Epitope-dependent Functional Effects of Celiac Disease Autoantibodies on Transglutaminase 2*

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Transglutaminase 2 (TG2) is a Ca\textsuperscript{2+}-dependent cross-linking enzyme involved in the pathogenesis of CD. We have previously characterized a panel of anti-TG2 mAbs generated from gut plasma cells of celiac patients and identified four epitopes (epitopes 1–4) located in the N-terminal part of TG2. Binding of the mAbs induced allosteric changes in TG2. Thus, we aimed to determine whether these mAbs could influence enzymatic activity through modulation of TG2 susceptibility to oxidative inactivation and Ca\textsuperscript{2+} affinity. All tested epitope 1 mAbs, as well as 679-14-D04, which recognizes a previously uncharacterized epitope, prevented oxidative inactivation and increased Ca\textsuperscript{2+} sensitivity of TG2. We have identified crucial residues for binding of 679-14-D04 located within a Ca\textsuperscript{2+} binding site. Epitope 1 mAbs and 679-14-D04, although recognizing separate epitopes, behaved similarly when assessing their effect on TG2 conformation, suggesting that the shared effects on TG2 function can be explained by induction of the same conformational changes. None of the mAbs targeting other epitopes showed these effects, but epitope 2 mAbs reduced the rate of TG2-catalyzed reactions. Collectively, these effects could be relevant to the pathogenesis of CD. In A20 B cells transduced with TG2-specific B-cell receptor, epitope 2-expressing cells had poorer uptake of TG2-gluten complexes and were less efficient in gluten epitope presentation to T cells than cells expressing an epitope 1 receptor. Thus, the ability of epitope 1-targeting B cells to keep TG2 active and protected from oxidation might explain why generation of epitope 1-targeting plasma cells seems to be favored in celiac patients.

TG2\textsuperscript{2} is a ubiquitously expressed, Ca\textsuperscript{2+}-dependent enzyme, which reacts with glutamine residues in a sequence-specific manner and covalently links the side chain carbonyl group to a primary amine (transamidation) (1). Typically, the amino group is provided by a protein lysine residue, in which case the reaction generates protein-protein cross-links, thought to be important for stabilization of extracellular matrix structures. Alternatively, the enzyme can hydrolyze the target glutamine residue, thereby converting it into a glutamic acid residue (deamidation). TG2 is implicated in the pathogenesis of a wide range of human diseases (2–5). Among these, the role of TG2 in the gluten-induced enteropathy CD is best characterized. By deamidating gluten peptides, TG2 is responsible for generating immunogenic epitopes, which are recognized by disease-causing CD4\textsuperscript{+} T cells in the context of HLA-DQ molecules, in particular the strongly disease-associated HLA-DQ2.5 variant (6). In addition, TG2 is a target for autoantibodies in CD, and it is believed that TG2-specific B cells are activated by gluten-specific CD4\textsuperscript{+} T cells following B-cell uptake and presentation of TG2-gluten complexes (7–11). We have previously generated a panel of human anti-TG2 mAbs from plasma cells of celiac patients (12). These antibodies displayed low mutation levels but were yet highly specific to TG2. They were found to mainly target four conformational epitopes clustered in the N-terminal part of the enzyme, referred to as epitope 1–4 (13). Of these, epitope 1 appears to be a dominant target across patients, which may indicate that B cells recognizing this part of TG2 have an advantage during activation (12, 13). None of the mAbs that were tested blocked TG2 enzymatic activity. In line with this observation, retention of TG2 activity is believed to be crucial for activation of TG2-specific B cells (12, 14). However, it is not yet known when or where these B cells are activated or where TG2-mediated deamidation of gluten occurs, because TG2 activity \textit{in vivo} appears to be tightly regulated (15).

TG2 is produced in the cytosol but can be transported to the extracellular environment (16, 17), where it binds non-covalently to the extracellular matrix. TG2 seems to have more interaction partners in the extracellular matrix, but the high affinity interaction with Fn is best characterized (18, 19). The enzymatic activity requires binding of multiple Ca\textsuperscript{2+} ions (1). TG2 has six Ca\textsuperscript{2+} binding sites (S1–S6), all located within the catalytic core domain of the enzyme. A cooperative binding mode was suggested for the binding of Ca\textsuperscript{2+}, indicating that the initial binding induces allosteric changes that modulate additional binding (20). TG2 is susceptible to oxidative inactivation
both in vitro and in situ in intestinal tissue sections (21–26). TG2 activity in vivo also appears to be tightly regulated (15), suggesting that TG2 in the extracellular environment may be subject to reoxidation regulation. Interestingly, oxidation does not occur in the presence of saturating amounts of Ca\(^{2+}\) (21). Oxidative inactivation is believed to result from disulfide bridge formation between Cys\(^{370}\) and either Cys\(^{230}\) or Cys\(^{371}\) (21). Notably, Cys\(^{230}\) is located within the S1 Ca\(^{2+}\) binding site. Thus, binding of Ca\(^{2+}\) and inactivation by oxidation involve the same parts of TG2 and appear to be mutually exclusive events.

Crystallization studies have shown that TG2 exists in at least two major conformations with different organization of the four structural domains. Binding of GDP, GTP, or ATP causes TG2 to adopt a "closed" conformation in which the two C-terminal domains bend down toward the N-terminal domain and fold over the active site in the catalytic core domain (27–29). This form of TG2 is catalytically inactive and believed to be the primary state intracellularly. In the presence of an active site inhibitor, on the other hand, TG2 was crystallized in an "open" conformation, where the four domains are aligned one after the other with minimal interaction between them, and the active site is exposed (30). The structure of TG2 in the presence of Ca\(^{2+}\) has not been solved. However, hydrogen/deuterium exchange analysis suggested that binding of Ca\(^{2+}\) induces structural changes in the core domain and that these changes are prevented by oxidation of the enzyme (31). In the absence of externally added effectors, recombinant human TG2 displays a heterogeneous conformation with a distribution of molecules between the open and closed conformations. Importantly, binding of anti-TG2 mAbs displayed epitope-dependent induction of conformational changes in TG2, indicating that antibody binding may have hitherto unappreciated functional effects on TG2.

In the present study, we address the effect of antibody binding on TG2 function by assessing oxidative inactivation and the Ca\(^{2+}\) sensitivity of the enzyme. We found that mAbs binding to epitope 1 consistently reduced oxidative inactivation and increased Ca\(^{2+}\) sensitivity of TG2. These effects were also observed for 679-14-D04, which recognizes a previously uncharacterized epitope. Here, we show that 679-14-D04 binds to an epitope distinct from epitope 1 and that the shared effects with epitope 1 mAbs are likely due to induction of similar conformational changes in TG2. Epitope 2 mAbs were found to reduce the rate of TG2-mediated deamidation, an effect that translated into less efficient uptake of TG2-gluten complexes when comparing gluten peptide presentation by epitope 1- and epitope 2-targeting transduced A20 B cells. Thus, the ability of epitope 1-targeting B cells to keep TG2 active and protected from oxidation might explain why generation of epitope 1-targeting plasma cells seems to be favored in celiac patients.

## Results

**Antibodies Mediate Epitope-dependent Protection of TG2 from Oxidative Inactivation**—To study enzymatic activity of TG2, we measured TG2-mediated deamidation of a synthetic gluten peptide by MALDI-TOF mass spectrometry under various conditions (21). Treatment of TG2 with GSSG induced formation of intramolecular disulfide bonds and loss of activity (Fig. 1A). Importantly, the activity could be restored by reduction with DTT as previously observed (21). We next addressed the effect of preincubating TG2 with anti-TG2 mAbs targeting different epitopes (12) prior to incubation with GSSG (Table 1). We observed that mAbs targeting epitope 1 (represented by mAb 679-14-E06 in Fig. 1B) prevented loss of enzymatic activity. This effect was not observed for mAbs targeting epitope 2 or epitope 3 (represented by mAbs 693-1-A03 and 763-4-A04, respectively, in Fig. 1B). Of two mAbs recognizing previously undefined epitopes, one (679-14-D05) was found not to have an effect, whereas the other (679-14-D04) showed an even stronger protective effect on oxidative inactivation than epitope 1 mAbs (Fig. 1C and Table 1). No effect was observed using non-TG2 binding human mAbs or the mouse anti-TG2 mAb CUB7402 (Fig. 1D and Table 1). The protective effect of epitope 1 mAbs and 679-14-D04 was observed over a range of concentrations and was comparable for full-length antibodies and for Fab fragments (Fig. 1, E and F, respectively, and Table 1). The protective effect of these mAbs was also observed by assessing TG2 activity as transamination of peptide substrate measured by CE-LIF (Fig. 2). These data demonstrate that antibodies binding to selected epitopes on TG2, including epitope 1, modulate the susceptibility of the enzyme to oxidative inactivation.

**Binding of TG2 to Fn Does Not Prevent Oxidative Inactivation despite Overlap with Epitope 1**—It has previously been demonstrated that epitope 1 overlaps with the Fn binding site of TG2 (13, 32). To address whether binding to Fn had a similar effect as the epitope 1 mAbs, TG2 was preincubated with either full-length Fn or a 45-kDa fragment of Fn that harbors the TG2 binding site (19, 33). Surprisingly, despite targeting overlapping regions on TG2, preincubation of TG2 with Fn did not protect TG2 from oxidative inactivation as observed for epitope 1 mAbs (Fig. 3 and Table 1). Thus, although epitope 1 overlaps with the Fn binding site, epitope 1 likely includes additional residues that may be crucial for the observed effect (34). Alternatively, the observed effect may derive from allosteric changes in TG2 induced by epitope 1 mAbs but not by binding to Fn.

**Epitope 1 mAbs and 679-14-D04 Increase the Ca\(^{2+}\) Sensitivity of TG2**—Because oxidation and Ca\(^{2+}\) binding appear to have opposing effects on the structure of TG2, we addressed whether the mAbs that prevent TG2 oxidation also affect the Ca\(^{2+}\) requirements for enzymatic activity. We first determined Ca\(^{2+}\) concentrations required for half-activity and full activity of TG2 (Fig. 4A). Measuring gluten peptide deamidation, we observed full activity of TG2 at 0.5 mM Ca\(^{2+}\), which is in line with previously reported values (35). We next determined TG2 activity at subsaturating concentrations of Ca\(^{2+}\) (0.2 mM) in the presence of different mAbs (Fig. 4B). Indeed, binding to epitope 1 mAbs and 679-14-D04 enhanced the activity of TG2 at this Ca\(^{2+}\) level, whereas other mAbs did not show this effect (Fig. 4B). Importantly, mAb binding was not sufficient to induce TG2 activity, as the enzyme still required Ca\(^{2+}\) to be catalytically active (not shown). The antibody-induced activity increase was more pronounced at higher concentrations of peptide substrate (Fig. 4, C and D). Thus, epitope 1 mAbs and 679-14-D04 appear to modify the deamidation rate by increasing the Ca\(^{2+}\) sensitivity of the enzyme.
Functional Properties of Anti-TG2 Autoantibodies

FIGURE 1. Deamidation activity of TG2 after oxidation in absence or presence of mAbs. A, deamidation activity was measured as mass shift of a substrate gluten peptide (maximum shift is 2.0 Da, corresponding to full peptide deamidation) using TG2 that had either been oxidized (Ox), kept in the reduced state (Red.), or reactivated (React.) by first oxidizing and then reducing. B, activity of TG2 oxidized in the absence or presence of anti-TG2 mAbs targeting epitope 1 (e1), 2 (e2), or 3 (e3). C, comparison of the deamidation activity of TG2 after oxidation in the presence of 679-14-D04 and epitope 1 mAbs. D, deamidation activity of TG2 after oxidation in presence of the TG2 negative mAbs 679-14-A04L and 679-14-A04H. E and F, comparison of the TG2 deamidation activity after 90 min after oxidation in the presence of mAbs and their corresponding Fab fragments. For A–D, representative results from one of at least two experiments are shown. E and F were performed once except analysis of reduced and oxidized TG2 that was performed twice where error bars indicate S.D.

Determination of the Binding Interface of TG2 and 679-14-D04 by Site-directed Mutagenesis—We have previously shown that epitope 1 mAbs and 679-14-D04 do not compete for binding to TG2 and that point mutations abrogating binding of epitope 1 mAbs do not influence binding of 679-14-D04 (31, 34). However, we found that epitope 2 and epitope 3 mAbs can compete with 679-14-D04 for binding (Fig. 5A), suggesting that the epitope of 679-14-D04 partly overlaps with epitope 2 and 3. Epitopes 1–4 depend on the N-terminal domain of TG2, because they do not bind the chimeric protein TG3-TG2 where the N-terminal domain of TG2 is replaced by the N-terminal domain of the homologous protein TG3 (36). However, this protein is recognized by 679-14-D04 (Fig. 5B), indicating that its epitope lies predominantly within the catalytic core domain of TG2. To identify residues important for recognition, we mutated selected positively or negatively charged residues in the core domain in proximity to or within Ca^{2+}-binding sites (20). We previously observed that binding of 679-14-D04 depends on TG2 conformation and that binding is stronger if the enzyme is locked in an open conformation or preassociated with Fn. To ensure optimal binding and equal conformation of the TG2 mutants, we therefore immobilized the mutants on Fn prior to incubation with 679-14-D04 and detection of bound antibody by ELISA. Mutation of residues His^{441}, Arg^{458}, and S5 (Arg^{433}–Asp^{438} (20)) and S2B (Glu^{447}–Glu^{454} (20)) did not affect binding, whereas the double mutant D151A/E155A targeting the S4 Ca^{2+}-binding site (Asp^{151}–Glu^{158} (20)) displayed dramatically reduced affinity for 679-14-D04 (Fig. 5C). Thus, the effects on TG2 activity that we observed for 679-14-D04 could derive from antibody mimicking of Ca^{2+} binding (Fig. 5, D and E). Of note, epitope 1 mAbs and 679-14-D04 have similar effects on TG2 function, even though they bind non-overlapping epitopes, and epitope 1 is not located within a Ca^{2+}-binding site. Binding to a Ca^{2+}-binding site is therefore not required to achieve the antibody-induced effects on TG2 function that we observe.

An additional finding from our mutagenesis studies was that the TG2 mutant R458A was no longer recognized by mouse anti-TG2 mAb CUB7402 in ELISA (Fig. 5F). This mAb was described to recognize a linear epitope in the core domain of TG2 consisting of residues 447–478 (37), in particular residues 451–454 as reported by Lai et al. (38). Our results, however, indicate that residue Arg^{458} is important for binding to CUB7402.

Binding of Both Epitope 1 mAbs and 679-14-D04 Induce a Closed TG2 Conformation—By monitoring the distribution of TG2 molecules between open and closed conformations using hydrogen/deuterium exchange mass spectrometry, we have previously reported that anti-TG2 mAbs can alter the confor-
mAbs reduced the initial reaction rate, but they do not prevent TG2-mediated deamidation. TG2 activity is believed to be crucial for activation of TG2-specific B cells in CD, because the active enzyme can form complexes with substrate gluten peptides, thereby allowing BCR-mediated uptake of gluten by TG2-specific B cells. Thus, following uptake of TG2-gluten complexes, TG2-specific B cells can present deamidated gluten peptides to T cells. We therefore tested whether the decreased activity observed upon binding to epitope 2 would affect the ability of epitope 2-targeting B cells to present deamidated gluten peptide to T cells after incubation with TG2 and native peptide. For this purpose, we generated transduced A20 B cells expressing HLA-DQ2.5, together with a BCR targeting epitope 1 or 2, membrane-bound 679-14-D04 or a TG2-negative BCR (Fig. 8A). When TG2 was present in solution, all TG2-reactive cells were able to take up TG2-gluten complexes and present deamidated gluten peptide to T cells (Fig. 8B, left panel). However, when excess TG2 in solution was washed away prior to incubation with gluten peptide, only cells expressing 679-14-D04 or epitope 1 BCR efficiently presented peptide to T cells, suggesting that TG2, which was bound to the epitope 2 BCR, did not readily form complexes with gluten (Fig. 8B, right panel). Based on these observations, it is conceivable that the decreased activity associated with epitope 2 binding can be explained by destabilization of the enzyme-substrate intermediate formed between TG2 and gluten peptide so that such mAbs can form complexes with gluten only in the presence of TG2, thereby activating B cells and possibly leading to an exacerbated immune response.

mAbs on TG2 activity after oxidation in the presence of mAbs, Fab fragments, or fibronectin.

### TABLE 1

| TG2+ | Epitope | Observed deamidation activity |
|------|---------|------------------------------|
|      | mAb 679-14-E06 | 1 + |
|      | mAb 679-14-E06 | 1 + |
|      | mAb 693-10-B06 | 1 + |
|      | mAb 693-10-B06 | 1 + |
|      | mAb 763-4-C06 | 1 + |
|      | mAb 693-1-B06 | 1 + |
|      | mAb 679-14-A05 | 1 + |
|      | mAb 679-1-F06 | 2 |
|      | mAb 693-1-F06 | 2 |
|      | mAb 693-1-A03 | 2 |
|      | mAb 763-4-B06 | 2 |
|      | mAb 693-10-A05 | 2 |
|      | mAb 763-4-A06 | 3 |
|      | mAb 693-1-B03 | 3 |
|      | mAb 693-1-E01 | 3 |
|      | mAb 693-10-A06 | 3 |
|      | mAb 693-1-D03 | 4 |
|      | mAb 679-14-D04 | 4 |
|      | Fab 679-14-D04 | 1 |
|      | Fab 679-14-D05 | 1 |
|      | Fn 45-kDa Fn | |
|      | TG2 neg mAb 693-2-F04 | |
|      | mAb 679-14-E06/H/679-14-A04L | |
|      | mAb 679-14-A04H/679-14-E06L | |
|      | Mouse mAb CUB7402 | |

14-D04 can be explained by the mAbs inducing similar conformational changes in TG2 despite binding to separate epitopes.

**Binding to Epitope 2 Decreases TG2 Deamidation Rate and Impedes TG2-Peptide Complex Presentation to T Cells**—When measuring the activity of unoxidized TG2 in the presence of anti-TG2 mAbs, we found that epitope 2 mAbs consistently reduced the initial rate of gluten peptide deamidation, whereas mAbs recognizing other epitopes did not have an effect (Fig. 7). The strongest effects were observed for the epitope 2 m Abs 763-4-B06 and the mouse mAb CUB7402. These observations are in accordance with previous observations made in our lab where we observed inhibitory effects of 763-4-B06 and CUB7402 in an assay in which TG2 was preincubated with mAb before addition of substrate and Ca$^{2+}$ (12). In the previous analysis, the remaining mAbs had no strong inhibitory effects. However, some antibodies were weakly inhibitory, and all other epitope 2 mAbs were of this category. Thus, it appears that epitope 2 mAbs reduce the initial reaction rate, but they do not prevent TG2-mediated deamidation. TG2 activity is believed to be crucial for activation of TG2-specific B cells in CD, because the active enzyme can form complexes with substrate gluten peptides, thereby allowing BCR-mediated uptake of gluten by TG2-specific B cells. Thus, following uptake of TG2-gluten complexes, TG2-specific B cells can present deamidated gluten peptides to T cells. We therefore tested whether the decreased activity observed upon binding to epitope 2 would affect the ability of epitope 2-targeting B cells to present deamidated gluten peptide to T cells after incubation with TG2 and native peptide. For this purpose, we generated transduced A20 B cells expressing HLA-DQ2.5, together with a BCR targeting epitope 1 or 2, membrane-bound 679-14-D04 or a TG2-negative BCR (Fig. 8A). When TG2 was present in solution, all TG2-reactive cells were able to take up TG2-gluten complexes and present deamidated gluten peptide to T cells (Fig. 8B, left panel). However, when excess TG2 in solution was washed away prior to incubation with gluten peptide, only cells expressing 679-14-D04 or epitope 1 BCR efficiently presented peptide to T cells, suggesting that TG2, which was bound to the epitope 2 BCR, did not readily form complexes with gluten (Fig. 8B, right panel). Based on these observations, it is conceivable that the decreased activity associated with epitope 2 binding can be explained by destabilization of the enzyme-substrate intermediate formed between TG2 and gluten peptide so that such
complexes cannot be taken up efficiently by TG2-specific B cells.

Although both 679-14-D04 and epitope 1 BCR allowed bound TG2 to react with gluten, only the latter was efficiently cross-linked to a fluorescently labeled gluten peptide through TG2-mediated transamination (Fig. 8C). This finding is in agreement with previous observations showing that epitope 1 mAbs, but not 679-14-D04, are efficiently cross-linked by TG2,
because binding via epitope 1 allows TG2 to act on the same Ig molecule to which the enzyme is bound (14). TG2-mediated cross-linking of gluten peptides to the BCR could be an alternative pathway for uptake of gluten by TG2-reactive B cells. Such a mechanism would thus give epitope 1 B cells an advantage over B cells targeting other TG2 epitopes, including the epitope of 679-14-D04.

**Discussion**

This work illustrates that activity and structure of TG2 are influenced by interaction with antibodies. Specifically, we demonstrate that celiac anti-TG2 mAbs targeting epitope 1 modulate TG2 activity both by preventing susceptibility to oxidative inactivation and by increasing Ca$^{2+}$ sensitivity of the enzyme. These two effects are likely interrelated, because Ca$^{2+}$ binding and oxidative inhibition appear to be opposing events. Thus, Ca$^{2+}$-induced conformational changes observed in unoxidized TG2 did not occur when the oxidized enzyme was incubated with Ca$^{2+}$, indicating that generation of disulfide bridges in the core domain interferes either with binding of Ca$^{2+}$ or Ca$^{2+}$-induced structural changes required for enzymatic activity (31). In agreement with this finding, oxidation of Cys230, Cys370, and Cys371 did not occur in the presence of saturating concentrations of Ca$^{2+}$ (21).

The effects on TG2 function observed for epitope 1 mAbs were also seen for one other mAb in our panel, 679-14-D04. This mAb targets an epitope that is distinct from epitope 1, and it showed even stronger effects on TG2 function than epitope 1 mAbs. Here, we have mapped the epitope of 679-14-D04 to the catalytic core domain of TG2 and found that it coincides with a Ca$^{2+}$ binding site. Despite targeting separate epitopes, we show that epitope 1 mAbs and 679-14-D04 induce similar conformational changes in TG2, which likely explains their shared effects on TG2 function. Epitope 2 mAbs, on the other hand, had a clearly different effect on TG2 conformation and were found to reduce the initial rate of TG2-mediated deamidation.

The role of antibodies in the development of CD is still unclear, and caution should be exercised in interpreting our observations in relation to CD pathogenesis. Notwithstanding, we would like to discuss some potentially important aspects. The differential effects of antibodies targeting different epitopes may explain why studies using polyclonal anti-TG2 sera from CD patients show conflicting and inconclusive results regarding the effects of antibodies on TG2 catalytic function (37, 39–50). Although our observations indicate that celiac anti-TG2 antibodies may modulate extracellular TG2 activity, our incomplete understanding of TG2 biology and activity in the extracellular matrix makes it difficult to properly assess this. In contrast, such effects are likely more important at the B-cell level. The different effects of epitope 1 and epitope 2 mAbs on TG2 conformation and activity translated into less efficient internalization and presentation of gluten peptides by A20 B cells transduced with an epitope 2 anti-TG2 BCR compared with cells expressing an epitope 1 or 679-14-D04 anti-TG2 BCR. Thus, B cells with a BCR that supports or retains TG2 activity through favorable conformational changes will receive more T cell help from gluten-specific T cells. Our findings may
therefore explain why epitope 1 antibodies appear to dominate the anti-TG2 response in CD patients.

The fact that antibodies targeting the same epitope as 679-14-D04 have not previously been described in CD is surprising, because B cells with this specificity presumably also should have an advantage in competition for T-cell help. One explanation for why only epitope 1 seems to be favored could be that epitope 1-targeting B cells acquire an additional advantage through binding of TG2 in an orientation that allows enzymatic cross-linking of gluten peptides to the BCR and, hence, more efficient gluten presentation as recently proposed (14). Indeed, we found that TG2 cross-linked gluten peptide more efficiently to epitope 1 BCR than epitope 2 BCR or membrane-bound 679-14-D04. Alternatively, epitope 1-targeting antibodies are more prominent because they are made up of stereotypic combinations of variable region gene segments with inherent TG2 reactivity. Thus, it was shown that most epitope 1 antibodies contain heavy chains using the IGHV5-51 gene segment in combination with certain light chain IGKV segments (13) and that these antibodies are commonly generated in CD patients (51). Precursor B cells targeting epitope 1 could therefore be more frequent than B cells targeting the epitope of 679-14-D04. It is also possible that B cells expressing certain TG2 epitopes are more likely to undergo negative selection because of self-reactivity than other TG2-reactive B cells and therefore require stronger stimulation to differentiate into plasma cells.

In summary, we have found that celiac antibodies targeting the epitope 1 of 679-14-D04 can affect both the deamidation and transamidation activity of TG2 by protecting the enzyme from oxidative inactivation and by increasing its Ca²⁺ sensitivity. These effects may have implications for the activation TG2-reactive B cells and help explain why certain epitope 1 antibodies are more likely to undergo negative selection and require stronger stimulation to differentiate into plasma cells. In addition, we have found that cell antibodies targeting certain epitopes can affect both the deamidation and transamidation activity of TG2 by protecting the enzyme from oxidative inactivation and by increasing its Ca²⁺ sensitivity. These effects may have implications for the activation TG2-reactive B cells and help explain why certain epitope 1 antibodies are more likely to undergo negative selection and require stronger stimulation to differentiate into plasma cells. Therefore, the mechanisms described here for CD might apply to other autoimmune conditions, and antibody or BCR-mediated modulation of the catalytic function of a target enzyme might be a key step in establishing an autoantibody response.
Experimental Procedures

Production and Purification of Anti-TG2 Autoantibodies and Fab Fragments—Cloning, expression, and purification of anti-TG2 mAbs was done as described previously (34). Unless otherwise indicated, the used mAbs were expressed as full-length human IgG1 molecules. Fab fragments were cloned with a similar procedure as previously described for Fab 679-14-E06 (34) by adding a stop codon after hinge residue 6 (PKSCD6, according to the ImMunoGeneTics numbering scheme) and purified using a protein L column (GE Healthcare) applying the previously described protocol for antibody purification. Selected mAbs representing epitope 1, 2, or 3 were engineered as human IgA1 by overlap extension PCR using the IgA1 version of mAb 679-14-E06 (12) as a constant region template, followed by subcloning into the expression vector between the Agel and HindIII restriction sites. IgA1 mAbs were purified on protein L.

TG2 Variants—Recombinant human TG2 produced in insect cells (obtained from Phadia) was used for all activity assays and in the hydrogen/deuterium exchange experiments. For comparison of WT and mutant TG2 by ELISA, recombinant enzyme produced in Escherichia coli was used (21, 31). Mutations were introduced into TG2 with the QuikChange site-directed mutagenesis kit (Stratagene) and produced and purified as previously described (34). Chimeric TG3/TG2 comprising the N-terminal domain of TG3 (residues 1–136) fused to the core and C-terminal domains of TG2 (residues 141–687) was generated by overlap extension PCR followed by subcloning into the pET-28a vector (Novagen) between the NdeI and HindIII restriction sites. Biotinylated TG2 used for flow cytometry was generated from E. coli-produced TG2 harboring a site-specific biotinylation BirA tag between the His tag and the TG2 sequence. The BirA tag was introduced by PCR amplification followed by subcloning into the pET-28a vector between the Ncol and HindIII restriction sites, and biotinylation of purified TG2 was carried out using the BirA biotin-protein ligase (Avidity) according to the instructions from the manufacturer.

Oxidation of TG2—To address the possible effect of anti-TG2 mAbs on oxidation of TG2, 24.6 pmol of TG2 was incubated with or without 60 pmol of mAb, 120 pmol of Fab fragment, or 30 pmol of Fn for 20 min at room temperature in activity buffer (100 mM Tris·HCl, pH 7.4) and then oxidized for 1 h (for mass spectrometry measurements) or 30 min (for capillary electrophoresis measurements) at 37 °C in the presence of 4 mM GSSG. Control samples were incubated for 1 h at 37 °C in the presence of 2 mM DTT. Reactivated samples were first oxidized as described above before incubation with 10 mM DTT for 30 min at room temperature.

Determination of Enzymatic Activity by MALDI-TOF Mass Spectrometry—TG2 (0.037 μg/ml) treated as described above was incubated at 37 °C with 12 μM of a synthetic gluten peptide (PQPQLPYPQPQLPY, where the underlined residues denote glutamine residues targeted by TG2, obtained from GL Biochem) in the presence of 10 mM CaCl2. Alternatively, TG2 was incubated with 30 μM of the peptide and 10 mM CaCl2 when addressing the initial rate of peptide deamidation in the presence of mAbs. At the indicated time points, 2–2.5 μl of the reaction mixture was quenched with an equal volume of 0.2% (v/v) TFA, and the quenched samples were mixed 1:1 (v/v) with 5 mg/ml α-CHCN matrix. Measurements were performed on an Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics). For each sample, 8 measurements of 200 shots were combined before the calculated centroid mass of a non-deamidated peptide control was subtracted from the determined centroid mass of the samples to obtain the mass shift introduced by deamidation. To address the effect of epitope 2 mAbs on TG2 deamidation activity without prior oxidation or reduction, the enzyme was preincubated with mAb as described above, followed by incubation at 0.037 μg/ml with 24 μM peptide and measurement of deamidation.

Determination of Enzymatic Activity by CE-LIF—CE-LIF measurements were performed by capillary zone electrophoresis (Agilent Capillary Electrophoresis System), which was coupled to an external laser-induced fluorescence detector (488 nm; Zetalif Evolution, Picosystems) using a modified method previously described by Stamnaes et al. (21). Native and modified (deamidated and transamidated) peptides were separated in a capillary of fused silica (75-μm inner diameter, ~50-cm total length, 0.5-cm window for laser induction after ~35 cm) after washing of the capillary with 1 M NaOH (15 min) and H2O (15 min) and equilibrating with running buffer (60 mM borate, 20 mM SDS, pH 9.3) (30 min). TG2-mediated transamidation of a FITC-labeled gluten peptide (FITC-Acp-PQPELPYPQQQLPY, where the underlined residue denotes the glutamine residue targeted by TG2, obtained from GL Biochem) and the amine donor 5-(biotinamido)pentylamine was assessed at the indicated time points by measuring the area under the peaks of the resulting electrophorograms. The peptide was incubated at 40 μM with or without (deamidation control) 400 μM 5-(biotinamido)pentylamine in the presence of 0.037 μg/ml TG2 and 10 mM CaCl2 in activity buffer at 37 °C. At each time point, 2.5 μl of the reaction mixture was taken out and kept at ~20 °C until measurement. The samples were diluted in running buffer to give a total volume of 100 μl and injected by 50 mbar pressure for 2 s. Measurements were performed at 25 °C with 20 kV and 100 μA using an electro-osmotic flow from cathode to anode. Ca2+ Sensitivity of TG2 in the Presence of mAbs—Possible effects of anti-TG2 mAbs on TG2 Ca2+ sensitivity were assessed using the peptide deamidation assay described above. TG2 (24.6 pmol) was preincubated with or without mAb (60 pmol) for 20 min at room temperature in activity buffer before the enzyme was incubated at 0.037 μg/ml with 12 μM of peptide in the presence of 0.2 mM CaCl2 at 37 °C. The optimal Ca2+ concentration used in the assay was initially determined by titrating the CaCl2 concentration.

ELISAs—For comparison of mAb binding to WT and single or double TG2 mutants, TG2 (3 μg/ml) was attached to a coated 45-kDa Fn (5 μg/ml) obtained from Sigma, whereas binding to chimeric TG3/TG2 was assessed after direct coating of the inhibitor-bound enzyme (3 μg/ml). Irreversible binding to inhibitor was done to make the enzyme obtain an open conformation. To achieve this, TG2 was incubated with 0.5 mM of the inhibitor Ac-P(DON)LFP-NH2 (where DON is the electrophilic amino acid 6-diazo-5-oxo-I-norleucine, obtained from Zedira) for 20 min at room temperature, followed by addition of CaCl2 to a final concentration of 5 mM and incubation for
another 20 min. Coating was carried out overnight at 4 °C in TBS, whereas TBS containing 0.1% Tween 20 was used for washing and incubation steps. Incubations were done at 37 °C. Binding to TG2 captured on the 45-kDa Fn was assayed using mAbs biotinylated with EZ-link sulfo-NHS-LC-biotin (Thermo Scientific) according to the manufacturer’s instructions, followed by detection with alkaline phosphatase-conjugated streptavidin (Southern Biotech). When TG2 was coated directly, bound mAb was detected using alkaline phosphatase-conjugated goat anti-human IgG (Southern Biotech). Competitive ELISA experiments were carried out by incubating coated inhibitor-bound TG2 (produced in insect cells) with anti-TG2 IgA1 mAbs targeting different epitopes for 30 min at 37 °C. Without removing the IgA1 mAbs, 679-14-D04 expressed as IgG1 was added to a final concentration of 0.03 μg/ml, and incubation was continued for 1 h. Detection of bound 679-14-D04 was carried out as described above.

T-Cell/B-cell Collaboration Assay—T-cell activation measured by release of IL-2 was used to assess B-cell uptake and presentation of TG2-gluten complexes. Murine A20 B lymphoma cells expressing HLA-DQ2.5 and 679-14-D04 IgD BCR were generated by retroviral transduction as previously described for the other BCRs using codon-optimized synthetic DNA (GenScript) (12, 14) A20 cells expressing HLA-DQ2.5 and TG2-reactive (679-14-E06, 693-1-F06, or 679-14-D04) or TG2-negative (693-2-F02) IgD BCR (14) were incubated at 10 million cells/ml with a combination of TG2 (1.2 μg/ml), CaCl₂ (2 mM), and various concentrations of a 33-mer gluten peptide (LQLQPFQPQLYPQYPQPQLYPQPQPFQPFQPFQF, where underlined residues denote glutenine residues targeted by TG2, obtained from GL Biochem) in RPMI for 1 h at 37 °C. Alternatively, the cells were preincubated with TG2 in PBS for 30 min on ice and washed with PBS to remove non-BCR-bound TG2. Washed cells were resuspended in RPMI and supplemented with CaCl₂ and peptide prior to incubation at 37 °C as described above. The cells were then washed with RPMI to remove free peptide, resuspended at 1 million cells/ml in 5% (v/v) FCS/RPMI, and incubated for 3 h at 37 °C. 25,000 hybridoma T cells specific to the deamidated DQ2.5-gliadin-α2 epitope were then added to 100,000 A20 cells, and the cells were cultured together at 37 °C. The next day, culture supernatants were collected and assayed for murine IL-2 secretion by ELISA—K. H., J. S., M. F. d. P., S. M., and R. I., designed the study and wrote the paper. K. H., J. S., M. F. d. P., S. M., and R. I. performed the experiments. All authors analyzed the results and approved the final version of the manuscript.

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