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Refining the Rules of Gliadin T Cell Epitope Binding to the Disease-Associated DQ2 Molecule in Celiac Disease: Importance of Proline Spacing and Glutamine Deamidation[1]

Shuo-Wang Qiao,[2]* Elin Bergseng,* Øyvind Molberg,* Günther Jung,[†] Burkhard Fleckenstein,* and Ludvig M. Sollid*

Celiac disease is driven by intestinal T cells responsive to proline-rich gluten peptides that often harbor glutamate residues formed by tissue transglutaminase-mediated glutamine conversion. The disease is strongly associated with the HLA variant DQ2.5 (*DQA1*05*, *DQB1*02*), and intestinal gluten-reactive T cells from DQ2.5-positive patients are uniquely restricted by this HLA molecule. In this study, we describe the mapping of two novel T cell epitopes of γ-gliadin and the experimental identification of the DQ2.5 binding register of these and three other γ-gliadin epitopes. The new data extend the knowledge base for understanding the binding of gluten peptides to DQ2.5. The alignment of the experimentally determined binding registers of nine gluten epitopes reveal positioning of proline residues in positions P1, P3, P6, and P8 but never in positions P2, P4, P7, and P9. Glutamate residues formed by tissue transglutaminase-mediated deamidation are found in position P1, P4, P6, P7, or P9, but only deamidations in positions P4 and P6, and rarely in P7, seem to be crucial for T cell recognition. The majority of these nine epitopes are recognized by celiac lesion T cells when presented by the related but nonassociated DQ2.2 (*DQA1*0201*, *DQB1*02*) molecule. Interestingly, the DQ2.2 presentation for most epitopes is less efficient than presentation by the DQ2.5 molecule, and this is particularly prominent for the α-gliadin epitopes. Contrary to previous findings, our data do not show selective presentation of DQ2.5 over DQ2.2 for gluten epitopes that carry proline residues at the P3 position. *The Journal of Immunology,* 2005, 175: 254–261.

C eliac disease (CD)[3] is a chronic disease of the small intestine caused by an inflammatory response to ingested wheat gluten proteins and related proteins of rye and barley. The lesion is characterized by villous atrophy, crypt hyperplasia, and infiltration of lymphoid cells in both the epithelium and lamina propria. CD has a strong HLA association; ~90% of the patients share the *cis*- or *trans*-encoded DQ2.5 variant (*DQA1*05*, *DQB1*02*), and the majority of the remaining patients are HLA-DQ8 (1). CD4$^+$ T cells reactive with the gliadin and glutenin subcomponents of gluten can be readily isolated from small intestinal biopsies of CD patients (but not of controls), and these cells are exclusively restricted by the disease-associated DQ2.5 or DQ8 HLA molecules. The activation of these gluten-reactive intestinal T cells probably represents a key event in the development of CD (1). From previous studies, it is clear that celiac lesion-derived, gluten-reactive T cells predominantly recognize peptides that cluster within the proline (Pro)-rich regions of gluten proteins (2), and these peptides contain glutamate (Glu) residues formed in vivo

through tissue transglutaminase (TG2)-mediated deamidation of glutamine (Gln) residues (1). The negative charges introduced by this deamidation process generally increase the binding affinity of gluten peptides to DQ2.5 (3, 4).

More than 15 different gluten peptides recognized by celiac lesion T cells have been identified (2, 3, 5–7). However, detailed mapping of the DQ2.5 binding registers by peptide binding analysis of lysine (Lys)-substituted analogs has only been performed for three epitopes: DQ2-αI, DQ2-αII, and DQ2-γI (3, 8). The binding data on these three epitopes demonstrated that the DQ2.5 molecule efficiently binds deamidated gluten peptides rich in Pro residues. Importantly, the recently resolved crystal structure of the DQ2.5 molecule in complex with the DQ2-αI epitope (9) provided atomic insight into how and why DQ2.5, in contrast to most other HLA class II molecules, has the ability to bind gluten peptides with unusual sequences. To increase the knowledge base of binding of gluten T cell epitopes to DQ2.5, we have performed detailed mapping of T cell stimulatory core sequences and the DQ2.5 binding register of three previously identified and two novel γ-gliadin peptides by testing a large series of synthetic peptides. In addition, we have analyzed the binding and T cell presentation of these epitopes by the HLA molecule DQ2.2 (*DQA1*0201*, *DQB1*0202*), which is not associated with CD (10). The results expand our experimental basis for understanding the binding of gluten peptides to DQ2 molecules.

Materials and Methods

Reagents and peptides

The majority of the peptides were prepared by multiple solid-phase peptide synthesis as described previously (11). In brief, the synthesis was done on a robotic system (Syro MultiSynTech) using Fmoc/O-*t*-butyl-chemistry on 2 chlorotrityl resin (Senn Chemicals) and diisopropylcarbodiimide and *N*-hydroxy-benzotriazole as coupling reagents. The identity of the peptides was confirmed by mass spectrometry, and purity was analyzed by reversed

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[3] Abbreviations used in this paper: CD, celiac disease; TG2, tissue transglutaminase; TCC, T cell clone; TCL, T cell line.

phase-HPLC. Some peptides were purchased from Pepscan. The peptides were aliquoted and lyophilized before storage at $-20°C$. Recombinant human TG2 was expressed as hexa-His (His_6-TG2) fusion protein in *Escherichia coli* and purified as described previously (12).

HLA-DQ2 molecules and peptide binding assays

Detergent-solubilized HLA-DQ molecules were purified from lysates of homozygous, EBV-transformed B lymphoblastoid cell lines (DQ2.5: CD114, *DQA1*0501/DQB1*0201* and DQ2.2: 9051 Pitout, *DQA1*0201/DQB1*0202*) as described previously (13). The *Mycobacterium bovis* 65-kDa heat shock protein-derived peptide (KPLLIIAEDVEGEY) and the HLA class I α46-60 peptide (EPRAPWIEQEGPEYW) were ^{125}I labeled by the chloramine-T method (14) and used as indicator peptides. Labeled peptides (30,000 cpm; 1–5 nM) were incubated with 100–500 nM purified DQ2.5 or DQ2.2 molecules overnight at 37°C in the presence of a mixture of protease inhibitors at pH 5.2 (13). Various concentrations of unlabeled peptides were added to inhibit the binding of the labeled indicator peptides. After incubation, DQ2 molecules and unbound peptides were separated on Sephadex G-50 Superfine (Pharmacia Biotech) mini spin columns as described previously (15). The radioactivity was counted, and the concentrations of competing peptides required to give 50% inhibition of binding of the indicator peptide (IC_{50}) were calculated. After an initial pilot with a 10-fold peptide dilution, at least two independent 3-fold peptide dilution experiments were done for each peptide.

T cell assays

Where indicated, the native gliadin peptides were preincubated with 150 μg/ml His_6-TG2 in 100 mM Tris (pH 7.4) with 2 mM $CaCl_2$ for 2 h at 37°C. Six EBV-transformed B lymphoblastoid cell lines were used as APCs: CD114, CD039, and CD125 were all derived from DR3-DQ2.5 (*DQA1*0501/DQB1*0201*) homozygous CD patients, whereas the cell lines 9050 Mou, 9051 Pitout, and 9047 PLH were all DR7-DQ2.2 (*DQA1*0201/DQB1*0202*) homozygous. Sixty thousand APCs irradiated with 75 Gy were coincubated overnight at 37°C in U-bottom, 96-well plates with various concentrations of peptides in RPMI 1640/10% FCS. After the incubation, the APCs were washed twice with cold RPMI 1640 to remove unbound Ags before 50,000 gliadin-specific T cells were added to each well, in RPMI 1640 with 15% heat-inactivated, pooled human serum. Sixteen T cell clones (TCCs) derived from the intestinal biopsies of nine DQ2.5$^+$ but DQ2.2$^-$ CD patients were used (specificity denoted in parentheses): TCC370.3.19E (αIII specific), TCC370.2.25 (αIII), TCC380.E2 (αI), TCC387.E9 (αI), TCC387.3 (γVI), TCC387.19 (γVII), TCC412.5.20 (αII), TCC423.1.3.8 (γI), TCC430.1.142 (αI and αIII), TCC430.1.135 (αII), TCC430.1.41 (γII), TCC430.1.134 (γIII), TCC430.1.112 (γIV), TCC436.5.4 (αII), TCC437.1.3.17 (γII), and TCC450.2.2.6 (αII). T cell proliferation was measured by the uptake of [^{3}H]thymidine (1 μCi/well; Hartmann Analytic), added 20 h before harvesting. Cells were harvested after 72 h onto glass fiber paper with an automated harvester (Mach III; Tomtec), and [^{3}H]thymidine incorporation was measured by liquid scintillation counting (Wallac MicroBeta TriLux 1450; PerkinElmer). Each Ag concentration was studied in triplicate. At least two independent experiments were performed for each assay.

Results

Characterization of the DQ2.5 binding registers of five γ-gliadin T cell epitopes

In this study, we determined the binding registers of three previously reported DQ2 epitopes, the DQ2-γII, DQ2-γIII, and DQ2-γIV epitopes, and two novel DQ2 epitopes from γ-gliadin, named DQ2-γVI and DQ2-γVII, based on T cell recognition of truncated peptides combined with HLA binding analysis of minimal T cell epitopes and Lys-substituted analogs. Lys is a disallowed anchor residue for binding to DQ2 in the P4 and P6 positions, whereas the side chain of P5 is oriented toward the solvent. For a correctly aligned peptide, decreased binding would be observed for Lys-substituted analogs at P4 and P6 but not at P5 (3). The results for each of these five T cell epitopes are presented below.

DQ2-γII epitope. Vader et al. (16) proposed previously IIQPEQPAQ as the binding frame of the DQ2-γII epitope, also referred to as Glia-γ30. In our competitive inhibition peptide binding assay, the peptide IIQPEQPAQ showed a >10-fold higher IC_{50} value compared with the 9-mer peptide IQPEQPAQL (Fig.

1*F*). This suggests IQPEQPAQL as the core region of nine residues for this epitope. This was confirmed by a limited Lys-scan with Lys substitutions in the proposed P4, P5, and P6 positions, with >20-fold increased IC_{50} values for the P4 and P6 Lys substitutes and an unchanged IC_{50} value for the P5 Lys-substituted peptide (Fig. 1*F*).

DQ2-γIII epitope. This epitope was first described by Arentz-Hansen et al. (2), reporting T cell recognition of the 13-mer FPQQPQQPYPQQP after TG2 treatment (Fig. 1*B*, ◆) (Gln residues found to be modified by TG2 are underlined). We demonstrate here that the minimal T cell epitope can be further narrowed to an 11-mer PEQPEQPYPEQ (Fig. 1*B*, ●) (predicted TG2 targeted sites are underlined). Typical IC_{50} data of Lys substitutes in the P4, P5, and P6 positions confirmed the EQPEQPYPE sequence as the core region of nine residues of this epitope (Fig. 1*G*). We next examined which of the three predicted TG2-mediated deamidation sites in positions P1, P4, and P9 were most important for the binding to DQ2.5 and T cell recognition. Peptide binding and T cell data of single Gln→Glu-substituted peptides revealed that the Gln residue in P4 (Fig. 1*B*, ▽) was the crucial deamidation site for T cell recognition, and the P4-substituted peptide also gave the lowest IC_{50} value (Fig. 1, *B* and *G*).

DQ2-γIV epitope. Previously, the shortest T cell stimulatory sequence for this epitope was narrowed to the 12-mer FSQPQQQF-PQPQ (Fig. 1*C*, ○) (2). We demonstrate here that T cell stimulation by a TG2-treated, C-terminally truncated 10-mer, FSQPQQQFPQ (Fig. 1*C*, ◆), is of comparable magnitude as the TG2-treated 12-mer. Both the peptide binding data of Lys substitutes in the P4, P5, and P6 positions (Fig. 1*H*) and the T cell recognition of the 9-mer SQPEQEFPQ (Fig. 1*C*, ◇) demonstrate that this is the core region of nine residues for this epitope. Within this 9-mer region, two Gln residues were identified as targets for TG2-mediated deamidation (2), and the identified register localizes these at the P4 and P6 positions. Although the T cell recognition of single Gln→Glu-substituted peptides at either of these two positions were significant and comparable with the TG2-treated native 12-mer peptide, the T cell recognition toward the synthetically double deamidated 12-mer peptide was nearly 100-fold stronger (Fig. 1*C*, ●). This indicates that deamidation to Glu at both positions is strongly preferred by the TCR. This enhanced T cell recognition cannot be solely explained by the slightly better peptide binding to DQ2.5 of the double-deamidated peptide compared with the single-deamidated peptides (Fig. 1*H*).

DQ2-γVI epitope. A T cell clone, TCC387.3, derived from an intestinal biopsy of a DQ2.5-positive celiac patient (CD387), was found to recognize a γ-gliadin-derived 20-mer peptide, LQPQQPFPQQPQQPYPQQPQ, in a TG2-dependent fashion (Fig. 1*D*, ○). Systematic screening with truncated variants of this peptide revealed that this TCC recognizes the TG2-treated 13-mer PQQPFPQQPQQPY (presumed TG2 target sites are underlined), which represents a novel γ-gliadin T cell epitope (Fig. 1*D*, ◆). Of note, TCC387.3 does not recognize the TG2-treated γ-III peptide FPQQPQQPYPQQP, which is also contained within the initial 20-mer peptide tested (data not shown). This epitope can be further narrowed to the 11-mer PEQPFPEQPEQ (Fig. 1*D*, ●). There are three possible 9-mer binding registers of this 11-mer, of which the EQPFPEQPE register is the most probable. However, a limited Lys-scan for the PEQPFPEQPEQ peptide with Lys residues in the proposed P4, P5, and P6 positions did not give the characteristic pattern of IC_{50} values found for other DQ2.5-binding peptides (Fig. 1*I*). A complete Lys-scan spanning the presumed P1–P9 positions was therefore undertaken. Unexpectedly, this complete Lys-scan brought no additional clarity, because only peptides with
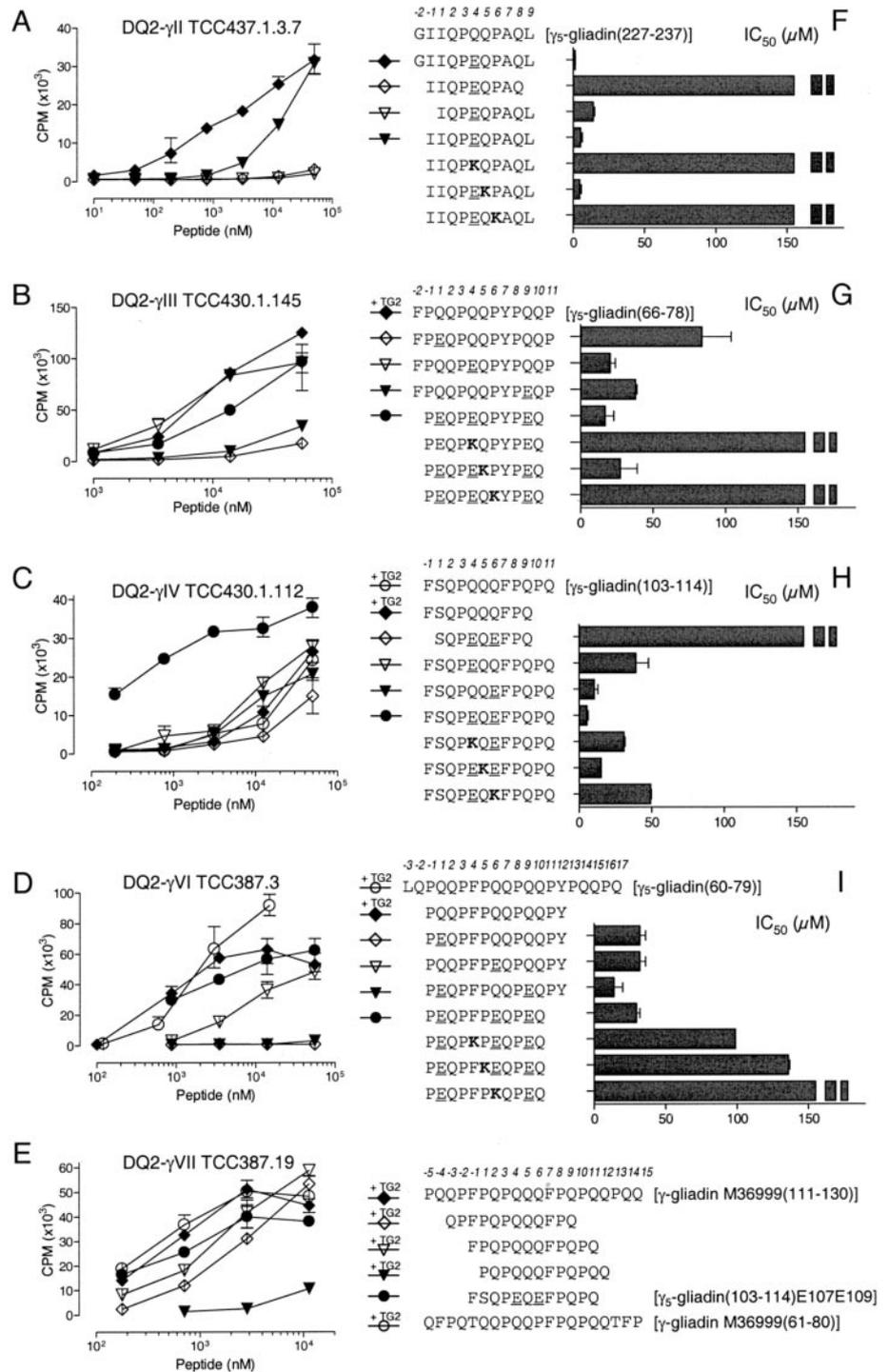
FIGURE 1. Characterization of five γ-gliadin epitopes. A panel of synthetic peptides was tested for recognition by specific T cell clones (*A–E*) and binding to DQ2.5 molecules in a cell-free, competitive inhibition binding assay with the radiolabeled *M. bovis*-derived peptide KPLLIIAEDVEGEY as an indicator peptide (*F–I*). The upper detection limit of the IC_{50} values was 167 μM due to limitation in peptide solubility. The T cell assays were all performed using a DQ2.5 homozygous, EBV-transformed B cell line from a CD patient (CD114) as APCs. All experiments were repeated at least twice. Error bars indicate the observed range within the triplicates for T cell results, or within minimum two peptide binding assays with 3-fold peptide titration. γ_5-gliadin accession no. AJ416339. CPM, counts per minute.

Lys substitution in the presumed P1, P2, and P7 positions gave reasonable binding to DQ2.5 (data not shown). These results could not be reconciled with known peptide binding data of DQ2.5 in any of the three possible binding registers of this 11-mer. Therefore, in lack of conclusive Lys-scan data, the suggested E̲QPF-PE̲QPE̲ 9-mer remains to be the most likely binding frame. T cell data with selected Gln→Glu substitutions show that of the total three targeted TG2 deamidation sites, only the Gln residue in the presumed P6 pocket is crucial for T cell recognition (Fig. 1*D*, ▽).

DQ2-γVII epitope. We found two TCCs that recognized a second novel γ-gliadin epitope, the DQ2-γVII epitope, TCC387.16, and TCC387.19. These two clones are probably sister clones, and

only results from TCC387.19 are shown here. TCC387.19 recognized several partially overlapping 20-mer peptides derived from the γ-gliadin protein M36999, all in a TG2-dependent fashion (data not shown). Based on one of these 20-mer peptides (PQQPFPQPQQQF PQPQQPQQ (Fig. 1*E*, ◆); presumed TG2 targets are underlined), we synthesized a panel of 12-mer peptides overlapping by 11 residues and covering the entire 20-mer sequence. Only three of these 12-mer peptides elicited T cell proliferation (Fig. 1*E*), and these data reveal PQPQQQFPQ as the core region of nine residues for this epitope. Interestingly, this nine-residue sequence differs from the core region of the DQ2-γIV epitope by only one Ser→Pro substitution at P1. The peptides F**SQPEQEFPQ**PQ (Fig. 1*E*, ●) (DQ2-γIV; core region of

FIGURE 2. Epitope recognition pattern of celiac lesion-derived, polyclonal T cell lines. Shown are the proliferative responses of each T cell line after stimulation with DQ2.5 APCs and TG2-treated, 100 μg/ml chymotrypsin-treated gluten, 2 μM α−33mer or 5 μM peptides that contain the different γ-gliadin epitopes. The following native peptides were used: α−33mer, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF; γ-I, PQQPQQSFPQQQRP; γ-II, GIIQPQQPAQL; γ-III/γ-VI, PQQPFPQQPQQPYPQQP; γ-IV, FSQPQQQFPQPQ; γ-VII, PQQPFPQPQQQFPQPQQPQQ. CPM, counts per minute.

nine residues are in bold) and **FPQPQQQFPQ**PQ (Fig. 1E, ▽) (DQ2-γVII; after TG2 treatment) elicited comparable proliferation of both TCC430.1.112 (DQ2-γIV-specific clone) and TCC387.19 (DQ2-γVII-specific clone) (Fig. 1E; data not shown for TCC430.1.112). The TCC387.19 also recognized another M36999-derived 20-mer peptide, QFPQTQQPQQPFPQPQQTFP, after TG2 treatment (Fig. 1E, ○), and the T**EQPEQPFPQ**P peptide was identified as the minimal sequence recognized by this TCC (data not shown). These observations illustrate the fact that some TCCs are cross-reactive to related sequences.

The γ-gliadin epitopes are recognized by celiac lesion T cells of several patients, but less frequently than the epitopes of α-gliadin

To ascertain the immunological relevance of the five characterized epitopes, we tested how frequently they were recognized by a panel of polyclonal, celiac lesion-derived T cell lines (TCLs). A total of 13 TCLs from 13 different CD patients that all displayed efficient responses to chymotrypsin digests of gluten were selected for testing. These lines were tested for recognition of TG2-treated peptides covering the five γ-gliadin epitopes, peptides containing the previously characterized γ-I epitope, and the α2-gliadin-derived 33-mer fragment (α−33mer) containing all the three α-gliadin epitopes. As shown in Fig. 2, the α−33mer was efficiently recognized by all 13 TCLs. In comparison, each of the six γ-gliadin epitopes was recognized by three to six TCLs, showing that

these are disease-relevant epitopes, albeit they are recognized less frequently by celiac lesion-derived T cells.

Pro residues are disallowed at positions P2, P4, P7, and P9, whereas critical Gln→Glu deamidations are found at positions P4 and P6

The alignment of nine gliadin T cell epitopes reveals a distinct pattern for the localization of Pro residues (Table I). The Pro residues are localized in positions P1, P3, P6, and P8, but never in positions P2, P4, P7, or P9. Notably, the DQ2-γI is the only epitope that does not have Pro at P3. All of the epitopes listed are dependent on TG2-mediated deamidation for T cell recognition, and the deamidation sites found to be critical for T cell recognition are denoted with a bold E in Table I. The other noncritical deamidation sites that nonetheless are targeted by TG2, as determined by mass spectrometry (2, 5) or by prediction from the TG2 sequence specificity (17, 18), are shown as underlined Q. Alignment of the epitopes in Table I demonstrates that the deamidation sites critical for T cell recognition are found in positions P4 and P6, or rarely in P7, as is the case for the DQ2-γI epitope.

Presentation of gliadin epitopes by DQ2.2 and their recognition by DQ2.5-positive celiac lesion T cells

Vader et al. (16) compared the T cell recognition of gluten epitopes when presented by DQ2.5- or DQ2.2-expressing APCs and found that T cells were unable to recognize epitopes with a Pro in the P3

Table I. *Alignment of the core region of three α- and six γ-gliadin T cell epitopes, presentation by DQ2.2 homozygous APC, and the IC$_{50}$ values in both DQ2.5 and DQ2.2 peptide binding assays*[a]

Gliadin Epitope		Peptide-Binding Register, P1-P9										Pro in P3	DQ2.2 T Cell Stimulation	IC$_{50}$ (μM)	
		1	2	3	4	5	6	7	8	9				DQ2.5	DQ2.2
α-I	QLQ	P	F	P	Q	P	E	L	P	Y		+	−	3.5	22
α-II		P	Q	P	E	L	P	Y	P	Q	PQLPY	+	(+)	19	42
α-III	PQPQL	P	Y	P	Q	P	E	L	P	Y		+	(+)	8	60
γ-I	PEQ	P	Q	Q	S	F	P	E	Q	Q	RP	−	+	4.3	8.8
γ-II	GI	I	Q	P	E	Q	P	A	Q	L		+	+	2.5	8.9
γ-III	FP	Q	Q	P	E	Q	P	Y	P	Q	Q	+	(+)	28	50
γ-IV	F	S	Q	P	E	Q	E	F	P	Q	PQ	+	+/−[b]	6.4	2.7
γ-VI	P	Q	Q	P	F	P	E	Q	P	Q	Q	+	−	71	>167
γ-VII		P	Q	P	E	Q	Q	F	P	Q		+	(+)	ND	ND
		Q	Q	P	E	Q	P	F	P	Q		+	(+)	ND	ND

[a] The sequences of deamidated 11–14-mer peptides used in peptide binding assays are aligned according to their peptide binding frames to DQ2.5. The Gln deamidation sites (by TG2) critical for T cell recognition are denoted by bold E, whereas underlined Q indicates other noncritical deamidation sites that nonetheless are targeted by TG2. Both groups of TG2-targeted Gln sites were synthetically converted to Glu in peptides used for peptide binding assays. For T cell recognition with DQ2.2 APC, + indicates equally well presentation by DQ2.2 compared with DQ2.5, (+) indicates presentation by DQ2.2 with lower efficiency compared with DQ2,5, and − indicates no T cell recognition with DQ2.2 APC at all. The radiolabeled HLA class I α 46–60 peptide EPRAPWIEQEGPEYW was used as an indicator peptide in both DQ2.5 and DQ2.2 peptide binding assays.
[b] DQ2.2 T cell stimulation of peptide FSQPEQEFPQPQ was seen with the cross-reactive γVII TCC387.19 (data not shown) but not with the γIV TCC430.1.112 (Fig. 3J).

position when these were presented by the DQ2.2 molecule. We performed similar types of experiments with the gliadin epitopes listed in Table I. To prevent autopresentation of the peptides by activated DQ2.5-expressing T cells, unbound peptides were removed by washing the APCs before the addition of the TCCs. As shown in Fig. 3, the T cells showed variable recognition of the different epitopes when presented by DQ2.2 APCs. One representative experiment for each TCC is shown, and other epitope-specific TCCs tested gave comparable results (data not shown). The peptides covering the DQ2-αI, DQ2-γIV, and DQ2-γVI epitopes were only recognized when presented by DQ2.5 APCs (Fig. 3, *A*, *D*, *J*, and *K*). In contrast, the DQ2-γI and DQ2-γII epitopes were

equally well recognized by the T cells irrespective of whether they were presented by the DQ2.5 or DQ2.2 APC (Fig. 3, *G* and *H*). The rest of the epitopes (i.e., the DQ2-αII, DQ2-αIII, DQ2-γIII, and DQ2-γVII epitopes) were most efficiently recognized when presented by the DQ2.5 APC, but all of them also elicited some T cell responses when presented by the DQ2.2 APC, at least at high peptide concentrations (Fig. 3, *B*, *C*, *E*, *I*, and *L*).

Presentation of the immunodominant α−33mer to specific T cells by DQ2.2

The immunodominant 33-mer peptide fragment derived from in vitro proteolytic digestion of recombinant α2-gliadin contains
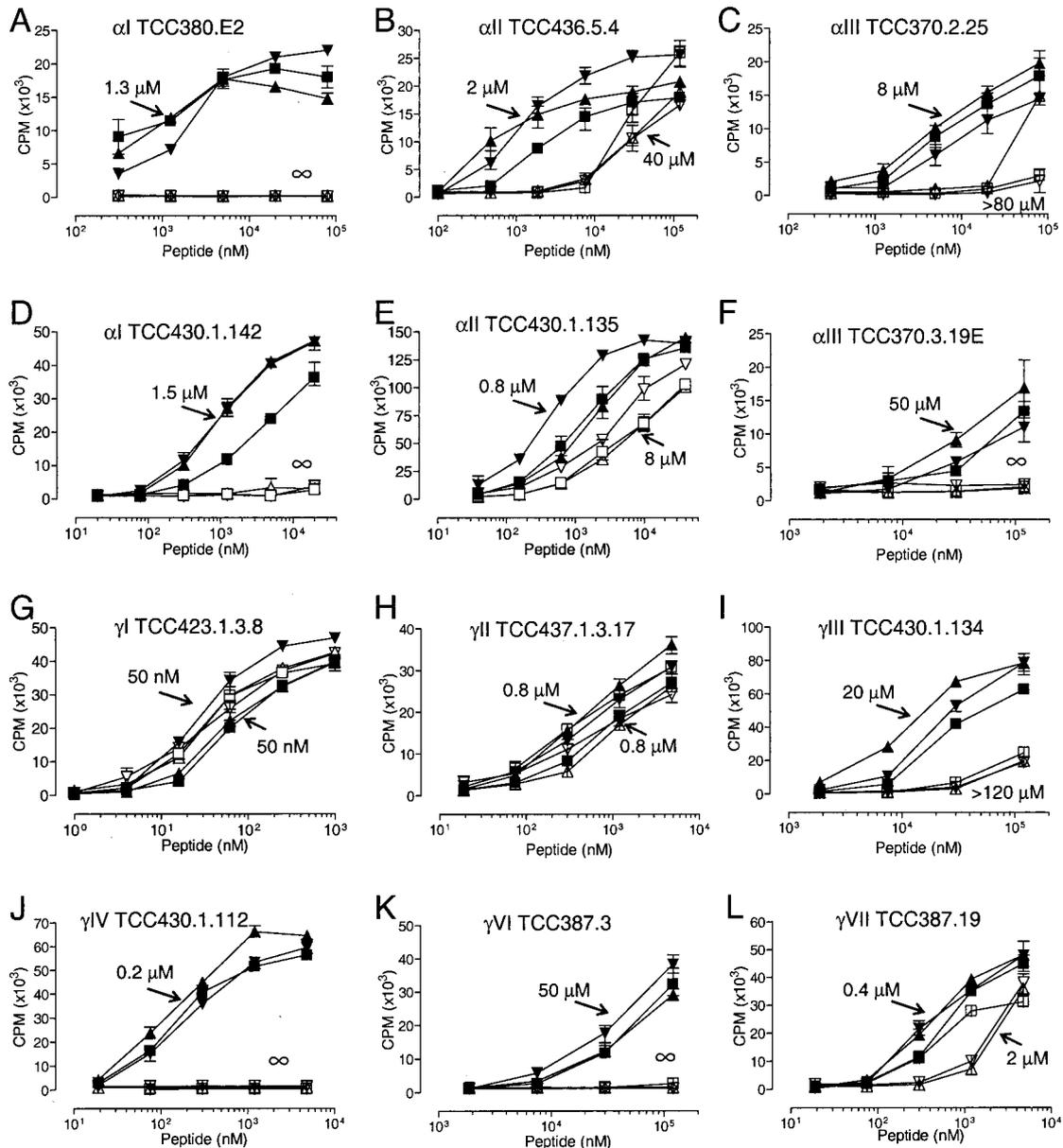
FIGURE 3. T cell recognition of three gliadin α-epitopes and six γ-epitopes with both DQ2.5 and DQ2.2 homozygous APCs. Three different EBV-transformed B cell lines were used in each group. Filled symbols represent DQ2.5 (*DQA1*0501/DQB1*0201*) homozygous APCs derived from three CD patients: CD114 (■), CD039 (▲), and CD125 (▼); open symbols represent three DQ2.2 (*DQA1*0201/DQB1*0202*) homozygous APCs: PLH (□), Mou (△), and Pitout (▽). All APCs were irradiated and incubated with peptides before the cells were washed twice on day 1 to remove unbound Ag. Epitope-specific T cell clones were then added. The following peptides were used (core regions of nine residues are underlined): α-I, LQL<u>PFPQPELPY</u>; α-II, <u>PQPELPYPQPQL</u>; α-III, PQL<u>PYPQPELPY</u>; γ-I, PEQPQQ<u>SFPEQERP</u>; γ-II, GII<u>QPEQPAQL</u>; γ-III, FP<u>EQPEQPYPEQ</u>; γ-IV, F<u>SQPEQEFPQPQ</u>; γ-VI, P<u>EQPFPEQPEQ</u>; and γ-VII, TG2-treated PQQPF<u>PQPQQQFPQ</u>PQQPQQ. Approximate EC$_{50}$ values are indicated. One representative experiment of at least two is shown. Error bars indicate the observed range within the triplicates in one experiment. CPM, counts per minute.

FIGURE 4. T cell recognition of the α−33mer and shorter peptides containing α-epitopes presented by both DQ2.5 and DQ2.2 homozygous APCs. The same six EBV-transformed B cell lines as in Fig. 3 were tested individually in duplicates. For clarity, the results within each group have been combined, and the mean and range are plotted here. The synthetically deamidated α−33mer LQLQPFPQPELPYPQ PELPYPQPELPYPQPQPF was presented by either DQ2.5 APCs (●) or DQ2.2 APCs (○), and shorter deamidated peptides containing the respective α-gliadin epitopes, the same as the ones used in Fig. 3, were presented by either DQ2.5 APCs (◆) or DQ2.2 APCs (◇). +, medium control for DQ2.5 APCs; ×, medium control for DQ2.2 APCs. All data shown are from one experiment with the same APCs and T cells. CPM, counts per minute.
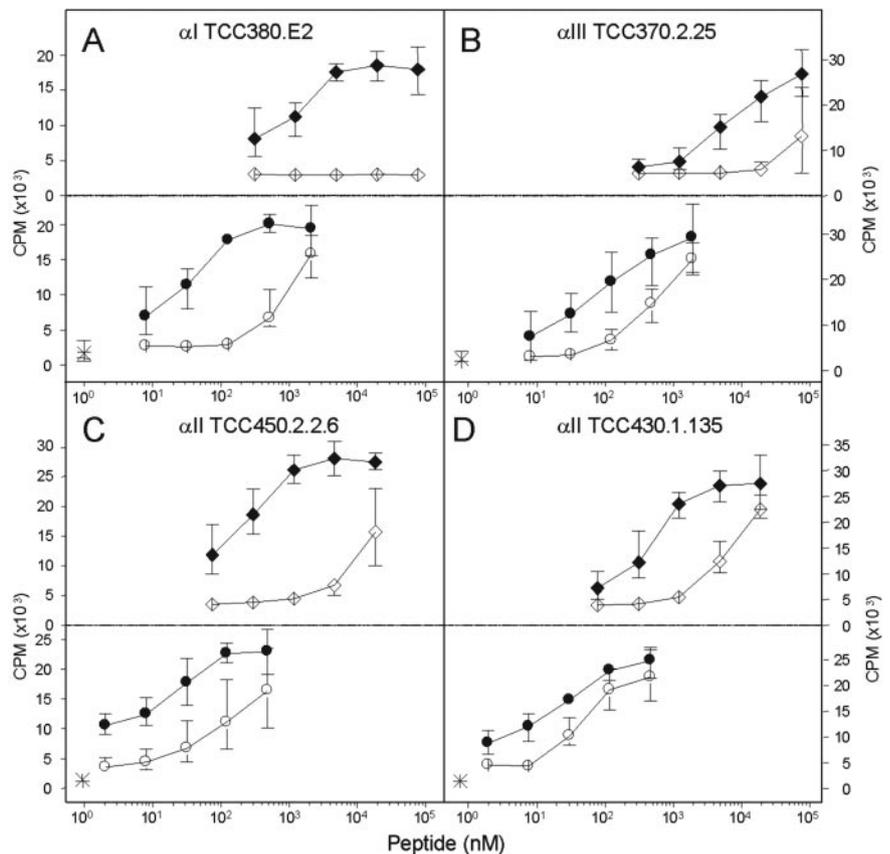
overlapping copies of all of the three α-gliadin epitopes and is shown to be a potent T cell stimulatory peptide (19). Therefore, we wanted to test how this naturally formed immunodominant α−33mer peptide was recognized by α-specific TCCs, when it was presented by DQ2.2 APCs compared with DQ2.5 APCs. Compared with most shorter α-epitope peptides that were hardly recognized by T cells when presented by DQ2.2 APCs, in general the longer α−33mer peptide was more efficiently presented by DQ2.2 APCs (Fig. 4). However, as shown by T cell data from some representative α-gliadin TCCs in Fig. 4, recognition of the α−33mer presented by DQ2.2 APCs was, in most cases, at least 1-log less efficient than recognition when presented by DQ2.5 APCs. Overall, the results show that T cell recognition of α-gliadin peptides presented by DQ2.2 APCs is a quantitative rather than an all-or-none phenomenon.

Discussion

In this study, we have characterized the DQ2.5 binding frames of three known and two novel γ-gliadin T cell epitopes. All of these five epitopes, as well as the three DQ2-restricted epitopes of α-gliadin and the DQ2-γI epitope previously fine-mapped by our group, were analyzed for peptide binding to the DQ2.5 and DQ2.2 molecules. This experimental assessment proved useful as it revealed errors in previous binding predictions and model constructions. Alignment of the core region of the identified epitopes shows that the gluten epitopes bind to DQ2.5 so that the Pro residues are avoided at positions P2, P4, P7, and P9 and that Glu residues formed by TG2-mediated deamidation are particularly critical for T cell recognition when localized at the P4 or P6 positions. In addition, we determined the ability of DQ2.2 homozygous APC and DQ2.5 APC to present the nine different epitopes to specific celiac lesion T cells. Overall, the data show that the disease asso-

ciated DQ2.5 molecule presents the gluten epitopes more efficiently than the nonassociated DQ2.2 molecule.

Gluten reactive CD4$^+$ T cell derived from celiac lesion biopsies recognize epitopes that are clustered in Pro-rich regions of gliadins (2). Importantly, the two novel epitopes found in this study also localize in Pro-rich regions, and this lends credence to the notion that T cell epitopes of gluten are contained in long Pro-rich fragments that survive gastrointestinal digestion similar to the highly antigenic 33-mer fragment of α2-gliadin (19). The core region of the nine gliadin T cell epitopes listed in Table I contain 2–4 Pro residues most often localized at the positions P3 (eight of nine epitopes) and P8 (seven of nine epitopes). Remarkably, the Pro residues are never localized at the P2, P4, P7, or P9 positions. The crystal structure of the deamidated DQ2-αI gliadin epitope bound to DQ2.5 revealed that the Pro residues of this peptide were positioned at the P1, P3, P5, and P8 positions (9). By this way, no hydrogen bonds between the amide nitrogens of the peptide main-chain and the conserved residues of the MHC (localized at P2, P4, P6, and P9) were lost. This finding gave rise to the idea that the DQ2.5 association with CD can be explained by the ability of DQ2.5 to make high-affinity interactions with deamidated gluten peptides despite the binding constraints imposed by the many Pro residues (9). This notion gains additional experimental support from the present study. The presence of Pro at position P6 and its absence at P7 is, however, not in accordance with localizations of Pro residues ruled by perseverance of the main chain hydrogen bonding network. The additional selectivity inferred by pocket preference could explain this positioning pattern with Pro fitting into the P6 pocket but not into the P7 pocket.

Although several Gln residues appear to be targeted by TG2 based on a prediction algorithm and/or experimental mass spectrometry data, our results show that only deamidation to Glu at the

P4/P6 and occasionally at the P7 positions are critical for T cell recognition. Peptide binding data (8, 20–23) and the crystal structure of DQ2.5 (9) have established that Lys-β71 of DQ2 is a key denominator for creating the preference for negatively charged anchor residues in the P4, P6, and P7 pockets. This is clearly a major factor explaining our observations. The discrepancy between the effect on DQ2 binding and T cell stimulation is, however, striking. For some of the γ-gliadin T cell epitopes (i.e., DQ2-γIII and DQ2-γIV) the T cell recognition of deamidated vs non-deamidated peptides is increased by >1000-fold, although their binding to DQ2.5 is only enhanced by 10-fold. Similar observations have been made previously for α-gliadin epitopes (3). Overall, the results suggest that the structure of the peptide-DQ2.5 complex is altered when the deamidations are located at the P4, P6, or P7 positions. The side chain of P7 is partly exposed to the TCR and could be itself responsible for this effect, whereas for P4 and P6 (which are completely buried in the αI-DQ2 structure) there are likely indirect effects. Interestingly, a similar phenomenon of altered T cell stimulation by substitution of a buried P6 residue (Glu→Asp) in a hemoglobin peptide bound to I-E^k is explained by an induced variance in the peptide P5–P8 main chain as well as a rotamer difference at the P8 side chain (24).

We also studied binding and T cell presentation of the gluten epitopes by the DQ2.2 molecule. It should be noted that the absence of T cell proliferation in our assays not necessarily implicates lack of Ag presentation by DQ2.2 APCs. Although the DQ2-γIV peptide FSQPEQEFPQPQ presented by DQ2.2 APCs failed to be recognized by the DQ2-γIV-specific TCC430.1.112 (Fig. 3J), the same peptide presented by DQ2.2 APCs was, however, efficiently recognized by the cross-reactive DQ2-γVII clone TCC387.19 (data not shown). This shows that the DQ2-γIV epitope can indeed be presented by DQ2.2 APCs as supported by peptide binding data (Table I). The lack of recognition by the DQ-γIV-specific TCC430.1.112 is rather explained by its TCR specificity than by poor peptide binding to the DQ2.2 molecule.

Genetic epidemiological studies have revealed that the DQ2.2 molecule in the absence of DQ2.5 is not associated with an increased risk for CD (reviewed in Ref. 10). The DQ2.5 and DQ2.2 molecules have identical β1 domains and differ by 10 aa in their α1 domains. Due to the sequence similarities between these two DQ2 molecules, there are minor differences in their peptide binding specificities (12, 23). van de Wal et al. (23) suggested that one important difference can be found at the P3 pocket: at this position, Pro is disfavored by DQ2.2 but not by DQ2.5. More recently, the same group has reported that the presence of Pro in P3 was negatively correlated with presentation of gluten epitopes to T cells by DQ2.2 molecules (16). These results differ from findings reported here. The DQ2-γII epitope (referred to as the Glia-γ30 epitope in Ref. 16), with a DQ2.5 binding register different from that previously predicted (16), has a Pro at the P3 position and is equally well recognized when presented by the DQ2.5 or DQ2.2 APCs. We also find that the DQ2-αII epitope, with a Pro at P3, is clearly presented by DQ2.2 APCs to T cells, which is in contrast to the negative DQ2.2 presentation reported by Vader et al. (16). Overall, we found that four of eight T cell epitopes with Pro in P3 are presented to T cells by DQ2.2 APCs, albeit often less efficiently than the presentation by DQ2.5 APCs. The simple rule that epitopes with a Pro residue at P3 are selectively presented by DQ2.5 therefore does not seem to hold true.

Why is it so then that individuals who carry DQ2.2 are not predisposed to CD although DQ2.2-positive APCs are capable of presenting six of nine gliadin epitopes investigated in this work? Two issues appear to be particularly relevant. First, not all gliadin epitopes may be equally important in the pathogenesis of CD. Ex-

tensive testing of multiple TCLs derived from celiac biopsies in our laboratory shows universal recognition of the naturally formed α−33mer that contains all three gliadin α-epitopes by TCLs derived from all CD patients (Ref. 19 and Fig. 2), whereas the gliadin γ-epitopes are less frequently recognized. When short 12-mer peptides containing the α-epitopes were presented by DQ2.2 APCs, there was little or none T cell recognition of the related DQ2-αI and DQ2-αIII epitopes and less efficient recognition of the DQ2-αII epitope compared with DQ2.5 APC (Fig. 1, A–F). In contrast, most γ-gliadin epitopes, as part of short peptides (11–20-mers), were recognized by T cells when presented by DQ2.2 APCs. Noticeably, the DQ2-γI and the DQ2-γII epitopes were equally well presented by DQ2.2 and DQ2.5 APCs. Therefore, the observations that γ-gliadin epitopes are infrequently recognized by CD TCLs and that most of them are efficiently presented by the nonassociated DQ2.2 molecule suggest that γ-gliadin epitopes may be less important in the anti-gluten T cell response in CD.

The concept of threshold introduced by Vader et al. (16) also seems relevant to explain why DQ2.2, in general, is not associated with CD. They suggested that there exists a threshold, governed by the number of gluten epitopes and the magnitude of the T cell response elicited by each epitope, that needs to be overcome to produce a pathological immune response to gluten. In the case of DQ2.2 individuals, the T cell response to gluten is usually insufficient to pass this threshold. Our and previous data demonstrate that the subtle differences in binding specificity between the DQ2.2 and DQ2.5 molecules mainly cause quantitative differences rather than an all-or-none effect on gluten peptide presentation. Although we found that most T cell epitopes (including the α−33mer) are presented by DQ2.2 APCs at high Ag concentrations, the T cell responses elicited by DQ2.2 APCs are usually much weaker. As shown in Figs. 3 and 4, Ag titration revealed that the T cell proliferation elicited by DQ2.2 APCs is often at least 1-log poorer compared with responses elicited by DQ2.5 APCs. The concept of a threshold effect is compatible with our data. It is interesting to note that even if the risk for developing CD associated with DQ2.2 is minuscule compared with that of DQ2.5, a recent study revealed that among the small group of DQ2.5- and DQ8-negative CD patients, individuals positive for DQ2.2 are overrepresented (41 of 61; 11 of 41 DQ2.2 homozygous) (25). This suggests that in the few DQ2.2-positive (and DQ2.5 negative) CD patients, the T cell response to gluten does reach a sufficient magnitude to cause disease. It will be interesting to characterize which gluten epitopes the intestinal T cell response is directed against in CD patients of this rare category. This should be the aim of additional studies.

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Disclosures

The authors have no financial conflict of interest.

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