Mutational Analysis Reveals a Complex Interplay of Peptide Binding and Multiple Biological Features of HLA-B27* [5]

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Molecular polymorphism influences the strong association of HLA-B27 with ankylosing spondylitis through an unknown mechanism. Natural subtypes and site-directed mutants were used to analyze the effect of altering the peptide-binding site of this molecule on its stability, interaction with tapasin, folding, and export. The disease-associated subtypes B*2705, B*2702, and B*2704 showed higher thermostability at 50 °C than all other subtypes and mutants, except some mimicking B*2702 polymorphism. The lowest values were found among pocket B mutants, most of which interacted strongly with tapasin, but otherwise there was no correlation between thermostability and tapasin interaction. Mutants resulting in increased hydrophobicity frequently acquired their maximal thermostability faster than those with increased polarity, suggesting that this process is largely driven by the thermodynamics of peptide binding. Folding, export, and tendency to misfold were influenced by polymorphism all along the peptide-binding site and were not specifically dependent on any particular region or structural feature. Frequent uncoupling of thermostability, folding/misfolding, and export can be explained by the distinct effect of mutations on the acquisition of a folded conformation, the optimization rate of B27-peptide complexes, and their quality control in the endoplasmic reticulum, all of which largely depend on the ways in which mutations alter peptide binding, without excluding additional effects on interactions with tapasin or other proteins involved in folding and export. The similarity of the generally disease-associated B*2707 to nondisease-associated subtypes in all the features analyzed suggests that molecular properties other than antigen presentation may not currently explain the relationship between HLA-B27 polymorphism and ankylosing spondylitis.

HLA-B27 is the major susceptibility factor for a group of chronic inflammatory diseases known as spondyloarthropathies, whose prototype is ankylosing spondylitis (AS). This is a disorder characterized by inflammation of the entheses and joints of the axial skeleton, followed by pathological new bone formation, ultimately leading to ankylosis (1). HLA-B27 is involved in triggering the inflammatory process, but the mechanism is unknown. Four main hypotheses, each based on a particular property of HLA-B27, are currently being investigated. The arthritogenic peptide hypothesis (2) is based on the antigen-presenting specificity of HLA-B27 and assumes that molecular mimicry between microbial and self-derived B27 ligands would trigger autoimmune T cell cross-reaction and tissue injury. The misfolding hypothesis (3) is based on the slow folding and tendency to misfold of HLA-B*2705 (4) and assumes that accumulation of misfolded B27 heavy chain (HC) could trigger inflammation by eliciting the unfolded protein response and activation of NFκB. The surface homodimer hypothesis (5, 6) is based on the expression of HC homodimers at the cell surface, following dissociation of HLA-B27-peptide complexes upon endosomal recycling (7), and the assumption that differential recognition of heterodimeric and homodimeric forms of HLA-B27 by leukocyte receptors (8) might have an immunomodulatory role of pathological consequences (9, 10). The B2-microglobulin (B2m) deposition hypothesis (11) assumes that tissue damage might arise from slow B2m deposition in the joints following dissociation of HLA-B27 at the cell surface. An attempt to unify these ideas was based on the pivotal role of peptide binding in determining not only the specificity of antigen presentation by MHC class I (MHC-I) molecules but also their stability, folding, and dissociation (12). Many studies have addressed the nature of HLA-B27-bound peptide repertoires and the influence of subtype polymorphism on peptide specificity (13, 14). X-ray diffraction analyses have unveiled the detailed binding mode of HLA-B27 ligands (15). The main anchor residue of HLA-B27-bound peptides is R2, whose side chain fits in the B pocket of the peptide-binding site, a feature conserved in all the major B27 subtypes. The A pocket, also conserved among natural subtypes, except in B*2703, interacts with the peptidic N terminus and makes a significant contribution to binding. The C-terminal residue, another major anchor of B27 ligands, interacts in the F pocket, which is variable among subtypes and responsible for much of their differential binding specificity. Mutations in the B pocket (4, 16) and other areas of the molecule (17) influence folding and export of HLA-B27.
Peptides bind to nascent MHC-I molecules and critically determine their folding and stability. Optimization of the MHC-I-bound peptide repertoires occurs in the peptide-loading complex (18), through the editing function of tapasin (Tpn), one of its constitutive components (19). Quality control mechanisms (20) prevent the export of suboptimal MHC-I-peptide complexes. These mechanisms, which are yet insufficiently characterized, involve the retention of suboptimally loaded MHC-I molecules in the endoplasmic reticulum (ER) and their retrieval from early secretory compartments, in a process mediated by Tpn (21, 22) and by the lectin chaperone calreticulin (23). Thus, the stability of B27-peptide complexes is a key feature linking peptide binding, folding, export, and post-ER dissociation. In this study, we used natural subtypes and mutants to define how molecular polymorphism all along the peptide-binding site affects the stability of HLA-B27 and its interaction with Tpn, and how these features relate to folding, export, and disease association.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Western Blot**—HMy2.C1R (C1R) is an HLA-A-negative human B cell line expressing low levels of HLA-B35 (24). Stable C1R transfectants (25–27) expressing 8 HLA-B27 subtypes (B*2701 to B*2707 and B*2709), 13 B*2705 mutants mimicking subtype polymorphism, and 12 pocket A or B mutants were used (Table 1). Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FBS (Invitrogen). ME1 (IgG1) is an anti-HLA-B7/B27/B22 monoclonal antibody (mAb) that recognizes HC-β2m-peptide complexes (28–30). HC10 (IgG2a) is a mAb that recognizes β2m-free MHC-I HC (31). It reacts both with monomeric and oligomeric HC forms (16). PaSta-1 (a kind gift of Dr. Peter Cresswell, Yale University) is a mAb specific for human Tpn (32). The anti-γ-tubulin mAb (IgG1) GTU88 (Sigma) was also used. Western blot analyses were performed exactly as described previously (17). The protein expression level of HLA-B27 variants in C1R transfectant cells, relative to B*2705, is shown in supplemental Fig. S1. For some of these variants, these data were reported previously (17) and are included here for comparison.

**Metabolic Labeling, Pulse-Chase Analysis and Immunoprecipitation**—Cells were incubated with 1-met/1-cys-free DMEM (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine for 45 min at 37 °C. They were pulse-labeled with 500–1000 μCi/ml [35S]Met/Cys (GE Healthcare) and chased at various times with RPMI 1640 medium supplemented with 1 mM cold L-Met and L-Cys at the same temperature. At each time point, cells were centrifuged, resuspended in 50 μl of PBS, frozen in liquid N2, and stored at −80 °C. Cells were lysed in 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science), centrifuged (16,000 × g, 10 min, 4 °C), precleared at 4 °C three times for 60 min with Sepharose CL-4B beads (Sigma) and 3 μl of normal mouse serum, and immunoprecipitated with specific mAb and protein-A-Sepharose beads (Sigma). Immunoprecipitates were normalized to equal trichloroacetic acid-precipitable 35S-labeled protein, washed three times with 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and analyzed by 10% polyacrylamide SDS-PAGE under reducing conditions. Endoglycosidase H (Endo H) (New England Biolabs, Beverly, MA) was added to the immunoprecipitates according to the manufacturer’s instructions. Radioactive samples were visualized by fluorography using DMSO-2,5-diphenyloxazole. The autoradiograms were scanned and quantified using the TINA 2.09e software (Isotopenmessgeräte GmbH). For co-precipitation of HLA-B27 with the anti-Tpn mAb PaSta-1, the same procedure was used except that metabolic labeling was carried out for 5 h.

**Thermostability Assays**—These were carried as described previously (33). Briefly, C1R transfectants were pulse-labeled for 15 min and chased at various times as described above. At each time point, the cell lysates were incubated for 1 h at various temperatures, immunoprecipitated with ME1, and analyzed by SDS-PAGE. The amount of heterodimer precipitated at each temperature at any given time was expressed as a percentage of the amount precipitated at 4 °C and plotted as a function of the temperature.

**Statistical Analysis**—Fisher’s exact test was used.

**RESULTS**

**Thermostability of HLA-B27 Subtypes and Mutants**

This was analyzed in a panel of C1R transfectant cells (Table 1). The dissociation of pulse-labeled HLA-B27-peptide complexes was assessed by immunoprecipitation with ME1 after incubation of the lysates obtained at 0-, 2-, and 4-h chase times, at temperatures ranging from 4 to 50 °C. The thermostability was measured as the percentage of the HC immunoprecipitated with ME1 at a given temperature, relative to the amount immunoprecipitated at 4 °C (Figs. 1 and supplemental Figs. S2–S6). This parameter reflects the global stability of the peptide cargo at a given time. B*2705 acquired very high thermostability (98 ± 3%) at 50 °C after 4 h of chase, in agreement with previous reports (19, 33). A similar behavior was observed for B*2702 and B*2704. All other subtypes analyzed formed less stable complexes, showing significant dissociation at 50 °C even at a 4-h chase time (Fig. 1A and supplemental Fig. S2).

In similar experiments, all pocket A (Fig. 1B and supplemental Fig. S3) and B (Fig. 1C and supplemental Fig. S4) mutants showed decreased thermostability, relative to B*2705. The thermostability of pocket A mutants, at 50 °C and a 4-h chase, was in the range of 57% (H171) to 67% (T163). The effect of mutations in the B pocket was more pronounced. Except for Q70, the relative thermostability of these mutants at a 4-h chase and 50 °C was in the range of 10% (A24V67) to 44% (V67). These results are consistent with the significance of pockets A and B in peptide binding and demonstrate their contribution to the molecular stability of HLA-B27.

Among the eight mutants mimicking B*2701 and B*2702 polymorphism (Fig. 1D and supplemental Fig. S5), those including the I80 and/or A81 changes showed a thermostability similar to or slightly lower than B*2705. N77 and, more prominently, Y74 and Y74N77 showed significantly decreased thermostability, as did all five mutants mimicking...
ing B*2704 and B*2706 polymorphism (Fig. 1E and supplemental Fig. S6).

The high thermostability of B*2702 and B*2704 may be explained by compensatory effects of I80 and A81 on the decreased thermostability induced by the N77 change, and between S77 and E152, respectively. The lower thermostability of B*2701 and B*2706 correlates with a similar behavior of Y74 and Y74N77 and of D114, Y116, and D114Y116, respectively.

The differences in thermostability among HLA-B27 variants were revealed mainly at temperatures above 37 °C and were much smaller at the physiological temperature (supplemental Figs. S2–S6). This does not mean that such differences are irrelevant in vivo, because they reflect real effects on the overall affinity of peptide binding that might affect molecular stability at physiological conditions such as, for instance, in acidic intracellular environments or at sites of inflammation where a temperature increase takes place.

Optimization of the Stability of HLA-B27 Variants

Shortly after synthesis, the B*2705-bound peptide repertoire is suboptimal, but Tnp-mediated editing ensures binding of ligands with higher stability (19). The optimization of the peptide cargoes of HLA-B27 variants was analyzed by measuring the thermostability increase as a function of time in pulse-chase experiments (supplemental Figs. S2–S6). The magnitude of this optimization can be appreciated by comparing the 50 °C values at 0 and 4 h of chase (Fig. 2). HLA-B27 subtypes showed variable degrees of thermostability increase (Fig. 2A). Among pocket A mutants (Fig. 2B), T163 reached its maximal stability shortly after synthesis, whereas all other mutants showed a significant time-dependent increase, which reached 4.7-fold in the case of H171. A comparable variability was observed among pocket B mutants (Fig. 2C), ranging from 2.1-fold (S67) to no optimization (i.e. Q70, A24, and A24V67). Among mutants mimicking subtype polymorphism (Fig. 2, D and E), little optimization was observed in mutants involving residues 77 and/or 80. In contrast, mutants involving residues 74, 114, and/or 116 and E152 showed a significant increase (about 1.6–1.9-fold), which was maximal (3.1-fold) for A81.

In all the cases tested, the thermostability increase occurred equally in the presence of brefeldin A (data not shown), suggesting that optimization of the molecular stability takes place in the ER. The magnitude of this process was unrelated to the maximal thermostability reached by any given variant and to the molecular location of the amino acid changes. However, a statistically significant correlation (p, 0.47 × 10^{-4}) was found between the effect of the mutation(s) on hydrophobicity and the increase in thermostability between 0 and 4 h of chase (Fig. 3). All eight mutations resulting in increased polarity showed a ≥1.7-fold thermostability increase with time (mean, 2.4 ± 1), whereas only 4 of 24 variants resulting in increased or unaltered hydrophobicity reached such a ratio (mean, 1.4 ± 0.3). This strongly suggests that the optimization rate of the molecular stability among HLA-B27 variants is, at least partially, driven by the destabilizing effect of solvent-exposed nonpolar residues in the MHC molecule, whose isolation from water through interaction with the peptide ligand would be thermodynamically favored.

Significant time-dependent increase of the thermostability at 50 °C was observed in multiple variants that showed high thermostability at 37 °C (i.e. B*2705, B*2702, B*2704, A81, etc.). This strongly suggests that the differences observed at 50 °C are physiologically relevant, because optimization of the molecular stability of B27-peptide complexes proceeds in vivo above thresholds that are not revealed by thermostability assays performed at 37 °C.

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**TABLE 1**

Amino acid changes of HLA-B27 subtypes and mutants

Mutants are designated with the single-letter amino acid code followed by the corresponding position number.

| Subtype | Amino acid change | Pocket | Mutant | Amino acid change | Pocket |
|---------|-------------------|--------|--------|-------------------|--------|
| B2705   | D74Y              | C/F    | Y9     | H9Y               | B      |
| B2701   | D77N              | F      | A24    | T24A              | B      |
|         | L81A              | F      | M45    | E45M              | B      |
| B2702   | D77N              | F      | N63    | E63N              | A/B    |
|         | T80I              | F      | S67    | C67S              | B      |
|         | L81A              | F      | S67    | C67S              | B      |
| B2703   | Y59H              | A      | S77    | D77S              | F      |
| B2704   | D77S              | F      | A81    | L81A              | F      |
|         | V152E             | E      | A24V67 | H114D             | D/E    |
| B2706   | D77S              | F      | E152   | V152E             | E      |
|         | V152E             | E      | T163   | E163T             | A      |
|         | H114D             | D/E    | S167   | W167S             | A      |
| B2707   | N97S              | C/E    | Y9070  | H9Y; K70Q         | B/C    |
|         | Y113H             | D*     | A24V67 | T24A; C67V        | B      |
|         | H114N             | D/E    | Y74N77 | D74Y; D77N        | C/F    |
|         | D116Y             | F      | N77I80 | D77N; T80I        | F      |
|         | S131R             | Loop   | N77A81 | D77N; L81A        | F      |
| B2709   | D116H             | F      | I80A81 | T80I; L81A        | F      |
|         |                   |        | D114Y116 | H114D; D116Y | D/E/F  |

* Residue 113 is oriented away from the peptide-binding site.
Interaction of HLA-B27 Variants with Tapasin

This was analyzed by co-precipitation of 5-h labeled subtypes and mutants with PaSta-1 and measured as the amount of HC, relative to Tpn, precipitated with the anti-Tpn mAb (HC/Tpn ratio) (Fig. 4). This parameter cannot distinguish between a weak interaction and a short lived one. Because peptide editing can occur at a fast rate, the HC/Tpn ratio does not necessarily reflect the degree of Tpn dependence or correlate with the time-dependent optimization of the peptide cargo, but it indicates the amount of HC bound to Tpn in the steady state. Among subtypes, only B*2705 and B*2709 showed high HC/Tpn ratios. The strong interaction of B*2709...
with Tpn was also observed in 721.221 cells (34, 35). The lower values in other subtypes (33–43%, relative to B*2705) were accounted for by similar effects of many of the mutations mimicking subtype changes, especially those affecting residue 77. Y116 behaved similarly to B*2709 (H116) and was the only mutant among those mimicking subtype polymorphism whose interaction with Tpn was increased, relative to B*2705. However, the effect of this residue was strongly influenced by co-occurrence of additional changes, as shown by the significantly lower HC/Tpn ratios of B*2706, B*2707, and D114Y116. All the A pocket mutations resulted in decreased HC/Tpn ratios, with H171 showing the smallest alteration. Among B pocket mutants, A24 and A24V67 showed very high HC/Tpn ratios, and Y9, S67, and M45 were similar to B*2705, and V67, Q70, and Y9Q70 showed decreased interaction (Fig. 4).

**Variable Relationships between Thermostability and Interaction with Tpn among HLA-B27 Variants**

These were classified in three groups according to their thermostability and HC/Tpn ratio (Fig. 5). Group 1 consisted of variants with high thermostability (B*2702, B*2704, I80, A81, and I80A81), all of which showed lower HC/Tpn ratio than B*2705. Group 2 consisted of variants showing decreased thermostability, and HC/Tpn ratio < 80%, relative to B*2705. This group included four subtypes (B*2701, B*2703, B*2706, and B*2707), all pocket A (N63, T163, S167, and H171), three pocket B (V67, Q70, and Y9Q70), and multiple mutants mimicking subtype polymorphism. Group 3 consisted of variants with strongly decreased thermostability (about 50% or lower) and HC/Tpn ratio < 80%, relative to B*2705. This group included B*2709, Y116, and most of the B pocket mutants. The average thermostability and HC/Tpn ratio in groups 1 (99 ± 3/62 ± 25), 2 (57 ± 13/51 ± 13), and 3 (32 ± 14/151 ± 60) showed an inverse relationship between both features only in group 3. The variants in group 1 showed that high thermostability can be achieved with very different degrees of Tpn interaction. The variants in group 2 showed that, for intermediate thermostability values, B27 variants often require a lower interaction with Tpn than B*2705 to load their optimal peptide cargo. Within group 3, the highest interaction with Tpn was associated with very low thermostability (A24, A24V67) or with the presence of a single change at position 116 (B*2709, Y116).

A comparison of Figs. 2 and 5 shows that the magnitude of the time-dependent optimization of the peptide cargo is inde-
Folding and Export of HLA-B27 Variants: Relationship to Thermostability

The folding and export features of several HLA-B27 subtypes and mutants were previously reported (17). Here, we have extended these studies to include B*2701, B*2703, mutants affecting pockets A and B, and those mimicking B*2701 polymorphism and to relate folding and export to the molecular stability of HLA-B27 variants. Two parameters were used to estimate folding. First, the ratio between unfolded and folded HC immunoprecipitated with HC10 and ME1, respectively, in pulse-chase experiments (H/M ratio). This reflects the folding rate and, at 0 h of chase, the folding efficiency of newly synthesized molecules. Second, the half-life of unfolded HC \( (t_{1/2}^{HC}) \) was estimated from the amount of HC10-immunoprecipitated material along the chase. This reflects the folding rate of the HC and the presence of misfolded HC that fails to progress toward formation of the canonic heterodimers. Export rates were assessed by measuring the acquisition of Endo H resistance, which takes place during transit of glycoproteins through the Golgi apparatus, in pulse-chase experiments. We have previously shown that the differences in HC protein expression among C1R transfectant cells (supplemental Fig. S1) do not provide a trivial explanation for the differences in folding and export among B27 variants (17).

HLA-B27 variants were classified in two groups according to their export rates as follows: 1) those similar or slower than B*2705 and 2) those with faster export rates (Fig. 6). Each group was in turn subdivided in three subgroups according to their folding features as follows.
Interplay of Biological Features in HLA-B27

Subgroup 1A, Variants with H/M Ratio and $t_{1/2}$HC $\geq$ 80% Relative to B*2705—For subgroup 1A, they show slow folding, and their large $t_{1/2}$HC suggests a strong tendency to misfold. This subgroup included B*2705 and four mutants (Y9, Q70, A81, and N77A81) with diverse molecular locations (pockets B and F) and variable thermostability, which showed a roughly inverse correlation with the export rates.

Subgroup 1B, Variants with H/M Ratio < 80% and $t_{1/2}$HC $\geq$ 100% Relative to B*2705—For subgroup 1B, they all had lower thermostability than B*2705, which also showed a generally inverse correlation with the export rates. These mutants may acquire a folded conformation shortly after synthesis but are delayed in the ER due to quality control. The large $t_{1/2}$HC, associated in most of the variants in this subset to low H/M ratios, probably reflects HC misfolding. The alternative possibility that a fraction of the B27-peptide complexes may fold quickly, although another fraction folds much more slowly, would imply the existence of two peptide subsets with sharply distinct affinities, which seems unlikely. This subgroup consists of 10 mutants, including 6 in the A (N63 and T163) or B pockets (A24, S67, A24V67, Y9Q70) and 4 mimicking subtype polymorphism (Y74, N77, D114, D114Y116).

Subgroup 1C, Variants with H/M Ratio and $t_{1/2}$HC < 80% Relative to B*2705—For subgroup 1C, this group consists of 4 mutants (M45, I80, Y116, and H171) distributed all along the peptide-binding site (pockets A, B, and F) and differing widely in thermostability. They fold quickly and probably undergo little or no misfolding but are apparently retained in the ER due to quality control. Unlike other mutants in this group, the large export rate of I80 is difficult to explain by a suboptimal peptide cargo, because this mutant reached high thermostability shortly after synthesis (Fig. 2D and supplemental Fig. S4). This suggests that factors other than peptide optimization might delay its exit from the ER.

Subgroup 2A, H/M Ratio and $t_{1/2}$HC $\geq$ 80% Relative to B*2705—For subgroup 2A, this subgroup consisted of only B*2702. The subtype was very similar to B*2705 in folding and thermostability, but its export rate was faster.

Subgroup 2B, Relative H/M Ratio < 80% and $t_{1/2}$HC $\geq$ 100%—For subgroup 2B, this subgroup consisted of B*2704 and Y74Y77. Their features suggest that a fraction of the molecules are folded and exported faster than B*2705, whereas some of the HC remains in the ER, presumably in a misfolded form.

Subgroup 2C, H/M Ratio and $t_{1/2}$HC < 80% Relative to B*2705—For subgroup 2C, this subgroup consisted of 10 variants, including 5 natural subtypes (B*2701, B*2703, B*2706, B*2707, and B*2709), 2 pocket A or B mutants (S167 and V67), and 4 mimicking subtype polymorphism (S77, Q152, I80A81, and N77I80), located in pockets E and F. Their fast folding and export suggest that they have decreased tendency to misfold. Most of these variants had lower thermostability and, except B*2709, lower interaction with Tpn than B*2705 (Fig. 5).

These results indicate that thermostability, folding/misfolding, and export of HLA-B27 are strongly influenced by polymorphism all along the peptide-binding site and are not specifically dependent on any obvious region or structural motif. The maximal stability attainable by each variant, or its optimization from shortly after synthesis, does not necessarily determine its folding and export rates or its tendency to misfold, as shown by the lack of correlation between the corresponding parameters. A possible exception is the set of mutants at the lowest end of the thermostability spectrum (< 40%), such as most in the B pocket, which show very slow export rates and a tendency to misfold.

DISCUSSION

Previous studies suggested that the structure of the B pocket was critical in determining the slow folding and tendency to misfold of HLA-B27 (4), which inspired the hypothesis that these features might determine its pathogenetic role in AS. Studies in transgenic rats confirmed a role of B*2705 misfolding in gut inflammation but not in promoting arthritis and spondylitis in these animals (36). In addition, comparative analyses of HLA-B27 subtypes showed an incomplete corre-
spondence between folding (17) or Tpn dependence (35) and association with AS. These studies pointed out to a higher complexity of the structural features determining folding and other biological properties of HLA-B27 and to a less obvious interplay with disease susceptibility, which deserved a more comprehensive analysis.

Mutations in the A and B pockets affect the anchoring of the peptidic P1 and P2 residues, respectively, which significantly contribute to peptide binding (37). Subtype polymorphism affects mainly the F pocket, which binds the peptidic C terminus, and other regions of the peptide-binding site (pockets C, D, and E) that interact with secondary anchor residues of HLA-B27 ligands (13, 38–41). These mutations are responsible for the distinct subtype-bound peptide repertoires of HLA-B27 ligands. The effects of mutations in the peptide-binding site on the biological features of HLA-B27 cannot be dissociated from their influence on peptide binding. However, the complexity and variety of effects observed in our mutational analysis revealed that the relation-

![Graph showing comparison of export rate, folding features, and thermostability among HLA-B27 subtypes and mutants.](image)

**FIGURE 6.** Comparison of the export rate (time of acquisition of 50% Endo H resistance), folding features (H/M ratio and $t_{1/2}$HC), and thermostability, at 50 °C and 4 h of chase, among HLA-B27 subtypes and mutants. All values are relative to $\beta^*$2705 (export rate, 52 ± 9 min; H/M ratio, 32 ± 11; $t_{1/2}$HC, 101 ± 12 min; thermostability, 98 ± 3%). HLA-B27 variants were classified in groups 1 and 2 according to their export rates (≥80 and <80%, respectively) and further subdivided in 3 subgroups each according to their folding features, as explained under “Results.” The data are means ± S.D. of at least three experiments.

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ship between the molecular stability and its optimization, folding/misfolding, quality control, and export of HLA-B27 variants is far from straightforward and suggests a sophisticated interplay among these features, which is modulated in diverse ways by molecular polymorphism.

**Molecular Stability Folding and Export—**Mutations induced a wide range of effects on the stability of HLA-B27, from the highest thermostability of B*2705, B*2702, B*2704, and some mutants mimicking B*2702 polymorphism to the lowest one of some pocket B mutants. These effects are primarily mediated by altering peptide binding (42–44), but additional effects on HC/β₂m interactions, which are particularly strong in HLA-B27 (45, 46), cannot be ruled out. That most variants with the lowest thermostability showed a strong interaction with Tpn is in agreement with the role of this chaperone in retaining in the ER those MHC-I molecules that fail to optimize their peptide cargo (47). Indeed, variants with thermostability <40% relative to B*2705 consistently showed large export rates. However, above this thermostability threshold, many variants were exported faster than the wild type, indicating that, despite binding peptides with relatively low stability, this was often sufficient for release from the peptide-loading complex and exiting the ER. The observed lack of correlation between thermostability, folding, and export can partially be explained because acquisition of an ME1-reactive conformation may occur with suboptimal peptides, driven by thermodynamic factors (48), and by the availability of many potential ligands in the ER. Because suboptimal B27-peptide complexes are retained in the ER and early secretory compartments (23, 47, 49), this explains that multiple variants exhibited fast folding but large export rates. However, this explanation apparently does not apply to I80 because this mutant folded with very high thermostability shortly after synthesis but showed a slow export. This suggests that mechanisms other than peptide optimization such as interaction with cargo receptors (20, 50), for example, may delay exit from the ER and/or trafficking to the Golgi apparatus in this mutant. More generally, these mechanisms might also contribute to the lack of correlation between export rates and the time-dependent thermostability increase observed in multiple variants.

In B*2705 slow folding is associated with a tendency to misfold. Misfolded HC forms covalent homodimers and multimers with noncanonical inter-chain disulfide bonds (16) and remains for a long time in the ER. We observed large t₁/₂HC values, in several variants undergoing either slow or fast folding which, particularly in the latter case, is strongly suggestive of misfolding. It is interesting that the AS-associated B*2704 subtype was 1 of only 2 variants showing low H/M ratio, high t₁/₂HC, and faster export than B*2705. This can be explained by assuming that stable B*2704-peptide complexes are formed and exported quickly, whereas a fraction of the HC remains misfolded in the ER. Formal confirmation of these assumptions would require a biochemical demonstration of ER-associated HC oligomers, which was not addressed in this study.

**Role of A and B Pockets—**The decreased thermostability induced by mutations in these pockets was more pronounced for pocket B, as expected from its major importance in peptide binding. The low stability of B pocket mutants was reflected in their generally large export rates and high HC/Tpn ratios. This strongly suggests that most of these mutants are retained in the ER through sustained interactions with Tpn. Slow export was also seen in some A pocket mutants (N63, T163, H171), but B*2703 and S167 folded and were exported quickly. The low t₁/₂HC of these two variants, and also of H171 and M45, suggests that they have little or no tendency to misfold, in agreement with a previous report concerning M45 (16). However, the large t₁/₂HC of most pocket B mutants suggests that many alterations in this pocket do not decrease, and probably exacerbate, misfolding. Finally, V67 and S67 differed significantly in their export rates and t₁/₂HC, and both mutants showed faster folding than A67 in a previous study (16). Thus, the effect of altering C67 in B*2705 depends on the particular mutation.

**Role of Residue 116—**Several studies carried out with B*4402 and B*4405, which differ only by having D or Y116, respectively, have shown that the latter subtype has decreased interaction with Tpn and faster export than B*4402 (19, 48, 51). Apparently, conflicting results were reported concerning the relative thermostability of the two subtypes in Tpn-proficient cells (19, 51). These studies suggested that more efficient peptide loading in the presence of Y116 determines lower binding to Tpn. An effect of Y116 in decreasing the interaction with Tpn, relative to D116, was also reported in B*0702 (52). However, among MHC-I molecules, the presence of an aromatic residue at position 116 correlated with increased binding to the transporter associated with antigen processing (53). In addition, B*1510 (Tyr¹¹⁶) and B*3503 (Phe¹¹⁶) showed stronger association with the transporter associated with antigen processing/Tpn than B*1518 and B*3501 (both with Ser¹¹⁶), respectively, which was associated with lower thermostability and slower export, at least in B*3503 (52, 54, 55). In HLA-B27, the D116Y change resulted in decreased interaction with Tpn, associated with lower thermostability and faster folding and export, only in those variants differing in additional positions, such as D114Y116, B*2706, and B*2707. However, a similar phenotype was observed in multiple variants differing from B*2705 in other positions, but not in residue 116. In contrast, when Y116 was the only change, the thermostability was lower than in B*2705 and the folding rate faster, but Tpn interaction was substantially higher, and the export rate was similar to the wild type. B*2709 (H116) also showed increased interaction with Tpn, associated with lower thermostability and faster folding and export than B*2705. Increased interaction of B*2709 with Tpn, associated in this case with slower export, was reported in a different cell line (34). Taken together, these studies indicate that the effect of residue 116 on Tpn binding differs among MHC-I molecules and is modulated by other mutations. Therefore, studies on the effect of this polymorphism in a given allotype should be extrapolated with caution to other allotypes. In HLA-B27, Y116 may favor the interaction with Tpn more or less independently of the effects of this residue on peptide binding. In the presence of other mutations, further alterations in peptide binding, or other interactive ef-
fects, might counteract the enhanced interaction with Tpn prompted by Y116.

Subtype Polymorphism and AS Susceptibility—The natural subtypes fall into two distinct groups as follows: The three major AS-associated subtypes B*2705, B*2702, and B*2704 bind highly stable peptide repertoires, and their large $t_{1/2}$HC values suggest that they have a tendency to misfold. A second group showed decreased thermostability, associated with fast folding and export, and low $t_{1/2}$HC, suggesting less or no HC misfolding. This group includes the two subtypes not associated with AS (B*2706 and B*2709), the AS-associated B*2707, and two subtypes whose association with AS is unclear (B*2701 and B*2703). The correlation between high thermostability and large $t_{1/2}$HC among subtypes was not observed among mutants. That subtypes with lower thermostability presented faster folding and export rates than B*2705 suggests that they exit the ER with less stable peptide cargoes, which might favor dissociation at the cell surface. However, this was not observed (34, 56), suggesting that under physiological conditions these subtypes might be sufficiently stable as to show no increased expression of unfolded HC at the cell surface. Nevertheless, the lower thermostability of B*2706 and B*2709 relative to most AS-associated subtypes does not support the $\beta_m$ deposition hypothesis for the pathogenesis of AS (11), because a prediction of this hypothesis was that disease-associated subtypes should be less stable than subtypes not associated with AS, which is the opposite of what was found in our analysis.

Among the mutants mimicking subtype changes, only those not involving charge alterations (I80, A81, 180 – A81) showed a thermostability comparable with that of B*2705. The high thermostability of B*2702, showing an acidic charge loss relative to B*2705 (D77N), implies a compensatory effect of the co-occurrence of other polymorphic residues, as discussed for Y116. A second group showed a thermostability comparable with that of B*2705. Thus, peptide specificity and the possibilities that this opens for subtype-specific molecular mimicry (60–63) would currently seem a more compatible feature with the known association of HLA-B27 subtypes with AS.

Acknowledgment—We acknowledge the excellent technical assistance of Ana Navarro (Instituto de Salud Carlos III, Madrid).

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