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Structural analysis of an HLA-B27 functional variant: Identification of residues that contribute to the specificity of recognition by cytolytic T lymphocytes

(class I antigens/polymorphism/gene conversion/peptide mapping)

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ABSTRACT The structure of a variant HLA-B27 antigen, B27.2, that is distinguished from the HLA-B27.1 and HLA-B27.3 subgroups by specific cytolytic T lymphocytes has been established by comparative peptide mapping and sequence analysis. There are only three amino acid substitutions between B27.1 and B27.2: aspartate-77, threonine-80, and leucine-81 in HLA-B27.1 are changed to asparagine-77, isoleucine-80, and alanine-81 in HLA-B27.2. These changes account for their single charge difference detectable by isoelectric focusing. The three clustered substitutions of HLA-B27.2 are identical to the corresponding residues in HLA-A24, so that both molecules become identical in their amino acid sequence between residues 72 and 96. This suggests that gene conversion may have occurred during the diversification of the HLA-B27 antigens. HLA-B27.2 has no changes in the α2 domain and is similar in its pattern of substitutions to the murine bm11 mutant. It is suggested that residues 77–81 are of major significance in determining the specificity of cellular recognition of class I HLA antigens. This study, together with the previous analyses of HLA-B27.1 and HLA-B27.3, completes the structural characterization of the three major HLA-B27 functional subtypes and establishes the molecular basis of their functional and serological differences.

Recognition of foreign antigens expressed on the surface of virus-infected or otherwise modified cells by cytolytic T lymphocytes (CTL) is generally carried out in association with shared class I histocompatibility antigens (1). These are polymorphic cell-surface glycoproteins encoded in the HLA-A, -B, -C loci of the human major histocompatibility complex (MHC) or in the equivalent H-2K, -D, -L regions of the mouse genome. They are composed of a MHC-encoded heavy chain, noncovalently bound to β_2-microglobulin, an invariant polypeptide (2). The heavy chain is organized in three extracellular domains designated as α1 (residues 1–90), α2 (residues 91–182), and α3 (residues 183–275); a transmembrane region; and an intracytoplasmic domain. Variable positions in the extracellular portion are located mainly in α1 and α2. These domains also carry most of the serological and CTL determinants. By contrast, α3 is much less polymorphic and is probably a major interactive site for β_2-microglobulin (3, 4).

A great knowledge of the structure of class I HLA antigens and of the molecular features of polymorphism has been attained as a result of extensive molecular analyses (5, 6). However, little is known about the relevance of particular regions of the molecule in determining the specificity of CTL recognition and MHC restriction. A way of approaching this issue takes advantage of the fact that various well-defined serological specificities may be further divided into several subtypes by specific CTL (7–14). Detailed biochemical analyses of some of these naturally occurring variants (15–19) have shown that the different subsets of a given serological specificity differ from each other in a very limited number of amino acid residues. Thus, they provide a unique tool for determining the amino acid sequence substitutions that affect the specificity of CTL recognition.

Although HLA-B27 is one of the best-defined serological specificities (20), at least three CTL-defined subgroups have been outlined with both allogeneic (11) and virus-immune B27-restricted CTL (12). These subgroups have been designated as B27W, -K, -C and B27.1, B27.2, and B27.3, respectively. A close correspondence exists between subgroups W and B27.1, K and B27.2, and C and B27.3 (12). In addition, these subsets may be distinguished on the basis of their differential reactivity with the monoclonal antibody B27M2 (12, 21). The HLA-B27 antigen is particularly relevant because of its very strong association with ankylosing spondylitis (22), this association being apparently equal for all B27 subtypes (11). We have recently determined the amino acid sequence of HLA-B27.1, the major subtype in Caucasians (23), as well as the structure of an HLA-B27.3 antigen, a subtype that is predominant in Orientals (19). In the present report, the structure of the HLA-B27.2 antigen has been determined by comparative peptide mapping with B27.1 and by radiochemical sequence analysis. This study completes the molecular characterization of the three major HLA-B27 functional subtypes and establishes the nature and location of residues of significance in determining the specificity of CTL recognition of this antigen.

MATERIALS AND METHODS

Cell Lines. The human Epstein–Barr virus-transformed B-lymphoblastoid cell lines LG-2 (HLA-A2,2; B27,27) and R34 (A2,9; B18,27) were used as the source of material for the radiochemical purification of HLA-B27.1 and HLA-B27.2, respectively. The R34 line was kindly provided by E. Gomard and J. P. Levy (Hospital Cochin, Paris).

Biochemical Analysis of HLA-B27.1 and -B27.3. Metabolic labeling, purification of radiolabeled HLA-B27 heavy chains, double label comparative peptide mapping, automated sequence analysis, and isoelectric focusing (IEF) were exactly as described (19).

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Abbreviations: CTL, cytolytic T lymphocytes; MHC, major histocompatibility complex; IEF, isoelectric focusing.

RESULTS

IEF of HLA-B27 from LG-2 and R34. Fig. 1 shows a comparison of the IEF pattern of the HLA-B27 heavy chains from LG-2 and R34 cells. The band pattern of B27.2 is displaced toward the basic end by the equivalent of a single charge difference with respect to B27.1. The multiple bands observed for each molecule are partially due to variability in sialic acid content (24) and probably also to other post-translational modifications (25–27). Neuraminidase treatment did not reduce heterogeneity but resulted in an identical shift of the band patterns of both molecules toward the cathode, as expected from the removal of sialic acid. Thus, the B27.2 molecule is more basic than B27.1 by the equivalent of 1 charge unit.

Comparison of LG-2 and R34 HLA-B27 Heavy Chains by Peptide Mapping. The strategy used for the structural characterization of the HLA-B27.2 molecule was based on its comparison with the B27.1 antigen from LG-2, whose sequence has been determined (23). Comparative peptide mapping by reverse-phase high-performance liquid chromatography was used because the suitability of this method for the structural analysis of other HLA variants, including HLA-B27.3, is well established (15–19).

Initially, leucine-, alanine-, lysine-, and tyrosine-containing tryptic peptide maps were obtained. These amino acids labeled peptides covering 94% of the extracellular portion of HLA-B27. Tyrosine- and lysine-labeled maps were identical (not shown), but both the leucine- and alanine-labeled maps presented a number of difference peaks (Fig. 2 *A* and *B*). Upon sequence analysis of the difference leucine-labeled peptides (Fig. 3*A*), it was shown that both L1 and L2 were labeled at step 3, suggesting that they were the B27.1 and B27.2 counterparts, respectively, of the peptide spanning residues 76–79. The elution position of L1 corresponds to that of peptide 76–79 from B27.1 in this chromatographic system (19). L3 was shown upon sequencing to be an identical peptide with ^{3}H and ^{14}C label at step 3 (not shown). This peptide spans residues 177–181 (19, 23). No other HLA-B27 peptides have leucine at position 3. L4 was a B27.1 difference peptide known to span residues 71–79 on the basis of its elution position and sequencing (19). Its B27.2 counterpart was L6, as indicated by the presence of leucine at step 8 upon sequencing (Fig. 3*A*). The sequence of L10 revealed ^{14}C radioactivity at steps 2 and 3 but ^{3}H label only at step 3 (Fig. 3*A*), suggesting that this peptide was that spanning residues 80–83 (TLLR in B27.1), because no other HLA-B27 peptide has two consecutive leucine residues at these positions. Thus, the B27.2 counterpart had at least a change involving leucine at position 2, but the chromatographic

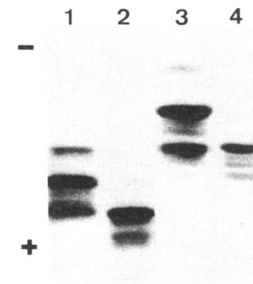

FIG. 1. IEF analysis of [^{35}S]methionine-labeled heavy chains of HLA-B27.2 (lane 1), HLA-B27.1 (lane 2), neuraminidase-treated HLA-B27.2 (lane 3), and neuraminidase-treated HLA-B27.1 (lane 4).

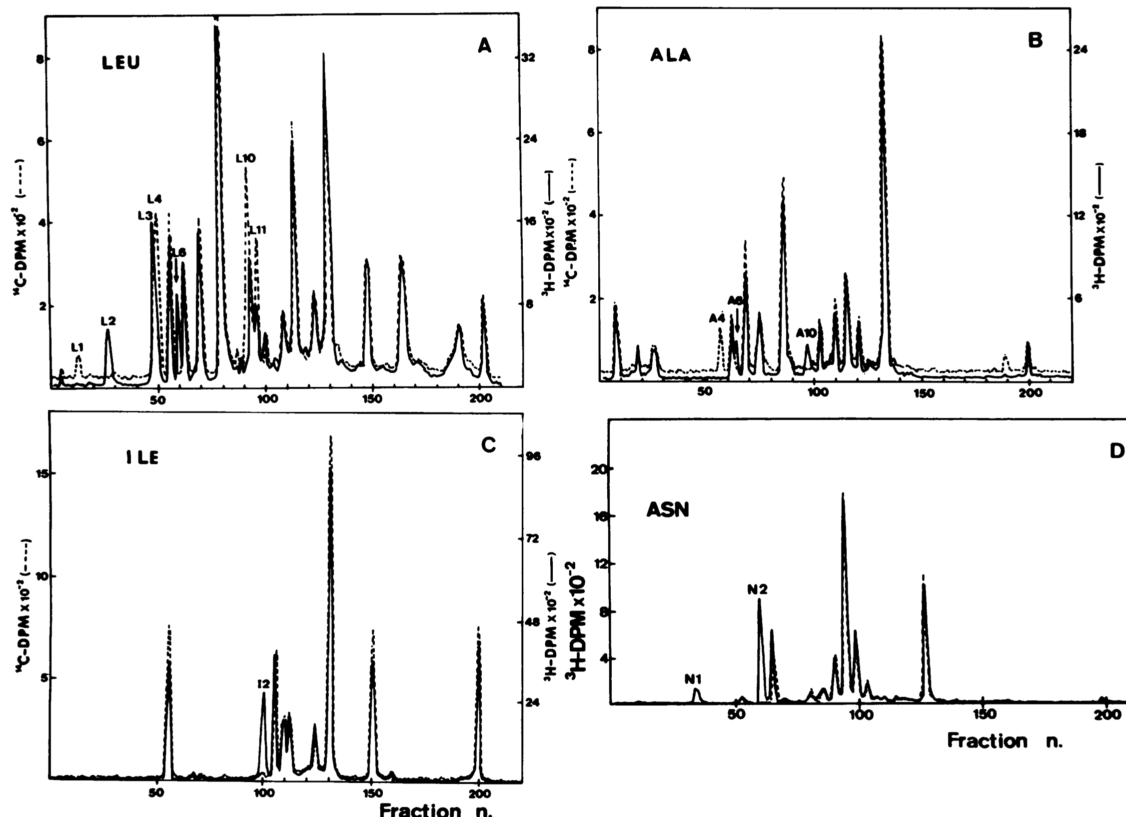

FIG. 2. Reverse-phase high-performance liquid chromatography comparison of (*A*) leucine-, (*B*) alanine-, (*C*) isoleucine-, and (*D*) asparagine-labeled tryptic peptides of HLA-B27.1 (---) and HLA-B27.2 (—). These proteins were labeled with ^{14}C and ^{3}H amino acids, respectively, and were chromatographed together. For asparagine labeling, [^{3}H]asparagine, was incorporated in both molecules. They were digested and chromatographed separately and their peptide maps were plotted together. Peaks are numbered according to their elution position within each peptide map. Difference peaks are specified.

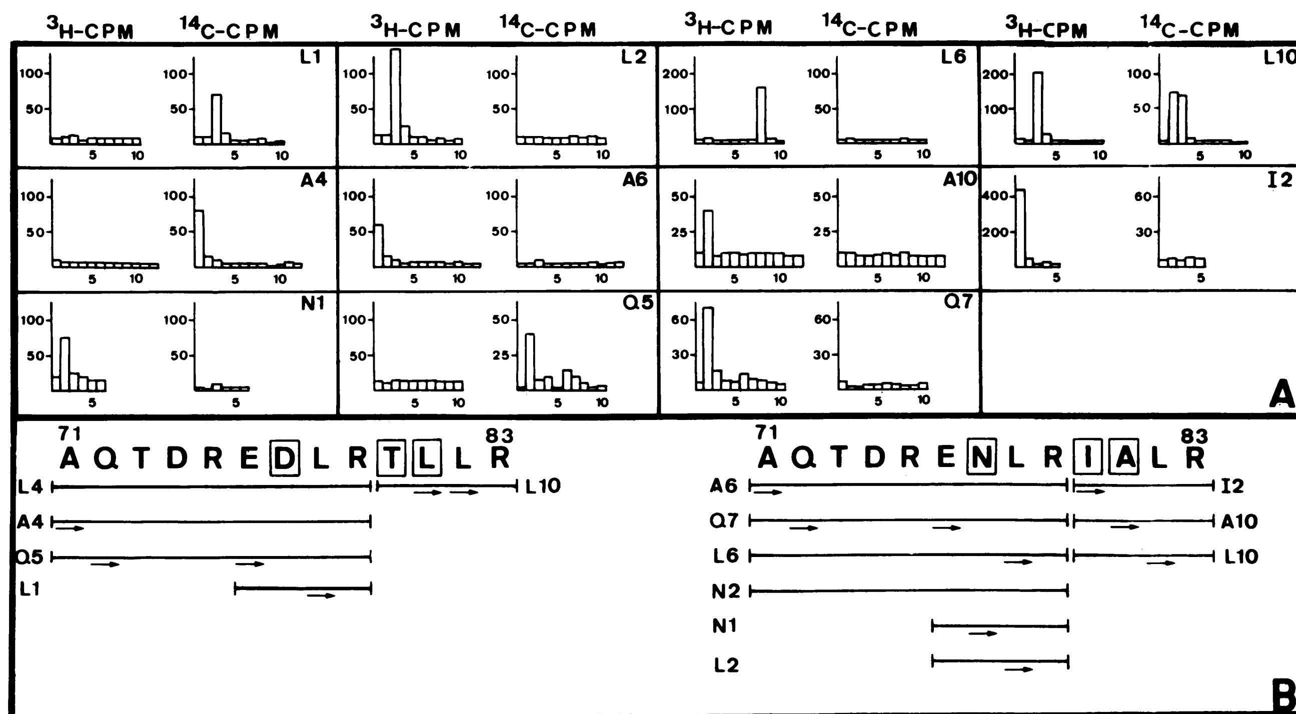

FIG. 3. (*A*) Radiochemical sequence analysis of the difference peptides. For each peptide, ^{3}H and ^{14}C radioactivity (cpm) are separately plotted versus cycle number. (*B*) Assignment of the difference peptides of HLA-B27.1 (left) and B27.2 (right). Arrows denote residues directly identified by radiochemical sequencing. Differences between both molecules are boxed. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

mobility of the peptide was unaltered. Sequencing of L11 indicated that its height difference was due to contamination with L10 (not shown). The alanine-labeled map revealed three difference peptides—A4, A6, and A10 (Fig. 2*B*)—whose elution positions corresponded to those of L4, L6, and L10, respectively. The presence of label at step 1 in A4 and A6 upon sequencing (Fig. 3*A*) confirmed that they were the peptide spanning residues 71–79 from B27.1 and B27.2, respectively. The sequence analysis of A10 revealed radioactivity at step 2. This result, together with the elution position of this peptide and the absence of a counterpart from B27.1, strongly suggested that A10 was the peptide spanning residues 80–83 from B27.2 and, therefore, that leucine in B27.1 was changed to alanine in B27.2.

Among all class I HLA antigens of known amino acid sequence, the only one with alanine at position 81 is HLA-A24 (28, 29), whereas all others have leucine at this position. Since HLA-A24 also has a hitherto unique isoleucine residue at position 80, we considered the possibility that isoleucine-80 might also exist in HLA-B27.2. Hence, an isoleucine-labeled tryptic map was obtained to compare B27.1 and B27.2 (Fig. 2*C*). A single B27.2 difference peak, I2, was found at the same elution position as L10 and A10, with no counterpart from B27.1. Its sequence analysis revealed radioactivity at step 1 (Fig. 3*A*), confirming the identity of the peptide as that spanning residues 80–83 and the presence of isoleucine at position 80 in B27.2, instead of the threonine residue present in B27.1 (23).

To determine the nature of the substitution within residues 76–79, a serine-labeled comparative peptide map was first obtained to probe the possibility of a substitution of aspartate-77 in B27.1 (23) to serine, because this residue is frequently found at this position in class I HLA antigens and it was also present in HLA-B27.3 (19, 28). However, the serine-labeled map revealed no difference peptides other than those due to metabolic conversion of serine to glycine that were also present in a control map of ^{3}H-labeled B27.1 vs. ^{14}C-labeled B27.1 (not shown). The HLA-A24 antigen is the only specificity of known sequence, with no aspartate or serine at position 77. Instead, asparagine is present at this position. Thus, an asparagine-labeled tryptic map was prepared for B27.1 and B27.2 (Fig. 2*D*). Two B27.2 difference peaks, N1 and N2, were obtained with no counterpart from B27.1. The elution position of these peaks corresponded with those of L2 and L6, respectively, indicating that N1 was the peptide spanning residues 76–79 and N2 was that spanning residues 71–79. Sequencing of the N1 peptide (Fig. 3*A*) confirmed the presence of asparagine at position 77 in B27.2, instead of the aspartate-77 present in B27.1. This change accounts for the single-charge difference between these two molecules observed by IEF (Fig. 1).

Finally, glutamine labeling was carried out in the absence of glutamine and glutamate to label both residues, so that the only two peptides not covered by previous maps (those spanning residues 18–21 and 45–48) were labeled. A tryptic map was obtained that completed the mapping of the extracellular portion of the molecule. Only two difference peaks were found: Q5 and Q7 (not shown). They were further purified by ion-exchange chromatography. These peaks corresponded to the B27.1 and B27.2 counterparts, respectively, of peptide 71–79, as assessed by the presence of radioactivity at steps 2 and 6 upon sequencing (Fig. 3*A*), corresponding to glutamine-72 and glutamate-76, respectively.

A summary of the assignments given to the various difference peptides, showing the residues directly identified by sequencing, is presented in Fig. 3*B*. All peptide differences may be accounted for by the three amino acid substitutions at positions 77, 80, and 81. All residues except the COOH-terminal arginine residues in the difference peptides 76–79 and 80–83 were directly identified by sequencing. Only an arginine to lysine substitution would be consistent with the observed tryptic cleavage. But this possibility was ruled out because the lysine peptide map was identical. Thus, it was concluded that no additional substitutions were present within these peptides.

DISCUSSION

The R34 cells carry an HLA-B27 antigen that is recognized by Epstein–Barr virus-immune B27-restricted polyclonal CTL specific for HLA-B27.2, but not by those that define the B27.1 or B27.3 subtypes (12). These cells are also lysed by bulk allogeneic CTL specific for subgroup B27K, but not for those that define the W and C subgroups (B. Breur and P. Ivanyi, personal communication). The structural differences between this molecule and the known amino acid sequences of the B27.1 (23) and B27.3 (19) antigens were established by comparative peptide mapping with HLA-B27.1. The only detected changes were aspartate to asparagine at position 77, threonine to isoleucine at position 80, and leucine to alanine at position 81. The possibility that additional substitutions with no effect on the elution profile or that some low-yield difference peptide could have gone undetected cannot be formally excluded. However, it is worth noting that the peptide 152–157, which includes critical residues that are found to vary among most CTL-defined variants (28), is clearly identified by its elution position (19) as L8, A8 (Fig. 2), and Q8 (not shown) and is identical between B27.1 and B27.2.

The pattern of amino acid changes between B27.1 and B27.2 provides an example in support of the hypothesis that gene conversion generates polymorphism in the HLA system (30, 31). All three differences are clustered in a short segment of the sequence. A minimum of three base changes within six consecutive nucleotides is required to account for the two substitutions threonine-80 to isoleucine-80 and leucine-81 to alanine-81. In addition, as mentioned above, all three variable residues 77, 80, and 81 become identical in B27.2 to those in HLA-A24. This is notorious because asparagine-77, isoleucine-80, and alanine-81 are unique in these two proteins when compared with all other class I HLA antigens of known sequence (28). Because of the nature of these substitutions, B27.2 and A24 become identical between residues 72 and 96. As noted by Nguyen *et al.* (29), the coding sequence of the HLA-A24 gene corresponding to residues 75–90 is identical in 46 consecutive nucleotides to that of the pHLA12.4 pseudogene (32). Since both of these genes were obtained from unrelated libraries, it seems clear that potential donor sequences may exist in an undetermined number of class I genes (or pseudogenes) in the human genome to account for the diversification of B27.1 and B27.2 by a mechanism analogous or identical to gene conversion.

Another example has been recently described for an HLA-A3 variant where the two detected differences with the known sequence of HLA-A3, at positions 152 and 156, make the corresponding region of the sequence identical to HLA-A24 (17). Thus, as it has been more extensively documented in the mouse (33–35), class I antigen variants illustrate the introduction of multiple changes by a single genetic event as a major driving force for the generation of HLA polymorphism and a mechanism with the potential to produce an accelerated diversification of MHC genes.

Interestingly, HLA-B27.2 presents no differences with B27.1 in the segment 147–157, a region that is believed to be critical for CTL recognition (16, 28). This is the only reported HLA variant defined with polyclonal CTL that has no substitutions in this area. An HLA-B7 variant, CF, has a single detected substitution at residue 116 (18). However, this variant has been distinguished from other B7 subtypes with cloned CTL but not with bulk effectors. In this respect, HLA-B27.2 is reminiscent of the mouse bm mutants because most of them, except bm1 (with changes at positions 152, 155, and 156), have their substitutions outside the region 147–157 (36). Thus, other polymorphic residues, besides those in this segment, determine the specificity of CTL recognition. The HLA-B27.2 antigen is strikingly similar to the bm11 mutant in that bm11 differs from the parental H-2K^b protein solely at positions 77 and 80 (S. Nathenson, personal communication). This mutant is far from H-2K^b by cellular typing with allospecific CTL (37, 38). In addition, it is one of the most dissimilar from H-2K^b in its specific inability to function as restriction element for H-2K^b-restricted CTL in a variety of antigenic systems including Sendai virus, minor histocompatibility antigens, and certain haptens (39–41). Thus, because of the drastic effect of substitutions within residues 77–81 on CTL recognition in both human and mouse, this segment emerges as another critical portion of the molecule in determining the functional specificity of class I antigens. Studies in the mouse suggest that the functional effect of changes in this area may not be as dramatic as those in the region 147–157. Thus, whereas bm1 behaves differently from H-2K^b as restriction element for every virus that has been tested, bm11 targets are killed by virus-immune H-2K^b-restricted CTL in some cases, such as with Moloney virus (36) and, to a lower degree, with simian virus 40 (42). However, an interpretation of these data in terms of the relative importance of segments 77–81 and 147–157 is biased by the possibility that the specific nature of the changes, and not only their location, may significantly influence the functional behavior of the mutants.

The relationship between the specific serological and CTL determinants of HLA-B27 may now be approached by comparing the pattern of substitutions between all three B27.1, B27.2, and B27.3 subgroups and the polymorphic areas of the molecule defined by sequence comparisons. We have previously suggested, on the basis of sequence comparisons with crossreactive specificities, that residues within segments 63–70, 77–83, and 113–116 are major contributors to the HLA-B27 serological allodeterminants (23). All three B27 subgroups differ in residues within segment 77–81 but are identical in the 63–70 and 113–116 segments (Fig. 4). This probably accounts for the fact that most conventional alloantisera do not distinguish among B27 subtypes, because many of the residues determining the specific serological determinants remain unchanged. However, some polyclonal alloantisera do distinguish between the CTL subtypes (43) as does the monoclonal antibody B27M2. This antibody reacts with B27.1 and, much more weakly, with B27.3; it is negative with B27.2 (12). It is now clear that the epitopes recognized by these antibodies include residues within the segment 77–81. Residue 77 is different in all three subtypes and B27.2 has two additional differences. This explains the reactivity pattern of the M2 antibody and the lowest degree of serological relatedness of B27.2.

A number of studies suggest that the bulk of the antibody and CTL responses against class I antigens may be directed against different determinants (refs. 6 and 28 and refs. therein). The fact that the three HLA-B27 subtypes may be

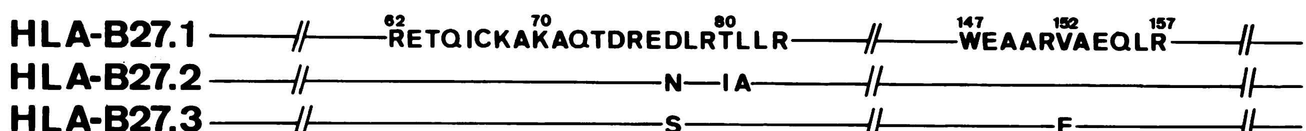

FIG. 4. Schematic comparison of the amino acid sequences of HLA-B27.1 (23), HLA-B27.2, and HLA-B27.3 (19), showing the location of the changes relative to the variable segments 62–83 and 147–157. The code for amino acids is the same as in Fig. 3*B*.

distinguished by both CTL and antibodies and that these subtypes differ from each other in residues within a highly variable region indicates that the epitopes recognized by alloantibodies and CTL are similar to some extent. Further studies are required to properly evaluate this important issue.

It is obvious that other polymorphic regions, in addition to those defined by the limited number of variants that have been characterized, must modulate the specificity of the recognition of HLA antigens by CTL; for example, in spite of the identity of B27.2 and A24 in residues 72–96, the three changes in this region with B27.1 are sufficient for B27.2-specific CTL to abrogate killing of B27.1 targets but not to crossreact with HLA-A24. In addition, HLA-B27 has been suggested to act as a dominant restriction element over other class I antigens in CTL responses against influenza and Epstein–Barr virus, regardless of the B27 subtype (44). Thus, other polymorphic residues besides those at 77–81 must contribute to the restriction properties of HLA-B27. Clearly, much more work is necessary to unveil the molecular mechanisms of the MHC-restricted antigen recognition by the T-cell receptors. But analyses such as those performed with the HLA-B27 antigens illustrate the value of HLA variants in the definition of functional sites of class I molecules and allow a progressive evaluation of the functional role of HLA polymorphism.

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