Celiac Lesion T Cells Recognize Epitopes That Cluster in Regions of Gliadins Rich in Proline Residues

HELENE ARENTZ–HANSEN,* STEPHEN N. MCADAM,* ØYVIND MOLBERG,* BURKHARD FLECKENSTEIN,* KNUT E. A. LUNDIN,*,† THOMAS J. D. JØRGENSEN,§ GÜNTHER JUNG,‖ PETER ROEPSTORFF,§ and LUDVIG M. SOLLID*

*Institute of Immunology and †Department of Medicine, Rikshopitalet, University of Oslo, Oslo, Norway; §Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark; and ‖Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

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Background & Aims: **Celiac disease is a gluten-induced enteropathy that shows a strong association with HLA-DQ2 and -DQ8. Gluten-specific T cells, invariably restricted by DQ2 or DQ8, can be isolated from celiac lesions. Such gut-derived T cells have a preference for recognition of gluten that has been specifically deamidated by tissue transglutaminase. Only a few gliadin T-cell epitopes have been identified by earlier work. The aim of this study was to perform a systematic characterization of DQ2-restricted T-cell epitopes in α- and γ-gliadins.** ***Methods:*** **Epitopes were identified by mass spectrometry analysis of peptide fragments of recombinant gliadins and by use of synthetic peptides.** ***Results:*** **We identified several new γ-gliadin epitopes and an additional α-gliadin epitope. Interestingly, these and the previously identified epitopes are not randomly scattered across the gliadins but cluster in regions of the proteins with high content of proline residues.** ***Conclusions:*** **Several DQ2-restricted T-cell epitopes exist in gliadin that are located in regions rich in proline. This likely reflects epitope selection at the levels of digestive and antigen-presenting cell processing, transglutaminase-mediated deamidation, and/or peptide binding to DQ2.**

Celiac disease is a food-sensitive enteropathy with a complex multifactorial etiology.[1,2] Both a major environmental factor (dietary wheat gluten and related proteins in barley and rye) and a major genetic factor (genes encoding for HLA-DQ2 and -DQ8) have been identified in this disorder, making it a valuable model for exploring the associations between HLA and disease. Intestinal $CD4^+$ T cells specific for wheat gluten play a central role in the development of the celiac enteropathy. These T cells, which can be readily isolated from the small intestine of patients with celiac disease but not of controls, are striking because they are almost entirely restricted by DQ2 (or by DQ8 in those few patients that express this molecule).[3,4] Most of these gluten-specific T cells are also distinctive in that they recognize gluten after it has been modified by tissue transglutaminase (tTG) and recognize poorly, if at all, native gluten peptides.[5,6] We have previously shown that tTG catalyzes the ordered deamidation of specific glutamine residues, converting them to glutamic acid. This introduces negatively charged residues into the gliadin peptide, thereby increasing the affinity of the peptides for DQ2.[7,8]

Gluten can be separated into gliadins and glutenins. The gliadins can be further subdivided into the α-, γ-, and ω-gliadins, with each subgroup consisting of a mixture of distinct proteins that differ by minor variations in amino acid sequence and are difficult to separate using biochemical methods.[9] The task of identifying gluten T-cell epitopes is complicated by the complex nature of this antigen and by the fact that most of these epitopes are posttranslationally modified by tTG. We previously described 3 DQ2-restricted gliadin T-cell epitopes: 2 in α-gliadins[7] and one in γ-gliadins.[10] Here we report the characterization of additional DQ2-restricted T-cell epitopes in gliadins by use of recombinant proteins and synthetic peptides. Interestingly, all the epitopes are located in distinct proline-rich clusters of the gliadin protein.

Materials and Methods

Subjects

Nine Norwegian adult patients with celiac disease were included in the study, which was approved by the regional ethical committee. Patients CD411 and CD467 were

Abbreviations used in this paper: HPLC, high-performance liquid chromatography; TCC, T-cell clone; TCL, T-cell line; tTG, tissue transglutaminase.

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Table 1. DQ2-Restricted Gliadin Epitopes

Epitope	Position	Sequence	Reference
DQ2-α-I	α-9 (60–68)E65	PFPQP**Q**LPY[a]	7
DQ2-α-II	α-2 (62–70)E65	PQP**Q**LPYPQ[a]	7
DQ2-α-III	α-2 (67–75)E72	PYPQP**Q**LPY[a]	This study
DQ2-γ-I	γ-5 (115–123)E121	PQQSFP**Q**Q**Q**[a,b]	10
DQ2-γ-II	γ-5 (228–236)E232	IIQP**Q**QPAQ[a]	Vader et al[e]
DQ2-γ-III	γ-5 (66–78)E68,E71	FP**Q**QP**Q**QPYPQQP[c]	This study
DQ2-γ-IV	γ-5 (102–113)E106,E108	FSQP**Q**Q**Q**FPQPQ[d]	This study
DQ2-γ-V	γ-5 (60–79)	LQPQQPFPQQPQQPYPQQPQ	This study

NOTE. Glutamine residues targeted by tTG are in bold.
[a]Only the 9–amino acid core regions are indicated. T cells may require longer peptides for recognition.
[b]tTG-mediated deamidation of the glutamine in position 121 is required for T-cell recognition.
[c]Analysis by electrospray ionization mass spectrometry using collision-induced dissociation experiments indicated tTG-mediated deamidation of glutamines in position 68 and positions 71 or 72.
[d]tTG-mediated deamidation of the glutamines in positions 106 and 108 is required for T-cell recognition.
[e]Vader W, Kooy Y, Van Veelen P, De Ru A, Harris D, Benckhuijsen W, Pena S, Mearin L, Drijfhout JW, Koning F. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. Gastroenterology 2002;122:1729–1737.

untreated, whereas patients CD370, CD380, CD423, CD429, CD430, CD432, and CD450 were on a gluten-free diet. All subjects expressed the disease-associated DQ2 molecule encoded by *DQA1*05/DQB1*02* alleles.

Amplification, Cloning, and Production of Recombinant Gliadins

By polymerase chain reaction amplification of genomic DNA isolated from the wheat strain Mjølner using γ-gliadin specific primers,[11] a panel of 11 unique, full-length γ-gliadin genes was obtained. At the protein level, these 11 genes translated into 5 distinct γ-gliadins (γ-1, γ-2, γ-3, γ-4, and γ-5; accession numbers AJ133613, AJ416336, AJ416337, AJ416338, and AJ416339, respectively), which all contained the known DQ2-γ-I and DQ2-γ-II epitopes (Table 1). The expression of the recombinant γ-gliadins and the recombinant α-2 gliadin in *Escherichia coli* has been described in detail elsewhere.[11]

Biochemical Purification of Fragments From Recombinant Gliadin Stimulatory for T Cells

Small T-cell stimulatory fragments from a chymotrypsin digest of the γ-5 recombinant gliadin were isolated by gel filtration (Superdex 200 HR 10/30 column; Pharmacia, Uppsala, Sweden), followed by guinea pig tTG treatment and subsequent ion exchange (Mono-Q PC 1.6/5) and reverse-phase high-performance liquid chromatography (HPLC) (μRPC C2/C18) on a SMART system (Pharmacia) as previously described.[7]

Preparation of antigen pepsin, pepsin-trypsin, or chymotrypsin digestion of crude gliadin was performed as described.[3,5] The peptides were either purchased from Research Genetics (Huntsville, AL) or synthesized at the Institute of Organic Chemistry (University of Tübingen, Tübingen, Germany) using Fmoc/OtBu chemistry and 2-chlorotrityl resin (Senn Chemicals AG, Dielsdorf, Switzerland).[12] Identity of the peptides was confirmed by electrospray mass spectrometry, and purity was analyzed by reverse-phase HPLC. Treatment of the peptides with guinea pig tTG (Sigma, St. Louis, MO) was performed at 37°C for 2 hours in phosphate-buffered saline and 1 mmol/L $CaCl_2$ using 100 μg/mL of tTG.

Gliadin-Specific T Cells and T-Cell Proliferation Assays

The generation of T-cell lines, T-cell cloning, and T-cell proliferation assays were performed as described elsewhere.[13] $DR3^+DQ2^+$ B lymphoblastoid cells (irradiated 80 Gy) were used as antigen-presenting cells. The HLA restriction of the T-cell clone (TCC) was determined by combining the use of the *DQA1*0501/DQB1*0301*-positive B-LCL SWEIG with and without an additional transfected *DQB1*0201* gene and by blocking with a DQ-specific monoclonal antibody (SPV-L3).

Mass Spectrometry

Mass spectrometry electrospray ionization mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, England), and matrix-assisted laser desorption ionization mass spectra were acquired on a Bruker Reflex II MALDI-TOF instrument (Bruker-Daltonik, Bremen, Germany). After purification, the samples were sprayed from nanoelectrospray needles (MDS Proteomics, Odense, Denmark) held at typically 800 V toward a skimmer cone (40 V). In collision-induced dissociation experiments (collision gas argon, manifold pressure $\sim 8 \times 10^{-5}$ mBar, collision energy 32–40 eV), product ions were analyzed by the orthogonal TOF analyzer.

Results

Identification of 3 New DQ2-Restricted T-Cell Epitopes in a Recombinant γ-Gliadin

Five individual recombinant γ-gliadins, which all contained the previously identified DQ2-γ-I and DQ2-

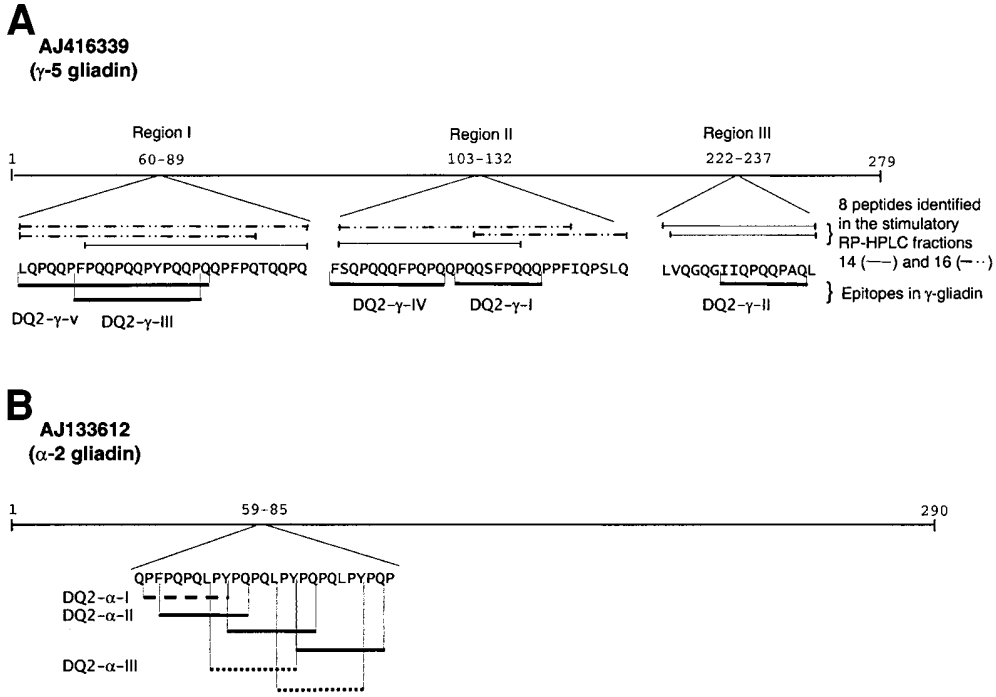

Figure 1. (*A*) Biochemical purification of 8 peptide fragments derived from the recombinant γ-5 gliadin. Peptides derived from the reverse-phase HPLC fraction 14 and 16 cluster in 3 different regions and are indicated above the sequence excerpts. The γ-gliadin epitopes DQ2-γ-I to DQ2-γ-V are depicted as *black bars* below the sequence excerpts. The 9–amino acid core region is given for the DQ2-γ-I and DQ2-γ-II epitopes, whereas the shortest peptides that elicit a T-cell response is indicated for the remaining epitopes. (*B*) Clustering of the epitopes within region 59–85 in the recombinant α-2 gliadin. The 9–amino acid core regions of the native gliadins that correspond to the epitopes DQ2-α-I, DQ2-α-II, and DQ2-α-III are indicated. Notably, the DQ2-α-II epitope is repeated 3 times and the DQ2-α-III epitope is repeated twice in the sequence. All 3 epitopes contain the 7–amino acid motif PQPQLPY. The glutamine residues targeted by tTG in each epitope are shown in Table 1.

γ-II epitopes (Table 1), were expressed in *E. coli*. To identify new epitopes present in the recombinant γ-gliadins, we chose a T-cell line (TCL) from patient CD411 (TCL 411E) that responded to all the tTG-treated recombinant gliadin proteins (γ-1 to γ-5) but not to the DQ2-γ-I and DQ2-γ-II epitopes. TCCs made from this TCL were used to identify positive fractions following purification of a chymotryptic digest of the γ-5 recombinant gliadin by subsequent steps of gel filtration, anion exchange, and reverse-phase HPLC. Two reverse-phase HPLC fractions (14 and 16) stimulated the TCC 411A. Fraction 16 also stimulated the TCC 411C. Analysis of these fractions by electrospray ionization mass spectrometry using collision-induced dissociation experiments identified 8 different peptides clustered in 3 different regions of the γ-5 recombinant gliadin (region I, II, and III; Figure 1*A*). Interestingly, both of these fractions contained peptides that overlapped with the previously identified γ-gliadin epitopes (Figure 1*A*).

Next, overlapping peptides spanning the 3 regions (I, II, and III) were synthesized and tested for T-cell recognition by clones derived from the TCLs 411E and 430.5; the latter was an intestinal TCL responsive to several peptides from these 3 regions.

Two types of T-cell reactivity patterns against peptides from region I were found. The TCC 430B and TCC 430C show the first type of reactivity pattern. These TCCs were reactive with the minimal peptide γ-5 (66–78) (defined as the DQ2-γ-III epitope; Table 1) in a strict tTG-dependent manner (Figure 2*A*).

TCC 411A and TCC 411B represent the second type of reactivity pattern against peptides of region I. These TCCs recognized a long peptide from γ-5 (60–79) (defined as the DQ2-γ-V epitope; Table 1); for these TCCs, treatment by tTG had no influence on T-cell recognition (Figure 2*B*). Testing of 11 peptides within the γ-5 (60–79) fragment that overlapped by 11 residues or more failed to stimulate TCC 411A or TCC 411B, implicating that these T cells may require peptides of 12 residues or longer for recognition. It should be noted that the tTG-dependent epitope DQ2-γ-III (γ-5 (66–78)) is contained within the γ-5 (60–79) fragment. Because we failed to identify a peptide shorter than γ-5 (60–79) capable of stimulating the TCC 411A and TCC 411B, we cannot formally rule out that these T cells recognize the same core region but in its unmodified form. A single type of reactivity pattern, represented by TCC 430A, was found against peptides of region II. This TCC recognized

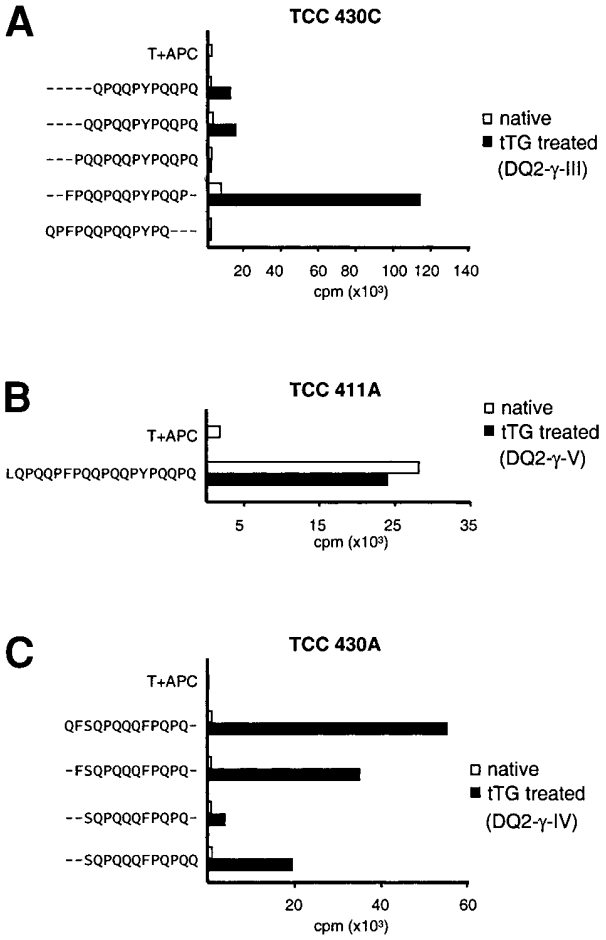

Figure 2. Recognition of the 3 new γ-gliadin epitopes (*A*) DQ2-γ-III, (*B*) DQ2-γ-V, and (*C*) DQ2-γ-IV by DQ2-restricted, intestinal TCCs. The peptides (10 μmol/L) were tested in their native form (□) or after treatment with guinea pig tTG (■).

the peptide γ-5 (102–113) (defined as the DQ2-γ-IV epitope; Table 1) in a strictly tTG-dependent manner (Figure 2*C*).

A Third α-Gliadin Epitope Clusters With the DQ2-α-I and DQ2-α-II Epitopes

During the screening of T cells generated within our laboratory, it became clear that a third α-gliadin epitope existed within the α-2 recombinant gliadin (accession number AJ133612).[11] Two TCCs (TCC 370A and 370B) were identified that were stimulated by the α-2 recombinant gliadin but failed to respond to either of the DQ2-α-I or DQ2-α-II epitopes. Because the pattern of epitope clustering observed with the DQ2-α-I or DQ2-α-II epitopes was also evident with the epitopes in the γ-5 recombinant, we wondered whether this new epitope might also cluster with the DQ2-α-I and DQ2-α-II epitopes. This was indeed the case, because both of the TCCs recognized peptide α-2(64–75)E72 (DQ2-α-III; Figure 1*B* and data not shown). The glutamine in position 72 is naturally targeted by tTG and is located in the same position within the repetitive 7-residue fragment as for the 2 other α-gliadin epitopes.

T-Cell Epitopes Cluster in Regions With High Proline Content

Most of the γ-gliadin epitopes and the α-gliadin epitopes contain deamidated glutamines and several proline residues. To our knowledge, there are only a few examples in which multiple prolines (more than 2) are found in the core region of other natural class II ligands (see http://www.syfpeithi.de). We used a sliding window analysis to plot the proline and glutamine content across the gliadins (Figure 3). Natural class II ligands are commonly between 10 and 34 residues long,[14] and we chose a window size of 20 residues (the picture remained

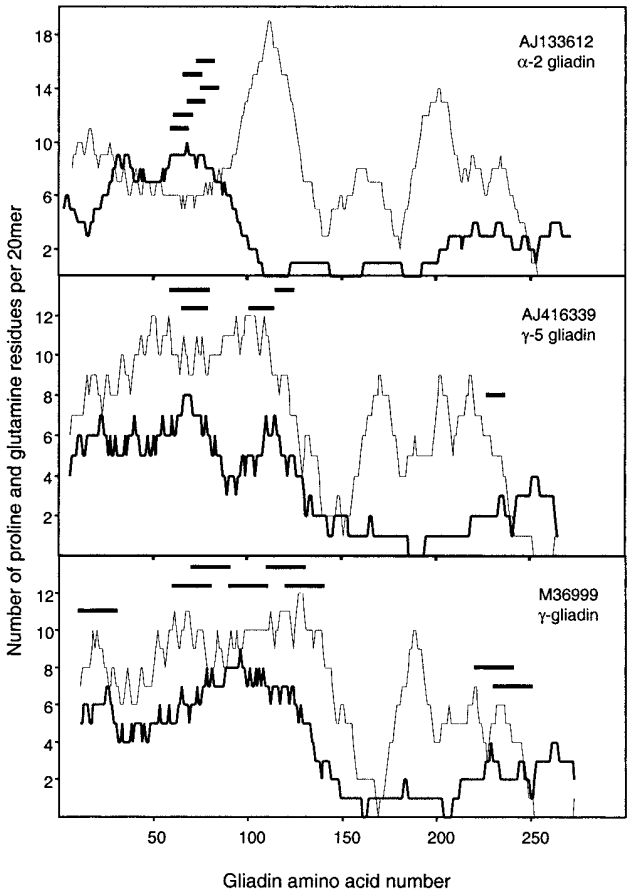

Figure 3. The plots represent the number of proline (*thick line*) and glutamine (*thin line*) residues across the recombinant gliadin α-2 (AJ133612), the recombinant gliadin γ-5 (AJ416339), and the γ-gliadin M36999. For the α-2 and the γ-5 gliadin, the horizontal bars indicate the individual epitopes within each gliadin (see Figure 1*A* and *B*). For the γ-gliadin M36999, the horizontal bars indicate the overlapping 20mer peptides that elicited an intestinal T-cell response (see Figure 4).

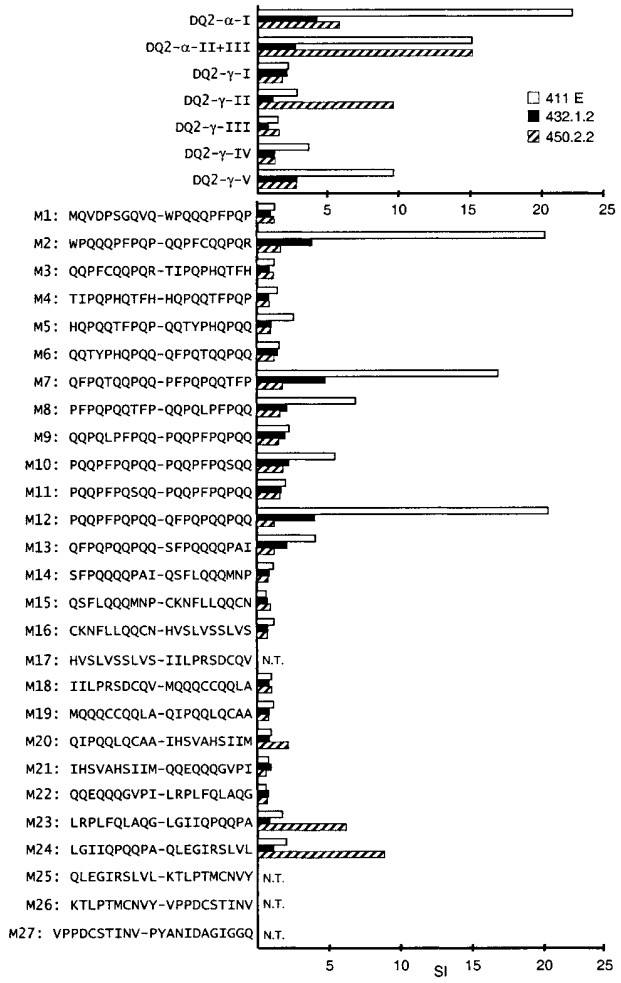

Figure 4. Testing of 3 polyclonal TCLs against 3 α-gliadin epitopes and 5 γ-gliadin epitopes (*upper panel*) and tTG-treated variants of 20mer peptides (10 μmol/L) overlapping by 10 residues covering most of the γ-gliadin M36999 (*lower panel*). Peptide M13 includes the DQ2-γ-I epitope, and the overlapping peptides M23 and M24 include the DQ2-γ-II epitope. Responses are given as the stimulation index, calculated by dividing the proliferative response to antigen by the background (T + APC; 411E, 812 cpm; 432.1.2, 12,527 cpm; 450.2.2, 1536 cpm). N.T., not tested.

similar if the window size was set anywhere between 15 and 30; data not shown). The plot from the α-2 recombinant is striking in that the DQ2-α-I, DQ2-α-II, and DQ2-α-III epitopes are scattered across a dominant peak that represents a region that has the greatest proline content (Figure 3, upper panel). There is much less correlation with the epitope distribution and the glutamine content. The γ-5 recombinant has 2 major regions of high proline content. The first region contains the DQ2-γ-III and the DQ2-γ-V epitopes, and the second region comprises the DQ2-γ-I and the DQ2-γ-IV epitopes. The DQ2-γ-II epitope is located in the C-terminal end of the γ-5 gliadin with some lower proline content (Figure 3, middle panel). Also, for the γ-5 gliadin, the epitope distribution correlates more with the proline content than the glutamine content.

T-Cell Epitope Clustering Confirmed Using Overlapping Synthetic Peptides

The identification of the epitopes that cluster within regions of high proline content was achieved using gliadins that had been first digested with either chymotrypsin or pepsin. Because these enzymes are unable to hydrolyze proline adjacent bonds, it could be argued that the pretreatment of our antigens may have biased the type of epitopes that we were able to identify; that is, there could be other epitopes in proline-sparse regions but these were destroyed by enzymatic digestion of the gliadin. We had a set of overlapping 20mer peptides available that covered nearly the complete sequence of another γ-gliadin (M36999),[15,16] so we screened 6 gliadin reactive polyclonal TCLs for recognition of these peptides after tTG treatment. The responses of the 3 lines with the broadest reactivity are shown in Figure 4 (see also Table 2). The TCL from patient CD411 (TCL 411E) made a strong response against the peptides M2, M7, and M12 and a weaker response toward M8, M10, and M13. Moreover, the TCL from patient CD432 (TCL 432.1.2) made a response to peptides M2, M7, and M12, whereas the TCL from patient

Table 2. γ-Gliadin–Derived 20mer Peptides Recognized by Intestinal TCLs

Peptide	Designation	Sequence[a]	Homology to epitopes
M2	M36999 (11–30)	WPQQQ**PFPQPQQPF**CQQPQR	DQ2-α-I
M7	M36999 (61–80)	QFPQTQQPQQ**PFPQPQQTF**P	DQ2-α-I,
		QFPQ**TQQPQQPFPQPQ**QTFP	DQ2-γ-IV
M8	M36999 (71–90)	PFPQPQQT**FPQQPQLPFPQQ**	DQ-γ-III
M10	M36999 (91–110)	PQQ**PFPQPQQPQ**QPFPQSQQ	DQ2-α-I
M12	M36999 (111–130)	PQQP**FPQPQQQFPQPQ**QPQQ	DQ2-γ-IV
M13	M36999 (121–140)	QFPQPQQ**PQQSFPQQQ**QPAI	Identical to DQ2-γ-I
M23	M36999 (221–240)	LRPLFQLAQGLG**IIQPQQPA**	DQ2-γ-II
M24	M36999 (231–250)	LG**IIQPQQPAQ**LEGIRSLVL	Identical to DQ2-γ-II

[a]Sequences homologous to epitopes given in Table 1 are in bold.

CD450 (TCL 450.2.2) only responded to peptides M23 and M24. Despite the high number of peptides recognized, all of them mapped to regions rich in proline residues. The high number of peptides recognized surprised us given the relatively restricted number of epitopes identified in previous studies; however, on closer inspection, we found that all of the recognized peptides had sequences that are identical or very similar to the previous identified epitopes (see Tables 1 and 2). The peptide M7 contains a sequence that is remarkably similar to the DQ2-α-I epitope. The TCC 380 E2, an intestinal TCC originally identified as a DQ2-α-I–specific clone, also made a response to the tTG-treated peptide M7, showing, for the first time, cross-reactivity on the T-cell level between α- and γ-gliadin (Figure 5). Moreover, cross-reactivity between different γ-gliadin–derived peptides was also shown. The TCC 430A, which had been identified as a DQ2-γ-IV–specific clone, also made comparable responses to the tTG-treated peptides M7 and M12 (data not shown). The peptide M7 also includes a sequence that is very similar to the DQ2-γ-IV epitope, as does peptide M12. The latter differs from the DQ2-γ-IV epitope by only a single S to P substitution. Because no attempts were made to identify the minimal sequences recognized, we have not yet designated epitope names for the possibly unique sequences represented among these overlapping peptides.

Discussion

An understanding of which T-cell epitopes are recognized in celiac disease and the processes leading to their selection should identify potential therapeutic targets for this disease and shed light onto the mechanisms responsible for the observed associations between HLA and disease. We previously identified 2 overlapping DQ2-restricted intestinal T-cell epitopes in α-gliadin that both contain multiple proline residues, and both require deamidation by tTG.[7] Although these 2 α-gliadin epitopes are clearly immunodominant,[7,17] it became apparent during the course of the present study that other epitopes may also exist. In this study, we have identified several new γ-gliadin epitopes and an additional α-gliadin epitope.

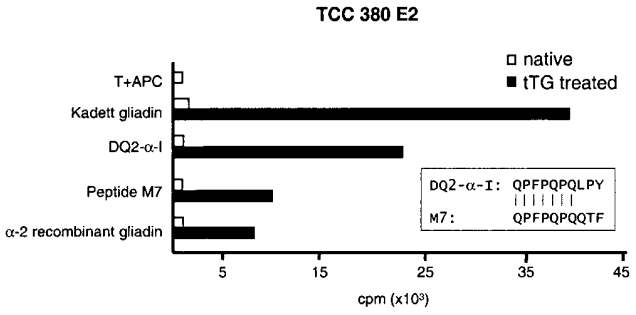

Figure 5. DQ2-restricted, intestinal TCCs display cross-reactivity between α-gliadin–derived and γ-gliadin–derived peptides. The TCC 380 E2 responds to the γ-gliadin peptide M7 and to the α-gliadin epitope DQ2-α-I and the α-2 recombinant gliadin. The peptides (10 μmol/L) were tested in their native form (□) or after treatment with guinea pig tTG (■). Native sequences of core region of the 2 peptides are given in the *inset*.

The identification of these new epitopes shows a picture of epitope clustering in both the α- and γ-gliadins. Intriguingly, these clusters correspond to regions of the gliadins rich in proline residues. This clustering is probably a result of a combination of several factors, but we see 3 that are particularly relevant. The first is related to antigen processing. Ingested antigens are peculiar in that they are subjected to both digestive enzymes and classical antigen-processing enzymes. Most mammalian peptidases/proteases are unable to cleave peptide bonds located amino or carboxy terminally to proline. Interestingly, peptides covering the DQ2-α-I, DQ2-α-II, and DQ2-α-III epitopes were recently shown to be highly resistant to digestive processing by pancreatic and brush border proteases due to their high proline content.[18] The importance of digestive processing for gluten antigenicity is supported by the observed proximal to distal gradient of the celiac lesion in the small intestine.[19] The presence of proline residues also influences the processing by antigen-presenting cells.[20,21] The second factor is the specificity of tTG. The spacing between the targeted glutamine and C-terminal proline residues plays an essential role in the specificity of tTG.[22,23] The spacing between glutamine and proline preferred by tTG (i.e., QXP but not QP or QXXP) is often found in the regions where the T-cell epitopes cluster. Notably, the deamidation patterns of most of the T-cell epitopes characterized here are in concordance with this newly described tTG specificity motif. The third factor is related to epitope selection by DQ2 molecules. It may be that DQ2 is superior in its ability to accommodate proline-rich peptides compared with other major histocompatibility complex class II molecules. Proline is the only natural amino acid present as a secondary amide, resulting in a kink in the polypeptide backbone and a loss of the main chain amide hydrogen that is a significant player in the hydrogen bonding network between the peptide and the major histocompatibility complex class II molecule.[24] Although crystal structure data of gliadin peptide DQ2 complexes will be required to fully understand the influence of multiple proline residues, our previous data have shown that substitution of proline within gliadin peptides can have a dramatic effect on their binding affinity for DQ2.[7,8]

Our finding that T cells can cross-react even between α- and γ-gliadins shows that unambiguous definition of distinct gliadin epitopes is difficult. The microheterogeneity found within the gliadin proteins, the repetitive nature of their sequence, and the dominance of a limited number of amino acids found in the gliadin proteins all combine to generate a tremendous number of similar and related sequences. It is thus likely that further intestinal T-cell gliadin epitopes will be identified in the future.

The demonstration that epitopes in α- and γ-gliadins recognized by celiac lesion T cells cluster in proline- and glutamine-rich regions deepens our understanding of why patients with celiac disease do not tolerate gluten. The association between high proline and glutamine density and gluten immunogenicity should provide a basis for novel approaches in the prevention and treatment of celiac disease.

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Address requests for reprints to: Ludvig M. Sollid, M.D., Institute of Immunology, Rikshospitalet, University of Oslo, N-0027 Oslo, Norway. e-mail: l.m.sollid@labmed.uio.no; fax: (47) 230 73822.

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