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HLA-DQ7 β_1 and β_2 derived peptides as immunomodulators

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Modulation of protein–protein interactions involved in the immune system by using small molecular mimics of the contact interfaces may lead to the blockage of the autoimmune response and the development of drugs for immunotherapy. The nonpolymorphic β-regions, exposed to the microenvironment, of the modeled HLA-DQ7, which is genetically linked to autoimmune diseases, were determined. Peptides 132–141 and 58–67, located at the β_1 and β_2 domains of HLA-DQ7, respectively, were tested for their involvement in the interactions with CD4$^+$ T lymphocytes. Linear, cyclic, and dimeric analogs that mimic the exposed surfaces of HLA-DQ7 were designed and synthesized. Their immunosuppressory activities, found in the secondary, humoral immune response to sheep erythrocytes (SRBC) in mice *in vitro*, ranged from 11% to 53%. The significance of the total charge of the peptides, the pattern of the hydrogen bonding, and the presence of secondary structure were investigated in relation to the immunomodulatory effect of the peptides. Two dimeric analogs of the HLA-DQ7 58–67 fragment, consisting of the two monomers covalently linked by a polyethylene glycol (PEG) spacer, able to mimic the superdimers, were also synthesized and studied. As the 58–67 segment is located at the β_1 region of HLA-DQ7, close to the major histocompatibility complex (MHC) groove, one may assume that the 58–67 peptide could accommodate the association between T-cell receptor (TCR) and human leukocyte antigen (HLA) by activating a co-stimulatory molecule of the TCR/HLA interaction. This hypothesis is supported by the confocal laser image of the fluorescein-labeled 58–67 peptide and by the fact that it is an immunostimulator at low concentration. **Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: peptide immunomodulators; HLA-DQ7 β_1; HLA-DQ7 β_2; solvent-accessible surface areas

Introduction

Interactions between proteins are very frequent and crucial for many biological processes. Modulation of such interactions may lead to the development of new drugs. A great example of interacting proteins is the immune system, involving large interfaces with many intermolecular contacts. Disruption of such interactions by small molecular mimics or inhibitors of these surfaces may lead to blockage of the autoimmune response and the development of drugs for immunotherapy.

The human leukocyte antigens (HLA-DP, HLA-DQ, and HLA-DR) class II molecules are $\alpha\beta$-heterodimers, integrally associated with membrane of antigen-presenting cells (APC) and playing a central role in the immune response by presenting peptide fragments of foreign (exogenous) antigens to the T-cell receptors (TCR) of the CD4$^+$ T cells. The CD4 co-receptor molecule of T cells enhances the binding process between the TCR and the class II molecule in low-affinity interactions and binds to the nonpolymorphic domains of the HLA-II molecules [1,2]. Furthermore, peptides derived from the β-chain of the HLA-DQ molecule suppress the humoral and cellular immune response [3,4], whereas peptides derived from the β-chain of the HLA-DP and HLA-DR molecules have immunomudolatory potency [5]. In addition, the analysis of the three-dimensional structure of the HLA class II molecules by X-ray crystallography [6] showed that the HLA molecules crystallize as superdimers $(\alpha\beta)_2$ instead of monomers. Dimerization of a peptide fragment, located in the β-chain of the HLA-DR molecule, blocked the immune response and resulted in enhanced immunosuppressory properties [7,8].

Although the main role of the immune system is to protect the organism against the pathogens, frequently the immune system turns against the self-antigens causing a wide range of diseases called autoimmune diseases. Many factors are involved in the development of an autoimmune disease, one of them is a genetic one and is related to the HLAs. It is well documented that some autoimmune rheumatic diseases such as Sjogren's syndrome (SS) and systemic lupus erythematosus (SLE) are genetically linked to DQ2 (HLADQA1*0501/DQB1*0201) and DQ7 (HLA-DQA1*0501/DQB1*0301) molecules [9–11].

The major objective of our study was to precisely determine and study the nonpolymorphic β-regions of the modeled HLA-DQ7 [12], which are exposed to the microenvironment and might be involved in the interactions with CD4$^+$ T lymphocytes. Linear, cyclic, and dimeric peptide analogs that mimic the exposed surfaces of HLA-DQ7 were designed, synthesized, and tested for their influence on the humoral immune response.

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Materials and Methods

Computational Methods

Prediction of the solvent-accessible surface areas (SASAs) of the HLA-DQ7 molecule

The determination of the SASAs of the modeled HLA-DQ7 molecule [12] was performed by using the GETAREA Web service [13]. The radius of the water probe was set to 1.4 Å. The SASAs were estimated per residue, and the β-chain of the DQ7 was mapped with 183 overlapping octapeptides, summing up the SASA values, to determine the peptide regions that present the higher percentages of exposed surface area.

Peptide Synthesis

The amino acid sequences of the synthesized peptides are listed in Table 1, whereas the parameters of the synthesis, purification, and characterization are summarized in Table 2. All the peptides were synthesized manually by the stepwise solid phase procedure [14–17].

Synthesis of the peptides 1–5

The synthesis of the peptides 1–5 was carried out on a Rink Amide AM resin (0.71 mmol/g) using the Fmoc methodology. The amino acids were introduced as Fmoc-Thr(tBu)-OH, Fmoc-Glu(0tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH (tBu, *tert*-butyl; Trt, trityl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl; Boc, *tert*-butyloxycarbonyl). Fmoc groups were removed using 20% piperidine/DMF. The coupling reactions were performed using a Fmoc-amino acid/HBTU/HOBt/DIEA/resin molar ratio of 3/3/3/6/1. DMF, used for couplings, was distilled in the presence of ninhydrin to remove traces of amines. Completion of the coupling reactions and deprotections were ensured by the use of the Kaiser ninhydrin test. Acetylation was performed using acetic anhydride in pyridine. The fluorescein-labeled peptide (5) was prepared by coupling of the 5-(and 6)-carboxy-fluorescein succinimidyl ester (NHS-carboxy fluorescein) to the *N*-terminal amino acid. The peptides were cleaved from the resin by treatment with TFA/TIS/H_2O (95/2.5/2.5) (TIS, triisopropylsilane). The resin was removed by filtration, the filtrate was evaporated under reduced pressure, and the product was precipitated with cold diethyl ether. Yields ranged from 50% to 90%. The crude peptides were purified by semipreparative RP-HPLC on a C18 column (25 cm × 10 mm, 5 μm, Supelco). Appropriate programmed gradients were applied using eluants A (H_2O/0.1% TFA) and B (CH_3CN/0.1% TFA). The purity of the peptides was checked by analytical HPLC on a C18 column (250 × 4.6 mm^2, Supelco), and the correct molecular masses were confirmed by ESI-MS (Micromass Platform LC). Finally, the peptides were lyophilized with acetic acid (Table 1).

Table 1. The amino acid sequences of the synthesized peptide analogs

Peptide	Amino acid sequences	Regions
1	FRNDQEETTG-NH_2	132–141
2	AEYWNSQKEV-NH_2	58–67
3	Ac-FRNDQEETTG-NH_2	132–141
4	Ac-AEYWNSQKEV-NH_2	58–67
5	Fl-AEYWNSQKEV-NH_2	58–67
6	F-Orn-N-D-E-E-E-T-T-G-NH2	132–141
7	FRProψ[CH_2NH]GQEETTG-NH_2	132–141
8[a]	PEG-(AEYWNSQKEV-NH_2)$_2$	58–67
9[b]	PEG-(AEYWNSQKEV-NH_2)$_2$	58–67

[a] Linker composed of 12 oxoethylene units.
[b] Linker composed of 7–18 oxoethylene units.

Synthesis of the peptides 6 and 7

Peptides 6 and 7 were synthesized on 4-methyl-benzyhydrylamine (MBHA) resin using the Boc strategy. Ornithine and glutamic acid were introduced as Boc-Orn(Fmoc)-OH and Boc-Glu(OFm)-OH, respectively, in the synthesis of peptide 6. The Proψ[CH_2NH]-Gly moiety was introduced as Boc-Proψ[CH_2NH]-Gly-OH in the synthesis of peptide 7. The other amino acids were introduced as Boc-Thr(Bzl)-OH, Boc-Glu(OBzl)-OH, Boc-Asp(OBzl)-OH, Boc-Arg(Tos)-OH (Bzl, benzyl; Tos, toluene-4-sulfonyl). Deprotection of Boc was achieved with 40% TFA/DCM. Couplings were performed using a molar ratio of Boc-amino acid/HBTU/HOBt/DIEA/resin of 3/3/3/9/1. Deprotection and coupling reactions were monitored by the Kaiser ninhydrin test. The cyclization reaction between the side chains of Orn and Glu was performed after deprotection of the side-protecting groups with 20% piperidine/DMF and coupling of the γ-carboxylic group of Glu and the $N^{\varepsilon}H_2$ group of Orn with HOBt/HBTU/DIEA. The peptides were cleaved from the resin with anhydrous hydrogen fluoride (HF) in the presence of anisole/phenol 1 ml/0.5 g as scavengers. Yields ranged from 87% to 90%. The crude peptides were purified by semipreparative RP-HPLC on a C18 column (25 cm × 10 mm, 5 μm, Supelco). The purity of the peptides was checked by analytical HPLC on a C18 column (250 × 4.6 mm^2, Supelco), and the correct molecular masses were confirmed by ESI-MS (Micromass Platform LC) (Table 1).

Synthesis of the dimeric polyethylene glycol (PEG) peptide analogs 8 and 9

PEG diacid comprising 12 oxoethylene units and two propionyl terminal groups was used for the synthesis of 8, whereas polydispersed PEG 600 diacid containing 7–18 oxoethylene units and two acetyl terminal groups was used for the synthesis of 9. Preparation of the pentafluorophenyl diester of PEG: pentafluorophenol (6.66 mmol) was added to a solution of PEG diacid (3.33 mmol) in dry ethyl acetate (30 ml), and the mixture was stirred in ice bath for 15 min. DCC (6.66 mmol), dissolved in ethyl acetate (0.5 ml), was added to the reaction mixture, and the precipitate was filtered and washed with ethyl acetate. The combined filtrates were evaporated *in vacuo*, and the resulting oily products were used for the synthesis of dimers 8 and 9.

Synthesis of peptide 8: after completion of the synthesis of the protected peptide Fmoc-Ala-Glu(OtBu)-Tyr(tBu)-Trp(Boc)-Asn(Trt)-Ser(tBu)-Gln(Trt)-Lys(Boc)-Glu(OtBu)-Val-Resin (0.0244 mmol, part of the resin used for analog 2 synthesis), the Fmoc-protective group was removed using 20% piperidine in DMF and the resin was washed thoroughly with DMF. PEG-pentafluorophenyl diester (0.0122 mmol), dissolved in DMF, was added to the resin in four portions during 48 h at room temperature. The peptide was cleaved from the resin using a mixture of TFA : H_2O : TIS (95 : 2.5 : 2.5) for 5 h and precipitated with cold diethyl ether. The crude peptide was purified by semipreparative

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Table 2. Parameters of the synthesis, purification, and characterization of the peptide analogs

Peptide	Yield (%)	RP-HPLC Gradient elution	t_R (min)	ESI-MS
1	Crude: 50[a]	A/B: 95 : 5	7.9	Calculated M^+: 1195.21
	Purified: 55[b]	A/B: 40 : 60		Found M^+: 1195.55
2	Crude: 82	A/B: 95 : 5	15.59	Calculated M^+: 1252.30
	Purified: 66	A/B: 30 : 70		Found M^+: 1252.35
3	Crude: 80	A/B: 95 : 5	13.2	Calculated M^+: 1237.25
	Purified: 50	A/B: 60 : 40		Found M^+: 1237.29
4	Crude: 85	A/B: 95 : 5	12.9	Calculated M^+: 1294.38
	Purified: 85	A/B: 30 : 70		Found M^+: 1294.49
5	Crude: 90	A/B: 90 : 10	25.2	Calculated M^+: 1610.64
	Purified: 45	A/B: 40 : 60		Found M^+: 1610.55
6	Crude: 87	A/B: 90 : 10	8.5	Calculated M^+: 1136.08
	Purified: 15	A/B: 30 : 70		Found M^+: 1136.18
7	Crude: 90	A/B: 90 : 10	8.8	Calculated M^+: 1106.20
	Purified: 30	A/B: 50 : 50		Found M^+: 1106.53
8	Crude: 83	A/B: 100 : 0	20.9	Calculated M^+: 3157.52
	Purified: 15	A/B: 20 : 80		Found M^+: 3157.53
9	Crude: 79	A/B: 100 : 0	18.5–21.5	Calculated M^+: 3129.49
	Purified: 13	A/B: 20 : 80		Found M^+: 3129.39

[a] Yield of synthesis.
[b] Yield of purification.

RP-HPLC on a ODS 120T TSKgel column (300 × 21.5 mm^2, 10 µm, TOSOH). The purity of the peptide was checked by analytical HPLC on a C18 column (250 × 4.6 mm^2, 5 µm, Vydac), and the correct molecular masses were confirmed by ESI-MS (MicrOTOF-Q, Bruker). Synthesis of peptide dimer 9 was similar except that PEG-pentafluorophenyl diester formed from polydispersed PEG diacid (average MW 600, Aldrich 40–7038) was used (Table 1).

Immunological Assays

The immunomodulatory activities of the peptides were tested in the mouse model of secondary, humoral immune response to sheep erythrocytes (SRBC) *in vitro*. The number of antibody-forming cells (AFC) was determined by the Jerne test modified by Mishell and Dutton [18]. Twelve-week-old CBA mice of both sexes were used.

CBA mice were sensitized with 0.2 ml of 1% SRBC suspension. Four days later, the animals were killed. The spleens were isolated aseptically and homogenized by pressing the organs through a plastic screen into Hanks' medium. After centrifugation, the splenocytes were treated with 0.83% ammonium chloride to lyze erythrocytes. Subsequently, splenocytes were washed twice by centrifugation in Hanks' medium and filtered through a sterile cotton wool to remove dead cells and debris. Finally, the cells were re-suspended in a culture medium consisting of RPMI 1640, supplemented with fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol, and antibiotics. The splenocytes were distributed in 24-well flat-bottom culture plates at a concentration of 5 × 10^6 cells/well, containing culture medium, followed by the addition of the antigen SRBC to the cell cultures at a dose of 0.1 ml of 0.005% suspension per well. Peptides were first dissolved in DMSO to the concentration of 10 mg/ml and then diluted by culture medium to the final concentrations of 100 and 10 µg/ml (the final DMSO concentrations were 1% and 0.1%, respectively). The AFC control was provided by 0.1% saline solution, whereas the influence of DMSO on the AFC number was established in a separate experiment with 0.1% and 1% of DMSO in the culture medium. As major histocompatibility complex (MHC) system is sensitive to peptides from the host and from the pathogens, we used the equimolar mixture of amino acids (we selected 14 amino acids constituting the investigated peptides) to study the effect of random sample on the AFC number. The amino acid mixture was examined in two experiments, in the first one, the culture medium (RPMI) was used to dissolve the sample, and in the second one, the protocol with DMSO was applied. No effect of the amino acids mixture on the immune response was found in either case (data not shown in Table 5).

After 4-day incubation, the number of AFC was determined. Data were statistically evaluated using Student's *t*-test and expressed as an AFC number per 10^6 splenocytes and regarded significant when $p < 0.05$. The percentage values of the inhibition of the immune response were calculated according to the equation:

$$\text{Percentage of immunosuppression} = 100\,[1 - (\text{experimental value/control})] \quad (1)$$

The control values used in this calculation were taken from experiments in which the same amount of DMSO as following from peptide solubilization was added to the cells. In the case of the direct DMSO experiment, as well as for the amino acids dissolved in RPMI, the immunosuppression scores were calculated using the control values for 0.1% saline solution.

When a peptide stimulated the immune response, negative values were presented.

Fluorescent Assays of the Peptide 5 using T, B, and Macrophage-like Cell Lines

The tests were performed using LBRM cell line (T cells), P-388D1 cell line (monocytes/macrophages), and 7TD1 cell line (B cells).

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Cells were cultured for 2 days in 24-well plates at density of 50 000 per well, before the test, in a culture medium (RPMI-1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, and antibiotics). The peptide was dissolved in DMSO at concentration of 10 mg/ml, then diluted in the culture medium to concentrations: 100, 10, and 1 µg/ml. The preparation was incubated with the cells for 1 h in a cell culture incubator (37 °C, 100% humidity). After the incubation, the cells were washed four times with Hanks' medium and re-suspended in 0.2 ml of the culture medium. The samples were analyzed in a fluorescent microscope (ZEISS Axiovert 25, 400×), and photographs were taken. The cell viability was checked by means of Trypan blue exclusion, the cells were 99% viable.

Results and Discussion

In a previous study, the modeling of HLA-DQ7 was presented based [12] on the crystal structure of HLA-DQ8, the HLA molecule of a high risk factor of type I diabetes, an autoimmune disease. The quality and reliability of the modeled HLA-DQ7 was confirmed by the Ramachandram plot and the TINKER molecular modeling software.

The nonpolymorphic β-regions of HLA-DQ7, which are exposed to the solvent and might be involved in the interaction with $CD4^+$ T lymphocytes, were determined using the GETAREA program. Initially, the SASA of each residue was calculated, and subsequently the β-chain of HLA-DQ7 was mapped with 183 overlapping octapeptides by summing the SASA of the comprising residues.

Two peptides were identified, p131 and p57 (Figure 1), spanning the HLA-DQ7 fragments: 133–140 (Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr) and 59–66 (Glu-Tyr-Trp-Asn-Ser-Gln-Lys-Glu). The first sequence is located at the β_2 and the second at the β_1 domain of the β-chain, close to the antigen-binding groove of HLA-DQ7 [19–21]. The molecular surface of the peptides was determined based on the modeled HLA-DQ7 and by using the Swiss-PDB Viewer program. It was found that the 133–140 sequence was characterized by cavities (exposed surface area 752.6), whereas the 59–66 sequence (exposed surface area 710.6) by fingers (Figure 2). In Figure 3, the α- and β-chains of the modeled HLA-DQ7, the

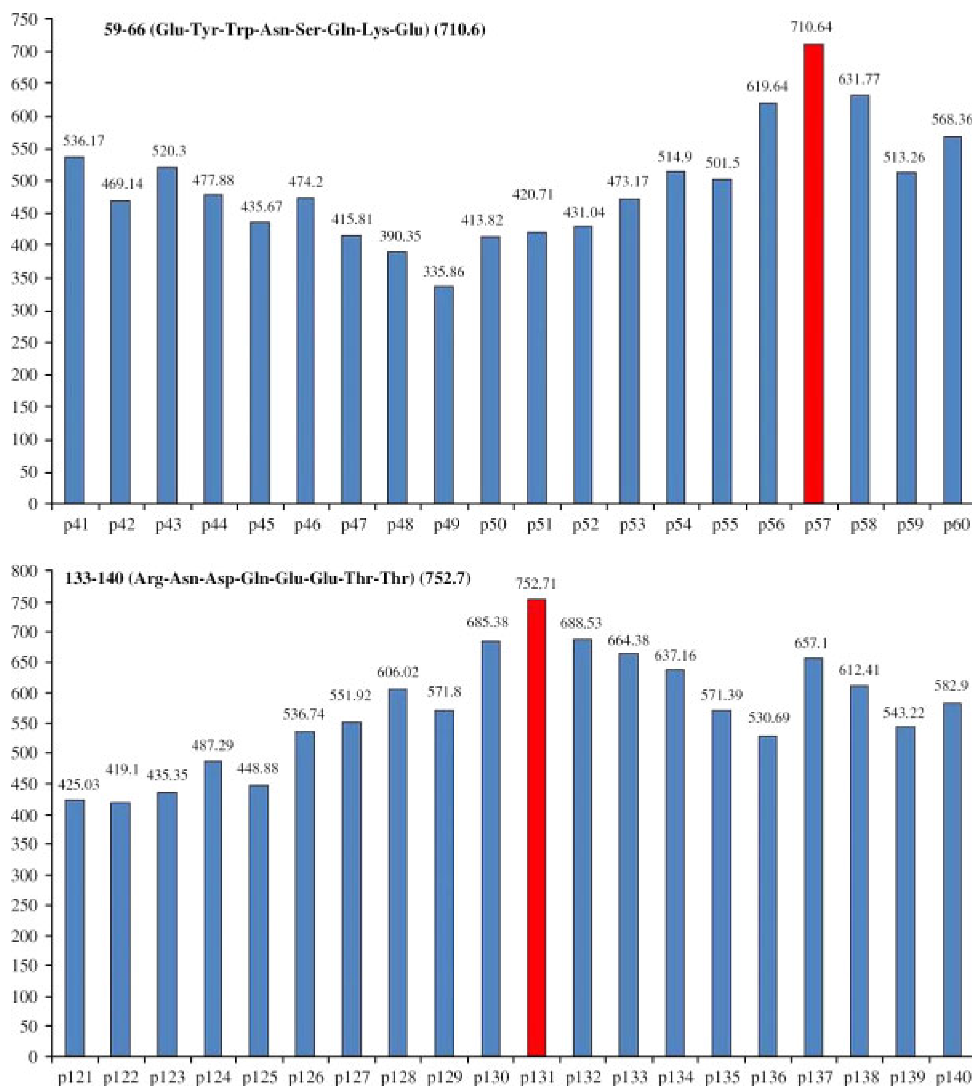

Figure 1. Percentages of the exposed surface areas of overlapping octapeptides. p57 (a) corresponds to the Glu-Tyr-Trp-Asn-Ser-Gln-Lys-Glu sequence β_1 59–66, % exposed surface area 710.64. p131 (b) corresponds to the Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr β_2 133–140, % exposed surface area 752.71. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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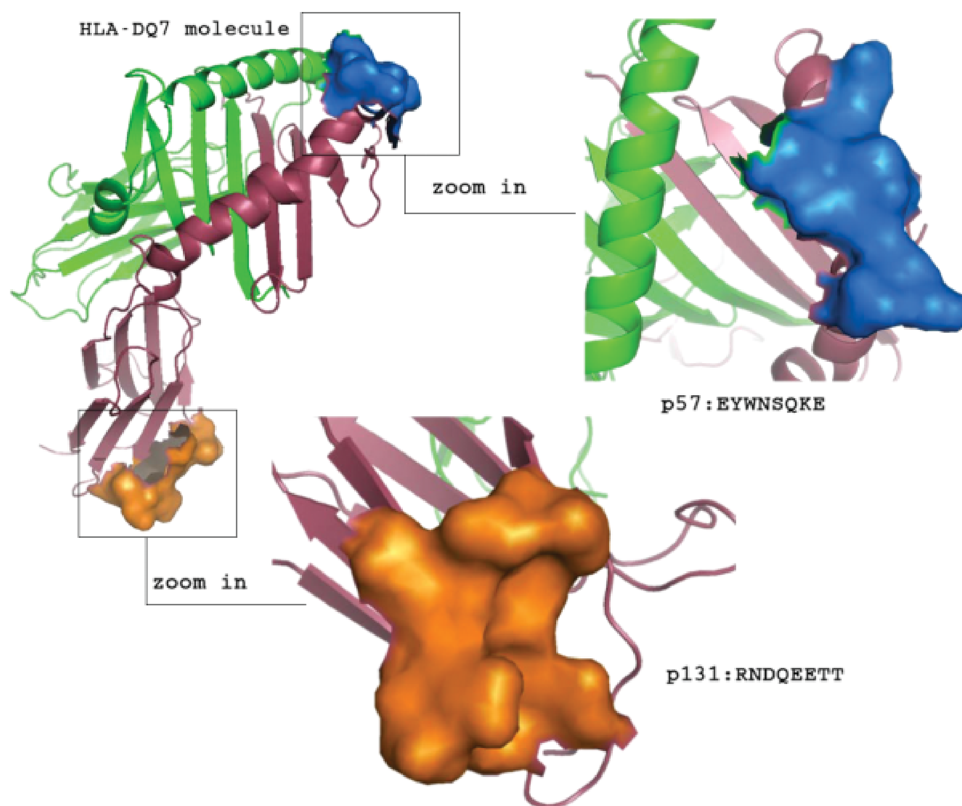

Figure 2. Molecular surfaces of the β_1 59–66 and β_2 133–140 of the modeled HLA-DQ7.

antigen-binding groove with a T-cell epitope [12], and the 133–140 β_2 and 59–66 β_1 domains exposed to the microenvironment are illustrated.

The 133–140 and 59–66 sequences were expanded by two residues: one at the *N*-terminus and the other at the *C*-terminus, respectively. The resulted sequences, of ten residues length, were synthesized as carboxy-amides either with free or acetylated *N*-terminal α-amino group (Table 1, peptide 1–4 analogs). A fluorescein moiety was inserted at the *N*-terminus of the peptide 5 analog to monitor its possible penetration into the cells.

Our concept for the design of the peptide 6 analog was to search for the presence of a folded structure in the 132–141 sequence and subsequently to stabilize it by the formation of a covalent bond. The dihedral angles φ, ψ, and ω of all residues within the peptide backbone, as well as the C^α(i)–C^α(i + 3) and O(i)–N(i + 3) distances were calculated using the tools of the Swiss-PDB Viewer program (Tables 3 and 4). In fact, the estimated distances between the C^α of Arg^{133} and the C^α of Gln^{136}, and between the oxygen of Arg^{133} and the nitrogen of Gln^{136} are lower than 7Å and 3.5Å, respectively. Therefore, the sequence 133–136 (Arg-Asn-Asp-Gln) fulfils the distance criterion for the occurrence of a β-turn between the Arg-CO (i) and the Gln-NH (i + 3). However, the φ and ψ values of Asn (i + 1) and Asp (i + 2) argue in favor of a β II′ turn.

In order to get a conformationally restricted analog, Arg^{133} was substituted by Orn and Gln^{136} by Glu and their side chains $N^\delta H_2$ and γ-COOH were cyclized. The central segment -Asn-Asp- (134–135) of the $\beta_{II'}$ turn Arg-Asn-Asp-Gln (133–136) was replaced by the pseudopeptide -Proψ[CH_2NH]-Gly to obtain the peptide analog 7 and study the influence of the pseudopeptide bond in its immunomodulatory effect [22].

Polyethylene glycol (PEG) dicarboxylic acid was used to form the dimeric analogs of the 58–67 fragment. The peptides were linked at *N*-termini by the PEG-dicarboxamide according to the method published previously for other peptides [8,23]. The linker in peptide 8 consists of twelve -CH_2CH_2O- (oxoethylene) units and two propionic acid fragments, whereas for the synthesis of peptide 9, a polydispersed PEG derivative containing 7–18 oxoethylene units and acetic acid fragments was used. The design of these peptide analogs was based on the three-dimensional structure of the human HLA-DR1. Crystallographic data pointed out that HLA-DR1 crystallizes as superdimer instead of monomer [6,7,24]. A superdimer structure was also observed in animal B cells [25]. It has been suggested that MHC class II molecules may interact with the TCR and $CD4^+$ co-receptor as a superdimer [26,27]. It may be therefore concluded that substances that are able to modulate the dimerization of receptors may control such a process and are potential immunomodulators. Dimeric ligands are known candidates for mediating dimerization of these types of receptors [28].

Previously, we observed a strong influence of the length of the linker on immunomodulatory activity of the resulting peptide dimer. Analogs with a linker too short to orient the peptide fragments in the same position as in the superdimer expressed the activity comparable to that of the monomeric peptide, whereas the application of proper length linker led to bivalent compound with increased potency. Interestingly, the use of a longer linker (even twice as long as the optimal one) produced analogs with high activity [7,8,23]. The comparison of the immunomodulatory activity of the synthesized dimers with linkers of different length may also serve as a tool to analyze the distance between the receptors.

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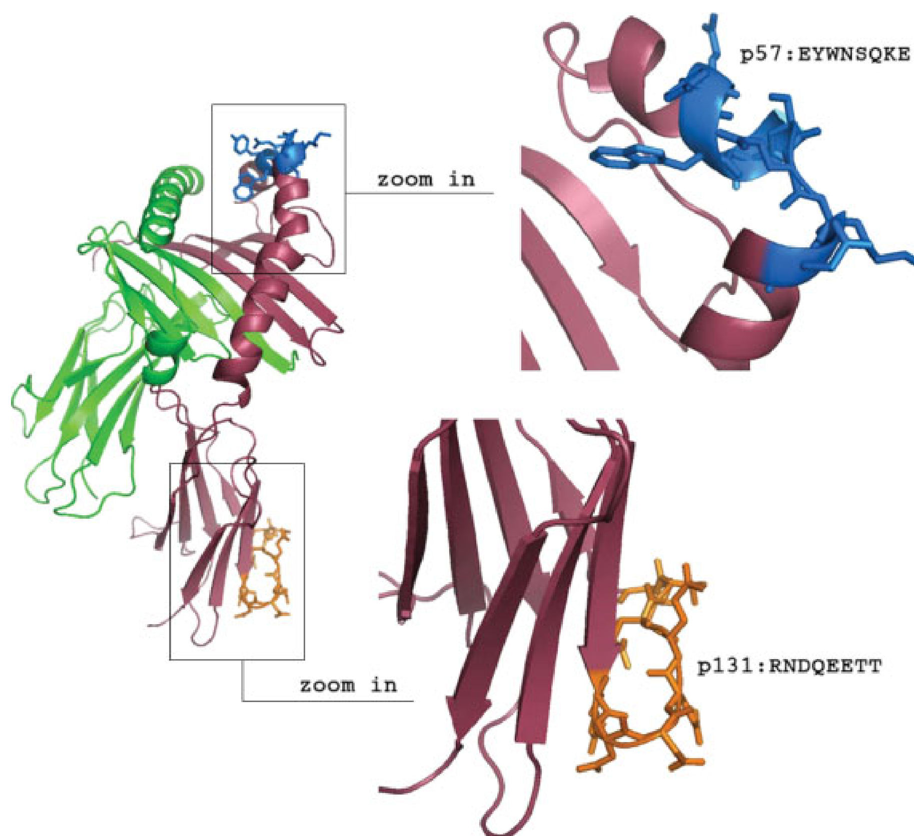

Figure 3. Illustration of the 59–66 and 133–140 region on the modeled HLA-DQ7.

Table 3. The dihedral angles (Degrees) φ, ψ, and ω of Phe-Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr-Gly (132–141)

Amino acid	ω	φ	ψ
132 Phe	176.29	−119.64	142.84
133 Arg	172.34	−122.44	101.33
134 Asn	−176.38	63.94	−108.52
135 Asp	179.54	−100.22	−5.57
136 Gln	−177.91	−111.70	145.60
137 Glu	175.13	−78.66	138.24
138 Glu	173.24	−86.09	119.82
139 Thr	178.74	−104.14	−39.05
140 Thr	−178.45	−98.86	158.07
141 Gly	−177.87	64.69	18.77

Table 4. Distances (Å) between (a) O(i)–N(i+3) and (b) C^{α}(i)–C^{α}(i+3) of Phe-Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr-Gly (132–141)

(a) O(i)	N(i + 3)	Distance
Phe	Asp	6.82
Arg	Gln	3.08
Asn	Glu	6.42
Asp	Glu	7.73
Gln	Thr	6.95
Glu	Thr	6.13
Glu	Gly	6.89
(b) C^{α}(i)	C^{α}(i + 3)	Distance
Phe	Asp	6.89
Arg	Gln	5.49
Asn	Glu	8.16
Asp	Glu	9.82
Gln	Thr	9.35
Glu	Thr	9.32
Glu	Gly	7.55

The dimeric analog of the HLA-DQ7 fragment should consist of two monomers covalently linked by a spacer with sufficient length to be able to position them in the same manner as in the superdimers. Because the superdimeric structure of HLA-DQ7 remains unknown, we examined the three-dimensional structure of the HLA-DR superdimers [6]. The distance between the Ala^{58} residues in HLA-DR1 dimer is 20 Å. Therefore, we selected a long flexible linker composed of 12 oxoethylene units (analog 8), which should be able to span the gap, allowing the peptide components to fit into the putative co-receptor. Moreover, to investigate the influence of the linker length, we synthesized a library of peptide dimers with spacers containing 7–18 oxoethylene units (polydispersed PEG diacid, analog 9).

The synthesized peptide analogs were studied for their immunomodulatory activities using the AFC test, and the results are summarized in Table 5. The peptides were dissolved in DMSO and then diluted by culture medium to the desired concentration.

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Table 5. Immunomodulatory activities of the synthesized peptide analogs

Peptides	Dose (µg/ml)	AFC × 10^{6a}	±SE[b]	p (Student's test)	Suppression (%)
	0[c]	1094	21	–	–
Cyclosporine A	1	400	23	<0.001	69
	10	250	20	<0.001	85
DMSO	0	1231	53	–	–
	0.1%[d]	1350	57	NS[e]	−10[f]
	1%[d]	1062	33	NS	14[f]
1	0	1094	21	–	–
	10	1587	78	<0.01	−32
	100	662	59	<0.001	30
2	0	1094	21	–	–
	10	1431	61	<0.01	−19
	100	800	44	<0.01	15
3	0	1094	21	–	–
	10	894	53	<0.01	25
	100	444	42	<0.001	53
4	0	1094	21	–	–
	10	1106	66	NS	8
	100	600	76	<0.01	36
6	0	1231	53	–	–
	10	1506	39	NS	−12
	100	1387	72	NS	−31
7	0	1231	53	–	–
	10	1743	12	<0.001	−29
	100	1125	44	NS	−6
8	0	1487	28	–	–
	10	1170	109	NS	28
	100	1140	68	<0.01	11
9	0	1094	21	–	–
	10	1087	51	NS	9
	100	687	116	<0.02	27

[a] The results are the average of four replicates.
[b] Standard error.
[c] 0.1% saline solution was used as a control.
[d] The concentration of DMSO in culture medium, as in experiments with peptides (See Materials and Methods).
[e] Statistically nonsignificant.
[f] The 0.1% saline control was used for suppression calculations.

The effect of peptides on the immune response is therefore combined with the influence of DMSO (a limited stimulation at lower dose and 14% suppression evoked by the 1% DMSO solution). To clarify the peptide participation in the overall immunomodulation, we calculated the suppression values against the respective DMSO controls.

Peptide analogs 1–4 (100 µg/ml) inhibited the humoral immune response of mice immunized with SRBC in the range from 15% to 53%. The acetylation of the amino terminus of both peptide fragments (analogs 3 and 4) induced stronger immunosuppression, compared with the corresponding free peptide 1 and 2 analogs. However, the application of lower doses (10 µg/ml) of peptides 1 and 2 resulted in stimulation of the immune response, thus giving these peptides a regulatory character. These effects evoked by different concentrations of peptide may suggest two mechanisms of action of these peptides: one, structure dependent, related to the stimulation of immune response, and the other acting through gradual saturation of antigen-presenting grooves of MHC by synthetic peptides, fragments of HLA-DQ7, and, consequently, reduction of immune response at higher doses. The suppression of the immune response by presentation of self-peptides was observed and examined several years ago [29,30].

The constrained analogs 6 and 7 showed stimulation of the immune response, with the activity of the cyclic analog 6 increasing with the administered dose. This suggests that the secondary structure of the analyzed analog may have a strong effect on the immunomodulatory activity. The rigid conformation of the cyclic analog 6 seems to be better suited to the stimulation of the immune response than the constrained but not fixed structure of the pseudopeptide analog 7, which at the higher dose seems to be less active. It is possible that the structure of these peptides prevents them from being presented as antigens, as the MHC groove prefers peptides in extended conformation [6]. Therefore, one of the possible explanations is that the cyclic peptide 6 cannot interact with MHC-presenting groove, but its conformation may be suitable for the hypothetical immunostimulatory interactions.

The dimerization of the 58–67 sequence by PEG diacid resulted in immunosuppression comparable at higher dose to that of the original sequence (analog 2), when the specific length linker (dimer 8) was used. The lower dose, however, evoked more significant immunosuppression. However, the activity of dimeric analog 9 (with polydispersed length linker) at the highest dose used was much higher than that of the dimer 8 and the monomeric peptide 2. The immunosuppression evoked by analog 9 was comparable to that of acetylated analog 4. It may be suggested that in this case the dimerization did not improve the immunosuppressory potency of the AEYWNSQKEV-NH_2 (58–67 fragment). However, stronger and dose-dependent inhibition of the response observed for the polydispersed dimer (analog 9) is worth attention. This may suggest that the length of the linker used for analog 8 was not a perfect one. It is possible that one of the shorter or longer linkers would be more suitable for this purpose. The superdimer structure in HLA-DR and DQ may not be identical, as well as their interaction with the co-receptor. A suggestion concerning a different organization of the HLA-DQ and DP superdimers was previously presented by Brown *et al*. [6].

It is interesting that *N*-terminally modified analogs (acetylated peptides 3 and 4 as well as the pegylated dimers 8 and 9) showed a stronger influence on the immune response than the peptides 1 and 2, containing a free α-amino group. The effect of acetylation on the immunosuppressory activity of peptides was thoroughly investigated before [31]. The acetylated analog 3 shows the strongest immunosuppresory activity in the experiment, which may be explained by the acidic character of the fragment 132–141, with one aspartic and two glutamic acid residues, combined with one arginine residue. As the 58–67 sequence contains two glutamic acid residues and one lysine, the presence of a free α-amino group has a significant impact on the total charge of the peptide. This may affect the pattern of hydrogen bonds responsible for the shape of peptide as well as its interactions with receptors and the stability of the peptide–receptor complex. The network of hydrogen bonds in the respective region of HLA-DR was recognized as important during the structural analysis of HLA [6,32,33].

Therefore, the character of the observed immunomodulatory activity may be divided into three categories: suppression of immune response (*N*-terminally modified analogs 3, 4, 8, and 9), stimulation of the immune response (constrained analogs 6 and 7), and the dose-dependent regulatory effects of peptides 1 and 2.

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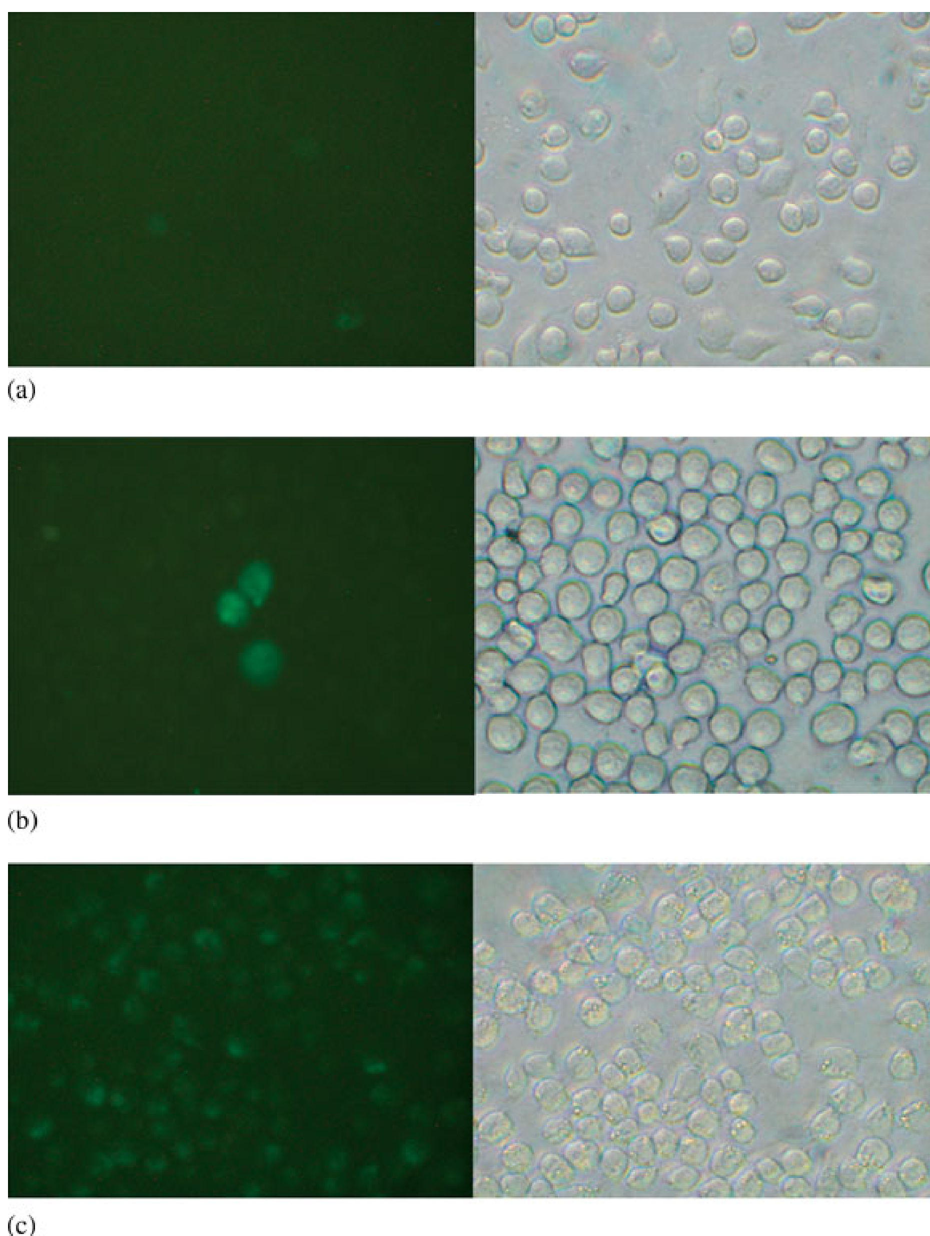

Figure 4. The binding of the fluorescein-labeled peptide 5 to T, B, and macrophage-like cell lines (the peptide concentration 100 µg/ml). (a) T-cell line LBRM; (b) B-cell line 7TDI; (c) macrophage-like cell line P-388D1. Left panel shows fluorescence effect, right panel – the cells in visible light.

As the 58–67 segment is located at the β1 region of the β-chain of HLA, close to the MHC groove (Figure 3), one may assume that peptide AEYWNSQKEV-NH_2 (58–67, analog 2) could accommodate the association between TCR and HLA by activating a co-stimulatory molecule of the TCR/HLA interaction. This hypothesis is supported by the fact that the confocal laser image (Figure 4) showed that the fluorescein-labeled peptide analog 5 was found both on the surface and inside the B cells and macrophages, both being the APC (7TD1 and P-388D1 cell lines, respectively). In majority of P-388D1 cells, a specific surface, local fluorescence and internal fluorescence of granules was confirmed at the concentration of 100 µg/ml of analog 5. The fluorescence was still observed at 10 µg/ml concentration of the compound; however, it was less intensive. At the concentration of 100 µg/ml, some 7TD1 cells (B cells) show total fluorescence, the internal fluorescence was specific. The decrease in concentration of analog 5 lowered the intensity of the fluorescence. For the LBRM cells (T cells), only a weak fluorescence was observed at higher dose of analog 5, with no fluorescence at the lower dose, probably indicating a small, nonspecific reaction. Because the peptide represents a fragment of the MHC class II antigen molecule, typically expressed on B cell and macrophages (APC) and not on T cells, the results confirmed the binding and recirculation of that fragment in B cells and macrophages but not in T cells.

Conclusions

The nonpolymorphic β-regions of the HLA-DQ7 molecule exposed to the microenvironment were identified based on its modeled structure. In order to investigate their interactions with $CD4^+$ T lymphocytes, peptide analogs, derived from 132–141 β_2 and

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58–67β_1, were designed, synthesized, and studied for their involvement in the immune response. Their immunomodulatory activity is highly dependent on the total charges of the peptide analogs, the pattern of their hydrogen bonding, and their secondary structure. Interestingly, the dimeric analogs of 58–67 β_1, located in the vicinity of the MHC groove, are likely to activate a co-stimulatory molecule of the TCR/HLA interaction.

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