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The Cys67 residue of HLA-B27 influences cell surface stability, peptide specificity and T-cell antigen presentation

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Running title: Functional role of Cys67 in HLA-B27
Summary

Cys67 of HLA-B27 is located in the B pocket, which determines peptide-binding specificity. We analyzed effects of the Cys67Ser mutation on cell surface expression, peptide specificity, and T-cell recognition of HLA-B*2705. Surface expression was assessed with antibodies recognizing either native or unfolded HLA proteins. Whereas native B*2705 molecules predominated over unfolded ones, this ratio was reversed in the mutant, suggesting lower stability. Comparison of B*2705- and Cys67Ser-bound peptides revealed that the mutant failed to bind about 15% of the B*2705 ligands, while binding as many novel ones. Two peptides with Gln2 found both in B*2705 and Cys67Ser are the first demonstration of natural B*2705 ligands lacking Arg2. Other effects of the mutation on peptide specificity were: 1) average molecular mass of natural ligands higher than for B*2705, 2) bias against small residues at peptide position (P)1, and 3) increased P2 permissiveness. The results suggest that the Cys67Ser mutation weakens B pocket interactions, leading to decreased stability of the mutant-peptide complexes. This may be partially compensated by interactions involving bulky P1 residues. The effect of the mutation on allore cognition was consistent with that on peptide specificity. Our results might help to understand the pathogenetic role of HLA-B27 in spondyloarthropathy.
Introduction

The molecular basis for the very strong association of HLA-B27 with ankylosing spondylitis (AS)\(^1\) (1), reactive arthritis (2), and other spondyloarthopathies remains unknown. Among the pathogenetic hypotheses proposed, a classical one assumes that cytotoxic T lymphocytes (CTL) activated in response to external antigen would eventually crossreact with a self-peptide constitutively presented by HLA-B27 (3). This hypothesis is supported by much indirect evidence (4-8). Alternatively, HLA-B27 heavy chain homodimers, whose formation \textit{in vitro} is dependent on an unpaired Cys67 residue (9), were suggested to act as non-canonical peptide presenting molecules, perhaps stimulating abnormal T-cell responses (10,11). It has also been proposed that HLA-B27 association to AS might be unrelated to peptide presentation, but rather to B27 heavy chain misfolding and triggering of endoplasmic reticulum stress responses (12-14).

The Cys67 residue of HLA-B27 is an integral part of its B pocket, which determines the specificity of the molecule for peptides with Arg2 (15). This residue plays a critical role in controlling the thermodynamic stability of soluble HLA-B27-peptide complexes (16). This same study claimed that mutation of Cys67 did not significantly affect the peptide binding properties of HLA-B27, but this conclusion was based on the affinity of a single HLA-B27 ligand and a few peptide analogs.

The pathogenetic role of Cys67 was tested on HLA-B27 transgenic rats. In this system, HLA-B27 triggers a spontaneous inflammatory disease with many features of human spondyloarthopathies (17). Rats transgenic for the Cys67Ser (C67S) mutant showed a disease phenotype similar to wildtype B*2705 transgenic rats of the same strain, suggesting that Cys67 has little influence on disease (18).

In this study we have addressed the role of Cys67 on cell surface expression,
peptide specificity, and T-cell recognition of HLA-B27. We demonstrate that the C67S mutation decreases the stability of HLA-B27-peptide complexes at the cell surface, induces distinct alterations in the endogenous HLA-B27-bound peptide repertoire, and influences T-cell allore cognition.

Materials and methods

Cell lines and DNA-mediated gene transfer.

HMy2.C1R (C1R) is a human lymphoid cell line with low expression of its endogenous class I antigens (19,20). B*2705-C1R transfectant cells were described elsewhere (21).

A genomic construct containing the C67S mutant of the B*2705 gene cloned into pUC19 (22,23) was a kind gift of Dr. Joel D. Taurog (University of Texas Southwestern Medical Center, Dallas, TX). This construct was designated as JHG1.1b. Exons 1 and 2 of the mutant were resequenced in our laboratory to confirm its correct structure. To obtain a C67S-C1R transfectant cell line, about 10^7 C1R cells were washed twice, and resuspended in 0.8 ml PBS. Ten µg of linearized JHG1.1b plasmid and 1 µg of linearized pSV2.neo were co-transfected by electroporation at 960 V, 300 µF in a BioRad Gene Pulser (BioRad Laboratories, Hercules, Ca). After 10 min at room temperature, cells were seeded in a culture flask in DMEM supplemented with 10% FBS. Twenty four hours after electroporation, 1 mg/ml G418 (Life technologies, Paisley, UK) was added to the cell culture. Surviving cells were tested for expression of the C67S mutant by flow cytometry as described below. C1R cell lines were cultured in DMEM supplemented with 7.5% FBS (both from Life Technologies).

RMA-S is a TAP-deficient murine cell line (24,25). RMA-S transfectant cells expressing B*2705 and human β2m have been previously described (26). These cells were
cultured in RPMI 1640 medium supplemented with 10% FBS.

**Monoclonal antibodies (mAb) and flow cytometry.**

The following mAb were used: W6/32 (IgG2a, specific for a monomorphic HLA-A,B,C determinant) (27), ME1 (IgG1, specific for HLA-B27, B7, B22) (28), BBM.1 (IgG2b, specific for human β2m) (29,30), and HC10 (IgG2a, specific for denatured and other forms of HLA class I heavy chain not associated to β2m) (31).

For flow cytometry analysis, about 2 x 10^5 C1R or RMA-S transfectant cells were washed twice in 200 µl of PBS, and resuspended in 50 µl of undiluted mAb supernatant. After incubating for 20 min, cells were washed 3 times in 200 µl of PBS and resuspended in 50 µl of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG rabbit antiserum (Calbiochem-Novabiochem GMBH, Schwalbach, Germany), incubated for 20 min and washed three times in 200 µl of PBS. All operations were done at 4ºC. Flow cytometry was carried out in an Epics Profile XL instrument (Coulter Electronics Inc., Hialeah, Fl.).

**Isolation of B*2705- and C67S-B27-bound peptides**

This was carried from 10^10 C1R transfectant cells lysed in 1% NP-40 in the presence of a cocktail of protease inhibitors, after immunopurification of HLA-B27 with the W6/32 mAb and acid extraction, exactly as described elsewhere (32). HLA-B27-bound peptide pools were fractionated by HPLC at a flow rate of 100 µl/min as previously described (33), and 50 µl fractions were collected.

**Mass spectrometry (MS) analysis and sequencing**

The peptide composition of HPLC fractions was analyzed by matrix-assisted desorption ionization time-of-flight (MALDI-TOF) MS using a calibrated Kompact
Probe instrument (Kratos-Schimadzu) operating in the positive linear mode, as previously described (33). Alternatively, a Bruker Reflex” III MALDI-TOF mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT” source in positive ion reflector mode was also used, as previously described (34).

Peptide sequencing was carried out by quadrupole ion trap nanoelectrospray MS/MS in an LCQ instrument (Finnigan ThermoQuest, San Jose, CA), exactly as detailed elsewhere (35,36). In some cases, peptide sequencing was also done by post-source decay (PSD) MALDI-TOF MS, as previously described (34).

In all cases peptide-containing HPLC fractions were dried and resuspended in 5 µl methanol/water (1:1) containing 0.1% formic acid. Aliquots of 0.5 µl or 1µl were used for MALDI-TOF or nanoelectrospray MS analyses, respectively.

**Synthetic peptides**

Peptides were synthesized using the standard solid-phase Fmoc chemistry, and were purified by HPLC. The correct composition and molecular mass of purified peptides were confirmed by amino acid analysis using a 6300 Amino Acid Analyzer (Beckman Coulter, Palo Alto, CA), which also allowed their quantification.

**Epitope stabilization assay**

The epitope stabilization assay used to measure peptide binding was performed as described (37). Briefly, B*2705 RMA-S transfectants were incubated at 26°C for 22 h in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. They were then washed three times in serum-free medium, incubated for 1 h at 26°C with various peptide concentrations without FBS, transferred to 37°C, and collected for flow cytometry after 4 h. B*2705 expression was measured using 50 µl of hybridoma culture supernatant containing the mAb ME1. Binding of the RRYQKSTEL peptide, used as
positive control, was expressed as C50, which is the molar concentration of the peptide at 50% of the maximum fluorescence obtained at the concentration range used (10^{-4} to 10^{-9} M). Binding of other peptides was assessed as the concentration of peptide required to obtain the fluorescence value at the C50 of the control peptide. This was designated as EC50.

**T-cell clones and cytotoxicity assay.**

A set of allospecific CTL clones raised against B*2705 were used. The generation, culture conditions, and fine specificity of these CTL clones has been previously described (38). Recognition of B*2705 and C67S by these CTL was analyzed using a standard 51Cr-release cytotoxicity assay, essentially as described elsewhere (39).

**Results**

**The C67S mutation alters cell surface expression and stability of HLA-B27.**

Cell surface expression of B*2705 on C1R transfectants was measured by flow cytometry with the ME1, W6/32, BBM.1, and HC10 mAb (Figure 1). W6/32 and ME1 recognize distinct epitopes on the heavy chain of the native HLA-B27 molecule (3,22,40). BBM.1 recognizes an epitope on human β2m (30), and HC10 recognizes an epitope on unfolded, but not native, HLA class I heavy chains (31). Whereas surface expression of C67S in our transfectants was lower than expression of B*2705 when measured with W6/32, ME1, or BBM.1, it was higher when measured with HC10. This result indicates that the ratio between unfolded and native HLA-B27 molecules on the cell surface was higher for C67S than for B*2705, suggesting lower stability of the mutant molecules.

**High overlap between the B*2705- and C67S-bound peptide repertoires.**

A systematic comparison of B*2705- and C67S-bound peptide repertoires was
carried out to determine the effect of the mutation on peptide specificity. The corresponding peptide pools were isolated by acid extraction, after immunoprecipitation of the B27 molecules with the W6/32 mAb, and fractionated by HPLC under identical conditions. The peptide composition of individual HPLC fractions was analyzed by MALDI-TOF MS. The MS spectrum of each HPLC fraction from one of the molecules was compared with the MS spectra of the correlative, previous and following HPLC fractions from the other molecule. This was done to account for slight shifts in retention times between consecutive chromatographic runs. Ion peaks with the same (±1) mass/charge (m/z) among the HPLC fractions compared were considered to reflect identical peptides shared by both molecules. Ion peaks in one HPLC fraction not found in the counterparts from the other molecule were considered to be peptides differentially bound to one molecule.

One example of such comparison is shown in Figure 2. HPLC fraction N. 161 from B*2705 was compared with HPLC fraction N. 160-162 from C67S. Of 14 ion peaks compared from the B*2705 fraction, 12 had counterparts in one or more of the HPLC fractions from C67S, and 2 were not detected in the mutant.

A total of 1193 peptides from B*2705 and 1175 peptides from C67S were compared in this analysis (Table I). Of the B*2705 ligands, 1005 (84%) were shared by the C67S mutant, and 188 (16%) were not found in the mutant. Conversely, 171 peptides from C67S (15%) were not found in B*2705. These results indicate that the C67S mutation is compatible with binding of a large majority, but not all, of the natural B*2705 ligands, and influences peptide specificity also by allowing binding of new ligands absent from the B*2705-bound pool.

Although MALDI-TOF MS is not a quantitative technique because ion peak signal
intensity is affected by multiple factors, an attempt was made to identify the extent in which peptide amount could be affected by the C67S mutation. Thus, to identify peptides common to both molecules but much more abundant in one of them, we selected ion peaks in each HPLC fraction whose intensity was higher than 50% of the maximum signal intensity in that fraction. Their amount was measured as the total number of millivolts (mV) corresponding to each ion peak in all the HPLC fractions in which it was detected. When the total intensity of a given ion peak was more than 10 times higher in one molecule than in the other, the corresponding peptide was assigned as a quantitative difference. One example (Figure 2), is the ion peak at m/z 1088.1, in HPLC fraction N. 161 from B*2705 (SRTPYHVNL). Of 252 peptides compared by these criteria (Table I) 25 (10%) were much more prominent in B*2705, and 31 (12%) predominated in C67S. These results suggest that, in addition to determining differential binding of some peptides, the C67S mutation also influences the amount of bound peptide in at least an additional 22% of the shared ligands.

The C67S mutant favors binding of bigger peptides.

The size distribution of natural ligands bound to B*2705 or C67S showed a gaussian pattern in both cases, with mean peptide sizes ([M+H]^+) of 1154 and 1185 Da, respectively (Figure 3A). This subtle size difference, which was reproducible in a second independent comparison (data not shown), became more obvious when the size distribution of peptides differentially bound to either B*2705 or C67S was compared (Figure 3B). Whereas the mean size of B*2705 peptide differences was 1089 Da, that of C67S differences was 1295 Da. Thus, peptides differentially bound to the mutant had an mean size 206 Da bigger than B*2705 differences. This is compatible with a slightly bigger length (1 to 2 residues), a bias towards bigger residues at some positions, or both.
The C67S mutant selects against peptides with small residues at position 1.

A total of 36 natural ligands bound to B*2705 or to the C67S mutant were sequenced by quadrupole ion trap nanoelectrospray MS/MS (Figure 4). Of these, 29 peptides, including 20 nonamers, 8 decamers, and 1 undecamer, were shared by both molecules. Among these shared ligands, 9 showed quantitative differences, 6 of them predominant in B*2705, and 3 of them predominant in C67S. In addition, 6 peptides, including 5 nonamers and 1 decamer were differentially bound to B*2705, and 1 nonamer was differentially bound to C67S.

In general there were no obvious differences between peptides common to B*2705 and C67S, and peptides found only or predominantly in B*2705, except at position (P) 1. Of the 12 peptides B*2705-specific or predominant in B*2705 (Figure 4), 11 (92%) had a small P1 residue: Gly, Ala, or Ser. Together, these three amino acids accounted only for 25% of the other shared ligands. In contrast, whereas basic P1 residues (Arg, Lys, and His) were found in 12 (60%) of the shared ligands, and in 2 of 3 peptides predominant in the mutant, these residues were absent from peptides differentially or predominantly bound to B*2705. These results strongly suggest that the C67S mutation disfavors binding of peptides with small P1 residues, while allowing basic residues at this position.

There were more peptides with Pro4 among those differentially or predominantly bound to B*2705 (58%) than among shared ones (15%). However, the significance of this is dubious since there was Pro at positions 4, 5, or 6 in 40% of the shared ligands (Figure 4).

Natural B*2705 ligands lacking Arg2.

Two peptides with Gln2 were sequenced from the C67S mutant, both of which
had counterparts in the B*2705-bound peptide pool (Figure 4). The MALDI-TOF MS spectra of HPLC fractions N.143 from C67S and B*2705 showed an ion peak at m/z 1056.4. The ion peak from the mutant was fragmented by quadrupole ion trap nanoelectrospray MS/MS, which allowed to determine the sequence of the corresponding peptide as RQTGIVLNR (M = 1055.6 Da) (Figure 5A). Assignment of this sequence and, in particular, distinction of Gln2 from the isobaric Lys residue was based on: a) match of this sequence, but not of that with Lys2, with a human protein in the database (Figure 4), b) detection of the y₈* fragment ion (m/z: 883.3), which is consistent with neutral loss of ammonia due to cyclation of the N-terminal Gln residue in the y₈” ion, and c) the MS/MS spectrum of the synthetic RQTGIVLNR peptide was identical to the peptide from the mutant (data not shown). The corresponding ion peak from B*2705 was sequenced by PSD-MALDI-TOF MS. Its spectrum (Figure 5B) was essentially identical to that of the synthetic RQTGIVLNR peptide (Figure 5C). This is the first demonstration that B*2705 binds peptides with Gln2 in vivo.

Similarly, The MALDI-TOF MS spectra of HPLC fractions N. 173 from C67S and B*2705 revealed an ion peak with m/z 1023.4. The sequence of this peptide obtained from the C67S mutant was RQVIPIIGK (Figure 4). In this case, PSD-MALDI-TOF or electrospray MS/MS spectra of the B*2705 counterpart could not be obtained, so that assignment of this peptide as a natural B*2705 ligand relies only on identity of molecular mass and retention time with the peptide from C67S.

**Extended B pocket specificity of the C67S mutant.**

A third peptide lacking Arg2 was found in HPLC fraction N. 155 from the mutant. In the MALDI-TOF MS spectrum of this fraction there was an ion peak at m/z
1105.4, which was not detected in the corresponding HPLC fractions from B*2705 (data not shown), so that this peak was assigned as a peptide difference from the mutant. The nanoelectrospray MS/MS spectrum of this peptide was consistent with the sequence: YKFSGFTQK (Figure 6A). Distinction between the isobaric Gln/Lys residues was based on: 1) unambiguous matching with a human sequence in the database (Figure 4), 2) lack of a prominent fragment ion corresponding to neutral loss of ammonia from the y8”+ or y8”2+ fragment ions, which would be expected if the peptide would have Gln2, due to cyclation of this residue when it becomes N-terminal in the y8” ions, and 3) identity of the MS/MS spectrum with that of synthetic YKFSGFTQK (Figure 6B), but not with the synthetic analog with Gln2 (Figure 6C). In this latter spectrum, prominent y8* fragment ions corresponding to neutral loss of ammonia from y8”+ and y8”2+ fragment ions were a clear differential feature distinguishing this spectrum from those of the C67S ligand and the synthetic YKFSGFTQK peptide.

This result demonstrates that the C67S mutation relaxes the specificity of the B pocket, allowing for residues other than Arg2 or Gln2.

**Gln2-, but not Lys2-containing peptides, bind efficiently B*2705 in vitro.**

The three peptide ligands lacking Arg2 found in the previous analyses were tested for cell surface binding to B*2705 in an epitope stabilization assay (Figure 7). The two peptides with Gln2 bound B*2705 with high efficiency (EC50: 1µM), and only slightly worse than an Arg2-containing B*2705 ligand, RRYQKSTEL (EC50: 0.5 µM), used as a control. In contrast, the Lys2-containing ligand found only in the mutant bound B*2705 with moderate efficiency (EC50: 36 µM), and over 30-fold less
efficiently than the Gln2-containing ligands.

**High allospecific T-cell epitope sharing between B*2705 and C67S.**

Since most allospecific CTL are peptide-dependent, another way to look at the effect of C67S on peptide specificity is to examine the crossreactivity of allospecific CTL raised against B*2705 with the C67S mutant. Thus, 11 anti-B*2705 CTL clones were tested for recognition of C67S-C1R transfectant target cells in a classical cytotoxicity assay (Table II). Seven CTL clones (64%) showed significant crossreaction (>50% relative lysis), whereas 4 CTL clones (36%) crossreacted more weakly (<40% relative lysis). These results indicate that many allospecific T-cell epitopes are conserved in the mutant. However, epitope sharing was lower than the peptide sharing determined by comparison of the B*2705- and C67S-bound peptide repertoires (Table I). This result suggests that some shared ligands may bind B*2705 and the mutant in different conformations, so that the corresponding T-cell epitopes are altered.

**Discussion**

The Cys67 residue is a structural feature of HLA-B27 shared by few other allotypes such as subtypes of HLA-B14, B15, B38, B39, and B73 (41). However, only HLA-B27 and the rare allotype HLA-B73 (42,43) also have Lys70, which confers to Cys67 enhanced chemical reactivity (44). Although this residue might be modified by homocysteine in vivo upon bacterial invasion (45), it seems that the overwhelming majority of HLA-B27 molecules in normal cells have a free Cys67. Since this residue is an integral part of the B pocket (15), it was expected to influence peptide binding and antigen presentation. Thus, in this study we investigated the role of Cys67 in: 1) cell surface expression and stability, 2) peptide specificity, and 3) T-cell allore cognition.

Early studies with HLA-B27 mutants bearing different substitutions at position
demonstrated that these mutations did not impair cell surface expression, but destroyed some mAb epitopes. These results were interpreted as a limited conformational effect of the mutations on the antigenic structure of HLA-B27 (22,23). Recent studies have shown that Cys67 controls the formation of HLA-B27 heavy chain homodimers retaining secondary structure, some peptide binding capacity, and reactivity with the conformation-dependent W6/32 mAb (9). More relevant to the present study, Reinelt et al. (16) demonstrated that Cys67 have a significant influence on the thermodynamic stability of B*2705-peptide complexes in solution. The C67S mutation decreased the thermal stability of the molecule, albeit less than other pocket B mutants, and promoted faster dissociation. These results are consistent with the inverted ratio of HLA-B27 associated fluorescence at the cell surface measured with W6/32, ME1, and BBM.1, relative to the fluorescence measured with HC10, between B*2705- and C67S-C1R transfectants. The increased HC10 reactivity observed with the mutant strongly suggests that a higher fraction of C67S-peptide complexes are dissociated at the cell surface relative to the wildtype. Although the molecular determinants of this decreased stability cannot be established from our experiment, a simple explanation of the result is that the strength of the interaction of many HLA-B27-peptide complexes is reduced in the mutant due to its altered B pocket structure, leading to decreased stability.

Assessment of the role of Cys67 in determining the peptide specificity of HLA-B27 required a systematic comparison of the endogenous peptide repertoires of the wildtype and mutant, which was undertaken in this study. Our results demonstrated that, although the C67S mutant still binds many of the natural B*2705 ligands, its peptide specificity was distinct from the wildtype in some significant aspects: 1) the mutant
failed to bind, within the detection limits of the MS techniques used, about 15% of the B*2705-bound peptides, while gaining the capability to bind a similar percentage of new ligands, 2) the mutant modulated, either positively or negatively, the amount of bound peptide for at least an additional 22% of natural B*2705 ligands; since conservative criteria were used to assess quantitative peptide differences among shared ligands, it is conceivable that this percentage may be significantly higher, 3) the mutant modulated residue preferences at P1, and 4) it increased permissiveness at the B pocket, allowing for P2 residues not found among B*2705 ligands.

The effect on P1 residues, consisting of a certain bias against small residues and towards basic ones, is presumably an indirect consequence of weaker pocket B interactions in the mutant. Basic P1 residues may provide additional strength to the interaction of peptides with HLA-B27, due to hydrogen bonding of the P1 residue with Glu63 in HLA-B27, as well as additional Van der Waals interactions of the aliphatic portions of the P1 side chains with Trp167, in the A pocket. This was observed, for instance, in H-2K\textsuperscript{b} complexed with an Arg1-containig peptide (46), or in HLA-B53 complexed with a Lys1-containing peptide (47). These interactions are not possible, or are weaker, with Gly and other small P1 residues. Stronger anchoring of the P1 residue could partially compensate for weaker pocket B interactions. Van der Waals interactions with Trp167 are also important with bulky aromatic P1 residues, such as the Tyr1 found in the only sequenced C67S-specific ligand. Significantly, an even stronger bias towards basic P1 residues than that observed in C67S, was reported for B*2703 (48,49). This allotype has an unusual mutation affecting an otherwise conserved residue in the A pocket, that weakens anchoring of the peptidic N-terminus (50,51). This suggests that weakening of canonical interactions in peptide-binding pockets make P1 side chain
interactions involving basic residues particularly relevant, consistent with our results in C67S.

An interesting observation concerning B pocket specificity in this study was the finding of natural B*2705 ligands with Gln2. This is the first demonstration of natural B*2705 ligands lacking Arg2. A previous publication which described a Gln2-containing ligand of B*2705 (52) was later retracted (53), and the description of this ligand is not believed to be valid (J. D. Taurog, personal communication). The finding was not totally unexpected, since previous studies from ourselves (54) and others (55) showed that some Gln2-containing peptides bind B*2705 in vitro with significant efficiency. Since the overwhelming majority of natural B*2705 ligands contain Arg2, identification of Gln2-containing peptides in the B*2705-bound pool was facilitated by detection of these peptides as more prominent ion peaks in correlative HPLC fractions from the mutant, perhaps reflecting increased allowance for this residue in C67S. For this reason, it cannot be inferred from our results that finding of 2 Gln2-containing peptides out of 35 sequenced ligands from B*2705 reflects the true proportion of peptides carrying the Gln2 motif in the B*2705-bound pool. That HLA-B27 presents peptides lacking Arg2 in vivo has obvious implications in studies aimed at predicting and identifying B27-restricted antigens such as, for instance, those from arthritogenic bacteria (56).

The effect of the C67S mutation on relaxing B pocket specificity was also demonstrated by sequencing of one ligand of this mutant not found in B*2705, which contained Lys2. This residue has never been found among natural HLA-B27 ligands.

The observed effect of the C67S mutation on allore cognition by B*2705-specific CTL shows that Cys67 plays a role in antigen presentation, and is consistent with the observed alterations of the peptide repertoire. The limited panel of CTL clones
available for our study imposes a cautious interpretation of the apparently lower T-cell crossreactivity with the mutant, relative to the homology of peptide repertoires. However, this result strongly suggests that the 15% lack of overlap between the peptide repertoires of B*2705 and C67S is not simply due to lack of sensitivity of our MS techniques for peptides with low abundance, since CTL would presumably detect peptide amounts undetected in our MS analysis (57). Rather, our CTL data are consistent with the possibility that some shared ligands are not antigenically identical when presented by B*2705 and the mutant. This would imply that, in addition to its effect on peptide binding, the C67S mutation may alter the conformation and antigenicity of some bound peptides.

In conclusion, our results indicate that Cys67 plays a role in determining the cell surface stability, peptide binding specificity, and T-cell recognition of HLA-B27. They also suggest that increased unfolding of the mutant at the cell surface, relative to the wildtype, may be due to decreased stability of C67S-peptide complexes, mainly as a consequence of weakened pocket B interactions.

Studies in transgenic rats indicate that the phenotype of HLA-B27-associated disease is at most modestly affected by the C67S mutation (18). This suggests that the effect of this mutation on peptide specificity has not much relevance for disease. It has been suggested that HLA-B27-associated disease may be a consequence of HLA-B27 misfolding (13). Failure of the C67S mutation to exacerbate disease in transgenic rats (18), in spite of decreased thermodynamic (16) and cell surface stability of the mutant, does not seem to support this model. However, both our study and that of Reinelt et al. (16) deal with HLA-B27-peptide complexes. Therefore, they do not necessarily imply an influence of the C67S mutation on intracellular folding of the HLA-B27 heavy chain. This should
be addressed in further studies.
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References

1. Brewerton, D. A., Hart, F. D., Nicholls, A., Caffrey, M., James, D. C., and Sturrock, R. D. (1973) *Lancet* 1, 904-907

2. Brewerton, D. A., Caffrey, M., Nicholls, A., Walters, D., Oates, J. K., and James, D. C. (1973) *Lancet* 2, 996-998

3. Benjamin, R. and Parham, P. (1990) *Immunol.Today* 11, 137-142

4. Zhou, M., Sayad, A., Simmons, W. A., Jones, R. C., Maika, S. D., Satumtira, N., Dorris, M. L., Gaskell, S. J., Bordoli, R. S., Sartor, R. B., Slaughter, C. A., Richardson, J. A., Hammer, R. E., and Taurog, J. D. (1998) *J.Exp.Med.* 188, 877-886

5. D’Amato, M., Fiorillo, M. T., Carcassi, C., Mathieu, A., Zuccarelli, A., Bitti, P. P., Tosi, R., and Sorrentino, R. (1995) *Eur.J.Immunol.* 25, 3199-3201

6. Lopez-Larrea, C., Sujirachato, K., Mehra, N. K., Chiewsilp, P., Isarangkura, D., Kanga, U., Dominguez, O., Coto, E., Peña, M., Setien, F., and Gonzalez-Roces, S. (1995) *Tissue Antigens.* 45, 169-176

7. Hermann, E., Yu, D. T., Meyer zum Buschenfelde, K. H., and Fleischer, B. (1993) *Lancet* 342, 646-650

8. Fiorillo, M. T., Maragno, M., Butler, R., Dupuis, M. L., and Sorrentino, R. (2000) *J.Clin.Invest* 106, 47-53

9. Allen, R. L., O’Callaghan, C. A., McMichael, A. J., and Bowness, P. (1999) *J.Immunol.* 162, 5045-5048
10. Allen, R. L., Bowness, P., and McMichael, A. (1999) Immunogenetics 50, 220-227

11. Edwards, J. C., Bowness, P., and Archer, J. R. (2000) Immunol. Today 21, 256-260

12. Mear, J. P., Schreiber, K. L., Münz, C., Zhu, X., Stevanovic, S., Rammensee, H. G., Rowland-Jones, S. L., and Colbert, R. A. (1999) J. Immunol. 163, 6665-6670

13. Colbert, R. A. (2000) Mol. Med. Today 6, 224-230

14. Colbert, R. A. (2000) J. Rheumatol. 27, 1107-1109

15. Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C. (1992) Cell 70, 1035-1048

16. Reinelt, S., Dedier, S., Asuni, G., Folkers, G., and Rognan, D. (2001) J. Biol. Chem. 276, 18472-18477

17. Hammer, R. E., Maika, S. D., Richardson, J. A., Tang, J. P., and Taurog, J. D. (1990) Cell 63, 1099-1112

18. Taurog, J. D., Maika, S. D., Satumtira, N., Dorris, M. L., McLean, I. L., Yanagisawa, H., Sayad, A., Stagg, A. J., Fox, G. M., Le O’Brien, A., Rehman, M., Zhou, M., Weiner, A. L., Splawski, J. B., Richardson, J. A., and Hammer, R. E. (1999) Immunol. Rev. 169, 209-223

19. Storkus, W. J., Howell, D. N., Salter, R. D., Dawson, J. R., and Cresswell, P. (1987) J. Immunol. 138, 1657-1659
20. Zemmour, J., Little, A. M., Schendel, D. J., and Parham, P. (1992) *J.Immunol.* 148, 1941-1948

21. Calvo, V., Rojo, S., Lopez, D., Galocha, B., and Lopez de Castro, J. A. (1990) *J.Immunol.* 144, 4038-4045

22. Taurog, J. D. and El-Zaatari, F. A. (1988) *J.Clin.Invest* 82, 987-992

23. El-Zaatari, F. A., Sams, K. C., and Taurog, J. D. (1990) *J.Immunol.* 144, 1512-1517

24. Ljunggren, H. G. and Karre, K. (1985) *J.Exp.Med.* 162, 1745-1759

25. Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H. G., Foster, L., and Karre, K. (1989) *Nature* 340, 443-448

26. Villadangos, J. A., Galocha, B., and Lopez de Castro, J. A. (1994) *J.Immunol.* 152, 2317-2323

27. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Williams, A. F., and Ziegler, A. (1978) *Cell* 14, 9-20

28. Ellis, S. A., Taylor, C., and McMichael, A. (1982) *Hum.Immunol.* 5, 49-59

29. Brodsky, F. M., Bodmer, W. F., and Parham, P. (1979) *Eur.J.Immunol.* 9, 536-545

30. Parham, P., Androlewicz, M. J., Holmes, N. J., and Rothenberg, B. E. (1983) *J.Biol.Chem.* 258, 6179-6186
31. Stam, N. J., Spits, H., and Ploegh, H. L. (1986) *J.Immunol.* **137**, 2299-2306

32. Paradela, A., García-Peydro, M., Vazquez, J., Rognan, D., and López de Castro, J. A. (1998) *J.Immunol.* **161**, 5481-5490

33. Paradela, A., Alvarez, I., García-Peydro, M., Sesma, L., Ramos, M., Vazquez, J., and López de Castro, J. A. (2000) *J.Immunol.* **164**, 329-337

34. Alvarez, I., Sesma, L., Marcilla, M., Ramos, M., Martí, M., Camafeita, E., and López de Castro, J. A. (2001) *J.Biol.Chem.* **276**, 32729-32737

35. Yague, J., Vazquez, J., and López de Castro, J. A. (1998) *Tissue Antigens* **52**, 416-421

36. Marina, A., García, M. A., Albar, J. P., Yague, J., López de Castro, J. A., and Vazquez, J. (1999) *J.Mass Spectrom.* **34**, 17-27

37. Galocha, B., Lamas, J. R., Villadangos, J. A., Albar, J. P., and López de Castro, J. A. (1996) *Tissue Antigens.* **48**, 509-518

38. Lopez, D., García Hoyo, R., and López de Castro, J. A. (1994) *J.Immunol.* **152**, 5557-5571

39. Aparicio, P., Jaraquemada, D., and López de Castro, J. A. (1987) *J.Exp.Med.* **165**, 428-443

40. Maziarz, R. T., Fraser, J., Strominger, J. L., and Burakoff, S. J. (1986) *Immunogenetics* **24**, 206-208

41. Parham, P., Adams, E. J., and Arnett, K. L. (1995) *Immunol.Rev.* **143**, 141-180
42. Parham, P., Arnett, K. L., Adams, E. J., Barber, L. D., Domena, J. D., Stewart, D., Hildebrand, W. H., and Little, A. M. (1994) *Tissue Antigens. 43*, 302-313

43. Vilches, C., de Pablo, R., Herrero, M. J., Moreno, M. E., and Kreisler, M. (1994) *Immunogenetics 40*, 166-166

44. Whelan, M. A. and Archer, J. R. (1993) *Eur.J.Immunol. 23*, 3278-3285

45. Gao, X. M., Wordsworth, P., McMichael, A. J., Kyaw, M. M., Seifert, M., Rees, D., and Dougan, G. (1996) *Eur.J.Immunol. 26*, 1443-1450

46. Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A., and Wilson, I. A. (1992) *Science 257*, 919-927

47. Smith, K. J., Reid, S. W., Harlos, K., McMichael, A. J., Stuart, D. I., Bell, J. I., and Jones, E. Y. (1996) *Immunity 4*, 215-228

48. Boisgérault, F., Tieng, V., Stolzenberg, M. C., Dulphy, N., Khalil, I., Tamouza, R., Charron, D., and Toubert, A. (1996) *J.Clin.Invest. 98*, 2764-2770

49. Griffin, T. A., Yuan, J., Friede, T., Stevanovic, S., Ariyoshi, K., Rowland-Jones, S. L., Rammensee, H. G., and Colbert, R. A. (1997) *J.Immunol. 159*, 4887-4897

50. Rojo, S., Aparicio, P., Hansen, J. A., Choo, S. Y., and Lopez de Castro, J. A. (1987) *J.Immunol. 139*, 3396-3401

51. Choo, S. Y., St.John, T., Orr, H. T., and Hansen, J. A. (1988) *Hum.Immunol. 21*, 209-219

52. Simmons, W. A., Summerfield, S. G., Roopenian, D. C., Slaughter, C. A.,
Zuberi, A. R., Gaskell, S. J., Bordoli, R. S., Hoyes, J., Moomaw, C. R., Colbert, R. A., Leong, L. Y. W., Butcher, G. W., Hammer, R. E., and Taurog, J. D. (1997) *J. Immunol.* **159**, 2750-2759

53. Taurog, J. D., Hammer, R. E., Moomaw, C. R., Slaughter, C. A., Roopenian, D. C., Zuberi, A. R., Gaskell, S. J., Bordoli, R. S., Colbert, R. A., Butcher, G. W., and Leong, L. Y. (1999) *J. Immunol.* **163**, 5741

54. Villadangos, J. A., Galocha, B., Garcia, F., Albar, J. P., and Lopez de Castro, J. A. (1995) *Eur. J. Immunol.* **25**, 2370-2377

55. Parker, K. C., Biddison, W. E., and Coligan, J. E. (1994) *Biochemistry* **33**, 7736-7743

56. Kuon, W., Holzhutter, H. G., Appel, H., Grolms, M., Kollnberger, S., Traeder, A., Henklein, P., Weiss, E., Thiel, A., Lauster, R., Bowness, P., Radbruch, A., Kloetzal, P. M., and Sieper, J. (2001) *J. Immunol.* **167**, 4738-4746

57. Sykulev, Y., Joo, M., Vturina, I., Tsomides, T. J., and Eisen, H. N. (1996) *Immunity* **4**, 565-571
Footnotes:

1Abbreviations used in this paper: AS, ankylosing spondylitis; CTL, cytotoxic T lymphocytes; C67S, Cys67Ser; C1R, HMy2.C1R; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PSD, post-source decay; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; m/z, mass-to-charge ratio; mV, millivolts; P, peptide position.
Figure legends

**Figure 1.** Flow cytometry analysis of HLA-B27 expression on C1R transfectant cells. About 2 x 10^5 untransfected C1R (thin solid line), B*2705-C1R (dotted line), or C67S-C1R cells (thick solid line) were stained with the indicated mAb, followed by FITC-conjugated rabbit-anti mouse IgG Ab. The background staining of untransfected C1R cells with W6/32 and BBM.1 is due to endogenous class I molecules of these cells (20). Peak heights were normalized in each panel to the peak height of untransfected C1R cells.

**Figure 2.** An example of the comparison between B*2705- and C67S-bound peptides. The MALDI-TOF MS spectrum of HPLC fraction N. 161 (upper panel) from B*2705 was compared with the spectra of HPLC fractions N. 160-162 from C67S (lower panels). Ion peaks from B*2705 detected in one or more of the HPLC fractions of the mutant are labeled with asterisk (*). Ion peaks from B*2705 not observed in the mutant are indicated with arrows. Peptides sequenced from these HPLC fractions are indicated (see also Figure 4). Two significant ion peaks from B*2705 fraction N. 161 (m/z: 1005.6 and 1055.6) were not included in the comparison as their m/z corresponded to Na^+ adducts of other ion peaks (m/z: 983.1 and 1033.4, respectively) in the same HPLC fraction. Maximum signal intensity in each of the four spectra was 289, 705, 435, and 152 mV, respectively.

**Figure 3.** Panel A: Distribution of m/z values of ion peaks in the MALDI-TOF MS spectra corresponding to the total number of B*2705- (solid line) and C67S-bound (dotted line) peptides compared. These were 1193 and 1175, respectively. The average m/z value for B*2705 and C67S ion peaks ([M+H]^+) was 1154 and 1185, respectively.
Panel B: Distribution of m/z values of ion peaks corresponding to B*2705 (black bars) or C67S (gray bars) peptide differences. These were 188 and 171, respectively. The average m/z value for B*2705 and C67S ion peak differences was 1089 and 1295, respectively.

Figure 4. Amino acid sequence of natural ligands from B*2705 and C67S. Peptides found in the peptide pools from both molecules (shared) or only in one of them (B*2705- or C67S-specific ligands) are indicated. Peptides labeled with one (*) or two (**) asterisks were assigned as quantitative differences predominant in B*2705 or the mutant, respectively. The human proteins and their accession numbers with which complete match of the ligands was found, and the molecule(s) from which each ligand was sequenced are indicated. Most ligands are reported here for the first time. When this is not the case, the corresponding reference is given. Peptide sequences were obtained by quadrupole ion trap nanoelectrospray MS/MS and/or by PSD-MALDI-TOF MS. The two shared ligands with Gln2 found in this analysis are boxed.

Figure 5. Panel A: Quadrupole ion trap nanoelectrospray MS/MS spectrum of an ion peak at m/z 1056.4 in HPLC fraction N. 143 from the C67S mutant. Detected fragment ions of the main b and y” series, prominent fragment ions of secondary series, and the deduced peptide sequence, are indicated. (see http://www.matrixscience.com for nomenclature of fragment ions). The y* and b_o ions are related to the corresponding y” and b ions by neutral loss of ammonia and water, respectively. Ion peaks related to the parental ion by neutral loss of ammonia or guanidine (g) are also indicated. Panel B: PSD-MALDI-TOF MS spectrum of an ion peak at m/z 1056.4 in HPLC fraction N. 143 from B*2705. The m/z value of assigned fragment ions is indicated. Panel C: PSD-MALDI-TOF MS spectrum of the synthetic RQTGIVLNR peptide. Conventions are as
in Panel B.

**Figure 6.** *Panel A:* Quadrupole ion trap nanoelectrospray MS/MS spectrum of the ion peak at m/z 1105.4 in HPLC fraction N. 155 from the C67S mutant. Conventions are as in Figure 5A. The areas of the spectrum around the m/z of the $y_8^-$ and $y_8^{2+}$ are expanded (insets) to show absence of the corresponding $y_8^*$ ions. The assigned peptide sequence is indicated. *Panel B:* nanoelectrospray MS/MS spectrum of the synthetic YKFSGFTQK peptide, showing virtual identity with the spectrum in Panel A. *Panel C:* nanoelectrospray MS/MS spectrum of the synthetic YQFSGFTQK peptide. Among other differences with the spectrum in Panel A, note the prominent $y_8^*$ (m/z: 925.2) and $y_8^{2+}$ (m/z: 462.7) ions (inserts).

**Figure 7.** Epitope stabilization assay showing the cell surface binding of the indicated peptides on B*2705-RMA-S transfectant cells. Data are means ± standard deviation of 3 independent experiments. EC50 values (see Materials and Methods) were the following: RRYQKSTEL: 0.5 µM, RQVIPIIGK: 1 µM, RQTGIVLNR: 1 µM, YKFSGFTQK: 36 µM.
Table I. *Comparison of B*2705- and C67S-B27-bound peptide repertoires*

|                  | B*2705 ligands | C67S ligands |
|------------------|----------------|--------------|
|                  | Total          | Common       | Specific     | Total          | Common       | Specific     |
|                  | 1193           | 1005 (84%)   | 188 (16%)    | 1175           | 1005 (85%)   | 171 (15%)    |

Quantitative differences between B*2705- and C67S-bound peptides

|                  | Total peptides compared | Predominant in B*2705 | Predominant in C67S |
|------------------|-------------------------|-----------------------|---------------------|
|                  | 252                     | 25 (10%)              | 31 (12%)            |
### Table II. Reactivity of B*2705-specific alloreactive CTL with the C67S mutant<sup>a</sup>

| CTL     | E:T | C1R | B*2705-C1R | C67S-C1R | C67S-C1R |
|---------|-----|-----|------------|----------|----------|
| 102DRF  | 1:1 | 5   | 63         | 7        | 11       |
| 12.8DM5 | 1.5:1| 10  | 35         | 8        | 23       |
| 139DRD  | 1:1 | 4   | 56         | 13       | 23       |
| 4S15    | 1:1 | 1   | 50         | 18       | 36       |
| 11SRY   | 1:1 | 0   | 27         | 14       | 52       |
| 33S15   | 1:1 | 6   | 45         | 24       | 53       |
| 33S69   | 0.5:1| 3   | 54         | 40       | 74       |
| 58GRK   | 1:1 | 0   | 67         | 41       | 61       |
| 27S69   | 1:1 | 3   | 71         | 48       | 68       |
| 20.8GRK | 1:1 | 5   | 27         | 34       | 126      |
| 37GRK   | 1:1 | 0   | 57         | 52       | 91       |

<sup>a</sup>Data are means of 2 to 9 independent experiments

<sup>b</sup>Percent lysis of C67-C1R relative to the specific lysis of B*2705-C1R cells.
Figure 2
Figure 3
| Peptide | Protein (Accession N.) | Sequenced from | Ref. |
|---------|------------------------|----------------|------|
| **Shared NONAMERS** | | | |
| GRYGGETKV | Splicing factor, arginine-serine-rich 7 (Q16629) | B*2705 | This study |
| RSSSIPITV | DNA replication licensing factor MCM3 (P33992) | B*2705 | This study |
| ARLQYALLTV | Beta V spectrin (Q9NR6) | Both | (61) |
| RRAGIKTVT | RNA-binding protein regulatory subunit (O14805) | C67S | This study |
| RRVEKHWRL | Eukaryotic translation initiation factor 2 gamma subunit (P41091) | C67S | This study |
| KRPKEANNF | 60S ribosomal protein L7 (P18124) | Both | (61) |
| SRLRNQSFV | Farnesyl-diphosphate farnesyltransferase (P37268) | B*2705 | This study |
| IRLPSQYNF | KIAA0906 protein (fragment) (P94890) | B*2705 | This study |
| QRYNPYSK | PX19 protein (Q9U13) | Both | This study |
| GRIDKPILK | 60S ribosomal protein L8 (P25120) | Both | This study |
| LVDPVNPFK | Alpha-globin gene (alpha-thalassemia allelic form) (P78461) | Both | This study |
| HRQVYITR | 40S ribosomal protein S25 (P25111) | Both | (62) |
| KRLVVFDAR | DNA-directed RNA polymerases I, II, and III 7.0 kDa polypeptide (P53803) | B*2705 | This study |
| **DECMERS** | | | |
| RQTGIVLNR | Spliceosome associated protein 145 (Q13435) | Both | This study |
| RQVIPIGK | KIAA0554 protein (fragment) (O60301) | C67S | This study |
| SRTFYHVNL | Proteasome subunit beta type 2 (P49721) | B*2705 | (61) |
| SRFPEALRL | 26S proteasome regulatory subunit S2 (Q13200) | Both | This study |
| GRYPGVSNY | Amyloid protein-binding protein 1 (Q13564) | B*2705 | This study |
| GRIGVITNR | 40S ribosomal protein S4, X isoform (P17250) | Both | (63) |
| KRGKAYNL | RNA helicase-related protein (O75619) | C67S | This study |
| **UNDENAMERS** | | | |
| KRFNADNLKL | Lymphocyte-specific helicase (Q60848) | C67S | This study |
| **B*2705-specific** | | | |
| **NONAMERS** | | | |
| ARSFGKTV | Chromosome 16p13 bac clone cii987sk-962B4 complete sequence, HTGS phase 3 (fragment) (P78421) | B*2705 | This study |
| ARIGVLSQV | Hypothetical 82.7 kDa protein RV1363C (Q10768) | B*2705 | This study |
| SREPVLQF | R31B14_3 (O60392) | B*2705 | This study |
| LRPNPLINGK | Small nuclear ribonucleoprotein SM D2 (P43330) | B*2705 | This study |
| GRIFGITYG | Chromosome-associated polyepitope-C (O95752) | B*2705 | This study |
| **DECMERS** | | | |
| ARIGKQVFGL | Ataxin-2-like protein A2LP (O95135) | B*2705 | This study |
| **C67S-specific** | | | |
| TKFGGPTQK | COX7RP1 (O14548) | C67S | This study |

**Figure 4**
Figure 5
Figure 6
