A single conformational transglutaminase 2 epitope contributed by three domains is critical for celiac antibody binding and effects

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ABSTRACT

The multifunctional, protein cross-linking transglutaminase 2 (TG2) is the main autoantigen in celiac disease, an autoimmune disorder with defined etiology. Glutamine-rich gliadin peptides from ingested cereals, after their deamidation by TG2, induce T-lymphocyte activation accompanied by autoantibody production against TG2 in 1-2% of the population. The pathogenic role and exact binding properties of these antibodies to TG2 are still unclear. Here we show that antibodies from different celiac patients target the same conformational TG2 epitope formed by spatially close amino acids of adjacent domains. Glu153 and 154 on the first alpha-helix of the core domain and Arg19 on first alpha-helix of the N-terminal domain determine the celiac epitope which is accessible both in the closed and open conformation of TG2 and dependent on the relative position of these helices. Met659 on the C-terminal domain also can cooperate in antibody binding. This composite epitope is disease-specific, recognized by antibodies derived from celiac tissues and associated with biological effects when passively transferred from celiac mothers into their newborns. These findings suggest that celiac antibodies are produced in a surface-specific way for which certain homology of the central glutamic acid residues of the TG2 epitope with deamidated gliadin peptides could be a structural basis. Monoclonal mouse antibodies with partially overlapping epitope specificity released celiac antibodies from patient tissues and antagonized their harmful effects in cell culture experiments. Such antibodies or similar specific competitors will be useful in further functional studies and in exploring whether interference with celiac antibody actions leads to therapeutic benefits.

Abbreviations: aa, amino acids, EMA, endomysial antibodies, HUVEC, human umbilical cord vein endothelial cells, mAb, monoclonal mouse antibody, ScFv, single-chain variable fragments, TG2, transglutaminase type-2

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INTRODUCTION

Transglutaminase 2 (TG2, 'tissue' transglutaminase, EC 2.3.2.13) is an ubiquitous cellular protein also present in the extracellular matrix where it catalyzes Ca^{2+}-dependent protein cross-linking via N'(γ-glutamyl)lysine bonds or the deamidation of glutamine residues. In addition, TG2 also has pleitropic intracellular functions as GTP-ase, protein disulphide isomerase, serine/threonine kinase and acts as adaptor on the cell surface for fibronectin, integrins, syndecan 4 and other matrix proteins regulating cell adhesion, differentiation and survival (1). TG's complex structure and high sensitivity to ligand-induced conformational changes (2) makes challenging to dissect the structural basis for these interactions, and only the fibronectin binding site on the loop with amino acids (aa) 94 and 97 (3) is known in details. Contradictory data exist also on the epitope recognized by TG2-specific antibodies produced in celiac disease (celiac sprue), an autoimmune condition resulting from intolerance to gluten-containing cereals (wheat, rye, barley) that induce after their TG2-mediated deamidation specific T lymphocyte activation, intestinal inflammation and villous atrophy in genetic susceptible individuals carrying HLA-DQ2 or DQ8 (4).

Celiac antibodies exert biological effects via TG2, partly by gain in catalytic activity, on epithelial cell differentiation (5) and transport (6), angiogenesis (7), vascular permeability (8), monocyte activation (9), cell cycle progression (10) and apoptosis (11) in cell culture experiments, and presumably also *in vivo*, but direct evidence for the latter has not been yet provided. Animal models remained incomplete (12) and IgA anti-TG2 antibodies similar to those in patients' serum (13) and bound in different tissues in a greatly specific pattern along vessels and TG2-rich endomysium (14) were not produced. A gluten-free diet normalizes the gut lesions and eliminates TG2 antibodies from both serum and tissues. Such a life-long diet is difficult to maintain and therefore exploration of alternative treatment modalities is in progress.

The monomeric human TG2 consists of four structural domains (N-terminal β–sandwich, core, and two C-terminal β–barrels) (1). In the presence of GDP, TG2 shows a closed conformation with the transamidating catalytic triad on the core domain in a hidden position (PDB code: 1KV3) (15). When TG2 is functioning as a Ca^{2+}-dependent transglutaminase, C-terminal beta-barrels are displaced by 120 Å and the structure becomes open and extended (2) (PDB code: 2Q3Z), at least transiently. In earlier studies celiac anti-TG2 antibodies bound to multiple (often complementary) fragments of human TG2 (16,17,18), but to none of linear TG2 peptides expressed in phage display (19). In another study, mutagenesis of the normally buried catalytic triad decreased antibody binding (20). Celiac antibodies do not bind well in Western blot (13) or in paraffin-embedded tissues indicating conformation dependency of antibody recognition. Here we describe a single conformational TG2 epitope important for all investigated celiac patient samples and potential target for immunotherapy. This celiac epitope involves the first alpha-helix of the N-terminal domain, the first alpha-helix of the core domain and, additionally, the C-terminal domain.

RESULTS

Binding of celiac antibodies is related to a calcium binding site on the core domain of TG2 but does not require calcium ions

We recently identified two adjacent novel Ca^{2+} binding sites on the core domain of TG2, S4 (aa 151-158) and S5 (aa 433-438), of which S4 strongly determines antigenicity for celiac antibodies (21). As these results were generated with multiple mutations of acidic glutamate and aspartate residues in these regions, we wished to precisely identify the anchor residues that form a celiac epitope. We prepared now TG2 molecules bearing D151N, E153Q, E154Q, E155Q, E158Q, E158L and R433S-E435S mutations separately. From these, only changes at residue 158 abolished the binding of celiac antibodies (Fig.1), but 158 is not surface-exposed and its mutations were found to have only indirect effect on the position of the helix formed by the S4 site amino acids. Lack of binding to TG2 denatured with urea (Fig. S1A) indeed confirmed that celiac autoantibodies do not bind to a linear epitope. A mutation on the surface in Glu153 had significant ($p<0.0001$) but only moderate effect, so Glu153 could be one potential anchor point for the binding of celiac antibodies, which, however, might need cooperation with other surface structures to form an epitope. Ca^{2+} ions themselves did not contribute to the formation of the celiac epitope as shown in supporting information (SI) text and Fig.S1B.

Analysis of further binding sites outside the core domain

We next used TG2 mutants each lacking one structural domain of TG2 (22) to locate celiac antibody binding sites further to S4. Previous studies (2,3) indicate that the N-terminal domain (I) expressed alone, and also domains III and IV, will adopt a functional conformation independently of the core (II) domain. When the domain mutants were applied in equimolar concentrations in immunoassays, both domain I and II turned out to be important for autoantibody binding (Fig.1), and antibody recognition was also influenced by the loss of domain IV (aa 585-687) but not at all

by the absence of domain III (aa 472-584). These results suggested, in accordance with previous data (16-18), that parts of celiac epitopes are found both on the N-terminal and C-terminal domains. Importantly, patient antibodies, however, did not bind to the mutant containing only domains I-III-IV but not domain II, so in the absence of the anchor points on the core domain the other celiac epitope parts were not capable to form a functional binding site.

Three anchor points determine a composite epitope formed by three distinct domains and two of these are sufficient for binding of celiac antibodies

Molecular modelling was used to evaluate whether cooperation of core domain Glu153 with other amino acids on the N-terminal and/or C-terminal domains might form functional epitopes. In the resting (closed) conformation of TG2 shown by X-ray crystallography (1KV3) (15), the N- and C-terminal parts of the molecule are close to each other and to the surface of the core domain. Core domain Glu153 is in the closest proximity to N-terminal domain Arg19 (12.9 Å) and C-terminal domain Met659 (16.8 Å), and moreover, Arg19 is also close to Met659 (7.7 Å) forming possibly a common conformational epitope (Fig.2). Each single mutation of these amino acids to serine (R, R19S; E, E153S; M, M659S) resulted in significant decrease in celiac antibody binding (26.5%, 28.8% and 56.9% remaining binding for R, E and M, respectively; Fig.3A) and combined mutations caused proportionally greater changes (6.6%, 21.7%, 14.6% and 13.4% remaining binding for RE, EM, RM and REM, respectively). The bindings of autoantibodies from consecutively diagnosed 58 childhood (Fig.3A) and 18 adult celiac patients (Fig.3B) as well as of all 8 tested single chain monoclonal antibody clones (ScFv) (23) originating from celiac patients (Fig.S3A) were abolished when mutants RE and REM were applied as antigens. Despite of the decreased binding of celiac antibodies, these mutant TG2 proteins bound normally a large set (22) of mouse monoclonal anti-TG2 antibodies, showed appropriately folded structures in CD spectra and functionality in fibronectin binding, transglutaminase and GTPase assays (Fig.S2A-D).

Further experiments with Factor XIII homologous mutants targeting Glu154 (SI Text and Fig.S3B,C) supported that celiac antibodies directly bind to the TG2 surface delineated by Arg19-Glu153-Met659. Anchor points homologous to Arg19, Glu153 and Glu154 were found by library search in those animal TG2 sequences and in human TG6 which are good celiac antigens (SI Table1), while blood coagulation Factor XIII, a related transglutaminase not antigenic in celiac disease (24), contains a positively charged Lys199 at position corresponding to Glu154.

To establish the relative importance of Arg19 and Met659 in celiac antibody binding, we studied fibronectin-bound TG2 (25) as a model of accessibility of TG2 epitopes in the extracellular matrix where antibodies primarily encounter the autoantigen in patients. Under these conditions, the mutations of Arg19 and Glu153 had similar effects as seen above (Fig.S3D), but mutation of Met659 alone did not alter celiac antibody binding and so celiac antibodies may bind to TG2 also in its catalytically active form when TG2 adopts an open-extended conformation where domain IV with Met659 swings out (2). Measurements on the position of Arg19 and Glu153 in this open form crystal structure (2Q3Z) did not show a difference in distance as compared to the closed conformation (1KV3)(Fig.S3E).

Antibodies of different celiac patients recognize parts of the same epitope

Although all celiac patient serum samples displayed a severely reduced binding to REM triple and RE double mutants, their reaction showed some variability with the point mutant where only the N-terminal anchor point (Arg19) had been changed (Fig.3). Celiac patient-derived monoclonal ScFvs expressed from phage libraries (23) belonged to two groups as well, one reacting with R19S and one not (Fig.S4A). However, ScFvs belonging to either group showed greatly reduced binding to Glu153 (E153S) or Glu154 (E154K) mutants and competed with each other for the binding to wild-type TG2 (SI Text and Fig.S4B). IgA and IgG antibodies differently recognizing R19S were purified from sera of celiac patients (25), and these competed as well with each other but not with

non-celiac human IgG (Fig.S4C). Further, we were able to develop a diagnostic ELISA with 98% sensitivity and 96% specificity for measuring IgG class celiac antibodies in subjects with selective humoral IgA deficiency by their concentration-dependent displacement effect (r=0.88) on a known celiac IgA tracer antibody (Fig.S4D,E).

Disease specificity of the composite TG2 epitope

During the development of celiac disease, both early cases during the latent stage when villous atrophy was not yet present and at diagnosing villous atrophy showed low reactivity to Glu153 and Arg19 mutants without statistical difference (Fig.4). Interestingly, no or only limited epitope spreading occurred during a follow-up for up to 17 years without proper diet or upon later diagnostic gluten challenge (Fig.S5). The serum samples from patients with other autoimmune diseases (SLE, Sjögren's syndrome, rheumatoid arthritis, See SI Text for details) containing non-celiac anti-TG2 antibodies but negative for anti-endomysial or anti-deamidated gliadin antibodies showed a clearly different binding pattern to the celiac epitope (Fig.4). Monoclonal TG2-specific mouse antibodies (mAb) 885 (Phadia, Uppsala, Sweden) which had previously been found to target Glu153 (Fig.S7) selectively interfered with the binding of celiac antibodies to wild-type TG2, but they did not compete with the binding of non-celiac TG2 antibodies (Fig.5). These data collectively suggest that celiac disease results in a particular and directed immune response towards TG2.

The composite TG2 epitope mediates *in vivo* tissue-binding of celiac antibodies and their biological effects

In order to explore whether the identified composite epitope is also important under *in vivo* conditions, we performed binding studies with antibodies isolated from celiac tissues (Fig.6). An extraintestinal tissue was chosen for this experiment to avoid contamination with non-specific IgA

present in gut plasma cells. Placenta specimens were available from two celiac mothers and both contained high amounts of TG2-bound maternal IgA antibodies in the wall of decidual blood vessels and on the surface of the chorionic villous structures (Fig.S6). This IgA was eluted with chloroacetic acid (14) and showed epitope targeting pattern identical to the typical one observed with celiac serum samples, as described above (Fig.6A). Further, frozen placenta sections were subjected to competition studies with mAb 885 and control mAbs. After incubation with excess amounts of mAb 885 but not with buffer only or with isotype control mouse antibodies (Dako), *in vivo* tissue-bound anti-TG2 IgA antibodies completely disappeared from the tissue as shown by immunostainings and were released into the buffer (Fig.6B). After incubation of celiac tissues with CUB7402 anti-TG2 mAb targeting a different TG2 epitope, celiac IgA remained unchanged and did not appear in the buffer (Fig.6B).

Since there is an altered vasculature in the celiac small bowel mucosa and celiac antibodies deposit around vessels, a defective angiogenesis was suggested to contribute to the architectural changes eventually leading to mucosal flattening (7). Therefore, we investigated the differentiation of commercial human umbilical cord vein endothelial cells (HUVEC) in matrigel as a standard angiogenesis assay (26). In this assay, purified IgA from autoimmune patients with non-celiac TG2 antibodies caused only slight non-specific descrease in endothelial tubule formation similarly to control IgA from antibody-negative healthy persons, but celiac IgA significantly decreased tubule length (Fig.6C) and formation (Fig.S7C) and this effect was prevented in the presence of mAb 885. Similar effect was observed on cell lengths when mAb 885 was co-administered with purified monospecific celiac ScFvs to HUVEC cells grown on collagen I (Fig. S7D).

Maternal celiac antibodies transferred into newborns have biologic effects

In parallel of antibody deposition in the placenta (Fig.6B), maternal IgG anti-TG2 antibodies were detected also in the umbilical cords and sera of the newborns, and on the surface of HUVECs

isolated from the umbilical cord (Fig.6E). These antibodies had the same epitope specificity as antibodies of their mothers (Fig.6A), but as expected, IgA autoantibodies did not cross the placenta. HUVEC cells exposed to maternal antibodies before birth displayed abnormalities in their shape and spreading, and lived for shorter time in culture compared to cells isolated from babies with antibody-negative celiac mothers (Fig.6E and S6). These cellular changes were similar to those we observed earlier when celiac IgA was added to normal HUVEC cultures (7).

DISCUSSION

The results presented here show a particular uniformity of gluten-driven autoantibody production in celiac disease towards one main conformational celiac TG2 epitope, characteristic for both serum antibodies and tissue-derived monoclonal antibodies. In contrast, TG2 antibodies from subjects with other autoimmune diseases prefer other binding sites. These findings make possible to design interfering compounds for further research and with potential to explore therapeutic use.

The main anchors points of this celiac epitope are Glu153 and Glu154 on the edge of the first alpha helix of the core domain of TG2, but they also need one more anchor point either on the N-terminal or C-terminal domains. The N-terminal anchor point is formed by the first helix containing Arg19. Arginins often form part of epitopes, and cooperation of Glu153 with Arg19 is predicted to be an energetically favorable binding site resulting in a large change in solvent-accessible surface area after antibody binding (27). The position of these two anchor points does not change during the opening of the enzyme upon its activation, so this epitope is accessible in tissues indepedently of the Ca^{2+}-driven conformational shift, especially when TG2 is bound to fibronectin. Celiac antibodies bind well to both the Ca^{2+}-bound and Ca^{2+}-free TG2 (Fig.S1B) and to open-form TG2 (8) and TG2 transamidates substrates even in the presence of bound celiac antibodies (28). These observations strongly contradict the earlier suggestion (20) that the catalytic triad, situated buried and exposed on the surface just when Ca^{2+} and substrates are available, would compose the celiac epitope.

As an alternative to Arg19 from the N-terminal domain, Glu153 also may cooperate with Met659 on the C-terminal domain, explaining earlier epitope mapping results with some C-terminal TG2 fragments (16,17). Accordingly, Arg19 was not indispensable for the binding of serum antibodies of all our celiac patients, nor for certain patient-derived phage antibodies provided the C-terminal domains were present. The competition between different ScFv-groups or natural IgA and IgG patient antibodies recognizing Arg19 differently and effective displacement

of diverse patient antibodies by mAb 885 targeting only Glu153 demonstrates that Glu153, Arg19 and Met659 constitute one composite epitope when the protein is in the closed conformation. Binding of celiac antibodies to a surface area close to the interface of adjacent domains may be the structural basis for certain conformation stabilizing effect responsible for the earlier observed gain in catalytic activity in the presence of celiac antibodies (8,25). The variable involvement of N-terminal and C-terminal domains explains why earlier studies typically found a sufficient binding if either the N-terminal or C-terminal TG2 parts were missing, but not if both were missing or if the core domain had been disrupted (16-18). We applied a special precaution to avoid the disruption of hydrogen bonds or the distorsion of the conformation, and the folded structure of our key mutants were confirmed by CD spectra. The results with our domain deletion mutant I-III-IV indeed confirm the essential role of the core domain, whereas individual variations in the extent of the actually targeted surface could explain some heterogeneity in binding to fragments with parts of the 3-dimensional epitope on several of them.

The present identification of a celiac disease-specific epitope, already characteristic in the early (latent) stage, has also diagnostic importance. A test that combines measurement of antibody binding to wild-type TG2 and to one with modified celiac epitope could distinguish in future between celiac-type and nonspecific anti-TG2 antibodies seen in other autoimmune diseases, tumors or tissue injury (29).

Our findings also show that gluten-derived gliadin peptides drive the celiac autoimmune response in an ordered and surface-specific way and epitope spreading is not common despite long-term disease. The primary antibody response in infants targets deamidated gliadin peptides (30) in which certain glutamines have been changed to glutamic acids. Some of these antibodies cross-reacted also with TG2 in an earlier study and TG2-specific mAbs recognized a complex of 3-dimensionally shaped deamidated gliadin peptides indicating molecular mimicry (22). Interestingly, the distance and spatial arrangement of the side-chains of Glu153 and Glu154 central

in the here described TG2 epitope are similar to the side-chains of Gln and Glu residues of the most typical immunogenic gliadin peptide (PQPELPY) docked into HLA-DQ2 (Fig.S8 and SI text). Further studies may confirm experimentally the pathologic relevance of this observation.

In the complex immunopathology of celiac disease where activated gliadin-specific T lymphocytes are held responsible for tissue damage (4), the role of anti-TG2 antibodies is still undefined and often debated. Some other autoantibodies are clearly pathogenic, e.g. in rheumatoid arthritis (31), pemphigus vulgaris (32), or myasthenia gravis (33). Celiac anti-TG2 antibodies have a number of adverse biological effects in cell culture and their tissue-binding to vessels was coincident in clinical studies with development of intestinal villous atrophy, liver, muscle and kidney damage, lymphadenopathy and brain atrophy (5-11,14). Passively transferred maternal antibodies targeting the celiac epitope affected the behaviour of endothelial cells prepared from the umbilical cords (Fig.6E, S6B) and some of the neonates also showed symptoms and low birth weight (SI Text). Further, the antibodies deposited on the surface of chorionic villi may alter TG2 activity (34), impair nutrient import and may be responsible for the altered pregnancy outcome (35) in celiac disease. TG2 also has non-enzymatic functions in cell adhesion, speading and survival (1,3) where autoantibody effects also can be directly mediated via the celiac epitope.

Celiac sprue is considered as a life-long disease and unless it is treated increased morbidity and mortality prevails (4). Since steady compliance with the gluten-free diet is difficult and often unsuccessful, other attractive alternatives have been proposed, e.g. oral protease supplementation for the gastrointestinal degradation of proline-rich gliadin peptides or inhibition of intestinal TG2 activity to prevent deamidation and binding of gluten peptides to HLA-DQ2 or HLA-DQ8 molecules, though proper TG2 inhibitors for such a purpose have not been found yet. Here we showed that tissue-deposited celiac autoantibodies, which could be associated with the multi-organ manifestation of the disease (14), can be displaced by a monoclonal antibody recognizing part of the main celiac epitope characterized in this study. Furthermore, this displacing antibody did not

alter the enzymatic activity of TG2 (Fig.S7) nor had the typical pathologic effects elicited by celiac antibodies in cell culture experiments and could even antagonize the latters. These findings raise the possibility that the adverse pathologic effect of celiac autoantibodies in patients can be reversed and prevented by either humanized monoclonal antibodies similar to mAb 885 or competitor compounds specifically designed to inhibit binding of autoantibodies to the celiac epitope of TG2, though such a therapeutic benefit may be diminished by pathologic consequences of an ongoing gluten-specific T cell activation.

In conclusion, the present identification of a disease-specific conformational epitope on the main autoantigen and its involvement in disease manifestations support the role of autoantibody response in disease pathomechanism. These findings may help design further studies to establish whether interference with the effects of celiac antibodies would have therapeutic potential.

MATERIALS AND METHODS

Patients

Serum samples from altogether 216 untreated and 22 treated celiac disease patients (aged 0.9-78 years) having Marsh grade III villous atrophy were used. Eleven subjects initially had preserved small bowel villous architecture but subsequently developed celiac type villous atrophy (early stage or latent celiac disease). Included non-celiac controls had normal small bowel villous architecture.

Molecular modelling

Crystal structures of human TG2 (PDB code: 1KV3; ref. 15) and TG3 (PDB code: 1VJJ; ref. 36) were used for modelling the full length TG2 as described in details in SI Methods.

Antigens, protein expression and purification

N-terminally His-tagged TG2 in pET-30 Ek/LIC Vector and domain deletion mutant TG2s were generated and purified as described (22). The other TG2 mutations were generated according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing using the ABI PRISM® 3100-Avant Genetic Analyzer.

Anti-TG2 ELISA and competition studies

The ELISA measurements were performed in duplicates as described previously (13) on Maxisorp (Nunc) microtiter plates coated with 0.6 μg TG2 or equimolar amounts of mutants in tris-buffered saline containing 5 mM $CaCl_2$ (*p*H 7.4). Further steps and competition experiments are described in details in SI Methods. Standard curves were prepared using dilutions of TG100 monoclonal anti-TG2 antibody (NeoMarkers) for each mutant and the binding of other antibodies was calculated by 4-parameter fit if binding to wild-type TG2 was 100%.

Immunofluorescent studies

Unfixed frozen sections were incubated with FITC-conjugated anti-human IgA or IgG (DAKO) in combination with double labelling for TG2 by anti-TG2 mAbs as described (14). For detecting competition, TG2-specific mAbs were added to the tissue for 30 minutes in PBS, and the incubation solutions were recollected and tested for patient IgA by ELISA after a purification step removing mAbs by protein-G conjugated magnetic beads (Dynabeads).

Human umbilical vein endothelial cells (HUVEC) preparation and cell culture experiments

HUVECs were prepared and cultured using standard techniques (37). In some experiments the cord vein was filled with DyLight 594-conjugated anti-human IgG (Jackson) before cell isolation and cells were double-stained after 24h in culture with TG100 anti-TG2 mAb and FITC anti-mouse antibodies. For the analysis of antibody effects, HUVECs were cultured in matrigel for 24 hours (SI Methods).

Statistical analysis

ELISA results were analyzed using GraphPad Prism Software and STATISTICA. For comparison of antibody binding to mutant TG2, data were analyzed using repeated measures ANOVA followed with Dunnett's Multiple post test, one way ANOVA followed by Tukey's post test, or Kruskal-Wallis test followed by Dunn's multiple comparison test as appropriate. A p value < 0.05 was considered significant.

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Conflict of interest

Debrecen University filed PCT WO2010/113025 A2 and 2010/116196 A2 based on these results and mAb 885 was deposited by Phadia at the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany.

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Figure legends

Figure 1. Binding of celiac IgA serum antibodies (n=7-28) in ELISA to wild-type (Wt) and mutant TG2s. Dash indicates median. ***, p<0.001, compared to Wt. S4 contains the combined mutations D151N-E153Q-E154Q-E155Q-E158Q (ref.21). Roman numerals indicate presence of domains (I-IV).

Figure 2. Three-dimensional view of TG2 in the closed conformation with the N-terminal β–sandwich shown in blue, catalytic (core) domain in red, β-barrel 1 in cyan and β-barrel 2 in pink. The bound GDP, fibronectin binding site (Asp94, Asp97) and catalytic triad (Cys277, His335, Asp358) are represented as ball-and-stick side chains. The amino acids of the putative celiac epitope with their lowest distances in Ångström are illustrated as surface representation (frame).

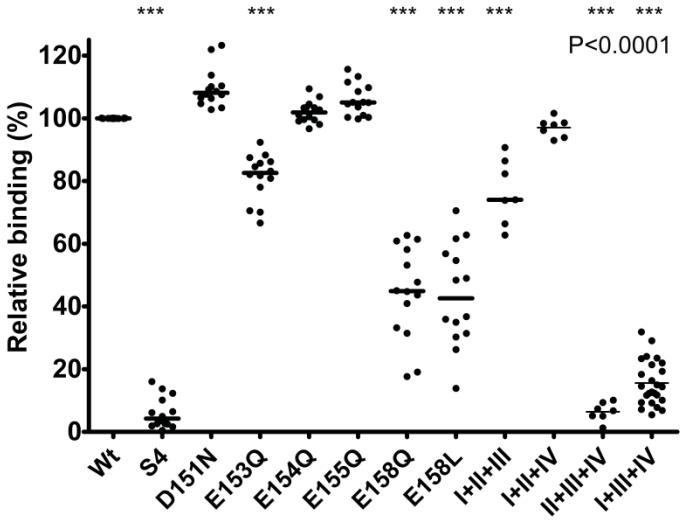
Figure 3. Binding of serum IgA from 58 celiac children (A) and from 18 celiac adults (B) to TG2 mutants in ELISA. Bound antibody concentrations were calculated from a calibrator curve (Fig.S2E) constructed from the concentration dependent binding of mouse monoclonal anti-TG2 antibody TG100. Binding to wild-type (Wt) is 100%. All samples were examined in duplicates. Dash indicates median. p<0.0001 represents significant differences between groups by ANOVA test. 433=R433S-E435S (irrelevant control mutant), R=R19S, E=E153S, M=M659S, RM=R19S-M659S, RE=R19S-E153S, EM= E153S-M659S, REM= R19S-E153S-M659S.

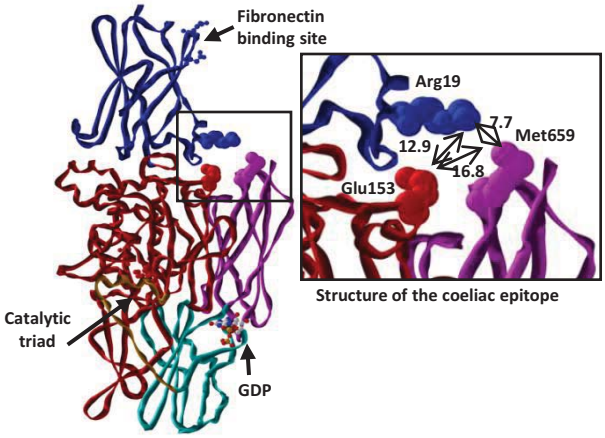
Figure 4. Binding to mutant TG2 proteins of serum IgA antibodies from patients with early stage celiac disease (●) without villous atrophy, manifest celiac disease (▲) with small bowel villous atrophy (n=11) and from patients with other autoimmune diseases (n=11) (o), measured by ELISA. The binding to wild-type (Wt) was set to 100%. Dash indicates median. ***, p<0.001, ns, not significant. R = R19S, RE = R19S-E153S, REM =R19S-E153S-M659S

Figure 5. Competition effect of mAb885 on the recognition of wild-type TG2 in ELISA by antibodies from celiac disease patients (n=6) and from non-celiac autoimmune patients with anti-TG2 antibodies (n=6). A. Remaining IgA binding in the presence of 18 µg/well mAb885 if the

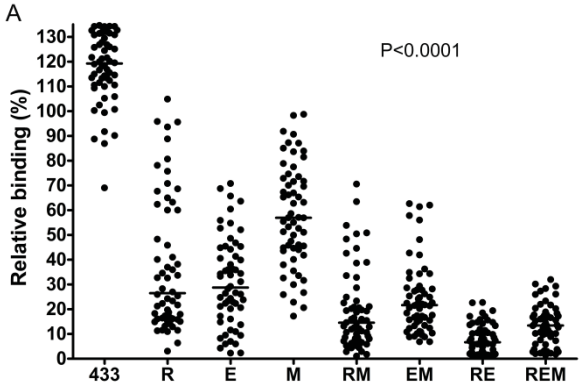
binding without mAb 885 was 100%. Values represent means ± standard errors. B. Comparison of the displacing effect of anti-TG2 mAbs 885, CUB7402, TG100 or H23 (18 µg/well) using one of the celiac serum samples. Representative values from three independently performed experiments.

Figure 6. Tissue binding and biological effects of celiac antibodies mediated by the celiac TG2 epitope. A. Epitope specificity of serum IgA (1,2), of IgA eluted from celiac tissues (IgA placenta 3,4) and of passively transferred maternal IgG from newborn serum measured using mutant TG2 proteins in ELISA. R=R19S, E=E153S, M=M659S, RE=R19S-E153S, REM= R19S-E153S-M659S, 433=R433S-E435S. B. Celiac IgA (green) in vivo deposited in the placenta and on the surface of chorionic villi (arrows) merge to yellow after incubation of the tissue sections with CUB7402 anti-TG2 mAb and double-stained by anti-mouse antibodies (red). After incubation with mAb 885 the IgA signal is no more visible and anti-TG2 IgA is detected in the buffer by ELISA. No IgA was released by incubation with isotype control mAb (IgG1) or CUB. C. IgA from celiac (CD) patients (n=5), autoimmune patients with non-celiac TG2 antibodies (n=6) and biopsied antibody-negative controls (n=3) were administered alone or together with mAb 885 to normal HUVECs in matrigel and vessel formation was measured. Median lengths of endothelial tubules ± SD are shown compared to wells without antibodies set to 100%. ns, not significant; ***, $p<0.001$. E. Morphology of HUVECs in culture from a newborn with prenatally bound maternal celiac IgG (red) on their surface (upper and middle) compared to HUVECs prepared from newborn with celiac mother on diet and negative for antibodies (lower panel). Bars=50 um.


***
***
***
***
***
***
P<0.0001
Relative binding (%)
120
100
80
60
40
20
0
Wt
S4
D151N
E153Q
E154Q
E155Q
E158Q
E158L
I+II+III
I+II+IV
II+III+IV
I+III+IV

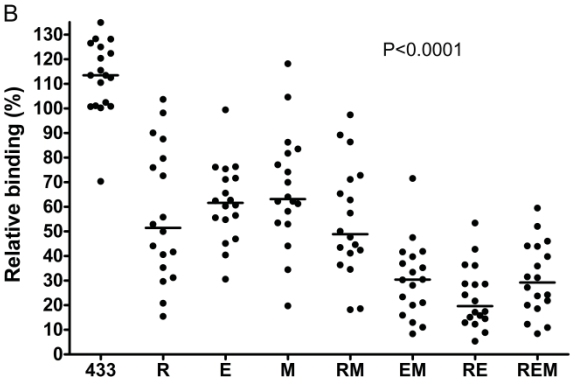

Fibronectin
binding site
Arg19
7.7
Met659
12.9
16.8
Glu153
Structure of the coeliac epitope
Catalytic
triad
GDP

A

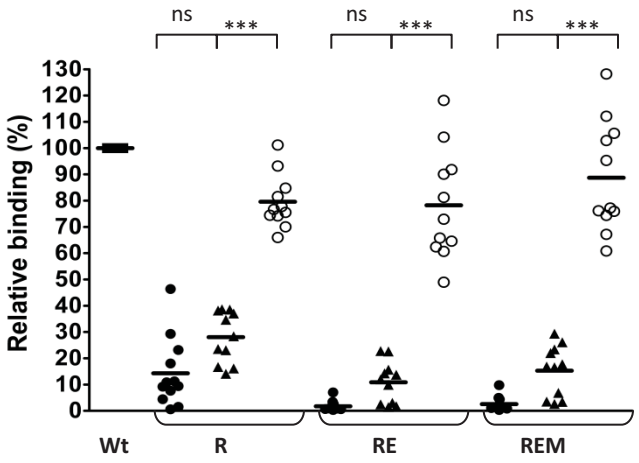


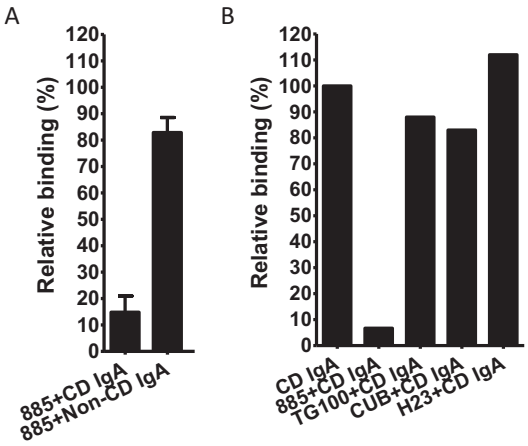
Median	119.2	26.5	28.8	56.9	14.6	21.7	6.6	13.4

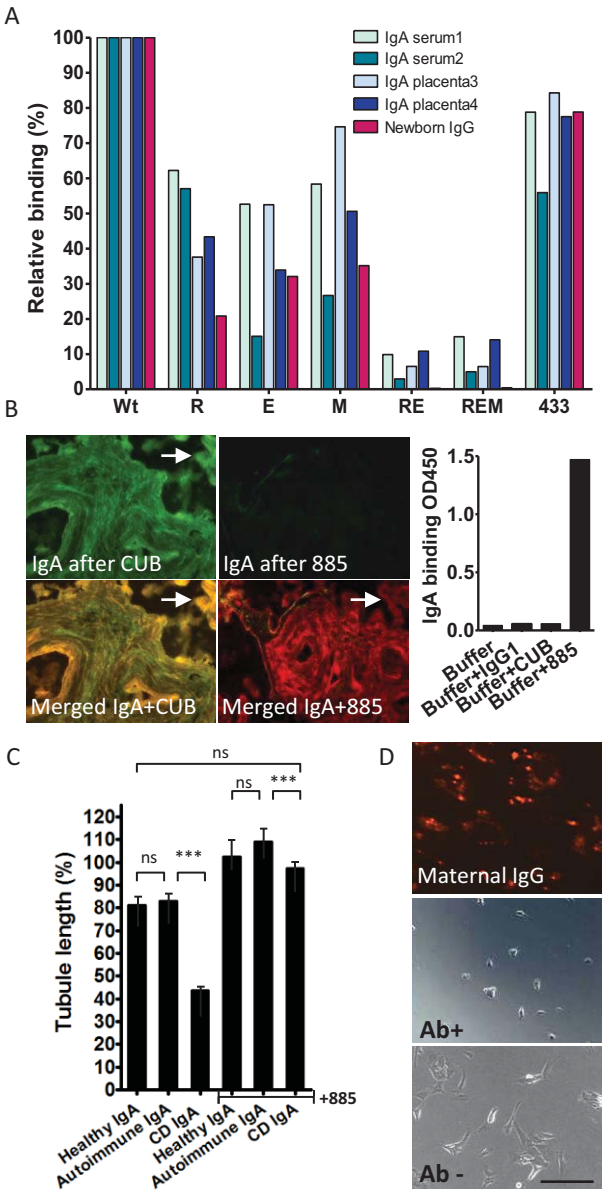
B



Median	113.4	51.4	61.6	63.1	48.9	30.4	19.6	29.2


ns
***
ns
***
ns
***
Relative binding (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Wt
R
RE
REM


A
Relative binding (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
885+CD IgA
885+Non-CD IgA
B
Relative binding (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
CD IgA
885+CD IgA
TG100+CD IgA
CUB+CD IgA
H23+CD IgA


A
Relative binding (%)
IgA serum1
IgA serum2
IgA placenta3
IgA placenta4
Newborn IgG
Wt
R
E
M
RE
REM
433
B
IgA after CUB
IgA after 885
Merged IgA+CUB
Merged IgA+885
IgA binding OD450
Buffer
Buffer+IgG1
Buffer+CUB
Buffer+885
C
Tubule length (%)
ns
***
Healthy IgA
Autoimmune IgA
CD IgA
+885
D
Maternal IgG
Ab+
Ab -