Gliadin T Cell Epitope Selection by Tissue Transglutaminase in Celiac Disease

ROLE OF ENZYME SPECIFICITY AND pH INFLUENCE ON THE TRANSMISSION VERSUS DEAMINATION REACTIONS

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Tissue transglutaminase (TG2) can modify proteins by transamidation or deamidation of specific glutamine residues. TG2 has a major role in the pathogenesis of celiac disease as it is the target of disease-specific autoantibodies and generates deamidated gliadin peptides that are recognized by CD4+ and DQ2-restricted T cells from the celiac lesions. Capillary electrophoresis with fluorescence-labeled gliadin peptides was used to separate and quantify deamidated and transamidated products. In a competition assay, the affinity of TG2 to a set of overlapping γ-gliadin peptides was measured and compared with their recognition by celiac lesion T cells. Peptides differed considerably in their competition efficiency. Those peptides recognized by intestinal T cell lines showed marked competition indicating them as excellent substrates for TG2. The enzyme fine specificity of TG2 was characterized by synthetic peptide libraries and mass spectrometry. Residues in positions −1, +1, +2, and +3 relative to the targeted glutamine residue influenced the enzyme activity, and proline in position +2 had a particularly positive effect. The characterized sequence specificity of TG2 explained the variation between peptides as TG2 substrates indicating that the enzyme is involved in the selection of gluten T cell epitopes. The enzyme is mainly localized extracellularly in the small intestine where primary amines as substrates for the competing transamidation reaction are present. The deamidation could possibly take place in this compartment as an excess of primary amines did not completely inhibit deamidation of gluten peptides at pH 7.3. However, lowering of the pH decreased the reaction rate of the TG2-catalyzed transamidation, whereas the rate of the deamidation reaction was considerably increased. This suggests that the deamidation of gluten peptides by TG2 more likely takes place in slightly acidic environments.

The food-sensitive enteropathy celiac disease (CD) is a chronic inflammatory disorder with a multifactorial etiology (1, 2). The disease is precipitated by dietary wheat gluten and related proteins in barley and rye. The ingestion of such proteins induces mucosal lymphocyte infiltration and villus atrophy. Subjects with active disease have autoantibodies specific for the enzyme tissue transglutaminase (3). CD shows a strong genetic association with the genes encoding for HLA-DQ2 and -DQ8 (1). Gluten-reactive CD4+ T cells isolated from the small intestine of CD patients are almost exclusively restricted by either of these HLA molecules (4, 5), and activation of such T cells is probably a critical event in the disease development (1). Interestingly, the gluten-reactive T cells of the celiac lesion predominantly recognize gluten peptides in which certain glutamines are converted to glutamic acid by deamidation (6). Evidence indicates that tissue transglutaminase (TG2) can mediate this deamidation in vivo (7–9). TG2 is best known for its ability to catalyze an acyl transfer reaction in which the carbamylamido group of a peptide-bound glutamine residue is the acyl donor and an appropriate primary amine is the acyl acceptor (10). The active site of TG2 comprises a catalytic triad built by cysteine 277, histidine 335, and aspartic acid 358 (11). In the first step, a glutamine residue forms a thiol ester with the active site cysteine, and ammonia is released (acylation). In the following rate-limiting transamidation step, the acyl group is transferred to the acyl acceptor amine forming an isopeptide bond (deacylation). However, the thiol ester bond can be also hydrolyzed, resulting in deamidation of the bound glutamine. In the literature the deamidation reaction is described to have a slower rate than the transamidation reaction (10).

To better understand how TG2 can be involved in formation of gluten T cell epitopes in CD, we have examined the affinity of various peptides to TG2, we have characterized the specificity of the enzyme, and we have studied the propensity of the enzyme to catalyze transamidation and deamidation reactions. We found that the affinity of various gliadin peptides to TG2 varies, and the peptides that stimulate T cells are among those with the highest affinity. The variation in affinity between the peptides can be explained by the fine specificity of the enzyme. The ratio of the deamidation to transamidation increased when pH was lowered beneath neutral pH. Our results suggest that the specificity of TG2 is involved in selection of gluten T cell epitopes and that the deamidation reaction of gluten peptides in celiac disease is taking place in slightly acidic environments.
EXPERIMENTAL PROCEDURES

Synthesis of Peptides and Peptide Libraries—Synthetic peptides and peptide libraries were prepared by multiple solid-phase peptide synthesis on a robotic system (Syro MultiSynTech, Bochum, Germany) using Fmoc/Ot-buty1 chemistry. Individual peptides were synthesized on 2-chlorotrityl resin (Senn Chemicals AG, Dielsdorf, Switzerland) as described previously (12). For fluorescein labeling, carboxyfluorescein (5 eq, Sigma) was coupled to the free N-terminal amino group of the resin-bound, protected peptide using diisopropylcarbodiimide (5 eq) as coupling reagent. Identity of peptides was confirmed by electrospray mass spectrometry, and purity was analyzed by reversed-phase-HPLC. Based on the sequence of the undecapeptide, TSEKSGTQLVT (13), 10 peptide libraries were synthesized on polystyrene A RAM amide resin (Rapp Polymere, Tübingen, Germany). In each library one of the amino acid residues flanking the central glutamine was replaced by a random-ized position (X), carrying all natural amino acids except cysteine. Randomized positions were introduced by double couplings with an equimolar mixture of 19 Fmoc-t-amino acids used in an equimolar ratio with respect to the coupling sites of the resin. Defined sequence positions were introduced using a 5-fold molar excess of single Fmoc-t-amino acids. An optimized synthesis and work-up protocol was used to obtain equimolar mixtures (14) as analyed by electrospray mass spectrometry (15).

Preparation of Antigen and Purification of Tissue Transglutaminase—The pepsin and trypsin digestion of crude gliadin and avenin was performed as described previously (8). Human TG2 was expressed as a glutathione S-transferase fusion protein in Escherichia coli using the vector construct as described previously (16). Guinea pig TG2 was obtained from Sigma.

Capillary Electrophoresis—All analyses were done on a Beckman MDQ capillary electrophoresis system equipped with a laser-induced fluorescence detector (488 nm). CE was carried out in a fused-silica capillary (25-cm length, 75-μm inner diameter) equilibrated by three rinsing steps using 100 mM sodium hydroxide, water, and electrophoresis buffer (20 p.s.i., 1.5 min each). Samples were injected by pressure (0.5 p.s.i., 1 s). Separations were performed at 22 kV at room temperature. All samples were running from the cathode to the anode. Two forms of CE using different electrophoresis buffers were applied: standard capillary zone electrophoresis (CZE) runs were done with 80 mM sodium borate, pH 9.3; for micellar electrokinetic chromatography (MEKC) 64 mM sodium borate, 20 mM sodium dodecylsulfate, pH 9.3 was used. The fluorescein-labeled peptide QLGFPFQPQFLPY (defined as F-IE, corresponding to α9-gliadin (57–68)), its deamidated derivative α9-IE (defined as F-IE), and the F-IE-TG2 complex were separated and quantified by MEKC. The transamination product of F-IE, which is obtained by using 5-biotinylpentylamine (5-BP) as an acyl acceptor, is defined as F-IE(5-BP). F-IE and F-IE(5-BP) were separated from F-IE by CZE.

Sample Incubation with TG2—F-IE was incubated at the concentrations indicated with 1 μM TG2 at 37 °C in 100 mM Tris/Cl, 2 mM CaCl2, pH 7.3 except in experiments in which the effect of pH was investigated. In competition assays different amounts of single unlabeled competitor peptides or peptic-tryptic digests of gliadin or avenin were added, and incubation was done for 18 min. To quantify deamidation versus transamination, 5-BP was titrated as a primary amine, and samples were incubated for different time periods. In case of CZE, samples were diluted at 1:3 by 100 mM Tris/Cl, pH 7.5, 10 mM EDTA. For MEKC, samples were diluted by 64 mM sodium borate, 20 mM SDS, pH 9.3. It was shown that under both conditions the enzymatic activity of TG2 was shut off immediately.

Sequence Specificity of Tissue Transglutaminase—Peptide libraries (1 mM) and 5-BP were incubated with 0.1 μM TG2 (guinea pig enzyme, Sigma), 100 mM Tris/Cl, pH 7.5 at 37 °C (1 h, 0.15 μM total volume). Samples were desalted by solid-phase extraction using C18 ZipTip (Millipore GmbH, Eschborn, Germany) and analyzed by direct infusion ESI-FTICR mass spectrometry on a passively shielded 4.7-T APXII ESI-MALDI-FTICR mass spectrometer (Bruker Daltonik, Bremen, Germany). The software XMASS version 5.0.10 (Bruker Daltonik) was used for data acquisition and processing. All samples were diluted in a solution of 0.1% trifluoroacetic acid in acetonitrile (50/50). ESI (Analytica of Branford, Branford, CT) was performed in the positive ion mode with a grounded capillary sprayer needle mounted 60° off-axis. No supporting nebulizer gas was used, and the flow rate was 1 μl/min. Transamidated reaction products were identified by their typical mass shift of +311.2 atomic mass units (+155.6 atomic mass units for doubly charged peptides) compared with the original peptides in the library. In each spectrum, the peak intensities of the converted products were divided by those of the corresponding unconverted peptides in the same sample to compensate for differences in ionization behavior and the partial lack of exact equimolar presentation between different peptides in the library. These ratios were defined as the “transamination rate.”

RESULTS

Separation and Quantification of TG2-catalyzed Reaction Products by CZE and MEKC—The synthetic peptides F-IE and F-IE(5-BP) were used to work out separation conditions by CZE. The deamidated peptide, which carries an extra negative charge, was base-line separated from F-IE and eluted 0.85 min later. Moreover, the formation of F-IE by incubation of F-IE with TG2 was demonstrated by CZE (data not shown). However, as we were also interested in separating and quantifying covalent F-IE-TG2 complexes (not discussed in this paper), we changed to MEKC, which allowed us to separate F-IE, the enzymatic deamidation product F-IE(5-BP), and F-IE-TG2 complexes (Fig. 1, A and B). The identity of F-IE(5-BP) and F-IE-TG2 complexes was confirmed by adding the synthetic peptide and a chemically labeled fluorescein isothiocyanate-TG2 adduct, respectively. The second product observed in Fig. 1B is most probably a double deamidated derivative, defined as F-IE(5-BP). Incubation of F-IE with TG2 in the presence of 5-BP led to the expected transamidation product F-IE(5-BP), which was separated from F-IE and deamidated products only by CZE (Fig. 1C). The identity of the obtained signals was proven by adding streptavidin-coated beads (Dynal). The major reaction product and a minor by-product were specifically removed, identifying them as F-IE(5-BP) and a double transamidated derivative F-IE(5-BP) (15).

Validation of the CE-based Analysis—The quantification of TG2-catalyzed deamination products by MEKC and the use of the fluorescence-labeled substrate F-IE was first validated by measuring the deamination of F-IE by TG2 in 100 mM Tris/Cl, pH 7.3, 2 mM CaCl2. Plotting the reaction velocity V versus [F-IE] revealed a kcat of 21 min−1 and a Km of 0.17 mM, resulting in a kcat/Km of 124 min−1 mM−1. Compared with the parameters obtained in a recent study for the non-labeled peptide (kcat = 23 min−1, Km = 0.35 mM) (18), our measured Km value of 0.35 mM is slightly lower, whereas kcat values are identical. The observed kcat/Km value is still in the order as expected and may partly reflect the fluorescence labeling of the peptide and different buffers used in both studies.

Comparison of Known Gliadin Substrates—For a further validation of the CE-based analysis, single unlabeled α- and γ-gliadin peptides were used to compete with the deamination of F-IE. These peptides are known as substrates of TG2 as they are converted to T cell epitopes by specific deamination of glutamine residues. The deamidated reaction product F-IE(5-BP) was quantified by MEKC, and by comparing results with and without competitors we calculated the competition efficiency over a broad concentration range. The calculated IC50 values obtained from these titration experiments ranged from 50 μM (for DQ2-α-II) to 235 μM (for DQ2-β-I) (Fig. 2). As controls, three peptides from ovalbumin, from hen egg lysozyme, and from the mouse α-light chain (λ) (515)-(89–107)A90 were used. These peptides have not been described as substrates of TG2 and carry only one (ovalbumin and hen egg lysozyme peptide) or no (α-light chain peptide) glutamine residues. For all three peptides only a weak competition ≤15% was observed at the highest competitor concentration used (550 μM), and IC50 values not determined.

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Competition and T Cell Recognition of Overlapping Peptides from γ-Gliadin M36999—The established competition assay and the validated CE analysis was used to measure competition of a set of 23 20-mer peptides overlapping by 10 residues and covering nearly the complete sequence of the γ-gliadin M36999 (19, 20). F-α1 was used as reporter peptide, and its deamidation by TG2 was quantified by MEKC. To limit the number of assays and CE runs, competition of each peptide was measured at a single concentration of 110 μM, a concentration where the titration curves obtained for the gliadin peptides showed the highest slope. The γ-gliadin peptides showed significant differences in their competition potency ranging from 73% (M13) to 9% (M4) (Fig. 3A). Interestingly, a sequence region (M36999 residues 61–140) was identified in which all peptides showed competitions >56% (M7–M13). The same set of 20-mer peptides was screened for recognition after TG2 treatment by six gluten-specific, polyclonal intestinal T cell lines generated from six different celiac disease patients, and the results are reported in detail elsewhere (21). Although these T cell lines recognized different numbers of peptides (from one to six) and varied in their response to the recognized peptides, the six T cell lines together recognized eight of the 23 γ-gliadin peptides: M2, M7, M8, M10, M12, M13, M23, and M24 (Fig. 3A). With the exception of M23 (46% competition), all these peptides showed competition higher than 58%, and five reside in the region M36999 (residues 61–140).

Sequence Specificity of Tissue Transglutaminase—The undecapeptide TSEKSQTPPLVT is an acyl donor substrate for TG2 (13). Ten peptide libraries randomized in positions −5 to −1 and +1 to +5 with respect to the central glutamine targeted by TG2 (Table I) were applied in transamidation assays using guinea pig TG2 and 5-BP as a primary amine. Transamidated reaction products were identified by ESI-FTICR mass spectrometry, and “transamidation rates” were determined.

These experiments allowed us to assess semiquantitatively the influence of all 19 amino acid residues in the 10 randomized sequence positions on TG2-catalyzed transamidation as summarized in Table I. From these titration experiments (using 10 μM F-α1) IC50 values of 94 and 210 μg/ml were obtained for the peptic-trypptic digest of gliadin and N,N-dimethylcasein, respectively. For the peptic-trypptic digest of avenin, an IC50 of at least 2000 μg/ml was estimated.

Competition of Peptic-Tryptic Digests of Gliadin and Avenin—Subsequently, more heterogeneous mixtures, as a peptic-trypptic digest of gliadin and avenin, and the known protein substrate N,N-dimethylcasein (22) were used as competitors. From these titration experiments (using 10 μM F-α1) IC50 values were estimated to be significantly higher than 550 μM.

After incubation with streptavidin-coated beads (Dynabeads M-280 Streptavidin) for 15 min at 25 °C (lower curve). All samples were diluted 1:33 with the corresponding electrophoresis buffer. RFU, relative fluorescence units.

**FIG. 1.** A, electropherogram of the fluorescein-labeled synthetic peptide QLQPFPQPQLPY (defined as F-α1) analyzed by MEKC (10 μM F-α1 in 100 mM Tris/Cl, pH 7.3, 2 mM CaCl2, diluted 1:33). No absorbance was observed from 0 to 4 min. The F-α1 synthetic product contained about 10% of the deamidated peptide F-α1E, which could not be separated by preparative HPLC. B, electropherogram obtained by MEKC after incubation of 10 μM F-α1 with 1 μM TG2 at 37 °C for 18 min in incubation buffer. C, electropherogram obtained by CZE after incubation of 10 μM F-α1 and 200 μM 5-BP with 1 μM TG2 at 37 °C for 2 h (upper curve). Also shown is the electropherogram of the same sample after incubation with streptavidin-coated beads (Dynabeads M-280 Streptavidin) for 15 min at 25 °C (lower curve). All samples were diluted 1:33 with the corresponding electrophoresis buffer. RFU, relative fluorescence units.
were preferred compared with hydrophilic and charged residues. Although no amino acid showed a pronounced effect in position –1, different residues induced different transamidation rates, and charged side chains decreased transamidation. The variance of all transamidation rates found for the 19 amino acids in an X position describes its impact on the sequence specificity of TG2. Thus, for the investigated undecapeptide, positions +2 and +3 were found as most important followed by positions –1 and +1 (Fig. 4).

Sequence Specificity of TG2 Explains the Competition Data of the γ-Gliadin Peptides—The data above strongly suggest that the spacing between the targeted glutamine and C-terminal proline residues dominates the specificity of TG2, i.e. QxP (where x, representing a variable amino acid, indicates the distance between Gln and Pro) strongly supports transamidation, whereas QP and QxxP abolish it (Table I). These motifs were used to predict targeted glutamine residues within the γ-gliadin peptides to explain their competition data (Fig. 3A). Glutamines within QP or QxxP were predicted as not targeted. Glutamates that are part of the QxP motif were predicted as targets, and a value of 1 was assigned to such peptides. Sequences containing QxP or QxxP were not found in these peptides. Glutamines, which are not followed by proline in positions +1, +2, or +3, were predicted as moderately targeted if at least three residues in positions –1, +1, +2, or +3 matched those listed under “+” in Table I. If so, a value of 0.5 was assigned to such sequences. The sum of the values assigned to a given peptide would then reflect the number of predicted glutamine residues that can be targeted by TG2. We found that the prediction correlated with the competition data of the overlapping γ-gliadin peptides (Fig. 3B). Especially for peptides M7–M13, prediction scores ≥2.5 were obtained that correlated with competition values >56%. Also, competition of C-terminal peptides M22–M24 (≥45%) was predicted, although smaller scores were obtained for these peptides. In contrast, for peptides M1 and M6 prediction scores of 2.5 and 3.0 were found, respectively, but both showed only moderate competition.

Deamidation in the Presence of Primary Amines—Using CZE, both TG2-catalyzed deamidation and transamidation of F-αI were quantified in the presence of 5-BP as a primary amine (Fig. 1C). Incubation of 10 μM F-αI with increasing amounts of 5-BP for 2 h resulted, as expected, in an increase of the transamidated and a decrease of the deamidated product (Fig. 5A). Notably, at an equimolar ratio between F-αI and 5-BP, deamidation was still superior to transamidation. Both reaction rates became similar at about 50 μM 5-BP. However, significant deamidation of F-αI was measured even at a 20- and 40-fold excess of 5-BP compared with F-αI. The amount of remaining F-αI (about 0.5 μM) was independent of the 5-BP concentration. Increasing the F-αI concentration at a fixed 5-BP concentration resulted in higher amounts of F-αI5BP (Fig. 5B). At equimolar conditions (5, 10, and 200 μM F-αI and 5-BP), 2.0, 4.0, 14.5, and 19.5 μM F-αI5BP was measured showing a decrease in the relative amount of F-αI5BP with increased substrate concentrations (40, 40, 29, and 9.8%). Nevertheless, an excess of 5-BP of 80-fold (for 5 μM F-αI), 40-fold (for 10 μM F-αI), 10-fold (for 50 μM F-αI), or 5-fold (for 200 μM F-αI) still resulted in significant generation of deamidated peptide. However, no deamidation was measured for 50 μM F-αI and 1000 μM 5-BP.

The data obtained from CZE were complemented with functional assays using three TG2-restricted intestinal T cell clones (TCCs) specific for the DQ2-αI5BP-epitope. These TCCs are highly specific for the deamidated peptide; they do not recognize the native α9(57–68) peptide or its transamidated derivative. In control experiments, we found that these TCCs recognized the free (DQ2-αI)5BP and labeled peptides (F-αI5BP) with similar sensitivity (data not shown). As shown for a representative TCC, the T cell proliferation was not significantly different in samples of 10 μM F-αI tested after incubation with TG2 in the presence of no or 10 μM 5-BP (Fig. 6). However, decreased proliferation was observed in the presence of higher concentrations of 5-BP (50 and 400 μM). Even at a 40-fold excess of 5-BP compared with F-αI, proliferation of the three TCCs clearly exceeded that measured for the background (incubation of 10 μM F-αI without TG2).

pH Dependence of TG2-catalyzed Deamidation and Transamidation—TG2 was incubated with F-αI and 5-BP in 100 mM Tris/Cl, 2 mM CaCl2 at various pH values ranging from 7.5 to 5.5. Separation and quantification of the deamidated (F-αI5BP) and transamidated reaction product (F-αI5BPP) by CZE showed that the ratio of the deamidation to transamidation reaction rates is influenced by pH (Fig. 7). Lowering the pH resulted in decreased formation of F-αI5BPP and increased formation of F-αI5BP. Determination of initial transamidation and deamidation reaction rates showed almost a bisection of the transamidation rate when pH was lowered from pH 7.5 to pH 6.0 (Fig. 8A). In parallel, however, the deamidation rate rose 10-fold, reaching nearly the velocity of transamidation. Furthermore, initial transamidation rates were compared with deamidation rates determined in the absence of 5-BP. In this case the deamidation rate stayed constant for both pH values (Fig. 8B).

DISCUSSION

The majority of T cells of the celiac small intestinal lesion recognize deamidated gluten peptides. This study provides details as to how the enzyme TG2 is responsible for this deamidation and demonstrates that the enzyme is directly involved in the selection of gluten T cell epitopes. Moreover, our results reveal that TG2-catalyzed gluten deamidation could possibly occur extracellularly as an excess of primary amines did not...
completely inhibit deamidation at pH 7.3. However, the deamidation is more likely to take place in a slightly acidic environment. Lowering of the pH resulted in a dramatic increase in the ratio of deamidated to transamidated products formed in the presence of primary amines. Deamidation of a glutamine side chain adds a negative charge to the peptide. Capillary electrophoresis, which separates mainly according to charge, functioned well for fast separation and quantification of educt and deamidated and transamidated reaction products. All reaction products of a fluorescein-labeled reporter peptide could be detected with high specificity and sensitivity with laser-induced fluorescence detection also in competition assays. This was especially relevant when heterogeneous competitor samples, like peptic-tryptic digests of gliadin or avenin, were used. Interestingly, the peptic-tryptic digest of avenin was found to be a much poorer substrate for TG2 than the peptic-tryptic digest of gliadin. This is likely related to the different toxicities of the two cereal proteins for celiac patients (23). A similar conclusion was recently reached in a study by Vader et al. (24).

We addressed the question of whether TG2 is involved in the selection of gliadin-derived T cell epitopes. We measured the competition of a set of overlapping γ-gliadin peptides and compared these data with the recognition of the same peptides by T cell lines generated from celiac disease patients. (N.T., these peptides were not tested.)

![Fig. 3](http://www.jbc.org/)

**A**, competition data of 20-mer γ-gliadin peptides overlapping by 10 residues. 10 μM F-αI and 1 μM TG2 were incubated for 18 min at 37 °C with each competitor peptide (110 μM). Samples were analyzed after dilution with electrophoresis buffer by MEKC. Asterisks indicate those peptides that are recognized after TG2 treatment by gluten-specific, intestinal T cell lines generated from celiac disease patients. (N.T., these peptides were not tested.)

**B**, predicted score of 20-mer γ-gliadin peptides as TG2 substrates. Each prediction of a glutamine as a target for transamidation/deamidation resulted in a value of 1 (bold and underlined Q) or 0.5 (underlined Q), and the sum of those values was assigned to each peptide.
intestinal T cell lines generated from CD patients. We also determined the specificity of TG2. The latter was analyzed by means of peptide libraries and mass spectrometry using guinea pig TG2 in a transamidation assay with 5-BP. Transamidated reaction products differ in mass from their educts by 311.2 atomic mass units, compared with 1 atomic mass unit for the deamidation reaction, and were therefore easier to identify when a peptide library was used as an educt. As the acyl donor substrate (glutamine-containing peptide) is bound to the enzyme prior to the primary amine, sequence specificity should be identical in the deamidation and transamidation reactions. The spacing between the targeted glutamine and proline in the C-terminal positions played a dominating role in the specificity of TG2. Our data (i.e. TG2 preferred mostly QxP but not QP or QxxP) are in accordance with a previous report on specificity of guinea pig TG2 where deamidation within synthetic substitution analogs of gliadin peptides was analyzed by tandem mass spectrometry (24). Unlike the previous report, we also found an influence of residues in position 1. The determined sequence specificity of TG2 nicely explains the variation in competition observed between the overlapping γ-gliadin peptides. Although these experiments do not directly prove the deamidation within these peptides, eight peptides were recognized after TG2 treatment by the T cell lines indicating that they indeed undergo TG2-catalyzed deamidation. Notably, these peptides were among those with the highest competition values signifying them as excellent substrates of TG2. From these results it can be concluded that TG2 itself is participating in epitope selection in CD.

At pH 7.3, TG2-mediated deamidation did occur even at an excess of primary amines (5-BP). In the small intestine, TG2 is predominantly expressed extracellularly in the subepithelial region just beneath the basal membrane (7) where the pH is likely around 7.3 and a variety of primary amines competing for the transamidation reaction are present. Our data indicate

### Table I

**Substrate specificity of guinea pig TG2**

| Peptide library | Position | Influence of aa in position X on the transamination of Gln (Q) |
|-----------------|----------|------------------------------------------------------------|
| XSEKSQTPLVT     | -5       | All<sup>a</sup>                                             |
| TXSEKSQTPLVT    | -4       | All<sup>a</sup>                                             |
| TSEKSQTPLVT     | -3       | All<sup>a</sup>                                             |
| TSEKSQTPLVT     | -2       | All<sup>a</sup>                                             |
| TSEKSQTPLVT     | -1       | ILMEHKR                                                   |
| TSEKSQTPLVT     | +1       | RKHYWFILVQASTED                                             |
| TSEKSQTPLVT     | +2       | STNGDEKR                                                  |
| TSEKSQTPLVT     | +3       | MNQADEHKR                                                  |
| TSEKSQTPLVT     | +4       | GP                                                        |
| TSEKSQTPLVT     | +5       | GP                                                        |

<sup>a</sup> All 19 amino acids present in the X position.

**Fig. 4.** Impact of sequence positions relative to the glutamine in determining the specificity of TG2. Given is the variance $\sum x^2 / (n - 1)$ of transamidation rates determined for the 19 amino acid residues present in a randomized position (where $x$ represents the transamidation rates and $n = 19$).

**Fig. 5.** Analysis of TG2-mediated deamidation of F-α1 in the presence of 5-BP as a primary amine using human TG2. Deamidated reaction products after a 2-h incubation at 37 °C were quantified by CZE. A, F-α1 (10 μM) was incubated with different amounts of 5-BP. B, incubation of 5, 10, 50, and 200 μM F-α1 with 5-BP ranging in concentration from 0 to 1 mM.
that DQ2-restricted T cell epitopes can be formed by TG2-catalyzed deamidation in this extracellular compartment in vitro. However, the ratio of deamidated to transamidated products was significantly increased when pH was lowered from 7.3. This suggests that deamidation in the gut is likely to occur in compartments with a slightly acidic pH. Apart from its extracellular expression, TG2 is also expressed in the epithelial cells including the brush border (7). The pH in the proximal small intestine is about pH 6.6 (25), which should allow a predominant deamidation of peptides in the brush border. Another possibility is that TG2 is endocytosed and is active during the initial pH decrease in early endosomes. This could be by endocytosis of surface immunoglobulin by TG2-specific B cells (26) or by endocytosis of TG2 expressed in the surface membranes of macrophages and dendritic cells (27–30). A substantial fraction of the active site of TG2 may be occupied by gluten peptides in the gut (18), and co-internalized free gluten peptides also could be subjected to deamidation.

Our data demonstrate that the rates of the transamidation and deamidation reactions are dramatically changed over a narrow pH range (from pH 6.0 to pH 7.3). The results resemble titration curves of an acid/base pair around a given pKₐ value. To allow the nucleophilic attack on the thiol ester intermediate, the amine must be unprotonated. As the pKₐ of lysine and 5-BP is around 10.5, slight pH changes in the range we observed would not lead to significant alteration in the ratio of their protonated and unprotonated ε-amino group as suggested in an earlier study to explain the decreased transamidation at low pH (31). Rather we propose a general base-catalyzed decylation mechanism for the transamidation reaction (32). A basic amino acid in TG2 (possibly histidine 335 of the catalytic triad) removes a proton from the amine substrate during the rate-limiting decylation step. Notably, the pKₐ of the imidazole group of a histidine in active sites of enzymes is in the range where we observed the pH effect on the transamidation reaction (33). Lowering of the pH below the pKₐ of this base would increase its protonation. Consequently the competing nucleophilic attack by water molecules is favored, explaining the increased deamidation rate. As the concentration of water molecules is pH-independent, deamidation in the absence of primary amines is not influenced by a pH shift.

Our data strongly suppose that TG2 is involved in the selection of epitopes recognized by T cells of the intestinal celiac lesion and indicate that TG2-catalyzed deamidation occurs in a slightly acidic environment. To define exactly where this is in the intestinal mucosa should be the focus of further studies. Detailed insight into the cell biology and biochemistry of this process may lead to the identification of new targets for therapy in CD.

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**Fig. 6.** Proliferation of the DQ2-restricted T cell clone 380 E2 in response to F-αl₅ measured by [³H]thymidine incorporation. F-αl₅ (10 μM) and human TG2 (1 μM) were incubated with 0, 10, 50, and 400 μM 5-BP at 37 °C for 2 h. In the sample without 5-BP, 5.5 μM F-αl₅ was determined. All samples were diluted as indicated on the x axis.

**Fig. 7.** pH dependence of the deamidation and transamidation activity of TG2. 50 μM F-αl and 200 μM 5-BP were incubated for 2 h at 37 °C with 1 μM human TG2 in 100 mM Tris/Cl, 2 mM CaCl₂ at various pH values ranging from 7.5 to 5.5. Separation and quantification of the deamidated (F-αl₅) (●) and transamidated reaction product (F-αl₅-5BP) (○) were achieved by CZE.

**Fig. 8.** Initial reaction rates of the transamidation and deamidation reactions at pH 7.5 and pH 6.0. F-αl (50 μM) and human TG2 (1 μM) were incubated with 5-BP (200 μM, black bars) or without 5-BP (open bars) in 100 mM Tris/Cl, 2 mM CaCl₂ at 37 °C. Formation of deamidated and transamidated reaction products at different time points was quantified by CZE.
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