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Augmentation of the Affinity of HLA Class I-binding Peptides Lacking Primary Anchor Residues by Manipulation of the Secondary Anchor Residues

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Abstract: A direct binding assay has been used to investigate the effect of the secondary anchor residues on peptide binding to class I proteins of the major histocompatibility complex. Based on predictions from a previous chemometric approach, synthetic peptide analogues containing unnatural amino acids were synthesized and tested for B*2705 binding. Hydrophobic unnatural amino acids such as α-naphthyl- and cyclohexyl-alanine were found to be excellent substituents in the P3 secondary anchor position giving peptides with very high B*2705-binding affinity. The binding to B*2705 of peptides optimized for their secondary anchor residues, but lacking one of the P2 or P9 primary anchor residues was also investigated. Most such peptides did not bind, but one peptide, lacking the P2 Arg residue generally considered essential for binding to all B27 subtypes, was found to bind quite strongly. These findings demonstrate that peptide binding to class I proteins is due to a combination of all the anchor residues, which may be occupied also by unnatural amino acids–a necessary step towards the development of peptidic or non-peptidic antagonists for immunomodulation.

Keywords: Antagonists; chemometry; major histocompatibility complex; molecular modelling; unnatural amino acids

ABBREVIATIONS

β_2m, beta 2 microglobulin; ANI, anisole; Cha, cyclohexylalanine; DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; EDT, 1,2-ethanedithiol; ES, electrospray; FAB, fast atom bombardment; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HLA, human leucocyte antigen; MHC, major histocompatibility complex; Nal, α-naphthyl-alanine; QSAR, quantitative structure–activity relationship.

INTRODUCTION

The class I proteins expressed by the MHC locus are highly polymorphic cellular glycoproteins and are known to bind within a well-defined binding groove peptides derived by proteolysis of intracellular proteins. The endogenous peptides are bound and

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transported to the outer surface of the cell where the MHC class I–peptide complexes expressed on the cell surface induce specific T-cell immune responses that are vital not only in viral and tumor immunity, but also in autoimmunity.

The peptide-receptor function that can deal with a large spectrum of structurally divergent peptides is achieved through a molecular mechanism recently elucidated by molecular crystallographic analyses of MHC class I proteins bound to peptides and by the sequencing of peptides eluted from the bound MHC class I proteins [1–4]. It is now clear that the binding specificity is governed by the complementary interactions of the sidechains of several 'anchor' amino acid residues of the bound peptide with the corresponding peptide sidechain-'anchoring' pockets located in the antigen-binding groove of MHC class I proteins [5–8]. In the case of human MHC class I protein HLA-B27, the bound peptides are mostly nonamers and the residues in positions 2 (P2) and 9 (P9) show limited variation and provide the 'primary' anchor residues [9, 10]. Direct binding studies of the MHC class I–peptide interaction have, however, indicated that the primary anchor residues are required for binding, but are not sufficient alone for high affinity binding [11–13]. In fact, crystallographic analyses indicate that, for B27-binding peptides, not only P2 and P9, but also positions 3 (P3) and 7 (P7), and possibly positions 5 (P5) and 6 (P6) are orientated so that their sidechains may interact with the peptide-binding pockets of the protein [7, 9].

To evaluate the contribution to the binding of these 'secondary' anchor residues, we have previously reported a QSAR study of B27-binding peptides using a chemometric approach. This method demonstrated the prominent effect of P3 on B27-binding and permitted us to design a peptide endowed with high B27-binding affinity [14]. The affinity was about two orders of magnitude greater than that of a known natural B27-binder. The approach used allowed us to obtain the largest amount of information from the lowest number of synthetic analogues with substitutions in multiple locations of a given starting compound. The information derived from the study is then used to design new compounds with enhanced activity and to provide a better understanding of the underlying biological mechanism involved.

In the present work, we used the sequence of the high affinity peptide resulting from the chemometric approach as the template for the design of synthetic B27-binding antagonists potentially useful as alelle-selective immunosuppressors [15–23]. We report here: (a) further optimization of the P3 secondary anchor residue for B27-binding by the use of unnatural amino acids, and (b) the role of the P2 and P9 primary anchor residues in the peptides optimized in such a way for B27-binding.

MATERIALS AND METHODS

Synthesis

General Procedure. Solvents and reagents for solid-phase synthesis were purchased from Millipore (Hamburg, Germany). Protected amino acids derivatives, HBTU, HOBt, DCC and DMAP were from Novabiochem (Läufelfingen, Switzerland). TentaGel SAC resin was obtained from Rapp Polymere (Tübingen, Germany) and Fmoc-Lys(Boc)-PEG PS from Millipore (Bedford, MA, USA). TFA was from Farmitalia-Carlo Erba (Milan, Italy), ANI, EDT and acetonitrile from Merck (Schuchardt, Germany).

The peptides were analysed by RP-HPLC on a Beckman System Gold with diode array detector under the following conditions: Vydac C_{18} column (0.46 × 15 cm); eluant A: 0.1% TFA–water, eluant B: 0.1% TFA-acetonitrile; gradient from 5% to 65% B over 20 min, flow 1 ml/min. When necessary, peptides were purified by preparative RP-HPLC on a Beckman System Gold with UV detection at 210 nm using a Vydac C_{18} column (2.2 × 25 cm); eluants A and B were used as indicated above; gradient from 15% to 30% B over 60 min, flow 8 ml/min. The amino acid composition was determined using a Carlo Erba 3A 30 amino acid analyser after hydrolysis with 6 N HCl for 22 h at 110°. FAB or ES mass spectra were performed on a VG Quattro mass spectrometer (Fisons Instruments, Altrincham, UK).

The peptides were synthesized by the solid-phase method using a Milligen 9050 automatic synthesizer with Fmoc/tBu chemistry and continuous flow technology [24, 25]. The average scale of each synthesis was 0.05 mmol, using either prefunctionalized resin (Fmoc-Lys(Boc)-PEG PS) for peptides 4, 5, 8 and 10 (Table I), or TentaGel SAC for the others. TentaGel SAC resin was esterified with Fmoc-Lys (Boc)-OH and Fmoc-Ala-OH using a preformed symmetrical anhydrides, obtained reacting 2 eq of protected amino acid with 1 eq of DCC in CH_2Cl_2, and DMAP as described [24]. The substitution levels of the support, determined by spectrophotometric assay, were 0.24 meq/g (Lys) and 0.23 meq/g (Ala). Both resins contain an acid labile linker: 4-hydroxymethyl-phenoxyacetic acid for PEG PS and 4-Hy-

droxymethyl-3-methoxyphenoxyacetic acid for TentaGel SAC. The rigid framework of both resins allowed us to apply a high-flow rapid protocol during the syntheses.

The sidechain of Glu was protected by tBu ester, that of Arg by Pmc group while the indole ring of Trp was protected by Boc group only during the synthesis of peptides 7, 8 and 9 [26]. We have previously observed that the interaction between the methoxybenzyl alcohol-based linker and the unprotected indole sidechain of Trp residues during acidolitic cleavage of the peptide from the resin is the origin of both low yield (due to reattachment of the peptide to the resin) and low purity (due to Trp sidechain alkylated by-products) [27]. This is a likely explanation for the low yield of peptide 3 (31%, crude; ~1%, purified), prepared using unprotected Trp. The typical procedure for the synthesis of the peptides is described below, taking as an example the most active one.

Synthesis of Peptide 10. The synthesis was performed starting from 350 mg of Fmoc-Lys(Boc)-PEG PS resin with a substitution level of 0.15 meq/g. Each amino acid coupling cycle include the following steps: (a) deprotection of *N*-α-Fmoc group with a 20% solution of piperidine in DMF for 3.5 min at 8.1 ml/min; (b) wash of the resin with DMF for 4.0 min at 4.6 ml/min; (c) recycle of the activated Fmoc-amino acid through the resin for 15 min at 8.1 ml/min; (d) wash of the resin with DMF for 5.0 min at 4.6 ml/min. Coupling reactions were carried out using a four-fold excess of an equimolar mixture of protected amino acid, HBTU and HOBt dissolved in 0.7 ml of DMF in the presence of two equivalents of 4-methylmorpholine. After the removal of the N-terminal Fmoc group the resin was washed by DMF, CH_2Cl_2 and ether and dried *in vacuo*. The dried peptide-resin was treated with 10 ml of a mixture of TFA/ANI/EDT 95:2.5:2.5 for 1.5 h at room temperature and the peptide was precipitated with cold ether and lyophilized to yield 50 mg of product. Amino acid analysis: Ser (exp. 1, found 0.88); Ala (3, 3.1); Val (2, 1.9); Phe (1, 1.0); Lys (1, 1.0); an additional peak is attributed to Nal. $[M+H]^+$: 989.5 (exp. 990.3).

The analytical data for all the peptides are reported on Table 1. All crude peptides were obtained in good yield (>80%), with the exception of peptide 3 (31%), as discussed above. Peptides whose HPLC purity was <95% were purified by preparative HPLC. The more difficult separation was that of the crude mixture of peptide 11, containing several deletion products, probably due to the high hydrophobicity of its C-terminal sequence.

HLA Class I Alpha Chain Refolding Assay

Peptides were tested for binding with HLA class I alpha chain prepared from cells of an HLA-A homozygous cell line BTB (A2, B27, C1) at four different concentrations in the range 0.003–30 μM and the peptide concentration (μM) that induces half maximum binding was determined and presented as a measure of relative binding activity. The assay method is based on the serological determination of the refolding of alkaline denatured HLA class I alpha chains induced by binding to specific peptides in the presence of excess HLA class I beta chain, i.e. β_2m [28]. In brief, BTB cells (100×10^6) were lysed with 1 ml of a Tris–HCl buffer (Tris 50 mM, pH 7.5) containing 0.5% NP40 and protease inhibitors.

The lysate was cleared by centrifugation and denatured by adjusting the pH to 11.6–11.7 by

Table 1 Analytical Characterization of Synthetic Peptides

No.	Sequence	MW[a]	$[M+H]^{+b}$	RT[c]	Yield (%)
1	ArgArgIleLysGluIleValLysLys	1169.5	1170.4	7.2	86
2	SerArgTrpAlaValValTrpAlaLys	1102.3	1103.0	10.7	22[d]
3	SerArgChaAlaValValTrpAlaLys	1069.1	1069.6	11.4	~1[d]
4	SerArgChaAlaValValPheAlaLys	1030.0	1030.6	11.3	>95
5	SerArgNalAlaValValPheAlaLys	1074.3	1074.5	11.6	30[d]
6	SerAlaTrpAlaValValTrpAlaLys	1017.2	1017.0	11.5	30
7	SerArgTrpAlaValValTrpAlaAla	1045.2	1046.2	11.9	38[d]
8	SerAlaChaAlaValValTrpAlaLys	984.0	984.6	12.3	85
9	SerArgChaAlaValValTrpAlaAla	1012.0	1012.6	12.5	>95
10	SerAlaNalAlaValValPheAlaLys	989.3	989.5	12.7	>95
11	SerArgNalAlaValValPheAlaAla	1017.3	1017.5	12.5	12[d]

[a] MW: molecular weight. [b] $[M+H]^+$: quasi-molecular ion in FAB- or ES-MS. [c] RT: retention time in HPLC, see Materials and methods for conditions. [d] Yield after preparative HPLC.

addition of 1 N NaOH. Half of it was then gel-filtered on a column (10 × 180 mm) of Sephadex G75 Superfine using the 50 mM Tris–HCl buffer pH 7.5 containing 0.1% NP40. Fractions (0.5 ml) were collected and tested for protein/peptide content by the ninhydrin reaction. The major fraction of the first protein peak, containing essentially pure unfolded alpha chains, were then pooled. The unfolded HLA class I alpha chains (20 μl) thus prepared were incubated with test peptides (10 μl) and excess β_2m (2 μl) at 25° for 16 h. The final β_2m concentration was 1 μM. The resulting alpha chain refolding was quantified by measuring the alpha chain activity, i.e. the antigenic properties characteristic of folded alpha chains, by a specific radioimmunoassay involving inhibition of the binding of ^{125}I-labelled HLA-A2 (folded alpha and beta chains dimer) and rabbit antiserum raised against HLA class I and adsorbed with β_2m [29]. The adsorbed antiserum reacts only with folded HLA class I alpha chains and not with the unfolded conformation. The relative binding activities reported in Table 2 were determined from the date plotted in Figure 1. The reproducibility of the assay is estimated to be ±10%.

Molecular Modelling

The atomic coordinates for the B*2705 and A*0201 molecules were obtained from the 1hsa [8] and 1hhi [30] entries in the Protein Data Bank at Brookhaven National Laboratory [31, 32]. for B*2705 only one of the complexes in the structure/entry was considered; i.e. a single alpha, β_2m, peptide complex. After addition of hydrogen atoms, the whole structure was energy-minimized in a shell of water molecules using the CVFF force field and the conjugate gradients algorithm in DISCOVER (Biosym Technologies). The final minimization was to 0.01 kcal/Å maximum derivative. Appropriate amino acid changes were made to the peptide to produce the model peptides 2 to 5, SRXAVVXAK using BIOPOLYMER (Biosym Technologies). When changing the P9 amino acid residue from Ala to Lys care was taken to pilot the new longer sidechain into the C/F pocket following the structure of the A*0201 molecule which, in the 1hhi entry, has a P9 Leu residue. Energy-minimization was then performed for all four of the new peptides including all protein residues and water molecules within 10 Å of the peptide as described above.

RESULTS AND DISCUSSION

Table 2 shows the sequences of the synthetic peptides used, along with the binding affinity to HLA-B27 subtype B*2705 determined by the HLA class I alpha chain refolding assay. Peptide 1, ArgArgIleLysGlyIleValLysLys, is a peptide derived from the sequence of a human heat shock protein, HSP89α(201–209) and has been identified among the endogeneous peptides isolated from HLA-B27 molecules [9]. This peptide carries the B*2705 primary anchor residues, P2-Arg and P9-Lys, and was chosen as a reference naturally occurring self peptide. Peptide 2, SerArgTrpAlaValValTrpAlaLys, is a peptide esigned to be optimal for B*2705-binding based on the data obtained by the chemometric approach [14].

Table 2 Relative Binding Activity of Synthetic Peptides to HLA-B27

No.	Sequence									RBA (μM)[a]
1	Arg	Arg	Ile	Lys	Glu	Ile	Val	Lys	Lys	5.8
2	Ser	Arg	Trp	Ala	Val	Val	Trp	Ala	Lys	0.011
3	_	_	Cha	_	_	_	Trp	_	_	0.060
4	_	_	Cha	_	_	_	Phe	_	_	0.060
5	_	_	Nal	_	_	_	Phe	_	_	0.018
6	_	**Ala**	Trp	_	_	_	Trp	_	Lys	>30
7	_	Arg	Trp	_	_	_	Trp		**Ala**	>30
8	_	**Ala**	Cha	_	_	_	Trp	_	Lys	>30
9	_	Arg	Cha	_	_	_	Trp	_	**Ala**	>30
10	_	**Ala**	Nal	_	_	_	Phe	_	Lys	0.083
11	_	Arg	Nal	_	_	_	Phe	_	**Ala**	>30

[a] RBA: relative binding activity. Peptides were tested for binding with HLA class I alpha chains at four different concentrations (0.003–30 μM; see Figure 1) and the peptide concentration (μM) that induces half maximum binding was determined and presented as a measure of relative binding activity.

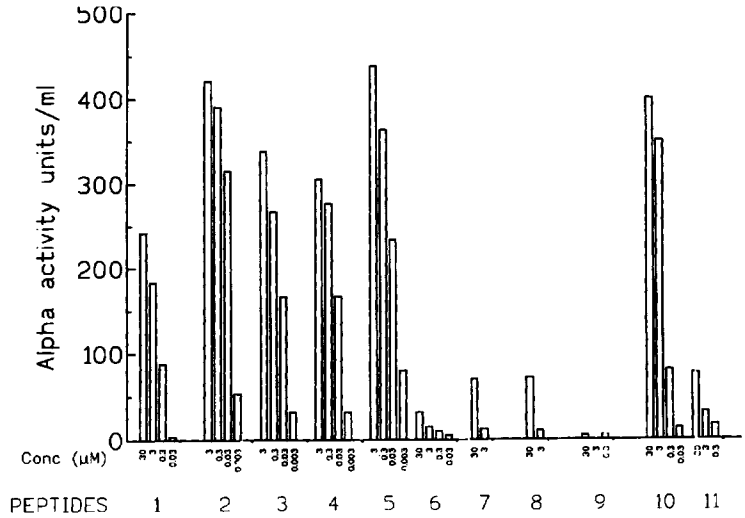

Figure 1. Binding activity of synthetic peptides at different concentrations (0.003–30 μM) to HLA-B27 presented as alpha activity units per ml of test sample.

It carries the same primary anchor motif as peptide 1 and is characterized by the bulky and hydrophobic Trp residues in P3 and P7. Its relative affinity, 0.011, was over 500 times better than peptide 1. This peptide was used here as the template for developing further peptides with optimized B27-binding affinity.

Replacement of the Secondary Anchor Residues with Unnatural Amino Acids and the Effect on Binding Activity

In peptide 3, SerArgChaAlaValValTrpAlaLys, a cyclohexyalanine residue replaces Trp in P3, all other positions being unmodified. The affinity, 0.060, was about five times lower than that of peptide 2. Peptide 4, SerArgChaAlaValValPheAlaLys, has an additional modification, Trp to Phe at P7. Nevertheless, this peptide gave the same affinity as peptide 3. In peptide 5, SerArgNalAlaValValPheAlaLys, a different unnatural residue, α-naphthylalanine, was used to replace cyclohexylalanine in P3, while maintaining Phe in P7. This peptide was found to have a much better affinity, 0.018, than peptides 3 and 4, almost comparable to peptide 2 used as the template peptide. All these modifications, namely Cha or Nal in P3 and Phe in P7, were dictated by a further refinement of our chemometric model [14]; the original requirement for a bulky and hydrophobic residue in both P3 and P7, was subsequently predicted to be more stringent in P3 than in P7, where in fact Trp can be replaced by Phe without affecting activity. On the other hand, a possible increase of affinity expected from the chemometric model when Trp in P3 is replaced with Nal was not observed. This may well be indicative that the maximum of affinity for B27-binding has been attained by the use of Trp in P3, and thus a further increment of binding affinity is not seen by the replacement with Nal, even though the latter is more hydrophobic and bulkier than Trp. It is also possible that their binding affinity cannot be distinguished, because the binding has reached the upper limit that the assay conditions used can detect.

Molecular modelling of the binding of peptides 2–5, based on the crystallographic structure B*2705 [8] indicated that the larger P3 and P7 sidechains appear to fill a depression in the binding groove rather than specific separate binding pockets. Smaller sidechains in P7 appear to fill the shallow E pocket formed by the alpha chain residues 114, 147, 152 and 156, but larger P7 sidechains, such as the Trp in peptide 2 or the Nal in peptide 5, extend towards the D pocket. In peptide 2 (Figure 2) and also in peptides 3–5, the models show that the aromatic (and Cha) sidechains in P3 and P7 are able to interact with each other by stacking. The areas of the surfaces in contact are approximately equal in all the models as the larger sidechains stack only partially. This interaction could perhaps contribute to stabilize the 'bulging' out of the groove of the intervening residues P4, P5 and P6.

Besides these findings, it is of particular interest to note that the presence of an unnatural residue in one of the anchor positions of a given peptide does not hamper the interaction with the corresponding MHC class I molecule. Simply speaking, a peptide anchor-

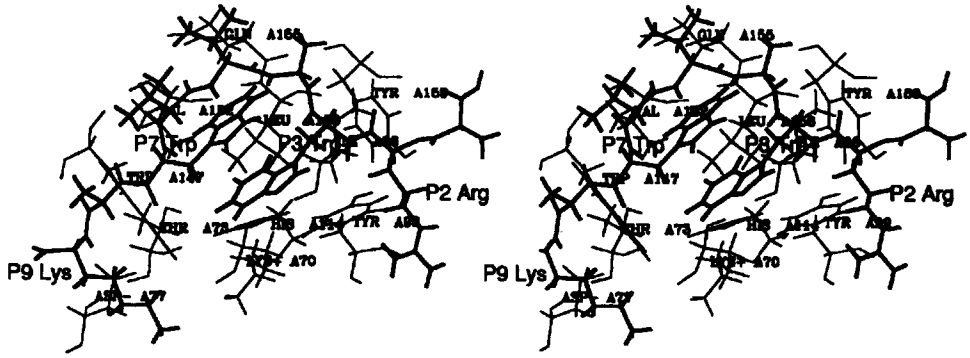

Figure 2 Stereoscopic model of peptide 2, SRWAVVWAK (heavy lines), in the binding groove of HLA-B*2705. Only selected residues of HLA-B*2705 relevant to the binding of the peptide P3 and P7 side chains are shown. The orientation was chosen to show how the P3 and P7 Trp sidechains can interact with each other.

ing pocket of MHC molecules can accommodate an unnatural amino acid, as also recently reported in the case of class II molecules [33]. This finding is significant as a first step toward peptidomimetics [34, 35] which aim at the development of non-peptidic antagonists for immunomodulation.

Significance of the Primary Anchor Residues in the Peptides Optimized for the Secondary Anchor Residues

Peptides 6–11 contain Ala substitutions in the primary anchor residue positions P2 and P9 (Table II). These substitutions were introduced into these peptides to investigate whether a very efficient set of secondary anchor residues endows a peptide with high affinity to an MHC molecule in the absence of one of the primary anchor residues. Ala was chosen because it had not been reported as an HLA-B*2705 primary anchor residue and thus it was supposed to give only a weak interaction, if any, with the corresponding pockets of B*2705 molecules.

Peptides 6 and 7 are Ala-P2 and Ala-P9 analogues, respectively, of peptide 2. Likewise, peptides 8 and 9 correspond to the same analogues of peptide 3, while peptides 10 and 11 to those of peptide 5. Among these six peptides, only peptide 10, SerAlaNalAlaVal-ValPheAlaLys, carrying Ala substitution in P2, retains a significant relative binding affinity, 0.083. This value is about four times lower than that of the parental peptide, peptide 5, but about 70 times higher than that of the reference compound, peptide 1. Therefore, the 'canonical' Arg residue in P2 is not mandatory for the interaction of peptides with B*2705 molecules, provided that the secondary anchor residues are suitably chosen from highly hydrophobic and bulky amino acids. Interestingly, the superiority of Nal compared with Cha and Trp as a secondary anchor residue was demonstrated when the dominant anchor residue was missing. These results are in line with recent reports that several natural MHC binders were found to be capable of inducing the correct folding of the relevant MHC molecules, even after both primary anchor residues were substituted with Ala [36, 37]. Thus, the presence of the primary anchor residues is not an absolute requirement for peptide binding. The present finding also indicates that both primary and secondary anchor residues independently contribute to the stabilization of MHC–peptide interactions. The independent role of the primary anchor residues has been shown for HLA-B7 by the direct peptide-binding analysis [38] and also for HLA-B27 by the induced mutations in peptide-anchoring pockets [39].

In conclusion, the combined effort of X-ray crystallography and sequencing of natural MHC binders led to the conceptual definition of the 'primary anchor residues', regarded as the residues primarily responsible for the binding of peptides to MHC molecules. Subsequently, the role of other residues in peptide binding was revealed. These residues, often referred to as 'secondary anchor residues', are thought to be required to obtain high affinity binding. In fact, we have shown here that the occupancy of the secondary anchor residue positions by proper amino acids is very important for high affinity binding of peptides to MHC molecules. This means that a peptide containing non-canonical amino acids in the primary anchor residues could bind efficiently to MHC molecules if suitable secondary anchor residues are present within its sequence.

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