The B pocket of the class I major histocompatibility complex-encoded protein HLA-B*2705 has recently been suggested to be responsible for the misfolding of this HLA haplotype and thus to induce susceptibility to autoimmune inflammatory diseases. Four mutants of the B*2705 heavy chain were refolded in the presence of three control peptides. The monitoring of the thermal unfolding of the B*2705-peptide complexes by circular dichroism spectroscopy showed that all heterotrimERIC mutants were markedly less stable than the corresponding complexes with the wild-type heavy chain. Among the four heavy chain mutations, the C67S change was investigated for unfolding and peptide binding properties because this position may mediate disulfide pair bridging and alter T-cell recognition of HLA-B*2705. Wild-type heterotrimers completely unfold in a single transition at mild acidic pH whereas increase of the pH to mild basic conditions induce only a partial biphasic unfolding. Cys-67 seems to play a crucial role in controlling the thermodynamic stability of the B*2705-peptide complexes as the C67S mutant unfolds faster and with a single transition, independent of pH. Fluorescence polarization and size exclusion chromatography of unfolding intermediates suggest that the peculiar unfolding of the B*2705 wild-type heavy chain cannot be explained by modified peptide binding properties but more likely by the formation of high molecular weight species.

There is a striking association between susceptibility to spondyloarthropathies (e.g. ankylosing spondylitis or AS) and expression of the class I MHC HLA-B27 allele (1, 2). Despite intensive research over the last decade, the pathogenic mechanism of the association is still unknown (3, 4). One of the most favored models for disease association postulates the binding to HLA-B27 of autoantigenic peptides, thus mimicking foreign antigens (5). This hypothesis is also supported by a recent finding for a specific T-cell reactivity to a self-peptide for AS patients (6). However, it cannot account for the differential susceptibility to several HLA-B27 alleles even though they select very similar peptide repertoires (7). Recent studies on transgenic mice seem to contradict the arthritogenic hypothesis. Mice lacking β₂m and thus a functional antigen presentation pathway can develop a spontaneous inflammatory arthritis (8). Moreover, this is also observed in the absence of MHC class II genes (9), indicating that B27-derived peptides might not be responsible for the disease association. This evidence has led to an increased number of alternative hypotheses that have been presented over the last few years (4, 10).

The B pocket of the HLA-B27 protein is considered to be the domain with the most influence on peptide selection. HLA-B27 shows a high specificity for Arg (position 2 of the peptide) in the B pocket of the binding site (11). In addition to the role played by the B pocket in peptide selection, this subsite is also believed to play a predominant role in protein misfolding. Mear et al. (12) recently showed that the replacement of the HLA-B27 B pocket with one from HLA-A2 could totally impair the misfolding phenotype of the protein. They proposed that misfolding and its consequences are a basis for susceptibility to spondyloarthropathies rather than allele-specific peptide presentation. The misfolding behavior of the protein could cause endoplasmic reticulum stress responses (13, 14) and therefore influence intracellular signaling pathways. HLA-B27 presents specific features because of a free Cys-67 at the top of the B pocket. This free Cys-67 has been shown to be highly chemically reactive (15), probably because of polarization from a neighboring Lys-70 (16). It has also been hypothesized that oxidative modification of this residue may play a role in B27-related disease (17). Recently Allen et al. (18) showed that a B*2705 heavy chain (HC) homodimer can form and be presented at the cell surface, which is dependent on Cys-67. The recognition of the HLA-B*2705 α₁ helix by the ME1 mAb is impaired by the formation of homodimers, consistent with a partial unwinding of the helix that could be caused by a disulfide bonding. The presentation of such a non-classical homodimer on the cell surface could induce altered T-cell responses, while potentially mimicking a MHC class II binding site, and lead to recognition by CD4 lymphocytes and autoimmunity. Peptides longer than 9 amino acids characteristic of MHC class II ligands have already been eluted from B27, which supports this theory (19).

However, it is still unknown which amino acids of the B pocket control the peculiar misfolding properties of HLA-B*2705. Therefore, we engineered a series of four pocket B mutants of the B*2705 HC and investigated peptide binding properties as well as thermal unfolding of the corresponding complexes with the three peptides. Circular dichroism-monitored thermal unfolding, fluorescence polarization spectroscopy, and size exclusion chromatography suggest that the ther-
modynamic stability of the B*2705 heavy chain is significantly decreased upon mutation of any of the pocket B residues. Cys-67 seems to play a major role in promoting unfolding to high molecular weight species while not affecting peptide binding properties.

**EXPERIMENTAL PROCEDURES**

**Cloning of the HLA-A*0201 Heavy Chain**—The heavy chain was cloned into pET-24b plasmid (Novagen) using the same protocol as described previously (20) for HLA-B*2705. Briefly, the gene was amplified by PCR starting from pQE-30 (Qiagen) containing the A*0201 heavy chain.2 The same primers that were used for B*2705 were used to introduce the two restriction sites Ndel and XhoI flanking the gene. After restriction, the fragment was ligated into the expression plasmid, which was then transformed into Escherichia coli DH5α strain for selection.

**B Pocket Mutants of the HLA-B*2705 Heavy Chain**—All the mutations of HLA-B*2705 heavy chain were introduced by the QuickChange site-directed mutagenesis kit (Stratagene) because it required only the change of a maximum of three neighboring base pairs. For this purpose, we amplified the pET-24b plasmid (Novagen) containing the B*2705 wild-type heavy chain with PCR. The corresponding primer pairs (forward and reverse) used for the four reactions are listed in Table I. The PCR products were then digested with DpnI and transformed into E. coli XL2-Blue strain (Stratagene) for selection. The sequence was confirmed by DNA sequencing (ABI 310 PRISM, Perkin Elmer).

**V67C Mutant of HLA-A*0201**—This mutation was introduced by the same method using the pET-24b-A0201 plasmid as template. The primers used are listed in Table I. The mutation was confirmed by DNA sequencing.

**Protein Expression and Purification**—The expression of the HLA-B*2705 or HLA-A*0201 heavy chains and of the relative mutants were performed in E. coli BL21-Codonplus(DE3)-RIL strain (Stratagene). The proteins were purified on a Ni2+-nitrilotriacetate-agarose column (Qiagen) followed by mass spectrometry and HPLC as previously described (20). Complete deprotection and cleavage from the fluorescein isothiocyanate. Complete deprotection and cleavage from the fluorescein-labeled peptide GRAFVTIK*K (* is the fluorescein label) a lysine with a Dde-protected side-chain was inserted at position 8. The fluorescein-labeled peptide GRAFVTIK*K was reconstituted in a buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 3 mM β-mercaptoethanol, 0.3 mM 2,2'-dithiodiethanol, pH 8.0) containing HLA-B*2705 HC (10 μM), β2m (20 μM), peptide (100 μM), and urea (6 M) against 1 liter of reconstitution buffer. After purification and concentration, the complex was refolded upon dialysis of 10 ml of reconstitution buffer. After purification and concentration, the complex was refolded upon dialysis of 10 ml of reconstitution buffer.

**Determination of Thermal Stability**—The thermal denaturation CD experiments were performed as previously described (20). Briefly, the HLA-B*2705 peptide complex was refolded upon dialysis of 10 ml of reconstitution buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 3 mM β-mercaptoethanol, 0.3 mM 2,2'-dithiodiethanol, pH 8.0) containing HLA-B*2705 HC (10 μM), β2m (20 μM), peptide (100 μM), and urea (6 M) against 1 liter of reconstitution buffer. After purification and concentration, the complex was used immediately for denaturation experiments. The stability of the complex was examined by thermal denaturation, unfolding was monitored by CD spectroscopy at a wavelength of 218 nm. The temperature was raised from 20 to 85 °C at a rate of 40 °C/h. The concentration of the complex was held constant at 1 μM. The melting points were determined following a standard protocol for thermal denaturation experiments (21). The melting points were averaged from at least two independent refolding experiments. Experimental error was estimated to be lower than 1 °C.

**Competition Experiments**—The HLA-B*2705/peptide complex was refolded by dilution of HC (1 μM), β2m (2 μM), labeled peptide (8 nM), and various amounts of competitor (typically 1 μM up to 100 μM) into 1 ml of dilution buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1 mM CHAPS, 0.3 mM 2,2'-dithiodiethanol, pH 8.0). After 36 h of incubation at room temperature, complex formation was confirmed according to a size exclusion HPLC assay (22). The ratio between bound and unbound labeled ligand was determined using fluorescence polarization. Polarization values were measured on a Polarion fluorescence-polarization microtiter plate (Hellma, Germany). The number of flashes was set to 200, total intensity was held at 65,000 rfu. Concentrations at half-maximal inhibition (IC50 values) were obtained by fitting polarization values versus total concentration of competitor to a dose-response model. The IC50 values were averaged from three independent experiments.

**Size Exclusion Chromatography**—Size exclusion chromatography was performed using a Superdex 200 HR 10/30 FPLC column (Amersham Pharmacia Biotech) pre-equilibrated in TBS containing 20 mM Tris, 150 mM NaCl, pH 8.0. Experiments were performed at 25 °C and concentrations were held at 1 μM. 100 μl of sample was loaded onto the column and eluted at 0.7 ml/min in TBS. Elution profiles of the proteins were monitored by UV-absorbance at 280 and 220 nm. Molecular weight was calculated by comparison of the elution retention times of MHC samples to those of marker proteins (data not shown).

**Peptide Dissociation from the MHC/Peptide Complex**—Details of these experiments will be described elsewhere.3 Briefly, the complexes were refolded following the dialysis protocol previously described (20) of 1 ml of protein-containing buffer against 100 ml buffer. We used the fluorescent-labeled peptide GRAFVTIK*K as ligand. FPLC chromatography was performed for purification, without a prior concentration step. After addition of a large excess of unlabeled competitor, the time course of peptide dissociation from the complex was monitored at 28 °C. All measurements were duplicates of independent experiments.

**RESULTS**

**Mutation of Any Pocket B Amino Acid Decreases the Thermal Stability of HLA-B*2705/Peptide Complexes**—To determine the stabilizing properties of B pocket amino acids, we first mutated separately the following four residues of the HLA-B*2705 HC: Thr-24 to Val, Gly-45 to Met, Tyr-99 to Phe, and Cys-67 to Ser. T24V and Y99F changes were chosen to be as conservative as possible. Met-45 is present in the HLA-A*0201 B pocket, which substituting the wild-type B*2705 B pocket impairs misfolding of this allele (12). Furthermore, position 45 is known to control allele-specific peptide binding (23). Last, the C67S mutation was chosen to be as conservative as possible while removing the free Cys.

For refolding HC-β2m-peptide heterotrimers, we used a peptide from the B27-restricted T cell epitope from the HIV1 gp120 protein GRAFVTIKG (K9) (24) and two analogs (GRAFVTIG and I9 and GRAFVTIGS or S9). The mutant-peptide complexes were assembled by a refolding assay starting from the recombinant proteins.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sense</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24V HLA-B*2705</td>
<td>Forward</td>
<td>5'-GCCCGGCTTACTGCTGGCCTAGTTG-3'</td>
</tr>
<tr>
<td>E45M HLA-B*2705</td>
<td>Reverse</td>
<td>5'-CACGTCGATCCAAGCAGGCGGCGG-3'</td>
</tr>
<tr>
<td>C67S HLA-B*2705</td>
<td>Forward</td>
<td>5'-GGGAGACACAGATCTCCAAGGCCAAG-3'</td>
</tr>
<tr>
<td>Y99F HLA-B*2705</td>
<td>Forward</td>
<td>5'-CTTGGCTTTGAGATCTCCAAGGCCAAG-3'</td>
</tr>
<tr>
<td>V67C HLA-A*0201</td>
<td>Forward</td>
<td>5'-CCAAGTAAATGTTGTCGCTAGTTG-3'</td>
</tr>
<tr>
<td>V67C HLA-A*0201</td>
<td>Reverse</td>
<td>5'-CCGAGGAGACACGAGATCTCCAAGGCCAAG-3'</td>
</tr>
<tr>
<td>V67C HLA-A*0201</td>
<td>Reverse</td>
<td>5'-CTTGGCTTTGAGATCTCCAAGGCCAAG-3'</td>
</tr>
</tbody>
</table>

2 D. Rognan and S. Krebs, unpublished results.

3 S. Dédier, T. Reinelt, S. Rion, G. Folkers, and D. Rognan, manuscript in preparation.
Biophysical Studies of HLA-B*2705/Peptide Complexes

FIG. 1. Influence of the mutation of B pocket amino acids on the thermal stability of HLA-B*2705 in complex with three peptides from the HIV1 gp120 parent sequence GRAFVTIGK (K9), GRAFVTIGI (I9), GRAFVTIGS (S9). The thermal denaturation of the complexes is monitored by CD spectroscopy at 218 nm, pH 8.2. £Tm, Tm (wild type) − Tm (mutant).

Bimbinant proteins and were analyzed for their thermal stability by means of CD spectroscopy. The wild-type HLA-B*2705/peptide complexes represent a wide range of thermal stabilities, with values varying from a high melting point of unfolding Tm (60.1 °C for K9), through medium (53.3 °C for I9) and to low values (43.4 °C for S9) (20). Each mutation appeared to be prejudicial to the thermal stability of the mutant-peptide complex with a detrimental effect ranging from about 2.8 °C to 22.6 °C in comparison to the wild-type complexes (Fig. 1). The strongest effects were observed for I9 and K9 complexes for which the destabilization values ranged from −8.1 °C to a maximum of −22.6 °C. The T24V or E45M mutations seemed to be extremely detrimental for the complexes loaded with K9 (16.1 to 22.6 °C destabilization) and less so for the complexes with I9 peptide (around −12 °C). Interestingly, the E45M mutant could still be refolded in the presence of Arg-2-containing peptides. The Y99F and C67S mutations destabilized more the complexes with I9 (−18.1 and −12.4 °C, respectively) than with K9 (−11.7 and −8.1). The smallest destabilization was observed for the complexes with S9. For all mutant/S9 complexes, only a modest destabilization ranging from 3–6 °C could be observed.

Thermal Denaturation of HLA-B*2705 Is Dependent on the pH and Residue at Position 67—The profile of thermal denaturation curves of HLA B*2705/peptide complexes was strongly dependent on pH value but independent of the nature of the complexed peptide. The complex of HLA-B*2705 with a reference peptide (I9; Ref. 20) was therefore denatured under mild alkaline, neutral, and mild acidic conditions (Fig. 2). Changes of the second transition are more easily monitored if the first transition is below 60 °C; therefore, the I9 peptide was chosen as ligand. Under mild alkaline conditions (pH 8.3) a single transition was observed, which led to partial unfolding, reflected by the loss of only a third of the original CD signal (Fig. 2A). Under neutral conditions, an additional step of unfolding was evident that resulted in an almost complete unfolding. Interestingly, the first transition, which is clearly peptide-dependent (data not shown), was unaffected by a pH modification; neither midpoint of transition nor degree of unfolding was changed. The novel intermediate state can be destabilized by lowering the pH to mild acidic conditions (pH 6.4) where both transitions seem to be merged (Fig. 2A). Only a single unfolding step was apparent with an almost complete degree of unfolding that was observed under neutral conditions.

The observation of the strong influence of pH on unfolding raised the question of whether this effect is common to other HLA alleles or if it is a specific property of the B*2705 subtype. Thus, we investigated the unfolding of HLA-A*0201, an allele for which no misfolding has been described (12). The thermal denaturation curves of HLA-A*0201 proteins complexed with the natural epitope ILMEHHKL (25) are presented in Fig. 2B. Under neutral conditions (pH 7.3) unfolding occurred in a single step with the degree of unfolding (>65%) significantly decreased compared with that observed for the reference HLA-B*2705/peptide complex under acidic conditions (~85%). Moreover, for HLA-A*0201, an increase of pH induced a two-step unfolding process. But in contrast to HLA-B*2705, the second step led to only minor additional unfolding of the complex with a total degree of unfolding of ~33%. For HLA-B*2705, the intermediate state could be stabilized by increasing the pH. At pH 9.4, the second transition was still observed but was shifted toward a higher Tm value.

Among the amino acids differing between HLA-B*2705 and HLA-A*0201 HC, Cys-67 was thought to have a major influence on the physiological properties of HLA-B*2705. We hypothesized that the observed differences in unfolding between HLA-B*2705 and HLA-A*0201 might be related to this unpaired cysteine. Thus, we engineered two HLA mutants for which a single point mutation had been introduced (C67S HLA-B*2705, V67C HLA-A*0201). As anticipated, these mutations induced a dramatic change in the unfolding process of the related HLA-peptide complexes. HLA-B*2705 C67S complexed with the I9 peptide now showed a single transition under neutral conditions (pH 7.3) resulting in about 65% unfolding (Fig. 2C). As for the HLA-B*2705 WT complex, an increase of pH value led to a two-step unfolding process, but similar to the HLA-A*0201 WT complex, with only a minor contribution by the second step toward the final degree of unfolding (~40%). No completed unfolding similar to that observed for the wild-type B*2705 HC (Fig. 2A) could be recorded for the C67S HC.

The unfolding of HLA-A*0201 V67C complexes under neutral conditions seemed to be unaffected by the mutation (Fig. 2D). However Cys-67 had a strong influence on the second unfolding step under alkaline conditions. The final degree of unfolding was significantly increased (to ~50%) at pH 8.4 and 9.4 compared with the HLA-A*0201 WT complex (~30%), thus shifting toward the unfolding characteristics of HLA-B*2705 WT complexes.

Cys-67 Promotes Unfolding of B*2705 toward High Molecular Weight Species—To gain further insight into the consequences of B*2705 unfolding, we characterized B*2705 WT and C67S folding intermediates. HLA-B*2705 WT and HLA-B*2705 C67S complexes were subjected to thermal denaturation under mild alkaline conditions (pH 8.0) to obtain the different unfolded states observed by CD spectroscopy and then subjected to SEC and native gel electrophoresis. Under native conditions, both the WT and the mutant HLA-I9 peptide complexes had identical elution times, indicating that both heavy chains form similarly folded complexes (data not shown). After heating to 73 °C, a difference between WT and mutant can again be observed (Fig. 3). For the WT protein, a peak at 11.1 min (corresponding to a mass of >500 kDa) reflects formation of high molecular mass aggregates. No peak corresponding to the heterotrimer, peptide-free B*2705 or HC could be detected and only a small b2m peak was observed.

For the C67S complex, aggregation also takes place, but the aggregates formed have a significant lower apparent molecular mass (~100 kDa). The peak at 20.8 min corresponds to 39 kDa, representing either native complex or peptide-free HC-b2m complex. Upon denaturation to 95 °C, precipitation occurred for the WT and mutant proteins, and only minor peaks indicating the native or peptide-free complex and b2m were observed. No peak corresponding to the recently described B*2705 HC dimer (18) could be identified in our experiments.
Analysis of thermal unfolding by native-polyacrylamide gel electrophoresis confirmed these observations (data not shown).

The C67S Mutation Does Not Affect Peptide Binding Properties of B*2705—To determine whether destabilization of B*2705-peptide complexes upon C67S mutation was correlated with peptide binding affinity, we measured the affinity of a series of four related peptides (Table II) to either the WT or C67S B*2705 mutant by a fluorescence polarization-based assay using a fluorescein-labeled reference peptide (GRAFVTIK*K). Analysis of binding data revealed no significant difference in binding between the WT and mutant (data not shown). For this reason, the IC50 values of the peptides can be compared directly between WT and mutant complexes.

A typical dose-response curve is shown in Fig. 4. The IC50 values obtained by fitting the experimental curve to a dose-response model are listed in Table II. As expected, the higher affinity (in the submicromolar range) was observed for the natural epitope (GRAFVTIGK) and its F3 analog. Replacement of Lys by Ile and Ser at P9 significantly decreased affinity by 1 and up to 2 orders of magnitude, respectively (Table II). Because of the low affinity of the S9 peptide, no complete competition could be observed in the concentration range used for this assay. Therefore, IC50 values for the S9 analog are given as an approximation only. It can be noted that the changes in peptide binding affinity are related to the melting temperatures of the corresponding B*2705/peptide complexes.

Surprisingly, the thermal destabilization recorded upon C67S mutation was not mirrored by a decrease in peptide binding affinities (Table II). Very similar IC50 values were observed for all four peptides to either the WT or the C67S B*2705 mutant. It can be noted that IC50 and Tm values are only related when a series of protein-peptide complexes containing identical HC are taken into account.

The C67S Mutation Increases Peptide Dissociation From the HC-$\beta$2m-Peptide Heterotrimer at 26 °C—The data from these experiments refer only to the dissociation of the labeled peptide from the MHC complex, as confirmed by complementary FPLC experiments (data not shown). Monitoring fluorescence polar-
TABLE II

<table>
<thead>
<tr>
<th></th>
<th>GRAFVTI G</th>
<th>GGRAFVTIGK</th>
<th>GRAFVTIG</th>
<th>GRAFVTIGS</th>
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<tr>
<td>WT</td>
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<td>60</td>
<td>57</td>
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<tr>
<td>C67S</td>
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<td>0.22</td>
<td>2.90</td>
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<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
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<td>41</td>
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</tbody>
</table>

Fig. 4. Determination of peptide binding affinity for HLA-B*2705 IC<sub>50</sub> values. Competition between a fluorescent peptide (GRAFVTIGK*K, *, fluorescein) and a peptide competitor for refolding the HC-peptide heterotrimer is monitored by fluorescence polarization. A typical titration curve is shown for the F3 peptide (GRFFVTI G, Table II). The experimental error of a single data point is 4% or less.

Fig. 5. Measurement of MHC-peptide complex stability at 26 °C by fluorescence polarization. For comparison of HLA-B*2705 WT and C67S, the labeled GRAFVTIGK*K peptide was used. The peptide dissociation kinetics was monitored in solution after addition of a large excess of the same unlabeled peptide to prevent peptide re-association. FP values (in mP units) decreases with release of the fluorescent peptide from the heterotrimer.

**Discussion**

In the current study we investigated the influence of the amino acids of the B pocket (Thr-24, Glu-45, Cys-67, Tyr-99) that are relevant for peptide binding (11) on the thermodynamic stability of several MHC-peptide complexes. Mutation of any of these amino acids resulted in loss of stability of up to 22 °C, which reflects an extreme influence of even a single amino acid mutation. There are no evident relationships between the number of intermolecular hydrogen bonds lost upon mutation and the observed destabilization. For example, the most destabilizing change (T24V) is the consequence of a single H-bond loss, according to the crystal structure of HLA-B*2705 (27). The Y99F mutation resulting in the loss of one direct and two water-mediated H-bonds is also very destabilizing and illustrates the importance of a bound water molecule in the B pocket (27). Surprisingly, modification of the polarity of the B pocket upon E45M mutation did not alter refolding of the B*2705 HC in the presence of Arg-2-containing peptides, although Arg-2 has been shown by x-ray diffraction to make a salt bridge to Glu-45, and despite the lack of evidence for an Arg-2 motif after pool sequencing, the peptide pool naturally bound to this mutant (28). Among the three peptides evaluated, the S9 complex was always the one that induced the least stable complexes. It has to be noted that the WT complexes loaded with S9 already have a low stability (20). It has been proposed that until a certain threshold of stability (T<sub>m</sub> of ~36 °C) has been reached, the mutation of such weakly stable complexes will have little influence on their thermodynamic properties. However, it should be stated that lower in vitro thermodynamic stability might not always be related to lower in vivo stability, because some potentially weakly stable complexes are not refolded in vivo, or the corresponding peptide is presented in very low amounts.

Interestingly, the C67S mutant destabilized the complex less drastically than the other three mutants of the B pocket. Some recent publications give great importance to Cys-67, because this residue is implicated in the formation of heavy chain homodimers. For this reason, we decided to focus our experiments on the C67S mutant. Our data show a significant difference between thermal unfolding of HLA-B*2705 and HLA-A*0201 complexes with regard to response to variation of pH. Shifting the pH toward alkaline conditions induces a two-step thermal unfolding. The first one is clearly peptide-dependent and is probably the consequence of peptide release from the heterotrimer. The second one, which appears at basic pH, is significantly increased for MHC proteins bearing a free cysteine (HLA-B*2705 WT, HLA-A*0201 V67C mutant). A simple explanation for these different unfolding processes can be clearly attributed to the amino acid at position 67 of HC with Cys-67 responsible for an increased final degree of unfolding after the second transition. Mutation of the amino acid at position 67 of the HC is reflected by almost complete interconguration of the unfolding profiles of HLA-B*2705 and HLA-A*0201/peptide complexes. It is already known that Cys-67 is
more acidic than expected, because it can be alkylated by an aziridine-containing peptide ligand whereas free Cys in solution cannot (15). Thus, any increase in the pH would favor the existence of Cys-67 as a very nuclophilic thiolate anion promoting oligomerization of the B*2705 HC.

Our hypothesis is supported by size exclusion chromatography of unfolded intermediates of B*2705 and its C67S mutant, obtained at basic pH. It clearly indicates that the mutation of Cys-67 results in the loss of high molecular weight unfolded states that characterize the unfolded B*2705 WT heavy chain. Surprisingly, the observed destabilization of the heterotrimer upon Cys-67 mutation is not reflected by a decreased affinity of peptide ligands to the mutant (Table II). We have shown that IC50 values for three analogous peptides are not significantly affected by mutation of Cys-67 to Ser. These data are contradictory to previous results (29) for which a correlation between the equilibrium dissociation constant (Kp) and thermal stability (T_m) of the complex is described. They can however easily be explained by the different experimental protocols used in both studies. Whereas Morgan et al. (29) correlates Kp values obtained from equilibrium dialysis experiments using a refolded peptide-free HC (29), our assay includes the refolding of the heterotrimer in the presence of the peptide and therefore does not lack the important contributions of protein-peptide interactions for complex refolding. Furthermore, as peptide loading of class I MHC complexes occurs in vivo during folding of the proteins in the endoplasmic reticulum (30), our assay is more likely to mirror physiological conditions and therefore is more suited to compare peptide selection of complexes containing different heavy chains subtypes.

The different contributions of Cys-67 to thermal stability and peptide binding suggest that Cys-67 is not directly involved in MHC-peptide interactions participating in the refolding of the HC—β2m-peptide heterotrimer. However, once the MHC-peptide complex has been formed, Cys-67, as well as any amino acid of the B pocket, plays an important role in maintaining a stable heterotrimer. Regarding these findings, our study strongly suggests that the nature of the selected peptide repertoire is not sufficient to explain the different associations of HC subtypes with autoimmune diseases (31). Whereas the unfolding behavior of HLA-B*2705 is clearly different from that of HLA-A*0201 because of the presence of a free cysteine, it is still not clear why the expression of several alleles has not been associated with high susceptibility to autoimmune disorders, Cys-67 being conserved in all 20 HLA-B27 alleles reported to date (10).

Furthermore, the results presented here confirm the important role of Cys-67 for in vitro properties of HLA-B*2705 complexes. This observation is an interesting example of the enormous influence of a single amino acid on the unfolding process of a protein. This finding is even more important in regard to the recently reported theory that postulates that misfolded MHC-proteins are causative for the association of HLA-B*2705 and autoimmune diseases (7, 30). Our data show that the HLA-B*2705 complex unfolds in a subtype-specific manner upon thermal denaturation. This observation suggests that not only heavy chain dimers (18) but also abnormally unfolded complexes may be the species responsible for autoimmune disease. Dimerization of class I MHC-HC seems to be a general property of HC containing unpaired Cys-residues (32) and therefore it cannot explain the peculiar association of B*2705 expression with autoimmune diseases. Triggering could either occur by altered TCR recognition or endoplasmic reticulum stress due to an increased level of protein aggregation (4, 33). It will be important to evaluate whether these specific properties of the complex are relevant for activation of CTL resulting in autoreactivity. However, the peculiar structural influence of Cys-67 on the thermodynamic property of HLA-B*2705 does not explain why the expression of a few B27 alleles is not associated with autoimmune disorders (10) even though Cys-67 is conserved for all B27 alleles. Rats transgenic for the C67S HLA-B*2705 mutant still develop arthritis, albeit with less severity than WT B*2705 rats (34). Furthermore, it remains to be determined whether only Cys-67 has the described influence or if mutation of further B pocket amino acids specific for HLA-B*2705 can induce similar effects.

Acknowledgments—We thank Dr. T. Reitinger for synthesis of the fluorescent-labeled peptide and Prof. J.A. López de Castro for critical reading of the manuscript.

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