Extended repertoire of permissible peptide ligands for HLA-B*2702

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Abstract

Recognition of self peptides bound to the class I major histocompatibility complex molecule HLA-B27 is thought to trigger proliferation of autoreactive T cells and result in autoimmune arthritic diseases. Previous work from other laboratories established that a predominant feature of endogenous peptides eluted from purified B27 is an arginine at position 2. We studied the binding of peptides containing both natural and unnatural amino acids by the subtype HLA-B*2702, with the goal of gaining insight into peptide binding by this B27 subtype that is associated with susceptibility to arthritic disease. A soluble form of B*2702 was depleted of endogenous peptides. We tested the binding of peptides substituted with cysteine, homocysteine, or an α -amino- ϵ -mercapto hexanoic acid side chain (Amh) instead of the naturally occurring arginine at position 2, to determine whether the peptide sulfhydryl residue could be covalently linked to cysteine 67 in the B*2702 binding cleft. Although none of the altered peptide sequences bound covalently to B*2702, the affinities of the homocysteine- and Amh-substituted peptides were close to that of the native peptide sequence. Substitutions at position 2 with other side chains, such as glutamine and methionine, also resulted in peptides that bound with only slightly reduced affinity. These results demonstrate that peptide side chains other than arginine at position 2 can be accommodated within the B*2702 peptide binding site with only minor reductions in affinity. This extended repertoire of permissible B27-binding peptides should be taken into account for a consideration of disease-associated peptide sequences.

Keywords: autoimmune disease; binding; HLA-B27; peptides; structure-based design; unnatural amino acids

Major histocompatibility complex (MHC) molecules are highly polymorphic integral membrane glycoproteins that play an important role in immune surveillance (Bodmer, 1978). During their assembly in the endoplasmic reticulum, class I MHC molecules bind peptide fragments derived from endogenously synthesized proteins, then transit to the cell surface where the peptide–MHC complex is displayed (Townsend & Bodmer, 1989). Crystal structures of human and murine class I MHC molecules demonstrated that class I proteins are folded into a shape ideally suited for peptide binding (reviewed in Bjorkman & Parham, 1990; Stern & Wiley, 1994, and references therein). Two long α -helices form the sides of a groove within the first two domains (α_1 and α_2) of the heavy chain of a class I molecule, and a β -pleated sheet forms the bottom. The class I light chain (β_2 -microglobulin; β_2m) and the other domain of the heavy chain (α_3) are underneath the α_1 and α_2 domains, propping them up in position to be recognized by a

T cell receptor on another cell. Peptides presented by class I molecules tend to be short (8–9 residues) and are derived either from normal cellular proteins (self peptides) or from viruses and other intracellular foreign agents (foreign peptides) (Townsend & Bodmer, 1989). The presence of foreign peptide–class I complexes at a cell surface targets the cell for lysis by a cytotoxic T-cell. Self peptide–class I complexes do not usually trigger lysis by T-cells because the immune system ensures self tolerance by several possible mechanisms (Marrack & Kappler, 1987; Qin et al., 1989).

Autoimmune diseases arise from a break-down in self tolerance (Todd et al., 1988). The human class I MHC molecule HLA-B27 is strongly associated with susceptibility to a variety of arthritic diseases (Brewerton et al., 1973, 1974). There are a number of subtypes of B27 (HLA-B*2701–B*2709), and at least four (B*2702, B*2704, B*2705, and B*2706) are linked to increased incidence of arthropathy (Benjamin & Parham, 1990). Approximately 70% of patients with reactive arthritis and more than 95% of ankylosing spondylitis patients carry the B27 gene, compared to 7% of the healthy population (Gilliland, 1987). A variety of evidence suggests that B27 itself is the disease susceptibility gene (Benjamin &

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Parham, 1990; Gaston, 1990; McMichael & Bell, 1991), including the demonstration that rats transgenic for the B27 gene spontaneously develop an arthritic disease with many features of ankylosing spondylitis (Hammer et al., 1990).

We are interested in understanding the properties of the B27 molecule itself, and of peptide binding by B27, that may contribute to the occurrence of an autoimmune reaction. The identification of a motif for peptides that bind to B*2705 and B*2702 (Jardetzky et al., 1991; Rötzschke et al., 1994), together with the available three-dimensional structure of HLA-B*2705 (Fig. 1) (Madden et al., 1991, 1992) facilitate studies of peptide binding by HLA-B27 subtypes. Sequence analysis of endogenous peptides eluted from B*2705 revealed a striking preference for nonameric peptides with an arginine at position 2 (P2), which was defined as an anchor position for B27-binding peptides (Jardetzky et al., 1991). The B*2705 and subsequent class I crystal structures showed that the class I preference for octamer and nonamer peptides is explained by the interaction of the main-chain atoms from the peptide N and C termini with conserved features of pockets A and F, which lie at opposite ends of the peptide binding groove (reviewed in Stern & Wiley, 1994). The intermediate pockets, B–E, are lined with residues that vary between alleles. In the case of B27-binding peptides, the arginine anchor residue is accommodated within pocket B, a mostly hydrophobic pocket ending near glutamic acid 45 and cysteine 67 (Fig. 1). The arginine side chain of the peptide forms a salt bridge with glutamic acid 45 (Madden et al., 1992). The side chain of the C-terminal residue of B27-binding peptides reaches into pocket F. The final residue of peptides that bind to most class I alleles tends to be aliphatic (Rammensee et al., 1993), although a dominance of positively charged residues at position 9 (P9) of B*2705-binding peptides was reported originally (Jardetzky et al., 1991). However, subsequent sequences of individual peptides and pools of peptides eluted from B*2705 showed hydrophobic as well as positively charged residues at the C-terminus, whereas sequences of peptides eluted from HLA-B*2702 suggested that the preferred C-terminal residue for peptides binding to this subtype is hydrophobic (Rötzschke et al., 1994). The F pockets of B*2702 and B*2705 differ by three residues at positions 77, 80, and 81 (B*2705 F pocket includes Asp 77, Thr 80, and Leu 81; the B*2702 F pocket includes Asn 77, Ile 80, and Ala 81; reviewed in Buxton et al., 1992). The presence of Asp 77 in B*2705 is likely to contribute to the observed preference for positively charged residues at P9 of B*2705-binding peptides (Rötzschke et al., 1994).

In the present study, we expressed a truncated, secreted form of HLA-B*2702 in Chinese hamster ovary cells (CHO) cells, and analyzed the binding of natural and unnatural peptides by this molecule. We designed peptides based upon the crystal structure of a HLA-B*2705–peptide complex (Madden et al., 1991, 1992). One of the goals of our studies was to determine if we could enhance the binding affinities of B*2702–peptide interactions by appropriate modifications at P2, because this residue is believed to be the predominant determinant of affinity and specificity for the binding of given peptide sequences to the B27 subtypes. In particular, we wished to determine if peptides containing a thiol group at P2 could be covalently linked to cysteine 67 of B*2702. We tested the binding of peptides substituted with a cysteine, a homocysteine, or an α -amino- ϵ -mercapto hexanoic acid side chain (Amh) instead of the naturally occurring arginine at P2 of the peptide. We find that peptides containing homocysteine or Amh bind to B*2702 with affinities that are only slightly reduced (two- to fivefold) compared to the naturally occurring arginine peptide, whereas the binding affinity of the cysteine-containing peptide is more than 100-fold reduced. However, the interaction of B*2702 with the homocysteine or Amh peptide is not covalent. Rather, most of the stabilization appears to arise from hydrophobic interactions via the methylene group of the homocysteine side chain. Indeed, we find that peptides substituted at P2 with other methylene-containing side chains also bind to B*2702 with affinities only moderately reduced compared to the arginine peptide. These results demonstrate the potential for significant promiscuity in the interaction of naturally occurring peptides with B27 *in vivo*, a feature that may account for its association with autoimmune arthritic diseases.

We also measured the affinities of C-terminal variants of naturally occurring peptide sequences to compare the affinities of peptides with aliphatic versus charged C-termini. Our results confirm that the presence of a hydrophobic C-terminal residue is important for high-affinity interaction with HLA-B*2702.

Results

*Expression of HLA-B*2702 in CHO cells and purification of protein*

A secreted form of B*2702 was expressed in CHO cells using a glutamine synthetase-based amplifiable expression system (Bebington & Hentschel, 1987). Soluble B*2702 was purified from

Fig. 1. Stereo figure of selected residues (marked with residue number and name, single-letter code) within binding pockets highlighted on the carbon- α backbone of the B*2705 peptide binding domain (Madden et al., 1991, 1992), with all non-hydrogen atoms of the model peptide sequence ARAAAAAA shown. Residue positions along peptide backbone are marked with numbers near the corresponding carbon- α atom. Figure generated with Xtalview using the coordinate file 1HSA obtained from the Protein Data Bank.

transfected cell supernatants on an immunoaffinity column. The purified protein elutes from a gel filtration column as a single species of about 50–55 kDa (data not shown), and SDS-PAGE analysis revealed bands migrating with apparent molecular masses corresponding to the truncated B*2702 heavy chain and β 2m (Fig. 2). N-terminal sequencing of the purified heterodimers yielded sequences corresponding to the first 10 residues of the mature B*2702 heavy chain and human β 2m (data not shown). In addition, arginine was observed at P2 of the sequencing cycle, consistent with previous observations that B27 shows a striking preference for associating with peptides containing an arginine at P2.

Assembly of peptide depleted B*2702

Purified preparations of class I MHC molecules are expected to be nearly completely occupied with mixtures of endogenous peptides, because class I MHC molecules bind peptide during their assembly in the endoplasmic reticulum (Townsend & Bodmer, 1989). However, studies of recombinant K^d /human β 2m heterodimers produced in CHO cells revealed that only ~30% of the purified protein contained endogenous peptides, presumably indicating a limiting supply of peptide available for class I molecules under conditions of vast overproduction (Fahnestock et al., 1992, 1994). To quantify the proportion of purified soluble B*2702 that is capable of binding exogenous peptide, and therefore presumably empty, we tested the binding of radiolabeled peptide to affinity purified B*2702 heterodimers. For binding assays, we used a tritium-labeled peptide of sequence RRYQKSTEL, which was identified by N-terminal sequencing of B27-associated peptides as a peptide derived from histones (Jardetzky et al., 1991). Purified B*2702 was incubated with ^3H -RRYQKSTEL, and labeled peptide bound to B*2702 was separated from free peptide. Based upon the known specific activity of the peptide, we estimate that only 1% of the purified B*2702 was capable of binding the exogenous labeled peptide (data not shown), suggesting that the vast majority of the secreted B*2702 contains endogenous peptides that were not displaced.

In order to enhance the percent of B*2702 molecules capable of binding exogenous peptides, we first attempted a denaturation–

Fig. 2. SDS-PAGE analysis of secreted HLA-B*2702 expressed in CHO cells after purification on a W6/32 immunoaffinity column. Lane 1, molecular weight standards; lane 2, HLA-B*2702 heavy chain (40–45 kDa) and β 2m (12 kDa).

Fig. 3. Specificity of RRYQKSTEL interaction with B*2702 after depletion of endogenous peptides. ^3H -RRYQKSTEL (0.8 μM) was incubated overnight at room temperature with 2.5 μM soluble B*2702 or soluble H-2K d . Peptide bound to protein was separated from free peptide subsequently using spin columns as described in the Materials and methods. Radioactivity bound to each protein sample is indicated in duplicate. **A:** Peptide depleted B*2702 following alkali treatment. **B:** B*2702 prior to alkali treatment. **C:** Empty K d produced by reassembly of denatured heavy and light chains in the absence of peptide.

renaturation protocol that had been successful for assembling empty H-2K d heterodimers (Fahnestock et al., 1992, 1994). However, the reassembled B*2702 heterodimers recovered after removal of endogenous peptides were aggregated (data not shown), and therefore not suitable for peptide binding experiments. We next employed a rapid alkaline denaturation procedure (Khilko et al., 1993) to yield a partially empty population of B*2702 heterodimers. Figure 3 shows that peptide binding by alkali-treated B*2702 is six- to sevenfold enhanced compared to untreated B*2702. The specificity of the interaction of the RRYQKSTEL interaction with B*2702 is demonstrated by the absence of binding of ^3H -RRYQKSTEL to empty H2-K d (Fig. 3).

Fig. 4. Representative Scatchard plot of ^3H -labeled peptide (RRYQKSTEL) binding to alkali-treated B*2702 heterodimers. The dissociation constant, K_D , is calculated as the negative of the inverse of the slope of the line. K_D derived from this experiment was 3.45×10^{-8} M and from an independent experiment was 5.11×10^{-8} M, yielding an average of $4.25 \pm 1.16 \times 10^{-8}$ M.

Measurements of binding affinities of a series of B27-restricted peptide analogues

Peptide depleted B*2702 heterodimers were used to obtain an equilibrium dissociation constant (K_D) by Scatchard analysis of data from binding experiments using the ^3H -RRYQKSTEL peptide (Fig. 4). Data from two independent experiments yielded an average K_D of $4.3 \pm 1.2 \times 10^{-8}$ M. A series of unlabeled peptides were next analyzed for their ability to compete for binding of the ^3H -RRYQKSTEL peptide. The concentrations of inhibitor peptides that produced 50% inhibition of labeled peptide binding were determined by plotting the percent inhibition of binding versus inhibitor concentration (Fig. 5). Dissociation constants for the interaction of inhibitor peptides with B*2702 were calculated using the concentrations required for 50% inhibition as described in the Materials and methods. As a test of the validity of the K_D values derived from the inhibition studies, an unlabeled version of RRYQKSTEL was used to compete for ^3H -RRYQKSTEL binding. The K_D

derived from the inhibition analysis was $14.7 \pm 4.3 \times 10^{-8}$ M (Fig. 5), demonstrating that the two methods of affinity constant derivation give comparable values.

We next investigated the binding properties of a series of peptides containing thiol groups at P2, in order to ascertain if a thiol group from the peptide can interact with Cys 67 of the B*2702 protein. Variants of RRYQKSTEL containing either cysteine, homocysteine (Hc), or Amh at P2 were synthesized and their ability to compete with the binding of ^3H -RRYQKSTEL to B*2702 was tested (Fig. 5). Table 1 summarizes the derived K_D values for this series of peptides, showing that substitution of homocysteine or Amh for arginine at P2 of the peptide affects the binding affinity by only about two to fivefold, whereas the substitution of cysteine for arginine results in a reduction by more than two orders of magnitude. We next analyzed the binding of the thiol-containing peptide with the highest affinity for B*2702 to see if it was binding covalently to B*2702. A tritiated version of RHcYQKSTEL was generated using a catalytic exchange procedure, and B*2702- ^3H -

Fig. 5. Competition between unlabeled peptides and ^3H -RRYQKSTEL for binding to peptide depleted B*2702—determination of the 50% inhibition values. Only data points corresponding to between 10% and 90% inhibition are plotted, because this portion of the inhibition curve can be approximated by a straight line (Müller, 1983). ^3H -RRYQKSTEL ($0.8 \mu\text{M}$) was incubated overnight at room temperature with $2.0 \mu\text{M}$ alkali-treated B*2702 and varying concentrations of the following unlabeled inhibitor peptides. **A:** RRYQKSTEL, RHcYQKSTEL, RAmhYQKSTEL, and RCYQKSTEL. **B:** RRYQKSTEL, RQYQKSTEL, RHsYQKSTEL, RMYQKSTEL, and RKYQKSTEL. **C:** GRLTKHTKF, GQLTKHTKF, GMLTKHTKF, and GKLTkHTKF. **D:** GRAFVTIGL, GRAFVTIGK, RRYQKSTEL, and RRYQKSTEK.

Table 1. Peptide category and sequence^a

Category	A.A. Structure	Sequence	I ₅₀ (μM)	K _D (nM)	Ratio	Sequence	I ₅₀ (μM)	K _D (nM)	Ratio
natural		RRYQKSTEL	2.3	147 ± 39	1.0	GRLTKHTKF	5.8	371 ± 56	1.0
R → C		RCYQKSTEL	231.5	19650 ± 7750	133.7				
R → Hc		RHcYQKSTEL	7.7	242 ± 151	1.6				
R → Amh		RAmhYQKSTEL	16.5	715 ± 128	4.9				
R → Hs		RHsYQKSTEL	41.1	2142 ± 41	14.6				
R → Q		RQYQKSTEL	11.3	554 ± 13	3.8	GQLTKHTKF	14.1	1173 ± 451	3.4
R → M		RMYQKSTEL	21.6	805 ± 285	5.5	GMLTKHTKF	21.7	938 ± 179	2.5
R → I		RIYQKSTEL	***	no binding	***	GILTKHTKF	***	no binding	***
R → K		RKYQKSTEL	259.4	8468 ± 5543	57.6	GKLTKHTKF	405.8	18840 ± 1822	50.7
R → E		REYQKSTEL	***	no binding	***	GELTKHTKF	***	no binding	***
P9 → L		P9LYQKSTEL	3.1	147 ± 39	1.0	GRAFVTIGL	3.3	121 ± 57	1.0
p9 → X		p9LYQKSTEL	34.4	1421 ± 292	9.7	GRAFVTIGK	54.9	1798 ± 1002	14.9

^aPeptide category and sequence are listed with the concentration at which peptide inhibited 50% of the binding of ³H-RRYQKSTEL (*I*₅₀; see Fig. 5 for determination of *I*₅₀ values). *K*_D values were calculated as described in Materials and methods using the *I*₅₀ values and a *K*_D of 4.3 ± 1.2 × 10⁻⁸ M determined by Scatchard analysis for ³H-RRYQKSTEL. "No binding" indicates that a peptide did not inhibit binding of the ³H-RRYQKSTEL at concentrations up to 0.5 mM. Ratio is calculated as the *K*_D of the parental peptide of the series divided by the *K*_D of the variant peptide. RRYQKSTEL is a B27-binding peptide derived from human histones (Jardetzky et al., 1991); GRLTKHTKF is derived from human ribosomal protein L36 (Röttschke et al., 1994); GRAFVTIGK is a HIV glycoprotein peptide believed to bind to B27 (Jardetzky et al., 1991).

RHcYQKSTEL complexes were formed. The radiolabeled complex was analyzed by SDS-PAGE under reducing and nonreducing conditions, and the gel was subjected to autoradiography. Under nonreducing conditions, we saw no high molecular weight bands corresponding to covalent B*2702–RHcYQKSTEL complexes, suggesting that the peptide does not bind covalently (data not shown). In addition, the presence of a reducing agent such as dithiothreitol did not enhance the dissociation rate of B*2702–³H-RHcYQKSTEL complexes (data not shown).

Because the relatively high-affinity interaction between RHcYQKSTEL and B*2702 could not be explained by formation of a covalent bond, we hypothesized that higher affinity of RHcYQKSTEL and RAmhYQKSTEL versus RCYQKSTEL for B*2702 could be the result of stabilizing aliphatic contacts provided by the extra methylene groups in the homocysteine and Amh side chains compared to a cysteine side chain. This hypothesis suggested that peptides with other aliphatic residues at P2 might bind to B*2702 with relatively high affinities. We therefore measured the affinities of variants of the RRYQKSTEL peptide that contained homoserine (Hs), glutamine, methionine, or isoleucine at P2 (Fig. 5B; Table 1; peptides RHsYQKSTEL, RQYQKSTEL, RMYQKSTEL, RIYQKSTEL). Table 1 shows results of experiments in which these peptides were analyzed for their ability to compete for the binding of ³H-RRYQKSTEL to B*2702. The glutamine- and methionine-

containing peptides (RQYQKSTEL and RMYQKSTEL) bind relatively well to B*2702, with only four- to sixfold reductions in affinity, whereas the homoserine-containing peptide (RHsYQKSTEL) shows an intermediate effect (15-fold reduced affinity), and the isoleucine-containing peptide (RIYQKSTEL) shows no detectable binding to B*2702. These results suggest that some aliphatic residues that are chemically quite dissimilar to arginine are tolerated with relatively small changes in binding affinity. By contrast, the conservative substitution of lysine for arginine at P2 (peptide RKYQKSTEL) resulted in a reduction in affinity of more than 50-fold. A peptide with a negative charge at P2 (REYQKSTEL) showed no detectable binding to B*2702.

Similar trends are observed with P2 variants of a peptide derived from the ribosomal protein L36 (peptide sequence GRLTKHTKF), one of the natural self-peptides found associated with B*2702 (Röttschke et al., 1994). Again, the glutamine and methionine variants (GQLTKHTKF and GMLTKHTKF) bind with only three- to fourfold reduced affinity compared to the wild-type sequence (Fig. 5C; Table 1), the lysine variant (GKLTHTKF) shows a greater reduction in affinity (55-fold), and the glutamic acid and isoleucine variants (GELTKHTKF and GILTKHTKF) showed no detectable binding.

We next derived affinities for peptides with C-terminal substitutions. In particular, we were interested in the relative affinities of

peptides with hydrophobic C termini compared to positively charged C termini, because both types of peptides had been reported to bind to B27 subtypes (Jardetzky et al., 1991; Rötzschke et al., 1994). The affinity of a lysine variant (peptide RRYQKSTK) was reduced by about 10-fold compared to the parental sequence (RRYQKSTEL). The same trend was observed in comparisons of two sequences based upon the HIV-derived peptide GRAFVTIGK (Jardetzky et al., 1991), which showed a 15-fold reduction in affinity compared to a variant containing leucine at the anchor position 9 (peptide GRAFVTIGL).

Discussion

The association of certain class I MHC alleles with autoimmune disease is thought to be related to their normal function in immune recognition. Class I MHC molecules bind and present peptide fragments of foreign and self antigens to T cells (Townsend & Bodmer, 1989). The strong linkage of HLA-B27 subtypes with susceptibility to arthritic disease may be due to the interaction of B27 with peptides derived during intracellular processing from self proteins found in the joints and spine, according to one model for disease pathogenesis (Benjamin & Parham, 1990). To prevent the recognition of self peptides and subsequent disease progression, the design of peptides to specifically block the binding sites of MHC molecules correlated with disease has been suggested (Adorini et al., 1990). From an analysis of the three-dimensional structure of a peptide-B*2705 complex (Madden et al., 1991, 1992), it might be possible to develop a set of potentially specific and high-affinity ligands targeted to the B27 subtypes. Because at least four of the B27 subtypes exhibit increased susceptibility to arthritic diseases, and the sequence differences between them cluster within the peptide binding site (Benjamin & Parham, 1990), a disease-inducing peptide must be capable of binding to multiple B27 subtypes. Benjamin and Parham (1990) analyzed class I HLA sequences to look for conserved features within B27 subtypes that are also specific to B27. They found a combination of six residues (His 9, Glu 45, Cys 67, Lys 70, Ala 71, and Gln 97) that is found in all B27 subtypes. The conserved residues are situated near the entrance to pocket B, a depression within the peptide binding site that centers around residue 45 (Madden et al., 1991, 1992).

Sequences of peptides eluted from HLA-B*2705 revealed that arginine predominated at P2 (Jardetzky et al., 1991), and its side chain was found to reach into pocket B, forming a salt bridge with Glu 45 (Madden et al., 1991, 1992). The cysteine residue within pocket B does not appear to be utilized in the interaction of B27-restricted peptides containing an arginine anchor at P2. However, the presence of an unpaired cysteine within this pocket in HLA-B27 has been suggested to play some role in autoimmune disease pathogenesis, perhaps by disulfide bonding with joint tissue-derived peptides containing cysteine residues (Benjamin & Parham, 1990). In order to evaluate directly the effects of substitutions of the arginine anchor residue of B27-binding peptides (including the substitution to cysteine), we devised a quantitative peptide binding assay for the interaction of peptides with B*2702. To our knowledge, this study is the first report of affinity constant measurements for peptides binding to B27.

For these assays, a soluble version of B*2702 was expressed in CHO cells and purified from transfected cell supernatants. In general, purified preparations of class I molecules contain mixtures of endogenous peptides, and the percent of class I molecules that can accept exogenous peptide is low (Chen & Parham, 1989). As ex-

pected, we observed that only ~1% of purified B*2702 was capable of binding exogenous labeled peptide. The finding that virtually all of the purified B*2702 heterodimers contain peptide is consistent with previous observations using the same CHO expression system for production of class I MHC molecules. In these studies, we noted that, when K^d was expressed at relatively low levels, comparable to those obtained for the B*2702 produced for this study (0.1–0.3 mg/L from transfected CHO cells grown on 10 cm plates), virtually all of the K^d heterodimers were occupied with endogenous peptides, whereas under conditions of much higher expression (9–10 mg/L from transfected CHO cells grown on 10 cm plates), only 30% of the K^d heterodimers were occupied with peptide (Fahnestock et al., 1992, 1994). The low percentage of empty B*2702 heterodimers made it necessary to produce a form of B*2702 that was depleted of endogenous peptides for binding studies using exogenous peptides. We reported previously a method to produce empty murine H-2K^d molecules using a biochemical separation of endogenous peptides from denatured class I heavy and light chains, followed by reassembly in the absence of added peptide (Fahnestock et al., 1992, 1994). Empty B*2702 molecules produced using this procedure were aggregated, suggesting that there may be an allele-specific or species-specific propensity for successful reassembly from denatured chains in the absence of peptide. We therefore adapted an alkali treatment procedure (Khilko et al., 1993) to produce partially empty B*2702 molecules that elute at the expected position from a gel filtration column, and that show a six- to sevenfold increase in ability to bind exogenous peptide compared to untreated B*2702.

For comparisons to substituted peptides, the binding affinity of a known B27-restricted peptide derived from human histones (sequence RRYQKSTEL (Jardetzky et al., 1991)) was determined by Scatchard analysis. A tritium-labeled version of the peptide (³H-RRYQKSTEL) was used in order to avoid introducing a bulky reporter group, such as iodine or biotin, that could interfere with peptide binding by introducing steric hindrance and ionic effects. Binding affinities of a series of unlabeled analogue peptides and other B27-binding peptides were then determined by quantitation of the inhibition of binding of the labeled peptide.

The effects of altering the arginine anchor residue at P2 of the peptide were examined first (Table 1). In agreement with the previously reported B27 peptide binding motif (Jardetzky et al., 1991), peptides containing arginine at P2 bound most tightly to B*2702. As expected, peptides containing glutamic acid at P2 showed no detectable binding to B*2702. The dramatic reduction of affinity upon substitution of arginine for cysteine at P2 suggests that cysteine-containing peptides would not bind to B27 *in vivo*; thus, the hypothesis that cysteine-containing self peptides in the joints pair with Cys 67 of B27 subtypes (Benjamin & Parham, 1990) is unlikely. Indeed, examination of the B*2705-peptide crystal structure (Madden et al., 1992, 1993) suggests that a cysteine at P2 could not reach far enough to pair with Cys 67 (the distance between the carbon- α atoms of Cys 67 and second residue of the peptide is 8.8 Å, compared to an optimal distance of 4.4–6.8 Å for disulfide bond formation (Richardson, 1981). In an attempt to construct a peptide potentially capable of covalent binding to B*2702, we next synthesized peptides in which two unnatural amino acids containing thiol groups were incorporated at P2 of the histone derived B27-binding peptide sequence. Homocysteine contains two methylene groups, which would be expected to lengthen its side chain by ~1.4 Å compared to cysteine, and Amh contains four methylene groups, and is therefore expected to be ~3.9 Å

longer than cysteine. Neither the homocysteine- or Amh-containing peptide appeared to bind covalently to B*2702, possibly because the thiol residues are not appropriately oriented to form a disulfide with Cys 67.

Although we did not find a peptide that could bind covalently to B*2702, both of the thiol-containing P2 variant peptides showed surprisingly high affinities for binding to B*2702—only two- to fivefold reduced in affinity compared to the arginine-containing peptide. These results suggested that a portion of the stabilization energy derived from the arginine side chain at P2 arises from aliphatic contacts of B27 pocket B residues with the methylene groups of arginine, in addition to ionic interactions with Glu 45. If so, we expected to find other aliphatic side chains that could substitute for arginine at P2 without a dramatic loss of affinity. Indeed, we found that nonconservative substitutions at P2 for residues such as glutamine or methionine were also permissible, resulting in a sixfold or lower loss in the binding affinity. Clearly, not all aliphatic side chains are permissible, because no binding was detectable with the isoleucine peptide at concentrations of up to 0.5 mM. Comparable affinity changes were found using analogous P2 variants of another B27-binding peptide (sequence GRLTKHTKF; derived from the ribosomal protein L36). The relatively small affinity loss resulting from the arginine to glutamine change observed in these studies is consistent with recent reports that peptides containing glutamine bind to B*2705 (Villadangos et al., 1995), and also consistent with the observation that peptides containing glutamine and valine are presented by B*2705 in transgenic rats (Feltkamp et al., 1996).

Previous studies have examined the effect of substitutions of the anchor residues of B27-binding peptides on the stability of B27-peptide complexes, using the half time of β 2m dissociation as a measure of stability (Parker et al., 1992). It was reported that the half time of β 2m dissociation of B27-peptide complexes formed with peptides containing glutamine or methionine at P2 is at least two orders of magnitude faster than B27-peptide complexes formed with the corresponding arginine-containing peptides. Our results with glutamine- and methionine-containing peptides show that the affinity reduction due to these substitutions is not nearly so dramatic, suggesting that the β 2m dissociation rate is not linearly correlated with relative affinities of different peptides for B27.

Anchor residue modifications for the interactions of peptides with other class I MHC molecules such as HLA-A2.1 (Ruppert et al., 1993) and H2-K^d (Fahnestock et al., 1994) have shown that relatively conservative changes in anchor residue type can result in significant changes in affinity. For example, when the P2 anchor residue in a K^d binding peptide is changed from tyrosine to phenylalanine, an 80-fold reduction in affinity is observed (Fahnestock et al., 1994). In the present study of peptide binding to B*2702, the conservative anchor residue modification of lysine for arginine had a greater effect on the affinity (55–60-fold reduction) of B27-binding peptides than did less conservative substitutions for aliphatic residues. For example, the modification of Amh for arginine resulted in only a fivefold reduction in affinity. The side chains of both lysine and Amh contain four methylenes, but differ in the capping groups (Table 1) (a positively charged amino group in the case of lysine and a negatively charged thiol in the case of Amh, under the slightly alkaline pH conditions of the experiments). The result that the lysine-substituted peptide binds with a 10-fold lower affinity compared to the Amh-substituted peptide suggests that a positively charged residue is not favored in the ϵ -position of a straight chain, and that the ϵ amino group of lysine

is not appropriately oriented to form a salt linkage with Glu 45, in contrast to the positively charged δ -guanido group of arginine.

Our results with P9 variants of peptides binding to B*2702 indicate a strong preference for a hydrophobic residue at this site, consistent with the results of Rötzschke et al. (1994). A 10-fold reduction in affinity is observed upon substitution of the naturally occurring leucine to a lysine in the histone-derived sequence RRYQKSTEL. Similarly, the HIV-derived peptide GRAFVTIGK binds poorly to B*2702 ($K_D \sim 1.8 \times 10^{-6}$ M); substitution of the C-terminal lysine to a leucine increases the affinity 15-fold. These results indicate that the C-terminal peptide residue is an important determinant of affinity in B*2702-peptide interactions.

Taken together, our results suggest that some aliphatic residues can be accommodated at anchor P2 for B*2702 binding peptides, but these residues must be straight chains (homocysteine, Amh, glutamine, methionine) rather than branched (isoleucine), and that two or more methylene groups are required (homocysteine is permissible, but cysteine is not). Our finding that substitution of arginine at P2 with glutamine or methionine in B*2702 binding peptides results in a relatively small reduction in affinity suggests that peptides containing glutamine and methionine are likely to be present among the endogenous B*2702-associated peptide pool. These results with B*2702 may also be applicable to other B27 subtypes because the B pocket is conserved among all B27 subtypes. This extended repertoire of permissible B27-binding peptides should be taken into account for a consideration of disease-associated peptide sequences.

Materials and methods

Expression of B*2702 in CHO cells

Secreted B*2702 heterodimers were produced in CHO cells using a glutamine synthetase-based amplifiable expression system (Bebbington & Hentschel, 1987). The cDNA for HLA-B*2702 was obtained in the plasmid pKKG5 from the laboratory of Dr. Andrew McMichael (University of Oxford, England). PCR was used to insert a 5' *Xho* I site, a 3' *Not* I site, and a stop codon after the codon corresponding to residue 284 of the mature protein. The resulting modified cDNA was sequenced and subcloned into the expression vector PBJ5-GS (Gastinel et al., 1992) that employs the glutamine synthetase gene as an amplifiable selection marker in the presence of the drug methionine sulfoximine (MSX). The cDNA-encoding human β 2m was introduced into a similar expression vector (Lin et al., 1990) lacking the glutamine synthetase gene, as described previously (Fahnestock et al., 1992). The heavy chain and β 2m expression plasmids were transfected into CHO cells using a lipofection procedure (Gibco/BRL). Transfected CHO cells were maintained in α minimal essential medium (α -MEM) supplemented with 5–10% dialyzed fetal bovine serum (DFCS), 100 units/mL penicillin/streptomycin, and 25 μ M MSX. When clones resistant to 25 μ M MSX began to appear, MSX concentration was raised to 100 μ M. Supernatants from MSX-resistant CHO clones were tested for the presence of secreted HLA-B*2702 heterodimers by immunoprecipitations using the monoclonal antibody W6/32, which recognizes properly folded human class I MHC heterodimers (Parham, 1979). SDS-PAGE analysis of the immunoprecipitates revealed a band migrating with an apparent molecular mass of ~45 kDa, corresponding to truncated B*2702 heavy chain, and a band at 12 kDa, corresponding to β 2m. Cell lines with the highest levels of B*2702 expression were identified on Coomassie

Brilliant Blue-stained SDS-PAGE gels of immunoprecipitations from equal volumes of supernatants from equivalent numbers of transfected cells.

Protein purification and characterization

A B*2702-secreting CHO cell line was grown to confluence in 50 10-cm plates. Supernatants were collected every two days for several weeks. Soluble B*2702 heterodimers were purified from the supernatants on a W6/32 immunoaffinity column. The affinity column was prepared by coupling the W6/32 monoclonal antibody to cyanogen bromide-treated Sepharose 4B (Pharmacia) at approximately 10 mg of antibody/mL of resin following the manufacturer's protocol. Supernatants were passed over the affinity column, which was then washed with 50 column volumes of 50 mM Tris, pH 7.4, 0.1% NaN₃, 5 mM EDTA. B*2702 protein was eluted from the W6/32 column with 50 mM diethylamine, pH 11.5, into tubes containing 1.0 M Tris, pH 7.4. Approximately 0.1–0.3 mg of purified B*2702 was recovered per liter of transfected cell supernatants (compared to 9–10 mg/L of a murine class I MHC heavy chain (H-2K^d) complexed with human β 2m secreted from transfected CHO cells grown on 10-cm plates (Fahnestock et al., 1994). Figure 2 shows an analysis of the purified B*2702 protein by SDS-PAGE.

*Assembly of empty B*2702 molecules*

We previously devised a procedure to assemble empty K^d by re-assembling empty heterodimers from guanidinium hydrochloride-denatured heavy and light chains from which endogenous peptides had been removed (Fahnestock et al., 1992). When this procedure was used to produce empty B*2702, only a small amount of empty protein was recovered, most of which appeared to be aggregated based upon its migration on a Pharmacia Superose 12 gel filtration FPLC column (data not shown). We therefore modified an alkaline denaturation procedure (Khilko et al., 1993) to make partially empty B*2702 molecules. Dithiothreitol was added to a final concentration of 1.3 mM to purified B*2702 (4–5 mg/mL). After 5 min of incubation on ice, 4 M K₂HPO₄, pH 12.0, was added to a final concentration of 0.25 M, and the incubation was continued for an additional 5 min. The protein was then applied to a Biospin 6 column equilibrated in borate buffer, pH 10.4, and centrifuged at 1,100 $\times$ g for 5 min. The flowthrough was collected and applied over a Biospin 30 column equilibrated in phosphate buffered saline, pH 7.5, and the column was centrifuged at 1,100 $\times$ g for an additional 5 min. This procedure resulted in partially empty protein that has six- to sevenfold higher peptide-binding capacity compared to untreated B*2702 (Fig. 3).

Peptide synthesis and labeling

Peptides containing only natural amino acids were synthesized using automated solid phase methodology either at the Protein and Nucleic acid Facility of the Beckman Center at Stanford University or at Anaspec Incorporated (San Jose, California). Peptides containing unnatural amino acids were custom synthesized by Peninsula Labs (homocysteine; Hc), Anaspec Incorporated (homoserine; Hs), or our laboratory (α -amino- ϵ -mercapto-hexanoic acid; Amh). The unnatural amino acid Amh was synthesized as described (Heeb et al., 1994). The peptide containing Amh (RAMHYQKSTEL) was

synthesized using an Applied Biosystems 433A peptide synthesizer (in Dr. Steve Mayo's laboratory at Caltech) to couple the C-terminal seven amino acids, into which Amh and the N-terminal arginine were incorporated manually. After synthesis, peptides were cleaved from the resin, deprotected using acid treatment, purified by reverse phase chromatography, and characterized by mass spectroscopy. The concentrations of lyophilized peptide samples were estimated spectrophotometrically after resuspension in water, using the extinction coefficient of tyrosine at 274 nm ($1,420 \text{ M}^{-1} \text{ cm}^{-1}$) or a Bicinchoninic acid assay.

Peptide-binding assays

A B27-restricted peptide derived from human histones (sequence RRYQKSTEL (Jardetzky et al., 1991) was tritiated by catalytic exchange (Amersham TR7 Tritium Labeling Service) to a specific activity of 0.28 mCi/mmol (³H-RRYQKSTEL). The radiolabeled peptide was purified by reverse phase chromatography on a pep-RPC column from Pharmacia, using an acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions containing the radiolabeled peptide were stored frozen at -80°C , and diluted to the specified (below) specific activity prior to the binding analyses. For Scatchard analyses, duplicate samples of alkali treated B*2702 (3–5 μM) were incubated overnight at room temperature with varying concentrations of ³H-RRYQKSTEL (0.062–2 μM) in phosphate buffered saline, pH 7.5. No change of binding values was observed with longer incubations; thus, equilibrium was established during this time period. Samples were loaded over Biospin 30 columns, and centrifuged at 1,100 $\times$ g for 5 min to separate peptides bound to B*2702 (flow-through) from free peptides. Samples (50–80 μL) from the flow-through were collected and transferred to a vial containing 10 mL of Safety-Solve (Research Products International Corp.) for scintillation counting on a Beckman LS 5000TD scintillation counter. The concentration of bound peptide at equilibrium was calculated using a specific activity of 0.81×10^6 counts per min/nmol. The concentration of free peptide was obtained by subtracting the bound peptide concentration from the total amount of peptide present in each experiment. The data were plotted as [bound]/[free] versus [bound] and the K_D calculated as $-(1/\text{slope})$.

For inhibition studies using unlabeled peptides, duplicate samples of alkali treated B*2702 (3–5 μM) were incubated overnight at room temperature with varying concentrations of unlabeled inhibitor peptides in phosphate buffered saline, pH 7.5. For each concentration of inhibitor, the percent inhibition was calculated by comparing the bound radioactivity to assays that did not include inhibitor, and the percent inhibition was plotted versus concentration of inhibitor on a logarithmic scale. The 50% inhibition value was determined from this graph and used to calculate the K_D of the inhibitor using the relationship (Cheng & Prusoff, 1973): $K_D = [I_{50}]/(1 + [^3\text{H-P}]/K_{D(\text{labeled peptide})})$; where $[I_{50}]$ is the concentration of inhibitor required for 50% inhibition, $[^3\text{H-P}]$ is the concentration of ³H-labeled peptide, and $K_{D(\text{labeled peptide})}$ refers to the dissociation constant determined for the labeled peptide by Scatchard analysis.

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