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J D Taurog, F A el-Zaatari

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### Research Article

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# In Vitro Mutagenesis of HLA-B27

## Substitution of an Unpaired Cysteine Residue in the $\alpha_1$ Domain Causes Loss of Antibody-defined Epitopes

Joel D. Taurog and Fouad A. K. El-Zaatari

Harold C. Simmons Arthritis Research Center, Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

### Abstract

The HLA class I molecules identified serologically as HLA-B27 are highly associated with ankylosing spondylitis and related human disorders. All known HLA-B27 amino acid sequences contain a cysteine residue at position 67; no other published HLA class I sequence contains a cysteine within the hypervariable region of the  $\alpha_1$  domain, which extends from amino acid residues 63–84. To investigate the role of this cysteine residue in the antigenic structure of HLA-B27, we isolated a genomic clone encoding a molecule of the HLA-B27.1 subtype and performed oligonucleotide-directed mutagenesis to convert the cysteine at position 67 to a tyrosine. When transfected into mouse L cells, both the wild-type and Cys<sub>67</sub> → Tyr<sub>67</sub> mutant B27 genes directed the synthesis and surface expression of molecules reactive with the monomorphic anti-HLA class I antibody W6/32. However, only the L cells transfected with the wild-type B27 gene reacted with the anti-B27 antibody ME1; L cells transfected with the mutant B27 were completely unreactive with this antibody. Experiments with hybrid exons created from the HLA-B27 and HLA-A2 genes yielded results consistent with the mapping of the ME1 epitope to the B27  $\alpha_1$  domain. A second anti-B27 antibody, GS145.2, also showed markedly reduced binding to the Cys<sub>67</sub> → Tyr<sub>67</sub> mutant. These studies document the importance of the unique Cys<sub>67</sub> residue in the antigenic structure of HLA-B27.

### Introduction

Human class I major histocompatibility molecules are glycoproteins expressed on the surfaces of nucleated somatic cells. Each consists of two noncovalently associated polypeptide chains: a 44,000-*M<sub>r</sub>* polymorphic heavy chain encoded by one of the multiallelic HLA-A, B, and C loci of the human MHC on chromosome 6, and an invariant 12,000-*M<sub>r</sub>* light chain,  $\beta_2$ -microglobulin, encoded on chromosome 15 (1). The HLA-encoded heavy chains are organized in three extracellular do-

main designated as  $\alpha_1$  (amino acid residues 1–90),  $\alpha_2$  (residues 91–182), and  $\alpha_3$  (residues 183–274); a hydrophobic transmembrane region; and an intracytoplasmic carboxy-terminal domain. Virtually all of the polymorphic variation within each locus and most of the variation among the three serologically identified class I HLA loci reside in the nucleotide and corresponding amino acid sequences of the  $\alpha_1$  and 2 domains. The polymorphism of the class I HLA molecules is thought to be related to their function as restriction elements for the specific recognition of foreign antigens by T lymphocytes.

The allelic products of the HLA-A, B, and C loci have been traditionally identified by alloantisera. However, several serologically well-defined alloantigenic specificities are divisible further into subtypes on the basis of a few amino acid differences in the  $\alpha_1$  and/or  $\alpha_2$  domains (2–6). HLA-B27, as defined by alloantisera, identifies a group of at least six HLA-B locus products that share the B27 allospecificity (5, 6).

Serologically defined HLA-B27 is associated with a group of idiopathic inflammatory human diseases, including ankylosing spondylitis (AS),<sup>1</sup> reactive arthritis, and acute anterior uveitis (7, 8). The strongest association is with AS; in Caucasian populations, the prevalence of HLA-B27 in the general population is ~ 8%, whereas in patients with primary AS it exceeds 90%. Attempts to correlate the incidence of AS with one or more of the B27 subtypes have not been successful; AS apparently is associated with most, if not all, of the B27 subtypes (7, 9).

This study was undertaken to begin to identify the features of the HLA-B27 sequence that correlate with the serologically defined B27 allospecificity and with disease association. One striking feature unique to B27, and shared by all six B27 subtypes whose sequences are known, is the presence of an unpaired cysteine residue at amino acid position 67 in the  $\alpha_1$  domain (6). In this communication, it is demonstrated that substitution of this cysteine with a tyrosine, the residue found at position 67 in the closely related allele HLA-B7 (10), eliminates the epitopes defined by two MAbs that react with all known B27 subtypes.

### Methods

*Isolation of genomic clones encoding HLA-B27 and HLA-B7.* Genomic DNA was isolated as previously described (11) from peripheral blood leukocytes of an AS patient who was homozygous for HLA-B27. DNA was digested with Eco RI; 6–7-kb fragments were ligated into the bacteriophage vector  $\lambda$  gt10, packaged, and used to create a size-se-

Address reprint requests to Dr. Joel D. Taurog, Harold C. Simmons Arthritis Research Center, Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas TX 75235.

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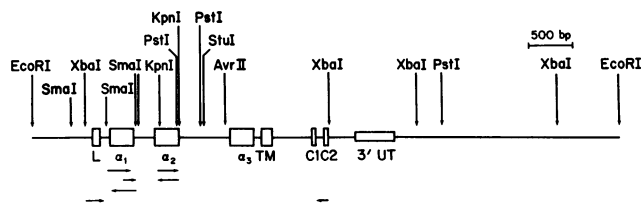
1. *Abbreviations used in this paper:* AS, ankylosing spondylitis; CTL, cytolytic T lymphocyte; FBS, fetal bovine serum; HAT, hypoxanthine-aminopterin-thymidine.

lected library in *Escherichia coli* strain C600 hfl<sup>-</sup> (Gigapack; Stratagene Cloning Systems, La Jolla, CA). The library was screened (12) with a probe, pHLA-1.1, that is relatively specific for the HLA-B locus under stringent conditions (13). 18 positive clones were obtained; 3 were plaque-purified and subcloned into pUC19. One clone, pD.1-B27 (Fig. 1), was selected for the experiments described in this communication. It was confirmed to encode a product of the B27.1 subtype (5, 14, 15) by extensive restriction mapping, DNA sequencing, and serologic identification of the gene product expressed in transfected cells. In a similar manner, a size-selected library was constructed from an HLA-B7<sup>+</sup> individual, and p12.1-B7, a genomic clone encoding HLA-B7, was isolated and similarly characterized.

**Site-directed mutagenesis.** A 2.2-kb Eco RI-Avr II fragment encoding exons 1–3 of pD.1-B27 was subcloned into Eco RI/Xba I-cut M13mp18 to produce the recombinant mB27m0-RA, and a 2.3-kb Avr II-Pst I fragment encoding exons 4–8 was subcloned into Xba I/Pst I-cut pUC19 to produce pB27-AP. Mutagenesis of mB27m0-RA was carried out as described in detail by Zoller and Smith (16), using the mutagenic 18-base oligonucleotide described in Fig. 2. Mutant recombinants in *E. coli* strain TG1 were identified by differential plaque hybridization with <sup>32</sup>P-end-labeled mutagenic oligonucleotide. One mutant, mB27ml-RA, was selected for further use (see Results). Three reconstituted B27 genes were then created: the mB27ml-RA insert was isolated as an Eco RI-Hinc II fragment (the Hinc II site residing in the M13 polylinker 3' to the insert) and ligated into Eco RI-Sma I-cut pB27-AP, to create the mutant pB27ml-RP. Alternative wild-type (pB27-RP) and mutant (pB27ml-RP) reconstituted B27 genes were produced by ligating the 2.0-kb Eco RI-Stu I fragments of pD.1-B27 and mB27ml-RA, respectively, into Eco RI/Sma I-cut pB27-AP. In these latter two constructs, ~ 200 bp was deleted from the 3' end of intron 3 (see Fig. 1).

**Exchange of Kpn I fragments between HLA-B27 and HLA-A2.** The gene encoding HLA-A2, contained on a 5.1-kb Hind III fragment and termed pHLA-2 (17), was the gift of Dr. H. T. Orr, University of Minnesota. We took advantage of the fact that the genes for HLA-B27 and HLA-A2 each contain two Kpn I sites, both located in the two genes at homologous positions in exon 3 (Fig. 1). The 212-bp sequences bounded by these two sites encode amino acids 114–182 in both gene products; 10 of these amino acids differ between the two sequences (Fig. 3). The two 212-bp Kpn I fragments were isolated, respectively, from the HLA-A2 gene subcloned into pUC9, and the pD.1-B27 gene subcloned into pUC13; they were then reciprocally ligated into the opposite Kpn I-restricted recombinant plasmids to create the respective constructs, pB27-KKA2 and pA2-KKB27. The orientations of the exchanged, religated 212-bp fragments were confirmed by restriction mapping.

**DNA sequencing.** Sequencing of the exons encoding the wild-type and mutant B27 α<sub>1</sub> and α<sub>2</sub> domains was accomplished by the dideoxy chain termination method (18) using 20-base oligonucleotide primers complementary to sequences flanking or contained within the exons. Oligonucleotides were custom-synthesized by solid phase phosphoramidite chemistry with a DNA synthesizer (380A; Applied Biosystems, Inc., Foster City, CA). They were purified by electrophoresis in 20%



**Figure 1.** Restriction map of the HLA-B27 genomic clone pD.1-B27. Arrows indicate the strategy for sequencing exons 1–3. Additional restriction sites, predicted from the sequence of Weiss et al. (14), were also used for verification of the constructs described in the text.

Amino Acid Number	64	65	66	67	68	69	70		
Amino Acid	Thr	Gln	Ile	Cys	Lys	Ala	Lys		
pD.1-B27	5'	ACA	CAG	ATC	TGC	AAG	GCC	AAG	3'
mB27m1-RA		ACA	CAG	ATC	TAC	AAG	GCC	AAG	
Mutagenic Oligonucleotide	3'	GT	GTC	TAG	ATG	TTC	CGG	T	5'
Amino Acid Change					...	Tyr	...		

**Figure 2.** Strategy for oligonucleotide-directed Cys<sub>67</sub> → Tyr<sub>67</sub> mutation of the B27 gene.

polyacrylamide/7.5 M urea gels. Sequence data analysis was performed with the software package Microgenie (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA).

**Southern blotting.** Southern blot hybridization of genomic DNA was carried out as previously described (11). Two probes were used: a 1.4-kb B7 cDNA (10), which hybridizes with broad specificity to human and mouse class I MHC genes; and an Xba I-Eco RI fragment containing the extreme 3' 700 bp of the pD.1-B27 gene (Fig. 1), which is HLA-B locus specific.

**DNA transfection of mouse L cells.** Thymidine kinase-deficient murine L cells, obtained from the American Type Culture Collection (ATCC; Rockville, MD), were grown in MEM containing 10% fetal bovine serum (FBS). Closed, circular test plasmid and the *Herpes simplex* thymidine kinase gene were cotransfected into L cells by the calcium phosphate precipitation method, as described in detail by Look et al. (19). Hypoxanthine-aminopterin-thymidine (HAT) resistant cells were harvested as bulk cultures ~ 3 wk later and maintained in HAT-supplemented medium.

**Mab and flow cytography.** The following murine Mab were used: W6/32, an IgG2a that reacts with all HLA-A, B, and C antigens (20); ME1, an IgG1 that reacts with HLA-B27, B7, Bw22, and Bw42 (21); and GS145.2, an IgG1 that reacts with HLA-B27 and to a lesser extent with B7 (22). MOPC 141 and P1.17, IgG myeloma immunoglobulins of no known specificity, were used as negative controls. W6/32, ME1, GS145.2, and P1.17 were purified from ascites by protein A Sepharose chromatography (23). MOPC 141 was used after precipitation in 40% saturated ammonium sulfate. Hybridoma cell lines secreting W6/32 and ME1 were obtained from the ATCC; GS145.2 ascites was the gift of Dr. K. Nelson, Genetic Systems, Inc., Seattle, WA. The specificity of the antibodies was confirmed by analysis of a panel of HLA-typed human mononuclear cells.

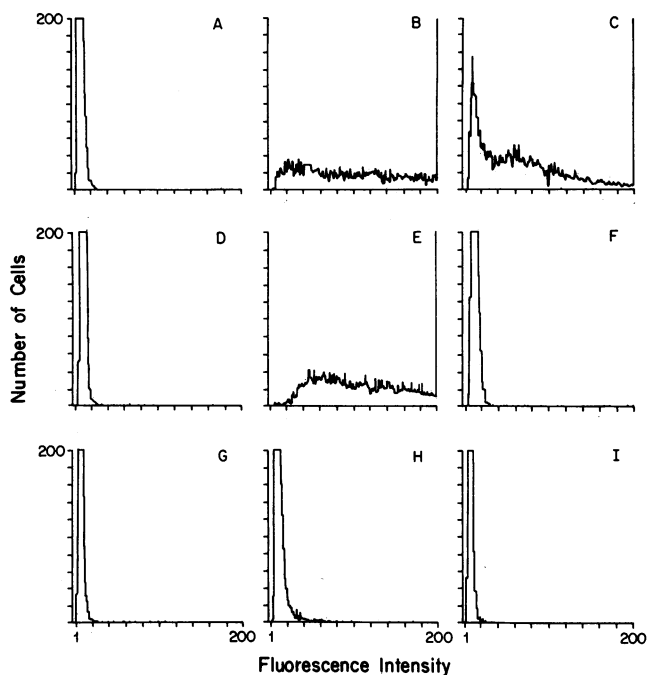
For analysis of transfected L cells, 1–1.5 × 10<sup>6</sup> freshly harvested cells were incubated 30 min on ice with the first antibody, washed twice, then incubated 30 min on ice in a 1:50 dilution of fluoresceinated goat-anti-mouse IgG H + L (Cappel Laboratories, Malvern, PA), and washed twice. Mab were used at a concentration of 20 μg/ml. Assay buffer was Dulbecco's PBS containing 5% FBS and 0.05% NaN<sub>3</sub>. In most experiments, cells were analyzed on a cytofluorograph (H50; Ortho Diagnostic Systems, Raritan, NJ). Cell sorting and some analyses were carried out on a FACStar (Becton, Dickinson & Co., Oxnard, CA). In both instruments, excitation was at 488 nm with an argon laser.

After initial transfection of L cells with pD.1-B27, 55% of the HAT-resistant cell population was positive with the anti-B27 antibody ME1. The cells were then subjected to two rounds of positive selection, and the resulting population was used for subsequent experiments. Some of the other transfected cell populations were used for analysis without sorting of the positive cells, as indicated in Results.

## Results

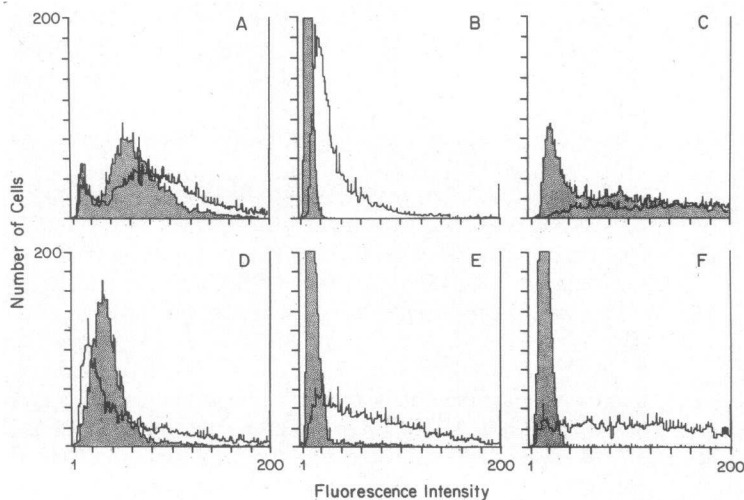
**Site-directed mutagenesis.** A putative mutant M13 clone, mB27m1-RA, was confirmed by DNA sequencing to contain the intended mutation in exon 2 (Fig. 4). The remainder of exons 2 and 3 of mB27ml-RA were also sequenced and shown not to vary from the wild-type B27 sequence. Plasmid constructs containing reconstituted mutant or wild-type genes





**Figure 5.** Cytofluorographic analysis of L cells transfected with the wild-type B27 gene pD.1-B27 (A-C) or the Cys<sub>67</sub> → Tyr<sub>67</sub> mutant B27 gene pB27ml-RP (D-F), compared with nontransfected L cells (G-I). Cells were incubated as described in Methods with 20 μg/ml, respectively, of control antibody MOPC 141 (A, D, and G); W6/32 (B, E, and H); or ME1 (C, F, and I), followed by fluoresceinated goat anti-mouse Ig. Analysis was performed on an Ortho H50 cytofluorograph. The ordinate peak height is 200, and the abscissa represents fluorescence intensity divided into a linear scale of 200 channels. The pD.1-B27 transfected population had previously undergone two rounds of selection for the brightest 5% of cells staining with, respectively, ME1 and GS145.2. The pB27ml-RP transfected population had undergone one round of selection with W6/32. Data were acquired from 5,000 cells; each pattern represents analysis of viable cells gated by forward- and side-scatter of light.

result of the manipulations used to reconstitute a functional class I gene. The binding of W6/32 to the bulk population of pB27ml-RP transfectants was relatively low (Fig. 6 B), probably because of lower than usual efficiency of transfection; however, the binding of ME1 to this population was actually below background and therefore truly negative.



**Figure 6.** Cytofluorographic analysis of L cells transfected with (A) the wild-type B27 gene pB27-RP, (B) the mutant B27 gene pB27ml-RP, (C) the B7 gene p12.1-B7, (D) the hybrid genes pB27-KKA2 and (E) pA2-KKB27, and (F) the A2 gene pHLA-2. Open curves represent fluorescence intensity with W6/32 and shaded curves represent fluorescence intensity with ME1; conditions of assay, ordinate and abscissa are the same as in Fig. 5. Results of control staining (all cells with MOPC 141, untransfected L cells with W6/32 and ME1) were the same as in Fig. 5, G and H (not shown). The cells transfected with p12.1-B7 had undergone two rounds of selection with W6/32; the other cells were from unselected bulk populations of the HAT-resistant colonies arising after transfection and selection.

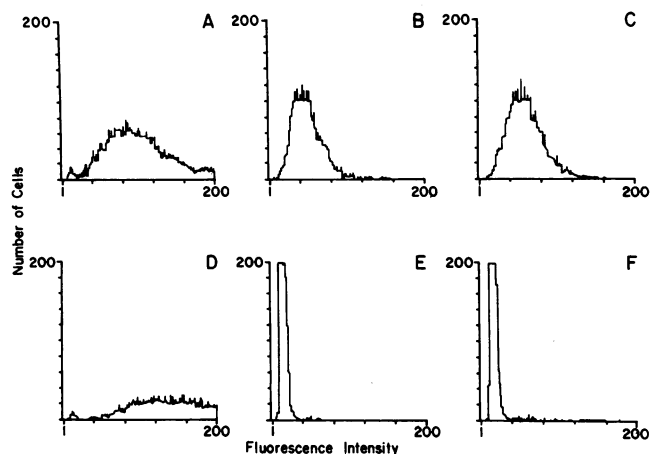
Like ME1, GS145.2 reacted strongly with the wild-type B27 transfectant but very poorly with the Cys<sub>67</sub> → Tyr<sub>67</sub> mutant product (Fig. 7). These results indicate that GS145.2, like ME1, recognizes a B27 epitope that is abolished by the non-conservative substitution at amino acid position 67.

To test whether ME1 also reacts with sites outside the α<sub>1</sub> domain, its binding to cells transfected with genes containing hybrid third exons was assessed. ME1 bound to cells transfected with the construct pB27-KKA2 (Fig. 6 D), indicating that the 10-amino acid substitutions in the α<sub>2</sub> domain of this gene product have little, if any, effect on the HLA-B27 epitope recognized by this antibody. Consistent with this interpretation was the finding that ME1 failed to bind to cells expressing either pA2-KKB27 or pHLA-2 (Figs. 6, E and F).

## Discussion

These experiments have demonstrated that the cysteine residue at position 67 of HLA-B27 plays a critical role in the epitopes recognized by the MAb ME1 and GS145.2, which react with all known HLA-B27 subtypes (6). In comparison, 10 amino acid substitutions in the carboxy-terminal three-fourths of the α<sub>2</sub> domain of HLA-B27 did not significantly affect the binding of the anti-B27 antibodies. This part of the α<sub>2</sub> domain thus appears not to participate in the B27 epitope recognized by ME1, although the involvement of polymorphic residues shared by B27 and A2 in this region (e.g. Leu<sub>156</sub>) cannot be excluded. These results are consistent with those of Sodoyer et al. (24), who used exon shuffling to map the ME1 determinant of HLA-B7 to the α<sub>1</sub> domain.

These findings demonstrate that a single amino acid substitution at a polymorphic position in a class I MHC molecule can produce a mutant molecule that is expressed normally and is reactive with antibodies recognizing both nonpolymorphic and polymorphic determinants on the parent molecule, and yet is unreactive with antibodies allospecific for the parent molecule. This is one of the first such demonstrations for an HLA-B molecule, for the α<sub>1</sub> domain of any HLA molecule, and for amino acid 67 of any class I MHC molecule. In other systems, Layet et al. (25) and Salter et al. (2) have demonstrated that the reactivity of three MAb with HLA-A2 and HLA-Aw69 depends on the presence of a tryptophan residue at position 107 in the α<sub>2</sub> domain. In a murine system, Koeller



**Figure 7.** Cytofluorographic analysis of L cells transfected with the wild-type B27 gene pD.1-B27 (A–C) or with the mutant Cys<sub>67</sub> → Tyr<sub>67</sub> mutant gene pB27ml (D–F). Cells were incubated with W6/32 (A and D), ME1 (B and E), or GS145.2 (C and F). Background staining with P1.17 was the same as in F (not shown).

et al. have demonstrated that the epitopes recognized by several monoclonal alloantibodies reactive with H-2D<sup>d</sup> map to amino acid positions 63, 65, 66, and 70 (26).

Because the mutant B27 molecule contained the same amino acid residue at position 67 that is found in the native B7 molecule, it might have been expected that the mutant would bind ME1, which reacts strongly with B7. However, in view of the fact that B27.1 and B7.1 differ by 10 amino acids in the  $\alpha_1$  domain, and by an additional 10 in  $\alpha_2$ , our results suggest that the ME1 determinants of B27 and B7 do not share strict identity of amino acid residues. Recent x ray crystallographic studies of HLA-A2 indicate that amino acid residues 58–84 assume an  $\alpha$ -helical conformation (27). This  $\alpha$  helix forms one side of the binding site for foreign antigen, with residues 63, 67, and 70 facing inward toward the antigen binding groove (28). All three of these residues differ between B27 and B7 and may differ in their effect on the ME1 epitopes of the two class I molecules.

GS145.2 also failed to bind to the mutant B27 molecule. GS145.2 is known to differ from ME1 with regard to reactivity with other HLA-B-locus products; its binding to B7 is weaker than to B27, and, unlike ME1, in panel assays it does not react with HLA-B22 (unpublished results and Nelson, K., personal communication). In studies of anti-HLA-murine MAb, Parham et al. (29) have identified four topographically distinct antigenic sites on the HLA-B7 molecule, only one of which is recognized by antibodies such as ME1 that also react with B27. Because each of these antibodies demonstrated a distinct affinity for B7 and a unique fine specificity in panel assays, it was postulated that this topographic region on B7 represents a cluster of overlapping epitopes that are shared by B7 and B27. Our data do not indicate directly whether GS145.2 also reacts with this topographic region on HLA-B7; however, the pattern of its reactivity with other HLA-B antigens indicates that its specificity is distinct from that of ME1.

Of particular note is the potential relevance of these findings to the unique association of HLA-B27 with human disease. Although it remains formally possible that this association is due to an allele in tight linkage disequilibrium with

HLA-B27, there is now substantial indirect evidence that the HLA-B27 molecule itself is directly involved in disease pathogenesis (7, 9). The six B27 subtypes identified by isoelectric focusing have been sequenced at the protein level (5, 6) and shown to display distinctive cytolytic T cell (CTL)-defined epitopes (5, 30, 31). The amino acid differences among the B27 variants that account for differences in CTL recognition have been mapped to positions 59, 74, 77, 80, 81, 114, 116, and 152. Because class I MHC molecules serve as recognition elements for CTL, and because only a small minority of HLA-B27<sup>+</sup> individuals ever develop B27-associated disease (7, 32, 33), efforts have been made to identify CTL-defined B27 subtypes that correlate specifically with disease. However, no significant correlation has been found between AS and any particular subtype of B27 (7, 9, 34), and AS remains most highly correlated with serologically defined B27, just as originally reported in 1973 (9, 35, 36).

Much of the sequence variability among HLA-A, B, and C molecules occurs in amino acids 62–83 in the  $\alpha_1$  domain and 94–116 in the  $\alpha_2$  domain (Fig. 3). Among the known HLA class I sequences, B27-unique residues shared by all the B27 subtypes are found at three positions: Cys<sub>67</sub>, Lys<sub>70</sub>, and Asn<sub>97</sub> (1, 5, 6, 37). Our study has demonstrated the importance of Cys<sub>67</sub> in two B27 serologic specificities. The x ray crystallographic model of the three-dimensional structure of HLA-A2 (27, 28) predicts that residue 67 makes contact with the ligand in the antigen-binding groove. It was demonstrated that a peptide fragment distinct from the HLA-A2 molecule occupied the antigen-binding groove in the crystallized A2, and it was postulated that such peptide fragments may be found constitutively in class I MHC molecules (29). Participation of HLA-B27 in disease pathogenesis may result from covalent interaction between the Cys<sub>67</sub> sulfhydryl group and one or more endogenous or exogenous ligands. With regard to potential exogenous ligands, studies by Geczy et al. (38, 39) have suggested that a B27-associated structure on leukocytes can be modified by bacterial products, and that this modification correlates with the presence of disease in B27<sup>+</sup> subjects. It would be of interest to know whether the B27 Cys<sub>67</sub> residue is involved in this modification process.

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