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STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF TRANSGLUTAMINASE 2 IN RELATION TO SIGNAL TRANSDUCTION AND COELIAC DISEASE

Róbert Király

Supervisor:

Prof. László Fésüs, M.D., Ph.D., Member of the H.A.Sc.

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

FACULTY OF MEDICINE, MEDICAL AND HEALTH SCIENCE CENTER

UNIVERSITY OF DEBRECEN

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INTRODUCTION

Coeliac disease (gluten sensitive enteropathy, GSE) is a gluten-induced chronic small intestinal disorder with a significant autoimmune component occurring in genetically predisposed individuals. The GSE is a unique autoimmune disorder because both the environmental trigger (gluten) and the main autoantigen (transglutaminase 2, TG2) are known and the removal of the gluten from the diet leads to complete improving of the disease. GSE has a multifactorial pathogenesis and its real prevalence may be as high as one in 100 individuals and susceptibility to GSE is related to the presence of HLA-DQ2 and DQ8 heterodimers. The clinical manifestations have a broad spectrum. Some patients develop a generalised malabsorption leading to a severe wasting already in early childhood while others may remain asymptomatic until adulthood. Clinically silent GSE can also appear as dermatitis herpetiformis (DH) or other diverse extraintestinal diseases. Today the only accepted treatment of GSE is the lifelong gluten-free diet (GFD) which decreases the high risk of very severe associated diseases.

Human tissue transglutaminase has been identified as the major autoantigen target of GSE-specific autoantibodies. The ubiquitous TG2, a unique multifunctional protein, catalyses the Ca^{2+}-dependent formation of ε-(γ-glutamyl)lysine isopeptide bounds between proteins accompanied by NH_3 release from reactive glutamines (transglutaminase, protein cross-linking) and the hydrolysis of GTP (GTPase, G protein signalling) and has some other enzymatic and activity-independent function. *In vivo* the Ca^{2+}- and nucleotide-binding can regulate reciprocally the enzyme functions of TG2. TG2 is present in various cell compartments including the cell nucleus and the inner and outer part of the cell membrane. It is involved in cell differentiation, apoptosis, phagocytosis, signal transduction, cell adhesion, extracellular matrix assembly, wound healing and is implicated in the pathophisiology of different diseases (coeliac disease, tumor growth and neurodegenerative disorders).

Antibodies in coeliac disease

A specific feature in coeliac disease is the presence of serum IgA-class endomysial antibodies (EmA) targeted against TG2. Although serum EmA has an almost 100% specificity for coeliac disease, approximately 10-20% of untreated coeliac disease patients remain negative for serum EmA. Most studies found that the lack of EmA is associated with mild histological lesions which would contradict the conception that EmA is a marker for early-stage coeliac disease without villous atrophy. The EmA is produced locally in intestine

mucosa and IgA autoantibodies are deposited here intraintestinally and extraintestinally. It could be hypothesised that anti-TG2 antibodies could be present in the small-bowel mucosa of patient with EmA negative untreated GSE. If it is true, the presence of anti-TG2 autoantibodies could be a very specific but not unique feature of GSE.

Biological effects of coeliac autoantibodies

Given the complex biochemical and cellular functions, the effect of anti-TG2 autoantibodies on activities of TG2 may have significance in the pathogenesis of GSE. Antibodies to TG2 induce periductal lymphocytic infiltrates in lacrimal glands of mice and inhibit the differentiation of human T84 crypt epithelial cells in vitro by interfering with TG2-dependent TGF-β activation. The coeliac antibodies induce cell proliferation by promoting the cell cycle S-phase entry of coeliac patient epithelial cells *ex vivo* and increase the permeability of the epithelial layer. These processes lead finally to crypt hyperplasia. Antibodies influence the function of mesenchimal cells inhibiting cell motility and enhancing matrix degradation by increasing expression of matrix metalloproteinases. Toll-like receptor 4 bound autoantibodies activate monocytes and monocyte-mediated cytotoxicity which are facilitated by gliadin and result tissue damage. The mucosal vasculature is disorganised in active coeliac disease which is typical in untreated GSE. Autoantibodies specifically targeting TG2 inhibit several steps of angiogenesis in vitro. 7% of the coeliac patients show neurological manifestation such as neuropathy epilepsy, brain atrophy and gluten ataxia. In patients with gluten ataxia the coeliac antibodies are also deposited in the brain vasculature. The blood-brain barrier has also increased permeability due to the anti-TG2 autoantibodies and the sera from untreated coeliac patients induce neural cell apoptosis, too. The presented results confirm the hypothesis that gluten-induced coeliac specific autoantibodies are important players of intraintestinal and extraintestinal coeliac pathogenesis.

TG2 strengthens the antigenicity of gliadin fragments by deamidation of glutamines which process is critical for T-cell recognition. For this reason, interference of the autoantibodies with the deamidation process could also influence the gluten-triggered immunological damage. In earlier studies, total IgA and IgG fraction of coeliac serum samples decreased transglutaminase activity in cell extract. Subsequent investigators did not find this effect to be significantly different from controls; but affinity-purified coeliac antibodies had moderate inhibiting capacity, which, however, was insufficient to block protein cross-linking. Furthermore, elevated TG2 expression and transglutaminase activity were reported in the subepithelial parts of the coeliac jejunal mucosa, which is the

predilection site for the anti-TG2 antibody deposition *in vivo*. Paradoxically, in earlier studies two kinds of antibodies could be produced against TG2, which either decreased or increased the activity of the enzyme. Therefore, it is a relevant and so far unclarified question how the anti-TG2 antibodies of coeliac patients influence enzymatic activities of this protein.

Ca^{2+}-binding of TG2

The nature of the actual enzymatic activity of TG2 is dependent on its structural state determined by the type and amount of bound ligands similarly to TG3. Liu *et al.* solved the X-ray structure of TG2 in its GDP-bound form. The enzyme consists four sequential domains: N-terminal β-sandwich, active sites containing core and two C-terminal barrels. When comparing the inactive GDP-bound form with the recently published structure of the enzyme which was crystallised with a substrate analogue covalently bound to its active site, a large difference can be observed. Presumably, this large conformational change is induced by Ca^{2+}-binding but the exact sites of bound Ca^{2+}-s in the structure of TG2 have not been determined. Thus search for Ca^{2+}-binding sites of TG2 and their characterisation is current.

Moreover, the Ca^{2+}-binding sites of TG2 could play important role in the binding of coeliac autoantibodies to TG2. Based on the previous results the Ca^{2+}-binding of TG2 is needed to promote the binding of coeliac antibodies to TG2, which process could be notable in the pathogenesis of GSE.

AIM OF THE STUDIES

1. Given the complex biochemical and cellular functions of TG2, the effect of anti-TG2 autoantibodies on these may have significance in the pathogenesis of GSE. The aim was to characterise both transglutaminase and GTPase activity of TG2 in the presence of coeliac antibodies in a detailed biochemical approach. It was also investigated whether the effects of the antibodies on TG2 could be correlated to various clinical manifestations and dietary treatment.

2. It has been supposed that anti-TG2 autoantibodies are always present in coeliac patients, sometimes earlier than the manifestation of the symptoms. Our aim was to confirm this theory by investigating whether TG2-specific IgA deposits can be found in the small-bowel mucosa even in seronegative coeliac patients.

3. Because TG2 is a unique multifunctional protein with Ca- and GTP-dependent activity and the calcium-binding could interact with autoantibody binding our aim was also to identify the exact calcium-binding sites of TG2 using site directed mutagenesis targeting the FXIIIa and TG3 homology sites and 2 other newly recognised sites with negative surface potential.

MATERIALS AND METHODS

IgG and IgA purification

Serum IgA and IgG antibodies were purified from serum samples of 25 endomysial antibody-positive patients with untreated coeliac disease and 3 endomysial antibody-negative non-coeliac control subjects (using Sepharose beads conjugated with protein G and agarose beads conjugated with jacalin). The coeliac patients were selected with the following clinical presentation types: (i) severe malabsorption presenting in early childhood, (ii) adult patients in a good general condition, (iii) adult patients with severe malabsorption, (iv) children with skin biopsy-proven DH, no enteral complaints, (v) IgA deficient children with similar clinical picture as those in group (i). Patients in Group G had only IgG class circulating endomysial antibodies, but not IgA. Patients from group (i) were also studied on a prolonged gluten-free diet period of over one year when they had clinical and histological recovery and were negative or borderline for serum endomysial antibodies. Patient serum samples and biopsies were used with the permission of the Ethical Committee of the Heim Pál Children's Hospital.

Transglutaminase enzyme preparations

Human TG2 enzymes from three different sources were used for our experiments in which we examined the effect of coeliac autoantibodies on TG2 activities. Recombinant TG2 was produced in *E. coli* as a Glutathione S-transferase fusion (GST-fusion) protein. As a source of cellular TG2, a human myeloid leukaemia cell line NB4 treated for 4 days with 1μM all-trans retinoic acid was used. TG2 was also obtained from human red blood cells.

For calcium binding study wild type (WT) recombinant TG2s were expressed in $(His)_6$-tagged form by Rosetta 2 cells subcloned into pET-30 Ek/LIC Vector. Site-directed mutants were constructed using the QuikChange Site-Directed Mutagenesis Kit. Mutant constructs were checked by restriction analysis and DNA sequencing. The $(His)_6$-tagged proteins were purified using ProBond Ni-NTA resin.

The cross-linking activity and purity of proteins were checked by Coomassie BB staining of SDS-poliacrylamide gels and by Western blots.

TG2-ELISA methods

For determination of the TG2-specific antibody content of purified immunoglobulins and the antigenicity and functional purity of mutant TG2 common ELISA methods was used. Wells were coated with the GST-fusion TG2 or the wild type or mutant $(His)_6$-tagged TG2.

The purified immunoglobulins were diluted and applied to the wells in different concentrations for the determination of dilutions which provide normalised TG2-specific antibody content for the experiments. Later two antibody concentrations were tested in transglutaminase activity measurements: (i) 15 µg/ml IgA or IgG where all coeliac antibodies produced maximum absorbance in ELISA ("equal immunoglobulin concentration"), and (ii) the dilutions where the coeliac antibodies produced 50% of the maximum absorbance in the ELISA assay ("normalised antibody concentrations"). The bound TG2-specific antibodies were detected with HRP conjugated rabbit anti-human IgA or IgG (Dako) followed by the addition 3,3',5,5'-tetramethylbenzidine substrate. The colour reaction was stopped with H_2SO_4 and absorbance was read at 450 nm. In antibody binding assays of mutant TG2s the monoclonal antibody-II (TG100) and HRP-conjugated anti-mouse IgG or untreated coeliac serum were used.

Methods for determination of transglutaminase activity

For determination of transglutaminase activity three different methods were used. The microtiter plate assay was based on the incorporation 5-(biotinamido)pentylamine into immobilised N,N-dimethylated casein. Amine incorporation was detected by streptavidine-alkaline phosphatase followed by adding p-nitrophenyl phosphate and measuring absorbance at 405 nm.

NH_3 liberated during the transglutaminase reaction in liquid phase was measured by a coupled glutamate-dehydrogenase reaction and consequent decrease of β-nicotinamide adenine dinucleotide phosphate (NADPH) (UV-test). The change of NADPH concentration was measured by following the decrease of absorbance at 355 nm.

The filterpaper assay is based on the incorporation of [1,4(n)-3H] putrescine into DMC. After precipitating the sample on filterpaper in cold trichloroacetic acid and extensive washings, the [3H]putrescine incorporation was measured using a β-counter.

Activity assay with fibronectin-bound TG2

A modified microtiter plate assay was developed to measure transamidating activity under conditions when only antibodies specifically bound to the enzyme are present. The wells were coated with human fibronectin. After washing the wells were coated with GST-fused recombinant TG2 or NB4 cell extracts. After washings the wells were incubated with jacalin purified total IgA and later the antibodies which did not bind to TG2 were washed

away. Then the wells were incubated with reaction mixture. Amine incorporation was detected as described above.

Investigation of target specificity of small bowel mucosal IgA deposits

Unfixed frozen duodenum sections from serum EmA-positive and EmA-negative coeliac patients were washed to dissolve nonspecific protein complexes. After further washings, the sections were stained for human IgA and TG2 using fluorescein isothiocyanate-labelled rabbit antibody against human IgA and monoclonal mouse antibodies against TG2 (CUB7402) followed by rhodamine conjugated anti mouse Ig antibodies.

Extracellular TG2 was removed from the sections with chloroacetic acid following the KSCN treatment; chloroacetic acid was used to disrupt the binding of TG2 to fibronectin and to remove TG2 from the tissues. The sections were thereafter similarly stained for remaining IgA and TG2.

In order to prove that extracellular IgA deposits in the small bowel of EmA-negative coeliac patients are targeted against TG2 unfixed frozen small bowel sections from coeliac and control patients were incubated with GST-TG2. After extensive washings, GST-TG2 bound to the tissue was labelled red by goat antibodies against GST followed by Alexa Fluor® 594-conjugated chicken antibodies against goat immunoglobulins. Human IgA in the tissue was labelled green as previously described. The used anti-GST antibody did not cross-react with natural TG2 in the tissues. In order to block the binding of GST-TG2 to tissue fibronectin, GST-TG2 was also added to the sections together with the 45kD gelatin-binding fragment of human fibronectin and monoclonal antibodies G92. These antibodies interact with the N-terminus of TG2 where one of the putative fibronectin binding sites is located.

GTPase activity assay and direct photolabeling

GTPase activity was determined by the charcoal method. The hydrolysed [32P]Pi was determined by β-counter. GTP labelling was performed as described by Begg and coworkers.

Molecular modelling and sequence alignment

The X-ray structures of human TG2, TG3 and Factor XIIIa (FXIIIa) were retrieved from the RCSB Protein Data Bank. The graphical analysis was made on Silicon Graphics Fuel workstation using GRASP and Sybyl program packages, RasMol, and VMD. The sequence alignments were performed using ClustalW.

Equilibrium dialysis

Ca^{2+}-binding was measured by equilibrium dialysis using ^{45}Ca isotope and 96-Well Equilibrium Dialyzer Plate. After the equilibration the radioactivity was measured with liquid scintillation counting using Tritosol. The results were normalised to the protein content of the sample determined by Bradford reagent and protein purity which was measured by Alpha Imager's Software. The free Ca^{2+} concentration was calculated by Maxchelator and Fabiato and Fabiato's computer program.

Isothermal titration calorimetry and inductively coupled plasma - optical emission spectrometry

The calcium binding properties of TG2 was measured using isothermal titration calorimetry (VP-ITC MicroCalorimeter, MicroCal) High purity recombinant TG2 was produced by ion exchange (HiTrap Q HP Column) for ITC measurements. The purity of eluted fractions was checked by SDS-PAGE and Coomassie staining. The pure recombinant TG2s were dialysed again ITC buffer with EDTA and then ITC buffer with Chelex 100. The ITC experiments were performed on 25^{o}C. The sample was prepared in the same way for inductively coupled plasma-optical emission spectrometry (ICP-OES). The concentration of calcium stock solution and made buffers was also checked by ICP-OES.

Circular dichroism analysis

Circular dichroism (CD) spectra were recorded on a Jasco-810 spectropolarimeter at 25^{o}C using quartz cuvette. CD deconvolutions were carried out by the continll, cdsstr and selcon3 analysis programs kindly provided by Dichroweb.

Statistical analysis

Analysis of Variance (ANOVA, one way), Wilcoxon signed rank test and Mann-Whitney U-test were used for statistical analysis using Microsoft Excel (Microsoft Inc.) and Analyse-it (Analyse-it Software, Ltd.).

RESULTS AND DISCUSSION

Coeliac antibodies can activate the transglutaminase activity particularly from untreated patients with severe manifestation. This effect could be crucial since we demonstrated that these antibodies are deposited in intra- and extraintestinal tissues of coeliac patients attached to TG2. Moreover, the patients in whom the antibodies were not detected in serum also had TG2 specific antibodies deposited in their bodies. Based on our results it looks that the specific TG2 antibodies are always present in untreated coeliac patients. Since these antibodies can regulate the enzyme activities of TG2, the study of the relation between the antibody binding and the calcium regulation of the enzyme is important and during this work we identified five non-canonical calcium binding sites of TG2.

Effects of coeliac autoantibodies on transglutaminase activity of TG2

TG2 activity and protein have been detected at critical sites of coeliac disease, such as the intestinal brush border and subepithelial compartments. In addition, TG2 is present at extracellular sites all around the body and anti-TG2 autoantibodies have been found deposited also at extraintestinal sites in coeliac patients. The potential effects of deposited antibodies are relevant in the progression of the disease; one special aspect is whether the TG2 dependent formation of modified gliadin peptides of elevated immunogenicity will be maintained. Furthermore, binding of antibodies may compromise interaction of TG2 with fibronectin and integrins disturbing cellular adhesion and spreading.

Coeliac patient antibodies (both IgA and IgG) enhanced transglutaminase reaction velocity of both recombinant and natural TG2 in assays with large and immobilised glutamine acceptor proteins, dose dependent manner (105.4-242.2%). Transglutaminase activity was more enhanced in the presence of IgA from childhood coeliac patients than with IgA from adults. Normalisation for the TG2-specific antibody concentrations in the assays clearly resulted in more homogeneous data compared to assays with the same IgA concentrations indicating that the activating effect of coeliac autoantibodies was directly related to the amount of TG2-specific antibodies. Furthermore, the activating effect of specific anti-TG2 autoantibodies also could be shown by our newly developed assay, where TG2 was bound to fibronectin and only antibodies directly binding TG2 remained in the assay system during the transamidation reaction. This experiment can be considered as equivalent to assays performed with affinity-purified antibodies, but avoids some drawbacks of the biochemical purification

process. This type of assay is the most relevant for substrates encountered in the extracellular matrix, where the enzyme works while being anchored to fibronectin.

The potential mechanism of activating effect

TG2 has a multistep catalytic mechanism where the formation of an acyl-enzyme intermediate and the release of ammonia define the initial velocity, while the reaction of the intermediate with water (deamidation) or with a primary amine to form peptide-bound γ-glutamyl derivatives have different constants. The first part of the reaction was accelerated by both coeliac and non-coeliac immunoglobulins as detected by the release of ammonia in UV-test. Therefore, the specific activating effect of the coeliac antibodies seems to result from their influence on the second part of the reaction.

Antibodies may stabilise conformation and may prevent inactivation by self-cross-linking or by other mechanisms. TG2 is indeed a very sensitive enzyme with rapid loss of transamidating activity in purified form and also outside of cells. Alternatively, antibodies also may facilitate access of substrates by modifying the conformation surrounding the active site. Autoantibody binding may facilitate proper folding of TG2 and assist to refold enzyme molecules which became less active by various reasons, including normal decay. Our findings may be explained by this mechanism, as the activating effect of coeliac antibodies reciprocally correlated with the initial specific activity of our enzyme preparations. The same trend was observed with both recombinant and naturally well folded red blood cell TG2. It is, therefore, unlikely that improper folding of recombinant TG2 in *E. coli* would have been the primary reason for the activating effect. In fact, the increase of TG2 activity has also been observed in the coeliac bowel mucosa, that is, *in vivo*.

The hypothesis that coeliac antibodies primarily act as molecular chaperones also might explain why no significant effect was observed both in this and other studies on TG2 activity where cell extracts were used as the source of the enzyme; under such circumstances other binding partners may already provide sufficient chaperoning for TG2. However, intracellular proteins are not present in the extracellular matrix where antibodies and TG2 interact, therefore the activating effect of autoantibodies may have biological and pathological significance.

Correlation of in vitro antibody effect with the clinical course

We found that IgA antibodies from both children and adults with severe clinical presentation were potent enhancers of transglutaminase activity, and this feature may be

associated with unfavourable clinical outcome of the disease. Furthermore, after clinical recovery on a gluten-free diet, immunoglobulins of the same patients did not show activating effect. The epitope mixture of patients diagnosed as adult may be more complex in consequence of epitope spreading. In our experiments, samples from sick adult patients had less activating effect in normalised concentrations, indicating that the activating antibody population represents a smaller and less dominating fraction in them, compatible with the delayed course of symptom development.

TG2 activity is normally tightly regulated in tissues. Its inappropriate activation may trigger cell death and extracellular matrix deposition and may counteract programmed inactivation of TG2 by nitric oxide during inflammation. Many coeliac subjects are clinically symptom-free despite a flat small-intestinal mucosa, but have increased cell proliferation and matrix turnover. Sustained TG2 activity may lead to a reduced rate of matrix turnover and can induce more severe clinical symptoms. In addition, under such conditions toxic gliadin peptides cross-linked by the enzyme to the matrix will be exposed longer to the immune system. It is still unclear whether autoantibodies can modulate TG2 functions inside the cells.

Effects of coeliac autoantibodies on GTPase activity of TG2

Penetration of IgA autoantibodies into subcellular compartments and into the nucleus has been described in some autoimmune diseases. Therefore, the inhibition of GTPase activity of TG2 observed for the first time in our study also might have some clinical relevance. Further studies are required to clarify whether coeliac antibodies can enter cells and influence cellular signalling in which TG2 participates.

Demonstration of TG2 specificity of IgA deposits

When the small bowel sections were incubated in vitro with human recombinant GST-TG2, binding of GST-TG2 was observed both to coeliac and non-coeliac tissue sections along fibronectin. This nonspecific binding to fibronectin could be blocked by preincubating GST-TG2 with soluble 45 kDa fragment of fibronectin and G92 monoclonal anti-TG2 mouse antibodies. Under these conditions, GST-TG2 bound to coeliac tissue sections in the specific pattern corresponding to deposited IgA, but did not bind to the duodenum sections from non-coeliac controls without extracellular IgA deposition. Small bowel sections from the seven EmA-negative coeliac patients gave similar results to the six EmA-positive coeliac samples. These experiments collectively demonstrate that coeliac IgA antibodies were specifically bound to TG2 target antigen in the duodenum samples of both serum EmA-positive and

serum EmA-negative coeliac patients with considerably high avidity. The EmA-negative coeliac disease patients were older and had more abdominal symptoms and complications than EmA-positive ones, which suggest that they had more advanced coeliac disease. During a long-standing immune reaction antibodies with increasing avidity are produced, which explain why adult coeliac patients may have lower serum EmA levels than children. In such way, long-standing coeliac disease might even result in seronegativity.

It has been proposed that coeliac autoantibodies might have biologic role in the immunopathology of the coeliac mucosal lesion, but the fact that these autoantibodies are not present in the serum of every coeliac patient contradicts this conception. The current result does not exclude the possible importance of autoantibodies in the pathogenesis of coeliac disease, since we showed that autoantibodies (equivalent to EmA) targeted against TG2 were deposited in the small bowel mucosa of even seronegative coeliac disease patients. Moreover, we also could demonstrate that the deposited IgA is functional towards TG2.

The used method could be applied for the diagnosis of seranegative coeliac disease instead of the time-consuming and laborious follow up or gluten challenge and also in the differential diagnosis of autoimmune enteropathy. Since the specific anti-TG2 autoantibodies are present in every coeliac person, it is obvious that they can modify the function of TG2 by interaction with each other, contributing to coeliac pathogenesis.

Calcium-binding of recombinant TG2

We presented that specific anti-TG2 autoantibodies are always present in coeliac disease and influence the enzyme activity of TG2 interacting with each other. Ca^{2+}-binding is needed for transglutaminase activity and can alter the binding affinity of coeliac autoantibodies to TG2. The Ca^{2+}-bound X-ray structure of TG2 has not been known but the exact knowledge of Ca^{2+}-binding sites of the enzyme and its relation with activities of TG2 is very important.

We determined that even after dialysis in EDTA-containing buffer the recombinant wild type TG2 binds tightly about 0.5 Ca^{2+} as measured by ICP-OES. This finding suggests that TG2 has a strong Ca^{2+}-binding site, where it contains a tightly bound Ca-ion which derived from the expression system. The equilibrium dialysis measurements showed that wild type recombinant enzyme can bind 6 Ca^{2+} similarly as known for the native erythrocyte TG2. ITC measurements confirmed our equilibrium dialysis and ICP-OES data. The curve of integrated heats shows 0.5 mol/mol Ca^{2+}-binding to TG2 with high affinity. The next 5 Ca^{2+} bind with very low and comparable affinity to the enzyme. The observed small heat changes

indicate that the enthalpy change of the Ca^{2+}-binding of the next 5 sites is very low. The known difference between the active and inactive form of TG2 suggests a big conformational change during the calcium activation process which could be accompanied by significant entropy change. This could be important in Ca^{2+}-binding and may also explain the small enthalpy change.

In the presence of Ca^{2+} TG2 can be in an active conformation and might work as a transamidase even during the equilibrium dialysis and ITC experiments. Wild type TG2 can crosslink itself in the absence of any other substrates. This process may alter the Ca^{2+}-binding properties of the TG2. Therefore, we examined the Ca^{2+}-bindig properties of C277S, active site mutant TG2 which lacks any transglutaminase activity to clarify the effect of self-polymerisation on Ca^{2+}-binding. Based on equilibrium dialysis data it can still bind approximately 6 Ca^{2+} although the binding is weaker than in case of the wild type TG2. The active site mutant also showed the same ITC response as the wild type enzyme. These results demonstrate that self-crosslinking does not have any significant influence on Ca^{2+}-binding of our recombinant wild type TG2.

Ca^{2+}-binding mutants of TG2

The known Ca^{2+}-binding domains, which are present in a lot of calcium-binding proteins do also not share significant similarities with the Ca^{2+}-binding motifs of transglutaminase family.

Based on the high sequence homology shared by transglutaminases and the available X-ray structures of Factor XIIIa and TG3 and their identified calcium binding sites, we used homology modelling and comparative molecular modelling studies to design 7 TG2 mutants, in which 5 different surface sites were altered by introducing single or multiple point mutations. The site mutants S1 and S3 were chosen based on homology to TG3 Ca^{2+}-binding sites. The S2 mutants were planned on the basis of homology to the Ca^{2+}-binding site of Factor XIIIa, although these have strong similarities to S2 Ca^{2+}-binding site in the TG3. In case of S2 and S3 sites we generated two separate mutants (S2A, S2B and S3A, S3B) since here the suspected calcium binding sites are formed by two opposing loops. S4 and S5 were selected based on surface patches characterised by higher local density of negatively charged amino acids on TG2. Mostly conservative amino acid replacements were performed to target Ca-binding specifically and to prevent significant conformational changes or structural disruptions.

For normalisation of protein expression and purity the binding of a monoclonal antibody (TG100) to each mutant was examined using an ELISA method since antibodies are more sensitive to conformational changes. The native state of the purified proteins were checked by circular dichroism spectroscopy.

Ca^{2+}-binding of mutant TG2 proteins

To compare the Ca^{2+}-binding of wild and mutant enzymes we decided to use 1.7 mM $[Ca^{2+}]_{free}$ in equilibrium dialysis. In case of wild type the exponential part of the binding curve reaches the maximum at this concentration. If the mutants have lower Ca^{2+}-binding properties compared to wild type we could see larger change on the exponential part of the binding curve than on other parts of this curve.

All Ca^{2+}-binding site mutant proteins bind significantly less Ca^{2+} than wild type at 1.7 mM $[Ca^{2+}]_{free}$. The experimental Ca^{2+}-binding values confirmed that each of the five mutagenised sites contributes to Ca^{2+}-binding of TG2. In case of TG2 every binding site is on the core domain of TG2 and they could influence each other leading to an energetically favourable arrangement of the enzyme structure. Our data that mutation of one site leads to the loss of more than one bound Ca^{2+} certainly support the assumption that there is a positive cooperativity among the Ca^{2+}-binding sites of TG2. Ahvazi *et al.* also found indications that the S2 and the S3 sites may cooperate.

Using ICP-OES we tested whether the S1 mutant protein also binds Ca^{2+} tightly after purification. The result clearly showed that the S1 mutant cannot bind Ca^{2+} after dialysis with EDTA while the wild type binds 0.5 mol of Ca^{2+}-ion/mol TG2 under this condition. This result means that TG2 has a Ca^{2+}-binding site with high affinity and this site is the S1.

Ca^{2+}-dependent transglutaminase activity of mutant TG2 proteins

The transglutaminase activity of TG2 is calcium dependent. In accordance, the transglutaminase activity of each mutant decreased to various extent and the S3, S4 and S5 mutants lost their activity completely in the microtiter plate as well as the filterpaper method. At higher Ca^{2+}-concentrations there were no significant increase of the activities which means that this cannot compensate the loss of a specific Ca^{2+}-binding side chains. Transglutaminase activity is inhibited by GTP and GDP and the mutants which have remaining activity showed GTP-sensitivity.

For TG2 the most important regulatory function of bound Ca^{2+} is the initiation of transglutaminase activity. The tightly bound Ca^{2+} on S1 is not enough for transglutaminase

activity in case of TG3. Additional Ca^{2+}-binding to S3 of TG3 is needed to open the active site and to form a substrate channel. In our study the measurable transglutaminase activity of TG2 S1 mutant suggests that although Ca^{2+}-binding to this site is important for this activity binding of Ca^{2+} to other sites also contribute to the induction of an active transglutaminase conformation. Binding of Ca^{2+} to S2 plays only a minor role in the formation of the active state of TG2 because mutation of S2 resulted in the highest residual transglutaminase activity. The loss of S3 calcium leads to an enzyme without transglutaminase activity suggesting that the binding of Ca^{2+} to S3 in TG2 plays a significant role in the induction of transglutaminase activity similarly to the case of TG3.

Ahvazi *et al.* found indications that the S2 and the S3 sites may cooperate in TG3. The S4 and S5 sites may have similar role in the process of fine tuning cooperativity since mutation of these sites also lead to the loss of Ca^{2+}-inducible transglutaminase activity.

How can weak Ca^{2+}-binding sites play such an important role in determining transglutaminase activity when structural measurements did not show significant changes of TG2 after Ca^{2+}-binding? It is a known feature that in the presence of interaction partners – phospholipids in case of C2 domain but it could be any appropriate substrate in case of TG2 – the affinity to Ca^{2+} is higher due to completed coordination spheres of Ca^{2+}. Further study is required to clarify whether substrates, other interacting partner or lipid molecules can regulate Ca^{2+} affinity and activity of the enzyme.

GTPase activity and GTP binding of mutant TG2 proteins

The GTPase activity and GTP binding of the mutant proteins was studied, since it is known that Ca^{2+}-binding influences GTP binding. Based on photoaffinity GTP labelling the S4 and S5 mutants did not bind GTP, S1 and S2A showed lower, while S2B, S3A and S3B similar GTP incorporation when compared to the wild type protein.

GTPase activity of the mutants correlated well with photoaffinity GTP labelling results except for the two mutants, S4 and S5, which showed 1.5-2-fold higher GTPase activity than the wild type protein. Most of the mutants have lower or similar GTPase activity compared to the wild type recombinant TG2. The presence of Ca^{2+} decreased the GTPase activity of proteins, except for S4 and S5, similarly to the wild type enzyme.

In the TG3 structure the Asp-324 amino acid, which coordinates the third Ca^{2+} directly and is located on a loop forming a part of the S3B site, is responsible for a switch between GTP and Ca^{2+}-binding and a different activity. Since Ca^{2+}-binding can decrease GTP binding and GTPase activity in case of TG2, too, the S4 and S5 sites could be responsible for fine

tuning of GTPase activity and the proper regulation of the distinct transglutaminase and GTPase activities. When these two sites could not bind Ca^{2+} GTPase activity of TG2 was not inhibited by increasing calcium concentration. The two mutated sites are sterically close to the hydrophobic pocket for GTP/GDP binding and they may conformationally influence GTP binding and GTPase activity of TG2. The mutations can result in a conformational state speeding up GDP/GTP exchange *via* decreasing the docking time of GTP and facilitating the release of GDP, which ultimately results in higher GTPase activity and lower GTP binding.

Antigenicity of mutant TG2 forms

The coeliac epitopes are conformational and the presence of Ca^{2+} can increase the binding of coeliac autoantibodies to TG2 – though there are some contradictory results. In an attempt to dissolve this controversy our Ca^{2+}-binding site mutants were tested in an ELISA system. The S1-S3 sites do not affect antibody binding because these mutants can equally well bind the coeliac antibodies as wild type. According to our results the S4 and S5 calcium binding sites may be needed to form the coeliac epitope on TG2 because S4 mutant showed significantly lower binding (11.5±8.2 % compared to wild type 100%) than other mutant. This epitope may require some calcium because the S5 mutant (which is in the proximity of S4) also shows decreased antibody binding which can be related to cooperativity between these two sites.

The binding of coeliac autoantibodies to TG2 is influenced by the presence or absence of Ca^{2+} in the case of guinea pig TG2. Therefore, we also examined the effect of Ca^{2+} and GDP on the binding of coeliac autoantibodies to mutant TG2s. The presence of EDTA, GDP or calcium failed to alter the antigenicity of the enzymes.

It has been recently reported that mutation of the transglutaminase catalytic triad of the active site decreased the binding of coeliac autoantibodies to the enzyme. In our experiments coeliac autoantibodies could bind to the C277S mutant with affinity similar to the wild type TG2. The active site of TG2 is buried in a normal inactive state and Ca^{2+}-binding induces the formation of an active conformation in which the substrate also can cover the active site. Moreover, extracellular TG2, which is the target of the autoantibodies, is not active under normal circumstances in spite of high Ca^{2+} concentrations there. All these data and information do not support the notion that the catalytic triad of TG2 has a role in the antigenicity toward coeliac antibodies. Clarification of the involvement of calcium binding sites of TG2 in the formation of the coeliac epitope may help us to understand the role of antibodies in the pathogenesis of GSE and could also be a potential target for therapy.

Evolution of Ca^{2+}-binding sites of transglutaminase enzymes

Members of the mammalian transglutaminase family have evolved through duplication of a single gene and subsequent redistribution to distinct chromosomes. Based on the available and presented data the description of subsequent evolution of the Ca^{2+}-binding sites of the human enzymes can be attempted. Sequence comparison clearly shows that the S2 Ca^{2+}-binding site is conserved in each transglutaminase and this by itself can determine Ca-dependency of transglutaminase activity since FXIIIa has only the S2-equivalent site. Similarly, the prostate enzyme (TG4) seems to have only this site; it is likely that these two secreted enzymes are sufficiently activated by Ca^{2+} through this site in the extracellular space where Ca^{2+} concentration is high. TG1 works in the terminally differentiating keratinocytes where Ca^{2+} concentration rises; sequence data show that in addition to S2 it may have a S1 site, too. It looks that intracellular transglutaminases need more sophisticated Ca^{2+} regulation. We propose that for intracellular transglutaminase activation the S1 site, which binds Ca^{2+} tightly, is essential, since all intracellular forms have potential S1 sites. Actually, sequence comparison suggests that even the red see bream and invertebrate drosophila transglutaminases have the S1 and S2 sites. Sequence comparisons explain why FXIIIa does not have S1: FXIIIa has a positively charged amino acid (Lys) in this calcium binding region. Similar sequence difference may preclude Ca^{2+} binding at the S1 site of TG4. There are some amino acids with apolar or positive side chains at the S3, S4 and S5 regions of FXIIIa, TG1 and TG4 and that of S4 and S5 in case of TG3 suggesting that they do not bind Ca^{2+} there. The S3 site is needed to open the substrate channel in intracellular transglutaminases though TG5 and TG7 probably lost this site; these two enzymes are located on another arm of the phylogenetic tree of transglutaminases as compared to TG2 or TG3 and TG6 and may use another site for this purpose. TG5, TG6 and TG7 also have S4 and S5 sites and therefore may have similar Ca^{2+} regulatory mechanisms as TG2 which perhaps explain how these transglutaminases may compensate for the loss of TG2 in knock-out mice.

SUMMARY

Coeliac disease (GSE) is the most frequent, chronic small intestinal autoimmune disorder with broad spectrum of manifestation in genetically predisposed persons. The main autoantigen of GSE is the transglutaminase 2 (TG2).

We identified that the specific TG2 autoantibodies also exist in small-bowel antibody deposits in seronegative patients. Our study confirms the hypothesis that the anti-TG2 autoantibodies are specific markers of GSE and are present in every coeliac patient.

Our finding raises the possibilities that TG2 and anti-TG2 autoantibodies can play important role in pathogenesis of GSE. We found that immunoglobulins from patients with severe malabsorption enhanced the transglutaminase activity of TG2. This activating effect was dose-dependent, most pronounced with immobilised glutamine-acceptor substrates, and correlated inversely with the basal specific activity of the enzyme and with dietary treatment. A similar activation could be demonstrated also with the TG2-specific fraction of autoantibodies and in transamidation activity assays which use fibronectin-bound TG2 and thereby mimic *in vivo* conditions. These results suggest that coeliac antibodies may stabilise the enzyme in a catalytically advantageous conformation.

GTPase activity of TG2 decreased in the presence of antibodies raising the possibility that inhibition of GTPase activity may affect cellular signalling.

Since the TG2 could be a key player in GSE and the Ca^{2+}-dependent function and structure relations were not completely characterised we examined the Ca^{2+}-binding properties of TG2. We identified 5 non-canonical Ca^{2+}-binding sites, out of which 3 by homology with known Ca^{2+}-binding sites of TG3 and Factor XIIIa and the other 2 with negative surface potentials using site directed mutagenesis. CD spectroscopy, antibody binding assay and GTPase activity measurements indicated that the amino acid substitutions did not cause major structural alterations. ^{45}Ca equilibrium dialysis and isothermal calorimetric titration showed that the wild type and active site deleted enzymes bind 6 Ca^{2+}. Each mutant binds less Ca^{2+} than these and mutation of a site resulted in the loss of more than one Ca^{2+} ions. All mutants were deficient in transglutaminase activity and similarly to the wild type enzyme GTP inhibited remnant activities. Similarly to the wild type form GTPase activities of the mutants were sensitive to Ca^{2+}-concentration except in case of S4 and S5 which exhibited increased GTPase activity. Testing reactivity of Ca^{2+} mutants with coeliac autoantibodies revealed that the S4 site strongly influenced antigenicity and the interaction of autoantibodies with TG2.

PUBLICATION LIST

List of publications used in the thesis:

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