β-Amino Acid Scan of a Class I Major Histocompatibility Complex-restricted Alloreactive T-cell Epitope*

Received for publication, March 29, 2001, and in revised form, May 7, 2001
Published, JBC Papers in Press, May 7, 2001, DOI 10.1074/jbc.M102772200

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An HLA-B27-restricted self-octapeptide known to react with an alloreactive T-cell receptor has been modified by systematic substitution of a β-amino acid for the natural α-amino acid residue, over the whole length of the parent epitope. All modified peptides were shown to bind to recombinant HLA-B*2705 and induce stable major histocompatibility complex-peptide complexes, but with some variation depending on the position of the β-amino acid on the peptide sequence. Alteration of the natural peptide sequence at the two N-terminal positions (positions 1 and 2) decreases binding affinity and thermodynamic stability of the refolded complex, but all other positions (from position 3 to the C-terminal residue) were insensitive to the β-amino acid substitution. All modified peptides were recognized by an alloreactive T-cell clone specific for the parent epitope with decreased efficiency, to an extent dependent on the position that was modified. Furthermore, the introduction of a single β-amino acid at the first two positions of the modified peptide was shown to be sufficient to protect them against enzymatic cleavage. Thus, β-amino acids represent new interesting templates for alteration of T-cell epitopes to design either synthetic vaccines of T-cell receptor antagonists.

Major histocompatibility complex (MHC)1 class I molecules are proteins that present a large repertoire of peptides on the cell surface where they can be recognized by cytotoxic T-lymphocytes (CTLs) (1). Under physiological conditions, only non-self-peptides, e.g. of viral origin, can activate CTL and thereby trigger an immune response. In contrast to the normal function of the immune system, CTLs can also be activated by self-antigens, which represent a pathological state resulting in severe autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, or autoimmune uveitis (2). The high association of several alleles with autoimmune diseases, e.g. HLA-B*2705 with spondyloarthropathy (3, 4), is not fully understood yet, but one of the most favored models postulates the allele-specific selection and presentation of autoantigenic peptides (5). For several of these diseases, self-peptides triggering CTL are described (6, 7), whereas in some cases like rheumatoid arthritis the relevant self-peptides are still unknown. MHC class I molecules are also involved in the acute rejection of allogeneic transplants (8, 9). Alloreactive T-cells are stimulated by allogeneic MHC molecules and often recognize epitopes containing both the presenting molecule and specific peptides (9).

Several antigens have been described to be involved in alloreactivity including MHC- and non-MHC-derived peptides. Current therapy for both autoimmune diseases and alloreactivity includes suppression of the entire immune system, e.g. by corticosteroids. However, the utilization of such immunosuppressive drugs is characterized by severe side effects. Consequently, much effort has been undertaken to establish alternative therapies that are based on the selective, antigen-specific modulation of the immune response. Several strategies have been developed including utilization of antibodies, soluble MHC complexes, and peptide vaccines (10–12). Among these strategies, peptide vaccines are of special interest as they have been reported to induce tolerance in mice and humans (6, 7) and, in contrast to protein-based therapeutics, are accessible by solid-phase synthesis. However, the therapeutic application of peptides is hindered by the rapid clearance from the serum because of the degradation by proteases (13). Therefore, it would be highly desirable to obtain peptidomimetics, which are enzymatically stable and bind with high affinity to MHC molecules. Furthermore, these compounds have to be recognized by the same pool of CTL as the parent peptide and induce the desired immunomodulatory effect. Many attempts have been undertaken to obtain peptidomimetics that fulfill these requirements, but only few successful results have been reported (14). The utilization of β-amino acids represents a systematic approach suited for the successive development of peptidomimetics (15). The side chains of β-amino acids are identical to the parent α-amino acids, which is of particular importance regarding the great influence of side chains on the complex stability (16). The modification of the backbone by introduction of the methylene moiety results in complete resistance of peptides composed solely of β-amino acids against proteolytic cleavage (15). Successful use of β-homoalanine for the design of non-natural MHC ligands has been reported previously by our group (17), but the introduction of the β-amino acid was limited to the middle part of the peptide, which is known to bulge out

* This work is supported in part by the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (Project 31–57307.99) and by Grants SAP99-0055 and FM99-0098 from the Spanish Ministry of Science and Technology (to J. A. L. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: MHC, major histocompatibility complex; CTL(s), cytotoxic T-lymphocyte(s); HPLC, high pressure liquid chromatography; HC, heavy chain; β-m, human β-2-microglobulin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Tm, melting point; Pn, position n; LC50, half-maximal lysis of the octamer epitope; Dde, 1-(4-4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl.
of the peptide binding groove (18) and thus only weakly interacts with the host MHC molecule (19).

Here, we report the systematic variation of the HLA-B*2705-restricted self-peptide by single replacement of all amino acids using the corresponding β-amino acid analogues. The presentation of the self-peptide chosen for modification (RRFFPYYV; one-letter code) was shown previously to be restricted by the HLA-B*2705 molecule and specifically recognized by the 27S69 T-cell receptor of an alloreactive T-cell clone (20). The influence of substitutions on complex stability, ligand affinity, proteolytic degradation, and CTL recognition was evaluated using circular dichroism, fluorescence polarization, HPLC, and a chromo-release cytotoxicity assay.

### MATERIALS AND METHODS

#### Peptide Synthesis—
Peptides were obtained by automated solid-phase peptide synthesis on an automated multiple peptide synthesizer (Syro Multi-Syn-Tech, Bochum, Germany) using standard Fmoc (N(9-fluoren-9-yl)methoxy carbonyl) protecting strategy. Protected β-amino acids were purchased from Fluka, Buchs, Switzerland. For synthesis of fluorescein-labeled peptide GRAFVTIKK (fluorescein) a lysine with a Dde-protected side chain was inserted at position 8. After synthesis and selective deprotection, Lysα was coupled to fluorescein isothiocyanate. Complete deprotection and cleavage from the resin was achieved by trifluoroacetic acid. Peptides were analyzed and purified by mass spectrometry and HPLC as described previously (16).

#### Protein Expression and Purification—
HLA-B*2705 heavy chain (HC) and human β2m-microglobulin (β2m) were cloned, expressed, and analyzed as described previously (16). Briefly, the proteins were expressed in an Escherichia coli BL21-Codonplus (DE3)-RIL strain (Stratagene) as polyhistidine-tagged fusion proteins. Purification of the proteins was performed by nickel affinity chromatography. The N-terminal polyhistidine tag of β2m was cleaved by thrombin after purification.

#### Thermal Denaturation—
The thermal denaturation assay was performed as described earlier (16). Briefly, the HLA-B*2705-peptide complex was refolded by dialysis of 10 ml of reconstituting 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 by 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 are averaged from at least two independent refolding experiments, and experimental error was estimated to be lower than 1 °C.

#### Binding Experiments—
The HLA-B*2705-peptide complex was refolded by dilution of HC (1 µM), β2m (2 µM), fluorescent peptide GRAFVTIKK (fluorescein) (8 mM), and various amounts of competitor (typically 1 mM-100 mM) 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 by a modified size-exclusion HPLC assay (22). The ratio between bound and unbound labeled ligand was determined by fluorescence polarization. Polarization values were measured on a Polarion fluorescence-polarization system (Tecan, Salzburg, Austria) using 200 µl of sample in a 96-well black microtiter plate (Hellma, Müllheim, Germany). Number of flashes was set to 200, and total intensity was held at 65000 relative fluorescence units. IC50 values were obtained by fitting polarization values versus total concentration of competitor to a dose-response model. The IC50 values are averaged from three independent experiments.

### RESULTS

#### β-Amino Acid Substitution Does Not Impair Formation of Stable MIC-Peptide Complexes—
To characterize the influence of amino acid substitution by corresponding β3-amino acids we mutated separately all positions of the HLA-B*2705-restricted octapeptide RRFPVVY (Table I). The influence of β3-amino acids on the stability of HLA-B*2705-peptide complexes was evaluated by thermal denaturation experiments using CD spectral measurements. The melting points are averaged from at least three independent experiments.

#### Peptide Sensitization Cytotoxicity Assay—
TAP (transporter in antigen processing)-deficient B*2705-T2 transfectant cells were incubated for 18–20 h at 26 °C in RPMI 1640 medium supplemented with 10% fetal calf serum (both from Life Technologies, Inc.) in the absence of peptide. Cells were then labeled for 90 min at 37 °C with 50 µCi of 51Cr, washed four times, resuspended in the same medium with 1% fetal calf serum, seeded in 96-well plates, blocked previously with bovine serum albumin at 1 mg/ml in sterile phosphate-buffered saline, and incubated for 30 min at room temperature with variable amounts of synthetic peptides. Effector CTL 27S69 cells (20) were then incubated with peptide-sensitized targets for 5 h at 37 °C on an effector:target ratio of 1:1 in the continuous presence of peptide, and the supernatants were subjected to γ-counting. Percent-specific 51Cr release was calculated as follows: (experimental lysis − spontaneous lysis)/maximum release − spontaneous lysis) × 100. Recognition of the natural CTL 27S69 epitope was quantified as the peptide concentration required to obtain half of the maximum lysis observed with this peptide in the concentration range used. Recognition of the β3-amino acid analogs was measured as the peptide concentration required to obtain the half-maximal lysis of the octamer epitope (IC50).

#### Peptide Degradation—
Peptide susceptibility to enzymatic cleavage was assessed using a previously reported protocol (23). Human sera were obtained by centrifugation at 2000 × g of blood samples collected from two healthy donors. The sera were stored at −20 °C. Prior to use, the sera were incubated for 5 min at room temperature followed by 5 min at 37 °C. 100 µl of serum was added to 20 µl of peptide stock solution (1 mM) and incubated at 37 °C. The reaction was stopped after 0, 1, 3, 6, 9, 12, and 18 min by addition of 12 µl of trifluoroacetic acid. Precipitated serum proteins were pelleted by centrifugation. Degradation was monitored by HPLC at a wavelength of 218 nm using 50 µl of the supernatants per injection. Separation was performed on a LiChro- 

### Table I

Amino acid sequence of modified peptides

| Peptide | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 |
|---------|----|----|----|----|----|----|----|----|
| Refb   | Arg| Arg| Phe| Phe| Pro| Tyr| Tyr| Val|
| βR1    | Arg| Arg| Phe| Phe| Pro| Tyr| Tyr| Val|
| βR2    | Arg| Arg| β-HArg| Phe| Phe| Pro| Tyr| Tyr| Val|
| βF3    | Arg| Arg| β-HPhe| Phe| Phe| Pro| Tyr| Tyr| Val|
| βF4    | Arg| Arg| Phe| β-HPhe| Pro| Tyr| Tyr| Val|
| βF5    | Arg| Arg| Phe| Phe| Pro| β-HPro| Tyr| Tyr| Val|
| βF6    | Arg| Arg| Phe| Phe| Pro| β-HTyr| Tyr| Tyr| Val|
| βF7    | Arg| Arg| Phe| Phe| Pro| β-HTyr| B-HTyr| Val|
| βF8    | Arg| Arg| Phe| Phe| Pro| β-HTyr| B-HVal| 4.2|

a) Midpoint of unfolding of the B*2705 heavy chain, measured by CD spectroscopy at 218 nm.

b) µM concentration of altered peptide competitor inhibiting 50% of binding of a fluorescent-labeled peptide to recombinant HLA-B*2705.
spectroscopy to monitor complex unfolding. A typical denaturation curve resulting from unfolding of the B*2705-RRFPYYV complex is shown in Fig. 1A. The midpoint of unfolding or melting points (T_m) observed for the different complexes are highly variable, which is reflected by differences between the T_m values of up to 12 °C (Fig. 1B). Furthermore, the influence of the substitutions depends strongly upon the position of the mutated amino acid. A decreased stability is observed upon modification of the two N-terminal residues of the peptide. For the βRRFPYYV βR1 peptide, the T_m value (39.7 °C) is 9 °C lower than that of the parent peptide. Mutation of the neighboring position 2 (P2) results in only minor destabilization; a T_m value of 46 °C was observed for RβRRFPYYV (βR2), which is a thermal destabilization of 2.5 °C when compared with the parent peptide (T_m = 48.5 °C). All other modifications from P3 to the C terminus result in altered peptides that stabilize the HLA-B27-peptide complex. An increase of stability of about 2 °C is observed for mutation of the amino acids Phe^4 (50.2 °C), Pro^5 (50.4 °C), and Val^8 (50.8 °C). The highest T_m values were measured for the peptides RRβFFPYV (51.5 °C) and RRFFPYβYV (51.8 °C), which reflects a stabilization of about 3 °C. No significant effect on complex stability is observed for substitution of Tyr^6 by its β3-amino acid analogue, the observed T_m of 49.1 °C being comparable with the that of the parent peptide.

**Modified Peptides Bind to Recombinant HLA-B*2705 with High Affinity**—The affinities of the modified peptides for recombinant HLA-B*2705 were evaluated by determination of IC_{50} values using an fluorescence-based competition assay. All peptides were able to compete for binding to HLA-B*2705 with the fluorescent peptide, which is reported to be a good binder.\(^2\) The specificity of ligand binding was confirmed by several control experiments (data not shown) and is reflected by the different dose dependences of competition observed for the modified ligands. A typical curve resulting from titration with the βF3 peptide (Table I) is shown in Fig. 2A. The final fluorescence-polarization value of about 55 millipolarization units corresponds to the value obtained for the free labeled ligand and mirrors complete competition by the non-labeled molecule. As seen in Fig. 2B and Table I, the effect of the β3-amino acid scan is highly dependent upon the position of the substitution. A significant decrease in affinity is observed for mutations at the N-terminal part of the peptide. For the βR1 and βR2 peptides IC_{50} values of 45.1 and 65.0 μM have been determined, respectively, which represents a 15–20-fold decreased affinity when compared with the parent peptide. In contrast, the substitution of other positions in the peptide results in IC_{50} values in the μM range that are comparable with that of the parent peptide (3.2 μM).

**Altered HLA-B*2705-Peptide Complexes Are Recognized by a Specific Alloreactive T-cell Clone with Decreased Efficiency**—The influence of β-amino acid substitutions at individual positions of the allospecific peptide epitope RRFFPYV on recognition by CTL 27S69 was analyzed by means of a peptide sensitization assay using the TAP-deficient B*2705-T2 transfectant cell line. All of the β-amino acid analogs were recognized by CTL 27S69 but less efficiently than the natural epitope (Fig. 3). Significant differences were observed in the effect of substitutions at different positions (Table II). The lowest effect, a decrease of about two orders of magnitude in LC_{50}, was obtained with βR1, βP5, and βV8. The most drastic decrease, about four orders of magnitude, corresponded to βY6 and βY7, followed by βF4. Intermediate affects, LC_{50} about three orders of magnitude lower than the unmodified epitope, were observed with βR2 and βF3.

The effect of β-amino acid substitutions on CTL recognition did not correlate with stability (T_m) or affinity (IC_{50}) of the corresponding B*2705-peptide complexes (Table I). For instance βY6, which was among the analogs recognized with lowest efficiency, had similar T_m and IC_{50} values as the natural epitope. Conversely βR1, which showed the smallest effect on CTL recognition, had significantly lower stability and affinity than the natural epitope for B*2705. Furthermore, significantly decreased recognition of βF3 was observed despite increased stability of the B*2705-βF3 complex.

These results indicate that substitutions of β-amino acids at individual positions of an allospecific peptide epitope decrease, but do not abrogate, CTL recognition. The effect is strongly dependent on the peptide position but is not a direct consequence of decreased affinity of the peptide analog for B*2705 or lower stability of the B*2705-peptide complex.

**Two Altered Peptides Show Enhanced Resistance to Proteolytic Cleavage**—The influence of the peptide modification on stability against proteolysis was evaluated by monitoring the successive degradation of the peptides by incubation with human blood serum. The degradation kinetics were followed by HPLC analysis using the corresponding peak area for peptide quantification. The unmodified reference peptide was rapidly degraded, less than 10% of the initial peak area being detected after 18 min. For the modified peptides, the time course of degradation showed to be highly dependent on the position of the substitution. No stabilization was observed for all modifications occurring between P3 and P8 positions of the parent peptide (Fig. 4). However, a significant resistance to proteolytic cleavage is observed for the peptides modified at the first two N-terminal residues (Fig. 4). For the βR2 peptide, about 74% of the initial peak area was recovered after 18 min. The substitution of the first amino acid showed to have the strongest influence on the degradation. For βR1, no significant decrease of the peptide area was observed after 18 min.
DISCUSSION

In this study we investigated the influence of systematic modification of a HLA-B*2705-restricted octapeptide by single substitution of all amino acids with their corresponding β3-amino acid analogues. As the thermal stability of MHC class I-peptide complexes is reported to be a good descriptor of the in vivo immunogenicity of the antigenic complex (24), all altered B27-peptide complexes were subjected to thermal denaturation experiments. The Tm values obtained showed to be highly dependent on the position of the mutation within the peptide. Upon modification of the central positions 4–6 a minor stabilization of about 1–2 °C is observed. These results are in agreement with the expected minor contributions of these residues to the total free energy of binding as the middle part of HLA-B*2705-bound peptides bulges out of the peptide binding groove (18) and does not strongly interact with the host MHC molecule. This structural feature explains why replacing this peptide region by organic spacers (polyesters (25), β-amino acids (17), polyethylene (19, 25)) does not influence either ligand binding or thermodynamic stability of the resulting MHC-ligand complexes. The importance of the correct hydrogen bonding at the N terminus of the peptide (18) is reflected by the observed destabilization upon mutation of the first (−2.9 °C) and the second (−2.2 °C) N-terminal residue. The β-amino acid scan shifts important hydrogen bond donors/acceptors (first two peptide bonds, arginine side chain) in the C-terminal direction. This effect is more deleterious for the βR1 peptide for which only the terminal ammonium and first side chain could be located in the binding groove, in an orientation similar to that of the reference peptide. All other atoms are translated toward the C-terminal residue in a region (pocket A, pocket B) of HLA-B*2705 very sensitive to small topological changes of the bound ligand. For the βR2 peptide, the destabilization noticed upon β-amino acid substitution is less pronounced as the important arginine side chain at P2, as well as the N-terminal P1 residue, share a bound orientation that should be analogous to that of the parent epitope. The increased stability observed upon substitution at P3 probably results from an improved accommodation of the aromatic side chains in the D pocket of the HLA-B*2705, a subsite known to favor bulky aromatic substituents (19, 25).

Interestingly, the substitution of the C-terminal residue induced the opposite effect resulting in an increased stability for RRFFPYβYV (+ 3.3 °C) and RRFFPYYβV (+ 2.3 °C). Some variation around the binding mode of the C-terminal residue

**FIG. 2.** Influence of peptide mutation on ligand affinity. IC50 values were determined by a fluorescence-polarization-based competition assay. A, typical titration curve for the peptide RRβFPPYYV (IC50 = 1.9 μM). The experimental error is 5 millipolarization units or less. B, influence of the position of the mutated amino acid on the IC50 values.

**TABLE II**

| Peptide         | LC50 ± S.D. | Molar ratio |
|-----------------|-------------|-------------|
| RRFFPYVV        | 2.5 × 10^-10 | 120         |
| βR1             | 3.0 ± 3.6 × 10^-8 | 720         |
| βR2             | 1.5 ± 1.0 × 10^-7 | 760         |
| βF3             | 1.9 ± 1.7 × 10^-7 | 3640        |
| βF4             | 9.1 ± 0.1 × 10^-7 | 140         |
| βP5             | 3.5 ± 2.5 × 10^-8 | 7600        |
| βY6             | 1.9 ± 2.0 × 10^-6 | 7200        |
| βY7             | 2.3 ± 3.1 × 10^-6 | 9200        |
| βY8             | 6.4 ± 3.5 × 10^-5 | 256         |

* Molar concentration of peptide required to get the half-maximal target cell lysis obtained with the reference peptide.

**FIG. 3.** Specific cytotoxicity of CTL 27S69 against B*2705-T2 cells sensitized with various amounts of the natural RRFFPYVV peptide epitope (solid circles) or each of the β-amino acid analogs. The natural B*2705 ligand, RRYYDLIEL, was used as a negative control (cross symbols). The effector:target ratio was 1:1. Data are means of three to seven independent experiments.

**TABLE II**

Specific lysis of B*2705-T2 cells sensitized with the alloreactive peptide or modified analogs

| Peptide         | LC50 ± S.D. | Molar ratio |
|-----------------|-------------|-------------|
| RRFFPYVV        | 2.5 × 10^-10 | 120         |
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| βP5             | 3.5 ± 2.5 × 10^-8 | 7600        |
| βY6             | 1.9 ± 2.0 × 10^-6 | 7200        |
| βY7             | 2.3 ± 3.1 × 10^-6 | 9200        |
| βY8             | 6.4 ± 3.5 × 10^-5 | 256         |

* Molar concentration of peptide required to get the half-maximal target cell lysis obtained with the reference peptide.
has already been shown by x-ray diffraction of class I MHC molecules (18). The last peptide amino acid can even be partly located outside the binding groove (26). Thus, alteration of the starting peptide at the C-terminal residues can be well accommodated and even enhance interactions with the binding groove. As the C-terminal residue controls for a large part to the overall thermodynamic stability of the MHC-peptide complex (16), β-amino acid substitution represents an interesting alternative for designing altered ligands.

In addition to the thermal stability, the affinity of a ligand to its host MHC molecule represents a parameter of high importance for the development of non-natural class I MHC ligands. Thus, we evaluated the IC50 values of the modified peptides using a new fluorescence-polarization-based competition assay. The IC50 value of the parent peptide (3.2 μM) is comparable with previously reported affinities for medium binders (27). All modified peptides were able to compete for binding with a fluorescent peptide. Structure properties observed for the binding affinity were very similar to that reported previously for the thermal stability of the resulting MHC-peptide complexes. Alteration of the first two N-terminal residues increases IC50 values by a factor of 15–20 (see Fig. 2 and Table I) whereas modification of the peptide sequence from P3 to the C terminus did not alter the binding properties of the altered peptides to the target MHC protein (Fig. 2B). Interestingly, our data are in contradiction with a recent report (28) indicating that P1, P2, PC-1, and PC residues (PC: C-terminal residue) of an HLA-A2 binding tumoral peptide are not permissive for β-amino acid substitution. The P1 modification was found to be less detrimental for binding than the P2 change, by opposition to measured thermal stabilities of the antigenic complexes. However, this contradiction can be explained by the differing set of protein-ligand interactions contributing to affinity and complex stability (27). In our binding assay, competitors compete with the reference peptide for promoting the refolding of the HC-βm-ligand heterotrimer. Thus, MHC-peptide interactions constitute only one aspect of the multistep refolding procedure in which both entropic and enthalpic contributions of the ligand play an important role. By contrast, thermal unfolding of already formed MHC-peptide complexes only takes into account the ligand release from the peptide binding groove. Our data illustrate a stronger contribution of residue P2 to complex refolding but a lower influence on complex stabilization compared with that of the N-terminal residue.

Molecular modeling of the RRFFPYVV epitope in complex with B*2705 (20) suggests that Arg2, Phe3, Tyr6, and Val10 are anchor residues, whereas Arg2, Phe4, Pro5, and Tyr7 are exposed at the surface of the complex. Ala scanning further indicated that Arg2 is not significantly involved in recognition by CTL 27S69, but Phe4, Pro5, and Tyr7 are critical for allorecognition (29). There was no correlation between the effect of β-amino acid substitutions on CTL recognition and the involvement of the corresponding residue in anchoring to B*2705 or in TCR interactions. Thus, of the residues for which β-amino acid substitutions had the lowest effect on CTL recognition, Arg2 is not critical for alloreactivity (29) but makes a significant contribution to affinity and stability of the B*2705-peptide complex (Table I). Pro5 is exposed and critical for CTL recognition, and Val10 is an anchor residue (20, 29). The highest effect on CTL recognition was observed upon substitution of two exposed residues (Phe4, Tyr5) or a hidden one (Tyr8). The lack of correlation between the effect of β-amino acid substitutions on CTL recognition and the nature of the peptide positions, or their contribution to affinity or stability of the B*2705-peptide complex, suggests that the conformational changes induced in the peptide following introduction of β-amino acids have independent effects on peptide binding and T-cell recognition.

The rapid clearance from blood serum, e.g. by enzymatic cleavage, is one of the major limitations for the pharmaceutical application of peptides. As peptides composed solely of β3-amino acids are completely stable against proteases (15), we investigated whether single β3-amino acids have an influence on the degradation upon incubation of the peptides with human blood serum. For the majority of the peptides a decrease similar to that of the parent peptide was observed. Typically, less than 20% of the initial amount was detected after 18 min of incubation. However, the βR2 peptide was significantly more resistant to degradation than the parent epitope. The strongest effect was induced for mutation of the N-terminal residue, which resulted in complete stability during the time of observation. This is particularly significant, because the βR1 substitution had the lowest effect on CTL allorecognition among those tested in this study. These data confirm the relevance of aminopeptidases for the cleavage of short peptides in blood serum (23). Moreover, our results show that single substitution by β3-amino acids analogues can already induce a significant protective effect against enzymatic degradation.

Our data reveal a strong influence of single β3-amino acids on parameters of high relevance for the immunogenic properties of the ligand. Furthermore, we could show that the introduction of β3-amino acids may be used as a general method to increase the resistance of short peptides against enzymatic degradation. Thus, the results of this study strongly suggest the utilization of β3-amino acids for the design of altered MHC ligands for therapeutic application.

Acknowledgments—We thank Dr. Richard Soll for help with peptide synthesis, Reto Bader for measuring mass spectra, and Alexander-Heckel for collecting blood samples.

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