The a activity of unfolded alpha chains without peptide (spontaneous refolding) was 130. This value has been subtracted. The control peptide X did not induce refolding, thus its activity was close to zero.

The first five peptides were selected from the sequence of Erb-B2. The number (peptide name) refers to the position, in the protein sequence, of the first amino acid of the nonapeptide. HIV derives from human immunodeficiency virus reverse transcriptase; peptides 92, 94 and 96 from *Parietaria officinalis*. The complete sequence of this protein is unknown, and numbers are arbitrary. IMP was selected from Influenza Virus Matrix protein.

X is a peptide with no anchor motifs for HLA-A0201, and was used as negative control.

Prediction of binding affinity of nonapeptides to HLA was made on the assumption that Lys, Ser, Gly, Pro, Asp, Glu, and Gln at position 4, as well as an hydrophobic residue at position 6, would favour the binding, and thus chain refolding.

displayed medium activity, while the two peptides of the last group, 845 and 96, exhibited low binding activity, as predicted. One single peptide (402) felt outside our predictions.

Thus, we tested whether the replacement of residue 4 by any other amino acid within the group would keep unchanged the ability of the peptide to be accomodated into the HLA groove, and assumed that any substitution by residues outside the group should have resulted in a decrease of the binding ability. Thus, several variants of the peptides were compared in their ability to bind to partially unfolded HLA-A0201 molecules.

Thus, Gly 4 in peptide 369 was replaced by either a threonine or a valine, Glu 4 in peptide 402 was replaced by either a serine or a valine, asparagine 4 in peptide 435 was replaced by aspartic acid, glutamine in peptide 689 was substituted by either a proline or an alanine, and Ala 4 in peptide 845 was replaced by a serine. Peptide HIV had three variants, i.e. a lysine, or an arginine, or a leucine, replacing glutamic acid, and peptide 92 had a variant with glycine substituting Pro 4. Several substitutions were made also in peptide 96 (only one reported).

The binding data, as shown in Table II, confirmed essentially our predictions: the binding affinity of modified peptides 369–4Thr, 369–4Val, 402–4Val, HIV-4Arg and HIV-4Leu was lower than that of the original peptides. There were no substantial differences between 402–4Glu and 402–4Ser, or 92–4Pro and 92–4Gly, while there was already a slight increase of binding affinity of peptide 845–4Ser, compared to 845–4Ala. Interestingly, peptides with lysine or aspartic acid at position 4 bound to HLA-A0201 with essentially the same affinity as the original peptides with uncharged residues. Substitutions in the low-affinity peptide 96 did not modify the binding ability.

A correlation among the seven amino acids that appear to increase the binding affinity of peptides to HLA-A0201 molecules could be found on the basis of the individual atomic temperature factors (i.e. B-factors) of their Cα atoms, that influence the degree of freedom of the bond and thus the chain flexibility of polypeptide chains. (Karplus and Schulz, 1985). The ranking of amino acids according to bond flexibility is reported in Table III, together with the ranking according to hydrophilicity (Hopp and Woods, 1981).

Table II. Binding of synthetic nonapeptides to HLA-A0201 soluble molecules, as determined with the α chain refolding assay.

Two sets of experiments were performed, under identical conditions, each in duplicates. For each experiment, the values reported are the mean of two determinations. (max. range + or – 14%). Peptides 369 and 402 were tested twice, as a control * indicates the original, unmodified control peptide.

First experiment: Peptide name	Binding affinity (α-chain refolding)
*369–4Gly	605
369–4Thr	510
*402–4Glu	420
402–4Val	300
*435–4Asn	440
435–4Asp	434
*689–4Gln	620
689–4Pro	595
689–4Ala	540
*92–4Pro	480
92–4Gly	466
Control (no peptide, to be subtracted)	95
Second Experiment	
*369–4Gly	786
369–4Val	600
*402–4Glu	515
402–4Ser	524
*845–4Ala	260
845–4Ser	322
*HIV-4Glu	798
HIV-4Lys	767
HIV-4Arg	521
HIV-4Leu	500
*96–4Thr	210
96–4Ser	220
Control (no peptide, to be subtracted)	125

(for binding affinity / α-chain refolding, see footnote in Table I).

Table III. Ranking of amino acids according to B-values of the Cα atoms (Line A) (Karplus and Schulz, 1985), and to hydrophilicity (Line B) (Hopp and Woods, 1981).

A)	Lys, Ser, Gly, Pro, Asp, Glu, Gln, Thr, Asn, Arg, Ala, Leu, His, Val, Tyr, Ile, Phe, Cys, Trp, Met.
B)	Arg, Asp, Glu, Lys, Ser, Asn, Gln, Gly, Pro, Thr, Ala, His, Cys, Met, Val, Ile, Leu, Tyr, Phe, Trp

At the left, the most flexible and, respectively, the most hydrophilic amino acid residues. Methionine and tryptophan respectively, are the most rigid and the most hydrophobic residue.

On the basis of this ranking, it is noteworthy that most peptides with high affinity for HLA-A0201 possess, apart few exceptions, "flexible" residues at position. 4. Arginine and asparagine, hydrophilic, but not flexible, do in fact seldom occur in high affinity ligands. A replacement of a flexible residue by a "rigid" amino acid results in a decrease of binding affinity, while replacements within the same group is substantially unrelevant. On the other hand, replacement of a rigid residue by a "flexible" residue seems to increase the binding affinity.

The residue at position 4 is of course not as decisive for binding as residues 2 and 9, but contributes, like other amino acids at different positions, to the stability of the complex. This is in agreement with the observation that a primary anchor residue motif, although necessary for HLA binding, is not by itself sufficient to guarantee high affinity (Ruppert *et al.* , 1993).

It is known that peptides inserted into the binding cleft of the HLA molecule are released upon acid treatment. Because of the breaking of the weak but multiple forces and interactions between residues of the peptide and amino acids of the HLA molecule, the groove likely assumes an open, looser structure, which under suitable circumstances, can accept exogenous peptides. The insertion of a new fragment obeys to rules, insofar the binding cleft can accomodate almost exclusively fragments that possess "canonical" anchor residues, free amino and carboxy termini, a fixed length (9 residues), and combinations of amino acids that do not create charge repulsions and steric hindrance with HLA residues within the cleft. Also chain flexibility at key positions should facilitate the accomodation of the fragment into the narrow space of the binding cleft which, after the binding of the peptide, tries to assume the original tight structure and conformation. In few words, a peptide may be fully accomodated only if it is able to adapt himself to the final shape of the groove. Sub-optimal chemical and physical properties, (composition, length or rigidity), interfere with a precise fitting of the peptide into the "print", and prevent the groove from re-assuming its tightened structure, as well as the complete refolding of the HLA molecule. Partial refolding indicates that the peptide does not exactly fit into the binding cleft.

For different HLA allotypes, the requirements for flexibility of the ligand may be different, or even fail, depending on the nature of HLA amino acids forming the inner structure and shape of the groove. It should be remembered, however, that flexibility and rigidity of polypeptide chains play a decisive role in several protein-protein interactions: as an example, mobility is a prerequisite for the recognition of protein domains by antipeptide antibodies (Westhof *et al.*, 1984; Chersi *et al.*, 1997).

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