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Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding

T.S.Jardetzky[1,2], J.C.Gorga[1], R.Busch[3], J.Rothbard[3], J.L.Strominger[1] and D.C.Wiley[1,2]

[1]Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 01238, [2]Howard Hughes Medical Institute, Harvard University, Cambridge, MA 02138, USA and [3]Imperial Cancer Research Fund, London, UK

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Major histocompatibility complex (MHC) glycoproteins play an important role in the development of an effective immune response. An important MHC function is the ability to bind and present 'processed antigens' (peptides) to T cells. We show here that the purified human class II MHC molecule, HLA-DR1, binds peptides that have been shown to be immunogenic *in vivo*. Detergent-solubilized HLA-DR1 and a papain-cleaved form of the protein lacking the transmembrane and intracellular regions have similar peptide binding properties. A total of 39 single substitutions were made throughout an HLA-DR1 restricted hemagglutinin epitope and the results determine one amino acid in this peptide which is crucial to binding. Based on this analysis, a synthetic peptide was designed containing two residues from the original hemagglutinin epitope embedded in a chain of polyalanine. This peptide binds to HLA-DR1, indicating that the majority of peptide side chains are not required for high affinity peptide binding.

Key words: influenza/major histocompatability complex/peptide binding/T cell epitopes

Introduction

The generation of an effective immune response to foreign antigens is regulated by the histocompatibility molecules of an individual (Zinkernagel and Doherty, 1974; Schwartz, 1985). The major histocompatibility complex (MHC) molecules on the surface of target cells present a 'processed' antigen to T cells and thereby elicit either a T helper or T killer response to the antigen. The T helper, class II MHC restricted, response controls the expansion and maturation of selected B cells (i.e. antibodies), while the T killer, class I MHC restricted, response is responsible for the destruction of infected cells. Both class I and class II molecules are recognized by the same basic set of T cell receptor genes, which are rearranged to form cell bound receptors analogous to antibodies (for a recent review, see Davis and Bjorkman, 1988). However, T cell receptors have the additional restriction that antigen is only recognized in the presence of a self MHC molecule.

For both class I and class II MHC molecules, it has been shown that the cellular response to intact antigens can be mimicked using the appropriate T cells, presenting cells and synthetic peptides (Ziegler and Unanue, 1982; Shimonkevitz *et al.*, 1984; Townsend *et al.*, 1986). This work suggested that peptides are the form of foreign antigen which interacts directly with the MHC molecules generating an antigen-MHC complex which is recognized by T cells (Benacerraf, 1978). This has subsequently been supported by the observation of peptide binding to purified murine MHC molecules *in vitro* (Babbitt *et al.*, 1985; Buus *et al.*, 1986; Watts *et al.*, 1986), and a more extensive comparison between MHC restriction and peptide binding *in vitro* supports the idea that the T cell immune response depends on the ability of Ia molecules to bind peptide antigens (Buus *et al.*, 1987). The determination of the crystallographic structure of the HLA-A2 molecule (Bjorkman *et al.*, 1987a, b) has provided further support for this MHC function and a model of the class II antigen binding site has been proposed (Brown *et al.*, 1988), based on the presence of similar sequence patterns of polymorphic and conserved amino acids in class II molecules aligned with class I molecules.

While the formation of peptide-MHC complexes neatly satisfies the requirement that one T cell receptor recognizes both antigen and MHC, it poses a new problem of antigen recognition by MHC molecules. While every individual has a very large number of antibodies and T cell receptors of different antigenic specificities, this is not the case for the MHC molecules. A limited set of MHC molecules must be able to recognize a universe of unknown antigens. Defining the elements of MHC binding specificity will be important in understanding this level of immune surveillance as well as in designing functional vaccines or other therapeutic reagents.

Here we describe the ability of a purified human class II molecule (HLA-DR1, in detergent-soluble and papain-cleaved forms) to bind two peptides derived from the influenza virus hemagglutinin (HA) and matrix (M) proteins, which have previously been defined as T cell epitopes in HLA-DR1 individuals (Lamb *et al.*, 1982a,b; Rothbard *et al.*, 1988). Single amino acid substitutions were made in the HA peptide and tested for binding, in order to determine residues of the peptide which define the specificity of the peptide-MHC interaction. We have attempted to simplify the problem of understanding peptide-MHC interactions by determining the minimum number of peptide side chains required to confer full binding to the HLA-DR1 molecule.

Results

Purified HLA-DR1 binds immunogenic peptides

The amino acid sequences of two HLA-DR1 restricted peptides are shown in Figure 1. The matrix epitope (M 17–31) and the hemagglutinin epitope (HA 306–318), both from influenza A virus, have been characterized previously (Lamb *et al.*, 1982a,b; Rothbard *et al.*, 1988) as HLA-DR1 restricted epitopes. The M 17–31 peptide and HA 306–318/D306 peptides were ^{125}I-labeled with Bolton–Hunter reagent (Bolton, 1986) and purified by gel filtration. Radioactive peptides were incubated with purified HLA-DR1

Influenza A Hemagglutinin 306-318	**PKYVKQNTLKLAT**
Influenza A Matrix Protein 17-31	**SGPLKAEIAQRLEDV**
Tetanus Toxin 763-775	**SGPDKEQIADEIN**
Ragweed Ra3 Protein 54-65	**REEAYHAADIKD**

Fig. 1. Peptide amino acid sequences. The single letter abbreviation of the amino acid sequences of two HLA-DR1 restricted peptides are shown (HA 306−318 and M 17−31). Below these, the sequences of two peptides which do not bind to HLA-DR1 are given: tetanus toxin (TT) 763−775 and ragweed Ra3 protein (Ra3) 54−65. Identical residues between M 17−31 and TT 763−775 are underlined.

and HLQ-DQw1 (Gorga *et al.*, 1987) for 48 h at 37°C, and binding was assayed by G-50 gel filtration as described for murine Ia molecules (Buus *et al.*, 1986). Both the matrix and hemagglutinin derived peptides bind to HLA-DR1, but not to HLA-DQw1 (Table I), consistent with the immunological data demonstrating HLA-DR1 restriction for these epitopes (Lamb *et al.*, 1982a,b; Rothbard *et al.*, 1988). The observed binding to HLA-DR1 could be inhibited by an excess of either of the two unlabeled peptides, indicating that the two epitopes compete for the same HLA-DR1 binding site (Table I). Similar levels of peptide binding were observed with papain-cleaved HLA-DR1 (data not shown).

The specificity of this peptide binding was further explored by testing other peptides for the ability to inhibit the interaction between M 17−31 and HLA-DR1. For example, the ragweed Ra3 54−65 and tetanus toxin 763−775 peptides do not inhibit the M 17−31 peptide binding to HLA-DR1 at high concentrations (100 μM, Table II). Figure 1 shows the sequences of both of these peptides. The tetanus toxin peptide, which has six residues identical with the M 17−31, has no detectable binding to the HLA-DR1 molecule. Further investigations with peptides from the circumsporozooite protein from Malaria indicate binding of CS 378−398, but not CS 103−122, CS 325−341 or $(NANP)_3$ (unpublished observations). Subsequent studies (Sinigaglia *et al.*, 1988) determined that CS 378−398 has the ability to stimulate a T helper response in the context of many DR alleles, including HLA-DR1. These data indicate that purified HLA-DR1 has the ability to specifically bind peptides, which can be shown to generate an HLA-DR1 restricted immune response.

Peptide binding kinetics with intact and papain-cleaved HLA-DR1

Buus *et al.* (1986) have shown that murine class II MHC molecules bind peptides with slow kinetics. A similar characterization of the binding kinetics for HLA-DR1 provides a comparison with the murine class II data, and allows a functional comparison between detergent-solubilized and papain-cleaved forms of the HLA-DR1 molecule.

The association rate data are shown in Figure 2A. The detergent-solubilized and papain-cleaved forms of HLA-DR1 show slow association rates, which are essentially indistinguishable within experimental error. The dissociation rate data are shown in Figure 2B. Again, the two forms of the HLA-DR1 molecule show similar, slow rates, although

Table I. Specific binding of antigenic peptides to HLA-DR1

Inhibitor[d]	Protein[b] (percent bound[a])			
	HLA-DR1		HLA-DQw1	
	[^{125}I]MP	[^{125}I]HA	[^{125}I]MP	[^{125}I]HA[c]
–	25.0	35.0	1.3	1.0
100 μM MP	1.4	2.3	ND[e]	ND
100 μM HA	1.5	1.4	ND	ND

[a]Peptide binding at pH 7.0 in PBS was assessed using a gel filtration assay (Buus *et al.*, 1986).
[b]HLA-DR1 and HLA-DQw1 were purified as described (Gorga *et al.*, 1987). Protein was at a final concentration of 2 μM.
[c]Peptides M 17−31 and HA-D306 were labeled with [^{125}I]Bolton−Hunter reagent and purified by gel filtration as described in Materials and methods.
[d]Peptides (MP: M 17−31; HA: HA 306−318) were synthesized as described in Materials and methods and included in the binding assays at the indicated final concentrations.
[e]Not determined.

Table II. Inhibition of M 17−31 binding to HLA-DR1 by peptides[a]

Peptide[b]	Inhibition[c]
HA 306−318	+++
M 17−31	+++
Ra3 54−65	–
TT 763−775	–
HA K313,K314	++

[a]Inhibition titrations were performed as described in Materials and methods.
[b]Peptides were synthesized as described in Materials and methods.
[c]Concentration range of peptide required for 50% inhibition of M 17−31 binding: +++, <1 μM; ++, 1−10 μM; +, 10−100 μM; −, >100 μM.

the detergent-solubilized form consistently appears to dissociate at a slightly faster rate. This difference in rate may be due to the presence of NP-40 in the intact HLA-DR1 experiments.

Although there may be small quantitative differences in the binding of peptides between detergent-solubilized and papain-cleaved forms of HLA-DR1, the data support the contention that papain cleavage of HLA-DR1 does not greatly affect the peptide binding function of the molecule. In addition, the conservation of the slow kinetics between mouse and human class II MHC molecules indicates the importance of the formation of stable peptide−MHC complexes.

A native PAGE peptide binding assay

Given the observation that peptide dissociation from MHC molecules is slow, the possibility of using non-denaturing PAGE for separating bound and free peptide was investigated. Figure 3A shows an inhibition titration of unlabeled HA 306−318, analyzed by native PAGE. Radioactive peptide−DR1 complex (lane b) runs in the same position as HLA-DR1 alone (lane a). The intensity of the band decreases as the concentration of inhibitory peptide is increased (lanes c−g). The level of inhibition can be quantitated by counting the isolated radioactive bands. These data are normalized to the maximum values found in the absence of inhibitor and are plotted in Figure 3B, along with a parallel titration measured by the gel filtration assay. The

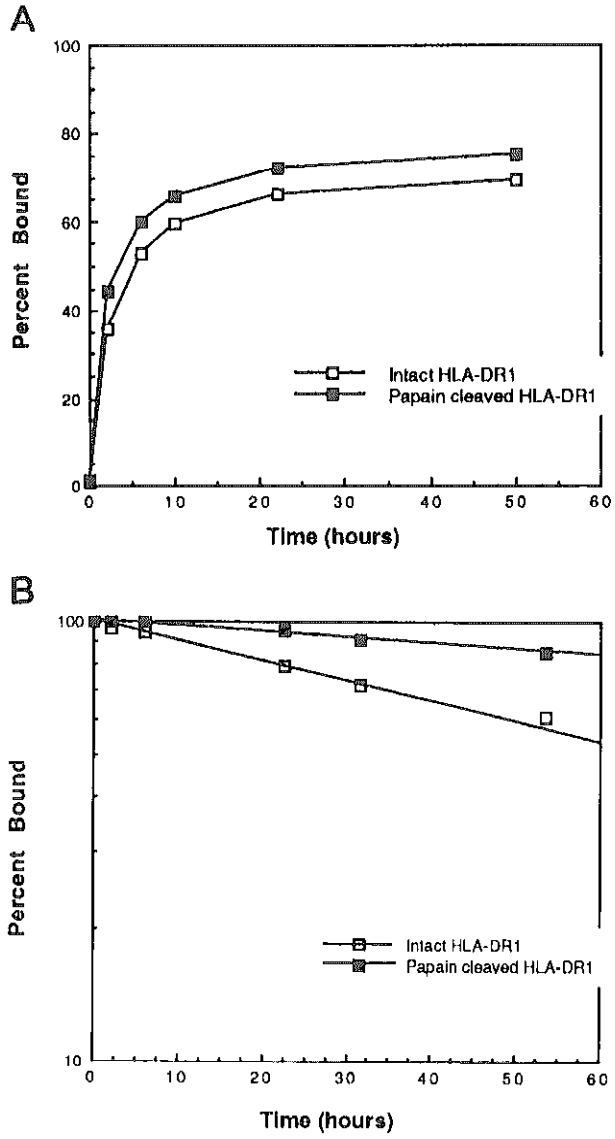


Fig. 2. Kinetics of intact and papain-cleaved HLA-DR1 peptide binding. (A) HLA-DR1 and papain-cleaved HLA-DR1 were incubated at 10 μM with ^{125}I-labeled M 17–31 at 37°C for the indicated time and complex formation was analyzed by G-50 gel filtration. (B) ^{125}I-labeled M 17–31–HLA-DR1 complex was isolated by gel filtration. The peak fraction containing diluted complex was adjusted to 100 μM unlabeled M 17–31 and incubated at 37°C. Aliquots were taken at the indicated times and re-run over the Sephadex G-50 column. The percent of maximum c.p.m. contained in the void volume fractions was used to calculate the percent dissociation. Data were fit to the equation $A = A_0\exp(-k_{off}t)$, with k_{off}(det–DR1) = 2.5×10^{-6} s^{-1} and k_{off}(pap–DR1) = 7.3×10^{-7} s^{-1}.

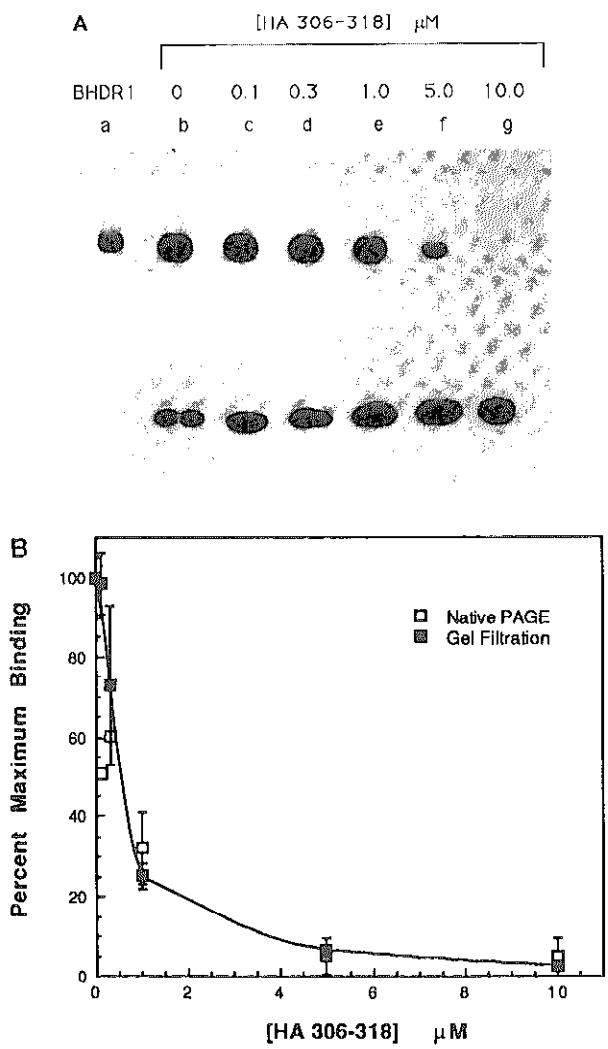


Fig. 3. Comparison of the native PAGE and gel filtration binding assays. (A) ^{125}I-Labeled DR1 and ^{125}I-labeled peptide–DR1 complex were loaded on a 12% native polyacrylamide gel and run using standard buffers (see Materials and methods). After electrophoresis gels were fixed, dried and exposed to Kodak X-OMAT X-ray film at −70°C. Lane (a): [^{125}I]Bolton–Hunter-labeled HLA-DR1 (BHDR1). Lane (b): ^{125}I-labeled M 17–31 and HLA-DR1 complex. Lanes (c)–(g): inhibition of labeled M 17–31 binding by increasing amounts of HA 306–318 peptide. (B) Quantitative comparison of gel filtration and native PAGE assays. Inhibition titrations were done as described in Materials and methods. Samples were analyzed by either gel filtration or native PAGE. In the PAGE assay, bands were localized by autoradiography, cut out and counted in a γ counter. Titration points were normalized to the maximum amount (% or c.p.m.) bound found in the absence of competing peptide. The values in the native PAGE assay were corrected for background by subtracting out the values found with peptide alone (typically ~2–3% of total signal). Error bars represent the average deviation derived from two experiments.

agreement between the two methods is good and this method has the advantage that many samples can be analyzed in parallel. It may also prove useful for the study of small quantities of mutant HLA molecules.

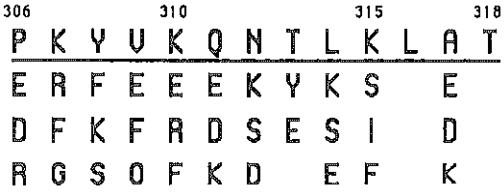
Single substitution analogs of the HA 306–318 epitope

Single-substitution analogs of the HA peptide are collected in Figure 4. The nature of the amino acid substitutions was chosen to test the ability of HLA-DR1 to discriminate between relatively small side chain differences (e.g. lysine to arginine at positions 307 and 310, and tyrosine to phenylalanine at position 308) as well as more radical changes in side chain character (e.g. lysine to glutamate at position 310, or tyrosine to aspartate or lysine at position 308), which includes the introduction of charged residues at hydrophobic positions.

Figure 5A shows the titration curves obtained for the peptides substituted at position 306. In this case, each of the peptides shows similar ability to bind HLA-DR1. This is true for the majority of the substitutions tested, except

DR1 molecule. The characteristics of HLA-DR1 binding

As the peptide HA-YAK forms slow-dissociating complexes



for those at position 308. The titration curves for these peptides are shown in Figure 5B. Substitution of tyrosine to phenylalanine results in a peptide with essentially unchanged binding characteristics. However, substitution to lysine, aspartate or serine at this position drastically alters the binding of peptide to HLA-DR1. The inhibition curves

T.S.Jardetzky *et al.*

The addition of a side chain could have three consequences: (i) increase affinity by interacting at a specific subsite of the MHC antigen binding site, (ii) decrease affinity by creating steric or other unfavorable interactions between peptide and MHC, and (iii) have no effects on the affinity of peptide to MHC. For example, Tyr308 apparently contributes binding energy, indicating a favorable interaction with the MHC molecule. In contrast, the double substitution of K313, K314 (Table II) decreases the affinity at a position where side chains are not crucial for high affinity binding, suggesting that a steric constraint reduces binding. An MHC molecule with fewer requirements for specific peptide side chains would bind a larger number of peptides, influencing the frequency with which it would present foreign antigens to the immune system.

These results also offer a plausible explanation for the observation that the CS 378–398 peptide from malaria can stimulate a response in the context of many different HLA-DR alleles (Sinigaglia *et al.*, 1988). All HLA-DR alleles share the same α chain, which contributes half of the antigen binding site (Brown *et al.*, 1988). Since very few peptide side chains may contribute to the binding energy, important side chains of the CS peptide may interact predominantly with the DR α chain. Based on the observations made with HLA-DR1, one could possibly design a simple polyalanine-based peptide to bind to all HLA-DR alleles. Allele specificity might then be achieved by adding amino acids which disrupt peptide interactions with the polymorphic HLA-DR β chain, providing a potentially simple route to the development of HLA-DR specific inhibitors.

Materials and methods

Preparation of HLA-DR1

HLA-DR1 and HLA-DQw1 were prepared as previously described (Gorga *et al.*, 1987). Briefly, for HLA-DR1, membranes from an HLA-DR1 homozygous B cell line (LG-2) were prepared and solubilized in 4% NP-40, 10 mM Tris, pH 8.0, 0.1 mM PMSF. This material was passed through a series of columns as described (Gorga *et al.*, 1987), using anti-DR antibody (LB3.1) coupled to protein A–Sepharose for the isolation of HLA-DR1. HLA-DR1 was eluted with 0.1% deoxycholate/50 mM glycine, pH 11.5, and fractions were immediately neutralized with 2 M glycine, pH 2. After papain digestion, separation of soluble HLA-DR1 from papain was achieved by gel filtration in 10 mM Tris, pH 8.0, 140 mM NaCl, 0.05% sodium azide. Protein was concentrated by vacuum dialysis and protein stock concentrations were generally 10 mg/ml.

Peptide synthesis

Peptides were synthesized using solid phase techniques (Barany and Merrifield, 1979) on an Applied Biosystems Peptide Synthesizer. Peptides were cleaved and extracted as described (Rothbard *et al.*, 1988). Peptides were purified by reversed-phase HPLC, lyophilized and subjected to amino acid analysis. Stock peptide solutions for binding studies were prepared and kept frozen at −20°C. Concentrations were determined by tyrosine absorption (Edelhoch, 1967) or by quantitative amino acid analysis.

Protein and peptide labeling

Protein and peptides were labeled with Bolton–Hunter reagent from New England Nuclear. Typically, 3 μg of peptide (or 10–20 μg of protein) was reacted with 100 μCi of ^{125}I-labeled Bolton–Hunter reagent in 10 μl of 50 mM sodium borate, pH 8.4, at 4°C for 16 h. Purification of labeled peptides and protein was carried out using 3 ml Sephadex G-15 gel filtration columns in an elution buffer of 10 mM potassium phosphate, pH 7.0, in phosphate-buffered saline (PBS; 20 mM potassium phosphate, pH 7.0, 130 mM NaCl). Typically, 2–20 μM HLA-DR1 was incubated with 100 nM Bolton–Hunter labeled M 17–31 in PBS, pH 7.0, containing 1 mM EDTA, 1 mM PMSF and 1 mM iodoacetamide ($IAANH_2$) in a total volume of 10–20 μl for 2 days at 37°C. Samples were loaded onto the gel filtration columns and eluted with PBS, collecting ~1.8 ml per fraction. Fractions were counted directly in a γ counter. The percent bound was calculated as the radioactivity eluting in the void volume of the column divided by the total radioactivity recovered. For studies with detergent soluble HLA-DR1, 0.5% NP-40 was included in the incubations and 0.1% NP-40 was used in the column running buffer.

PAGE

SDS–PAGE and native PAGE experiments were carried out with a BioRad Mini-Protean electrophoresis system. Separation gels were generally 12% in acrylamide and stacking gels were 6%. Native separation gels contained a final concentration of 0.38 M Tris, pH 8.2, and stacking gels contained a final concentration of 0.125 M Tris, pH 6.8. Running buffer was made up of 3 g Tris and 14.4 g glycine. Gels were electrophoresed for 2 h at 130 V, fixed for 30 min in 10% acetic acid and dried onto Whatman 3MM paper. Autoradiography was done at −70°C using Kodak X-OMAT AR X-ray film.

Inhibition titrations

Inhibition titrations for HA 306–318 analogs were set up in standard buffer (PBS, pH 7.0, 1 mM EDTA, 1 mM PMSF, 1 mM $IAANH_2$) with papain-cleaved HLA-DR1 at a final concentration of 2 μM in 10 μl. Inhibitory peptides and ^{125}I-labeled M 17–31 were mixed together initially and HLA-DR1 was added to start the binding reactions. Samples were incubated at 37°C for 48 h and 10 μl of non-denaturing sample buffer was added before electrophoresis. Aliquots of 10 μl of each sample were analyzed by native PAGE. After autoradiography, bands were located and cut out of the gels and counted directly in a γ counter. Three parallel samples provided the maximum value of radioactive peptide associated with HLA-DR1, with an average deviation of 10–15%. Two lanes of peptide in the absence of HLA-DR1 provided the background levels of radioactivity in the gels, and this was typically <2% of the maximum value for bound peptide. The percent of maximum bound values reported is calculated as [(sample c.p.m.)−(background)]/[(maximum c.p.m. bound)−(background)]. The results are the average of two to three experiments and error bars indicate representative average deviations.

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