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New conformationally restricted analog of the immunosuppressory mini-domain of HLA-DQ and its biological properties

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Abstract

Our previous studies revealed that the nonapeptide fragment of HLA-DQ located in the β164–172 loop of the Thr-Pro-Gln-Arg-Gly-Asp-Val-Tyr-Thr sequence suppresses the immune humoral and cellular responses [30]. Based on the crystal structure of HLA-class II molecules we designed and synthesized a cyclic analog with restricted conformation, cyclo(Suc-Thr-Pro-Gln-Arg-Gly-Asp-Val-Lys)-Thr-OH (Suc = succinyl) by reacting a Lys side chain with a succinylated N-terminus. The cyclization product more potently suppresses the cellular immune response than its linear counterparts and is efficiently cleaved by trypsin. The results indicate that the β164–172 loop may serve as a functional epitope on the HLA class II surface for intermolecular binding. © 2000 Published by Elsevier Science Inc.

Keywords: Histocompatibility antigen; Thymopentin analogs; Immunosuppressors; RGD sequence; Cyclic peptide analog; Proteolytic stability

1. Introduction

Human major histocompatibility complex (MHC) class II genes are expressed primarily on specialized antigen-presenting cells such as macrophages, B-lymphocytes, and dendritic cells. The encoded products, known as human leukocyte antigens (HLA) class II, consist of noncovalently coupled heterodimers of α (32 kDa) and β (28 kDa) glycoproteins grouped in at least three subsets designated HLA-DR, HLA-DQ, and HLA-DP. $CD4^+$ helper cells recognize antigen-derived peptides bound to HLA class II molecules exposed on the cell surface of antigen presenting cells. The coreceptor molecule CD4 enhances the binding between the T-cell receptor and the class II molecules, particularly in low-affinity interactions. A major advance in understanding the molecular basis of MHC class II function was the determination of a three-dimensional structure of the extracellular domains of the HLA class II molecule DR1 [4,25]. The crystal structure of HLA-DQ has not been solved yet. However, high homology (>60% identity) of the sequences enables the construction of three-dimensional models of HLA-DQ based on mutational analyses of the HLA-DR crystal structures [9,15,31].

The β2-domain of HLA-DQ contains the RGDVY (167–171) sequence, which is very similar to thymopentin (pentapeptide RKDVY, an active fragment (32–36) of thymopoietin, an immune system activator produced in thymus). The thymopentin-like region is situated on the opposite side of the molecule than the antigenic peptide–binding site and in a close spatial proximity to the site identified as interacting with the T-cell coreceptor CD4. The presence of a thymopentin-like fragment in such an important protein of the immune system like HLA-DQ suggests that the protein may possess its own immunomodulatory potency located in the site responsible for association of the protein with its respective partners. The fragment contains also the RGD sequence, which is known to be important in several proteins in mediating cell adhesion interactions. According to the three-dimensional model of HLA-DQ, the RGD sequence is located in the loop of the antigen molecule exposed toward the solvent and therefore may be involved in interactions with other proteins. These interactions may additionally enhance the binding between the $CD4^+$-cell receptor and the class II molecules.

We found that a nonapeptide fragment of HLA-DQ (164–172, TPQRGDVYT) strongly suppresses the cellular

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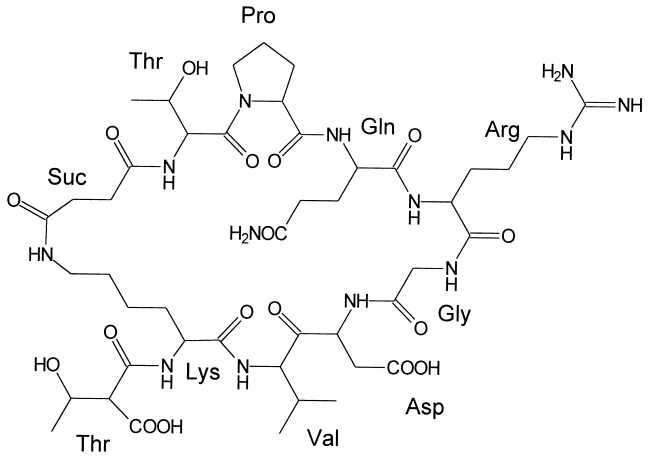

Fig. 1. Chemical structure of the cyclic peptide 1.

and humoral immune responses. The shorter fragments, QRGDVYT and RGDVYT, show a weaker potency than the nonapeptide, whereas QRGDVY, RGDVY, and RGDV show fragile immunosuppression in the cellular response only [30–32]. The counterparts' fragments of HLA-DP and HLA-DR molecules show immunosuppressive properties similar to the HLA-DQ fragments [33]. The nonapeptide fragment of HLA-DQ selectively inhibits adhesion of platelets to fibrinogen [31] suggesting that HLA-DQ itself may be involved in such process in the case of T cells. We also found that the immunomodulatory properties of the investigated peptides are connected with changes in their conformational preferences in solution i.e. with their tendency to form β-turn conformations [31].

In the present study, we designed and synthesized conformationally constrained analog of the nonapeptide HLA-DQ fragment to mimic the thymopentin-like loop of HLA-DQ. The objective was to obtain the peptide that may interact with hypothetical receptors of the thymopentin-like mini-domain of HLA-DQ. The chemical structure of the synthesized constrained peptide 1 is given in Fig. 1. The peptide was tested for its immunologic and antiadhesive properties as well as for its resistance to enzymatic degradation. We used the CD spectroscopy to analyze conformation of the peptide in solution. The biologic and conformational properties of peptide 1 were compared with its linear counterparts: the reference compound, Ac-Thr-Pro-Gln-Arg-Gly-Asp-Val-Lys(ϵ-Ac)-Thr-OH (peptide 3) and H-Thr-Pro-Gln-Arg-Gly-Asp-Val-Tyr-Thr-OH (peptide 2), nonapeptide fragment 164–172 of HLA-DQ.

2. Experimental

2.1. Materials

Trypsin (type III from bovine pancreas) was purchased from Sigma (T8253), and the derivatives of amino acids for peptide synthesis were purchased from Nova-Biochem. The side-chain protecting groups for BOC-amino acids were: benzyl for Thr, cyclohexyl for Asp, tosyl for Arg, Fmoc for Lys, and 2-bromobenzyloxycarbonyl for Tyr. Merrifield resin (1.06 mmol/g) was purchased from Sigma. BOC-Thr(OBzl)-Merrifield resin (0.75 mmol/g), was obtained by standard procedure [8]. The solvents for peptide synthesis were purchased from POCH and Riedel-de-Haën. Tris and trifluoroacetic acid were obtained from Bio-Rad and Riedel-de-Haën, respectively.

2.2. Peptide synthesis

The synthesis of the linear compound 2 was published previously [31]. The reference peptide 3 was synthesized by solid–phase method utilizing BOC synthetic procedure. The strategy of synthesis of the cyclic peptide 1 was similar to the one described by Schiller [23]. The peptide was assembled on solid support, using standard BOC synthetic procedure, using the Fmoc group to protect the side-chain of Lys. Succinic residue was incorporated by reaction with succinic anhydride (3 fold excess) for 30 min in the presence of diisopropylethylamine after deprotection of the N-terminal Thr^1 residue. The Fmoc protecting group of the side chain of the Lys^8 residue was removed with 25% piperidine in DMF solution (1×1 min + 1×20 min), and the resin was washed with DMF. The peptide on the resin was cyclized by forming an amide bond between the N-terminal Suc and the side chain of Lys in DMF/dichloromethane (1:1, v/v, 15 ml/g of resin) at room temperature in presence of 1-hydroxybenzotriazole (3 equiv), diisopropylethylamine (6 equiv), and BOP (3 equiv). The cyclization was completed within 12 h based on the ninhydrin test. The peptide was cleaved from the resin using trifluoromethanesulfonic acid and *m*-cresol solution in trifluoroacetic acid (1:1:8, v/v/v) at 0°C for 30 min. and at room temperature for 90 min., extracted with trifluoroacetic acid, followed by precipitation with diethyl ether. The products were purified by preparative HPLC on an Altech Econosil C_{18} column, 22×250 mm, using a linear gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid (gradient 0.5%/min, flow rate 7 ml/min) and transformed into acetate form. Final products ware obtained with more than 99% purity estimated by analytical HPLC. The amino acid compositions were checked by analysis with an AAA-851 analyzer (Mikrotechna, CSRS). Molecular masses and sequences of synthesized peptides were confirmed on a Finnigan MAT TSQ 700 mass spectrometer equipped with a Finnigan electrospray ionization source.

2.3. Immunologic tests

The immunomodulatory activities of the peptides (humoral immune response) were tested by the direct plaque-forming cells (PFC) test. The footpad test was used for determination of the delayed type hypersensitivity (DTH, cellular immune response). The details of all tests have been

Table 1
Direct plaque-forming cells (PFC) numbers in the mouse spleen cell cultures of mice immunized with sheep red blood cells (SRBC) and treated with peptides 1, 2, and 3

Peptide	Dose (μg/well)	PFC/10^6	± SE[a]	P (Student test)	Suppression (%)
1	Control[b]	1627	17		
	1	1517	17	<0.01	6.8
	10	1922	57	NS[c]	(−18.1)
	100	1167	15	<0.001	28.3
2	Control	1627	17		
	1	1731	17	NS	(−6.4)
	10	1363	25	<0.001	16.2
	100	1190	12	<0.001	39.2
3	Control	1567	28		
	1	1394	23	<0.01	11.0
	10	1173	25	<0.001	25.1
	100	999	8	<0.001	36.2

[a] The results are expressed as a mean ± SE (standard error) of four wells.
[b] Control 0.9% saline solution.
[c] Non significant.

described previously [6,34]. All data obtained in the biologic experiments were statistically elaborated using the Student P test. To make the comparison of the data easier we included the percentage values of the inhibition of the immune responses. The values were calculated according to the equation:

$$\% \text{ of immunosuppression} = 100\,(1 - \text{experimental value/control})$$

When a peptide stimulated the immune response, negative values were presented. The results of the immunologic tests are summarized in Tables 1 and 2.

PFC number was determined by the Jerne test modified by Mishell and Dutton [12]. The animals used were 8–10-week old 129/Iiw and CBA/Iiw mice; the antigen was sheep red blood cells (SRBC) and the solvent was 0.9% saline solution. 2.0–4.0 mg of the peptide were dissolved in 0.2–0.4 ml of 0.9% saline and the solutions were diluted to the desired concentration with 0.9% saline. In the experiments, 0.1–0.2 ml of a peptide solution was added to the cell culture wells. The results are expressed as a PFC number per 10^6 splenocytes.

Table 2
The influence of peptides 1, 2, and 3 on the inductive phase of delayed type hypersensitivity (DTH)

Peptide	Dose (μg/mouse)	Units[a]	± SE[a]	P (Student test)	Suppression (%)
1	Control[b]	10.5	0.5		
	20	6.5	0.4	<0.001	38.1
	200	5.3	0.7	<0.001	49.5
2	Control	10.5	0.5		
	20	8.8	0.7	NS[c]	(16.2)
	200	8.1	0.9	<0.05	22.9
3	Control	11.0	0.4		
	20	11.0	0.5	NS	(0)
	200	9.8	0.3	<0.05	10.9

[a] The results are expressed as a mean ± SE of six mice. 1 unit = 10^{-2} cm.
[b] Control 0.9% saline solution.
[c] Non significnt.

DTH reaction was measured using the methodological approach of Lagrange [11]. DTH was induced in 129/Iiw mice. The animals were immunized by i.v. treatment with SRBC in 0.9% saline. The peptide solution was administered 24 h after the sensitizing dose of the antigen to check its influence on the inductive phase of DTH. The results are expressed as an increase in number of footpad thickness units (DTH test; 1 unit = 10^{-2} cm).

2.4. CD spectroscopy

CD spectra were recorded on a Jasco J-600 spectropolarimeter. Peptides were dissolved at concentrations of approximately 70 μg/mL and the spectra were recorded in water and trifluoroethanol (TFE) at room temperature. A rectangular quartz cuvette of 1 mm pathlength was used. Each spectrum represents the average of eight scans. Data are presented as molar ellipticity [Θ].

2.5. Proteolytic assays

Proteolytic stability of peptides 1–3 against trypsin was measured using analytical HPLC utilizing the method described previously [27]. Briefly, the peptides and trypsin were incubated at room temperature in Tris-HCl buffer (pH 7.8). Aliquots of the reaction solution were collected at intervals, and the reaction was arrested by lowering the pH and cooling to 4°C. The aliquots were injected directly onto a HPLC column and the eluent was monitored by the absorbance at 223 nm.

Identification of degradation product peaks was made by manual collecting of eluent from individual HPLC peaks followed by analysis by mass spectrometry (Finnigan MAT TSQ 700 mass spectrometer equipped with an electrospray ionization source). Collision-induced dissociation (CID) of positive ions was used to confirm structures of non-cyclic degradation products. The CID was performed on the Q0 octapole transfer lens and was accomplished by increasing the octapole-offset voltage from −3 to −33V.

3. Results

3.1. Peptides

We synthesized the nonapeptide fragment 164–172 of HLA-DQ H-Thr-Pro-Gln-Arg-Gly-Asp-Val-Tyr-Thr-OH (2), the cyclic analog, cyclo(Suc-Thr-Pro-Gln-Arg-Gly-Asp-Val-Lys)-Thr-OH (1) and its linear counterpart, Ac-

Thr-Pro-Gln-Arg-Gly-Asp-Val-Lys(ε-Ac)-Thr-OH (3). The chemical structure of cyclic compound 1 is shown in Fig. 1. The peptides were prepared by manual solid-phase techniques. Details of the synthesis of peptide 1 are given in the experimental part. The starting linear nonapeptide was cyclized on the solid support to minimize dimerization. The purified monomer was identified by its molecular weight using an electrospray mass spectrometer. The cyclic monomer was obtained with the yield of 70%, estimated by analytical HPLC of the crude product.

3.2. Immunologic tests

Synthesized peptides 1–3 were investigated for their activity in the regulation of both the cellular and the humoral immune response. Each experiment was repeated at least twice, giving similar results. Although the immunologic evaluation for linear compound 2 was published previously [30] we repeated them in order to compare precisely the potency of the linear and cyclic analog. The immunosuppressory potencies values of peptide 2, obtained previously in both PFC and DTH tests, were slightly higher than received in the present test.

The influence of the peptides on the humoral immune response in mice (Table 1) was assayed in vitro by counting the number of spleen cells that formed the plaques with sheep red blood cells (PFC-test). The results are expressed as a mean ± SE of four wells. Peptides 1, 2, and 3 evoked suppression of the humoral immune response measured by the PFC number. The compounds show similar potency at a dose of 100 μg/well.

The cellular immune response (Table 2) was assayed in vivo by determination of the influence of the peptides on the inductive phase of the delayed type hypersensitivity (DTH). The results are expressed as a mean ± SE of six mice. Although notable, dose-dependent immunosuppressive effects were observed for the linear peptides 2 and 3 the first one shows remarkable higher potency. However, immunosuppressory potency of the cyclic peptide 1 measured at a dose of 200 μg per mouse is noteworthy. Thus, the cyclization selectively increases the suppression of the cellular immune response, although the chemical modifications introduced to analogs 1 and 3 are not favorable for the immunosuppression.

3.3. CD spectroscopy

Conformational investigations of peptides 1, 2, and 3 were performed by CD method using TFE and water as solvents. Essentially, the spectra in TFE solution are similar to each other, although the intensities of the negative bands are smaller in case of the cyclic peptide 1. Spectrum of peptide 1 in TFE solution (Fig. 2B) is practically the same as in water (Fig. 2A), but the intensity of the band at 220 nm is lower in water than in TFE. Although the spectra are somewhat similar to the CD spectrum of the α-helix, the

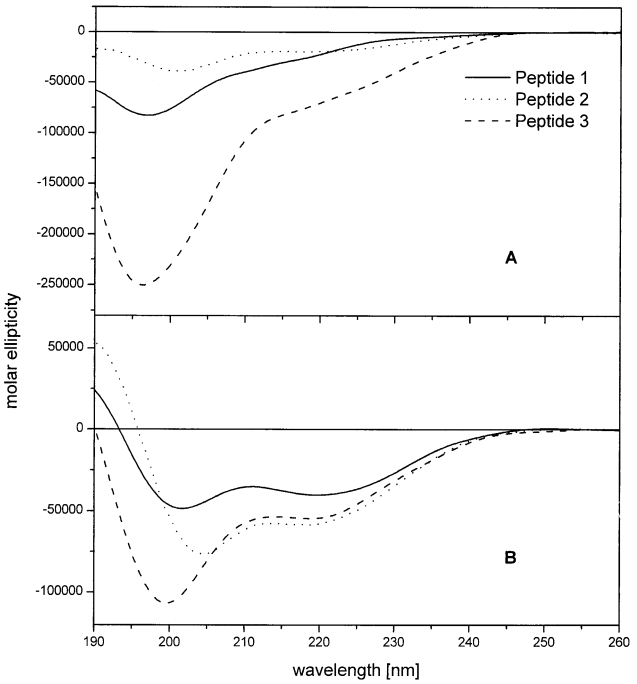

Fig. 2. The CD spectra of cyclic peptide 1 and the nonapeptide fragment of HLA-DQ in water (panel A) and trifluoroethanol (panel B).

helical conformation of the cyclic nonapeptide 1 with 31-atoms ring is rather doubtful. The negative maximum at about 220 nm results possibly from the presence of the β-turn conformation (type I or III) in the conformational equilibrium [16,17]. The CD spectra of linear peptides 2 and 3 dissolved in water are characterized by a weak negative shoulder at 220 nm and a negative band at 200 nm, typical of disordered structures [35], (Fig. 2A). High intensity of the negative bands at 200 nm in case of the doubly acetylated peptide 3 imply that the removal of the terminal basic amino group of Thr and/or the phenolic side chain of Tyr destabilized the peptide conformation in solution.

3.4. Proteolytic stability

HPLC profiles of the synthetic peptides after 1- and 40-min incubation with trypsin are depicted in Fig. 3. The percentage of the hydrolyzed peptides was estimated from peak areas in the HPLC profiles. The cleavage of linear peptide 2 and its cyclic analog 1 as a function of time is shown in Fig. 4. The results stress that cyclic peptide 1 hydrolyze significantly faster than its linear analog 2. The experiments were performed on peptides 1 and 2 separately (Fig. 4A and Fig. 4B, respectively) and repeated on their mixture (Fig. 4C), giving similar results. This indicates that the difference in proteolytic stability of peptides 1 and 2 are not associated with conditions of the experiment (like subtle differences in pH, temperature, etc.), since these conditions should affect both of the components at the same time. Proteolytic stability studies on a mixture of peptides were

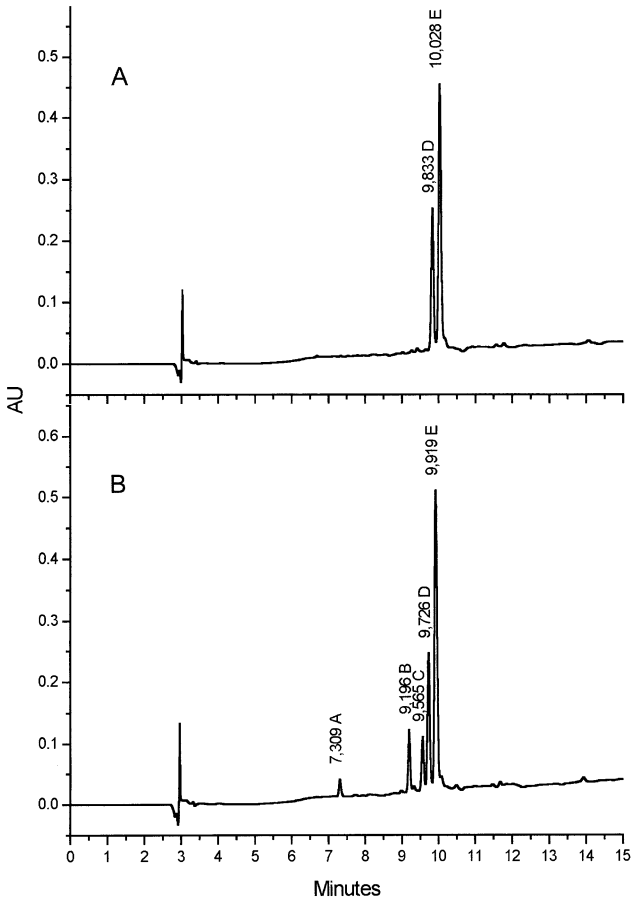

Fig. 3. HPLC profiles of the mixture of peptides 1 and 2 after 1 min (Panel A) and 40 min (Panel B) of incubation with trypsin. The details of the digestion are described in the Experimental Section. Peaks D and E corresponds to the intact peptides 1 and 2, respectively, while peaks A, B and C are their tryptic products, H-Thr-Pro-Gln-Arg-OH, H-Gly-Asp-Val-Lys(Suc-Pro-Gln-Arg-OH)-Thr-OH, and H-Gly-Asp-Val-Tyr-Thr-OH, respectively.

practical for this comprehensive study, because the substrates and the degradation products possess different retention time. The results obtained for the linear peptide 3 (not shown) were very similar to obtained for compound 2.

The cleavage sites of immunosuppressors 1 and 2 by trypsin were investigated by analyzing by electrospray mass spectrometry of the degradation products of linear and cyclic compounds. As can be expected, trypsin catalyzes hydrolysis of the peptide bond at the carboxyl end of Arg^4 of linear compound 2, since two degradation products with molecular weights of 500.3 and 553.2 correspond to H-Thr-Pro-Gln-Arg-OH and H-Gly-Asp-Val-Tyr-Thr-OH, respectively. Cyclic peptide 1 gave only one tryptic degradation product with molecular weight of 1100.5, which is exactly 18 Da higher than that of peptide 1. This indicates, that trypsin catalyzes hydrolysis of one of the endocyclic peptide bonds only, since the hydrolysis product does not break apart into two products. The degradation product was characterized with electrospray mass spectrometer by collision-induced dissociation (CID) of its positive ions. The

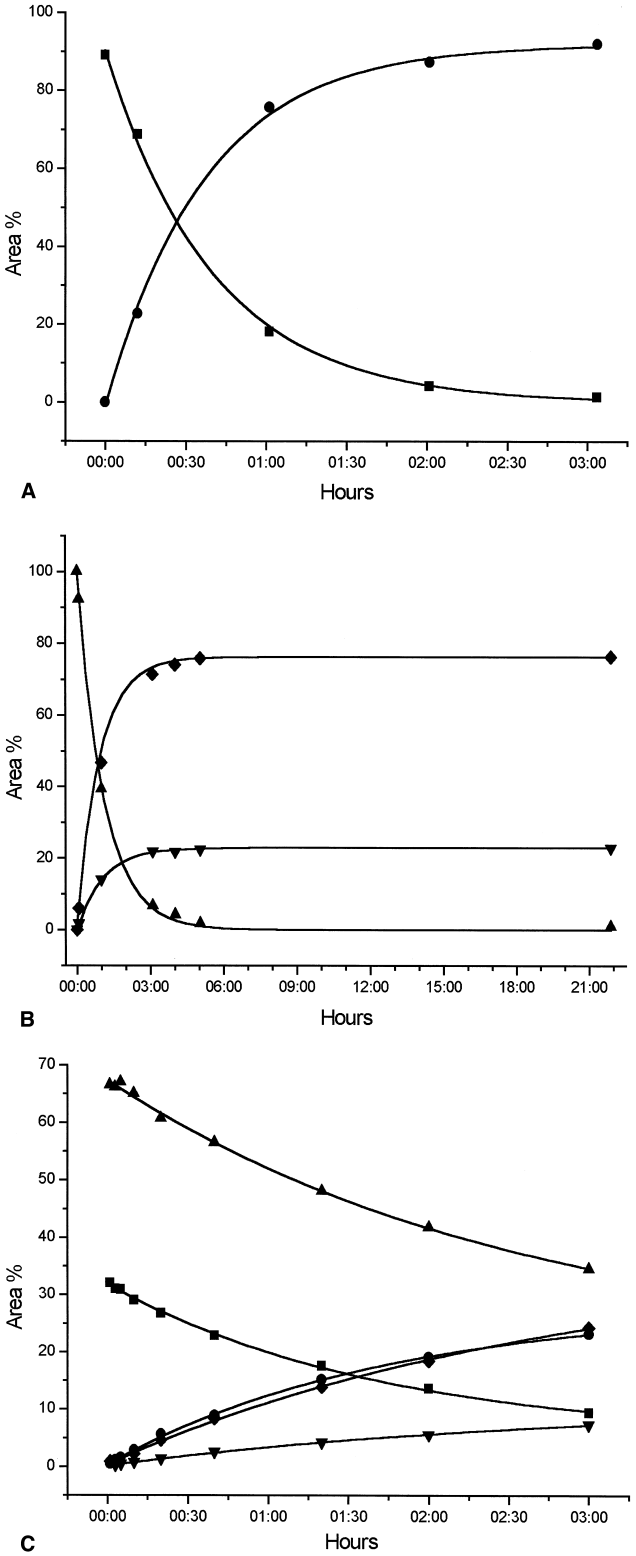

Fig. 4. Degradation profiles of peptides 1 (■) and 2 (▲). The degradation products are: H-Gly-Asp-Val-Lys(Suc-Pro-Gln-Arg-OH)-Thr-OH (●) from compound 1, and H-Thr-Pro-Gln-Arg-OH (▼) and H-Gly-Asp-Val-Tyr-Thr-OH (◆) from compound 2. The experiments were performed on peptides 1 and 2 separately (panel A and B, respectively) and repeated on their mixture (panel C).

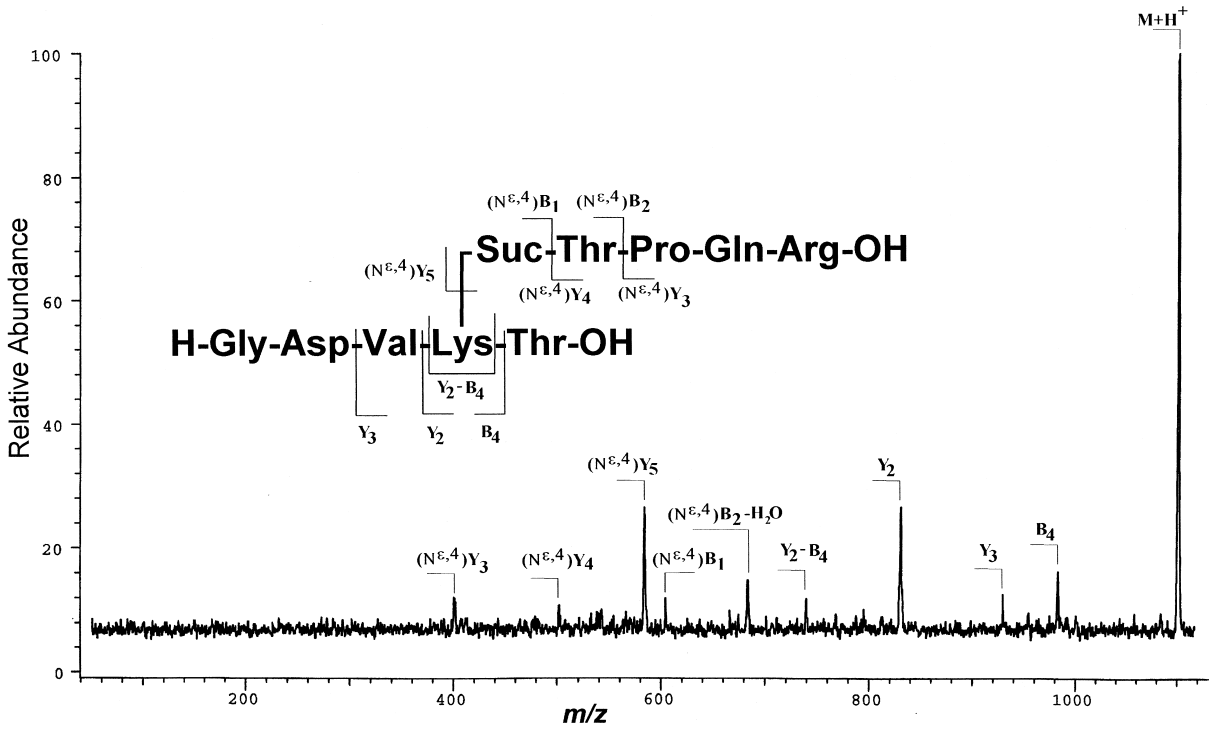

Fig. 5. Collision-induced dissociation mass spectrum of the product generated by incubation of peptide 1 with trypsin.

CID-MS spectrum (Fig. 5) reveals a partial sequence information sufficient to state, that only one degradation product, with the sequence H-Gly-Asp-Val-Lys(Suc-Pro-Gln-Arg-OH)-Thr-OH was formed. The CID-MS spectrum of the product obtained by hydrolysis of peptide 1 in the presence of trypsin and its structure-ascribed fragments is presented in Fig. 5.

The nomenclature used to identify the peptide cleavage products has been described by Roepstorff and Fohlman [19]. In short, the B_n ions represent the N-terminal fragments which have been severed at the amide bond, where n designates the residue number counting from the N-terminus and the Y_n ions represent corresponding C-terminal fragments from the C-terminus. Recently, a nomenclature system for fragment ions of cyclic peptides was also proposed [14]. According to our best knowledge, the CID-MS spectrum presented in this paper is the first published of the peptide branched at the lysine side chain. As can be seen in Fig. 5, the CID spectrum is a result of fragmentation of the bonds not only in the main-chain of H-Gly-Asp-Val-Lys-(Suc-Pro-Gln-Arg-OH)-Thr-OH but also in the peptide that acylate the lysine ϵ-amino group. Therefore, we used amended nomenclature in order to describe CID-fragments of the side-chain peptide, in which an ion representation is prefixed by symbols indicating the position of substitution (atom and residue number). For example, $(N^{\epsilon,4})Y_3$ designate Y_3 fragment of the peptide crosslinked to the ϵ-amino group of the Lys^4 residue of the main backbone. The proposed nomenclature is consistent with the IUPAC-IUB ICBN [10] nomenclature concerning acylation of a side-chain amino group of peptides.

4. Discussion

Interactions between proteins play an important role in many biochemical processes. Although interactions between proteins generally involve large interfaces with many intermolecular contacts, peptides that mimic small binding epitopes may blockage the interactions. Therefore, the search for potential functional epitopes that could be targeted for the design of new inhibitors of proteins interactions is very intensive.

The immunosuppressory region of the β2-domain of HLA-DQ molecules is located on the loop projected outward from the HLA molecules toward the solvent and, therefore, they may be involved in the interactions with some specific receptors, although its function remains unknown. The region can serve as an important functional epitope on the HLA class II surfaces for intermolecular binding. The synthesized peptides that are able to mimic this epitope may interfere with antigen-receptor interface and consequently inhibit their interactions. The immunosuppressive properties suggest that our peptides inhibit some interactions that are essential for activation of $CD4^+$ T lymphocytes. We found previously that the immunosuppressory fragments of HLA-DQ do not affect cell lines production of various cytokines [32]. This indicates that the immunosuppressive action of the nonapeptide cannot be

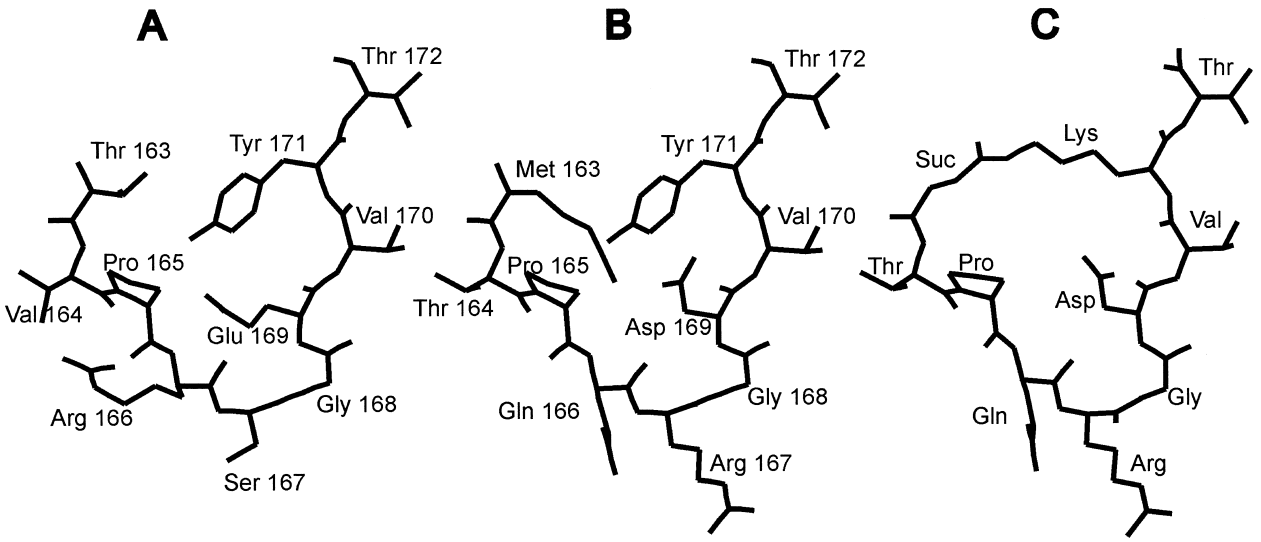

Fig. 6. A. Structure of 163–172 fragment of the β2-domain of HLA-DR1 molecule (Stern et al., 1994). B. Model of the decapeptide 163–172 fragment of HLA-DQ based on mutational analyses of the HLA-DR crystal structure (same projection as in Fig. 6A). It is most noteworthy that the side chains of residues 163 and 171 are oriented toward each other. C. Structure of peptide 1 formed by replacing the Met and Tyr residues by Suc and Lys, respectively, connected to each other by a lactam bridge.

interpreted as the result of blocking of the response of T cells to the tested cytokines. Similar results were also published recently by Boytim et al. [2]. The authors found that the synthetic HLA-DQ (65–79) fragment, located on the α domain, inhibits the proliferation of human T cells but not affects expression of IL-2 or IL-2R. All these confirm our suggestion that MHC class II-derived peptides can directly interfere with interactions of the intact antigens with some coreceptors and modulate T cell responses.

Covalent cyclization of peptides is an important tool in structure-function analysis of bioactive peptides, because it constrains the molecule to enrich or exclude the receptor-bound conformation [18]. The cyclic structures have been often generated by joining the side chains of appropriately substituted amino acids by covalent bonds. These approaches restrict the conformational space available to the resulting products and, in some cases, have produced remarkably active and/or metabolically stable peptide analogues [24,27–29]. However, side chain cyclization can succeed only when the modified positions do not interfere with the binding of the analog to the receptor or enzyme. Therefore, most attempts to evaluate the feasibility of conformational restriction produced cyclic structures in which the modified side chains of the hormone or enzyme inhibitor were found to project away from the receptor.

The receptor-bound conformation of the nonapeptide should be similar to the corresponding epitope region of HLA-DQ, basing on the assumption that both of the molecules interact with the same coreceptor. We decided to design and synthesize a cyclic analog of the nonapeptide TPQRGDVYT, which mimics sterically the region of HLA-DQ. We began the design by examining the three-dimensional model of the decapeptide 163–172 fragment of HLA-DQ based on mutational analyses of the HLA-DR crystal structures [4,25] (Figs. 6B and 6A, respectively). The close proximity of the side chains of Met^{163} and Tyr^{171} in the model (Fig. 6B) supported the idea that it should be possible to form a cyclic structure by judiciously connecting the side chains. We chose residues Met^{163} and Tyr^{171} for cyclization since the side chains are oriented toward the interior of the HLA-DQ molecule. Therefore, it seems unlikely that these residues are critical for interaction with the putative HLA-DQ receptor and replacing them by other residues should not affect the potency. For the same reason the introduced bridge should not interfere with the receptor. Furthermore, the side chains of residues 163 and 171 are oriented toward each other, what makes them favorably for cyclization. The distance between the α atoms of the residues is 9.7Å and the molecular modeling suggested that it could be easily spanned by an 8-atom linker.

For synthetic ease, we replaced Met and Tyr residues by Suc and Lys, respectively and joined them by means of a lactam bridge (Fig. 6C). The peptide synthesis and cyclization was carried out on solid support to avoid polymerization. The relatively high yield of the cyclic monomer (70%, estimated by analytical HPLC of the crude product) indicates that the starting linear nonapeptide may easily adopt conformation in which the carboxyl group of the N-terminal Suc residue is in a close proximity to the ϵ amino group of the Lys residue.

Two effects have to be considered in the cyclization. First, the modification may induce a conformational change of the peptide, affecting the binding potency. Second, the lactam bridge formation requires altering of chemical profile of peptide 2 by removal of the terminal basic amino group of Thr and the phenolic side chain of Tyr. To analyze

the contributions of these two effects, we synthesized the peptide 3, which contains the chemical moieties present in the cyclic peptide 1. The mutations reduced moderately the potency measured in the cellular response assay, and do not affect the humoral immune response. Therefore, the increase of potency observed in case of compound 1 is caused by cyclic constrained rather than other structural changes.

Molecular modeling studies (data not shown) suggested that cyclic nonapeptide 1 could closely mimic the conformational feature of the β-turn in a model of the epitope region of HLA-DQ. To confirm our design principle, the structure of the cyclic peptide 1 was determined by CD spectroscopy (Fig. 5). Spectrum of peptide 1 in TFE solution is similar to its spectrum in water, suggesting that the peptide molecule is rigid and its conformation is not too much affected by a solvent. The negative maximum at about 220 nm probably results from the presence of the β-turn conformation in the conformational equilibrium [17]. This finding is consistent with the expected β-turn conformation, with Arg167 and Gly168 in $i + 1$ and $i + 2$ positions, respectively in the thymopentin-like loop of the HLA-DQ model. Linear peptide 2 exhibited more flexible conformation in water solution as suggested by CD analysis and consequently displayed decreased biologic potencies in comparison with constrained peptide 1.

High potency of peptide 1, particularly in the DTH test, suggests that the stable β-turn conformation mimicking the native protein surface region is important for the immunosuppressive activity of the peptide. The peptide may block the interactions of the mimicked epitope with some receptors. CD4 is one of known coreceptors that interact with MHC class II molecules, leading to enhanced responses of $CD4^+$ T cells. Numerous studies have been performed to determine the regions of HLA class II involved in CD4 binding. It has been found that CD4 binds mostly to nonpolymorphic determinants of class II MHC [5,21,37]. A peptide derived from sequences within the β1-domain of HLA-DR (41–55), as well as two peptides derived from sequences within the β2-domain (121–135 and 141–155) inhibit CD4-class II adhesion [3]. Zagury et al. [37] have also found that the synthetic peptide derived from the $\beta-$chain of HLA-DP (140–149) induces immunosuppression by inhibition of the $CD4^+$ cell immune activation. Satoh et al. [20] demonstrated that synthetic cyclic heptapeptide that mimics the CD4 domain 1 CC′ surface loop of the CD4 molecule effectively blocked the CD4-MHC class II interaction. The peptide possessed a significant immunosuppressive activity in vitro and in vivo and was resistant to proteolytic degradation. All these findings suggest that the peptides with sequence involved in the MHC class II interaction with CD4 are sufficient to induce a downstream negative regulatory signal.

According to the crystal structure of HLA-DR molecule as well as the conformational models of the other HLA classes, the immunosuppressive region is located within the surface loop, in a close spatial proximity to the CD4 binding site. The proximity suggests that the loop may also be involved in the interactions with the CD4 molecule. However, the loop may also be responsible for interactions with other unknown coreceptors. Therefore, the synthesized peptide may inhibit adhesion of HLA class II molecules to their coreceptors, although another mechanism of immunosuppression, cannot be excluded either [13].

Our results clearly demonstrate that the cyclization of the nonapeptide fragment of HLA-DQ did not increase its resistance to digestion catalyzed by trypsin. Mass-spectrometry analysis proved that the peptide bond between Arg^4 and Gly^5 was hydrolyzed in the presence of the enzyme. This indicates that cyclic peptide 1 is even a better substrate for trypsin than linear peptide 2. It has been proved, that trypsin exhibits specificity for a particular substrate and inhibitor conformation near the scissile bond [36]. The susceptibility of cyclic peptide 1 to be a subject of the proteolytic digestion may be connected with its tendency to accept a conformation needed for proteolysis. We compared the crystal structure of CMTI-I, which is known tight-binding trypsin inhibitor (from the X-ray crystal structure of the complex formed between bovine β-trypsin and CMTI-I [1]) with the structure of cyclic peptide 1, to examine the possibility that peptide 1 possesses a restricted substrate-like conformation. As can be seen in Fig. 7 conformations of backbones of S_1-S_4 and P_1-P_4 residues (nomenclature that of Schechter and Berger [22]) in peptide 1 and CMTI-I, respectively are very similar. Despite the differences in conformational angle values of the side chains, the general run of the peptide backbone of the tetrapeptide fragments is similar in both cases, what is evidenced by relatively small deviations of the positions of peptide backbone atoms (Cα, C′ and N) of both fragments; RMS (root-mean-square) deviation was found to be 0.8 Å (results not shown). Thus, the easy proteolytic digestion of peptide 1 supports in fact our suggestion that its privileged conformation in solution may be close to that of the conformation of the immunosuppressory mini-domain of HLA-DQ. It seems also possible that the Arg^{167}-Gly^{168} peptide bond in intact HLA-DQ molecules may also interact with an active site of the enzyme and consequently be hydrolyzed in presence of trypsin or other serine proteases.

In summary, we attempted to design a conformational constraint to preserve the conformation of the nonapeptide fragment loop of HLA-DQ. We synthesized analog where the side chain of appropriately substituted residue in position 8 was joined to the N-terminus by means of succinic bridge. For synthetic ease, we replaced the Tyr^8 residue by Lys connected with the succinic residue by the amide bond. Our results demonstrate that the conformationally restricted synthetic peptide mimicking the HLA-DQ (164–172) strongly inhibit the cellular immune response and is cleaved efficiently by trypsin. Our results indicate that the loop may serve as an important functional epitope on the HLA class II surface for intermolecular binding.

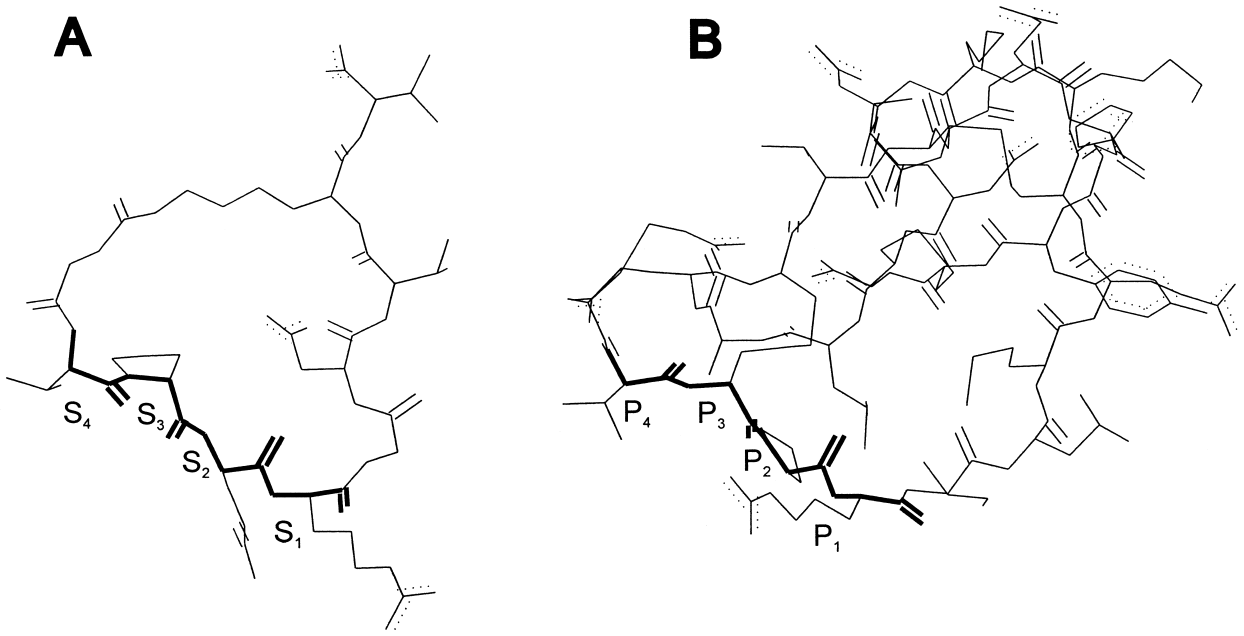

Fig. 7. Comparison of the three-dimensional structures of peptide 1 (same as in Fig. 6) and CMTI-I (from the X-ray crystal structure of the complex formed between bovine β-trypsin and CMTI-I, Bode et al. 1989). Backbones of S_1-S_4 and P_1-P_4 residues of peptide 1 and CMTI-I are bolded.

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