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Immunogenic Peptides Can Be Detected in Whole Gluten by Transamidating Highly Susceptible Glutamine Residues: Implication in the Search for Gluten-free Cereals

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ABSTRACT: Tissue transglutaminase (TG2) plays a central role in celiac disease (CD) pathogenesis by strongly enhancing the immunogenicity of gluten, the CD-triggering antigen. By deamidating specific glutamine (Q) residues, TG2 favors the binding of gluten peptides to DQ2/8 molecules and, subsequently, their recognition by cognate T cells. Six peptides were previously identified within wheat gliadin whole extracts by tagging the TG2-susceptible Q residues with monodansylcadaverine (MDC) and nanospray tandem mass spectrometry (nanoESI-MS/MS). The immunogenicity of these peptides was next tested in gliadin-specific T-cell lines established from CD intestinal mucosa. Four peptides, corresponding to known epitopes of α- and γ-gliadins, induced cell proliferation and interferon (IFN)-γ production. Interestingly, one of the two non-T-cell stimulatory peptides corresponded to the 31−49 α-gliadin peptide implicated in the innate immune activation in CD mucosa. This study describes a strategy for identifying immunogenic gluten peptides potentially relevant for CD pathogenesis in protein extracts from wheat and other edible cereals.

KEYWORDS: *celiac disease, cereals, gluten epitopes, tissue transglutaminase*

INTRODUCTION

Celiac disease (CD) is a complex enteropathy precipitated by the ingestion of gluten from wheat, barley, and rye and associated with the HLA Class II alleles DQ2 and DQ8.[1] A large body of evidence indicates that CD is a T-cell-mediated disease caused by pro-inflammatory responses against gluten peptides. The isolation of HLA-DQ2 or -DQ8 restricted, gluten-reactive T cells from the intestinal mucosa of celiac patients, and recently from peripheral blood following oral gluten challenge, has strengthened the key role of adaptive immunity in celiac lesions.[2−6] However, the exact identification of pathogenic gluten peptides has been hampered by the structural complexity of this important storage protein. In fact, gluten is a mixture of hydrophobic polypeptides characterized by a high content of proline and glutamine residues that renders these proteins resistant to proteolysis by gastrointestinal enzymes.[7] Furthermore, reflecting the peculiar features of their primary sequence, gluten proteins contain very few of the negatively charged residues that are mandatory for peptide binding to HLA-DQ2 and DQ8 molecules.

Since the 1997 discovery that tissue transglutaminase (TG2) is an important autoantigen in CD,[8] significant advances have been made in elucidating its role in CD pathogenesis. Under normal conditions, TG2 is a ubiquitous enzyme involved in tissue healing that catalyzes protein cross-linking through the transamidation of glutamine and lysine residues.[9] Under particular conditions, TG2 can also catalyze the deamidation of specific glutamine (Q) residues to glutamic acid (E). This represents a key feature of the immune network responsible for the induction and maintenance of CD, as TG2 deamidation favors the binding of gluten peptides to HLA-DQ2/8 molecules, setting the stage for the activation of gluten-specific CD4+ T cells.[10]

To date, a number of gluten peptides that stimulate intestinal CD4+ T cells have been identified in several studies performed on different celiac populations, indicating that the repertoire of gluten peptides is greatly heterogeneous.[6,11−15] Among the immunodominant gluten peptides, the α-gliadin-derived 33-mer is perhaps the most investigated. This peptide contains six overlapping sequences able to activate celiac T cells and is highly resistant to digestion by gastrointestinal proteases, thus reaching the small intestinal mucosa in its entire immunogenic form.[12] In addition, whereas the pathogenic role of CD4-mediated cells in CD is well-defined, several other studies have recently pinpointed the involvement of an innate, non-T-mediated immune response in intestinal mucosal atrophy.[16] More specifically, the α-gliadin 31−43 peptide or its extended

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Table 1. Gluten Peptides Analyzed in This Study

Peptide	Position	Sequence[a]	Antigenic Determinant(s)
18-mer	Glia-α2 (71-89)	QPQPYPQPQL PYPQPQPF	PYPQPQLPY, PQPQLPYPQ (30)
25-mer	Glia-α2 (64-89)	PQLPQFLQPQPYPQPQLPYPQPQPF	PYPQPQLPY, PQPQLPYPQ (30)
14-mer-1	γ-Glia (105-118)	PQQQTLQPQQPAQL	LQPQQPAQL[b]
14-mer-2	γ-Glia (173-186)	PQQPFPSQQQQPLI	FPSQQQQPL[b], PFPSQQQQP[b]
19-mer	Glia-α (31-49)	LGQQQPFPPQQPYPQPQPF	
21-mer	Glt (19-39)	SHIPGLERPSQQQPLPPQQTL	

[a]tTG-susceptible Q residues are underscored (Q). [b]9-mer predicted epitopes.

sequence α-gliadin 31−49 induces a marked pro-inflammatory maturation of lamina propria mononuclear and dendritic cells in both organ cultures of celiac mucosal explants and cell culture systems.[17−20]

By tagging the TG2-susceptible Q residues with a MDC fluorescent probe, we previously identified six peptides within a pepsin-trypsin digest of wheat gliadins by nanoESI-MS/MS that were highly susceptible to deamidation by TG2.[21] Interestingly, among these peptides, two sequences of 18-mer and 25-mer lengths were truncated versions of the well-described 33-mer,[12] and one peptide corresponded to the innate immune eliciting peptide 31−49.[16]

The aim of the present study was to investigate the immunostimulatory properties of the six identified peptides in celiac patients to functionally validate the MDC-Q-TG2 peptidomic approach. The Q-MDC-tagged identified peptides and the known immunogenic 33-mer[12] were synthesized and treated with TG2 under deamidating conditions. Both native and TG2-deamidated peptides were tested for immune recognition using a panel of HLA-DQ2 adult CD patients. Immune reactivity was evaluated through gliadin reactive T-cell lines derived from intestinal mucosa of CD subjects.

MATERIALS AND METHODS

Patients. Jejunal biopsies were obtained from 15 adult CD patients (mean age = 29.1 years, range 18−49 years). Seven patients were serum-positive for anti-TG2 antibodies and had an atrophic mucosa. Eight patients were serum-negative for anti-TG2 antibodies and had been on a gluten-free diet for at least two years. The patients were typed for the DQA1, DQB1, and DRB1 genotypes using commercial HLA-typing kits (Dynal, Oslo, Norway), and all were HLA-DQ2.5 (DQA*0501, DQB*0201) positive. The patients were enrolled at the Gastroenterology Unit of a local hospital in Avellino and gave their fully informed consent to the study. The study protocol was approved by the Ethical Committee of San G. Moscati Hospital, OsSc registry no. 06/09, trial no. 234, 12/21/2007.

Peptide Synthesis. *N*-Fluorenyl-9-methoxycarbony (N^{α}-Fmoc)-L-amino acids, *N*′-tetramethyluroniumhexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), diisoproylethylamine (DIEA), and piperidine reagents and *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), and diethyl ether solvents were purchased from Iris Biotech (Marktredwitz, Germany). The Fmoc-L-amino acid−polyethyleneglycol solid phase (AA-PEG-PS) supports used for SPPS were supplied by Perseptive Biosystems. All other reagents required for washings, cleavage, and chromatography analysis were obtained from Sigma-Aldrich (St. Louis, MO, USA). Automated continuous-flow solid phase peptide synthesis (SPPS) were performed following the Fmoc orthogonal protecting strategy[22] on a PerSeptive Biosystems Pioneer Peptide Synthesis System GEN600611, following the manufacturer's protocol. Syntheses were carried out on Fmoc-L-Leu-PEG-PS (0.20 mmol/g) for 14-mer-1 and 21-mer, on Fmoc-L-Ile-PEG-PS (0.17 mmol/g) for 14-mer-2, and on Fmoc-L-Phe-PEG-PS (0.18 mmol/g) for 18-mer, 19-mer, 25-mer, and 33-mer. After automated synthesis, the peptides were manually cleaved from the solid support by treatment with trifluoroacetic (TFA)/triethylsilane (TES)/thioanisole (TA) (94:3:3), for 2 h. The peptides were purified by high-performance liquid chromatography (HPLC), and their identity was assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF MS) (Voyager DE-PRO, PE-Biosystems, Framingham, MA, USA). The samples were then lyophilized and stored at −20 °C until use.

Enzymatic Reactions. Wheat flour was obtained from an Italian hexaploid wheat variety (cv. Strampelli). Gliadin was extracted in 70% ethanol according to the method fo Wieser et al.[23] Peptic-tryptic digestion of gliadin (PT-gliadin) was performed as previously described.[21] TG2-mediated deamidation of PT-gliadin and peptides was carried out by incubating the substrate at 37 °C for 4 h with TG2 (at 1:10 ratio enzyme/substrate) in 5 mM Tris-HCl buffer (pH 6.8), containing 5 mM $CaCl_2$, 10 mM NaCl, and 10 mM dithiothreitol. The peptides/PT-gliadin were then lyophilized and stored at −20 °C until future usage. The peptides and PT-gliadin, either native or deamidated, were solubilized in deionized water at concentrations of 0.5 and 2.0 mg/mL, respectively, and thereafter diluted in culture medium at the indicated concentrations.

Binding Assays to HLA-DQ2 and -DQ8. Peptide binding assays were performed as previously described.[24] Briefly, CD-associated HLA-DQ2.5 and DQ8 molecules were purified from homozygous MATT (DQA1*0501/B1*0201) and PREISS (DQA1*0301/B1*0302) B-lymphoblastoid cells lines (EBV-B-LCL), respectively, by affinity chromatography using a pan anti-DQ SPV-L3 monoclonal antibody. Peptide binding assays were performed by incubating purified HLA-DQ molecules (5−500 nM) with various concentrations of tested peptide in the presence of 0.1−1 nM ^{125}I-radiolabeled probe peptide for 48 h. MHC binding of the radiolabeled peptide was measured using a TopCount (Packard Instrument Co., Meriden, CT, USA) microscintillation counter. The peptide concentration yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was then calculated. The peptides were typically tested at six different concentrations covering a 100,000-fold dose range in three or more independent assays.

Generation of Gliadin-Specific T-Cell Lines and T-Cell Assays. T-cell lines (iTCLs) were generated from small intestinal mucosa as described previously.[25] Briefly, mucosal cells were stimulated for two to three cycles with irradiated autologous peripheral blood mononuclear cells and deamidated PT-gliadin (50 μg/mL). IL-15 (R&D System, Minneapolis, MN, USA) was added every 2−3 days at 10 ng/mL. iTCLs were assayed for a response to PT-gliadin/peptides by detecting both IFN-γ production by ELISA and measuring cell proliferation, as previously described.[11] Briefly, T cells (3×10^4) were incubated with autologous EBV-B-LCL (1×10^5) and PT-gliadin (50 μg/mL), or gluten peptides (30 μg/mL), in 200 μL complete medium (X-Vivo plus 5% human serum, Lonza-BioWhittaker, Verviers, Belgium) in U-bottom 96-well plates. Cell supernatants were collected after 24 or 48 h for cytokine measurement, as indicated. Cell proliferation was measured through incorporation of [^{3}H]-thymidine (0.5 μCi/well, Amersham-Pharmacia, Milan, Italy). PT-gliadin and peptides were assayed in duplicate and in at least three independent experiments. Cytokines were analyzed in the cell supernatants by ELISA or intracellularly by flow cytometry. As criterion for peptide positive recognition, we arbitrary chose a 2-fold response (either as IFN-γ production and/or proliferation) over

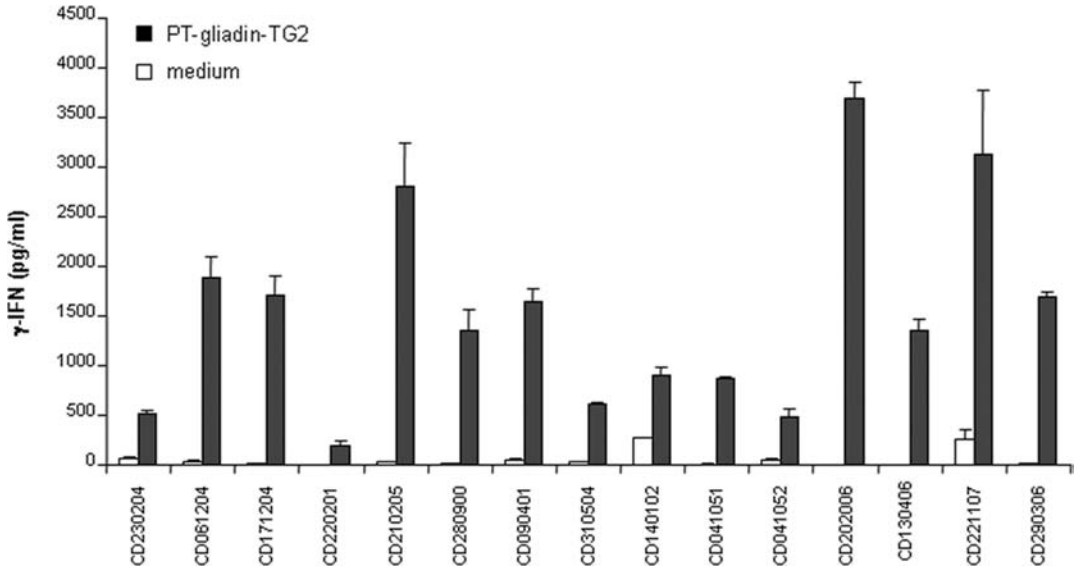

Figure 1. Response to TG2-deamidated gliadin in T-cell lines from celiac mucosa selected for this study. The MDC-Q-TG2 peptidomic approach was validated as a tool to detect immunogenic gliadin peptides in long-term T-cell lines generated from small intestinal mucosa of 15 CD patients. T cells (3×10^4) were assayed for IFN-γ production in response to deamidated PT-gliadin (50 μg/mL). Autologous EBV-B cells were used as APC. IFN-γ was detected in cell supernatant after 48 h by ELISA. The results are shown as the mean ± SD of duplicate wells.

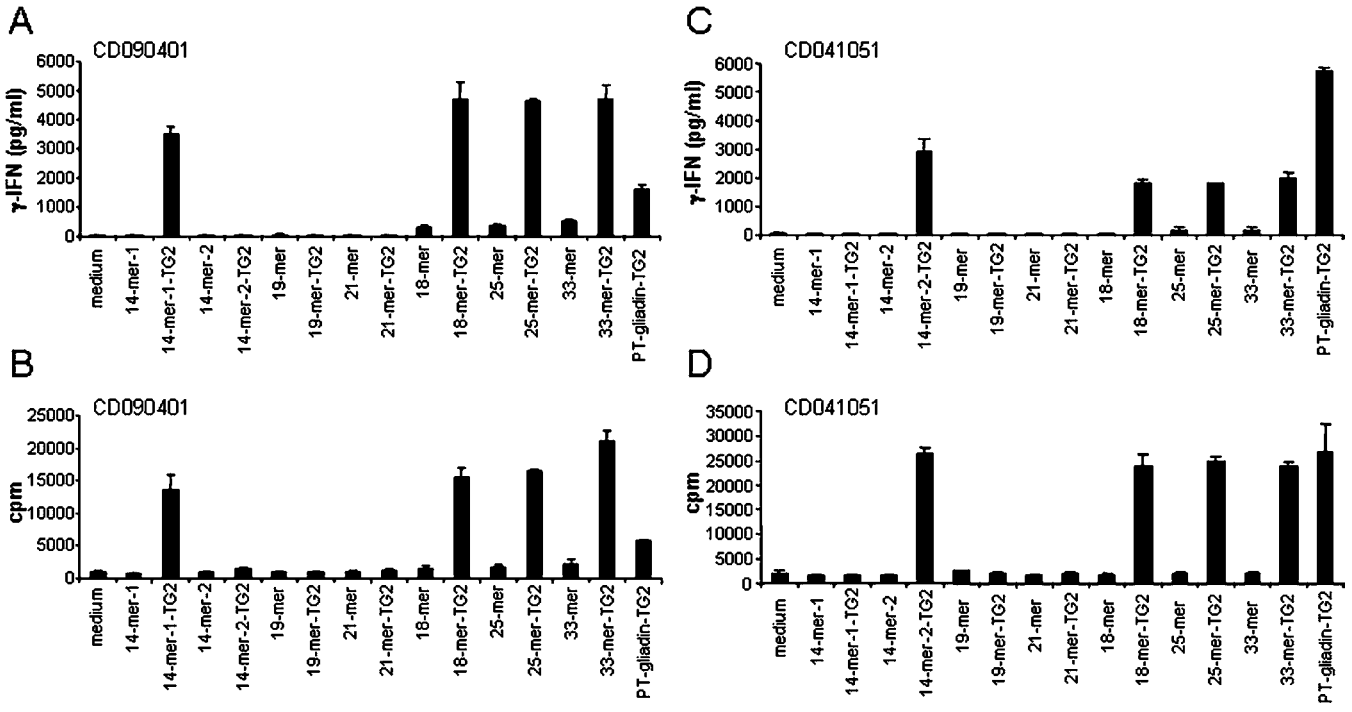

Figure 2. MDC-Q-TG2 peptidomic approach detects highly immunostimulatory T-cell peptides in the whole PT-gliadin. The profiles of immune response of DQ2 positive CD patients CD090401 (A, B) and CD041051 (C, D) to the 14-mer-1, 14-mer-2, 18-mer, and 25-mer identified after probing the PT-gliadin digest with MDC in presence of TG2 are shown. Intestinal T cells were incubated with autologous EBV-B-LCL cells and either native or TG2-deamidated peptides (30 μg/mL) and PT-gliadin (50 μg/mL). Both γ-interferon production (A, C) and cell proliferation (B, D) were measured after 48 h of antigen incubation. The results are shown as the mean ± SD of duplicate wells.

background values (response to medium alone), as previously described.[11]

RESULTS

Immunostimulatory Properties of Highly Tissue-Transglutaminase Susceptible Gliadin Peptides. Six gliadin peptides were previously identified as the most reactive TG2 substrates among the complex gliadin fraction of a wheat variety through a peptidomic approach (Table 1).[21] This procedure was based on first tagging peptides with a MDC fluorescent probe and then identifying the tagged peptides by MS/MS analysis. TG2 substrate peptides were de novo synthesized in their native form, and each was incubated with TG2 in a deamidation condition buffer. MS/MS analysis showed that all peptides were deamidated at the Q sites previously binding MDC. Three of six peptides were derived

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Table 2. Recognition of Gliadin Peptides Identified by MDC-Cross-Linking in PT-Gliadin

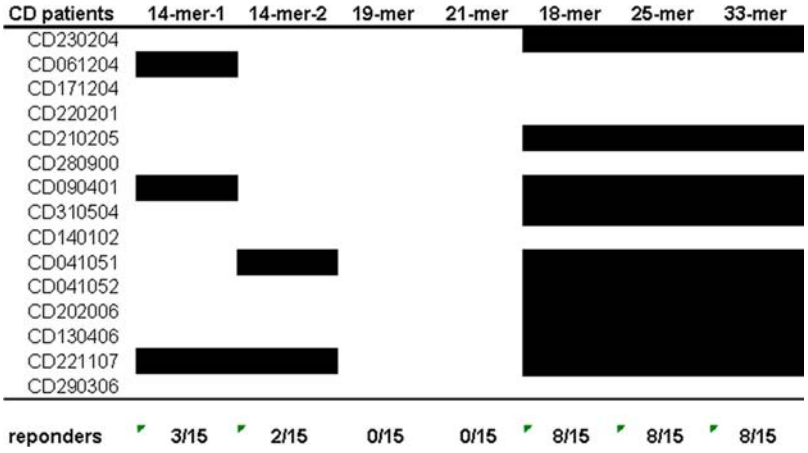

Table 3. Sequence Homology with Known Gluten Immunogenic Peptides

LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	33-mer	Known epitope (7)
PQLPQFLQPQPYPQPQLPYPQPQPF	25-mer	
QPQPYPQPQLPYPQPQPF	18-mer	
I I QPQQPAQL	DQ2.5-glia-γ2	Known epitope (15)
PQQQTLQPQQPAQL	14-mer-1	
PFSQQQQPV	DQ2.5-glut-L1	Known epitope (15)
PQQPFPSQQQQPLI	14-mer-2	

from α-gliadin (18-mer, 25-mer, and 19-mer) and two from γ-gliadins (14-mer-1 and 14-mer-2), and the remaining peptide matched a glutenin sequence (21-mer) (Table 1). The occurrence of glutenin in the gliadin fraction is not surprising, as the procedure of ethyl alcohol fractionation could lend itself to a cross-contamination of gliadins by glutenins, as previously reported.[26,27]

In the present study, both native and TG2-deamidated peptides, including the known immunodominant 33-mer, were assayed for the capacity to stimulate long-term gliadin-reactive T-cell lines generated from the intestinal mucosa of 15 DQ2-positive CD patients. These iTCLs, >95% of which are CD3+CD4+ T-cells (data not shown), were selected on the basis of their high IFN-γ production, and/or cell proliferation, to deamidated PT-gliadin (Figure 1).

Four peptides, 18-mer, 25-mer, 14-mer-1, and 14-mer-2, were highly antigenic in at least two patients (Figures 2; Table 2). More specifically, the 18-mer and the 25-mer were antigenic in 8 of 15 of the analyzed patients, the 14-mer-1 was active in 3 of 15, and the 14-mer-2 was stimulatory in only 2 patients (CD041051 and CD221107) (Figure 2C,D and data not shown). No activity was noted for the 19-mer and 21-mer peptides in any of the 14 patients analyzed.

Sequence Homology Analysis with Known Gliadin Epitopes. We next evaluated whether the four antigenic peptides had any sequence homology to the known T-cell epitopes. Extensive analysis revealed that both the 18-mer (QPQPYPQPQLPYPQPQPF) and the 25-mer (PQLPQFLQPQPYPQPQLPYPQPQPF) were truncated analogues of the immunodominant α-gliadin 57−89 (33-mer) peptide. Furthermore, they contained two of the three epitopes previously described within the 33-mer (DQ2.5-glia-α1b PYPQPQLPY and DQ2.5-glia-α2 PQPQLPYPQ; Table 1)[28−30] Interestingly, the multiepitopic and proteolysis-resistant 33-mer peptide itself was not identified among the most prominent Q-MDC-transamidated peptides from the whole Strampelli wheat PT-gliadin, but rather 2 truncated versions were identified. In addition, the 14-mer-1 peptide (PQQQTLQPQQPAQL) was largely homologous to a previously described peptide from γ-gliadin, DQ2.5-glia-γ2-(IIQPQQPAQL) (Table 3),[12] whereas the 14-mer-2 peptide, active in two patients (CD041051 and CD221107), harbored an FPSQQQQ sequence that was analogous to a known T-cell glutenin epitope (DQ2.5-glut-L1, PFSQQQQ), identified by Vader et al.[15]

The sequence homology evaluation of the remaining transamidated peptides, the 19-mer (LGQQQPFPPQQPYPQPQPF) and the 21-mer (SHILGPERPSQQQPLPPQQTL), indicated that the 19-mer corresponds entirely to the α-gliadin 31−49 sequence, previously reported to elicit an innate immune response in the celiac mucosa.[17−19] Although this peptide contains one Q-residue (sequence GQQQP) susceptible to deamidation and transamidation by TG2 (Table 1), neither the native nor the deamidated forms stimulated the T-cell lines. Finally, the 21-mer mapping to the 19−39 region of low molecular weight (LMW) glutenin that was inactive in our assays did not correspond to any known gliadin sequences.

Binding to CD-Associated HLA DQ2 and DQ8 Heterodimers. The presence of a deamidable Q residue in the consensus sequence QXP greatly favors the binding of gluten peptide to HLA-DQ2/8 molecules, but this is not necessarily sufficient for fitting the stringent criteria of the HLA-DQ2/8 binding pockets.[13,26] Accordingly, we investigated whether the negligible T-cell stimulatory activity of the 19-mer and 21-mer peptides in our cohort of celiac patients could be due to low or absent binding affinity for HLA-DQ2/8 molecules and, conversely, whether the high immunogenicity

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Table 4. Binding to Purified DQ2.5 and DQ8 Molecules

Peptide	DQ2.5	DQ8	DQ2.5	DQ8
	native peptide		deamidated peptide	
18-mer	6435[a]	-	232	-[b]
25-mer	4893	-	299	8783
33-mer	2586	-	44	3565
14-mer-1	4893	-	610	4594
14-mer-2	7403	-	6312	2951
19-mer	-	-	-	-
21-mer	-	-	-	-

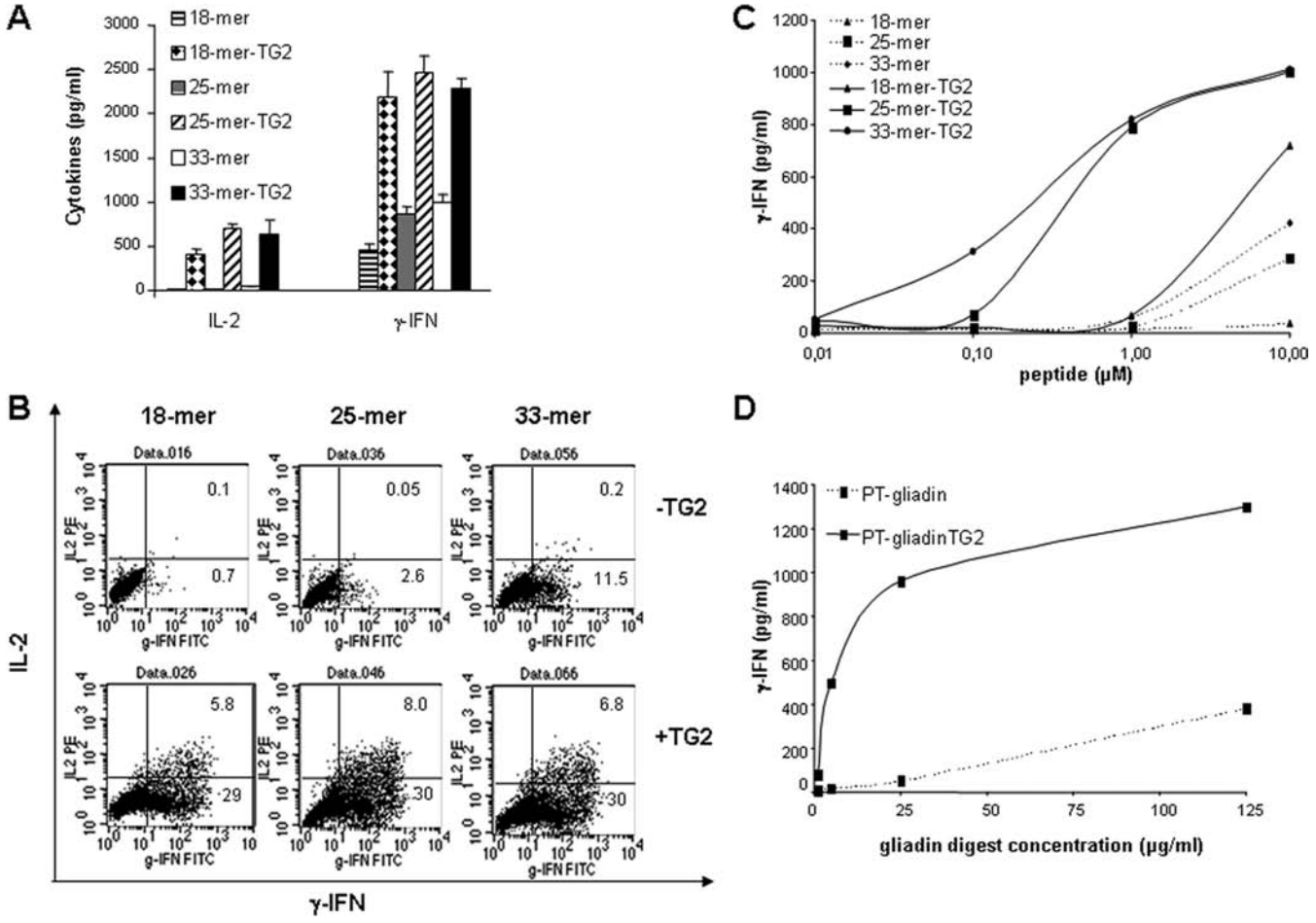

Figure 3. Cytokine production profile in response to both TG2-deamidated and native gliadin peptides. (A) Cytokine production was measured in iTCLs from patient CD230204 after stimulation with the 33-mer peptide and its truncated 18-mer and the 25-mer analogues. Intestinal cells were stimulated with autologous EBV-B-LCL cells overnight pulsed with native or TG2-deamidated peptides (30 μg/mL), and cell supernatants were collected after 24 (IL-2) or 48 h (IFN-γ). (B) Intracytoplasmic levels of IFN-γ and IL-2 were measured in iTCL from patient CD230204 after 16 h of stimulation with peptide-pulsed (30 μg/mL) autologous EBV-B-LCL cells. (C) Dose curve of the IFN-γ response of iTCLs from patient CD230204 to native and deamidated peptides or (D) to native and deamidated whole gliadin PT-digest (30 μg/mL). IFN-γ was measured in the cell supernatant after 48 h of cell stimulation with titrated peptides/PT-gliadin.

of remaining peptides might reflect strong DQ2 binding affinity. Interestingly, the 18-mer and 25-mer peptides, which were active in 53% of DQ2+ patients, bound DQ2.5 molecules with very high affinity, whereas the 14-mer-1 and 14-mer-2 peptides, which were less frequently recognized by DQ2+ celiacs, bound to both HLA-DQ2 and -DQ8, but with low affinity. Finally, both the 19-mer and 21-mer peptides were very poor binders to HLA-DQ2 and DQ8 molecules independent of TG2 deamidation, thus providing an explanation for their lack of T-cell stimulatory activity in celiac patients (Table 4). Our data suggested that binding affinity to HLA-DQ strictly influences the stimulatory properties of peptides and their recognition frequency in T cells from celiac intestinal mucosa.

Gliadin Peptides Highly Susceptible to Transamidation by TG2 Primarily Stimulate T Cells in Their Deamidated form. As the propensity of gluten peptides to be substrates of TG2 has been reported to strictly correlate with their capacity to stimulate T-cell responses in CD patients,[14] we next investigated whether the peptides identified through the MDC-Q-TG2 procedure were exclusively immunostimulatory in their deamidated (Q-E) forms. We chose to test the 33-mer and its truncations among the 6

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peptides identified because they were the most active in our study population. A T-cell line (patient CD230204) reactive to the 33-mer and to its truncated versions was stimulated with optimal peptide concentrations (30 μg/mL), and IL-2 and IFN-γ productions were measured either in cell culture supernatant by ELISA (Figure 3A) or intracellularly by flow cytometry (Figure 3B). Native peptides were able to induce IFN-γ production but almost no IL-2. Interestingly, the non-deamidated 33-mer was the most active compared to its truncated analogues. As expected, the magnitude of the responses induced by native peptides was much lower than those obtained in response to the deamidated ones. Furthermore, no substantial differences were observed among the three peptide analogues in inducing IFN-γ when deamidated, at least at high concentrations (30 μg/mL) (Figure 3A,B). We further evaluated the stimulatory properties of both the native and deamidated peptides and the whole gliadin digest in a dose−response curve (Figure 3C,D). The native 33-mer and 25-mer were stimulatory only at a high concentration (10 μM), in contrast with the deamidated forms of these two peptides, which were very potent in inducing cell activation. A similar reactivity profile was observed when the same T-cell line was tested against whole gliadin digest plus/minus TG2 treatment (Figure 3D). Altogether, our results confirm the previous findings that the stimulatory properties of gluten epitopes strictly increase after deamidation[9,10] and that the native forms of the known gluten peptides may display immunogenic properties, but only at very high concentrations.

DISCUSSION

In this study, we validated the transamidating activity of TG2 as a tool to detect relevant T-cell immunostimulatory gluten peptides in whole wheat extracts. Four of the six peptides, identified as the most reactive substrates of TG2-mediated transamidation within a proteolytic digest of whole gluten from a common hexaploid wheat,[21] markedly stimulated celiac mucosa T cells. A search of published studies and of protein data banks, such as the National Center for Biotechnology Information (NCBI) and the Immune Epitope Data Base (IEDB), revealed that these immunogenic peptides contain known T-cell epitopes. More specifically, the 18-mer and 25-mer contain the DQ2.5-glia-α1b (PYPQPQ(E)LPY) and DQ2.5-glia-α2 (PQPQ(E)LPYPQ) α-gliadin epitopes, the 14-mer-1 contains a highly homologous sequence of DQ2.5-glia-γ2 (IQPEQPAQL) epitope of γ-gliadins, and the 14-mer-2 (PQQPFPSQQQQPLI) was very similar to a glutenin sequence (PQQPPFSQQQQPLL) harboring the DQ2.5-glut-L1 (PFSQ(E)QQ(E)QPV) epitope.[15] Loci encoding gliadin proteins, particularly of hexaploid species, originate from gene duplications and translocations that result in insertion and deletion of peptide sequences and amino acid substitutions.[31] Because of this, it is very common to find peptides in distinct wheat species that differ by a few amino acids.[28] Furthermore, these extensive gene polymorphisms and sequence homologies make the molecular characterization of gluten T-cell epitopes quite challenging. In addition, gluten peptides are very poorly immunogenic in their native forms, mostly due to their weak binding to CD-associated HLA DQ2 and DQ8, which preferentially bind peptides with negatively charged amino acids. This conundrum was solved when, under particular conditions, TG2 was demonstrated to deamidate specific glutamine (Q) residues and enhance the binding of gluten peptides to HLA DQ2/DQ8.[8,9] In the presence of acyl acceptors such as MDC, TG2-susceptible Q residues may be transamidated. TG2-mediated transamidation has revealed a valid tool for monitoring and picking up CD-relevant T-cell peptides, as demonstrated by our and other studies,[14] or to neutralize the T-cell stimulatory property of gluten.[25] Our biochemical strategy was developed to find the peptides among a complex gliadin digest that were most susceptible to TG2-mediated transamidation. Surprisingly, with our biochemical approach the multiepitopic and proteolysis resistant 33-mer was not detected among the most reactive peptides as substrate to TG2-mediated transamidation. One possible explanation is that 33-mer, as well as several other dominant epitopes, might be contained in MDC-Q-tagged picks with lower fluorescent intensity that were not analyzed herein. Dorum et al.,[14] by using 5-biotinamido-pentylamine (5-BP) as acyl acceptor of TG2 transamidation, selectively characterized immunogenic peptides in cereal whole protein extracts, showing another successful example of the peptidomic approach for finding toxic gluten peptides. Compared to ours, the method by Dorum is an extensive analysis of all peptides tagged by 5-BP and implicates a step of enrichment with magnetic beads and column purification before the mass spectrometry analysis of the 5-BP-tagged peptides. Interestingly, in line with our findings, the 33-mer peptide was not found in the study of Dorum et al.; as well, even upon the enrichment procedure of immune stimulatory gluten peptides, instead two isoforms of 33-mer were detected in the whole gluten enzymatic mixture. Although other approaches have been developed to quantify the panel of toxic peptide content in bread wheat, which are based on the analysis of genome and transcript frequency,[32] the MDC-Q-TG2 technique has the advantage that it can be applied to the whole gluten proteome. In addition, it can be used to detect which gluten T-cell peptides survive the proteolysis by gastrointestinal enzymes, including the brush border prolylendopeptidases,[7] thus being potentially toxic for CD patients.

Several other studies have indicated that gluten contains peptides able to activate components of the innate immune system that contribute to enterocyte damage and concomitant tissue remodeling.[17−19] Among these "toxic" sequences, the 31−43 peptide and its long version, 31−49, are among the most extensively studied.[17−19] Interestingly, the MDC-Q-TG2 peptidomic technique identified the 31−49 peptide in the PT digest of whole gliadin. Notwithstanding the presence of a deamidated Q in position 4, which could fit well in the DQ2 binding register, p31−49 was recognized by none of the 14 celiac intestinal T-cell lines examined in this study. Interestingly, a binding assay to purified HLA-DQ molecules indicated that p31−49 does not bind either DQ2.5 or DQ8, thus explaining its complete lack of immunogenicity in celiac patients, as previously reported by ours and other studies.[11,14]

The deamidation of specific Q residues by TG2 greatly increases the capacity of gluten peptides to stimulate celiac T cells,[9−11,33] and our data confirm that the deamidated forms of all four immunogenic peptides identified in this study were several-fold more active than the corresponding native sequences. Studies in HLA-DQ8 transgenic mice have suggested that native gluten peptides are able to stimulate cognate T cells and that TG2 activity contributes to peptide immunogenicity through an amplification loop of responses induced by native peptide.[34,35] Of note, we found the same hierarchy in the capacity to stimulate T cells in both the native and deamidated versions among the 33-mer and its truncated analogues. The native 33-mer and 25-mer were stimulatory

only at high concentrations, in contrast to the deamidated forms that were very potent in inducing cell activation even at low concentrations. Because it cannot be formally excluded that some Q-E modifications might occur during peptide synthesis, or intracellularly, other studies are required to shed light on the immunogenicity of native gluten sequences and if the nondeamidated peptides might have a role in the early phase of intestinal mucosa lesions in CD patients.

In conclusion, we demonstrated that peptides relevant for CD pathogenesis could be identified in whole gliadin extracted from wheat flour by an enzymatic approach based on the transamidation activity of TG2. This procedure might be suitable for searching for alternative cereal sources containing fewer gluten toxic peptides, such as some cultivars of ancient diploid wheat.[36,37] For example, our method could be applied as a first screening to select wheat cultivars with low MDC incorporation in an enzymatic gluten digest followed by more extensive mass spectrometry and immunogenicity analysis of potential toxic sequences.

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ABBREVIATIONS USED

TG2, tissue transglutaminase; CD, celiac disease; MDC, monodansylcadaverine; nanoESI-MS/MS, nanospray tandem mass spectrometry; PT, peptic−tryptic; INF, interferon; iTCLs, T-cell lines; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; N^{α}-Fmoc, *N*-fluorenyl-9-methoxycarbonyl; HBTU, *N*′-tetramethyluronium-hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; DIEA, diisoproylethylamine; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; SPPS, solid phase peptide synthesis; AA-PEG-PS, amino acid-polyethyleneglycol solid phase; TFA, trifluoroacetic; TES, triethylsilane; TA, thioanisole

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