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Increasing the potency of MHC class II-presented epitopes by linkage to Ii-Key peptide

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Abstract

We previously found that peptide Ii77-92 from the immunoregulatory Ii protein significantly enhances the binding of antigenic peptides to MHC class II molecules. Now a series of hybrids have been constructed linking LRMK, the active core region of the Ii77-92 peptide, to an antigenic epitope of cytochrome *C*. In vitro T cell hybridoma stimulation by some of these hybrids is up to 250 times more potent than by the antigenic peptide. The biological activities of the hybrids were tested in terms of length and composition of the linker. Simple spacers containing a polymethylene bridge (-HN-CH_2-CH_2-CH_2-CH_2-CO_2-) were fully active in these hybrids which can enhance vaccination with MHC class II-presented epitopes.

Keywords: Ii-key peptide; Hybrids; Peptide vaccines; MHC class II

1. Introduction

Our objective is to increase potency in presentation of major histocompatibility complex (MHC) class II-restricted antigenic epitopes to T cells. Peptide vaccines presented by MHC class II molecules have not been as widely exploited as peptide vaccines presented by MHC class I molecules, in part because the binding affinities are much less, for example 0.1 μM vs. 0.1 nM [1]. By coupling an immunoregulatory segment of the MHC class II-associated invariant chain (Ii), the Ii-Key peptide, to pigeon cytochrome *C* (PGCC)81-104, we have increased the potency 250 times in stimulating PGCC-specific T hybridomas.

The Ii protein blocks the antigenic peptide binding site of MHC class II molecules at their synthesis in the endoplasmic reticulum, where ambient peptides are readily bound to MHC class I molecules. After directing intracellular trafficking of Ii protein/MHC class II molecule complexes to a post-Golgi compartment, the Ii protein is digested and released, and antigenic peptides become bound to MHC class II molecules [2–6].

We have found previously that the peptide Ii 77-92 (LRMKLPKPPKPVSQMR), referred to as Ii-Key, enhanced presentation of antigenic peptides by living or fixed antigen presenting cells to T cell hybridomas [7,8]. The activities of 160 homologs of Ii77-92 were characterized in a murine T hybridoma activation assay as well as in an antigenic peptide binding and release assay, using immunopurified exomembranal forms of HLA-DR1. Those studies revealed a 'core' LRMKLPK structure which had greater potency than the original 16-amino acid peptide [9]. Even the LRMK segment retained moderate activity, although less than LRMKLPK. In testing the activities of various C-terminal deletion homologs of Ii-Key, it was found that the length of the C-terminus of the homologs defined two classes of Ii-Key peptides [10]. While both longer and shorter Ii-Key peptides accelerated binding of biotinylated hMBP peptide into 'empty'

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DR1 molecules, longer peptides more efficiently facilitated the loading of biotinylated hMBP peptide onto DR1 molecules previously charged with unlabeled hMBP. Finally, competitive peptide binding assays demonstrated that the Ii-Key binding site was distinct from the antigenic peptide-binding site [10]. This latter finding was of particular significance as it provided compelling evidence for the existence of an allosteric site controlling antigen presentation that is away from the antigen binding trough of MHC Class II molecules.

We now report the novel finding that a hybrid physically linking the LRMK portion of the Ii-Key with antigenic peptide has enhanced potency in antigenic peptide presentation. Furthermore, hybrids with chemically simplified linkers were also investigated and found to be effective.

2. Materials and methods

2.1. Synthesis of peptides

The peptides of Table 1 were synthesized by Commonwealth Biotechnologies Inc., 601 Biotech Drive, Richmond, VA 23225, USA. The purity and composition of each was confirmed by high performance liquid chromatography separation and mass spectrometry. The peptides were dissolved in phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.1 M NaCl).

2.2. T cell proliferation assay

Mitomycin *C*-treated CH27 cells (A^kE^k) antigen presenting cells (APC) were generated by incubating 5×10^6 cells/ml for 20 min at 37°C with 0.025 mg/ml of mitomycin *C* (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM)/10 mM *N*-2(hydroxyethylpiperazine-N′[2-ethanesulfonic acid]) (HEPES), followed by two washes with 4 vol. of DMEM–5% fetal calf serum (FCS), 10 mM HEPES. The T cell hybridoma Tpc9.1, which is specific for PGCC 81-104 peptide presented on the murine class II MHC allele E^k were irradiated 2200 rad before each assay. For the primary culture assay, 2×10^4 mitomycin *C*-treated APC, 1×10^5 T hybridoma cells and serial 1:4 dilutions from 3 μM of the peptides containing antigenic epitopes were cultured at pH 7.2–7.4, in complete DMEM–5% FCS, 10 mM HEPES, 1X nonessential amino acids (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 5×10^{-5} M 2-mercaptoethanol. Wells containing only T hybridoma cells (T) + APC were included to monitor for background T cell activation; and wells containing T + APC + antigenic peptides peptide were included to monitor for baseline antigen-specific T hybridoma activation. Supernatants (10 μl) from each culture were removed after 24 h and were assayed for their effect on growth of 1×10^4 interleukin-dependent HT-2 lymphoblastoid cells [added in 160 μl complete Roswell Park Memorial Institute (RPMI) 1640 buffer–5% FCS, respectively], as measured by incorporation of tritiated thymidine, added at 1 μCi/well during the last 5 h of a 24 h HT-2 assay. For all assays the reported value is the mean of triplicate wells, with a mean standard error of usually less than ± 10%.

3. Results

3.1. Design of hybrids with a variable spacer between the Ii-Key core motif and an antigenic epitope

The design rationale was drawn from X-ray crystallographic pictures of Ii protein-derived peptides and antigenic peptides in the antigenic peptide binding groove of MHC class II molecules. These concepts, explained below, were tested in hybrids in which the variables were the length and composition of the

Table 1
Design of hybrid peptides with variable spacers between the Ii-Key core motif and an antigenic epitope[a]

Hybrid number	Sequence		Symbol
	Ii Spacer	Antigen	
	Ac-	IAYLKQATAK -NH_2	×
1	Ac- LRMK- ava-	IAYLKQATAK -NH_2	○
2	Ac- LRMK- ava- ava-	IAYLKQATAK -NH_2	□
3	Ac- LRMK- LPKS-	IAYLKQATAK -NH_2	•
4	Ac- LRMK- LPKS-AKP-	IAYLKQATAK -NH_2	■
5	Ac- LRMK- LPKS-AKP-VSK-	IAYLKQATAK -NH_2	◆

[a] The single letter amino acid codes are as follows: A = L-alanine, D = L-aspartate, E = L-glutamate, F = L-phenylalanine, H = L-histidine, I = L-isoleucine, K = L-lysine, L = L-leucine, M = L-methionine, N = L-asparagine, P = L-proline, R = L-arginine, Q = L-glutamine, T = L-threonine, and Y = L-tyrosine. Ava = 5-aminopentanoic acid [ε-amino-*n*-valeric acid].

spacer between the Ii-Key core motif and an antigenic peptide (Table 1).

The shortest active sequence of Ii-Key peptides (LRMK) was a constant in this series, although this tetrapeptide had about half maximal activity of the best Ii-Key homolog LRMKLPK when tested in a T hybridoma activation assay [9]. The antigenic epitope, PGCC 95-104 IAYLKQATAK, was also a constant in the series of hybrid peptides. A theoretical Ii-Key/antigen hybrid construct was proposed from analysis of X-ray crystallographic images of the human leukocyte antigen (HLA)-DR1 antigenic binding site occupied by the cleaved leupeptin-induced peptide (CLIP) from Ii [11] and the influenza virus hemagglutinin (HA) peptide [12], respectively. This theoretical hybrid construct consists of the CLIP sequence crossing over to HA at the initiation of the antigenic epitope of the HA sequence.

This rationale for hybrid construction was tested specifically with hybrid No. 5. That construct was composed of the Ii-Key core sequence, LRMK and the PGCC antigenic epitope IAYLKQATAK linked with a spacer consisting of Ii protein residues LPKSAKPVSK (81–90). In this homolog, the amino acids of Ii protein are in exact registry with the CLIP, as residues of that Ii protein-derived peptide overlay residues of the HA peptide (307–319), when respective X-ray crystallographic images of CLIP and HA (indicated above) are superimposed. Specifically, methionine^{99} of CLIP is in the P1 hydrophobic pocket of the HLA-DR1 MHC class II molecule as is the Leu^{87} of the HA fragment. It should be noted that the LRMK core of Ii-Key [9] is distal to the N-terminus of the longest CLIP peptide [13].

3.2. Polyprolyl type II helical secondary structure

In order to appreciate the rationale for the design of the remaining hybrid peptides, it is useful to consider the secondary structure and alignment of the Ii protein and antigenic peptides as polyprolyl type II (PPII) helices, within the groove of the antigenic peptide binding groove.

X-ray crystallographic images show that the CLIP and antigenic peptides each coils in the antigenic peptide binding site as a PPII helix. In this type of helix, the amino acid repeat frequency per turn is 3.0 amino acids, in contrast to α-helices with 3.2 amino acids per turn. Along the longitudinal axis of the helices, the PPII helix is 'stretched out' about twice the distance per turn as found in α-helices. Consequently, PPII helices do not have the inter-turn hydrogen bonds which stabilize α-helices. Specifically, in an α-helix the peptidyl backbone imido proton of residue i forms a hydrogen bond to the peptidyl backbone carbonyl of residue $i + 3$. Due to this internal stabilization along the turns of a peptidyl backbone, α-helices form energetically relatively strong local secondary structures. Those helices then fold within proteins both upon each other and onto other local secondary structures.

In contrast, the extended PPII configuration provides for a much looser secondary structure. This type of structure has been employed in proteins as recognition units for protein–protein interactions. Such PPII helices are recognized, for example, by Src homology region 3 domains in proteins of second messenger pathways [14]. As indicated above, antigenic epitopes are also coiled as PPII structures in the antigenic binding site of MHC class II molecules for T cell recognition. Such PPII structures allow greater surface area for display of variable side chains of the antigenic sequence than would be possible for an α-helix. Given the triangular cross-sectional structure of a PPII helix, residues along one ridge of the antigenic peptide PPII helix bind into hydrophobic pockets at the base of the antigenic peptide binding cleft in the MHC II class II molecule. The side chains along the other two ridges of the PPII helix are exposed in shallow pockets along the surface of the MHC molecules for interaction with the T cell receptor. This configuration roughly doubles the number of potential atomic interactions between the antigen and MHC class II and T cell receptor molecules relative to the interactions that would be possible were the antigen coiled as an α-helix.

Finally, modeling studies indicate that the location of the allosteric site, for interaction with the Ii-Key core, might be entirely outside the antigen binding site of MHC class II molecules. In the antigenic peptide binding trough of MHC class II molecules (delineated by two anti-parallel α-helices) the PPII helical configuration of the bound peptide extends N-terminally at least five residue positions beyond the first residue of the commonly identified antigenic epitope. X-ray crystallography studies indicate that P^{87} of the Ii sequence (LRMKLPKPPKP^{87}) occurs at the end of the trough formed by the two anti-parallel α-helices [11,12].

3.3. Experimental hypotheses regarding structural requirements in spacers

In modeling possible interactions of the hybrid peptides consisting of the Ii-Key core structure (LRMK) and the antigenic epitope IAYLKQATAK, there are several hypotheses regarding contacts between the MHC class II molecules and specific atoms of the spacer (Table 1). In one hypothesis, atoms of the amino acid side chains in the spacer form hydrogen bonds with specific residues of the MHC class II molecule only when the spacer is coiled as a PPII helix. This view was tested with hybrid No. 5, in which the full 10 amino acid residues immediately C-terminal to

LRMK in the sequence of Ii protein constituted the spacer. That segment preserved the natural overlay of the CLIP residues and the HA antigenic peptide observed upon superimposition of the two X-ray crystallographic images. If only this hybrid in the series were biologically active, one could conclude that specific MHC class II residues in the trough (N-terminal to that holding the antigenic sequence) must hydrogen bond to specific groups in the spacer.

An alternative model requires no specific interactions between the spacer and MHC class II molecule. In hybrid No. 4, the first seven residues immediately C-terminal to LRMK in the sequence of the Ii protein were present as the spacer. In hybrid No. 3, only the first four residues immediately C-terminal to LRMK in the sequence of Ii protein were used as the spacer. If hybrids Nos. 4 and 3 had activities comparable to that of hybrid No. 5, then the secondary structure of the intervening segment as a PPII helix would not be critical.

Another test of the same idea was with hybrids Nos. 1 and 2, in which the spacer amino acid residues were replaced with ε-amino-valeric acid (ava) residues. Hybrid No. 2 used two ava residues and hybrid No. 1 used one ava residue. These hybrid peptides were homologs, respectively, of hybrids 4 and 3. The stretched out ava residue (-HN-CH_2-CH_2-CH_2-CH_2-CO_2-) approximates the length of the backbone of a tripeptidyl unit. If hybrids Nos. 1 and 2 were active, one can conclude that there are no functional requirements for specific interactions of atoms of the spacer with the MHC class II antigenic peptide binding trough.

The hybrid peptides were acetylated at the N-terminus and amidated at the C-terminus, to inhibit activity of exopeptidases.

3.4. Biological activities of the hybrids

The potency of antigenic epitope presentation to a T cell hybridoma was tested using the series of hybrids indicated in Fig. 1. A T cell hybridoma which is specific to the hornworm moth cytochrome *C* epitope IAYLKQATAK was stimulated with that antigenic peptide or with one of the hybrids. Cultures of an antigen presenting cell and T cell hybridoma were incubated with serial dilutions of the antigenic peptide (1:4 from 3 μM). Response was measured by tritiated thymidine uptake in an HT-2 culture into which supernatants of the antigenic stimulation culture had been transferred after 24 h of stimulation. The endpoint for half maximal response to the antigenic peptide was about 20 nM. The endpoints for half-maximal stimulation with hybrids were up to 250 times lower. The activity of hybrids using a methylene spacer are comparable to hybrids in which the spacer is the natural sequence of Ii protein. Thus, in clinically useful vaccine hybrids the spacer can be composed of a simple, flexible link.

An effective therapeutic is possible from the covalent hybridization of the Ii-Key core sequence, for example LRMK, through a flexible chain to a selected antigenic epitope. The flexible chain can be extended in length from three to six peptidyl units and can be composed of simple repeating units which do not hydrogen bond in any spatially distinct manner to the MHC class II molecule. In fact, such simple flexible spacers are preferred since less than optimal activity is found using a spacer composed of the natural 10 amino acid sequence of Ii protein between LRMK and the putative crossover site of CLIP and an antigenic peptide, as indicated by crystallographic data.

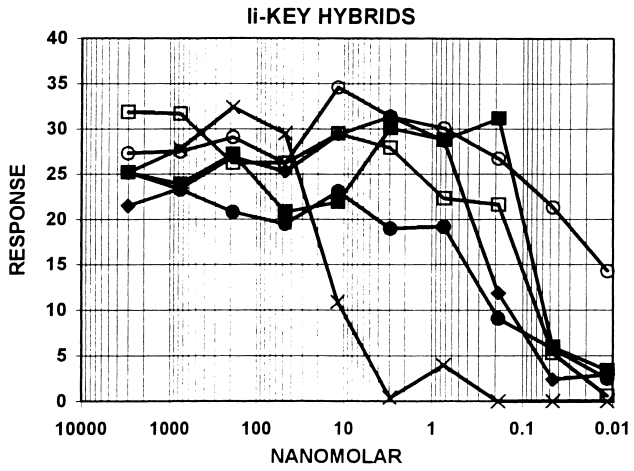

Fig. 1. Enhanced antigenic response by hybrids between Ii-Key core sequence and antigenic peptide. The immunological response to the antigenic epitope measured by tritiated thymidine uptake (*y* axis in thousands of counts/min), is presented as a function of the dilution factor of the hybrid (1:4 serial dilutions from a 3 μM stock solution). The symbols for the respective hybrids and their structures are presented in Table 1.

4. Discussion

Hybrids consisting of antigenic peptide — flexible linker — Ii-Key peptide have up to 250 times the potency of the respective antigenic peptide in a T hybridoma response assay measured at half-maximal response. The linker ε-amino-valeric acid, with four methylene (-CH_2-) groups, is fully active and preferred over amino acids of the Ii protein sequence due to simplicity of synthesis.

We previously showed that Ii-Key core peptides promote both the release of bound biotinylated antigenic peptides, and the exchange of a second peptide into

the antigenic peptide binding site [8,9]. Covalent linkage of the Ii-Key core sequence with the antigenic peptide does not appear to lead to a competition between these two functions, but instead to an increased level of binding and recognition of antigenic determinants. This could occur due to increased representation of the antigenic epitope on the surface of the APC, or to some favorable structural change in the MHC–antigenic peptide–T cell receptor complex induced by the presence of the Ii-Key epitope. Such a favorable structural change could lead to either attraction or repulsion of various cell surface molecules interacting with the hybrid containing MHC class II complex. Such effects have been reported when antigenic epitopes are linked to antibodies reacting with T cell surface molecules other than T cell receptors [15]. Substitution of antigenic epitopes into the CLIP also creates antigen specific stimulants with properties like those reported here [16]. Those compounds do not include the Ii-Key core segment and do not address the use of simple flexible spacers.

Ii-Key hybrids might accelerate the development of MHC class II-restricted antigenic peptide vaccines. Addition of Ii-Key spacer during the synthesis of peptide libraries, by combinatorial methods, will greatly increase the potency of the products. When the sensitivity of indicator assays are limiting, for various reasons, the greatly enhanced potency of the components in the peptide/peptidomimetic libraries will afford better pickups and/or SAR studies. This result is obtainable whether the readout is cellular proliferation or a biophysical measurement.

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