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Thermodynamic Stability of HLA-B*2705·Peptide Complexes

EFFECT OF PEPTIDE AND MAJOR HISTOCOMPATIBILITY COMPLEX PROTEIN MUTATIONS*

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Designing synthetic vaccines from class I major histocompatibility complex (MHC)-binding antigenic peptides requires not only knowledge of the binding affinity of the designed peptide but also predicting the stability of the formed MHC-peptide complex. In order to better investigate structure-stability relationships, we have determined by circular dichroism spectroscopy the thermal stability of a class I MHC protein, HLA-B*2705, in complex with a set of 39 singly substituted peptide analogues. The influence of two anchoring side chains (P3 and P9) was studied by peptide mutation and appropriate site-directed mutagenesis of the HLA-B*2705 binding groove. The side chain at P9 is clearly the one that contributes the most to the thermal stability of the MHC-peptide complexes, as destabilization up to 25 °C are obtained after P9 mutation. Interestingly, structure-stability relationships do not fully mirror structure-binding relationships. As important as the C-terminal side chain are the terminal ammonium and carboxylate groups. Removal of a single H-bond between HLA-B27 and the terminal peptide moieties results in thermal destabilization up to 10 °C. Depending on the bound peptide and the location of the deleted H-bond, the decrease in the thermal stability of the corresponding complex is quantitatively different. The present study suggests that any peptidic amino acid at positions 3 and 9 promotes refolding of the B27-peptide complex. Once the complex is formed, the C-terminal side chain seems to play an important role for maintaining a stable complex.

Class I MHC[1] molecules play a key role in the intracellular recognition of pathogens by continuously presenting antigenic peptides to CD8+ T cells (1). The molecular rules underlying peptide recognition by class I MHC proteins have been fairly well elucidated by x-ray diffraction of several MHC-peptide complexes (2) and determination of allele-specific binding motifs of MHC-bound self-peptides (3, 4). Most of the peptides selected by class I molecules are nonapeptides and present anchoring backbone atoms at both termini and MHC-binding side chains at positions 2 (P2) and at the C terminus (P9). Auxiliary anchors at P1 and P3 usually fine tune peptide recognition (5). Each anchoring side chain interacts with one of the 6 polymorphic MHC pockets (6), whose locations have been conserved along evolution but whose physicochemical properties are highly variable and thus ensure allele specificity (7). As peptide-free class I MHC molecules are unstable under physiological conditions, binding of the ligand is concomitant to refolding of the MHC-peptide complex (8). Therefore, determining the exact binding affinity of a peptide to its host MHC receptor is not an easy task. At least two different binding assays have been described in the literature. The most commonly used is an epitope stabilization cell assay (9) in which peptide-free MHC molecules are artificially produced at 26 °C (10) and then loaded with a peptide ligand to promote assembly of the binary complex at the cell surface. Subsequently, one can perform a classical displacement of a radiolabeled peptide with a competitor to determine the inhibition constant of the competing ligand (11). A second type of binding assay involves refolding of recombinant β2m and heavy chain (HC) (12) in the presence of a peptide ligand and a competitor (13, 14). The latter assay is likely to be closer to physiological conditions since it better describes competition occurring *in vivo* within a peptide pool. The way binding affinity is measured is important for studying structure-binding relationships and determining rules governing epitope selection and peptide immunogenicity. Most studies agree to conclude that immunogenic peptides are generally found among the top binders (15) but several exceptions to this rule have already been reported (16, 17). Hence, the stability of the MHC-peptide complex has been proposed to be a better descriptor of immunogenicity than peptide binding affinity (18). As only few structure-stability data are available (19–21), we have undertaken a study in which two critical positions of a class I MHC-binding peptide have been systematically modified and their influence on the stability of the resulting MHC-peptide pairs evaluated. HLA-B*2705 was chosen as class I MHC protein because its expression is highly associated to the triggering of autoimmune inflammatory diseases with a still rather obscure mechanism (22). An HLA-B27-restricted epitope (GRAFVTIGK, one-letter code) from the HIV1 gp120 protein (23) was used as reference peptide. To mirror as closely as possible physiological conditions, a refolding assay was used to assemble HC-β_2m-peptide heterotrimers and their thermal stability examined by CD spectroscopy. We present herein the effect of peptide and HLA mutation on the thermal stability of the reconstituted heterotrimers.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides were obtained by automated solid-phase peptide synthesis using an automated multiple peptide synthesizer (Syro Multi-Syn-Tech, Bochum, Germany) using a standard Fmoc

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[1] The abbreviations used are: MHC, major histocompatibility complex; HC, heavy chain; β_2m, β_2-microglobulin; HLA, human leukocyte antigen; PCR, polymerase chain reaction.

(*N*-(9-fluorenyl)methoxycarbonyl) protecting strategy. Peptides were analyzed and purified by analytical or preparative reverse-phase high performance liquid chromatography on a LiChrospher RP-18 column (Merck) and on a C18 column (Waters), respectively. The following gradients were used: from 10 to 60% A over 30 min or from 0 to 50% A over 30 min at the flow rate of 0.6 ml/min on the analytical column and from 10 to 40% A over 30 min at the flow rate of 15 ml/min on the preparative column. The binary solvent system (A/B) was as follows: 0.1% trifluoroacetic acid in acetonitrile (A) and 0.1% trifluoroacetic acid in water (B). The absorbance was detected at 220 nm. Electrospray ionization mass spectrometry was performed for all final peptides on a Finnigan mass spectrometer.

*Cloning, Expression, and Purification of the HLA-B*2705 Heavy Chain*—The region coding for amino acids 1 to 274 of the HLA-B*2705 heavy chain was modified from *pB27* (gift of Dr. K. C. Parker, National Institute of Health, Bethesda, MD) by polymerase chain reaction (PCR) using the oligonucleotides primers: forward 5′-CGATAT**CATATG**GG-ATCCCACTCCATGAGG-3′ and reverse 5′-CGCCGC**CTCGAG**CCAT-CTCAGGGTGAGGGGC-3′ (all primers are from Microsynth GmbH). Bold sequences are coding for the inserted *Nde*I and *Xho*I restriction sites flanking the *B*2705* gene. The resulting fragments were digested with *Nde*I and *Xho*I, and ligated into the expression plasmid pET-24b(+) (Novagen). Selection was performed in *Escherichia coli* DH5α and the sequence confirmed by sequencing (ABI 310 PRISM).

For expression of the C-terminal polyhistidine tag fusion protein we used *E. coli* BL21-Codonplus(DE3)-RIL strain (Stratagene). Bacteria were grown at 37 °C in LB broth containing 34 μg/ml chloramphenicol and 50 μg/ml kanamycin. Protein expression was induced with 0.24 mM isopropyl-β-D-thiogalactopyranoside and the cells harvested after 3.5 h. The protein was overexpressed as inclusion bodies mainly. Bacterial pellets were resuspended in 8 M urea, 20 mM Tris, 150 mM NaCl, pH 7.5 (8 M urea buffer), and centrifuged to remove insoluble material. The crude extract was then filtered and passed over a Ni^{2+}-nitrilotriacetate-agarose column (Qiagen), and after washing with 8 M urea buffer containing 30 mM imidazole, the heavy chain was eluted with the elution buffer (8 M urea buffer containing 250 mM imidazole).

Cloning, Expression, and Purification of Human β_2-Microglobulin—β_2-Microglobulin was cloned into pET-15b (Novagen), as a fusion protein with a N-terminal polyhistidine tag. The gene coding for β_2-microglobulin was modified from *pGex-β2m* (24) by PCR with the oligonucleotide primers: forward 5′-CCAAGC**CATATG**ATCCAGCG-TACTCCAAAGATTC-3′ and reverse 5′-CCAAGC**GGATCC**TCACAT-GTCTCGATCCCACTTAACTATC-3′. Bold sequences code for *Nde*I and *Bam*HI restriction sites. The fragment was digested with *Nde*I and *Bam*HI, the insert ligated into the plasmid pET-15b and transformed in *E. coli* DH5α for selection.

Expression was performed in the *E. coli* BL21-Codonplus(DE3)-RIL strain. Bacteria were grown for 3.5 h after induction with 0.24 mM isopropyl-β-D-thiogalactopyranoside at 37 °C in LB broth containing 34 μg/ml chloramphenicol and 100 μg/ml ampicillin. After harvesting the cells, the bacterial pellets were resuspended in 8 M urea buffer and centrifuged. The protein was purified on a Ni^{2+}-nitrilotriacetate-agarose column (Qiagen) with 8 M urea buffer containing 30 mM imidazole. The His-tag was cleaved by thrombin (Serva, 80 units/liter cell culture) after successive dilutions of urea and adduct of 2.5 mM $CaCl_2$ to the buffer. After cleavage, β_2-microglobulin was directly eluted with the same buffer.

Site-directed Mutagenesis—All the mutations of the B*2705 heavy chain were introduced by point mutation with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The single mutants (Y7F, Y171F, Y84F, and T143A) were obtained by PCR, starting from the wild type heavy chain in pET-24b. The double mutants were obtained from the two single mutants Y171F and T143A (to be modified into Y7F/Y171F and Y84F/T143A) in pET-24b. This method requires the use of two complementary primers for the PCR reaction. The following pairs have been used: Y7F-forward, 5′-CCCACTCCATGAGGTTTTTCCACA-CCTCCG-3′ and Y7F-reverse, 5′-CGGAGGTGTGGAAAAACCTCATG-GAGTGGG-3′; Y171F-forward, 5′-GGCTCCGCAGATTCCTGGAGAA-CGGG-3′ and Y171F-reverse, 5′-CCCGTTCTCCAGGAATCTGCGGAG-CC-3′; Y84F-forward, 5′-CCCTGCTCCGCTTCTACAACCAGAGCG-3′ and Y84F-reverse, 5′-CGCTCTGGTTGTAGAAGCGGAGCAGGG-3′; T143A-forward, 5′-GCGGCTCAGATCGCCCAGCGCAAGTGG-3′ and T143A-reverse, 5′-CCACTTGCGCTGGGCGATCTGAGCCGC-3′.

The PCR products were transformed after digestion with *Dpn*I into *E. coli* XL2-Blue strain (Stratagene) for selection. All the mutations were confirmed by DNA sequencing. Expression of all the mutants were performed in *E. coli* BL21-Codonplus(DE3)-RIL strain as described previously for the wild type heavy chain.

Protein Identification—Protein concentration was determined using the Bradford protein assay (Bio-Rad). The proteins were identified by N-terminal sequencing (Edman degradation performed at the sequencing service of the ETH Zurich). Purity was checked by SDS-polyacrylamide gel electrophoresis performed in 10% acrylamide for heavy chains or in 15% acrylamide for β2m and stained with Coomassie Brilliant Blue R-250 (Fluka). The heavy chain has an apparent mass of 34 kDa and β2m of about 12 kDa.

*HLA-B*2705·Peptide Complex Refolding*—Refolding of the MHC-peptide complex was performed by dialysis of 10 ml 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 (10 μM), β_2-microglobulin (20 μM), peptide (100 μM), and urea (6 M) against 1 liter of reconstitution buffer. After 36 h at 4 °C, the solution was concentrated to 1 ml with an ultrafiltration unit (Ultrafree 15 MWCO: 10 kDa, Millipore). Purification was carried out on a gel-filtration column (Superdex 75 16/60, Amersham Pharmacia Biotech) pre-equilibrated in analysis buffer (20 mM Tris, 150 mM NaCl, pH 8.0). The purified complex was concentrated on a Ni^{2+}-agarose column and eluted with analysis buffer containing 250 mM imidazole. Imidazole was then removed from the samples by gel filtration (P6-Gel, Bio-Rad) using an analysis buffer containing 0.5 mM imidazole to prevent aggregation due to the poly-His tag of the heavy chain. The eluted fractions were immediately used for denaturation experiments.

CD Spectroscopy—Stability of the refolded complex was examined by thermal denaturation experiments. Unfolding was monitored by CD spectroscopy at a wavelength of 218 nm as the temperature was raised from 20 to 85 °C at a rate of 40 °C/h. The experiments were performed on a Jasco J-720 polarimeter using a water-jacketed 5-mm sample cell. Samples were measured using the above described analysis buffer containing 0.5 mM imidazole. Concentration of the complex was determined photometrically and was held at about 1 μM.

The unfolding of the complex was regarded as a two-state transition (25). Therefore, the melting points were determined following a standard procedure for thermal denaturation experiments (26). Due to the different level of cooperativity of thermal unfolding, thermodynamic values (ΔH_m and Δc_p) were not taken into account for the comparison of different ligands. The melting points are averaged from two or three independent refolding experiments and the error was estimated to be lower than 1 °C.

RESULTS

The presence of a C-terminal polyhistidine tag on the B*2705 HC while facilitating the purification of HC does not impair proper refolding of a reference heterotrimer as (i) a peptide-dependent peak could be detected by FPLC gel filtration (Fig. 1, *A* and *B*); (ii) the isolated peak when analyzed by SDS-polyacrylamide gel electrophoresis is consistent with the presence of heavy and light chains (Fig. 1*C*), (iii) the heterotrimer formed with the B27-restricted T cell epitope from the HIV1 gp120 protein (GRAFVTIGK) (23) presents a midpoint of thermal unfolding (T_m = 60.1 °C, Fig. 1*D*) at a temperature similar to that reported for the native B*2705-GRAFVTIGK complex (27).

Influence of P3 and P9 Side Chains on the Thermal Stability of HLA-B27·Peptide Complexes—To study the influence of peptide side chains on the thermal stability of B27-peptide complexes, a limited choice of positions is indeed possible. Intermediate residues (P4 to P8) are unsuited candidates for at least three reasons: (i) they bulge out of the binding groove in most of known crystal structures (2); (ii) peptide binding motifs identified by pool sequencing of naturally bound peptides show pronounced variability at these positions (3); (iii) they can be replaced by polyglycine (28, 29) or organic fragments (24, 29–32) without significant changes in both affinity and complex stability. Auxiliary anchors (P1, P3) are more interesting as they fine-tune the allele-specific peptide selection (33). We have chosen P3 as representative of auxiliary anchors because previous reports suggest an important role of this side chain in B*2705 binding (34, 35). Among the remaining two strong anchors (P2, P9), we selected P9 for a systematic modification. B27 binding requires the presence of an arginine side chain at P2 (3, 36, 37). Gln is the only other residue that is tolerated for

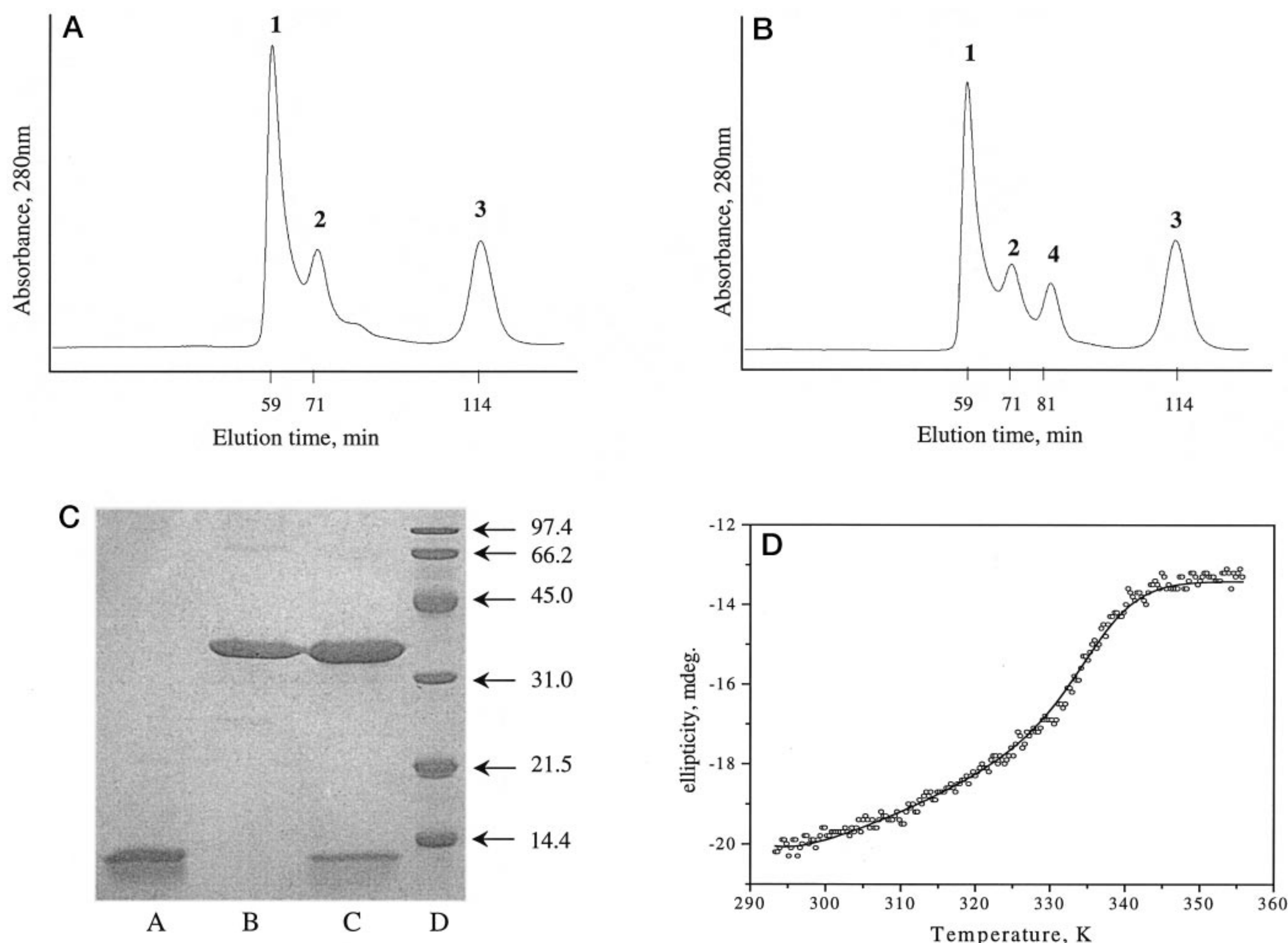

FIG. 1. **Characterization of the HC-β_2m-peptide heterotrimer.** The reference peptide (GRAFVTIGK) is a B*2705-restricted viral epitope from the HIV1 gp120 protein (23). Fast protein liquid chromatogram of refolded products without (*A*) and with the reference peptide (*B*). *Peaks 1* and *2* correspond to heavy chain aggregates, *peak 3* represents the β_2m light chain, and *peak 4* is only obtained upon refolding in the presence of a B*2705-binding peptide; *C,* SDS-polyacrylamide gel showing β_2m (*lane A*), B*2705 heavy chain (*lane B*), and the contents of FPLC peak 4 (*lane C*). Positions of molecular weight markers (in kDa) are indicated in the *right margin*; *D,* thermal denaturation by CD spectroscopy of the heterotrimer eluted in peak 4 (T_m = 60.1 °C).

B*2705 but at the cost of a reduced stability of the MHC-peptide complex (28, 38). Most of the P2 variants will probably not induce any detectable refolding of the HC-β2m-peptide heterotrimer. Therefore, we decided to choose P9 for further investigation.

The auxiliary anchor at P3 was first systematically varied and its influence on the thermal stability of the resulting B*2705-peptide complexes studied (Fig. 2*A*). Most of the complexes obtained were equally stable (averaged T_m = 56.4 ± 3.65 °C) with a very narrow distribution range of melting temperatures (from 48.9 to 61.0 °C). Only 4 residues (Pro, Asp, Glu, and Arg) are relatively disfavored at P3 resulting in complexes with T_m values about 50 °C. The 16 remaining amino acids lead to comparable melting temperatures (about 57 °C) and no clear structure-stability relationships can be derived from the present data. From glycine to lysine, a vast array of physicochemical properties has been scanned without significantly influencing the thermal stability of the B*2705 HC. Small (Ala and Ser) as well as large side chains (Met) are equally preferred. The polarity of the P3 residue does not seem to play a major role in establishing thermally stable complexes (compare Ile and Thr, for example).

In opposition to P3 changes, mutation of the main P9 anchor drastically affects the stability of the MHC-peptide pair (Fig. 2*B*). T_m values are distributed over a much broader range (from 36.7 to 60.1 °C) than for the above described P3 mutation. The mean T_m value, averaged over the 20 possible residues (averaged T_m = 47.5 ± 7.4 °C) is lower than that observed for P3 variants. The 20 natural amino acids at P9 can be clustered into four groups corresponding to B*2705-peptide complexes of decreasing thermal stabilities: (i) class A: positively charged residues (Lys and Arg) with T_m about 60 °C; (ii) class B: bulky aliphatic (Val, Ile, Leu, and Met) and aromatic residues (Tyr and His) with T_m about 53–55 °C; (iii) class C: small aliphatic (A), bulky aromatic (W), and polar uncharged residues (Ser, Thr, Cys, and Gln) with T_m about 43 °C; (iv) class D: negatively charged (Asp and Glu), conformationally peculiar amino acids (Gly and Pro), and Asn, with T_m about 37 °C. For the last peptide group, the stability of the resulting complex is slightly lower than that reported for other peptide-free class I MHC heavy chains (T_m about 40 °C) (21, 39) although a HC-β2m-peptide heterotrimer could be detected for each of the latter peptides.

Role of the N- and C-terminal Peptide Atoms—To check the importance of peptide N- and C-terminal ends, we selectively mutated four MHC side chains that anchor, in the known B*2705 crystal structure (37), the peptide ammonium and carboxylate moieties (Tyr^{7}, Tyr^{171}, and Tyr^{84}, Thr^{143}, respectively; see Fig. 3*A*). Mutations were chosen to be as conservative as possible (Tyr to Phe, Thr to Ala) and result only in the loss of

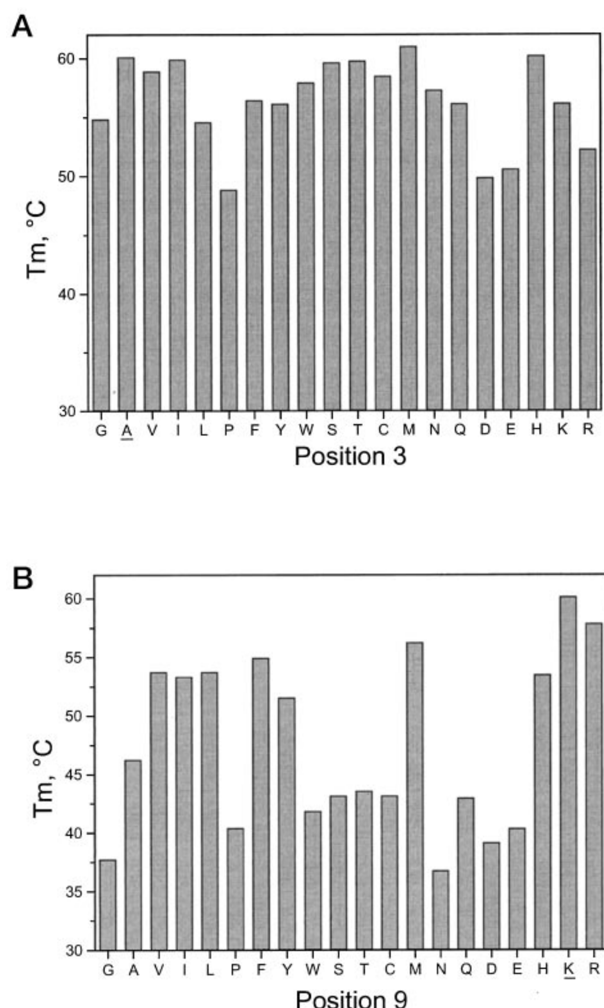

FIG. 2. **Influence of two peptide anchoring side chains on the thermal stability of B*2705 in complex with 20 singly substituted peptide analogues.** Thermal denaturation is monitored by CD spectroscopy of the complex at 218 nm, pH 8.0 (see "Experimental Procedures") *A,* stabilizing role of the P3 position (GRXFVTIGK, X, any natural α-amino acid); *B,* stabilizing role of the P9 position (GRAFVTIGX). The residue occurring in the parent peptide are *underlined*.

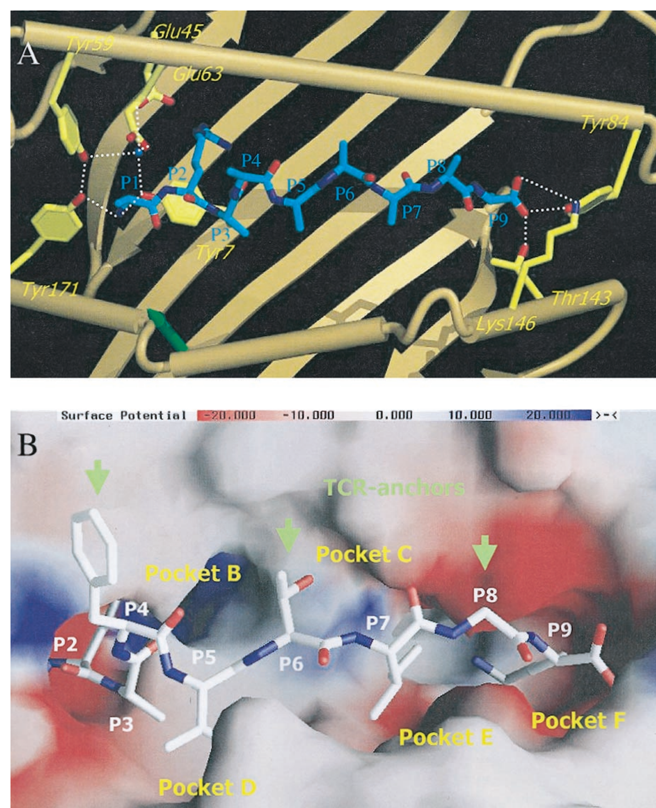

FIG. 3. **Structure of a HLA-B*2705·peptide complex.** *A,* intermolecular interactions between HLA-B*2705 and peptide terminal atoms in the x-ray structure of a HLA-B*2705·peptide complex (37) (Protein Data Bank entry, 1hsa). Only the hydrogen bonds (*white broken lines*) between the peptide (*cyan*) N- and C-terminal ends and the MHC binding groove (*yellow*) are displayed. The protein backbone is displayed as *cylinder* (α-helices), *arrows* (β-strands), and *tubes* (random coils). A disulfide bridge is displayed in *green* and a bound water molecule as a *blue ball*. The figure has been prepared using PREPI (44) and rendered with Pov-Ray (45). *B,* electrostatic potential in *kt/e* of the HLA-B*2705 crystal structure displayed at the molecular surface, by the GRASP package (46). The reference GRAFTVIGK peptide has been modeled in the binding groove by homology to the x-ray structure of the B*2705-bound ARAAAAAAA peptide (Protein Data Bank entry: 1hsa). *Green arrows* indicate putative T cell receptor anchors. Peptide positions and MHC pockets are indicated, excepted for peptide position 1 (P1) and its respective pocket A, which are both masked by the protein surface.

one MHC-peptide hydrogen bond for each individual change. The influence of the four mutations was observed for three peptides inducing MHC-peptide complexes of decreasing stabilities (the reference GRAFVTIGK peptide or A3, GRAFVTIGI or I9, GRAFVTIGS or S9, recall Fig. 2, *A* and *B*).

Each mutation was detrimental to the thermal stability of all investigated complexes (Fig. 4*A*). Depending on the peptide loaded, the loss of a single H-bond resulted in a decrease of the stability up to 10 °C. Interestingly, the mutation effect depends on the location of the mutated residue and on the bound peptide (Fig. 4*A*). For the class A peptide (A3), three mutations (Y7F, T143A, and Y171F) are destabilizing (more than 5 °C) and the last one (Y84F) almost negligible. In contradiction with this observation, all mutations are very destabilizing for the I9 peptide (class B) with rather similar effects (ΔT_m about 10 °C) except for the Y84F change for which only a 5 °C destabilization effect is observed. For the class C peptide (S9), the destabilization seems to mainly occur after pocket A mutations (Y7F and Y171F, ΔT_m about 5 °C) whereas pocket F mutations (Y84F and T143A) are less detrimental (Fig. 4*A*).

However, the consequence of a single mutation at each end of the binding groove could be compensated by a slight conformational change of the B*2705 heavy chain allowing the building of new H-bonds, especially with the neighboring Tyr^{59} and Lys^{146} side chains, see Fig. 3*A*) To determine whether the B*2705 protein may conformationally adapt to the proposed individual mutations, double changes (Y7F/Y171F and Y84F/T143A) were performed and their effect on the stability of the resulting complexes investigated with two peptides (A3, I9) investigated (Fig. 4*B*). Note that the S9 peptide was not used here for loading as the double mutations would probably lead to complexes of low stability and poor refolding. The double mutants could be refolded in the presence of the two selected peptides and the resulting complexes presented wavelength CD spectra similar to that of the corresponding wild type and single mutant complexes (data not shown) indicating that double mutations did not affect the conformation of the refolded heterotrimer. As for the single mutations, the double changes affect more the thermal stability of the resulting complexes with the I9 peptide than with the A3 peptide. Interestingly, the effect of the double mutation at pocket F on MHC-A3 complexes are nearly synergistic with respect to the individual changes. For the MHC-I9 complex, 80% of the summed contributions of individual changes are recovered upon double mutations.

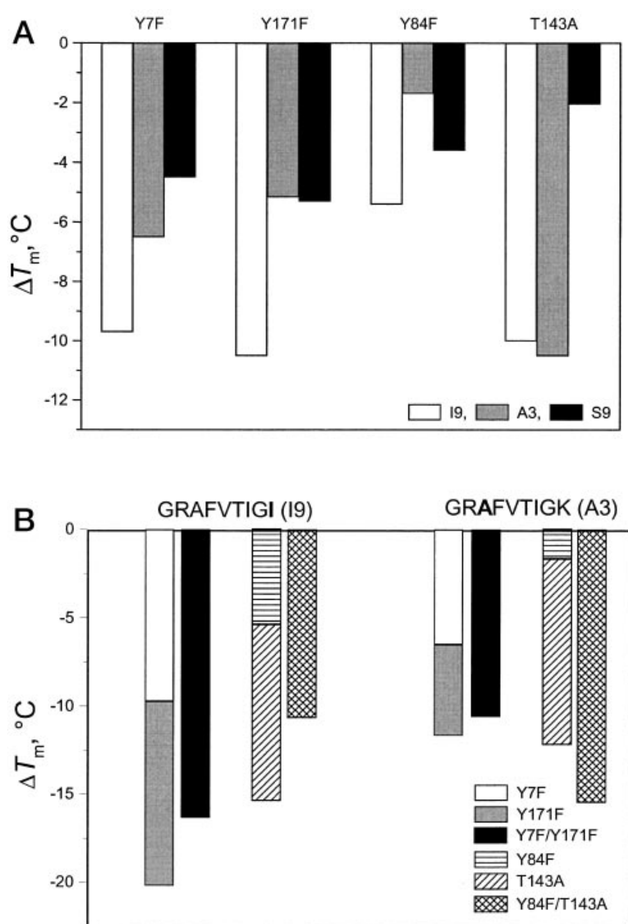

FIG. 4. **Influence of the peptide terminal ammonium and carboxylate groups on the thermal stability of B*2705 in complex with three HIV1 gp120 peptide analogues (GRAFVTIGI, GRAFVTIGK, and GRAFVTIGS).** Thermal denaturation is monitored by CD spectroscopy of the complex at 218 nm, pH 8.0 (see "Experimental Procedures"). $\Delta T_m = T_m$ (wild type) $- T_m$ (mutant). *A,* influence of single pocket mutations (pocket A: Y7F, Y171F; pocket F: Y84F, T143A. *B,* influence of double mutations Y7F/Y171F and Y84A/T143A.

DISCUSSION

In contradiction with a recent report (40), we succeed in refolding a recombinant B*2705 HC in the presence of β_2m and a B*2705-restricted peptide. Two peptide positions (P3 and P9) were selected for systematic modification as they have recently been shown to play a major role in B*2705 binding (33). The invariant Arg[2] could not been varied as this modification will lead to heterotrimer assemblies that are almost impossible to refold and purify. For example, the switch of the Arg[2] side chain from the Cα to the backbone N atom (peptide to peptoid) totally impairs heterotrimer assembly.[2]

Only a few side chains at position 3 (Asp, Glu, Arg, and Pro) significantly destabilize the MHC-peptide pair. This observation is in full agreement with already described epitope stabilization binding data (33). Pro[3] is likely to limit the conformational freedom of the N-terminal P1-P3 tripeptide and probably impairs proper binding of Arg[2] to pocket B. The detrimental effect of negatively charged residues at P3 can be easily explained by looking at the crystal structure of HLA-B*2705 (36), as the corresponding pocket D interacting with the P3 side chain presents a strong negative electrostatic potential at its upper surface (Fig. 3*B*). Furthermore, the pocket D is probably not deep enough to accommodate the bulky Arg side chain. However, structure-stability relationships observed for this peptide series do not fully mirror known structure-binding data (29, 33, 34) especially for hydrophobic and aromatic residues which are not preferred over Ala at P3, although pocket D is very hydrophobic in its deeper part. In the current stability study, aromatic (Phe, Tyr, and Trp) and small side chains (Ala, Ser, and Thr) at P3 induce similar thermal stabilities of the resulting complexes, whereas epitope stabilization assays rank aromatic residues far before small amino acids (33). Three reasons may explain this discrepancy. First, full occupancy of pocket D may be required for a strong binding but not to maintain a stable MHC-peptide binary complex. In favor of this hypothesis, one observes that electron density arising from the peptide pool co-crystallized with HLA-B*2705 does not entirely fill pocket D (36). Another plausible explanation for these peculiar structure-activity relationships is the possible interplay between P3 and Val[5] side chains in the current peptide series, that might both point toward the same pocket D if P3 is a small residue (Fig. 3*B*). An A3F change should induce a stabilization effect in the light of known binding data. However, this would require a conformational change of the peptide backbone at position 5 in order to prevent steric repulsion between P3 and P5 side chains. Hence, the net stabilizing effect of a Phe[3] side chain (with a Val[5] side chain pointing outwards the binding cleft) would be similar to that of Ala[3] with a Val[5] side chain pointing toward the binding groove. This would explain why small-sized polar side chains (Ser, Thr, and Cys) are also compatible in the current series with a stable MHC-peptide assembly (Fig. 2*A*). Similar interdependence of anchor side chains have been reported by x-ray diffraction of human (HLA-A2) (41) and murine (H-2K^b) (42) class I MHC molecules. A last possible explanation is that peptides bearing a very strong P9 anchor (*e.g.* Lysine) are less sensitive to P3 changes. Compensating effects between P3 and P9 anchors have recently been described for HLA-B27-binding peptides (33).

Systematic variation of the P9 residue shows that the overall stability of B*2705-peptide complexes is highly dependent on the C-terminal side chain. The two basic residues (Lys and Arg) are intrinsically the best amino acids at the C terminus. Hydrophobic aliphatic residues are also favored at P9 which is in total agreement with known binding data. Surprisingly, we found that Phe is also ranked among the top amino acids at P9 in contradiction with epitope stabilization assays (33) but in agreement to another refolding study (43). As the P9 side chain is deeply bound to pocket F and almost entirely buried upon binding, side chain interdepence cannot be invoked here. A logical explanation to these discrepancies is that a Phe/Arg P9 side chain contributes to the refolding and stability of the heterotrimer but does not readily enter the F pocket of a peptide-free B27 binding groove. As expected from previous binding data, Gly, Pro as well as acidic and polar side chains are clearly disfavored at P9 which interacts with a negatively-charged pocket F (Fig. 3*B*).

The wide variation found between the two extremes (Lys and Asn, ΔT_m about 25 °C) demonstrates that the P9 side chain may control in part the overall stability of the B27-peptide complexes. As P9 can be considered as an isolated anchor, far from the N-terminal tripeptide part, we wondered whether peptide release might systematically start at the C terminus. Hence, a peptide with a dissociated C-terminal residue would present about two-thirds of its total surface (from P4 to P9) out of the binding groove and its full release from the MHC groove would be consequently facilitated. However, P9 side chain release might probably require disruption of the conserved H-

[2] S. Dédier, S. Reinelt, T. Reitinger, G. Folkers, and D. Rognan, unpublished data.

bonds between the C-terminal carboxylate atoms and the B27 binding groove (Tyr[84] and Thr[143]; see Fig. 2*A*). Therefore, we engineered a couple of B*2705 mutants for which side chains interacting with both peptide termini have been mutated. Thus, we can compare thermal destabilization upon selective H-bond removal at either the N- or the C-terminal peptide end. The obtained results suggest that both termini are similarly important for B*2705 stabilization. The removal of a single H-bond out of the 4 that anchor peptide terminal atoms to the MHC molecule corresponds in term of thermal destabilization (5 to 10 °C) to the mutation of a top class P9 side chain (Lys and Arg) to a second or third class residue (Ser, Cys, and Thr, for example). This result is in agreement with a previous report on HLA-A2-binding peptides for which the terminal ammonium and carboxylate atoms had been replaced by methyl groups (25). However, our study presents the advantage to determine the effect of single H-bond removal at both ends of the binding groove. This effect is dependent on the bound peptide and on the location of the lost interaction. A class A peptide (A3 analogue) is less sensitive than a class B ligand (I9 analogue, Fig. 4*A*). Surprisingly, the consequence of a single mutation was less dramatic for the class C peptide (S9 peptide) especially upon pocket F mutation. It must be noted that the S9 peptide already induces a wild type complex of low stability.

Taken together, it is, however, somewhat difficult to explain all individual changes. Tyr[84] seems to be the MHC side chain that generally contributes least to the stability of the MHC-peptide complex, whereas Tyr[7] and Tyr[171] are important in all cases. For the Y7F mutation, the destabilization is probably enhanced by the destruction of a water-mediated pentagonal H-bond network (Fig. 3*A*). Interestingly, the mutation effects are cumulative as the effect of double mutation at both ends of the binding groove corresponds roughly to the summed contribution of individual changes. For the I9 peptide, the 20 °C destabilization observed upon Y7F/Y171F mutation (T_m = 37 °C) is analogous to that observed after modification of the terminal amino group by a methyl moiety for HLA-A2-binding peptides (25) and corresponds to the loss of two intramolecular H-bonds.

These findings raise the question why the effects of protein mutations depend on the bound peptide? It is straightforward to understand that a class A peptide (A3) is least affected as it bears a strong Lys[9] anchoring side chain. Suppression of H-bonding at the rim of pocket F do not impair a deep anchoring of the side chain in the same pocket. As expected, far away mutations at pocket A only partially destabilize the corresponding complex. For a weaker binder (I9), all mutations are much more destabilizing even for mutations in pocket A. This observation would suggest that P9-pocket F interactions control for a major part of the overall stability of the MHC-peptide pair. However, this hypothesis would be contradicted when comparing the mutation effects on complexes with S9 and I9 peptides. If Ser[9] is less suited than Ile[9] for pocket F stabilization (Fig. 2*B*) and if P9-pocket F interactions control the whole complex stability, why are mutation effects lower for the B*2705-S9 complex than for the B*2705-I9 complex? To reconcile our stability hypothesis and the observed results, one might argue that the stability of all complexes between B*2705 mutants and the two peptides (I9 and S9) are nearly identical (about 43 °C for I9 and 39 °C for S9, whatever the mutation). Thus, such complexes have reached the lowest stability compatible with refolding of the heterotrimer. One should not forget that peptide-free class I MHC heavy chains unfolds at a rather similar temperature (from 39 to 45 °C) (21, 39). Thus there is a lower T_m limit for unstable MHC-peptide pairs. For the weakest binder (S9), the C-terminal Ser residue is only weakly bound to the wild type pocket F and already gives a complex of very weak stability (T_m = 43 °C). Then, further mutation of this subsite has no dramatic consequences as long as the lowest stability threshold has not been reached. From our studies, this lower limit is at least 36 °C, the lowest stability observed with a wild type HLA-B*2705 (Fig. 2*a*).

That even double B*2705 mutants refold in the presence of a weak binding peptide suggests that all residues at P3 and P9 are probably compatible with B*2705 refolding at the condition that the P2 anchor is an arginine. Once refolded, the nature of the C-terminal side chain probably controls the overall stability of the MHC-peptide complex. The crucial importance of the P9 anchor for promoting stable MHC-peptide complexes should be similar for other peptide subsets with a different HLA specificity but sharing a similar location of anchor residues (P2, P3, and P9) with B27-restricted peptides. From the lowest melting temperature observed in this peptide series, we can conclude that a peptide-free B*2705 heavy chain is less stable than two class I MHC heavy chains (H-2K^d, HLA-Aw68) and as stable as HLA-A2.1 for which low stability complexes (unfolding at 35 °C) have been recently described (20).

A better understanding of molecular rules governing MHC-peptide stability is essential for designing modified ligands (agonists or antagonists) targeted to interfere with the CTL recognition. Our study suggests that special care should be given to the C-terminal peptide residue of most class I MHC-restricted peptide vaccines for ensuring an optimal stability and immonogenicity of the resulting MHC-peptide pair.

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