

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY (Ph.D.)**

**STRUCTURAL ANALYSIS AND CLINICAL SIGNIFICANCE OF
CELIAC DISEASE ANTIBODY EPITOPES ON
TRANSGLUTAMINASE 2**

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Structural analysis and clinical significance of celiac disease anti-body epitopes on transglutaminase 2

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1. INTRODUCTION

Clinical features of celiac disease

Celiac disease (CD) is known from ancient Roman times as a disease of the gut causing malabsorption. In light of recently accumulated knowledge, CD is today considered as a systemic disorder with pathologic immune reaction to ingested gluten proteins of wheat, rye and barley. In subjects with genetic predisposition, gluten-derived gliadin peptides induce chronic intestinal inflammation, villous atrophy and the production of disease-specific autoantibodies against the enzyme type-2 transglutaminase (TG2).

In Europe and in the United States the prevalence of CD can be estimated as high as 1%. CD is a multigenic disorder with high rate of familiar occurrence. The HLA locus has the strongest genetic influence on the disease; the primary genetic association in CD is with DQ2 and to a lesser extent to DQ8. These alleles are also commonly found in healthy individuals, which indicate that these HLA genotypes are necessary but not sufficient for disease development and additional genes have been suggested to contribute to CD development.

Clinical manifestations of CD are highly variable. In children, diarrhea, abdominal distension and failure to thrive are common. In adults, the classic presentation of the disease is chronic diarrhea, may be with abdominal pain, weakness and malabsorption. The majority of patients have milder symptoms and some patients are asymptomatic (silent CD), but also these do have manifest mucosal lesion and after introduction of a gluten-free diet patients report favorable changes in their conditions. Latent CD patients show normal small-bowel villous architecture for a while, but at one other time of their life they develop small-bowel villous atrophy and crypt hyperplasia.

Dermatitis herpetiformis is the best known among the extraintestinal manifestations of CD. In addition to anti-TG2 antibodies and enteropathy, this is characterized by papulovesicular rash and granular IgA deposition in the dermal papillae targeting epidermal transglutaminase (TG3). Neurologic disorders are also common extraintestinal features of CD (gluten ataxia) and CD can be associated with a number of autoimmune diseases like type I diabetes mellitus, autoimmune thyroid diseases and Sjögren's syndrome. Due to the absence or protean nature of symptoms, large proportions of CD remain unrecognized, which can cause certain cancers like non-Hodgkin lymphoma, enteropathy-associated T-cell lymphoma and small intestinal adenocarcinoma.

Serology has become a highly relevant part of the diagnosis of CD: detection of anti-TG2 IgA antibodies is recommended as the first diagnostic step followed by endoscopy and biopsy in positive

cases. The anti-TG2 tests are highly sensitive for CD (97%) and specificity is greater than 90% in the case of human TG2 antigen applied in enzyme-linked immunosorbent assay (ELISA). However several studies described false negative and false positive anti-TG2 IgA results. Another widely used method is detection of serum autoantibodies on tissue sections containing TG2 in endomysial or reticulin structures of monkey esophagus or human umbilical cord (EMA test) and its specificity is nearly 100%. New antibody tests with deamidated forms of gliadin peptides (DGPs) as antigens were also introduced with 94% diagnostic efficiency.

The diagnostic criteria were first formulated by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) in 1970 and included three intestinal biopsies. These criteria were modified in 1990 including one biopsy showing villous atrophy and recovery on a gluten-free diet. In 2011 new criteria were announced: small bowel biopsy will no longer be compulsory in children showing symptoms compatible with CD and presenting with very high positivity for both anti-TG2 and endomysial serum antibodies and HLA DQ2 or DQ8.

Due to our present-day knowledge of celiac disease, the only accepted treatment is lifelong gluten-free diet. There is regular relapse with the reactivation of the immune process whenever the diet is stopped. This strict diet requires the elimination of wheat, rye and barley, which may severely affect the lifestyle and social activities of the patient. So there is an apparent need for alternative therapies.

Molecular mechanisms in the pathogenesis of celiac disease

The pathogenesis of CD includes multiple interactions between environmental, genetic and immunologic factors. The external trigger of the disease is gluten, which activates the innate and adaptive immune system and leads to mucosal damage and production of anti-gliadin and anti-TG2 antibodies. Interestingly, anti-TG2 antibodies are no longer produced after gluten is excluded from the patient's diet, despite the fact that TG2 is still available to the immune system.

Some gliadin peptides are resistant to digestive enzymes and could enter the lamina propria, where they will be processed and presented by antigen presenting cells via their HLA-DQ2 or DQ8 molecules. The deamidation of the non-charged glutamine residues to glutamic acid by TG2 enhances the binding to DQ2 and will trigger more potent immune reaction by the expansion of gluten-specific CD4+ T cell repertoire. These gluten-specific CD4+ T cells will secrete pro-inflammatory cytokines and thus stimulate fibroblasts and cytotoxic T cells to produce matrix metalloproteinases. These processes will lead to villus atrophy. Activation of gluten-specific CD4+ T cells also results in

clonal expansion of gluten-specific B-cells and release of gluten-specific antibodies, while TG2-specific B-cells may become activated through intermolecular help by the same CD4+ cells. However, the exact mechanism of production of anti-TG2 antibodies is still not clarified in experiments.

Pathologic effects of autoantibodies

A number of recent studies suggest that TG2-specific autoantibodies may also play a role in the pathogenesis of the disease as they deposit on the extracellular surface of cells and in the matrix where TG2 is abundantly available in a fibronectin-bound form. Celiac IgA antibodies inhibit the differentiation of intestinal epithelial cells, induce epithelial cell proliferation and specifically increase the transepithelial passage of gliadin peptides *in vitro*. Autoantibodies of celiac patients can also activate monocytes by binding to toll-like receptor 4 and can induce monocyte-mediated cytotoxicity and thus provoke tissue damage. CD-specific IgA antibodies were also shown to disturb the initial steps of angiogenesis *in vitro* in human umbilical cord vein (HUVEC) cultures and to increase blood vessel permeability for macromolecules and lymphocytes.

Since TG2 plays an important role in the pathogenesis of CD and TG2-specific autoantibodies bound to the enzyme can be found in the gut and in other organs, modification of the enzymatic activity of TG2 by these antibodies can be an important factor in the disease development and progress. Controversial data have been published on the effect of celiac autoantibodies on human TG2; inhibitory, partially inhibitory and activation effects were also described and the outcome was dependent on the reaction environment and substrate properties as well. However, we showed earlier that celiac antibodies can bind to TG2 even when the active site is occupied by an inhibitor.

Transglutaminase 2

TG2 is a ubiquitous enzyme; it catalyzes Ca²⁺-dependently the post translational modification of proteins by incorporation of primary amines into the γ -carboxamide group of glutamine residues or by the cross-linking of proteins via ϵ -(γ -glutamyl) lysine bonds. In addition, it can catalyze deamidation of glutamine to glutamic acid, bind guanine nucleotides and hydrolyze guanosine-triphosphate (GTPase, G protein function) and it has several other enzymatic and activity-independent functions. Although TG2 is mainly a cytosolic protein, it can be found in the nucleus, and it is also secreted to the cell surface and to the extracellular matrix (ECM). On the cell surface it is located in complex with fibronectin (FBN) and integrins, and this interaction facilitates adhesion,

spreading and motility of cells. TG2 plays a role in wound healing, angiogenesis, cell differentiation, apoptosis, phagocytosis, as well as in the assembly, remodeling and stabilization of ECM.

TG2 is 687 amino acids (aa) long, 76 kDa protein with four domains (I-IV); N-terminal β -sandwich (aa 1-139), catalytic core domain (aa 140-460) and C-terminal β -barrels 1 and 2 (aa 472-585 and aa 586-687). Two conformations of the enzyme have been revealed by crystallizing it either complexed with guanosine-diphosphate (GDP; 'closed' conformation, in physiological conditions, the transamidation function of TG2 is inactive) or with an inhibitor that mimics inflammatory gluten peptide substrates ('open' conformation).

The first domain of the molecule contains the FBN- and the integrin-binding sites. In the GDP-bound form the catalytic triad (Cys277, His335 and Asp358) is deeply buried within the core domain and a unique guanine-binding site can be found in a cleft between the catalytic core and the first β -barrel. TG2 binds six Ca^{2+} ions of which the binding sites for two were described in our laboratory in an earlier study. S1 is a strong Ca^{2+} -binding site (aa 229-233), while S4 (aa151-158) and S5 (aa434-438) bind only weakly Ca^{2+} , but their mutations affect the antigenicity of TG2 for celiac antibodies.

Epitope mapping of transglutaminase 2 for celiac binding sites

Celiac TG2 epitopes were investigated by several groups, with various methods, but no such unique epitope has been found so far and it is not clear either whether only one or several such epitopes exist. The major conclusions from these measurements were (i) neither the C-terminal nor the N-terminal domains are essential for antigenicity, but if both are missing, the celiac antibody binding is lost; (ii) the N-terminal region of the molecule may harbor one epitope; (iii) the C-terminus can be another important site; (iv) the core domain can also harbor one epitope; (v) truncation of the core domain is not tolerated, which indicates that the conformation of this part is important. Serum samples from the same patients sometimes recognized several complementary fragments. Based on these results it was suggested that the antibody response may be dispersed and variable according to the subjects. In another study TG2 containing mutations of the catalytic triad had severely reduced antibody binding capacity. Further studies conducted by TG2 fragments displayed on the surface of phages also indicated that the conformation of the protein might have a high importance to form a functional binding site for the celiac antibodies, as it was not possible to identify the epitopes of celiac patient derived monoclonal antibodies by these means either.

2. AIMS OF THE STUDY

1. Our aim was to search for the exact location of the binding sites of celiac anti-TG2 antibodies by molecular modeling and site directed mutagenesis and to establish whether a celiac disease-specific binding site exists.
2. A second aim was to compare the binding properties of antibodies from different patient groups, from different ages and from different tissue sources to see which antibody specificities are clinically relevant.
3. We also aimed to establish whether the found TG2 epitopes are involved in the biological effects of celiac disease antibodies, which are the downstream consequences of the binding and whether any of the effects could be inhibited by a site-specific inhibitor.
4. We also wished to investigate a possible molecular mimicry between TG2 and gliadin peptides that could shape antibody specificities and could have a role in disease pathogenesis.

3. MATERIALS AND METHODS

Patients

Serum samples from altogether 216 untreated and 22 treated celiac disease patients (aged 0.9-78 years) having villous atrophy at diagnosis were used for the epitope mapping of TG2. Eleven CD subjects initially had preserved small bowel villous architecture, thus the diagnosis of celiac disease could not immediately be made (latent celiac disease). Further 74 CD patients (median age, 7 years; range, 1.6–39.7 years) and 65 EMA negative non-celiac controls with normal small bowel villous architecture (median age, 13 years; range, 0.9–42.4 years) took part in the diagnostic evaluation of DGP peptides.

Autoimmune sera

Eleven serum samples from adult patients with established other disease diagnoses were selected from routine clinical samples based on their anti-TG2 antibody content. These patients had systemic lupus erythematosus (n=3), rheumatoid arthritis (n=2), and Sjögren's syndrome (n=6). None had gastrointestinal symptoms suggestive of celiac disease, nor serum endomysial antibodies or antibodies against deamidated gliadin peptides. The reactivity with human recombinant TG2 was confirmed by using two different recombinant constructs.

Expression and purification of recombinant TG2 proteins

Full-length human recombinant N-terminally histidine-tagged TG2 (aa 1-687) in pET-30 Ek/LIC Vector was expressed in Rosetta 2 *E. coli* cells. TG2 mutations were generated according to the QuikChange Site-Directed Mutagenesis Kit. Domain deletion mutant TG2s were created by the same technique with a template pcDNA 3.1/myc-His mammalian expression vector containing the following combinations of TG2 structural domains: A/ I+II+III (aa 1-584), B/ I+II+IV (aa 1-471 and 585-687), C/ II+III+IV (aa 139-687), D/ I+III+IV (aa 1-138 and 472-687), and E/ histidine-tag only, without the sequence of the enzyme. The sequence of the mutants was confirmed by DNA sequencing using the ABI PRISM® 3100-Avant Genetic Analyzer.

Rosetta 2 cells were transformed with the expression vectors containing wild type or mutant TG2s and grown in LB at 37°C to an OD600 0.6-0.8. Cells were grown the presence of 0.3 mM isopropyl

β -D-thiogalactoside, harvested and resuspended in lysis buffer. After cell lysis and centrifugation supernatants were applied to ProBond Ni-NTA resin and purified according to the manufacturer's instructions. After elution with imidazole, protein fractions were concentrated and used for further investigations.

Measurement of transglutaminase and GTP-ase activity

Transglutaminase activity was measured with microtiter plate assay based on the incorporation 5-(biotinamido) pentylamine into immobilized *N,N*-dimethylated casein and GTPase activity was measured with charcoal method.

Molecular modeling

Crystal structures of human TG2 (PDB code: 1KV3) and TG3 (PDB code: 1VJJ) were used for modeling the full length TG2. Homologous model was built by Modeller using the multiple template option of the program. Graphical analysis was made on Silicon Graphics Fuel workstation using Sybyl program package –with the help of Péter Bagossi- and VMD.

Anti-TG2 ELISA

Microtiter plates were coated with 0.6 μ g TG2 in 100 μ L of tris-buffered saline (TBS) containing 5 mM CaCl_2 (pH 7.4) (Ca-TBS). When denatured TG2 was investigated, TBS contained 8 M urea or 6M Guanidine-HCl during the coating. The plates were washed 3 times with TBS containing 0.1% (v/v) Tween20 and 10 mM EDTA (TTBS+EDTA). All antibodies were diluted in TTBS+EDTA. Serum samples, Single Chain Variable Fragments (ScFv) derived from celiac patients or monoclonal antibodies (TG100, CUB7402, 895, 4G3; G92; H23) were incubated for 1 h at room temperature. Plates were washed and incubated either with horseradish peroxidase-conjugated rabbit anti-human IgA or IgG or with monoclonal antibody (MAb) recognizing SV5 tag at the C-terminus of the ScFv, followed by HRP-conjugated anti-mouse IgG for 1h at room temperature. The color reaction was developed by adding 100 μ L 3,3',5,5'-tetramethylbenzidine substrate and then stopped with 50 μ L 1 N H_2SO_4 . The absorbance was read at 450 nm.

Standard curves were prepared using three dilutions of TG100 monoclonal anti-TG2 antibody for each mutant and the binding of other antibodies was calculated by 4-parameter fit if binding to wild-type TG2 was 100%.

Fibronectin-TG2 ELISA

Microtiter plates were coated with 0.3 µg human fibronectin diluted in bicarbonate buffer pH 9.6 for 1h at room temperature. The plates were incubated with 0.8 µg TG2 in TBS containing 5 mM CaCl₂ and 0.1% (v/v) Tween 20 and the assay continued as above.

Competition ELISA assays

Microtiter plates were coated with 0.6 µg wild type (Wt) TG2 in 100 µL of TBS containing 5 mM CaCl₂ (pH 7.4). Mixture of M13 phage-conjugated ScFvs and increasing amounts of soluble ScFvs were simultaneously added and bound phage-ScFvs were detected with anti-M13 phage antibody conjugated with HRP. In other assays, wells were incubated with celiac serum and increasing amounts of purified total celiac IgG antibodies and binding of IgA and IgG antibodies were detected. Competition assays were also carried out with celiac serum added to the plate together with increasing amounts (up to 18 µg) of monoclonal mouse antibodies (885, CUB7402, H23) and bound IgA was measured.

IgG and IgA purification

For competition assays IgG antibodies were purified from serum samples of CD and healthy control subjects with Protein G conjugated Sepharose beads and IgA purification was performed by anti-human IgA (α-chain specific)-agarose according to the manufacturers' instructions.

Immunofluorescent studies

Unfixed sections of patient tissues were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgA or IgG. These stainings were also combined with double labeling for TG2 by anti-TG2 MABs (CUB7402 and 885). For secondary antibodies, Alexa 594-conjugated anti-

mouse antibodies was used. For detecting competition, TG2-specific MAbs were added to the tissue for 30 minutes in phosphate buffered saline (PBS), and the incubation solutions were recollected and tested for patient IgA by ELISA.

Elution of IgA from tissue sections

Unfixed cryosections were washed twice with PBS and elution buffer containing 0.25% chloroacetic acid with 0.2 M/L NaCl, pH 2.7 was applied to the sections for 20 minutes. The eluates were neutralized with 1 M/L imidazole, concentrated on 10K membranes and the buffer was changed to PBS.

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

HUVEC cells were prepared and cultured using standard techniques. HUVEC cells were mixed with Matrigel and grown in 24-well dishes with complete endothelial medium EGM1 containing 2% fetal bovine serum with or without 1 ug/well MAb 885. After 4 hours, 1 ug/well purified patient or control IgAs were added. After further 24 hours ten images were taken per well and the length of formed endothelial tubules was analyzed by Image J software. Results were expressed as % median tubule length of basal values with 95% confidence intervals using as reference the geometric mean of tube length in wells without antibodies. In some experiments HUVECs were plated on collagen I together with or without the investigated antibodies and grown for 24 hours. Cell lengths were evaluated by Image J software in similar ways. These cellular experiments were performed by our collaborator Sergio Caja in Tampere, Finland.

DGP-ELISA

DGP-reactive antibodies were measured from patient serum samples by Quanta Lite™ Celiac DGP Screen test kit according to the manufacturer's instructions. This kit contains ELISA plate coated with synthetic peptides corresponding to deamidated gliadin peptides and a combined HRP conjugate that recognizes both IgA and IgG class human antibodies. The same plates were also probed with mouse monoclonal TG2-specific antibodies using the buffers provided in the kit. The mouse antibodies were recognized by HRP-conjugated anti-mouse antibodies and the reaction was revealed as written in anti-TG2 ELISA.

Competition studies with DGP ELISA

Monoclonal TG2-specific antibodies were added to the commercial DGP-ELISA plates in the presence of increasing amounts of human recombinant TG2 constructs or celiac patient serum samples. The His-tagged construct devoid of the sequence of the enzyme was used as negative control. The OD450 signal of the blank was defined as 100% inhibition, and the signal without added TG2 or celiac serum was defined as 0% inhibition.

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.

4. RESULTS

The celiac transglutaminase 2 epitopes are conformational

Several previous attempts were made to identify the celiac epitopes of TG2 and the results suggested that the bindings of CD antibodies are dispersed and several parts of the molecule can take part in the formation of the epitope(s). In our initial approach we also considered that CD antibodies cannot recognize TG2 in Western-blot experiments after usual SDS/PAGE which destroys the three-dimensional structure of the molecule, so if the bindings of CD antibodies depend on the intact fold of TG2, the CD epitope can be conformational.

To establish which proportion of celiac antibodies in polyclonal patient serum samples bind to linear epitopes or to conformational binding sites we used guanidine-hydrochloride and urea, which agents have chaotropic properties and denature the enzyme. We coated the ELISA plates with TG2 diluted in buffers containing these agents and wells coated with TG2 in CaCl_2 were applied as controls. TG100 monoclonal anti-TG2 antibody with a linear binding epitope was used to prove that denatured TG2 was present in equal amounts on the plate.

The binding abilities of celiac IgAs to TG2 were abolished when the enzyme was denatured, while the binding was unaltered when the enzyme remained intact in the presence of CaCl_2 . This result suggests that almost all celiac autoantibodies are conformational and they practically do not bind to linear epitopes.

The celiac epitope is related to Ca^{2+} -binding sites of TG2 but Ca^{2+} ions do not form part of the epitope

During our previous work, Site 4 (S4, amino acids 151-158) and Site 5 (S5, amino acids 433-438), Ca^{2+} -binding sites on the core domain were identified which may play a role in the binding of celiac antibodies. S4 is located at the first alpha helix of the core domain, relatively close to S5. In the case of S4 the binding of CD IgAs was diminished to $11.6 \pm 8.5\%$ (if binding to Wt TG2 is 100%), so we decided to search for the anchor points of the celiac epitope in this area. During this work we prepared TG2 point mutants holding D151N, E153Q, E154Q, E155Q amino acid changes separately and the binding of serum IgA from celiac patients was measured in ELISA. Only changes at residues 153 decreased the binding of celiac antibodies significantly ($p < 0.0001$, median 82.6% remaining binding for E153Q).

Identification of anchor points outside the core domain

We further investigated the additional anchor points of the celiac epitope on the other domains of TG2 and we expressed TG2 mutants each lacking one structural domain of TG2. When the domain mutants were applied in equimolar concentrations in immunoassays, both domain I (aa 1-139) and II (aa 147-460) turned out to be important for autoantibody binding (median 6.7% and 14.4% for remaining binding of mutant C=II.+III.+IV. domains; and D=I.+III.+IV. domains; respectively). The binding was also influenced to some extent also by the loss of domain IV (aa 585-687; median 74.0% for remaining binding of mutant A=I.+II.+III. domains). In contrast, antibody binding proceeded normally even in the complete absence of domain III (aa 472-584; median 97.9%).

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

Next, we used molecular modeling to evaluate whether cooperation of core domain Glu153 together with other amino acids on the N-terminal and/or C-terminal domains might form a conformational and functional epitope or epitopes. The N- and C-terminus of the molecule are close to each other and also to the surface of the core domain. Glu153 on core domain is in the closest proximity to Arg19 on the N-terminal domain (12.9 Å) and to Met659 on the C-terminal domain (16.8 Å). Besides these, Arg19 is also close to Met659 (7.7 Å), so this three amino acids possibly form a common conformational epitope. Therefore, mutant TG2 molecules containing changes of these amino acids to serine as single point mutations (R, R19S; E, E153S; M, M659S) or in combination (RE, EM, RM, and REM) were created.

Recombinant TG2s were expressed in histidine-tagged forms and purified with nickel affinity chromatography. The protein fractions were checked by SDS/PAGE and Western blotting. Binding properties of mutant TG2 proteins were tested with a large set of mouse monoclonal anti-TG2 antibodies (CUB7402 with epitope aa 447-478; 895 with epitope aa 649-687; 4G3 with epitope aa 1-165; G92 with epitope aa 1-14 and H23 with epitope aa 433-438). All mutants could bind effectively the monoclonal antibodies with different TG2-epitopes.

We determined the transglutaminase activities of the mutants. Mutant R showed Ca²⁺-dependent TGase activity ~30% higher than wild type (Wt), while the other mutants showed decreased TGase activity ranging from ~21% (EM mutant) to ~73% (RM mutant) if the activity of the Wt is 100%. GTPase activities of the mutants were measured by the charcoal method. The specific activity of the Wt enzyme was set to 100%. The GTPase activities of mutant RM, EM and REM were more than

two fold higher than the activity of the Wt. The single mutants and double mutant RE showed only 24-50%. In FBN-TG2 ELISA the mutants bound monoclonal antibodies in the same extent as Wt.

The bindings of autoantibodies from consecutively diagnosed 58 childhood and 18 adult celiac patients were tested with the R, E and M mutants. Each single mutation resulted in significant decrease in celiac antibody binding (median 26.5%, 28.8% and 56.9% remaining binding for R, E and M, respectively) and double and triple mutations caused proportionally greater changes. The EM mutant still showed 21.7% binding capacity, RM had 14.6%, while triple mutant REM showed roughly the same 13.4%. Surprisingly, the lowest binding capacity was displayed by the double mutant RE with a median of 6.6%, ranging from 1.1%-22.7% in the case of celiac children. Antibodies of celiac adults showed similar binding pattern, but slightly higher binding rates compared to children's antibodies.

Although the investigated TG2-specific serum autoantibodies represent polyclonal immunoglobulin molecules it seems that the majority bind to the identified celiac epitope. To further investigate whether additional immunoglobulin clones with different binding site(s) exist, we obtained single chain variable fragments (ScFv) from our Italian collaborator partner (Daniele Sblattero, University of Trieste, Trieste, Italy). These ScFvs were selected against recombinant human TG2 from a total antibody library created from DNA isolated from lymphocytes of celiac intestinal biopsy samples. These ScFvs are monospecific, bearing only one binding site on the enzyme and represent an independent patient group compared to the Hungarian celiacs. Binding of 8 ScFvs were tested and all were abolished when mutants RE, REM and D (I.+III.+IV domains of TG2) were applied as antigens (mean 13.0%, 14.0% and 5.4% remaining bindings).

Investigation of mutants based on homology with factor XIII

Further aim was to determine one single amino acid which plays a key role in the built up of the coeliac epitope. Based on the homology with blood coagulation factor XIII (fXIII), which is not an antigen in CD, we have compared the primary amino acid sequence and structure of the identified site in the core domain of TG2 with fXIII to identify one amino acid difference, which could be responsible for the low antigenicity of fXIII. Amino acid Glu154 in TG2 and the corresponding Lys199 in fXIII have been identified and amino acid Glu154 in TG2 was changed to lysine (mutant 154K) to create a homologous structure to fXIII. This replacement was also combined with amino acid changes in the other domains (combination of E154K with R19S and M659S changes: mutant RKM).

These mutants were also analyzed by Western blot and their enzymatic activities were also measured. Both mutants had decreased TGase activity; the GTPase activity of mutant 154K was abolished, while mutant RKM showed 78% compared to the Wt. The mutants could bind to FBN in the same extent as Wt TG2.

One amino acid change to lysine (E154K) significantly decreased the binding of celiac sera to TG2 in ELISA (mean 66.6% remaining binding). Comparing these with single mutant E, the binding pattern is similar, since both contain the mutation in this site of the core domain. Triple mutant RKM showed similarly decreased binding as mutant REM. Again, adult CD samples had slightly higher binding compared to those from children.

Binding of celiac antibodies to fibronectin-bound TG2 as a model of epitope exposure in the extracellular matrix

The reactivities of celiac antibodies to different TG2 mutants were also tested with fibronectin-TG2 ELISA. TG2 appears on the cell surface complexed with FBN and integrins that is why FBN-TG2 ELISA was also used to mimic these conditions. Beside this, FBN-TG2 ELISA measures only the FBN-bound TG2, so it is a further purification step of the enzyme on the plate and with this coating the possible unfolding of the TG2 on the plastic can be avoided. The celiac antibodies showed similar binding pattern to the FBN-bound mutants as to the enzymes plated directly on the surface of the ELISA plate. The binding to the single mutant M was slightly decreased or slightly elevated (47.1%-140.8%, median 99.6% respectively, if the binding to Wt is 100%). Decreased binding was observed with mutant E and EM (median 69.9% and 69.1%, respectively), while the reduction was more striking when the mutant contained amino acid change R19S. It was just 40.7% in the case of single mutant R and under 20% with double mutant RE and triple mutant REM.

Based on these it seems that mutation of Met659 alone did not alter celiac antibody binding. This anchor point on the C-terminal (IV) domain is less important 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. 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).

Antibodies of different celiac patients recognize parts of the same epitope

Celiac patient-derived monoclonal ScFvs expressed from phage libraries belonged to two groups: one reacting with R19S and one not. However, both these groups showed greatly reduced binding to Glu153 (E) or Glu154 (154K) mutants, which raised the question whether they recognize separate epitopes or simply bind differently to a common surface area. We addressed this question in competition studies measuring the binding to wild-type TG2 of a phage-linked ScFv (reacting with R19S) with a recognizable M13 phage tag and increasing amounts of soluble ScFvs representing the above groups were added. Soluble ScFvs reacting with R19S (clone 4.1) inhibited the binding of phage-linked ScFv by 76%, but soluble ScFv not reacting with R19S (clone 3.7) also competed effectively with the binding of the phage antibody reacting with R19S causing 40% inhibition. Using phage-linked ScFv not reacting with R19S, 30% inhibition could be obtained with both groups of soluble ScFvs.

For another approach, natural IgA and IgG antibodies purified from sera of celiac patients were used by adding increasing amounts of IgG and constant amounts of IgA to the plate covered with Wt TG2 and the bound IgA and IgG were measured in parallel wells. Celiac IgA and celiac IgG1 were reactive with R19S, while celiac IgG2 was not reactive with R19S; purified IgG fraction of a healthy person was used as control. The celiac IgA and IgG antibodies from different groups could compete with each other, while the control IgG did not have any inhibitory capacity.

Disease specificity of the composite epitope

We compared the binding pattern of celiac disease serum samples with that of serum samples from patients with anti-TG2 antibodies due the other autoimmune diseases (SLE, Sjögren's syndrome, rheumatoid arthritis). The non-celiac group showed a clearly different binding pattern to the celiac epitope; and these subjects were also negative for antibodies against deamidated gliadin peptides in ELISA test as well as in the endomysial assay (EMA) that detects anti-TG2 antibodies with tissue sections in a celiac-specific manner. In order to investigate whether the celiac epitope targeting already exists in the early preclinical stage of CD, serum samples from patients without villous atrophy but with circulating anti-TG2 antibodies (latent cases) were obtained. These serum IgAs showed low reactivity to Glu153 and Arg19 mutants similarly to those from patients with overt disease. These data collectively suggest that celiac disease results in a particular and directed immune response toward TG2 and this pattern of epitope recognition has diagnostic predictive value.

Similar TG2 epitope specificity of circulating antibodies and of those bound to patient tissues *in vivo*

High avidity antibodies might be trapped in the tissues explaining seronegative celiac cases in the minority of patients, but these might be more important for inducing pathology. We eluted patient IgA antibodies from tissue sections and after purification tested their binding epitopes with the panel of relevant mutant TG2 proteins. We obtained placenta samples from two seropositive celiac mothers and both contained high amounts of TG2-bound maternal antibodies in the decidual parts and on the surface of the chorionic villous structures. These tissue samples provided enough material for use in ELISA measurements after elution of IgA with chloroacetic acid. IgG (but not IgA) class anti-TG2 antibodies deposited also in the umbilical cords and appeared in the infants' serum. The antibodies from the neonates' sera and antibodies and IgAs eluted from placentas showed similar epitope specificity pattern as serum antibodies of the mothers.

Monoclonal antibody targeting Glu153 interferes with celiac antibody binding *in vitro*, displaces them from patient tissues and prevents their biologic effects

A mouse monoclonal anti-TG2 antibody clone (MAb 885, Phadia, Uppsala, Sweden) was found to target Glu153, thus a part of the celiac epitope. When wild-type human TG2 was incubated with three different celiac patient serum in the presence MAb 885, only 6.9-10.1% of natural polyclonal celiac IgA could bind whereas similar amounts of anti-TG2 MAb TG100, CUB7402 or H23 having an irrelevant epitope did not have significant interfering effect. Furthermore, IgA samples from celiac patients (n=6) were specifically competed out by MAb 885 (remaining binding 14.8%, if the binding without MAb 885 is 100%), while non-celiac IgA from patients with other autoimmune diseases (n=6) could bind to Wt TG2 also in the presence of MAb 885 (remaining binding 82.8%).

Further, competition studies were performed with MAb 885 and control MAbs on frozen celiac patient placenta sections containing deposited IgA. MAb 885 was able to bind to tissue sections and could be recognized along extracellular TG2. In parallel, tissue-bound IgA completely disappeared from the tissue and could be detected in the buffer by ELISA. Incubation with buffer only, with isotype control mouse antibodies or with CUB7402 anti-TG2 MAb (with a different TG2 epitope-specificity) did not alter the binding of *in vivo* tissue-bound anti-TG2 IgA antibodies.

We tested the biological effects of MAb 885 by measuring the transamidating activity of TG2 with microtiter plate method. In line with our earlier findings, celiac antibodies caused an increment

(150.4% if activity of Wt TG2 without antibodies is 100%), while MAb CUB7402 and TG100 blocked catalytic activity of the enzyme reaction (5.6% and 21.6%, respectively), MAb 885 did not have any modulating effect (89.6%) when the same antibody amount was used (5 µg/ml) as used in the case of the other MAbs.

We also tested the effect of MAb in angiogenesis assay. Purified IgA fractions of celiac, non-celiac (from autoimmune patients) or healthy control serum samples were added to HUVEC cells cultured in matrigel in the presence or absence of 885 antibodies. Purified IgA from autoimmune patients with non-celiac TG2 antibodies and control IgA from antibody-negative healthy persons caused only slight non-specific decreases in endothelial tubule formation (median 83.1% and 81.3%, if tubule length without antibodies is 100%) but celiac IgA significantly decreased tubule length (median 43.7%, $p < 0.001$ compared to autoimmune IgA) and formation. This effect was prevented if MAb 885 were also present (there is no statistical difference between tubule length in the presence of healthy control IgA and tubule length in the presence of celiac IgA co-administered with MAb 885).

Structural similarities of the TG2 epitope and HLA DQ-docked gliadin peptide

Since our studies show that the majority of celiac antibodies prefer one specific epitope, one should speculate how this knowledge helps us to understand the pathogenesis of the disease. Considering the features of CD it is well known that the trigger of the disease is gliadin, which provokes the patients' immune system to produce autoantibodies against a self-antigen TG2. So we investigated whether there are some similarities between the structures of gliadin and TG2.

Although short gliadin peptides in solution likely have unordered secondary structures, the crystal structure of an immunogenic gliadin peptide (LQFPQPPELPY) is available showing it as docked into the antigen binding groove of HLA-DQ2 (pdb: 1S9V). According to the model, amino acids Gln6, Glu8 and Leu9 of the gliadin peptide seem to interact with the residues in the antigen binding groove of HLA-DQ2. The distance of side-chain carbon atoms of Gln6 and Glu8 (9.84 Å) and their spatial arrangement is similar to the distance measurable between the side-chains of Glu153 and Glu154 forming the celiac epitope in the TG2 structure (9.36 Å), and the distance of Glu8 to Leu9 in the gliadin peptide is similar to that between Glu154 and Val431 of TG2 with a good overlap of these side-chains when the two structures are superimposed.

Investigation of the DGP antigen by nonceliac monoclonal TG2-specific antibodies

To further investigate the potential similarity between TG2 and gliadin epitopes, 74 EMA-positive untreated celiac disease patients were tested by the Celikey (Phadia, Freiburg, Germany; measures anti-TG2 antibodies) and the Celiac DGP Screen (Inova Diagnostics, San Diego, CA; using deamidated gliadin peptide (DGP) as antigens) tests. All of the samples were positive for both TG2 antibodies and DGP antibodies as well.

Next we explored whether some antigens in the DGP test could be similar to TG2 and would be recognized also by other nonceliac TG2-specific antibodies. We obtained 13 different monoclonal mouse TG2-specific antibodies with different binding sites as characterized by earlier studies, spanning all 4 domains of TG2. We applied them in the DGP test and three of them could recognize DGP on the ELISA plate. MAb1 has a linear binding site in the core domain of TG2, whereas the epitopes of MAb2 (aa 637–648) and MAb3 (aa 649–687) are in the C-terminal domain. Since the binding sites on TG2 of the three cross-reactive MAbs are different this finding suggests the presence of multiple epitopes in DGP that can resemble to TG2.

Competition of transglutaminase and DGP for antibody binding

Next, we investigated whether the binding of the TG2-MAbs to DGP was specific and could be inhibited by transglutaminase or its fragments. In the presence of soluble full-length recombinant human TG2 the bindings of MAb1, MAb2, and MAb3 to DGP were completely inhibited and the inhibition was dose dependent. The empty His-construct and TG2 constructs without the binding domains for the MAbs did not inhibit the binding, which indicates that the binding was specific for the specific TG2-binding sequence of the MAbs. Full length recombinant TG2 or any of the MAbs could not inhibit the binding of celiac patient samples to DGP, which indicates that celiac IgA contain additional antibody clones to DGP epitopes that are different from TG2.

5. DISCUSSION

TG2 plays a key role in the pathogenesis of celiac disease as the main autoantigen, it can catalyze the deamidation of gliadin peptides and thus strengthen the immune response. Growing evidence is available that the autoantibodies can have a pathogenetic role as well. Celiac IgA antibodies can enhance the transamidating activity of TG2, alter epithelial transports, disturb angiogenesis, induce morphologic changes and affect the cell cycle in fibroblasts *in vitro*. Identification of the main epitope allowed us to find a specific competitor antibody which offers a research tool to further dissect these pathophysiological processes and also to explore whether interference with these processes at the level of antibody binding could be of therapeutic potential.

In this study three amino acids were identified by molecular modeling as the constituents of the main celiac epitope. The main anchor point of the epitope is Glu153, which is situated in the first α -helix of the catalytic core (II.) domain and can cooperate with Arg19 in the first α -helix of the β -sandwich (I.) domain. Arginin is a good candidate anchor residue in protein-protein interactions in general, and the binding site involving these two above mentioned helices results in the largest solvent-accessible surface area reduction upon binding. The third residue which can take part in the formation of the epitope, is Met659 and it is located in the second β -barrel (IV.) domain. Based on this analysis, mutant TG2s were generated and the binding properties of celiac antibodies from different origins were investigated. Binding of both celiac IgA antibodies and celiac patients-derived monoclonal antibodies to double mutant RE was significantly reduced, while further amino acid changes (triple mutants) did not diminish the binding further, suggesting that changes in two of these amino acids are enough to abrogate the binding of the autoantibodies. Similarly, when TG2 was applied in FBN-bound form, mutation M659S did not alter the antibody binding significantly. In the activated, open conformation of the enzyme the C-terminal residues (both β -barrels) are displaced by 120 Å, while the positions of amino acids Arg19 and Glu153 do not change during the process. This suggests that the identified epitope is independent from the conformational shift occurring upon activation and can be supported by the observation that celiac antibodies could bind to the Ca^{2+} -bound TG2, to TG2 without Ca^{2+} and to the inhibitor-bound TG2 as well.

According to our findings Glu153 and Arg19 effectively determine the epitope; however, only Glu153 seems to be crucial for antibody-binding. Some celiac serum IgAs and a part of the monoclonal scFvs could effectively bind to mutant R19S. This is in line with earlier findings, where proper binding was observed if either the N- or the C-terminal part of TG2 were truncated, but the binding disappeared if both of these or the core domain was lacking. In line with this knowledge we

also showed that the domain mutant lacking the core domain but containing both the N- and C-terminal domains was not a recognizable antigen for celiac antibodies and thus the anchor residue on the core domain is essential for binding.

To confirm that the conformations of our other mutants were not affected by the mutagenesis, activity measurements were performed. The transglutaminase activity of mutant R was 30% higher than the Wt, which proves that the mutant is properly folded. Such increased crosslinking activity could be found in the case of factor XIII for SNP V34L. The activities of the other mutants were variably decreased, some mutations (E and 154K mutations) colocalize with one of the putative Ca²⁺-binding regions of the molecule and this may explain by itself the diminished activity. Three of the mutants (RM, EM, REM) could hydrolyze GTP in a higher extent than the Wt. According to earlier data in the literature the removal of the last 52 amino acids from the C-terminal increases GTP hydrolysis activity. All of these mutants contain the M659S amino acid change, which residue is situated in the C-terminal domain of the molecule, so we may notice a similar enhanced GTPase activity. The FBN-binding capacities of all of the mutants are the same as the Wt enzyme's capacity suggesting the proper folding of the mutants and CD spectra of the key mutants do not show unordered segments.

The identification of several possible anchor points (Arg19, Glu153, Glu154, Met659) of the celiac epitope raises the question whether these amino acids are able to form one joint epitope or these residues represent distinct epitopes. Interestingly, these amino acid changes were important for all natural serum antibodies representing polyclonal antibody populations and intact Arg19 and Met659 in the core domain deficient mutant were not sufficient to ensure binding in the absence of Glu153. Glu153 was also important for all monospecific ScFv antibodies, but they showed variability in the binding properties to mutant R and could be divided into two groups: R-reactive and R-non-reactive antibodies. They could effectively compete with each other from the same, as well as from the other group, which indicates that they bind to the same common epitope. This was true also for the celiac IgA and IgG type antibodies.

Recombinant human TG2 is widely used in the diagnosis of CD in clinical ELISA kits with good sensitivity and specificity. However several studies on TG2-based tests described false positive anti-TG2 IgA results in the absence of IgA endomysium positivity and false negative IgA TG2 results with IgA endomysium antibody positivity. False positive TG2 antibody results are relatively common in the clinical settings and this severely restricts the use of TG2 antibody positivity as the sole diagnostic test. Patients with other autoimmune diseases, tumors, cardiac failure, neurological

disorders, psoriasis and liver diseases may exhibit low levels of antibodies reacting with TG2. This can be explained by the fact that relatively high amount of TG2 can be found in tissues intracellularly, which can be liberated by cell damage and can provoke antibody production independent from CD.

For our experiments, we obtained non-celiac serum samples with anti-TG2 antibodies from patients with other autoimmune diseases. These cases were found to have a diagnosis of systemic lupus erythematosus, rheumatoid arthritis or Sjögren's syndrome. These samples contained antibodies against TG2, but they did not show EMA staining or positivity for DGP antibodies. In line with this, they showed markedly different binding pattern to TG2-mutants, and the alterations at Arg19, Glu153 and M659 did not influence their binding, nor could they be competed out with a TG2 epitope specific monoclonal antibody (MAb 885). It is thus possible to design a new diagnostic kit including wild type TG2 protein in which the celiac epitope is intact and a test TG2 in which the celiac epitope is impaired. As celiac autoantibodies cannot bind to the test molecule or bind only at a reduced level, this type of test can distinguish real CD antibodies from anti-TG2 antibodies in other diseases. Such a kit could be designed on several platforms like ELISA, radioimmunoassay (RIA), immunoprecipitation, or label-free binding assays, like BIACORE for clinical use.

Utilization of the epitope specific ELISA also may help the diagnosis in the preclinical stage of the disease (latent celiac cases), because these patients had antibodies with the same epitope specificity as manifest cases. This subgroup of the disease often called as 'potential' CD also, since patients may present villous atrophy and crypt hyperplasia after several years, whilst other organs can be severely damaged (gluten ataxia, cardiomyopathy, diabetes mellitus, hypothyreosis). Thus, the early diagnosis of these patients is highly important to avoid these associated diseases.

The existence of a single disease-specific autoantigenic epitope also may change our knowledge on celiac disease pathogenesis. Interestingly, even several decades of active disease did not shift the main epitope specificity in CD adults, although epitope spreading is normally a regular feature in many autoimmune disorders. In this respect, peculiar properties of celiac antibodies have been already noted by other groups: all CD antibodies are characterized by the VH₅ usage and the rate of somatic hypermutation is very low for celiac antibodies. We also experienced the stability of the epitope targeting pattern in long-term followed non-compliant patients. These results collectively suggest that gliadin peptides induce a specific reaction toward a particular surface of TG2. Our epitope modeling studies and further experiments with DGP and monoclonal TG2 antibodies show that some gliadin peptides and TG2 can have similar surface properties and suggest that molecular

mimicry could take part in the presentation of some peptides. This possibility does not exclude the hapten-carrier mechanism, rather complements it and can be a triggering initial feature in the disease process. In fact, our experimental results show that celiac patient samples contain IgA antibodies against a number of deamidated gliadin peptides, also against these, which are not cross-reactive with TG2.

Celiac sprue is considered as a life-long disease and unless it is treated increased morbidity and mortality prevails. In fact, compliance with the strict gluten free diet is difficult and additional effective treatment options may be of help to neutralize the consequences of dietary transgressions. The common target epitope of CD-specific autoantibodies raises the possibility that antibody interference could be used in future as an adjuvant therapeutic modality. Here we showed that tissue-deposited celiac autoantibodies, which could be associated with the multi-organ manifestation of the disease can be displaced by a monoclonal antibody recognizing part of the main celiac epitope characterized in this study. Furthermore, this displacing antibody did not have the typical pathologic effects elicited by celiac antibodies in cell culture experiments and could even antagonize the latters. Monoclonal antibody therapy interfering with a key cytokine, growth factor or cell receptors is today a common therapeutic strategy in chronic inflammatory, immune and malignant disorders, like rheumatoid arthritis, Crohn's disease and some tumors. Our results give initial tools to explore such a treatment possibility also in celiac disease.

6. SUMMARY

Celiac disease is a pathologic immune reaction to ingested gluten and it is characterized by gliadin-specific T lymphocytes and the production of disease-specific autoantibodies against the enzyme type-2 transglutaminase (TG2).

In our work we identified the main celiac epitope of TG2, which is conformational and located at the surface of the enzyme where amino acids from three domains of TG2 are close to each other. The epitope is related to one of the Ca^{2+} -binding site of the enzyme, but the Ca^{2+} ions themselves are not involved in the composition of the epitope and in the binding of celiac serum antibodies. Core domain Glu153 and N-terminal domain Arg19 determine the celiac epitope which is accessible both in the closed and open conformation of the protein. Met659 on the C-terminal domain and Glu154 can cooperate in antibody binding. The epitope is relevant and accessible also under tissue conditions when TG2 is bound to fibronectin and was crucial also for coeliac antibodies isolated from tissues or passively transferred to newborns from celiac mothers. Using patient-derived monoclonal single chain antibody fragments we proved in competition experiments that the identified amino acids form one common main epitope. Serum samples from patients with other autoimmune diseases (systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis) contained anti-TG2 antibodies for which this epitope was not important and showed a clearly different binding pattern to mutants with altered celiac epitope. This indicates that the epitope characterized in this study is celiac disease specific. Our findings also show that the main epitope of TG2 is an antigen already in the early (latent) stage of the disease. Monoclonal mouse antibodies with partially overlapping epitope specificity released celiac antibodies from patient tissues but did not alter the enzymatic activity of TG2 and did not cause similar harmful biological effects in cell culture experiments as celiac antibodies. Such antibodies might have a therapeutic potential.

We found that there is a possible structural homology between deamidated gliadin peptides (DGP) and TG2; moreover, non-celiac monoclonal TG2-specific antibodies can recognize DGP. However, according to competition studies celiac IgA also contain additional antibody clones to DGP epitopes that are different from TG2.

Identification of the binding site of celiac antibodies allows the development of even more specific diagnostic tests and interference with celiac antibody binding might offer some additional therapeutic benefit. The three dimensional homology of DGP and TG2 suggests that molecular mimicry can be an important factor in celiac disease pathogenesis.

7. PUBLICATIONS

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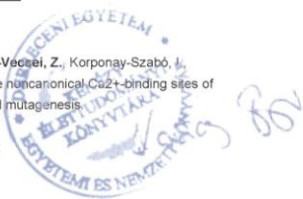
Candidate: Zsófia Simon-Vecsei
Neptun ID: BERGU1
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Simon-Vecsei, Z.**, Király, R., Bagossi, P., Tóth, B., Dahlbom, I., Caja, S., Csósz, É., Lindfors, K., Sblattero, D., Nemes, É., Maki, M., Fésüs, L., Korponay-Szabó, I.: A single conformational transglutaminase 2 epitope contributed by three domains is critical for celiac antibody binding and effects.
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3. Király, R., Csósz, É., Kurtán, T., Antus, S., Szigeti, K., **Simon-Vecsei, Z.**, Korponay-Szabó, I., Keresztessy, Z., Fésüs, L.: Functional significance of five noncanonical Ca²⁺-binding sites of human transglutaminase 2 characterized by site-directed mutagenesis.
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4. Myrsky, E., Caja, S., **Simon-Vecsei, Z.**, Korponay-Szabó, I., Nadalutti, C., Collighan, R., Mongeot, A., Griffin, M., Maki, M., Kaukinen, K., Lindfors, K.: Celiac disease IgA modulates vascular permeability in vitro through the activity of transglutaminase 2 and RhoA.
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