Immunogenetics (2005) 57: 8–15
DOI 10.1007/s00251-005-0780-8

ORIGINAL PAPER

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T-cell recognition of HLA-DQ2-bound gluten peptides can be influenced by an N-terminal proline at p-1

Received: 8 October 2004 / Revised: 4 January 2005 / Published online: 16 February 2005

Abstract Recent research has implicated a large number of gluten-derived peptides in the pathogenesis of celiac disease, a preponderantly HLA-DQ2-associated disorder. Current evidence indicates that the core of some of those peptides is ten amino acids long, while HLA class II normally accommodates nine amino acids in the binding groove. We have now investigated this in detail, using gluten-specific T-cell clones, HLA-DQ2-specific peptide-binding assays and molecular modelling. T-cell recognition of both a γ-gliadin peptide and a low-molecular-weight glutenin peptide was found to be strictly dependent on a ten-amino acids-long peptide. Subsequent peptide-binding studies indicated that the glutenin peptide bound in a conventional p1/p9 register, with an additional proline at p-1. Testing of substitution analogues demonstrated that the nature of the amino acid at p-1 strongly influenced T-cell recognition of the peptide. Moreover, molecular modelling confirmed that the glutenin peptide binds in a p1/p9 register, and that the proline at p-1 points upward towards the T-cell receptor. Database searches indicate that a large number of potential T-cell stimulatory gluten peptides with an additional proline at relative position p-1 exist, suggesting that the recognition of other gluten peptides may depend on this proline as well. This knowledge may be of importance for the identification of additional T-cell stimulatory gluten peptides and the design of a peptide-based, tolerance-inducing therapy.

Keywords Celiac disease · Gluten epitope · HLA-DQ2

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Introduction

Celiac disease (CD) is a multifactorial inflammatory disorder caused by an uncontrolled T-cell response directed against wheat gluten and analogous grain storage proteins. The HLA-class II molecule HLA-DQ2 (α1*0501, β1*0201) is the most important susceptibility locus for CD, as roughly 95% of all CD patients are DQ2-positive (Sollid et al. 1989). The role of HLA-DQ2 in presenting gluten-derived peptides to $CD4^+$ effector T cells is well established. HLA-DQ2 selectively binds peptides with large hydrophobic residues at positions p1 and (especially) p9. At positions p4 and p7, negatively charged anchors are preferred, and at p6 a proline residue or a negative charge (van de Wal et al. 1996). While native gluten hardly contains any negatively charged amino acids, these can be introduced by the enzyme tissue transglutaminase (tTG), that selectively deamidates glutamine residues in gluten, resulting in peptides that bind to HLA-DQ2 with high affinity (Molberg et al. 1998; van de Wal et al. 1998a).

Peptide binding to MHC class II molecules is accomplished by the accommodation of side chains of amino acids in the bound peptide in the respective pockets of the peptide-binding groove of MHC molecules. In addition, a network of hydrogen bonds stabilizes the binding of the peptide in the groove over the entire contact length (Koelle et al. 1997; Vartdal et al. 1996). All known crystal structures of class II MHC molecules show that the peptide-binding core consists of nine amino acid residues (the so-called p1/p9 register).

A large number of HLA-DQ2-restricted, T-cell stimulatory gluten epitopes that are implicated in the pathogen-

esis of celiac disease have been identified (van de Wal et al. 1998b; Arentz-Hansen et al. 2000; Molberg et al. 2001; Vader et al. 2002b). However, relatively little is known about their particular MHC-binding characteristics. In a previous study, we reported algorithms that predicted T-cell stimulatory gluten peptides (Vader et al. 2002a). These algorithms were based on requirements for binding to HLA-DQ2 and the specificity of the enzyme tissue tTG. Two types of algorithms were used, one predicting nine-mer peptides, the other predicting ten-mer peptides. Strikingly, T-cell responses were only observed against a series of gluten peptides, which were found with the algorithm searching for ten-mer peptides:$X_1X_2X_3Q_4X_5P_6Q_7X_8P_9$(YFWIL)$_{10}$. Moreover, in a recent study the binding of a ten-mer peptide to MHC class II molecules was demonstrated and suggested to be the consequence of a bulge in the bound peptide (Yassai et al. 2002). Gluten molecules are very proline-rich, and proline is known to introduce a bend in the polypeptide backbone of proteins and peptides, which might facilitate the binding of ten-mer gluten peptides to HLA-DQ2. Since the described predictive algorithm identified some 40 potential T-cell stimulatory peptides in the gluten database, this can indicate that such ten-mers may be an important part of the repertoire of T-cell stimulatory gluten peptides. We have therefore investigated the requirements for binding of these putative ten-mer binding peptides to HLA-DQ2 in detail.

Materials and methods

T-cell lines and clones

The gluten-specific T-cell clones were generated from small intestinal biopsies of celiac disease patients and have been described previously (Vader et al. 2002b).

Peptides

Peptides were prepared using standard Fmoc chemistry. Their identity was confirmed by MALDI-TOF mass spectrometry and HPLC.

Tissue tTG treatment

tTG treatment was performed by incubating the peptides (500 μg/ml) with guinea pig tTG (100 μg/ml, Sigma) in buffer (50 mM triethylamine-acetate, 2 mM $CaCl_2$, pH 6.5) for 4 h at 37°C.

T-cell proliferation assay

Proliferation assays were performed in triplicate in 150 μl RPMI-1640 (Gibco) supplemented with 10% human serum in 96-well, flat-bottom plates (Falcon) using 10^4 gluten-specific T cells stimulated with 10^5 irradiated HLA-DQ2-matched allogeneic PBMCs (3,000 RAD) in the presence or absence of antigen peptides (10 μg/ml). After 48 h at 37°C, cultures were pulsed with 0.5 μCi of ^{3}H-thymidine and harvested 18 h later.

Peptide-binding assay

Ninety-six-well FluoroNunc plates were coated with the HLA-DQ-specific mAb SPV-L3, 2 μg/well in 100 μl carbonate buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6) for 2 h at 37°C, subsequently blocked for 1 h at 37°C, with 0.2% solution of gelatin in PBS. HLA-DR3/DQ2-positive, EBV-transformed B-cells were grown, and a sample was checked for proper HLA-DQ2 expression by FACS analysis, using the SPV-L3 mAb. Subsequently, the remainder of the cells were lysed in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1% NP-40 and protease inhibitor mix (Complete, Roche), 4°C, 4×10^6 cells per 1 ml. Cell debris was removed by centrifugation (4°C, 2,000 g, 15 min). Such prepared lysates were mixed with an equal volume of ice-cold 1% solution of BSA in PBS and pipetted into the SPV-L3-coated plates in 100-μl aliquots. After an overnight incubation at 4°C, the plates were washed and 50 μl binding buffer (0.1% NP-40, 0.1% Tween, 33.6 mM citric acid, 72 mM Na_2HPO_4, pH 5.5 and Complete protease inhibitor mix) was added to each well. A titration range of peptides to be tested (concentration range 600–1.0 μM) were prepared in 10% DMSO containing a fixed amount of the biotin-labelled indicator peptides Glt-156 or MHCIα (46–63) at concentrations of 2.5 μM and 1.2 μM, respectively. Subsequently, 50 μl of the samples was applied to the SPV-L3/HLA-DQ2-coated plates. Following a 48-h incubation at 37°C, each well was washed extensively. Subsequently, 100 μl 1,000× diluted streptavidin-europium in assay buffer (both Wallac) was added and incubated for 45 min at room temperature. After extensive washing, 150 μl/well of enhancement solution (Wallac) was applied, and the plates were read in a time-resolved fluorimeter (1234, Wallac) 15–30 min thereafter. IC_{50} values were calculated based on the observed competition between the test peptides and biotin-labelled indicator peptides and indicate the concentration of the tested peptide required for half-maximal inhibition of the binding of the indicator peptide. Each IC_{50} value was determined in three independent experiments, and the average is presented.

Homology modelling

Homology modelling of the complex between HLA-DQ2 and the Glt-156 peptides was performed essentially as previously described (Reichstetter et al. 2002). The recently determined crystal structure of DQ2 was used as the base molecule (Kim et al. 2004). The program Quanta (Accelrys, San Diego, Calif., USA) was used to obtain a complete structure by providing the missing amino acid residues from the crystal structure (e.g. β105-112) as well as missing atoms from certain residues. The deamidated α-gliadin an-

tigenic peptide in this structure (PFPQPELPY) with its four proline residues adopts a conformation in which the p9Tyr residue lies outside the p9 pocket in a niche formed by β57Ala, β60Tyr, and β61Trp. We reasoned that this mode of anchoring the p9 residue could not be correct for all peptides that bound to DQ2, especially those of high affinity, e.g. the MHCIα (46–63) peptide with an affinity of 163 nM (van de Wal et al. 1997). We therefore aligned the crystal structures of HLA-DQ2 and HLA-DQ8 on the polypeptide backbone atom positions of their α and β chains and used the coordinates of the insulin peptide in the HLA-DQ8 structure as the proper ones for the Glt-156 peptide. The p9 pocket of DQ2, the most spacious p9 pocket of any MHC II molecule known to date, is formed by α68His, α69Asn, α72Ser, α73Leu, α76Arg, β9Tyr, β30Ser, β37Ile and β57Ala (van de Wal et al. 1997). We reasoned that for a peptide with intermediate to high affinity for HLA-DQ2, as the Glt-156 peptide, the p9 pocket had to be occupied by the Phe residue. Energy minimizations proceeded via 1,000 steps of the steepest gradient method followed by 1,000 steps of the conjugate gradient method of the program Discover (Accelrys) with no cross-terms. Graphical representations were performed via the WebLabViewer program (version 3.5) of Accelrys. The energy-minimized structure thus obtained contains the peptide in a polyproline II helical conformation, as expected for all peptides bound to MHC II molecules (Jardetzky et al. 1996).

Database search

To screen for potential gluten T-cell epitopes, the PIR Non-Redundant Reference Protein Database (PIR-NREF) was used http://www.pir.georgetown.edu/pirwww/search/pirnref.shtml). The *Triticum aestivum* sequence register was searched with help of the PIR-supplied pattern search tool.

Result and discussion

Minimal epitopes required for T-cell stimulation

To determine the minimal peptide size required for T-cell recognition, we synthesized a set of partially overlapping peptides corresponding to eight T-cell stimulatory gluten peptides. Subsequently, these peptides were treated with tissue tTG, which introduces the negative charges required for HLA-DQ2-binding, and tested the T-cell stimulatory properties of these peptides with appropriate gluten-specific T-cell clones.

For most of the tested T-cell stimulatory gluten epitopes, the minimal core sequences required for T-cell recognition confirmed previous results (Arentz-Hansen et al. 2002; Vader et al. 2002a,b, 2003) and were found to be nine amino acids long (Table 1). In contrast, the minimal core of the Glt-156 peptide required for T-cell stimulation was found to consist of ten amino acid residues (Fig. 1). Similarly, a requirement for a ten-mer was also found for the Glia-γ2 epitope (Vader et al. 2002a) and Table 1).

Table 1 Minimal gluten T-cell stimulatory epitopes

Epitope name	Minimal epitope[a]	Amino acid length
Glia-γ30 (222–236)	IIQPQQPAQ	9-mer
Glu-5 (unknown)	QXPQQPQQF	9-mer
Glia-γ1 (138–153)	PQQSFPQQQ	9-mer
Glia-α2 (62–75)	PQPQLPYPQ	9-mer
Glia-α9 (57–68)	PYPQPQLPY	9-mer
Glt-156 (40–59)	PFSQQQQSPF	10-mer?
Glia-γ2 (89–102)	PFPQQPQQPF	10-mer?

[a] *Underlined* glutamine residues that are deamidated by tissue transglutaminase

Alignment of the Glt-156 and Glia-γ2 peptides allows two alternative binding registers (Table 2). In the first binding register, termed p1/p10, the two negative charges that are introduced as the result of the activity of the enzyme tissue tTG are found at position p4 and p7, which favours binding of these peptides to HLA-DQ2. In the second binding register, termed p-1/p9, the presence of phenylalanine at position p1 and p9 and an E at p6 could facilitate binding to HLA-DQ2. In order to confirm the importance of the N-terminal proline in the Glt-156 peptide for the T-cell recognition, we tested the impact of amino acid substitutions at this position. Substitution of the proline with serine, alanine, phenylalanine and glutamic acid strongly reduced T-cell recognition (Fig. 2a).

Subsequently, we determined the need for the phenylalanine at the C-terminus of the Glt-156 peptide. For this purpose, homologues were synthesized, in which the phenylalanine was substituted with proline, glutamine, alanine, glutamic acid, leucine or tyrosine, and the T-cell stimulatory properties of these peptides were tested in two independent experiments (Fig. 2b, c). While the conservative tyrosine and semi-conservative leucine substitutions only moderately reduced the T-cell stimulatory properties, these properties were strongly diminished or completely abolished by non-conservative proline, glycine, alanine and glutamic acid substitutions (Fig. 2b, c).

To exclude the possibility that the substitutions of the C-terminal phenylalanine affected the deamidation of glutamine at the putative p7 residue, and thereby abrogated the T-cell recognition, we checked the effect of the amino acid replacements on the deamidation pattern. As expected, the deamidation was compromised by the introduction of a proline, since a proline located three amino acids from the C-terminal of a glutamine is known to inhibit the deamidation of this glutamine (Vader et al. 2002a). The replacement with a glycine, however, had no influence on the deamidation (not shown). As the T-cell stimulation was compromised by both substitutions, this confirms the significance of the bulkiness of the amino acid on the C-terminus for the T-cell recognition.

Together, these results indicate that a ten-mer peptide is required for T-cell recognition of the Glt-156 peptide. Similar results were obtained for the Glia-γ2 peptide (not

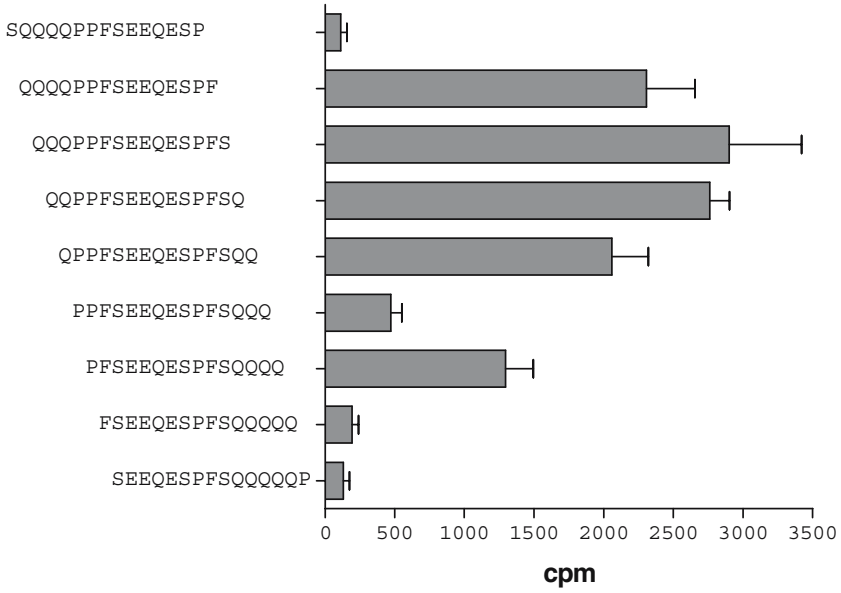

Fig. 1 Minimal core sequence of Glt-156 epitope capable of stimulating T-cell proliferative response

shown). We therefore investigated the possibility that the peptide binds in a p1/p10 register in more detail.

Minimal epitopes required for HLA-DQ2 peptide binding

To distinguish between the two possible peptide-binding registers we have carried out peptide-binding studies to HLA-DQ2. Since the Glia-γ2 is known to be a relatively poor HLA-DQ2 binder (Vader et al. 2003), these studies were carried out with the Glt-156 epitope.

First, we checked the HLA-DQ2-binding capacities of Glt-156 with C-terminal substitutions (F→P or F→G) in a cell-free HLA-DQ2 peptide-binding assay. Both substitutions were found to result in significantly higher IC_{50} values compared to the wild-type peptide (Table 3), indicating a lower binding capacity as the result of the substitutions. We therefore conclude that the C-terminal phenylalanine functions as an anchor residue in the p9 pocket of HLA-DQ2.

To address the question which amino acids may serve as anchors at the N-terminus of the peptide in the p1 binding pocket, we have substituted both the N-terminal proline and phenylalanine with other amino acids and determined the HLA-DQ2 binding capacities of these homologue peptides.

Substitutions of N-terminal proline had no effect on HLA-DQ2 binding capacity (data not shown), indicating that this proline is not strongly involved in interactions between the peptide and HLA-DQ2 molecule. Substituting the phenylalanine with other hydrophobic amino acids only slightly decreased binding. Replacements with charged residues, however, had a very pronounced negative effect (Table 4), indicating that the phenylalanine side chain is buried in the hydrophobic p1 pocket, docking the N-terminus of the peptide in the HLA molecule.

To further confirm that the N-terminal proline is not required for binding to HLA-DQ2, we have carried out a binding test using an overlapping set of Glt-156-based peptides and determined the minimal HLA-DQ2 binding core sequence (Table 5). Indeed, contrary to the minimal T-cell stimulatory epitope, the minimal binding core was shown to consist of only nine amino acids and did not include the N-terminal proline.

Finally, we modelled the Glt-156 and the Glia-γ2 peptide, using the recently solved HLA-DQ2 crystal structure (Kim et al. 2004). In agreement with this crystal structure, optimal docking of the Glt-156 and Glia-γ2 peptides into the HLA-DQ2 peptide-binding groove was found with the conventional p1/p9 register (Fig. 3 and not shown). As expected, the total calculated energy of the complex in the conventional p-1/p9 register is lower than in the p1/p10 register, or a register in which the N-terminal proline forms the p1 anchor, and the C-terminal phenylalanine forms the p9 anchor with a presumed bulge between p7Glu and p8Ser (data not shown). The modelled structure of the complex shows that several features of the DQ2 molecule can exhibit

Table 2 Alignment of peptides sequences with HLA-DQ2-binding motif

Position	p-1	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10
Preferred		FWYILV			DEVLI		PAE	DE		FYLWI	
Glt 156		P	F	S	E	E	Q	E	S	P	F
Glia γ-2		P	F	P	E	Q	P	E	Q	P	F
Glt 156	P	F	S	E	E	Q	E	S	P	F	
Glia γ-2	P	F	P	E	Q	P	E	Q	P	F	

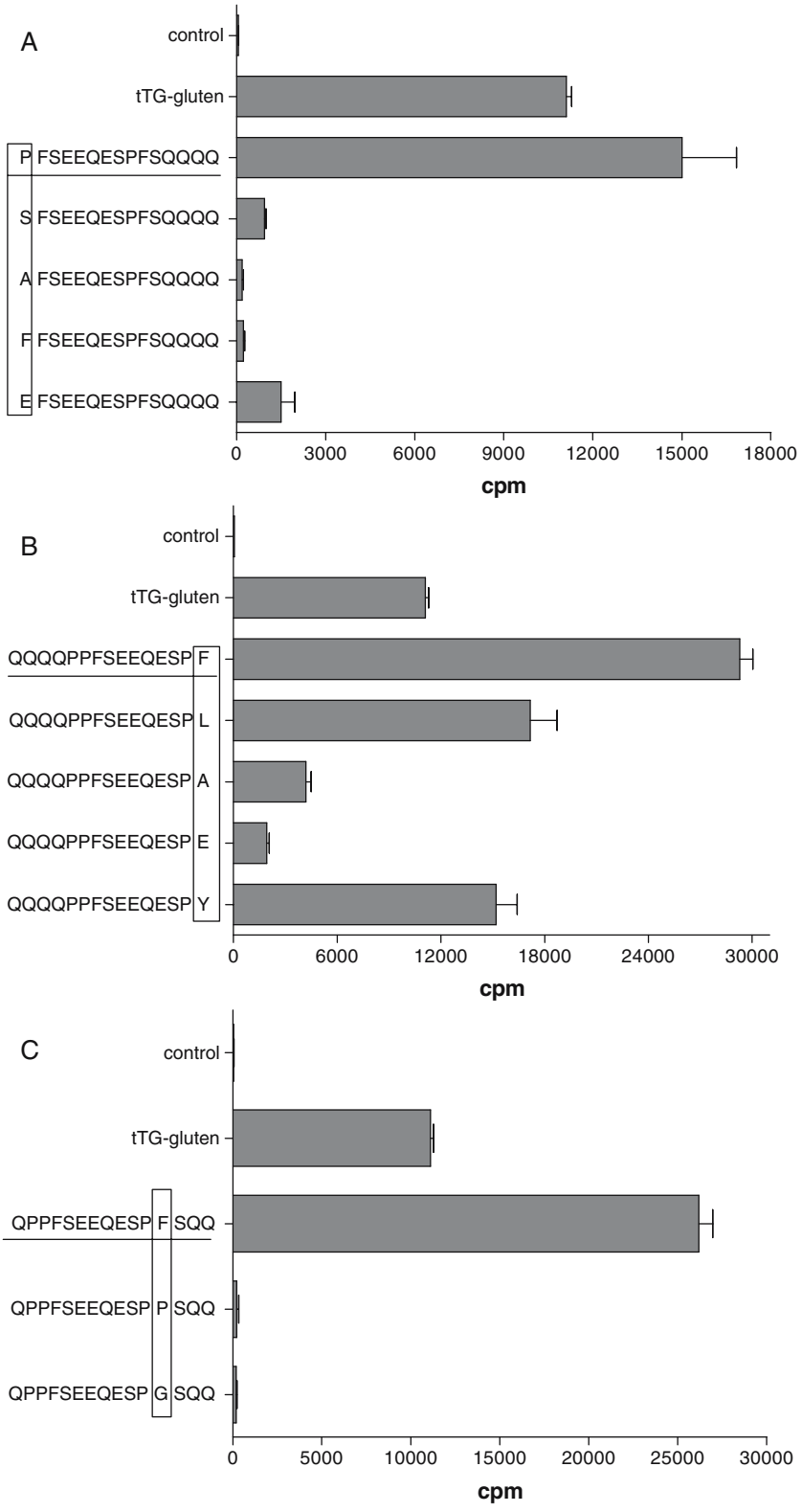

Fig. 2 Influence of substitutions of N-terminal proline and C-terminal phenylalanine on proliferation of gluten-specific T-cell clone. *tTG-gluten*: peptic/tryptic gliadin digest, treated with tissue transglutaminase

Table 3 Influence of p9 substitution on HLA-DQ2 binding

Amino acid sequence	IC_{50} [μM]
QPPFSEEQESP**F**SQ	3.5
Q P P F S E E Q E S P **P** S Q	48.0
Q P P F S E E Q E S P **G** S Q	28.0

profound influence on the binding of the Glt-156 peptide. Thus, we find the two phenylalanine residues inside pockets 1 and 9, the p4Glu deeply buried in the p4 pocket and p6Glu pointing parallel to the β-sheet floor. Moreover, the hydrogen-bonding interactions of the constant MHC II residues and the Glt-156 peptide backbone are all in place, justifying

Table 4 Influence of p1 replacement on HLA-DQ2 binding

Amino acid sequence	IC_{50} [μM]
QPP**F**SEEQESPFSQU	3.0
Q P P **L** S E E Q E S P F S Q Q	3.8
Q P P **A** S E E Q E S P F S Q Q	10.0
Q P P **K** S E E Q E S P F S Q Q	50.0
Q P P **D** S E E Q E S P G S Q Q	100.0

Table 5 Minimal core sequences facilitating HLA-DQ2 binding

Amino acid sequence	IC_{50} [μM]
SQQQQPPFSEEQESP	49.0
QQQQPPFSEEQESPF	0.9
QQQPPFSEEQESPFS	2.1
QQPPFSEEQESPFSQ	3.2
QPPFSEEQESPFSQQ	2.1
PPFSEEQESPFSQQQ	3.1
PFSEEQESPFSQQQQ	6.4
FSEEQESPFSQQQQQ	14.7
SEEQESPFSQQQQQP	>500

the high binding affinity measured for this peptide. Interestingly, the proline at p-1 is pointing upwards, which may explain its impact on T-cell recognition (Fig. 3b). In addition, removal of the proline has a small impact of the position of the p1 anchor residue (Fig. 3b). Moreover, the presence of p-1 proline adds a potential hydrogen-bonding interaction of the positively charged α-amine group of the peptide with the carbonyl oxygen of HLA-DQ2 α-chain residue 53. This interaction may have a small impact on the IC_{50} value (Table 5).

Database search

Proline is a highly abundant amino acid in gluten, constituting about 12% of wheat glutenins and 17% of wheat gliadins (in barley this is 15% and 23%, respectively) (Wieser et al. 1980). We have already demonstrated in our previous paper that among the epitopes identified by ten-mer-predicting algorithms, only those that contained proline at the N-terminus (position p-1) were capable of evoking T-cell responses (Vader et al. 2002a). To check how

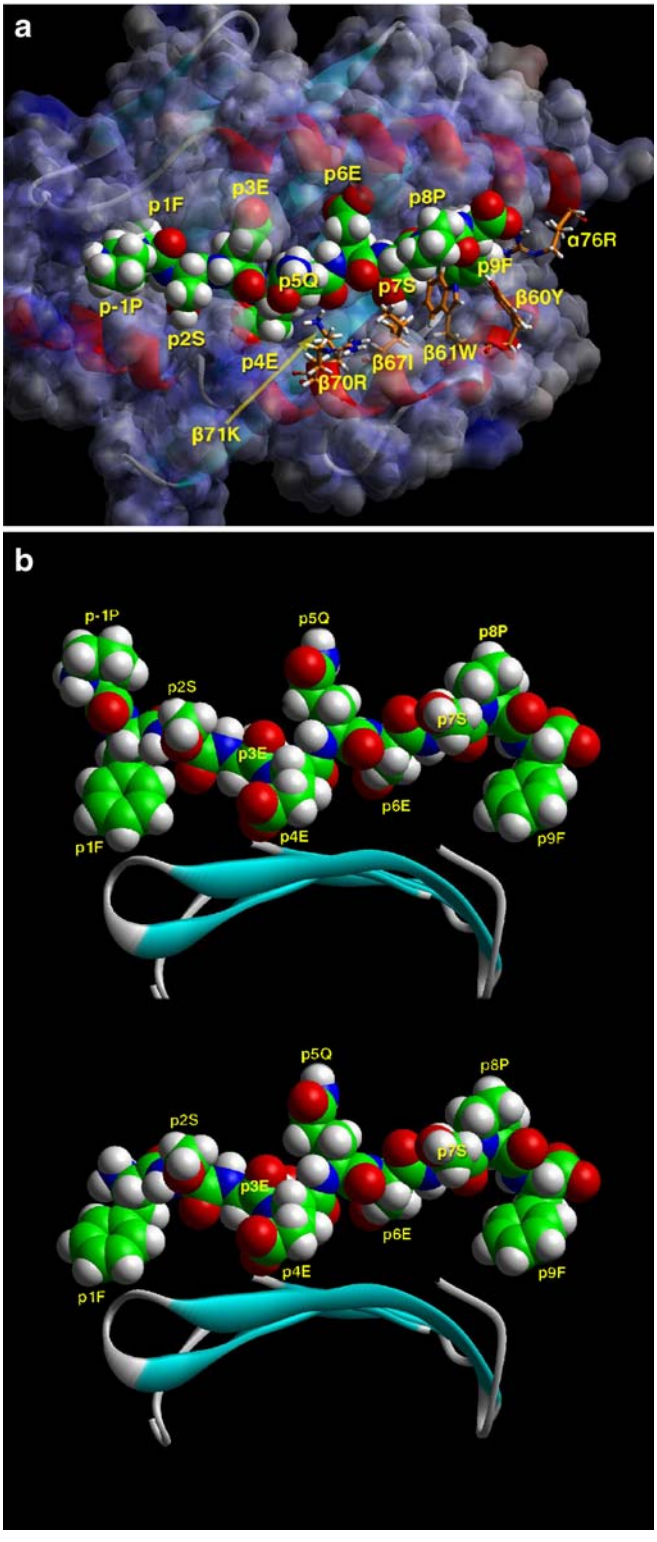

Fig. 3 Computer modelling of the Glt-156 peptide in the HLA-DQ2 molecule. The peptide in the groove is shown in space-filling mode with the following color conventions: *green* carbon, *blue* nitrogen, *red* oxygen, *white* hydrogen. Positive electrostatic surface potential is depicted as a *blue surface*, negative potential as a *red surface* and intermediate values as a *grey surface*. For the HLA-DQ2 protein, the α helix is shown in *red*, the β-pleated sheet in *turquoise* and the random coil in *grey*. **a** T-cell receptor (TCR) view of the peptide emerged in the HLA-DQ2 peptide-binding groove. p1 and p9 phenylalanine residues are buried in respective pockets, docking the sides of the peptide. p-1 proline is easily accessible to the TCR. **b** Side view depicting the Glt-156 peptide (top) and non-immunostimulating peptide FSEEQESPFS at the level of the β-sheet floor in the exact orientation found in the groove. Note that residues p1F, p4E, p6E and p9F are buried in the respective pockets, while the p-1Pro is pointing upwards. The major effect arising from the p-1 deletion is the absence of a residue in that space, and the positive charge at the amino-side of p1Phe, instead of p-1Pro. There are also slight movements of p1Phe and TCR-exposed residues, e.g. p3 and p4, that might influence TCR recognition, but such an effect would be secondary to the impact of the lack of the p-1Pro

Table 6 Results of database search with algorithms predicting potential epitopes binding to HLA-DQ2 with proline in the p-1 position

Algorithm[a]	Glutenins	Gliadins	Number of hits	Distinct sequences
P F X X Q X Q X P [FYLWI]	37	26	168	14
X* F X X Q X Q X P [FYLWI]	14	5	22	9
P Y X X Q X Q X P [FYLWI]	13	16	29	3
X* Y X X Q X Q X P [FYLWI]	0	0	0	0

[a] *X* any amino acid, *X** any amino acid except proline

many potential gluten epitopes contain proline at the p-1 position, we designed four algorithms with glutamine at the anchor positions p4 and p6, phenylalanine or tyrosine at the anchor position p1, a bulky hydrophobic amino acid (F, Y, L, W or I) at the anchor position p9. The proline at position p8 was meant to facilitate the deamidation of glutamine at the p6. Using these algorithms, we screened a protein database and found 17 potential epitopes with proline at position p-1. These epitopes were repeated almost 200 times in more than 60 different gluten molecules (Table 6). The repertoire of identified sequences with other amino acids at p-1 was more limited, consisting of nine potential epitopes repeated only 22 times in 19 gluten molecules. Thus, gluten contains many potential T-cell epitopes with N-terminal proline at p-1 position.

Concluding remarks

The results show that certain gluten epitopes, although binding to HLA-DQ2 in the canonic p1/p9 nonamer register, require a p-1 proline for optimal T-cell recognition. The prominence of the peptide-flanking residues for the T-cell receptor (TCR) interaction and consequently, epitope immunogenicity, has been already well established (Moudgil et al. 1998). Arnold et al. (2002) demonstrated that recognition of peptide flanking residues is a common event. In contrast, in the few available MHC II peptide/TCR complex crystal structures, the CDR3 regions of the TCR α and β chains align over the p5 residue of the bound peptide (Hennecke et al. 2000; Hennecke and Wiley 2002; Rudolph and Wilson 2002). This has led to the suggestion that the specificity for peptide recognition is dependent on TCR contact with the central p5 residue. In other cases, however, TCR recognition of bound peptide was found to be specific for p2/3 and p7/8 residues (De Oliveira et al. 2000). Thus, TCR recognition of HLA class II bound peptides is not uniform. Here we show that TCR recognition of gluten peptides can be influenced by an N-terminal proline at p-1. Since there is a high number of potential gluten epitopes with a N-terminal flanking proline, we suggest that this phenomenon should be taken into account while searching for new gluten epitopes or designing novel, peptide-based, tolerance-inducing therapies for celiac disease.

Acknowledgements This study was supported by the Dutch Organization for Scientific Research (ZonMw grant 912-02-028), a grant from the EU (BHM4-CT98-3087), the 'Stimuleringsfonds Voedingsonderzoek LUMC' and the Centre for Medical Systems Biology (CMSB), a center of excellence approved by the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research (NWO). G.K.P. was supported by grants from the Technological Educational Institute of Epirus Research Committee, and from the European Union's 3rd Framework Program for Regional Development (Program EPEAEK, Scheme 'Archimedes'). We wish to thank Dr. C.Y. Kim for kindly providing the coordinates of the HLA-DQ2 complex and Mr. Demetrios Kyrkas for technical support. We thank Dr. Tom Ottenhoff for critical reading of the manuscript.

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