Changes in the Repertoire of Peptides Bound to HLA-B27 Subtypes and to Site-specific Mutants Inside and Outside Pocket B

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Summary

HLA-B27 subtypes share many structural features, including their pocket B, which interacts with a conserved Arg residue at the second position of B*2705-bound peptides. Subtypes differ among each other at other locations in the peptide binding site. In this study, metabolic labeling and radiochemical pool sequencing were used to address the following issues: (*a*) presence of the Arg 2 (R2) motif among peptides bound to the various HLA-B27 subtypes; (*b*) influence of mutations inside and outside pocket B on this motif; and (*c*) the degree of similarity among the peptide pools bound to the various B27 subtypes. Sequencing of Arg-labeled peptide pools extracted from B*2701 to B*2706, and from two site-directed mutants of B*2705 with changes outside pocket B, indicated that all of these molecules bind peptides with Arg at position 2. Peptides from several mutants with changes altering the structure of pocket B, and from one mutant at the pocket B rim, also retained the R2 motif. However, this was absent in the peptide pool extracted from the M45 mutant, in which the negative charge of pocket B, conferred to HLA-B27 by Glu45, was canceled. These results indicate that alterations outside pocket B, and even disruption of the network of hydrogen bonds that stabilizes Arg binding in pocket B, do not impair binding of peptides bearing the R2 motif, but a nonconservative substitution at position 45 does. As a substantial fraction of anti-B*2705 cytotoxic T lymphocyte (CTL) clones crossreact with the M45 mutant (Villadangos, J., B. Galocha, D. López, V. Calvo, and J.A. López de Castro. 1992. *J. Immunol.* 149:505) this result suggests that determinant mimicry by nonidentical peptides may frequently account for unexpected CTL crossreactions. Metabolic labeling with various other amino acids and radiochemical sequencing revealed similarities, but also substantial differences, among the peptide pools from the various HLA-B27 subtypes. This strongly suggests that many peptides bind to multiple subtypes, but significant subsets of peptides bound to a given HLA-B27 subtype do not bind to other subtypes or do so with greatly altered efficiency. These results indicate the importance of polymorphism outside pocket B in modulating peptide binding to HLA-B27.

Class I HLA molecules bind endogenous peptides and present them at the cell surface to CTL. X-ray diffraction studies have shown that the peptide binding site is a groove contributed by the α1 and α2 domains and formed by an eight-stranded β-pleated sheet topped by two large α-helical regions (1, 2). This groove accommodates peptides, generally nonamers, in extended conformation (3, 4). For each class I molecule the peptide binding site can be described in terms of a series of pockets, six designated A to F in HLA-A2 (5), that interact with individual peptide side chains. HLA polymorphism modulates the number, shape, and polarity of these pockets (6), and, in this way, determines the peptide binding specificity of each class I HLA protein.

Crystallographic analyses of peptide/MHC complexes from HLA and H-2 molecules have revealed that most of the contacts between the MHC protein and the bound peptides are established with the peptide main chain and with both peptide termini. Other contacts involve some of the peptide side chains and the various pockets of the MHC molecule (7–9). The structure of these pockets determines the peptide side chains they can accommodate. The highly restrictive character of some pockets imposes the anchoring peptide motifs that characterize the type of peptides that can bind to a particular HLA molecule (10). Pocket B, which binds peptide side chain 2 (P2), is extremely well fit in HLA-B27 for interacting with Arg side chains, and there is no evidence that other residues can be accommodated (7, 11). Thus, Arg at position 2 (R2) is the major peptide motif among HLA-B27-bound peptides.

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A critical issue for understanding the rules governing peptide binding is the effect of variations in the structure of pockets on their capacity for accommodating particular peptide side chains. For instance, all HLA-B27 subtypes have the same pocket B structure, although some of the residues forming this pocket are very polymorphic among HLA-B antigens. By contrast, HLA-B27 subtypes differ from one another by one or a few residues located in other pockets (12). Thus, the peptide binding specificity of HLA-B27 antigens must be assessed by examining the effects of changes in pocket B, and in other pockets, on the nature of bound peptides. In this study we have examined the effect of mutations in pocket B on the capacity of binding peptides with the R2 motif. In addition, we have compared the peptide pools bound to various HLA-B27 subtypes. The results indicate that changes outside pocket B do not alter the R2 motif. Some structural changes in pocket B, but not the change of Glu45 to Met, are also compatible with the R2 motif. In spite of sharing R2, the peptide pools bound to the various subtypes are substantially different, indicating the role of other pockets in the specificity of peptide binding by HLA-B27.

Materials and Methods

Cell Lines and Mutants. Genomic DNA containing B*2701, B*2703, and B*2706 generated by site-directed mutagenesis (13) were transfected into the class I–deficient HMy2.C1R cells by electroporation as previously described (14), followed by selection with 200 μg/ml of hygromycin B (Sigma Chemical Co., St. Louis, MO). The B*2705 and B*2702 transfectant C1R cell lines were previously described (14). Transfectant cell lines expressing high amounts of these HLA-B27 subtypes (Table 1) were used as source of material.

The following site-directed mutants derived from B*2705 were also used: Y9, A24, M45, N63, V67, Q70, A24V67, Y9Q70, and E152. These are designated with the one-letter code of the amino acid(s) introduced followed by the corresponding residue number(s). Each mutant was expressed on HMy2.C1R cells after transfection. Most of these mutants and transfectants have been previously described (14, 15). Y9, Q70, and Y9Q70 were obtained and transfected by the same procedure. HLA-B27-negative populations in Y9 and Y9Q70 were removed, following instructions from the manufacturer, by positive selection of the transfected population with goat anti–mouse antibody conjugated to Dynabeads M-450 (Dynal, Oslo, Norway), after incubating the cells with the ME1 (anti-B27, -B7, -B22) mAb (16). The expression levels of all the mutants, as estimated by FACS® (Becton Dickinson & Co., Mountain View, CA) analysis, are shown in Table 1.

The Wewak I (HLA-A11, 24; B*2704, 62) and JY (HLA-A2; B7) LCL were used as source of material for B*2704, and for HLA-A2 and HLA-B7, respectively. C1R transfectants and LCL were grown in DMEM supplemented with 5% heat-inactivated FCS and in RPMI 1640, 25 mM Hepes buffer, and 10% FCS (all from Gibco Laboratories, Paisley, UK) respectively. The B*2701, B*2703, and B*2706 transfectants were continuously maintained with 300 μg/ml of hygromycin.

Isolation of Biosynthetically Labeled HLA-bound Peptides. 2×10^7 cells were labeled in 10 ml of RPMI 1640 lacking a given amino acid (RPMI 1640 select-amino acid kit; Gibco Laboratories), supplemented with 5% dialyzed FCS and 250 μCi of that ^{3}H-labeled amino acid (Amersham Corp., Buckinghamshire, UK), and incubated overnight at 37°C in 5% CO_2. For [^{35}S]Met labeling, Met-deficient MEM (Gibco Laboratories) was used. The cells were then washed twice in 20 mM Tris/HCl buffer, 150 mM NaCl, pH 7.5, and were lysed in the same buffer containing 0.5% NP-40 (Sigma Chemical Co.) and a mixture of protease inhibitors: 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin (all from Boehringer, Mannheim, Germany), 100 μM iodoacetamide, 3 ng/ml EDTA, 2 mM PMSF (Sigma Chemical Co.), and 0.2% NaN_3 (Merck, Darmstadt, Germany). After 1 h on ice the lysate was centrifuged in a microfuge for 45 min at 4°C. The supernatant was divided in two aliquots, and 100 μl of ME1 (16) or PA2.1 (anti-HLA-A2) (17) mAb coupled to Sepharose was added to each aliquot. After 3 h at 4°C in a rotary wheel, the immunoprecipitates were washed eight times: twice with NET buffer (0.05 M Tris/HCl, 0.15 M NaCl, 5 mM EDTA, 0.02% NaN_3, pH 7.4), containing 0.5% NP-40 and 1/10 saturated NaCl; twice with NET, 0.5% NP-40, 1/20 saturated NaCl; twice with NET, 0.5% NP-40; and twice with NET alone.

Washed immunoprecipitates were boiled at 100°C in 10% acetic acid, for 10 min. After centrifugation for 5 min in a microfuge, the supernatant was passed through a microconcentrator (Centricon-3; Amicon Corp., Danvers, MA) previously washed with 10% acetic acid. The peptide fraction was concentrated in speed-vac to a volume of 100 μl. HPLC profiles of the internally labeled peptide pools from B*2705 immunoprecipitates showed a highly complex pattern, indicating that the bulk of B27-bound peptides, and not a few major ones, were recovered with this procedure (not shown).

Radiochemical Pool Sequencing. Concentrated peptide pools extracted from each HLA immunoprecipitate were sequenced with 0.5 mg of OVA (Sigma Chemical Co.) carrier protein and 3 mg BioBrene Plus (Applied Biosystems Limited, Warrington, UK), in a gas-phase protein sequencer using the MAC program. Each sequencer fraction was mixed with 3 ml of Universol ES (ICN Biochemicals, Irvine, CA) for counting on a liquid scintillation counter.

Results

Assessment of the R2 Motif in HLA-B27-bound Peptides by Radiochemical Sequencing. The distribution of Arg residues among the peptides bound to B*2705 was examined by radiochemical sequencing of the peptide pool after metabolic labeling with [^{3}H]Arg, specific immunoprecipitation of HLA-B27, and acid extraction of the immunoprecipitated material (Fig. 1). The radioactivity profile showed a major peak at cycle 2, and signal significantly above background at cycles 1 and 3. On the basis of crystallographic and biochemical analyses (7, 11), the vast majority, if not all, of the endogenous peptides bound to B*2705 contain Arg at their second position. Thus, radioactivity at position 2 reflects the presence of Arg in essentially all the peptides in this pool. Radioactivity at cycle 1 ranged from 34 to 44% of that recovered at cycle 2 in three independent experiments. This is only slightly lower than the proportion of Arg residues at position 1 among 14 reported sequences from endogenous and viral peptides known to bind to B*2705, which was 50% (11). The radioactivity at cycle 3, relative to cycle 2, ranged from 32 to 51% in the three experiments. Because of carry-over from the previous cycle, the presence of Arg in this position cannot be assigned. Radioactivity at cycle 3 was somewhat higher than the carry-over obtained upon radiochem-

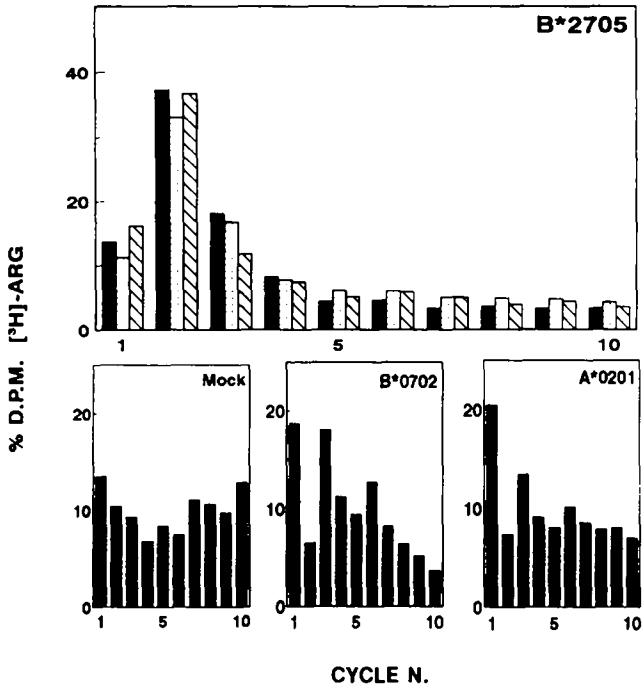

Figure 1. Radiochemical sequence analysis of the Arg-labeled peptide pools from HLA-B27 (B*2705), HLA-B7 (B*0702), and HLA-A2 (A*0201). ^{3}H dpm are plotted vs. cycle number as the percent of the total counts recovered in all cycles that are found at each given cycle. Peptides were extracted from specific immunoprecipitates obtained from metabolically labeled C1R transfectants (B*2705), untransfected C1R cells (mock), and the JY (B*0702, A*0201) LCL, using the PA2.1 (for HLA-A2) and ME1 (for all others) mAbs. For B*2705, results from three independent experiments are represented. Total dpm loaded into the sequencer were 18,000–20,000 (B*2705 and B*0702), 6,800 (A*0201), and 8,000 (mock). Total dpm recovered after each sequencer run were: 1,974, 2,424, and 2,036 (B*2705), 443 (mock), 2,990 (B*0702), and 572 (A*0201). The dpm recovered in the control sequence correspond to background, as they did not exceed 60 dpm at any cycle.

ical sequencing of single peptides (18, 19), but was in the range obtained with pool sequencing of cold material (10). The absence of radioactivity at other cycles indicates that Arg is much less frequent at other positions than at position 2, as also observed among B*2705-bound peptides of known sequence (11).

The specificity and sensitivity of this method for assessing the R2 motif was further examined by sequencing the Arg-labeled peptide pools from HLA-B7 or HLA-A2. The former antigen is predicted to lack pocket B, due to the presence of Tyr67 (7). HLA-A2 has a hydrophobic B pocket that binds aliphatic peptide side chains, predominantly Leu (5, 10). With the peptide pool from HLA-B7, specific signal was observed at cycles 1, 3, and 6, but not at cycle 2. Similarity, no radioactivity at cycle 2 was found upon sequencing the peptide pool from HLA-A2 (Fig. 1).

These results show that radiochemical sequencing of Arg-labeled peptide pools can be used to analyze the presence of the R2 motif in HLA-B27-bound peptides and to detect this residue at other positions.

Binding of Arg-containing Peptides to HLA-B27 Variants with Unaltered Pocket B. The same approach was used to analyze the distribution of Arg residues along the sequence of the peptides bound to the B*2701 to B*2706 subtypes, as well as to the E152 mutant. All these molecules have the same pocket B structure but differ from each other at one or more positions in other pockets (Table 1). The E152 mutant was examined because it is not recognized by most of the anti-B*2705 alloreactive CTL (14, 20).

Cells expressing each of these proteins were metabolically labeled with [^{3}H]Arg, and the corresponding B27-bound peptide pools were subjected to radiochemical sequencing. As shown in Fig. 2, the radioactivity profiles of all the peptide pools were similar to each other and to the peptide pool from B*2705 (Fig. 1). The major radioactivity peak was found at position 2, demonstrating that the R2 motif is conserved among the peptides bound to all of these HLA-B27 variants. In addition, radioactivity at cycles 1 and 3, relative to cycle 2, ranged from 43 to 52% and from 32 to 57%, respectively. These values are comparable to those obtained in different experiments with B*2705-bound peptides, and therefore do not reflect detectable differences among the various peptide pools. Finally, no significant Arg-associated radioactivity was observed at other cycles.

These results indicate that the HLA-B27 subtypes and E152 mutant all bind peptides with the R2 motif. Furthermore, subtype polymorphism, or the E152 mutation, does not result in a detectable alteration of the frequency of Arg residues at other peptide positions.

Binding of Arg-containing Peptides to HLA-B27 Pocket B Mutants. Arg-labeled endogenous peptides were also isolated from a series of HLA-B27 mutants with one or two changes in pocket B (Table 1) expressed on C1R transfectant cells, and subjected to radiochemical pool sequencing. The results (Fig. 3) are described below.

First, the predominance of Arg at position 2 was maintained in all mutants except M45. In this mutant no Arg was detected at cycle 2, beyond that attributable to carryover from cycle 1. This indicates that the substitution of Glu for Met at position 45 in HLA-B27 abrogates or greatly reduces the capacity of binding peptides with the R2 motif. By contrast all other pocket B mutants retained this capacity.

Second, conspicuous radioactivity was found at cycle 1 in all mutants except N63. This result indicates that most of the mutants retained the capacity of binding peptides with Arg at position 1, but the N63 mutation results in nearly undetectable amino-terminal Arg among the peptides bound to this mutant.

Third, except for M45 and N63, the ratio of radioactivity between cycles 1 and 2 ranged from 48 to 70%. This ratio was 35–52% in the peptides from B*2705 and other HLA-B27 molecules with unaltered pocket B (Figs. 1 and 2). The somewhat higher ratio found in some of the mutants, such as A24 (0,69) and A24V67 (0,70), is compatible with relaxed structural constraints in their B pockets, so that peptides with some residue(s) other than Arg at position 2 might also be accommodated. However, the possibility that some

Table 1. *HLA-B27 Subtypes and Mutants*

Subtype/mutant	Amino acid changes	Pocket*	Mean channel ± SD‡
B*2705	-		155 ± 17
B*2701	D74→Y74	C/F	142 ± 30
	D77→N77	C/F	
	L81→A81	C/F	
B*2702	D77→N77	C/F	155 ± 22
	T80→I80	C/F	
	L81→A81	C/F	
B*2703	Y59→H59	A	151 ± 22
B*2704	D77→S77	C/F	148 ± 19§
	V152→E152	E	
B*2706	D77→S77	C/F	141 ± 29
	H114→D114	D, E	
	D116→Y116	C/F	
	V152→E152	E	
Y9	H9→Y9	B	138 ± 17
A24	T24→A24	B	126 ± 16
M45	E45→M45	B	131 ± 27
N63	E63→N63	A, B	160 ± 20
V67	C67→V67	B	150 ± 23
Q70	K70→Q70	B	157 ± 23
A24V67	T24→A24	B	119 ± 17
	C67→V67	B	
Y9Q70	H9→Y9	B	120 ± 16
	K70→Q70	B	
E152	V152→E152	E	134 ± 34
HMy2.C1R	-		24 ± 16

* Pocket assignments are based on references 5 and 7. The K70 side chain in HLA-B27 is oriented away from pocket B and forms a salt bridge with D74 (7).
‡ FACS® analysis of transfectant cell lines, using the anti-HLA-B27 mAb ME1.
§ B*2704 FACS® analysis was performed on the Wewak I LCL.

of the mutations in pocket B indirectly result in binding of an increased number of peptides with amino-terminal Arg, regardless of their effect on the R2 motif, cannot be ruled out.

Fourth, as in B*2705 and other subtypes, no significant radioactivity was observed beyond cycle 3 in any of the mutants.

HLA-B27 Subtypes Bind Related but Substantially Different Peptide Sets. The relatedness among the B*2701- to B*2706-bound endogenous peptides was examined by radiochemical sequencing of the Lys-, Leu-, Ile-, Val-, and Met-labeled peptide pools. This approach reveals the amount of an amino acid at one particular position, relative to other positions, among the peptides bound to a given HLA molecule. It does not reflect the absolute frequency of that residue at a particular position, as only a fraction of the total peptide pool is usually labeled with any given amino acid.

Sequencing of the Lys-labeled peptide pool (Fig. 4) from B*2705 revealed radioactivity not attributable to carry-over, at cycles 1, 3, 4, 5, and, less clearly, 6 and 8. No Lys could be assigned at cycles 2, 7, and 9. This pattern was only partially maintained in the peptide pools from other B27 subtypes. For instance, radioactivity at cycle 4, relative to cycles 1, 3, and 5, was substantially higher in B*2701 than in B*2705. In B*2702, Lys was less abundant at position 1, and significantly more at cycle 8, relative to positions 3–6, than in B*2705. Lys was predominant at positions 1, 3, and 5 in B*2704, and at 1 and 3, but not 5, in B*2706.

Sequencing of Leu-labeled peptide pools revealed predominant radioactivity peaks at cycles 3 and 9, as well as radioactivity not accounted for by carry-over at cycles 5–7 in B*2705. Other subtypes showed a somewhat similar distribution of Leu, but significant differences in the relative proportions of this residue were also evident, e.g., the increased relative proportion of Leu at cycle 7 in B*2704 and at cycle 4 in B*2706.

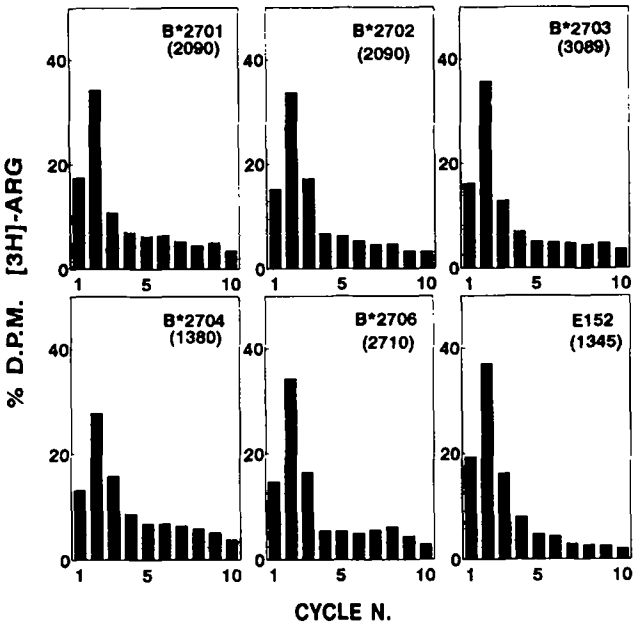

Figure 2. Radiochemical sequence analysis of the Arg-labeled peptide pools from the B*2701 to B*2706 subtypes and the E152 mutant. These were obtained by immunoprecipitation from C1R transfectants, except B*2704, which was obtained from the Wewak I LCL. Data are plotted as explained in Fig. 1. dpm loaded into the sequencer were 20,000 (B*2701, B*2702, B*2703, and B*2706), 12,000 (B*2704), and 18,000 (E152). Total dpm recovered after sequencing are given in parenthesis within each panel.

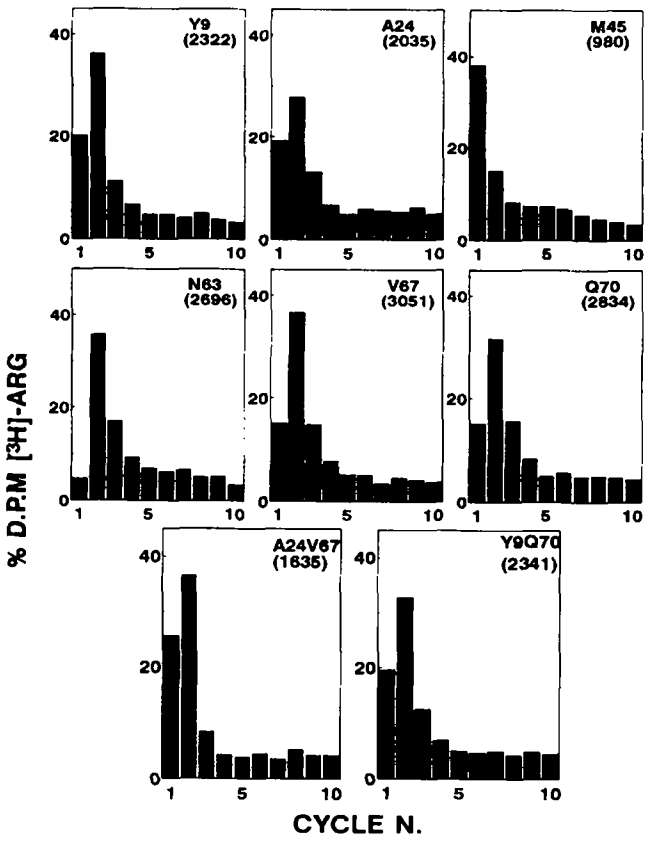

Figure 3. Radiochemical sequence analysis of Arg-labeled peptide pools from HLA-B27 mutants obtained from internally labeled C1R transfectant cells. Data are plotted as explained in Fig. 1. dpm loaded into the sequencer were 20,000 (Y9, N63, V67, Q70, and Y9Q70), 16,000 (A24V67), 15,000 (A24), and 12,000 (M45). Total dpm recovered after sequencing are given in parenthesis within each panel.

The Ile-labeled pool from B*2705 showed a predominant distribution of this residue at positions 3, 5, 6, and 7 upon sequencing. Some relevant radioactivity was also clear at cycle 1. Again, within a general similarity, peptide pools from other subtypes showed some differences. For instance, the relative abundance of Ile at position 1 was highest in the peptide pool from B*2706, which also showed increased signal at cycle 9. The relative amount of Ile at cycle 4 was also higher in B*2704 and B*2706.

Pool sequencing from B*2705 allowed us to assign Val at every peptide position except 2, 7 (due to carry-over), and 9. As with other amino acids, the general pattern was conserved in the peptide pools from other subtypes, although changes in the relative amounts of Val at the various positions were clear. The most significant difference was the virtual absence of Val-associated radioactivity at cycle 3 in the peptide pools from B*2704 and B*2706. Other conspicuous differences were in the ratio of dpm at cycles 7 and 8 in B*2705 compared with B*2701 to B*2703, and the significantly higher signal at cycle 1 in B*2706, compared with other subtypes, especially B*2705.

Met appeared predominantly at cycles 1, 3, and 5 in B*2705, and a similar pattern was observed among the peptides from B*2701, B*2702, and B*2703, although its abundance at cycle 1, relative to other cycles, was lower in the latter subtype. Other significant differences were observed in B*2704 and B*2706.

An idea of the sensitivity of this approach for assessing the relatedness among peptide pools from different HLA molecules can be obtained by comparing the B27-associated sequencing profiles with those from HLA-A2-bound peptides (Fig. 4). Clear differences were observed in the distribution of particular residues in A2- relative to B27-bound peptides. An example is the significant presence of Ile and Met, and the great predominance of Leu, at position 2 relative to other peptide positions.

Discussion

The results in this study show that radiochemical pool sequencing of metabolically labeled peptides can be used to establish the anchoring side chain motifs of the endogenous peptides bound to a given HLA molecule, as well as to detect differences among the peptide pools bound to closely related HLA subtypes. The anchoring motif appears as a highly predominant radioactivity peak at a given position when the peptide pool is labeled with the corresponding amino acid, and is associated with lack of significant radioactivity at that position when the peptides are labeled with other amino acids. If an anchoring motif is not a single amino acid, but several ones, a prominent radioactivity signal at the corresponding

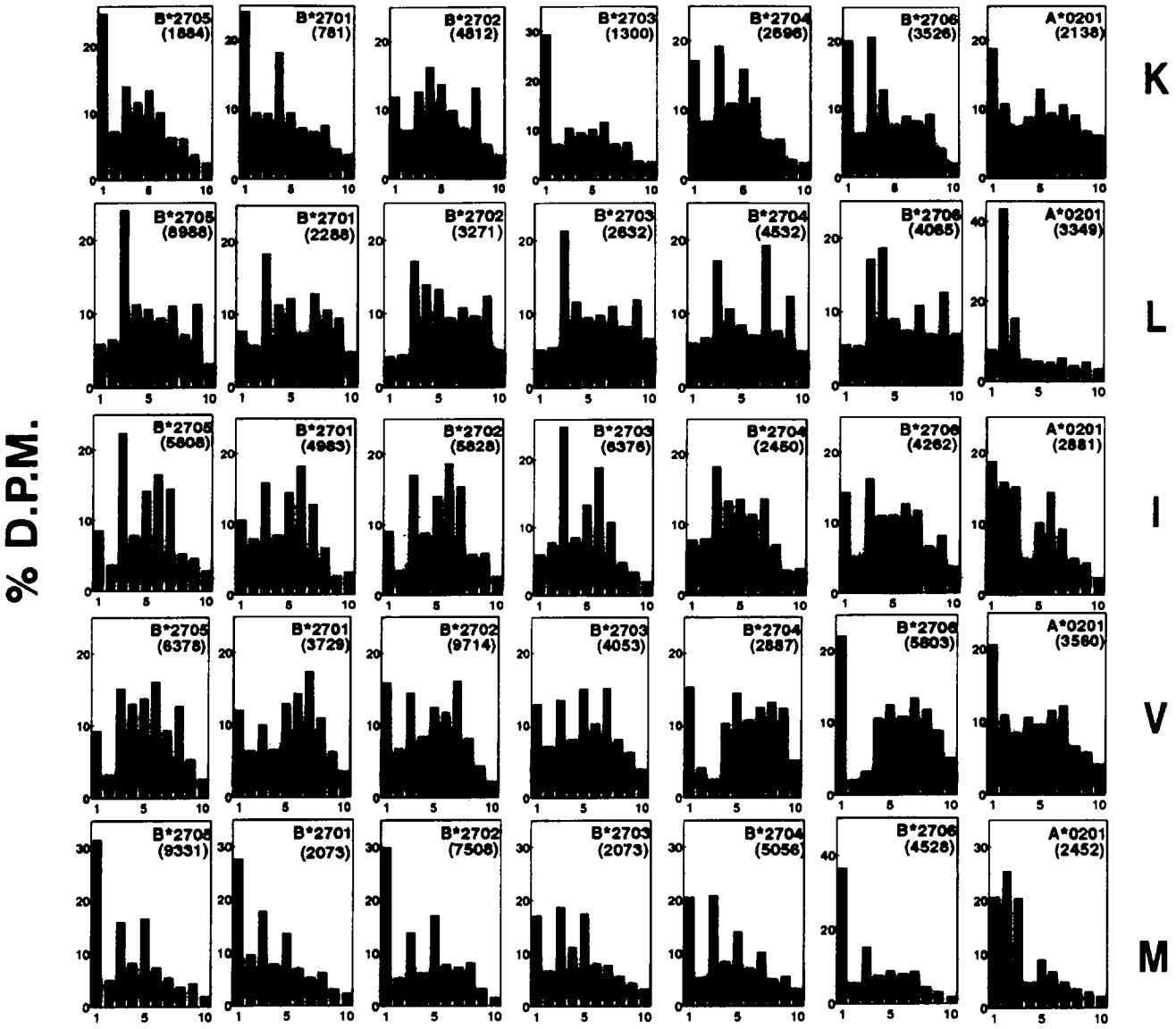

Figure 4. Radiochemical sequence analysis of internally labeled peptide pools from B*2701 to B*2706 and from A*0201. The labeled amino acid used in each set of experiments is denoted by its single-letter code at the end of each row. Thus, K, L, I, V, and M designate the set of sequences of Lys-, Leu-, Ile-, Val-, and Met-labeled peptide pools, respectively. Data are plotted as explained in Fig. 1. Radioactivity loaded into the sequencer ranged from 6,000 to 34,000 dpm. This difference results from multiple factors, including the amino acid used, its specific activity, the fraction of the total peptide pool labeled with a given amino acid, and the average number of labeled residues among relevant peptides. Total dpm recovered after sequencing are given in parenthesis within each panel. Leu- and Ile-associated radioactivity at cycle 2 in the B*2701 peptide pool appears to be higher than expected from carry-over. Although no assignment can be done, the possibility that this might reflect the occurrence of these residues at cycle 2 in some peptides should be left open.

position is observed with those amino acids, as shown for HLA-A2.

Radioactivity peak heights depend on the relative frequency of a particular residue along the various peptide positions. Thus, peptide pools from different HLA specificities bearing different anchoring motifs are easy to distinguish because the corresponding anchorage residues will give very prominent radioactivity at the corresponding position. By contrast, labeling with residues that are frequent at many positions may obscure existing differences among peptide pools.

Jardetzky et al. (11) have reported a predominance of basic amino acids at position 9 in B*2705-bound peptides. They were not detected with this method, probably reflecting the loss of sensitivity inherent to sequencing carboxy-terminal peptide residues. This loss is very dependent on the nature of the two last residues of the peptide. However, radioactivity at cycle 9 was sometimes observed, for instance with Leu-labeled peptides.

As expected from the precise fit of pocket B in B*2705 for Arg (7), the peptides bound to the B*2701 to B*2706

subtypes possessed the R2 motif, indicating that this is solely dictated by the structure of pocket B and is not significantly influenced by subtype polymorphism. The frequency of Arg1, relative to Arg2, was not significantly altered either by subtype polymorphism, even in B*2703, which differs from B*2705 by having His instead of Tyr at position 59, in pocket A (12). This result indicates that His59 does not interfere with the presence of Arg at peptide position 1, and is compatible with crystallographic data (7) showing that residue 59 interacts with the peptide amino terminus, the P1 side chain pointing away from pocket A.

The absence of the R2 motif in the peptides bound to the M45 mutant agrees with the crystallographic model showing the importance of charge complementarity between Glu45 in HLA-B27 and the guanidinium group of the peptidic Arg2 (7). If peptides having the R2 motif can still bind to the M45 mutant, they must be a minor fraction of the M45-bound peptide pool or bind at very low cell surface density. Nevertheless, in a previous study (15) we reported that a substantial fraction (>40%) of anti-B*2705 alloreactive CTL crossreacted with the M45 mutant. If it is assumed that peptides are involved in allorecognition, a likely explanation for such frequent crossreactivity is that, to a large extent, it is mediated by unrelated peptides having the same or similar side chains at those positions that interact with the TCR. This possibility was previously suggested to explain unexpected crossreactions with the M45 mutant (15, 21). Thus, although residual binding of R2-containing peptides might perhaps account for CTL crossreaction with M45 in some cases, the present results strongly suggest that determinant mimicry by nonidentical peptides may be frequent. This is compatible with the extensive variability allowed to HLA-bound peptides at those positions not interacting with HLA pockets (10, 11).

It has been proposed that ankylosing spondylitis may be related to a B27-directed autoimmune T cell response triggered by external pathogens carrying crossreactive T cell determinants (22). The idea that T cell determinant mimicry by nonidentical peptides may not be infrequent adds a new dimension to this hypothesis, as crossreactivity would not necessarily involve a bacterial peptide highly homologous to a B27-bound endogenous peptide, but just one sharing certain residues amenable to interacting with the TCR.

Conservation of the R2 motif among the peptides bound to the Y9 and A24 mutants was somewhat surprising on the basis of structural evidence showing the involvement of His9 and Thr24 in the network of hydrogen bonds that stabilizes the peptide R2 side chain in pocket B (7). Both the Y9 and A24 mutations disrupt this network. Nevertheless, it is clear from our data that such disruption is still compatible with many peptides carrying the R2 motif. Thus, straight extrapolations from x-ray models or from model building calculations regarding the effect of single residue changes on peptide binding must be done with great caution, as these effects may not be easy to predict. Conservation of the R2 motif does not imply that all the peptides that bind to B*2705 will also bind to these mutants. Indeed, A24 was not recognized by 50% of the anti-B*2705 CTL tested in a previous study (15), suggesting that many peptides bound to B*2705 cannot bind to this mutant, at least in a way that can be recognized by the same CTL. Although the Y9 mutation can also affect binding of some peptides, we have observed very frequent crossreactivity between this mutant and B*2705 with alloreactive CTL (our unpublished data), suggesting that most endogenous peptides that bind to B*2705 can still bind to the Y9 mutant. The conservation of the R2 peptide motif in the V67 and Q70 mutants is consistent with the relatively conservative nature of the substitution at residue 67, which also has little effect on allorecognition (15), and with the orientation of the side chain at position 70, away from pocket B (7). These data do not exclude the possible presence of other P2 side chains in some of the peptides bound to these mutants, as some of the mutations could relax the restrictive character of the B pocket.

Glu63 in HLA-B27 is located in the rim of pocket B and interacts with peptidic main chain atoms (7). The conservation of the R2 motif among the peptides bound to the N63 mutant indicates that Glu63 is not required for maintaining this motif, and is consistent with crystallographic data showing no interaction of this residue with the R2 side chain. The strikingly low signal for Arg at position 1 among the peptides bound to the N63 mutant strongly suggests a direct effect of this mutation on disrupting the contacts that stabilize amino-terminal Arg residues. Such disruption would affect the interaction between Glu63 and Arg62, and through this, the interaction between Arg62 and Glu163, which interacts with the peptide R1 (7).

Comparison of the sequence profiles of the peptide pools from B*2701 to B*2706, suggests that B27 subtypes share a significant number of bound peptides, but many R2-containing peptides either bind to only some subtypes or they bind to all the subtypes but with greatly different efficiency. This is in agreement with functional studies showing frequent crossreactivity, but also differential recognition, of HLA-B27 subtypes with anti-B*2705 CTL (14, 23). Substantial differences among peptide pools from two HLA-A2 subtypes have also been recently reported (24). Differences among the peptide pools from B27 subtypes are due to the location of subtype polymorphism in areas of the molecule that interact with bound peptides. However, the differences seen in sequence patterns do not only affect those peptide residues interacting with the pockets in which subtype changes are located, but also other peptide positions, including those whose side chains point away from the groove. Thus, sequence patterns reflect the global alteration of the peptide pool. The present results demonstrate the importance of pockets other than the B pocket in shaping the repertoire of peptides that can bind to a particular class I HLA protein. This must be emphasized because it means that, in spite of the major contribution of the interaction with the peptide main chain and the R2 side chain, many peptides having the R2 motif will bind to HLA-B27 variants only if the structure of other pockets is maintained unaltered.

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