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

Thesis for the degree of doctor of philosophy (Ph.D.)

RÓBERT KIRÁLY

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by

RÓBERT KIRÁLY

Supervisor: 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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ABBREVIATION LIST

AGA – antigliadin antibody

ANOVA – analysis of variance

ATRA – all-trans retinoic acid

BSA – bovine serum albumin

CD – circular dichroism

CTLD – C-type lectin-like domain

DC – dendritic cell

DH – dermatitis herpetiformis

DMC – N,N-dimethylated casein

ELISA – enzyme linked immunosorbent assay

EmA – endomysium autoantibody

FXIIIa – Factor XIIIa

GFD – gluten-free diet

GSE – coeliac disease (gluten sensitive enteropathy)

GST – glutathion-S-transferase

HLA – human leucocyte antigen

HRP – horse radish peroxidase

ICP-OES – inductively coupled plasma-optical emission spectrometry

IEL – intraepithelial lymphocyte

Ig – immunoglobulin

IL – interleukin

ITC – isothermal titration calorimetry

LPMC – lamina propria mononuclear cell

MIC – MHC class I chain-related gene

RBC – red blood cell

SDS PAGE – SDS-polyacrylamide gel electrophoresis

TG2 – transglutaminase 2 (transglutaminase type 2; tissue transglutaminase)

TG3 – transglutaminase 3 (transglutaminase type 3; epidermal transglutaminase)

TLR – Toll-like receptor

1. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A coeliakia (GSE) a vékonybél leggyakoribb, genetikailag fogékony egyénekben kialakuló krónikus autoimmun betegsége, melynek igen sokféle klinikai manifesztációja lehet. A GSE fő autoantigénje az egyedülállóan sokarcú transzglutamináz 2 (TG2).

Kimutattuk, hogy az anti-TG2 antitest lerakódások negatív szerológiai vizsgálatok esetén is jelen vannak a vékonybélben, megerősítve azt a hipotézist, hogy a betegekben mindig képződnek anti-TG2 autoantitestek. Ezért jogosan vetődik fel a lehetőség, hogy a TG2 és az anti-TG2 antitestek fontos szerepet játszanak a coeliakia patogenezisében illetve manifesztációjában. Munkánk alapján a súlyos felszívódási zavarral rendelkező betegek autoantitestei dózisfüggő módon fokozzák a TG2 transzglutamináz aktivitását. Hasonló aktiválás volt megfigyelhető egy *in vivo* körülményeket utánzó tesztben, ahol a fibronektinhez kötött TG2 transzglutamináz aktivitását vizsgáltuk specifikus TG2 ellenes autoantitestekkel. Eredményeink alapján valószínűsíthető, hogy a coeliakiás autoantitestek stabilizálják az enzimet a katalitikusan kedvező konformációban. Az autoantitestek gátolják az enzim GTPáz aktivitását, így befolyásolhatják a TG2 jelátviteli funkcióit is.

A TG2 coeliakia patomechanizmusában lévő szerepének további tisztázásához megvizsgáltuk az enzim Ca^{2+}-kötő sajátságait, mivel annak Ca^{2+}-függő funkciója és az e mögött lévő szerkezeti tulajdonságok befolyásolhatják az autoantitestek kötődését a TG2-hoz. ^{45}Ca equilibrium dialízis és izoterm mikrokalorimetriás titrálás azt mutatta, hogy a vad típus és a keresztkötő aktivitásra nem képes aktív hely mutáns egyaránt 6 Ca^{2+}-t köt. Homológ modellezés, negatív felületi potenciálú helyek kiválasztása és irányított mutagenezis segítségével azonosítottunk 5 különböző Ca^{2+}-kötő (S1-S5) helyet, melyek szerkezetileg nem kanonikus Ca^{2+}-kötő helyek és multiplex kötésre is képesek lehetnek. Minden ezeket érintő mutáns Ca^{2+}-kötő képessége csökkent, annak ellenére, hogy CD spektroszkópia, antitest kötési vizsgálat és GTPáz mérés alapján elmondhatjuk, hogy ezek a mutációk nem okoztak szignifikáns szerkezeti változást. Egy kötőhely mutációja egynél többel csökkentette a kötött Ca^{2+}-ok számát, ami a kötőhelyek közötti kooperativitásra utal. Az S1 hely nagy affinitású kötőhely, a többi alacsony affinitással köti a Ca^{2+}-t. Minden mutánsnak csökkent a transzglutamináz aktivitása, ami GTP-vel gátolható volt. A vad típushoz hasonlóan a GTPáz aktivitások Ca^{2+}-függőek voltak kivéve az S4 és S5 mutánsokat, amelyeknek jelentősen emelkedett a GTPáz aktivitása. Az S4 hely jelentősen befolyásolja a TG2 antigenitását, azaz szerkezeti kapcsolatban van a TG2 coeliakiában fontos epitópjaival és ez az új ismeret lehetőséget ad azok pontosabb lokalizálására.

2. INTRODUCTION

After the end of the last Ice Age, about 10,000 years ago, people learnt that gathering and hunting are not the only ways to get food. During the Neolithic revolution people discovered plant cultivation and started to harvest cereal crops like wheat. People who could not tolerate wheat in their diet became ill with coeliac condition. The coeliac disease 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 remission of the disease.

2.1. COELIAC DISEASE

Coeliac disease, also spelled as celiac disease, non-tropical sprue or gluten sensitive enteropathy (GSE) is a lifelong, gluten-induced, chronic small intestinal disorder with significant autoimmune component occurring in genetically predisposed individuals.

The first report of childhood and adult coeliac disease was written in the first century AD by Aretaeus the Cappadocian, a Greek physician. He illustrated the coeliac state and his work were edited and translated by Francis Adams (1796-1861). The word coeliac, first appears in his translation which derives from the Greek koilia (abdomen). The next clinical description comes from Samuel Gee (1839-1911) in 1888. His suggestion was that the regulation of farinaceous food could be the main part of the treatment. Both Christian A Herter (1865-1910) in 1908 and Sir Frederick Still (1868-1941) in 1918 mentioned that bread can aggravate the symptoms. In 1950 Willem-Karel Dicke (1905-1962) showed that the status of the patients improves if wheat, rye and oats flour were excluded from the diet. JH Van de Kamer as well as Charlotte M Anderson (1915-2002) confirmed this and found that gluten fraction is the toxic part of wheat (Anderson CM *et al.*, 1952). In this way since 1950 the gluten free diet became the treatment of GSE.

John W Paulley (1918-2007) described the inflammatory changes of the small intestine and based on this the biopsy became the standard means of diagnosis. Later, the increasing of IgA level in the coeliac disease (Kenrick KG and Walker-Smith JA., 1970) suggested an immunological pathomechanism.

Today we know that coeliac disease is associated with very wide spectra of diseases and shows significant autoimmune processes. Some pathophysiological aspects of coeliac

disease are well characterised and can be useful to study other autoimmune disorders which make the research of coeliac disease current (Losowsky MS., 2008).

2.1.1. Clinical features of GSE

2.1.1.1. Epidemiology

The evaluated prevalence of GSE has significantly been changed in the last thirty years. The biopsy technique and serology tests have improved and the revealed incidence of disease have increased. Formerly, the number of recognised coeliac patients were low because they were diagnosed for abdominal symptoms which underestimated the true number of patients. Since many patients have atypical and diverse symptoms to determine the real prevalence was difficult (Fasano A and Catassi C., 2001).

The widespreading of highly sensitive and specific serological tests allowed for population screenings and the prevalence was 1:150 in Europe and 1:266 in the world (McLoughlin R *et al.*, 2003).

Recently some very systematic screening studies show even higher prevalence (1:99) in Finnish schoolchildren (Mäki M *et al.*, 2003) and it seems the total prevalence of coeliac disease has doubled in Finland during the last two decades. The data analysis showed that environmental factors are responsible for the increasing prevalence (Lohi S *et al.*, 2007). These environmental factors could be breast feeding and timing of the commencement of gluten ingestion, viral infection that promote the secretion of interferon alpha, and smoking (Green PH and Jabri B., 2003).

The epidemiological changes of GSE suggested to imagine coeliac disease as an iceberg model, built originally by Richard Logan in 1991 (Fig. 1). The tip of the iceberg is the clinically apparent disease. However, up to 90% of patients remain undiagnosed during childhood as clinical symptoms may be absent or non-specific for long time (Ravikumara M *et al.*, 2007). Coeliac disease can appear with mild, severe or atypical symptoms, or it can be present in a silent form with only mucosal lesion. The next part of the iceberg represents the individuals with initially normal small bowel villous architecture with high density of intraepithelial lymphocytes and/or who are positive for tissue antibodies while eating normal amounts of gluten, some of them may progress from this latency to over disease later in life (Mäki M and Collin P., 1997).

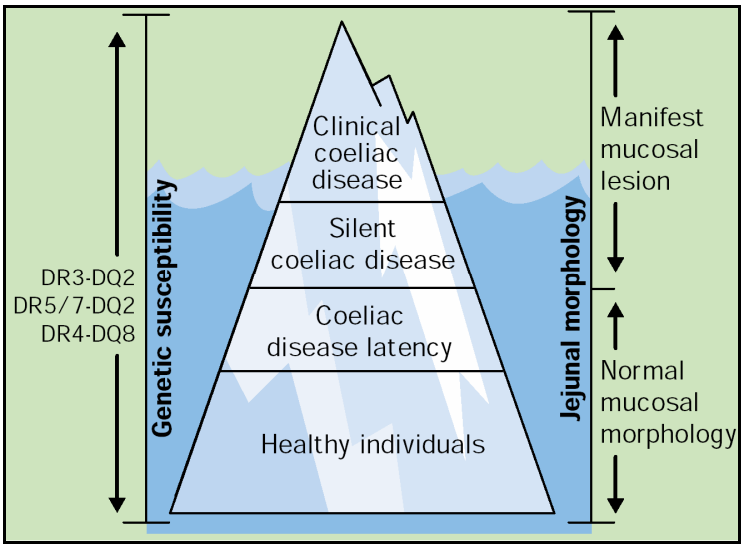


Figure 1. The coeliac disease iceberg. Figure taken from Mäki M and Collin P., 1997.

2.1.1.2. Genetics

Coeliac disease shows high incidence in first degree relatives ranging from 8 to 17.7% (Dube C *et al.*, 2005; Biagi F *et al.*, 2008). Follow-up and twin studies suggest close genetic linkage and the necessity of family screening of coeliac patients. Susceptibility to GSE is related to the presence of HLA-DQ2 and DQ8 heterodimers which are present in more than 90% of coeliac patients (Green PH and Jabri B., 2003). Several other genes were identified which can sensitise the human population for GSE but the role of these genes in the pathogenesis is not clarified. Mainly interleukin genes and immune regulator genes were found to be associated with coeliac disease, but also with other inflammatory conditions (psoriasis, inflammatory bowel disease), suggesting that these are not specific for coeliac disease and only reveal a higher predisposition to inflammation. A genome-wide scan found association of a region in chromosome 5 (5q31-33) to coeliac disease (IL4, IL5, IL9, IL13, IL17B, NR3C1) (Adamovic S *et al.*, 2008). Myosin IXB gene on chromosome 19q13 was also found to be associated with coeliac disease. This gene could be a shared risk factor because it was associated with inflammatory bowel disease, systemic lupus erythematosus and rheumatoid arthritis as well. (Koskinen LL *et al.*, 2008). One other candidate gene, the CTLA-4 gene located on 2q33 is a cell surface protein providing negative signal for T-cell activation. On 2q33 there are some other genes which can also modify the T cell activation (CD28, IL-1 cluster) (Djilali-Saiah I *et al.*, 1998). A recent study also found some associated genes which are important regulators of immune responses including e.g. CCR3, IL12A, CCR3, SH2B3 and TAGAP. Some of these genes

are associated with other autoimmune diseases which suggest that T-cell activation can play a role in every autoimmune disorder (Hunt KA *et al.*, 2008).

2.1.1.3. Clinical manifestations

The clinical manifestation of GSE is very diverse and depends on the age of the patients and the duration and extent of disease. In children the classical form of GSE occurs below the age of 4 years with abdominal distension, diarrhoea, failure to thrive and muscle wasting. Vomiting, irritability, anorexia, pallor and oedema due to hypoproteinemia and even constipation are common symptoms. The untreated disease can cause short stature, rickets, pubertal delay, iron and folate deficiency with anemia (Catassi C and Fabiani E, 1997). In older children or adolescents recurrent abdominal pain, hypertransaminasemia, aphtosus stomatitis, arthralgia, defects in dental enamel or behavioural disturbances such as depression, low performance in school and irritability could be the atypical features of GSE.

In adults GSE is diagnosed at higher rate in women. The relative prevalence is also higher (2.13%) in elderly population aged 52-74 than whole population (Vilppula A *et al.*, 2008). Some patients remember that they had some symptoms in their childhood but many have no history of symptoms which suggest that GSE can develop in adulthood. Typical symptoms are episodic diarrhoea, flatulence, abdominal discomfort, weight loss. Most of the adult patients have only mild or atypical symptoms: abdominal pain, iron-deficiency anemia, vitamin deficiency, osteoporosis, bone fractures, aphtosus stomatitis, infertility, psychiatric and various neurological conditions.

Some patients, who are apparently asymptomatic, could have silent form of GSE with typical flattened intestinal mucosa and sometimes autoantibodies. The common features are iron deficiency, behavioural disturbances, suboptimal fitness and reduced bone mineral density. Some months after the beginning of the diet the patients often report favorable changes in their physical conditions. This suggests that also these silent coeliac cases might benefit of treatment and without that may be exposed to the risk of worsening or to the development of other coeliac associated diseases.

The specificity of circulating serum antibodies for coeliac disease (anti-endomysial and anti-transglutaminase 2 antibodies) is very high (about 99%). The antibody positive persons without pathologic small bowel biopsy have latent or potential coeliac disease with the possibility to develop coeliac symptoms some years later.

Many medical conditions are significantly associated with GSE. Dermatitis herpetiformis (DH) is a skin disorder. It is a chronic, extremely itchy rash consisting of papules and vesicles. A skin biopsy and direct immunofluorescent investigation of the skin are performed in most cases and IgA deposits at the apex of the dermal papillae can be detected. All the patients with DH have HLA-DQ2 or DQ8 haplotype and about 75% have small-bowel villous atrophy with crypt hyperplasia. During a gluten-free diet the mucosal lesion and rash recover and additional dapsone treatment is effective on skin lesion. Based on these findings dermatitis herpetiformis can be a manifestation of GSE or silent GSE (Reunala TL., 2001). It is clear that the risk of autoimmune diseases (type 1 diabetes, autoimmune thyroiditis, Sjögren's syndrome, rheumatoid arthritis) is higher in GSE than general population; however, studies show contradictory results (Viljamaa M *et al.*, 2005; Ventura A *et al.*, 1999).

In some cases very serious complications of GSE may appear: intestinal adenocarcinoma, enteropathy associated T-cell lymphoma or refractory sprue (GSE does not improve on gluten-free diet). Patients with GSE have 80-fold risk for adenocarcinoma compared to the general population. These malignant diseases have very poor prognosis. Considering the possible consequences of gluten intake the correct and early diagnosis is very important (Green PH and Cellier C., 2007; Fasano A and Catassi C., 2001; Farrell RJ and Kelly CP., 2002).

2.1.1.4. Diagnosis of coeliac disease

During the better understanding of the clinical presentation of the disease, more sensitive, specific and less invasive diagnostic methods have been developed. Before 1970 unspecific tests – assays of digestive and absorptive function of the small intestine – were used to confirm the diagnosis of clinically typical GSE. In 1970 the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) formulated the first diagnostic criteria of GSE which required at least 3 phases: (i) confirmation of the clinical diagnosis by duodenal biopsy that should show flat mucosa, (ii) after recovery on a gluten-free diet the second biopsy showing normal mucosa, (iii) third biopsy with flat mucosa due to gluten challenge at the return of clinical symptoms or other signs of disease. In 1990, these criteria were simplified and the duodenal biopsy that shows characteristic small-bowel mucosal lesion with villous atrophy, crypt hyperplasia, intraepithelial lymphocytosis and the clinical recovery on a gluten-free diet remained only necessary. The positive

serological tests are not essential but suggestive to perform biopsy (Walker-Smith JA *et al.*, 1990).

To describe histologically the degree of abnormalities in the coeliac mucosa the Marsh classification was established (Fig. 2). Marsh stage 0 is the normal mucosa; in Marsh stage 1 the number of intra-epithelial lymphocytes is increased, usually exceeding 20 per 100 enterocytes; in Marsh stage 2 crypts of Lieberkuhn begin to proliferate which can lead to partial or complete villous atrophy in Marsh stage 3. Marsh stage 4 indicates hypoplasia of the small bowel architecture (Marsh MN., 1992).

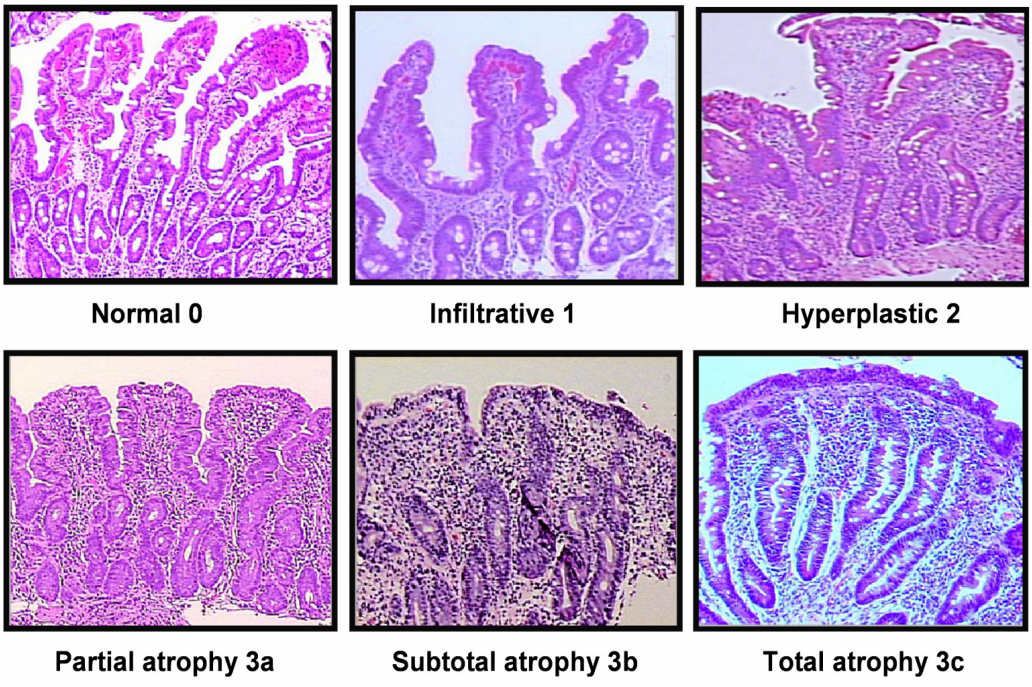


Figure 2: The different stages of coeliac disease based on Marsh classification. Figure taken from Horvath K: Recent Advances in Pediatrics, 2002.

After the identification of immunological aspects of GSE the development of serological tests helped to learn that the GSE can occur during adulthood. One is the antigliadin antibody (AGA) test which seemed to be promising by measuring AGA IgG and IgA but later did not prove to be satisfactory in clinical practice. However, combined use of gliadine-derived nonapeptides (PLQPEQPFP and PEQLPQFEE) had 94% diagnostic efficiency compared with the 81% diagnostic efficiency of conventional gliadin antibody test (Schwertz E *et al.*, 2004).

The presence of serum endomysium autoantibody (EmA) is almost 100% specific for coeliac disease and correlates with the degree of villous atrophy, even if the subject does not have any symptoms. Today this is the most widespread and accepted method of serological coeliac tests (Sategna-Guidetti C *et al.*, 1993).

The discovery of the main autoantigen in coeliac disease (Dieterich W *et al.*, 1997) led to the development of enzyme-linked immunosorbent assays (ELISA). In these assays guinea pig TG2 and later recombinant human TG2 were used for coating as antigen. These new ELISA tests are easier, cheaper, do not need high qualification for testing and show high specificity (95-98%) and sensitivity (90-98%) (Korponay-Szabó IR *et al.*, 2008). Moreover, based on the interaction of autoantibodies and TG2 there is commercial point-of-care quick test from fingertip blood which can help to explore new cases and monitoring the diet less invasively (Korponay-Szabó IR *et al.*, 2005).

Recently a new commercial ELISA test using deamidated gliadin peptide antigens was developed. This test seems to work as well as transglutaminase based serological tests suggesting structural similarities between gliadin derived antigens and transglutaminase 2 (Schwertz E *et al.*, 2004; Korponay-Szabó IR *et al.*, 2008).

Case with negative serological test or equivocal biopsy results are challenging and in these circumstances HLA typing may be of help. If the subject does not possess HLA-DQ2 or HLA-DQ8 haplotypes the chance of GSE is very small.

2.1.1.5. Patients without autoantibodies?

The recent serological tests (mainly EmA) work with very high specificity and selectivity and they give reliable data for diagnosis; still about 10-20% of untreated patients are negative for serum EmA (McMillan SA *et al.*, 1991; Dickey W *et al.*, 2000). Most studies found that the lack of EmA is associated with mild histological lesions (Abrams JA *et al.*, 2004; Tursi A *et al.*, 2001; Rostami K *et al.*, 1999) which would contradict the conception that EmA is a marker for early-stage coeliac disease without villous atrophy (Kaukinen K *et al.*, 2001).

EmA-binding patterns closely correspond to the distribution of TG2 in tissues and EmA and anti-TG2 antibody titers show correlations (Korponay-Szabó IR *et al.*, 2000; Korponay-Szabó IR *et al.*, 2003; Sulkanen S *et al.*, 1998; Mäki M *et al.*, 2003) indicating the TG2 is the target antigen for the EmA reaction. EmA is produced locally in intestinal mucosa (Marzari R *et al.*, 2001; Piccarelli A *et al.*, 1996) and IgA autoantibodies are deposited here intraintestinally and extraintestinally (Korponay-Szabó IR *et al.*, 2004). 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 and uniform feature of GSE.

2.1.1.6. Treatment

The only accepted treatment of GSE is the lifelong gluten-free diet (GFD). This means the elimination of wheat, rye and barley from the diet. Oats are not uniformly recommended due to possible contamination with other cereals. The gain of knowledge about coeliac pathogenesis leads to some attempts to develop non dietary therapies (Dewar D *et al.*, 2004).

2.1.2. Pathogenesis of coeliac disease

The pathogenesis of coeliac disease is multifactorial. It results from the multiple interaction of gluten, immune, genetic and environmental factors. The early initial administration of gluten before 4 month of age increased the risk of disease development. Probably, gluten introduction together with breast feeding after 7 months can minimise the risk of coeliac disease. Gastrointestinal infection and rotavirus infection also increase the risk of GSE (Persson LA *et al.*, 2002; Ivarsson A *et al.*, 2002; Stene LC *et al.*, 2006).

2.1.2.1. The trigger: gluten

Gluten is the protein fraction of cereals which is responsible for cohesion and stickiness properties essential to bake bread. The ethanol soluble fraction of gluten consists of prolamins and insoluble glutenins. These prolamins: gliadin (wheat), secalin (rye) and hordein (barley) are the major triggering factors. Avenin, the prolamin of oats is not toxic, and in small amount can be tolerated (Srinivasan U *et al.*, 1996) but some patients who introduced oat in their diet showed coeliac symptoms (Lundin KE *et al.*, 2003).

Gliadin is a heterogeneous mixture of proteins containing at least 40 components which are divided into α-, β-, γ- and ω-gliadins. Gliadins contain very high amounts of glutamine and proline residues similarly to secalin and hordein. These amino acids increase the resistance of gliadin peptides to intestinal digestion. Interestingly, a 33-mer peptide was identified, which is resistant to degradation by gastric pancreatic and intestinal proteases and could be the primary initiator of the inflammatory response to gluten in GSE.

This peptide is a very good substrate of the transglutaminase 2 and potent stimulator of gut-derived T-cell clones (Shan L *et al.*, 2002).

Gliadin can increase the zonulin release of enterocytes which is a modulator of the tight junction permeability affecting through intracellular signalling of enterocyte. The 33-mer and other oligopeptides of gliadin can pass through the intestinal barrier paracellularly (Clemente MG *et al.*, 2003). The intestinal infection also can increase the permeability of intestinal barrier. Gliadin peptides getting into the epithelium and lamina propria interact with TG2 and antigen presenting cells (Green PH and Cellier C., 2007).

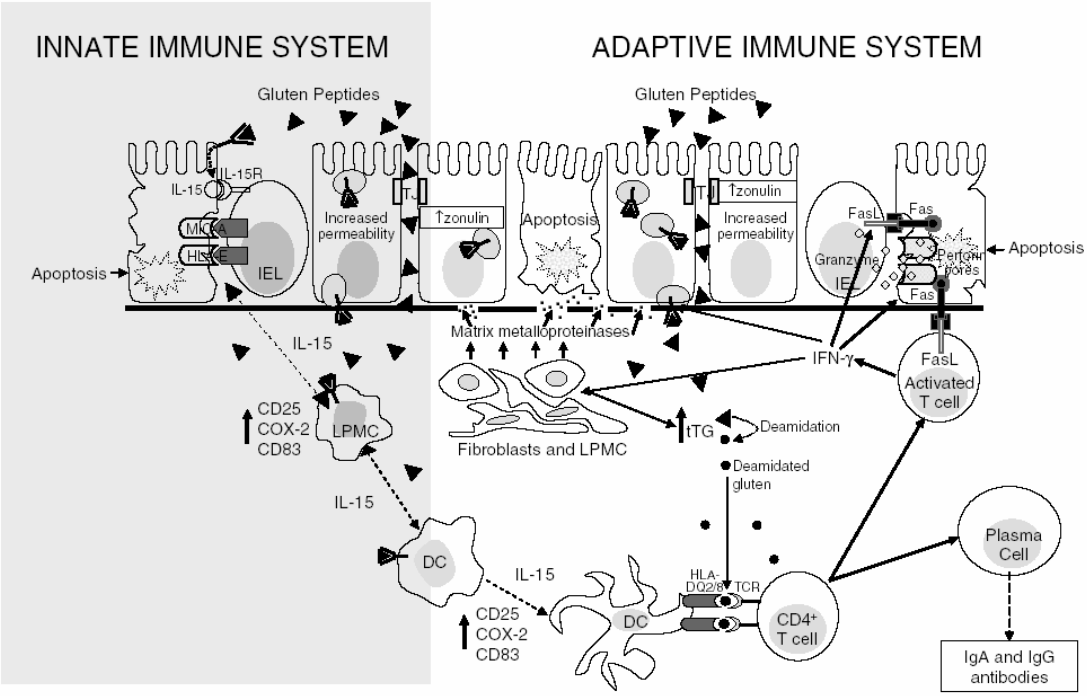


Figure 3. Epithelial transport and immune recognition of gluten in coeliac disease. Figure taken from Ciccocioppo *et al.*, 2005. (DC, dendritic cell; FasL, Fas ligand; HLA, human leucocyte antigen; IL, interleukin; IEL, intraepithelial lymphocyte; LPMC, lamina propria mononuclear cell; TCR, T cell receptor; TJ, tight junction; tTG, transglutaminase 2)

2.1.2.2. Immune responses in mucosa, innate immune response in coeliac disease

The different gliadin peptides play different roles in the coeliac pathogenesis. Several gliadin fractions and synthesised gliadin-derived oligopeptides were tested determining their toxic or immunogenic properties with or without TG2 treatment. When some fragments cause damage on proximal and distal intestine without specific T-cell activation it is known as the toxicity of gliadin, and when the fragments specifically induce the T-cell lines it is called gliadin immunogenicity (Ciccocioppo R *et al.*, 2005).

In the early phase of the disease several gliadin fragments can induce an innate immune response. Because the specific immune response is HLA-DQ2 and –DQ8 dependent but not every subject who carries this HLA fall ill, Londei *et al.* hypothesise that the key moment in the pathogenesis of GSE is the innate immune response and the key effector molecule is interleukin-15 (IL-15) which could be a potent target in therapy of refractory coeliac disease and T-cell lymphoma (Londei M *et al.*, 2005).

In active GSE the transport of toxic and immunogenic gliadin fragments is increased transcellularly and paracellularly. After gluten exposure IL-15 was rapidly induced only in coeliac patients and it was produced mainly by monomyeloid cells and epithelial cells. IL-15 is involved in the migration and expansion of intraepithelial lymphocytes (IEL) which is abnormally increased in GSE (Maiuri L *et al.*, 2001). The gliadin fragments carried in enterocytic vesicles can get through the basal membrane and activate the dendritic cells (DC) and lamina propria mononuclear cells (LPMC) which will produce IL-15 and other stress protein. Thus, under stress and inflammatory stimuli enterocytes express MHC class I chain-related gene (MIC) and HLA E molecules which were recognised by natural killer receptors of intraepithelial lymphocytes resulting apoptosis of enterocyte (Fig. 3).

2.1.2.3. Adaptive immune response in coeliac disease

Other extracellular TG2 deamidated gliadin fractions are bound to HLA class II molecules DQ2 or DQ8 on antigen-presenting cells and will be recognised by gliadin-reactive CD4+ T-cells in the lamina propria. The peptide binding of DQ2 is well characterised. Studying the interaction between DQ2 molecule and epitope from γ-gliadin using crystallography study it was recognised that negative charge is preferred in the anchor of the peptide at some positions. Gliadin does not have significant amounts of negative charged amino acids but the deamidation of glutamine residues of gliadin by TG2 results in negative glutamic acids in the appropriate position. Several native and TG2 treated deamidated gliadin fragments were tested and the deamidated gliadin peptides have higher immunogenecity than non deamidated ones (Dewar D *et al.*, 2004).

After presenting the bound deamidated gliadin peptide by DC, T-cells produce proinflammatory cytokines, mainly interferon-γ and stimulate both cytotoxic T-cells and fibroblasts to release metalloproteinases. These processes lead to the damage of extracellular matrix and basal membrane. Fas ligand and granzyme produced by stimulated T-cells cause also enterocyte apoptosis. Characteristic crypt hyperplasia and villous injury

is attributed mainly to this T-cell activation, but detailed steps are not fully known. Activated CD4+ cells induce lymphocyte B differentiation to antigen producing plasma cell resulting specific anti-gliadin and particularly anti-TG2 autoantibodies (Fig. 3) (Ciccocioppo R *et al.*, 2005).

The production of anti-TG2 autoantibodies in the intestine and their spreading in the whole body alone suggest that the multifunctional TG2 could be involved in the development and manifestation of coeliac disease.

2.1.3. Role of autoantibodies in the pathogenesis of coeliac disease

2.1.3.1. Production of autoantibodies

Circulating antibodies are demonstrated for instance against gliadin peptides, desmin, actin, calreticulin in coeliac patients, but anti-TG2 antibodies are the most typical and specific for coeliac disease (Shaoul R and Lerner A., 2007). It is not clear whether in coeliac disease there are diverse types of antibodies composing a pool which contains antibodies with different specificity or at the beginning of GSE there are antibodies against certain viral or gliadin peptides and these show epitope spreading and cross-reactivity with some self protein which share homology with the first antigen (Zanoni G *et al.*, 2006).

Earlier the presence of autoantibodies was thought to be an epiphenomenon of GSE. Interestingly, the trigger of the GSE is the gliadin but the autoantibodies are targeted particularly against a self-protein, TG2. Already in 1994 arose the hapten-carrier model (Mäki M., 1994). The gliadin can be complexed to the coeliac autoantigen, TG2 (Dieterich W *et al.*, 1997; Fleckenstein B *et al.*, 2004) and both can be processed together by antigen presenting cells. Then the autoantibodies direct mainly against self TG2 and not only gliadin because gliadin peptides are bound to TG2 and neo-epitopes of TG2 can become uncovered based on the recently published X-ray structure of TG2 together with inhibitor mimicking gliadin peptides (Pinkas DM *et al.*, 2007).

The molecular mimicry is another theory: Initially the antibodies might be targeted against gliadin peptides and appear early during the development of coeliac autoimmunity. The deamidated gliadin peptides share common epitopes with TG2 and have similar three-dimensional appearance to epitopes on TG2. Through molecular mimicry after epitope spreading TG2 becomes the main autoantigen and an autoimmune disease can be detected (Korponay-Szabó IR *et al.*, 2008). In patients with dermatitis herpetiformis anti-TG3 is

present (Sárdy M *et al.*, 2002) and in patients with gluten ataxia anti-TG6 autoantibodies are demonstrated (Aeschlimann D *et al.*, 2007). The appearance of the antibodies against the other members of the transglutaminase family may also explain the numerous extraintestinal manifestation of GSE. Interestingly, Zanoni *et al.* demonstrated that coeliac antibodies can bind to VP-7 protein of rotavirus and human Toll-like receptor 4 (TLR4). The rotavirus can cause gastroenteritis inducing immunity and might also initiate coeliac disease by molecular mimicry (Zanoni G *et al.*, 2006).

The autoantibodies are produced locally in the intestinal mucosa (Marzari R *et al.*, 2001) and can be present in the circulation and deposited in the intestinal mucosa and around the blood vessels. Patients in early phase of GSE with normal villous morphology and even seronegativity also have these deposits on normal gluten containing diet (Korponay-Szabó IR *et al.*, 2004; Kaukinen K *et al.*, 2005; Salmi TT *et al.*, 2006).

2.1.3.2. Biological effects of autoantibodies

The coeliac autoantibodies have specific biological effects. In experimental studies, antibodies to TG2 induced periductal lymphocytic infiltrates in lacrimal glands of mice (Freitag T *et al.*, 2004) and inhibited the differentiation of human T84 crypt epithelial cells in vitro by interfering with TG2-dependent TGF-β activation (Halttunen T and Mäki M., 1999). The coeliac antibodies induce cell proliferation by promoting the cell cycle S-phase entry of coeliac patient epithelial cells *ex vivo* (Barone MV *et al.*, 2007) and increase the permeability of the epithelial layer. These processes may lead finally to crypt hyperplasia. Antibodies influence the function of mesenchymal cells inhibiting cell motility and enhancing matrix degradation by increasing expression of matrix metalloproteinases. TLR4 bound autoantibodies activate monocytes and monocyte-mediated cytotoxicity which is facilitated by gliadin and results tissue damage. Based on these, Zanoni and coworkers suggested that TG2 specific autoantibodies might be a link between the innate and adaptive immune response (Zanoni G *et al.*, 2006; Lindfors K *et al.*, 2008). 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 (Myrsky E *et al.*, 2008). 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 sera from untreated coeliac patients induce neural cell apoptosis, too.

The presented results support the importance of gluten-induced coeliac specific autoantibodies in the intraintestinal and extraintestinal pathogenesis of coeliac disease.

2.1.3.3. Effect of coeliac antibodies on activity of TG2

Given that the autoantibodies attach *in vivo* in the gut and other organs to their autoantigen, human TG2 (Korponay-Szabó IR *et al.*, 2004; Salmi TT *et al.*, 2006; Dieterich W *et al.*, 1997), the effect of anti-TG2 autoantibodies on TG2 functions may have significance in the pathogenesis of GSE due to the complex biochemical and cellular functions of TG2 (see later in the 2.2 chapter of dissertation). In physiological condition the transglutaminase activity is latent within the cells, but prominent in the extracellular matrix, where TG2 works in a fibronectin-bound form (Verderio EA *et al.*, 2003). TG2 strengthens the antigenicity of gliadin fragments, which interact with TG2 with higher selectivity than other known natural substrates, by deamidation of glutamines which process is critical for T cell recognition in coeliac disease (Molberg O *et al.*, 1998; Arentz-Hansen H *et al.*, 2000; Shan L *et al.*, 2002). For this reason, interference of the autoantibodies with the deamidation process could also influence the gluten-triggered specific immunological damage.

In earlier studies, total IgA and IgG fraction of coeliac serum samples decreased transglutaminase activity in cell extract (Esposito C *et al.*, 2002). 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 (Roth EB *et al.*, 2003; Dieterich W *et al.*, 2003). Furthermore, elevated TG2 expression and transglutaminase activity were reported in the subepithelial parts of the coeliac jejunal mucosa (D'Argenio G *et al.*, 1989; Esposito C *et al.*, 2003), which is the predilection site for the anti-TG2 antibody deposition *in vivo* (Korponay-Szabo IR *et al.*, 2004).

Paradoxically, in earlier studies two kinds of antibodies could be produced against TG2, which either decreased or increased the activity of the enzyme (Fesus L and Laki K., 1977). Therefore, it is a relevant and so far unclarified question how the anti-TG2 antibodies of coeliac patients influence the enzymatic activities of this protein. It is also a question whether the effects of the antibodies on TG2 could be correlated to various clinical manifestations and dietary treatment.

2.2. TRANSGLUTAMINASES

The first transglutaminase was identified and published by Heinrich B Waelsch (1905-1966) in 1957 (Sarkar NK *et al.*, 1957). Since then there have been more than 5000 article and nearly 500 review connections with transglutaminases.

2.2.1. Transglutaminase family

Transglutaminases (protein-glutamine γ-glutamyltransferases, EC 2.3.2.13) are a family with high homology of acyl-transfer enzymes that catalyses various posttranslational modifications for instance transamidation (crosslinking, amine incorporation, acylation), esterification, hydrolysis (deamidation, isopeptide cleavage). Ca^{2+}-dependent formation of (γ-glutamyl)polyamine bounds between γ-carboxamide group of glutamine and ε- amino group of peptide bound lysine or polyamines is a particularly important activity of transglutaminases. Forming isopeptide bound this enzyme group is very important for instance in blood coagulation, skin-barrier formation, apoptosis, wound healing, extracellular-matrix assembly (Lorand L and Graham RM., 2003).

Table 1. Genetically identified human transglutaminases. Table taken based on Lorand L and Graham RM., 2003

Protein	Synonyms	Residues (Mr, kDa)	Tissue expression	Localization	Function	Gene	Gene map locus	Inherited disease
FXIII subunit A (FXIIIa)	Fibrin-stabilizing factor, Laki-Lorand factor, Pro-fibrinoligase, plasma pro-TG	732 (83)	Platelets, astrocytes, dermal dendritic cells, chondrocytes, placenta, plasma, synovial fluid	Cytosolic, extracellular	Blood coagulation, bone growth	F13A1	6p24-25	FXIII deficiency
TG1	TG_K, keratinocyte TG, particulate TG	814 (90)	Keratinocytes, brain	Membran cytosolic	Cell-envelop formation	TGM1	14q11.2	Lamellar ichthyosis
TG2	TG_C, tissue TG, endothelial TG, erythrocyte TG, Gh, Ghα	686 (80)	Ubiquitous	Cytosolic, nuclear, membrane, cell surface, extracellular	Multiple	TGM2	20q11-12	Unknown
TG3	TG_E, epidermal TG, callus TG, hair follicle TG, bovine snout TG	692 (77)	Squamous epithelium, brain	Cytosolic	Cell-envelop formation	TGM3	20q11-12	Unknown
TG4	TG_P, prostate TG, vesiculase, dorsal prostate protein 1 (DP1), major androgen-regulated prostate secretory protein	683 (77)	Prostate	Unknown	Semen coagulation in rodents	TGM4	3q21-22	Unknown
TG5	TG_X	719 (81)	Ubiqitous except for the CNS and lymphatic system	Unknown	Unknown	TGM5	15q15.2	Unknown
TG6	TG_Y	Unknown	Unknown	Unknown	Unknown	TGM6	20q11	Unknown
TG7	TG_Z	710 (?)	Ubiquitous	Unknown	Unknown	TGM7	15q15.2	Unknown
Band 4.2	B4.2; ATP-binding erythrocyte membrane protein band 4.2	690 (72)	Red blood cells, bone marrow, fetal liver and spleen	Membrane	Membrane skeletal component	EPB42	15q15.2	Hereditary spheocytosis

Based on the reaction mechanism transglutaminases are members of the papain like superfamily of cystein proteases (Makarova KS *et al.*, 1999) which have a highly conserved catalytical triad of Cys-His-Asp or Cys-His-Asn. The enzyme family has eight enzymatically active and one inactive members based on the human genome (Table 2). The 4.2 band protein is inactive due to substitution of active site cystein for alanin and is found in erythrocyte membrane. The lack of 4.2 band protein resulted in haemolytic anaemia (Sung LA *et al.*, 1992). The Factor XIIIa (FXIIIa) plays pivotal role in blood coagulation (Takahashi N *et al.*, 1986), TG1, TG3 and TG5 take part in the cell envelop formation of skin (Hitomi K, 2005), TG4 is essential in fertility in the seminal fluid (Dubbink HJ *et al.*, 1996). The functions of TG6 and TG7 have not been characterised, yet (Griffin M *et al.*, 2002).

2.2.2. Transglutaminase 2

Transglutaminase 2 (TG2), also known as tissue transglutaminase, transglutaminase c or G_h protein, is a unique multifunctional protein with diverse biological functions. Human TG2 contains 687 amino acids and is a 76 kDa protein. 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, spreading, angiogenesis, wound healing and is implicated in the pathophysiology of different diseases (coeliac disease, tumor growth and neurodegenerative disorders) (Fesus L and Piacentini M., 2002; Griffin M *et al.*, 2002; Lorand L and Garham RM., 2003). Interestingly, $TG2^{-/-}$ mice show normal phenotype, but moderate glucose intolerance, defective clearance of apoptotic cells and decreased fibroblast and granulocyte function and wound healing were demonstrated (Szondy Z *et al.*, 2003).

TG2 has several kinds of enzymatic activities. Originally, TG2 was recognized as Ca^{2+}-activated transglutaminase enzyme (Sarkar NK *et al.*, 1957) performing post-translation protein modification by incorporation of small amines into proteins (Fig. 4). It can also form ε-(γ-glutamyl)lysine isopeptide bounds between polypeptide chains, and deamidate glutamine side chains at low pH and the absence of appropriate substrates accompanied every one by NH_3 release from reactive glutamines. Only under test tubes condition TG2 demonstrate isopeptidase activity (Folk JE., 1983; Fesus L and Piacentini M., 2002).

In connection with transglutaminase activity of TG2 Balajthy *et al.* published that inhibition of transamidation activity of TG2 during the differentiation process leads to a decreased amount of protein cross-links in the nucleus and inhibit the development of certain neutrophil cellular functions and the neutrophils of $TG2^{-/-}$ mice show decreased response to inflammatory stimuli.

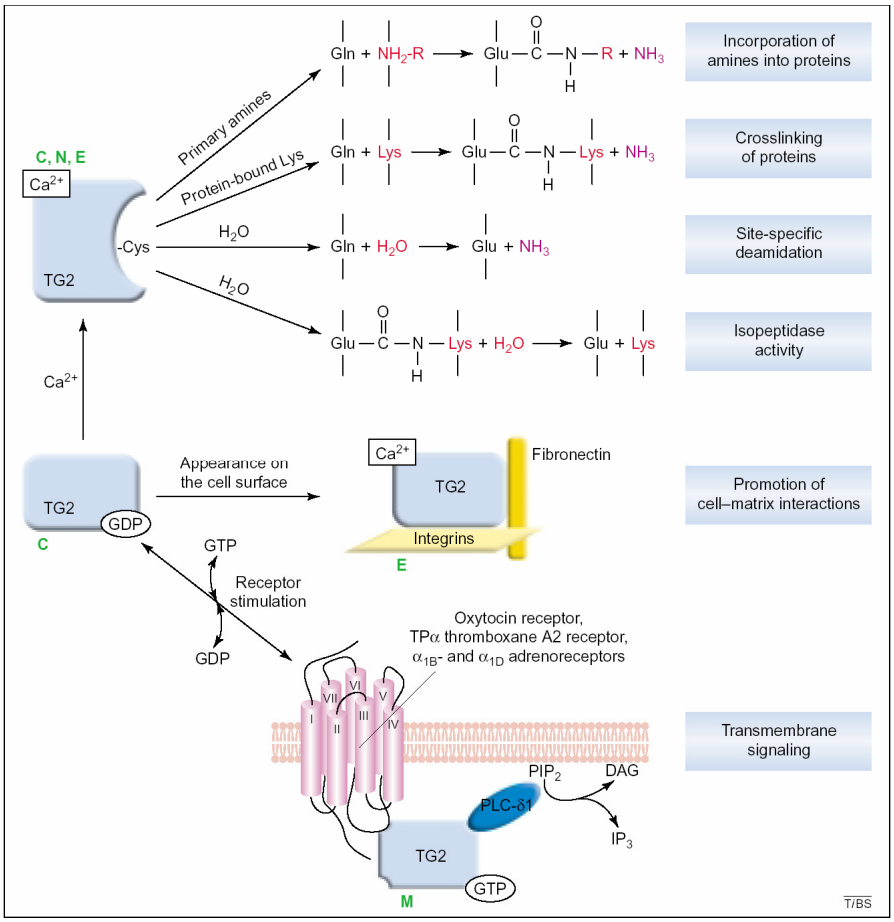


Figure 4. Classical biochemical activities of transglutaminase 2. Figure taken from Fesus L and Piacentini M., 2002. The explanation of the figure could be find in the text.

GTP and GDP inhibit the transamidating activity of the enzyme (Achyuthan KE and Greenberg CS., 1987; Bergamini CM *et al.*, 1987). Later, the ability of TG2 to hydrolyze GTP and ATP (Lee KN *et al.*, 1989; Lai TS *et al.*, 1996), and its role in mediating signal transduction through G-protein coupled receptors based on GTPase activity was also discovered (Im MJ *et al.*, 1997). In addition, TG2 demonstrate protein kinase (Mishra S and Murphy LJ., 2004; Mishra S *et al.*, 2006) and protein disulfide isomerase (Hasegawa G *et al.*, 2003) activities, too. TG2 also acts as a BH3-only protein

interacting with proapoptotic factors by a mechanism which is not entirely clarified yet (Verderio EA *et al.*, 2003; Rodolfo C *et al.*, 2004). Fibronectin-bound surface TG2 serve as a co-receptor for integrins (Akimov SS *et al.*, 2000) playing an important role in the adhesive functions of cells (Zemskov EA *et al.*, 2006).

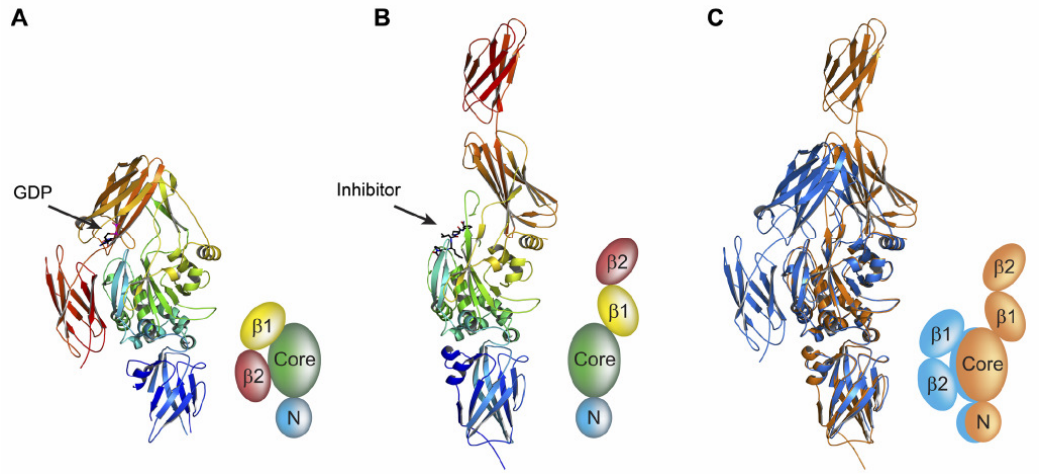


Figure 5. Structures of GDP- and inhibitor-bound TG2. The crystal structures of GDP-bound (A) and inhibitor-bound (B) form of TG2. (C) The N-terminal β-sandwich and catalytic core domains of the two structures are superimposed, highlighting the conformational change. The GDP-bound structure is shown in blue and the inhibitor-bound structure in gold. Figure taken from Pinkas *et al.*, 2007.

2.2.3. Structure 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 (Bergamini CM., 2007) similarly to TG3 (Ahvazi B *et al.*, 2003). TG2 can exist in a closed (compacted) and open conformation. Liu *et al.* solved the X-ray structure of TG2 in GDP bound form (Liu S *et al.*, 2002). The enzyme consists four sequential domains: N-terminal β-sandwich, catalytical core and two C-terminal β-barrels. The active site (Cys-277, His-335 and Asp-358) is at the base of a cavity and is buried by two loops. One of the loops contains Tyr-516 which is conserved in transglutaminases and forms hydrogen bond with Cys-277 that blocks the entry of substrates until activation. The Trp-241 is another well conserved residue of transglutaminases and is essential in the catalysis of transglutaminase reaction stabilising the transition state and the intermediates.

On the opposite side to the catalytical triad, TG2 has a unique guanidine nucleotides-binding site which is located in a hydrophobic pocket formed by the core and

the first β-barrel (amino acids 476-482 and 580-583 of first β-barrel and Lys-173 and Phe-174 on core domen). Begg and coworkers published that GTP binding to Arg-580 is critical for TG2 transition to the compact, catalytically inactive conformation and the Cys-277-Tyr-516 interaction increase the rate of this transition. Preventing the GTP binding of Arg-580 by site-directed mutagenesis abolishes the regulator function of guanidine nucleotides in intact cells (Liu S *et al.*, 2002; Iismaa SE *et al.*, 2003; Begg GE *et al.*, 2006 and 2006b). This effect is also confirmed by four identified isoforms of human TG2 which demonstrated significantly lower affinity to nucleotides and parallelly increased crosslinking activity (Lai TS *et al.*, 2007).

When comparing the inactive GDP-bound form with the recently published X-ray structure of the enzyme crystallised with a substrate analogue covalently bound to its active site (Fig. 5), a large difference can be observed (Pinkas DM *et al.*, 2007). Presumably, this large conformational change is induced by Ca^{2+}-s but the exact sites of bound Ca^{2+}-s in the structure of TG2 could not be determined. However, a structural study using spectroscopical techniques demonstrates that GTP-binding causes bigger changes in the structure than Ca^{2+}-binding. The GTP induces the formation of a more compact and stable structure which shows higher resistance against proteolytic degradation (Di Venere A *et al.*, 2000; Achyuthan KE and Greenberg CS., 1987). Moreover, there were no large differences between the zymogen and activated Ca^{2+}-bound form in case of FXIIIa and TG3 determined by X-ray christallography. Concerning TG3, only the binding of a third Ca^{2+}-binding induces a channel formation which give possibilities for substrates to achieve the active site. Based on high similarity between TG3 and TG2 a parallel process could be presumed. To conclude, probably the Ca^{2+}-binding promotes the docking of appropriate substrates which can open the closed TG2 conformation (Ahvazi B and Steinert PM., 2003b; Fox BA *et al.*, 1999)

Interestingly, the Ca^{2+}-bound form of TG2 shows higher binding of coeliac autoantibodies (Roth EB *et al.*, 2003). Thus the search for Ca^{2+}-binding sites of TG2 and their characterisation is current.

2.2.4. Ca^{2+}-binding motifs of proteins

Ca^{2+} plays essential role in the living organism particularly in regulation of large number of biological processes through calcium binding of proteins. The basis of interaction between Ca^{2+} and proteins is an electrostatic one which needs negative or

partially negative charge on the protein surface. Ca^{2+} affinity is dependent on Gibbs' free energy between bound and unbound states. The affinity has an entropic contribution. The bound Ca^{2+} releases the waters from its coordination sphere which is a thermodinamically favourable event but this needs high dehydration enthalpies. The main drift for Ca^{2+}-binding might be the formation of electrostatic interactions between the ion and the protein residues or other ligands.

There are canonical and non-canonical structures which bind Ca^{2+} but all of them have a commonality: at the binding site they have high negative surface potential derived particularly from Asp or Glu residues. Ca^{2+} as 'hard' metal ion can coordinate six or seven ligands with a negative character or charge in a pentagonal bipyramidal arrangement. There are some well known canonical Ca^{2+}-binding domain structures: EF-hand domains, C-type lectin-like domains (CTLD), calcium-dependent phosphatidylserine-binding domains (C2, Annexin and Gla domain) and EGF-like domains which were characterised in detail by X-ray diffraction and NMR spectroscopy. EF-hand domain is composed of two α-helices with a conjunctive loop known as helix-loop-helix structural unit. First, the flexible N-terminal part of the loop binds the Ca^{2+} by 5 ligands and then to complete the coordination spheres of ion the C-terminal helix will be reposited providing two ligands by the N-terminal Glu or Asp. The Asp is conserved in the loop and Gly is the main linker between Asp-s (Gifford JL *et al.*, 2007; Grabarek Z., 2006).

The CTLD domain has a double-loop structure which is stabilised by two disulfide bridges. Mainly EPN, QPD or EPT and WND motifs on the flexible long loop are involved in the binding of four Ca^{2+}-s. The coordination of Ca^{2+} in CTLD positionals other side chains and loops which are involved in carbohydrate binding and the bound carbohydrate also contributes to the Ca^{2+} coordination. (Zelensky AN and Gready JE., 2005)

C_2-domain (identified in protein kinase C) is called as Ca^{2+}-dependent lipid binding domain and consists of two four-stranded β-sheets. The loops are involved in the binding of three Ca^{2+}-s through five aspartate side chains, one serine chain and three carbonyl groups. The affinity of Ca^{2+}-s to these binding sites is lower due to incomplete coordination but binding of phospholipids can complete the coordination spheres (Rizo J and Südhof TC., 1998). The Ca^{2+}-binding sites of annexins are located on the core domain which contains four annexin repeats. Here also a loop is involved in Ca^{2+}-binding with a typical G-X-G-T-{38}-(D/E) consensus sequence but within annexin family there are more unique calcium binding sequences and moreover the high calcium concentrations can result in forming new binding site (Liemann S and Huber R., 1997). The Gla, γ-

carboxyglutamate-rich, mainly helical domain can be found in prothrombin and Factors VII, IX and X; it is a small domain containing 9-12 γ-carboxyglutamate. The role of bound Ca^{2+} is also the anchoring of protein to membrane (Lemmon MA., 2008). The EGF-like domain also can bind Ca^{2+}. It is a thirty to forty amino acid residues long and includes six cysteine residues which have been shown to be involved in three disulfide bonds to stabilise its structure (Malby S *et al.*, 2001; Smallridge RS *et al.*, 2003).

2.2.5. Ca^{2+}-binding motifs of crystallised transglutaminases

Within the transglutaminase family the calcium-bound X-ray structure of human blood coagulation Factor XIIIa (FXIIIa) and human epidermal transglutaminase (TG3) are known which have not shared significant similarities with canonical Ca^{2+}-binding motifs. FXIIIa has one Ca^{2+}-binding pocket. Based on its X-ray structure, the main-chain oxygen of Ala-457 is the main protein ligand in Ca^{2+}-binding. The other direct coordinators are five waters which are within 4.0 Å of the ion. Asn-436, Asp-438 Glu-485 and Glu-490 are also involved in the formation of this negatively charged site and they are hydrogen-bonded to the water molecules (Fox BA *et al.*, 1999). TG3 has three Ca^{2+}-binding sites: Site 1 is formed by residues Asn-224-Asn-229. Here Ca^{2+} is coordinated *via* direct contacts with the main-chain carbonyl oxygen atoms of Ala-221, Asn-224, Asn-226 and Asn-229, the carbonyl side chain oxygen of Asn-224 and a water molecule. Site 2 is located on the near of the end of the catalytic core domain and is homologous to sole known, earlier called as the EF-hand like, heptacoordinated Ca^{2+}-binding site of FXIIIa. In TG3, the side chain carbonyl oxygen groups of Asn-393, Glu-443 and Glu-448, the main-chain carbonyl oxygen atoms of Ser-415 and two directly coordinated water molecules are involved in ion binding which is different from those of FXIIIa. At site 3, Ca^{2+} is coordinated by direct contacts of carbonyl side chain oxygens of Asp-301, Asp-303 and Asp-324, the side chain atoms of Asn-305, the main-chain carbonyl oxygen atoms of Ser-307 and a water molecule (Ahvazi B *et al.*, 2002).

The similarities of the above mentioned sequences are very high in FXIIIa, TG3 and TG2. Moreover, site 2 is well conserved in all transglutaminases. These suggest that the three Ca^{2+}-binding sites could also be present in TG2. Bergamini's results clearly show that TG2 can bind 6 Ca^{2+} (Bergamini CM., 1988), so there have to be further binding sites. Indeed, TG2 has several negatively charged amino acids with high surface potential which might serve as Ca^{2+}-binding sites (Ambrus A *et al.*, 2001).

3. 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.

4. MATERIALS AND METHODS

4.1. Materials

All materials were purchased from Sigma unless otherwise indicated.

4.2. Patients

For TG2 activity measurements serum IgA and IgG antibodies were purified from serum samples of 25 endomysial antibody-positive patients with untreated coeliac disease. All these patients had severe jejunal villous atrophy (Marsh grade IIIb or IIIc), and the diagnosis of coeliac disease was established according to the criteria of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (Walker-Smith JA and Murch S., 1999). The coeliac patients were selected from those with the following clinical presentation types: (i) Group E: severe malabsorption presenting in early childhood (n=5, median age 1.4 years, range 1.2-3.2), (ii) Group A: adult patients in a good general condition (n=5, median age: 30.3 years, range 16.6-51.7), (iii) Group S: adult patients with severe malabsorption (n=5, median age 25.4 years, range 23.2-32.2), (iv) Group D: children with skin biopsy-proven DH, no enteral complaints (n=5, median age: 9.5 years, range 5.0-16.6), (v) Group G: IgA deficient children with similar clinical picture as those in Group E (n=5, median age 5.6 years, range 4.2-9.3). Patients in Group G had only IgG class circulating endomysial antibodies, but not IgA. Patients from Group E were also studied on a prolonged gluten-free diet (GFD) period of over one year when they had clinical and histological recovery and were negative or borderline for serum endomysial antibodies. Further, IgA and IgG were also purified form the serum samples of three endomysial antibody-negative non-coeliac control subjects aged 4-14 years who had normal small-intestinal mucosa (Group C).

For investigation of target specificity of small-bowel mucosa IgA deposits, sections from seven serum EmA-negative and six EmA-positive untreated coeliac patients were used.

For determination of antigenicity of mutants TG2 serum samples from 64 untreated coeliac patients were used.

All patients had a small intestinal biopsy confirming the diagnosis.

Patient serum samples and biopsies were used with the permission of the Ethical Committee of the Heim Pál Children's Hospital, Budapest.

4.3. IgG and IgA purification

IgG antibodies were purified from serum samples of GSE and control subjects using Sepharose beads conjugated with protein G (AP Biotech) according to the manufacturer's instructions. Antibody fractions were eluted with 0.1 M glycine-HCl buffer, pH 2.8 and collected into tubes containing 1 M Tris-HCl buffer, pH 8.0 to prevent denaturation. Phosphate buffered saline, pH 7.2 (PBS) was added to the flow-through which was subsequently used to purify IgA using agarose beads conjugated with jacalin. Bound IgA was eluted with 0.2 M galactose in PBS. The Ig fractions were concentrated using Centricon tubes (Millipore) and the buffer was exchanged for 0.1 M Tris-HCl, pH 7.5. The protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad) using human immunoglobulin (Calbiochem) as standard.

For some central experiments, total IgA antibody fractions were further affinity-purified with the TG2 present in the reaction itself and nonspecific antibodies were washed away before starting the activity assay.

4.4. 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 described previously (Ambrus A *et al.*, 2001). Circular dichroism spectra indicated that the protein has achieved a correct folding similar to natural TG2. Purity of the enzyme preparations used for the measurements was over 85% as judged by Coomassie blue staining of SDS-poliacrylamide gels showing only some degradation products and GST.
As a source of cellular TG2, a human myeloid leukaemia cell line NB4 (Lanotte M *et al.*, 1991) treated for 4 days with 1μM all-trans retinoic acid was used. The cells were harvested at the peak of TG2 expression. The cell pellet was sonicated in 20 mM Tris-HCl pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM ethylenediamine-tetraacetic acid (EDTA) and Complete^{TM} (Roche) protease inhibitor. After spin centrifugation the supernatant was used for the experiments.

TG2 was also obtained from human red blood cells (RBC). RBC were twice washed in 5 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, haemolysed in distilled water and diluted in 5 mM Tris-HCl with 0.5 M NaCl. Haemoglobin was removed from the crude lysate using DEAE-Cellulose, and the TG2 containing fraction was eluted from

the gel by 0.5 M NaCl. Presence of FXIIIa in the RBC lysate was excluded by non-reactivity with specific antibody.

For calcium binding study wild type recombinant TG2s were expressed in $(His)_6$-tagged form by Rosetta 2 cells (Novagen). pGEX-2T-TG2 DNA was amplified with specific primers (primer 1, 5'- gac gac gac aag atg aga att cag acc atg gcc gag gag ctg g – 3', and primer 2, 5'- gag gag aag ccc ggt tga att cgg tta ggc ggg gcc aat gat gac – 3'), and subcloned into pET-30 Ek/LIC Vector. Site-directed mutants were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutant constructs were checked by restriction analysis and DNA sequencing (ABI PRISM). Rosetta 2 (Novagen) strains were transformed with wild or mutant TG2 containing pET-30 Ek/LIC-TG2 vectors. The cells were grown in LB at 37 ^{o}C to OD_{600} 0.6-0.8. To induce the expression of $(His)_6$-tagged proteins, the cultures were grown for 5 hours at 20 ^{o}C in the presence of 0.3 mM isopropyl β-D-thiogalactoside, then the cells were harvested by centrifugation at 4 ^{o}C. The cells were resuspended in lysis buffer [binding buffer: 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 20 mM imidazole, 20 mM β–mercaptoethanol; containing 10% (v/v) glycerol and 1% (v/v) Triton X-100] and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysis was performed by sonication followed by centrifugation for 25 minutes at 20000 g. Supernatants were diluted 2 times with binding buffer, and loaded onto ProBond Ni-NTA resin (Invitrogene). The column was washed with 40 column volume binding buffer containing 800 mM NaCl and 20 mM imidazole, then 15 column volume binding buffer containing 500 mM NaCl and 30 mM imidazole. The recombinant protein was eluted with 10 column volume eluting buffer, binding buffer containing 250 mM imidazole. The eluent was concentrated with Amicon Centricon-YM 50 MW (Millipore) and the buffer was exchanged to storing buffer [20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol]. All purification steps were performed on ice or at 4^{o}C. The protein concentration was determined using Bradford method (Bio-Rad).

The self-crosslinking activity and purity of proteins were checked by Coomassie BB staining (Fermentas) of SDS-poliacrylamide gels and by Western blots using goat polyclonal anti-TG2 antibody (Upstate) diluted 1:20 000 and anti-goat antibody conjugated with horseradish peroxidase (HRP) (Sigma), 1:30 000. The bands were revealed by Chemiluminescent ECL Detection System (Millipore).

4.5. Determination of TG2-specific antibody content of purified immunoglobulins

A transglutaminase 2 antibody ELISA described by Dieterich *et al.* (Dieterich W *et al.*, 1997) was used with the modification that wells were coated with 0.5 µg of GST-fusion recombinant TG2 per well. The purified immunoglobulins were diluted in PBS containing 1% BSA and applied to the wells in concentrations ranging from 30 µg/ml to 58.6 ng/ml for the determination of dilutions which provide normalised TG2-specific antibody content for the experiments. Bound TG2-specific antibodies were detected with peroxidase conjugated rabbit antihuman IgA or IgG (Dako) diluted 1:4000 in PBS containing 1% BSA, followed by the addition 3,3',5,5'-tetramethylbenzidine substrate. The colour reaction was stopped with 50 µl 1N H_2SO_4 and absorbances were read at 450 nm. All samples were tested in duplicate.

4.6. Microtiter plate assay of transglutaminase activity

The microtiter plate assay based on the incorporation 5-(biotinamido)pentylamine (Molecular Probes) into immobilised N,N-dimethylated casein (DMC) was used as described (Madi A *et al.*, 1998) with the following modifications: Transglutaminase activity was measured using 5 mM $CaCl_2$, 0.2 µg GST-fused TG2 (or 3 µg NB4 cell extract or 1 µg haemoglobin-free red blood cell lysate, which had equivalent activity) in the presence of 15 µg/ml or normalised antibody concentrations of human IgA or IgG, or 5 µg/ml anti-TG2 monoclonal mouse antibodies (CUB7402, NeoMarkers). The enzyme and antibodies were preincubated for 10 minutes at room temperature in the Tris-HCl buffer. The reaction was started by adding 50 µl of this preincubation mixture to the other reactants and was performed at 37°C for 30 minutes. Amine incorporation was detected by streptavidine-alkaline phosphatase followed by adding 200 µl of 25 mM p-nitrophenyl phosphate and measuring absorbance at 405 nm. Enzyme activity values were obtained from ΔA_{405}/min of colour development between 10 and 30 minutes.

Microtiter plate method was also used for determination of transglutaminase activity of $(His)_6$-tagged form of wild and mutant TG2-s with the next modification: transglutaminase activity was measured using 5 mM $CaCl_2$ and 0.4 µg TG2 without preincubation. The reaction was started with 50 µl enzyme mixture and was performed at 37°C for 30 minutes.

4.7. Kinetic assay to measure deamidating activity of TG2 (UV-test)

NH_3 liberated during the transglutaminase reaction in liquid phase is measured by a coupled glutamate-dehydrogenase reaction and consequent decrease of β-nicotinamide adenine dinucleotide phosphate (NADPH) (Muszbek L *et al.*, 1985). The total volume of reaction mixture was 200 µl containing 10 mM dithiotreitol (DTT), 30 mM ethyl-amine, 0.75 mM adenosine 5'-diphosphate, 7.5 mM α-ketoglutarate, 0.8 mM NADPH, 22.5 U/ml glutamate dehydrogenase (EC 1.4.1.4, Roche), 5 mM $CaCl_2$, 5 mM glutamine substrate peptide (representing a sequence from α2-plasmin inhibitor; provided by L Kárpáti, Dept. of Clinical Biochemistry and Molecular Pathology, University of Debrecen, Debrecen, Hungary), 2 µg GST-fused TG2 and 150 µg/ml or ten times of normalised concentrations of IgA or IgG in 120 mM HEPES buffer, pH 7.5 (Hevessy Z *et al.*, 2000). After 10 minutes preincubation at room temperature (enzyme and antibodies in the buffer) the reaction was performed at 37°C for 30 or 40 minutes. The change of NADPH concentration was measured by following the decrease of absorbance at 355 nm. Linear range rates were used to determine values of enzyme activity.

4.8. Filterpaper assay of transglutaminase activity

The assay is based on the incorporation of [1,4(n)-3H] putrescine (30 Ci/mmol, PerkinElmer Life Sciences Inc.) into DMC as described previously (Balajthy Z *et al.*, 1997). The 100 µl reaction mixture contained 3 µg of NB4 cell extract or crude RBC lysate and 30 µg/ml antibodies. After preincubating TG2 and antibodies in the buffer for 10 minutes at room temperature, the reaction was started with addition of $CaCl_2$ and incubated at 37°C for 5 minutes. After precipitating 25 µl of the sample on filterpaper in cold trichloroacetic acid and extensive washings, the [3H]putrescine incorporation was measured using a β-counter.

To compare the transglutaminase activity of $(His)_6$-tagged wild and mutant TG2 filterpaper method was used without preincubation. Reaction mixture contained 5 µg of recombinant TG2. The reaction was started by the addition of $CaCl_2$ (5 mM final concentration) and incubated at 37°C for 5 minutes.

4.9. 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 100 µl of 1 µg/ml human fibronectin from Sigma in carbonate

buffer pH 9.6 for 1 hour at room temperature. After washing with TTBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) the wells were coated with 3 µg GST-fused recombinant TG2 or 7 µg NB4 cell extracts (see above) in buffer A (20 mM Tris-HCl buffer pH 7.2, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 8 mM $CaCl_2$) for 30 minutes at 4°C. After washings with buffer A containing 0.1% Tween 20, the wells were incubated with 3 µg jacalin purified total IgA for 20 minutes at 4°C, and antibodies which did not bind to TG2 were washed away with buffer A with 0.1% Tween 20. Then the wells were incubated with 200 µl of 1 mM 5-(biotinamido)-pentylamine substrate in 0.1 M Tris-HCl buffer pH 8.5 containing 5 mM $CaCl_2$, 10 mM DTT. Amine incorporation was detected as described above. The reaction was calcium dependent and was able to measure transglutaminase activity both recombinant and cell extract derived TG2-s (Fig. 6).

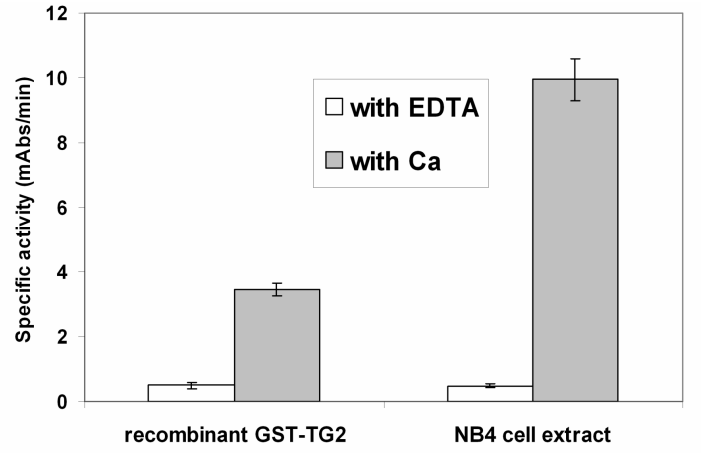


Figure 6. Transamidating activity of fibronectin-bound GST-fused recombinant and NB4-derived natural TG2 in the presence of 10 mM EDTA or 5 mM calcium. Modified microtiter plate assay without adding patient antibodies.

4.10. GTPase activity assay and direct photolabeling

GTPase activity was determined by the charcoal method as described (Lee KN *et al.*, 1989) with the following modifications: The 100 µl reaction mixture contained 1 µg of GST-fusion TG2, 30 µg of coeliac immunoglobulins in 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 9.9 µM GTP and 0.1 µM [γ-32P]GTP (3000 Ci/mmol, Institute of Isotopes Ltd.). Blank was determined without the enzyme. Reaction was stopped by the addition of 700 µl of 6% (w/v) activated charcoal in ice-cold 50 mM NaH_2PO_4, pH 7.5, centrifuged and released [32P]Pi was determined by counting of 400 µl of the supernatant.

GTPase activity of $(His)_6$-tagged forms of wild and mutant TG2 were determined with minor modifications: The released $[^{32}P]P_i$ was determined by counting of 150 µl of the supernatant. GTP labelling was performed as described (Begg GE *et al.*, 2006).

4.11. Control experiments with purified antibodies

The purified IgA preparates were checked for the presence of IgG and vice versa by ELISA test. No significant cross-contaminations were found (data not shown). When the purified antibodies were used instead of the DMC, amine incorporation was not observed using the filterpaper assay. The microtiter plate assay was used to exclude the possibility that the antibody preparations or pure GST alone would have transglutaminase activity.

4.12. Investigation of target specificity of small bowel mucosal IgA deposits

Unfixed frozen duodenum sections from six serum EmA-positive and seven EmA-negative coeliac patients were washed in PBS, pH 7.4, and incubated for 30 minutes with 0.1 M sodium citrate buffer (pH 5.0) or with 0.5-1 M potassium thiocyanate (KSCN), which dissolves nonspecific protein complexes as a chaotropic agent. After further washings in PBS, the sections were stained for human IgA using fluorescein isothiocyanate-labelled rabbit antibody against human IgA and monoclonal mouse antibodies against TG2 (CUB7402) followed by rhodamine conjugated anti mouse Ig antibodies.

In further experiments, extracellular TG2 was removed from the sections with 0.25% chloroacetic acid (Fluka Chemie AG) in 0.2 M NaCl, pH 2.7 (Korponay-Szabó IR *et al.*, 2004) 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, we investigated whether they can bind labelled TG2 added to the tissue (Fig. 7). Glutathione S-transferase-tagged full-length human recombinant TG2 (GST-TG2) was expressed in *E.coli* as previously described (Ambrus A *et al.*, 2001). Unfixed frozen small bowel sections from coeliac and control patients were washed in PBS and incubated for 15 minutes at room temperature with GST-TG2 at a concentration of 0.01 mg/ml. After extensive washings, GST-TG2 bound to the tissue was labelled red by goat antibodies against GST (Pharmacia Biotech) followed by Alexa Fluor® 594-conjugated chicken antibodies against goat immunoglobulins

(Molecular Probes). Human IgA in the tissue was labelled green as previously described. The anti-GST antibody used 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 (Sigma F-0162; 0.2 mg/ml) and monoclonal antibodies G92 (0.4 mg/ml) (Trejo-Skalli AV *et al.*, 1995). These antibodies interact with the N-terminus of TG2 where one of the putative fibronectin binding sites is located (Jeong JM *et al.*, 1995).

The study protocol was approved by the Ethics Committee of Tampere University Hospital and informed consent was obtained from all study subjects.

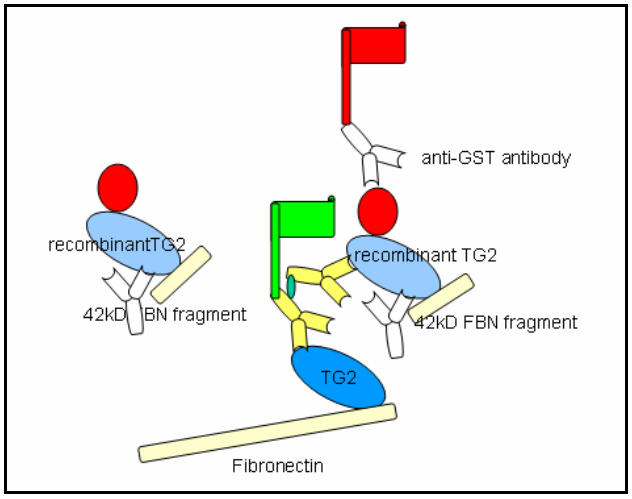


Figure 7. In vitro binding of recombinant TG2 to deposited specific IgA in coeliac disease

4. 13. Molecular modelling and sequence alignment

The X-ray structures of human TG2, TG3 and Factor XIIIa were retrieved from the RCSB Protein Data Bank (www.rcsb.org/pdb). The graphical analysis was made on Silicon Graphics Fuel workstation using GRASP and Sybyl program packages (Tripos, St. Louis, MO), RasMol, and VMD (Humphrey W *et al.*, 1996). The sequence alignments were performed using ClustalW (Larkin MA *et al.*, 2007).

4. 14. Equilibrium dialysis

Ca^{2+}-binding was measured by equilibrium dialysis with modification of a published procedure (Bergamini CM., 1988). Recombinant TG2 (~1.7 mg/ml) was dialysed for 48 hours at 4^{o}C in 96-Well Equilibrium Dialyzer Plate (MWCO10kDa)

(Harvard Bioscience) against 150 µl dialysis buffer (50 mM Tris-HCl and 5 mM mercaptoethanol, pH 7.5.) supplemented with 8.3 µCi of $^{45}CaCl_2$/ml (PerkinElmer) containing different concentrations of cold $CaCl_2$. After the equilibration the radioactivity was measured with liquid scintillation counting using Tritosol (Fricke U., 1975). The results were normalised to the protein content of the sample determined by Bradford reagent (Bio-Rad) and protein purity which was measured by Alpha Imager's Software (~85-90%). The free Ca^{2+} concentration was calculated by Maxchelator and Fabiato and Fabiato's computer program (Patton C *et al.*, 2004; Fabiato A and Fabiato F., 1979).

4.15. Isothermal titration calorimetry and inductively coupled plasma - optical emission spectrometry

The calcium binding properties of TG2 were measured using isothermal titration calorimetry (VP-ITC MicroCalorimeter, MicroCal) in the Department of Biophysics and Radiation Biology, Semmelweis University Budapest contributed by Krisztián Szigeti. High purity recombinant TG2 was produced by ion exchange for ITC measurements. After affinity chromatography the sample buffer was changed to FPLC A binding buffer containing 50 mM Tris-HCl pH 7.8, 50 mM NaCl and 1 mM EDTA. After filtration the protein was loaded to HiTrap Q HP Column using ÄKTA Prime equipment (Amersham Pharmacia Biotech). TG2 was eluted by linear gradient of FPLC B buffer (FPLC A buffer containing 1 M NaCl). The purity of eluted fractions was checked by SDS-PAGE and Coomassie staining (Fermentas). The appropriate fractions were concentrated using centrifugal concentrators (Millipore) and was dialysed again ITC buffer (25 mM Tris-HCl, pH 7.5 and 1 mM 2-mercaptoethanol) with 0.5 mM EDTA and then two times ITC buffer with Chelex 100 at 4°C for 6-6 hours. The ITC experiments were performed on 25°C. At beginning 40 µM TG2 were in the sample chamber and 2 mM $CaCl_2$ solution in ITC buffer were injected according these scheme: 5 x 2 µl, 6 x 5 µl, 5 x 15 µl, 7 x 20 µl and 2 x 25 µl.

The sample was prepared in the same way for inductively coupled plasma-optical emission spectrometry (ICP-OES, Analab Ltd. Debrecen, Hungary) experiments. This measurement was performed with the contribution of Mihály Braun, PhD. The concentration of calcium stock solution and made buffers was also checked by ICP-OES.

4. 16. Antibody binding assays of mutants TG2

For determination of antigenicity and functional purity of TG2, ELISA was used similar to the test described previously (Sblattero D *et al.*, 2000). Microtiter plates

(ImmunoPlate Maxisorp, Nunc) were coated with 0.6 μg TG2 in 100 μL of 50 mM Tris-buffered saline (TBS) containing 5 mM $CaCl_2$ or 2 mM EDTA (pH 7.4). The plates were washed 3 times with TTBS, TBS containing 10 mM EDTA, 0.1% (v/v) Tween 20, followed by an hour incubation at room temperature with monoclonal antibody-II (TG100; NeoMarkers) diluted in TTBS in 1:1000 or with 1:200 diluted serum samples obtained from untreated coeliac patients who had high concentrations of anti-TG2 IgA autoantibodies. The plate was washed 3 times with TTBS, and then it was incubated for 1 hour at room temperature with HRP-conjugated anti-mouse IgG (1:5000, Sigma) or anti-human IgA (1:5000, Dako). After washing the colour reaction was developed by adding 100 μL 3,3',5,5'-tetramethylbenzidine substrate and then stopped with 50 μL 1 N H_2SO_4 . The absorbance was read at 450 nm.

4. 17. Circular dichroism analysis

Circular dichroism (CD) spectra were recorded on a Jasco-810 spectropolarimeter at room temperature using 0.02 and 0.1 cm quartz cuvette in the far UV and in the near UV, respectively. For CD measurements the sample buffer was changed to 50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA. CD spectra are plotted as mean residue molecular ellipticity [θ] (deg cm^2 $decimol^{-1}$)/ wavelength [λ] (nm). CD deconvolutions were carried out by the continll, cdsstr and selcon3 analysis programs kindly provided by Dichroweb (Lobley A *et al.*, 2002; Whitmore L and Wallace BA., 2004 and 2008). The CD measurement and analysis of the spectra were performed together with Tibor Kurtán, PhD.

4. 18. Statistical analysis

Enzyme activity measurement data within and between different patient groups were analysed using Analysis of Variance (ANOVA, one way), Wilcoxon signed rank test and Mann-Whitney U-test using Microsoft Excel (Microsoft Inc.) and Analyse-it (Analyse-it Software, Ltd.).

5. RESULTS

5.1. EFFECT OF COELIAC AUTOANTIBODIES ON TRANSGLUTAMINASE 2 ACTIVITY

5.1.1. Determination of normalised TG2-specific antibody concentrations

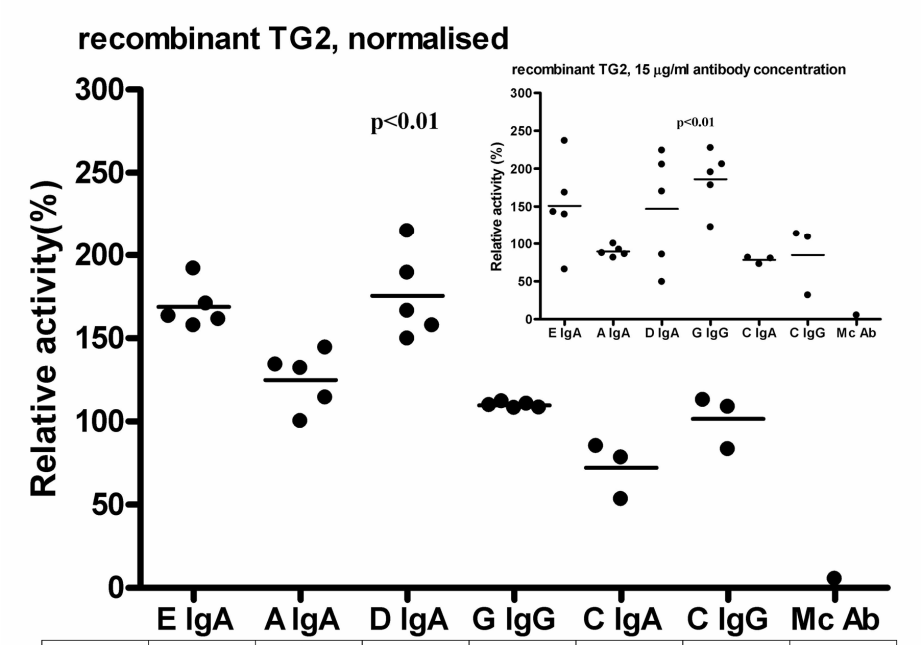
TG2-specific antibody contents of the purified immunoglobulin preparations were determined by ELISA using dilution curves (data not shown). Two antibody concentrations were tested in transglutaminase activity measurements: (i) 15 µg/ml IgA or IgG where all coeliac antibodies produced maximum absorbance 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"). In the latter cases coeliac antibodies were used in up to 15-60 times higher dilutions than the control antibodies (Table 2). In different assays the established molar ratio of enzyme and antibody contents were kept constant.

Table 2. Anti-TG2 antibody titres and normalised concentrations of purified IgA or IgG preparations used for the measurements

Coeliac children (untreated)	E1	E2	E3	E4	E5
anti-TG2 antibody titre (AU)	26.7	106.4	53.2	53.2	106.4
used IgA concentration (µg/ml)	3.75	0.94	1.88	1.88	0.94
Coeliac children after dietary treatment(GFD)	GFD E1	GFD E2	GFD E3	GFD E4	GFD E5
anti-TG2 antibody titre (AU)	<5	6.7	<5	13.3	<5
used IgA concentration (µg/ml)	15	15	15	7.5	15
Coeliac adults	A1	A2	A3	A4	A5
anti-TG2 antibody titre (AU)	26.7	13.3	26.7	13.3	26.7
used IgA concentration (µg/ml)	3.75	7.5	3.75	7.5	3.75
Coeliac 'sick' adults	S1	S2	S3	S4	S5
anti-TG2 antibody titre (AU)	10.0	13.3	212.8	26.7	106.4
used IgA concentration (µg/ml)	10	7.5	0.47	3.75	0.94
DH children	D1	D2	D3	D4	D5
anti-TG2 antibody titre (AU)	53.2	26.7	53.2	53.2	26.7
used IgA concentration (µg/ml)	1.88	3.75	1.88	1.88	3.75
IgA deficient children	G1	G2	G3	G4	G5
anti-TG2 IgG antibody titre (AU)	434.8	212.8	833.3	212.8	106.4
used IgG concentration (µg/ml)	0.23	0.47	0.12	0.47	0.94
Healthy controls (n=3)					
anti-TG2 antibody titre (AU)	<5 (IgA), 13.3 (IgG)				
used IgA concentration (µg/ml)	15				
used IgG concentration (µg/ml)	7.5				

5.1.2. Effects of antibodies on transglutaminase activity of recombinant TG2 using the microtiter plate and deamidation assays

In the microtiter plate assay which measures cross-linking of amines into immobilised large glutamine acceptor protein substrates, normalised concentrations of both IgA and IgG antibodies from coeliac patients increased the transglutaminase activity of TG2 to 105.4-242.2% compared to antibodies from the control group, and to 109.7-175.1% compared to the enzyme without any antibody (Fig. 8). Transglutaminase activity was more enhanced in the presence of IgA from childhood coeliac patients (Groups E, D) than with IgA from adults (Group A). The differences between different groups were statistically significant ($p<0.01$).



	E IgA	A IgA	D IgA	G IgG	C IgA	C IgG	Mc Ab
mean:	168.9	124.9	175.1	109.7	80.2	104.1	5.25
n:	5	5	5	5	3	3	1

Figure 8. Effect of purified antibodies (IgA or IgG) from coeliac patients and from healthy donors on the transglutaminase activity of GST-tagged recombinant human transglutaminase 2 (TG2) using the microtiter plate method. TG2 (0.2 µg) was incubated with normalised concentrations of antibodies (Table 2) or 15 µg/ml antibodies (insert). TG2 activity is shown as a percentage of basal activity (no immunoglobulin addition, 15.5-136.0 ΔAbs_{405}/min/mg protein) of TG2. E: coeliac children, A: coeliac adults, D: coeliac children with dermatitis herpetiformis, G: coeliac children with IgA deficiency, C: healthy control children, Mc Ab: 1 µg CUB 7402 monoclonal antibody. Data are presented as means from three separate experiments performed in triplicates from each individual. Data were analysed using ANOVA. Dash indicates mean. Pure GST did not have transglutaminase activity.

Normalisation of antibody concentrations for the specific antibody titre implicated the use of the IgG coeliac antibodies in the up to 60-fold dilutions compared to control IgG where the activating effect was only slight (mean 105.4%); however, it was noted that these IgG antibodies had the highest specific antibody content and hence the most potent activating effect (mean 186.1%) at 15 µg/ml antibody concentrations (Fig. 8, insert).

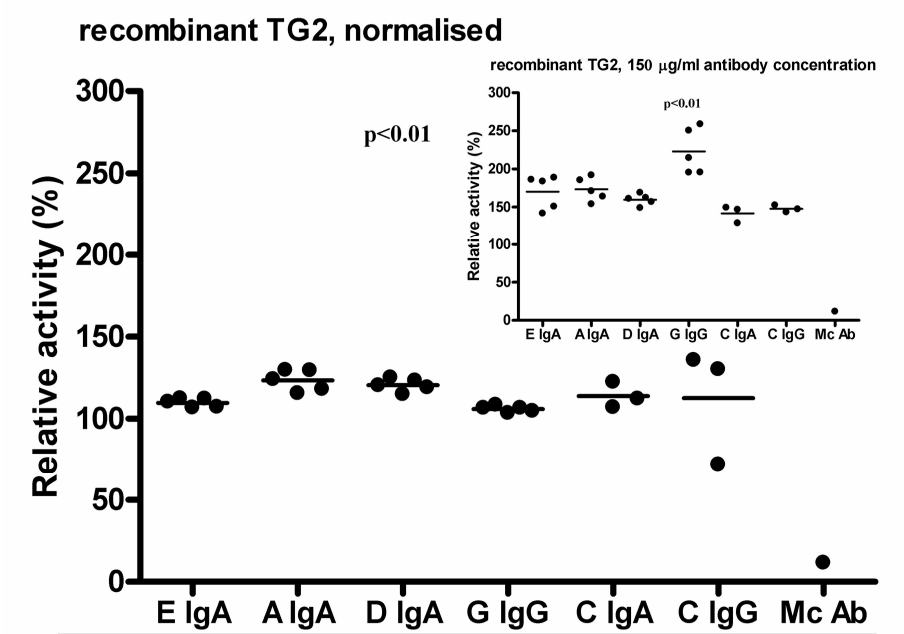
Normalisation for the TG2-specific antibody concentrations in the assays clearly resulted in more homogeneous data compared to assays with the same IgA concentrations (15 µg/ml), indicating that the activating effect of coeliac autoantibodies was directly related to the amount of TG2-specific antibodies measured by ELISA.

The presence of non-coeliac control IgA or IgG slightly decreased transglutaminase activity of the pure enzyme to 79.6% and to 84.9% (Fig. 8, insert) at 15 µg/ml antibody concentrations, while CUB7402 monoclonal anti-TG2 antibodies almost completely inhibited (5.3%) transglutaminase activity.

Interestingly, some coeliac antibodies had slightly inhibitory effect at 15 µg/ml (E5 82.9%, compared to control subjects), but the same antibody population showed activating effect (157.7% compared to control IgA) at normalised antibody concentrations with higher dilutions.

Use of further antibody dilutions (with antibodies from E1 and E2 patients) and comparison of the results at 15 µg/ml and normalised concentrations showed that the activating effect of the coeliac antibodies was dose-dependent (data not shown).

In the kinetic deamidation UV-test, which measures kinetics of deamidation of an added glutamine containing peptide in liquid phase, both coeliac and control antibodies enhanced ammonia release (Fig. 9). This liquid phase assay, which measure the first phase of the transglutaminase reaction, required ten times higher amounts of TG2, and therefore also the antibodies were used in ten times higher concentrations. Coeliac antibodies showed only slight activating effects at normalised antibody concentrations. However, at equal immunoglobulin concentrations the effect of coeliac antibodies on enzyme activity ranged from 113.5% to 148.7% compared to the presence of non-coeliac immunoglobulins (Fig. 9, insert). The differences between different groups were statistically significant ($p<0.01$). The CUB 7402 monoclonal antibody inhibited the deamidation to 11.6% remaining activity.



mean:	**109.7**	**123.3**	**120.5**	**105.9**	**113.8**	**112.6**	**11.6**
n:	**5**	**5**	**5**	**5**	**3**	**3**	**1**

Figure 9. Effect of purified serum antibodies (IgA or IgG) from coeliac patients and from healthy donors on transglutaminase activity of recombinant TG2 as measured by the release of ammonia. TG2 (2 µg) was incubated with ten times of normalised (Table 2) antibody concentrations or 150 µg/ml antibodies (insert). TG2 activity was calculated as percentage of basal activity of TG2 (no immunoglobulin addition, 2.7-6.9 ΔAbs_{355}/min/mg protein). Data are presented as means from two separate experiments done in duplicate. Data were analysed using ANOVA. Dash indicates mean.

5.1.3. Correlation of in vitro antibody effect with the clinical course

In order to establish whether the activating effect of coeliac antibodies had relevance for the clinical outcome and it was associated with the presence of severe enteral complaints, the effects of IgA purified from Group E patients before and after a gluten-free diet were compared. In addition, IgA antibodies from untreated adult patients with severe malabsorption (Group S) were also studied in the microtiter plate assay (Fig. 10A and 10B). There was a clear decrease in effects of antibodies on transglutaminase enzyme activity in Group E patients on diet in parallel with the decline of transglutaminase-specific antibody content (Table 2) and clinical improvement after diet. The antibodies from severely sick adult patients (Group S) enhanced transglutaminase enzyme activity more than the antibodies from adult patients in a good clinical condition (Group A) at 15 µg/ml concentrations (Fig. 10A). However, Group S antibodies had less pronounced effects at normalised antibody concentrations than Group E antibodies (Fig. 10B).

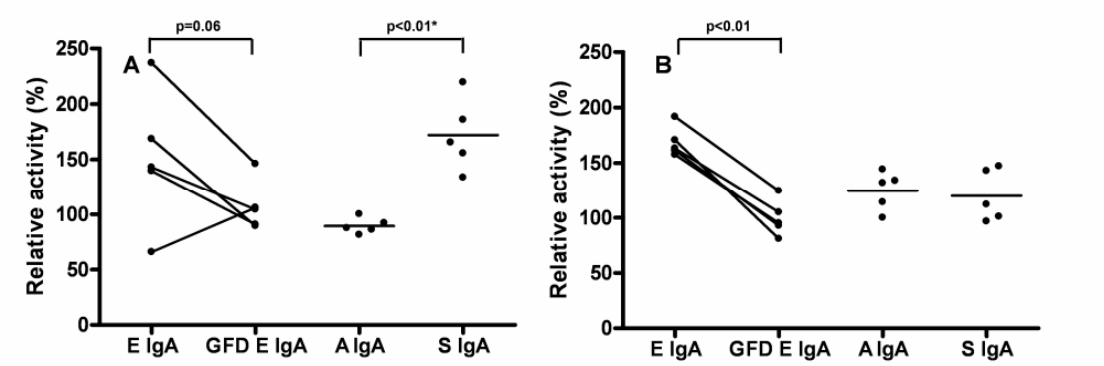


Figure 10. Effect of purified IgA antibodies from sick childhood and adult coeliac patients on the transglutaminase activity of recombinant human transglutaminase 2 (TG2) using the microtiter plate method. TG2 (0.2 µg) was incubated with 15 µg/ml antibodies (A) and normalised (Table 2) antibody concentrations (B). TG2 activity is shown as a percentage of basal TG2 activity without immunoglobulin addition. E: coeliac children, GFD E: coeliac children on a gluten-free diet, A: coeliac adults in good condition, S: coeliac adults with severe malabsorption. Data are presented as means from three separate experiments performed in triplicates from each individual. Data were analysed using Wilcoxon signed rank test and the (*)Mann-Whitney U-test. Dash indicates mean.

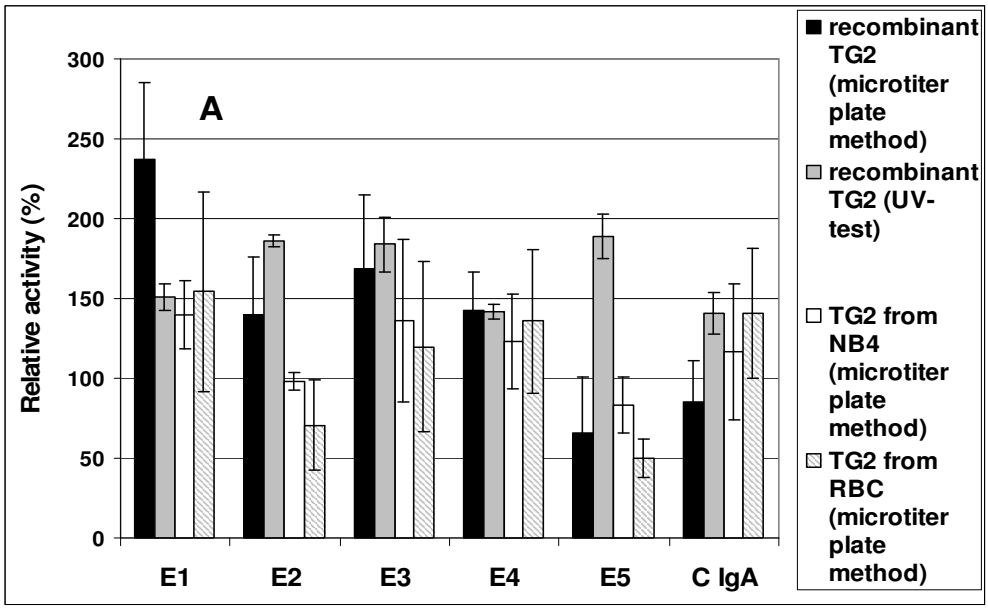
5.1.4. Effects of the antibodies in cell extracts

In order to establish whether the coeliac antibodies have similar effects also on natural human TG2 in biological samples where also other biological constituents are present, measurements were repeated also with TG2 in NB4 and red blood cell extracts.

Using the microtiter plate method, the effects of both coeliac patient and monoclonal antibodies were less pronounced and did not show significant difference using ANOVA analysis (84.7-154.4% in NB4 and 42.9-156.5% in red blood cell extracts, and see Fig. 11). The relative differences between different coeliac patient groups have also changed; activation of the enzyme was higher for Group A antibodies while Group D antibodies were rather inhibitory. Also the inhibitory effect of the CUB 7402 monoclonal antibody was greatly reduced for both enzyme preparations (76.4% in NB4 and 47.4% in red blood cell extracts).

Since it was not possible to perform the UV-based assay with cell lysates because of disturbed transparency at 355 nm, the filterpaper method was applied as a liquid phase assay. In this assay, which detects TG2-dependent amine incorporation into dimethylated casein all coeliac antibodies slightly decreased transglutaminase activity of TG2 both in NB4 and red blood cell extract (activity 95.4-84.1% and 92.1-59.7% compared to controls, respectively), similarly to the commercial monoclonal anti-TG2 antibody (activity 64.3% and 59.8%, respectively). These results show that presence of other biological constituents

has significantly modified inhibitory and activating effects of both coeliac and commercial antibodies.



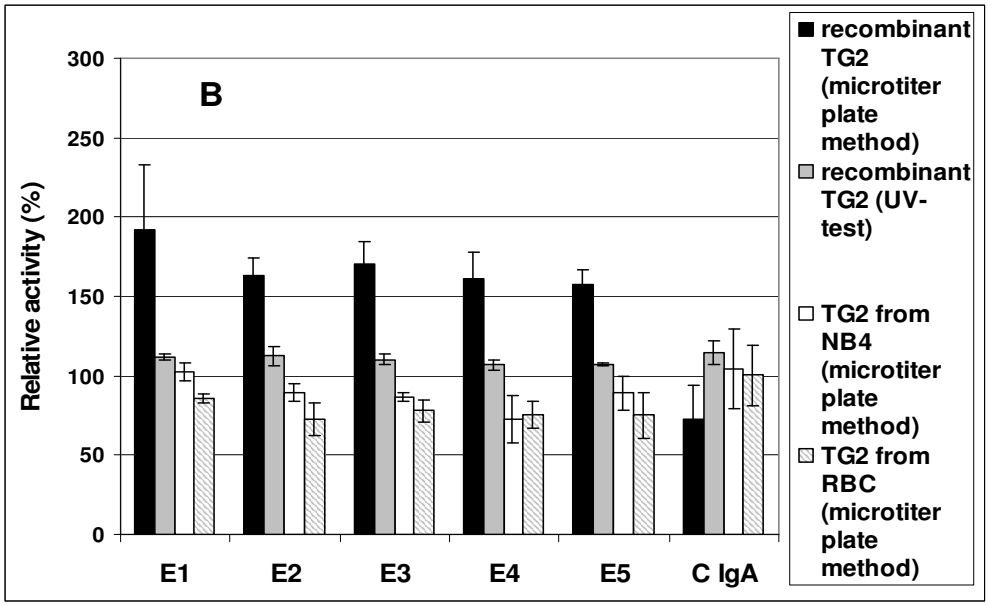


Figure 11. Comparison of the effects of purified serum antibodies from young children with early and severe malabsorptive manifestation on transglutaminase activity of TG2 using different assays and different sources of the enzyme. TG2 activity was calculated as a percentage of basal activity of TG2 without immunoglobulin addition; the values were 6.3-21.1 ΔAbs_{405}/min/mg protein of NB4 cell extract and 11.3-56.1 ΔAbs_{405}/min/mg protein of TG2 from red blood cells (RBC). Bars are representing individual patients (E1-E5) and control IgA (C) with SD. The measurements were done at 15 µg/ml antibody concentrations (A) and normalised antibody (B) concentrations.

5.1.5. Determination of the effect of specific anti-TG2 autoantibodies on transglutaminase activity of TG2

In further experiments, we sought to directly prove that the activating effect observed with the total IgA and and IgG fractions also can be reproduced by the TG2-specific antibody fraction.

A new assay was developed based on the high affinity between the fibronectin and TG2 (Hang J *et al.*, 2005), in order to keep TG2 in a catalytically active condition bound to the plate *via* fibronectin, similarly as is the case in the extracellular matrix. Then the immunoglobulin preparates were affinity-purified with the TG2 antigen within the assay itself, by washing out non-bound antibodies. When transglutaminase activity was measured in this way, a significantly increased activity was found with group E antibodies both in assays applying recombinant TG2 and natural TG2 from NB4 cells (Fig. 12). Thus, this assay method also has successfully eliminated the disturbing effect observed in crude cell extracts, by also washing out other biological constituents after coating natural TG2 to the plate. When group E patients were on a gluten-free diet, the activating effect disappeared or significant decreased. In this assay, the specific anti-TG2 autoantibodies from severely sick adult patients also enhanced transglutaminase enzyme activity more than the antibodies from adult patients in good clinical condition, and the CUB 7402 monoclonal antibody inhibited the transglutaminase activity of recombinant TG2 and NB4 derived TG2 to 19.7% and 59.5% residual levels, respectively.

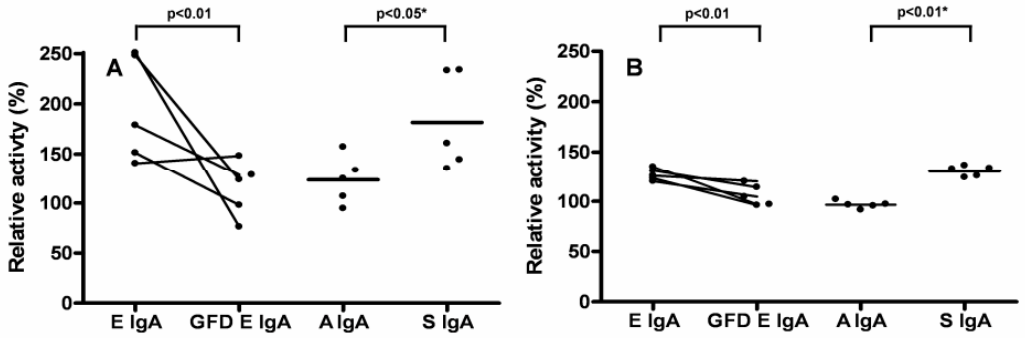


Figure 12. Effect of specific anti-TG2 autoantibodies from sick childhood and adult coeliac patients on the transglutaminase activity of fibronectin bound recombinant human transglutaminase 2 (TG2). (A) and NB4 cell extracts derived TG2 (B) using the modified microtiter plate method. TG2 activity is shown as a percentage of TG2 activity with control immunoglobulin addition. E: coeliac children, GFD E: coeliac children on a gluten-free diet, A: coeliac adults in good condition, S: coeliac adults with severe malabsorption. Data are presented as means from three separate experiments performed in triplicates from each individual. Data were analysed using Wilcoxon signed rank test and the (*)Mann-Whitney U-test. Dash indicates mean.

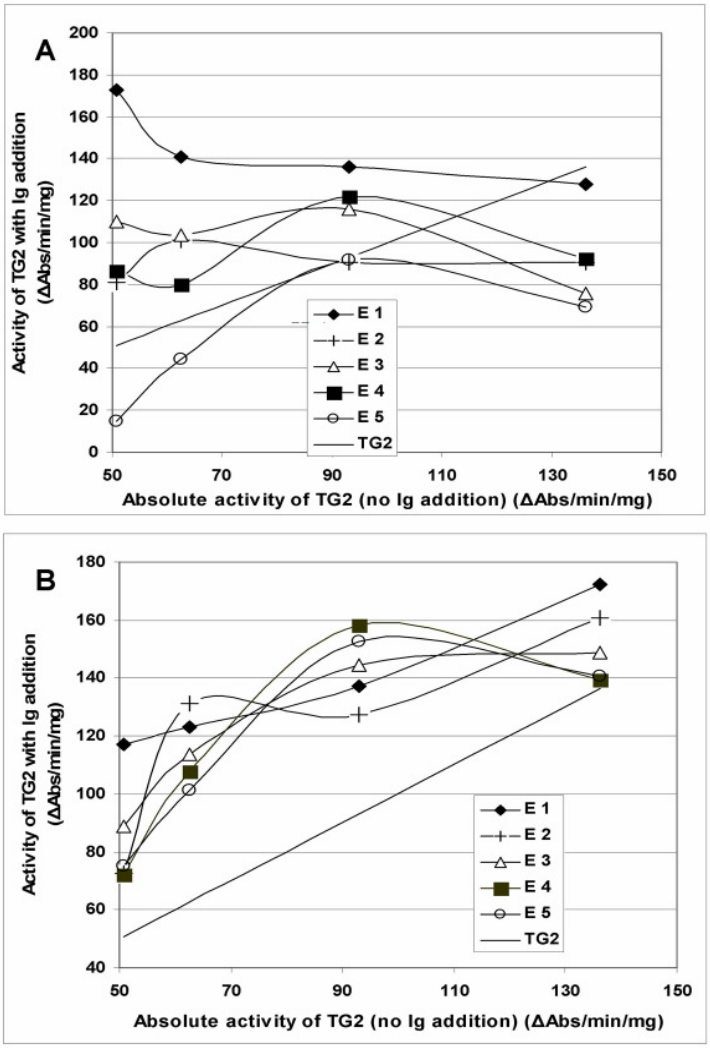


Figure 13. Dependence of enzyme activating effect of purified coeliac antibodies (Group E1-5) on basal TG2 enzyme activity. The measurements were made at 15 µg/ml antibody concentrations (A) and normalised antibody (B) concentrations using the microtiter plate method.

5.1.6. Correlation between activating effect of autoantibodies and varying activity of TG2 preparations

In the solid phase assay the activating effects of coeliac antibodies on transglutaminase activity varied from one series of independent experiments to the other: when the basal activity of the recombinant TG2 was lower, the activating effect of Group E antibodies at 15 µg/ml antibody concentrations was more pronounced (e.g. 338.2% for E1 at 50.9 ΔAbs/min/mg specific activity versus 146.2% at 93.0 ΔAbs/min/mg specific activity) while even a slight inhibitory effect occurred using the enzyme with the highest specific activity (Fig. 13A). A similar trend was observed with the Group E antibodies also

in the measurements with normalised antibody concentrations (Fig. 13B) and when using TG2 in red blood cell lysate (data not shown).

5.1.7. Effects of antibodies on GTPase activity of TG2

The coeliac antibodies and CUB 7402 monoclonal antibody equally inhibited the GTPase activity of TG2 (Fig. 14). Activity ranged from 73.4% to 67.0% as compared to control antibodies ($p<0.01$).

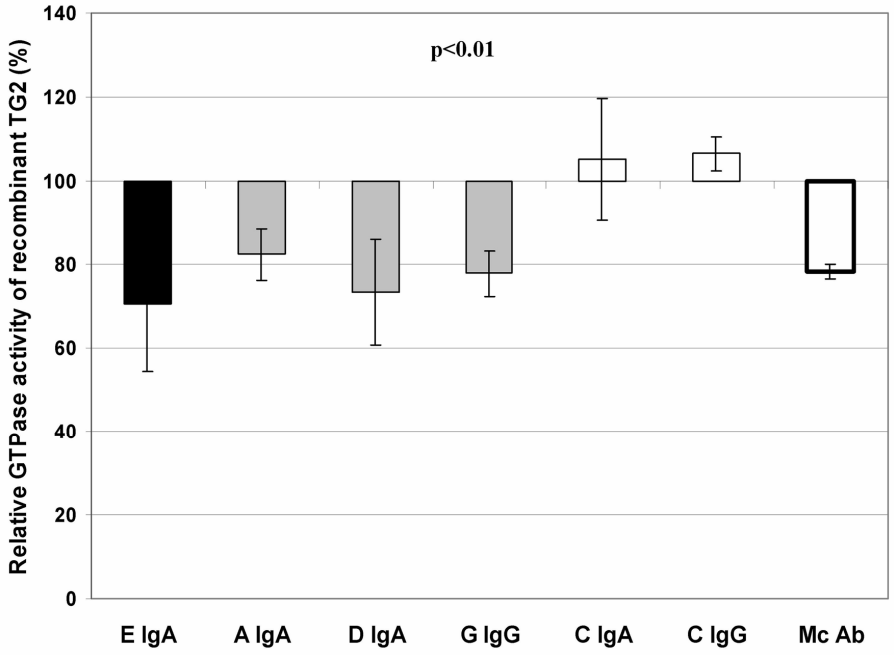


Figure 14. Effect of purified serum antibodies (IgA or IgG) from coeliac patients and from healthy donors on GTPase activity of TG2. In each case 1 µg TG2 was incubated with 30 µg coeliac or 2 µg CUB 7402 monoclonal antibodies. Bars show the mean values for two different patients of the same clinical group. GTPase activity was calculated as percentage of activity (188.0-276.5 pmol GTP/min/mg protein) in the absence of antibodies. Data are presented as means (SD) from two separate experiments done in triplicate. Data were analysed using ANOVA.

5.2. DEMONSTRATION OF TG2 SPECIFICITY OF IGA DEPOSITS

The small bowel mucosal subepithelial and pericryptal IgA deposits along TG2 in both EmA-positive and EmA-negative coeliac disease patients remained unchanged after citrate buffer and 0.5-1 M KSCN treatments (Fig. 15A). In contrast, the amount of IgA deposits substantially decreased in eight samples and almost completely disappeared in five samples (Fig. 15B) if the sections were treated additionally with chloroacetic acid which removes TG2 from its fibronectin binding sites. The amount of detectable TG2 also decreased in parallel (Fig. 15C). Chloroacetic acid had similar effects in EmA-positive and EmA-negative samples.

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 (Fig. 15D-E), but did not bind to the duodenum sections from non-coeliac controls without extracellular IgA deposition (Fig. 15F). 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.

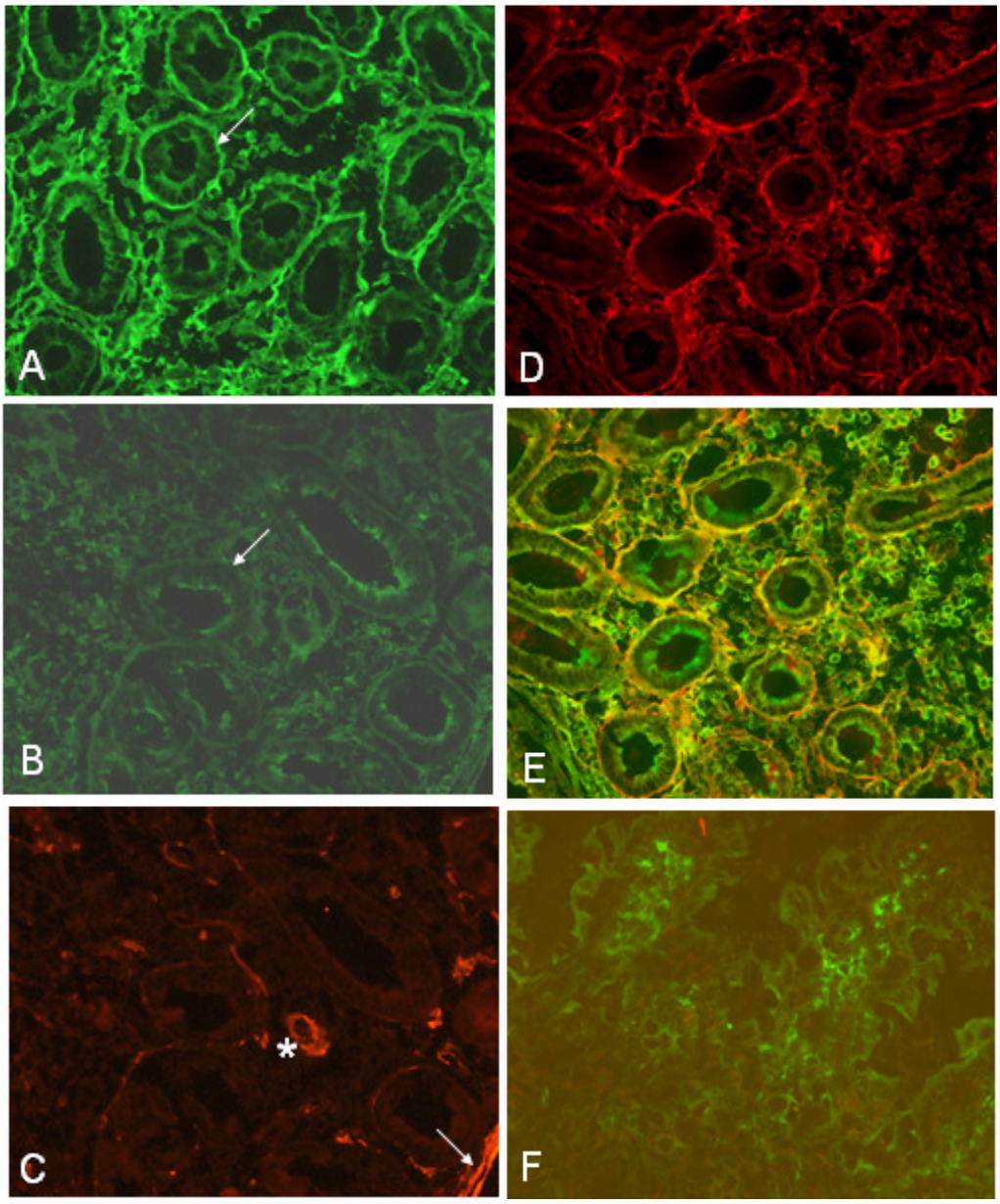


Figure 15. Investigation of specificity of deposited IgA for transglutaminase 2 (TG2). (A) Pretreatment of coeliac duodenum section with 0.5 M KSCN does not affect extracellularly deposited IgA (green). After incubation with chloroacetic acid, which removes intrinsic TG2 from its fibronectin binding sites, most deposited IgA (B) and extracellular TG2 (red, C) disappear from the sections (arrows). There is some TG2 visible only in vessels (asterisk) and smooth muscle cells (arrow). Exposure time for B and C was four times longer than for A. (D-E) GST-tagged human recombinant TG2 shown in red by anti-GST antibodies binds to coeliac duodenum section (D) along extracellularly deposited IgA shown in green (E). Merging of green and red labels to yellow indicates co-localisation. Direct binding of GST-TG2 to fibronectin was blocked with 45kDa fibronectin fragment and with monoclonal antibodies against the N-terminal of TG2. (F) No binding of GST-TG2 to non-coeliac control duodenum. Arrow shows the crypt region. Natural TG2 in the tissue is not recognised by the anti-GST antibody. Bar=50 µm.

5.3. CHARACTERISATION OF THE Ca^{2+}-BINDING SITES OF TRANSGLUTAMINASE 2 BY SITE DIRECTED MUTAGENESIS

5.3.1. Ca^{2+}-binding of recombinant TG2

As a start of the Ca^{2+}-binding experiments we determined that even after exhaustive dialysis in EDTA-containing buffer the bacterially-expressed wild type TG2 binds tightly 0.45±0.03 mol of Ca^{2+}-ion/mol TG2 as measured by inductively coupled plasma-optical emission spectrometry (ICP-OES). This finding suggests that TG2 also contains a tightly bound Ca-ion. To determine the Ca^{2+}-binding properties of recombinant wild type TG2, equilibrium dialysis measurements were performed. The results showed that wild type enzyme can bind about 6 Ca^{2+} (Fig. 16A) similarly as known for to the native erythrocyte TG2. The affinity constant of the hyperbolic saturation curve was calculated to be 560 μM.

Isothermal titration calorimetry (ITC) measurements confirmed our equilibrium dialysis and ICP-OES data (Fig. 16B). The curve of integrated heats shows 0.5 mol/mol TG2 Ca^{2+}-binding with high affinity. The next 5 Ca^{2+} bind with very low and comparable affinity to the TG2. The observed small heat changes indicate that the enthalpy change of the Ca^{2+}-binding of the next 5 sites is very low. The difference between active and inactive form of TG2 show big conformational change during the activation process which could be accompanied 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 (Fig. 17). This process may alter the Ca^{2+}-binding properties of TG2. Therefore, we examined Cys-277-Ser (C277S) mutant TG2 to clarify the effect of self polymerisation on Ca^{2+}-binding. C277S is an active-site mutant which lacks any transglutaminase activity and does not have the ability to crosslink itself (Lee KN., 1993). Based on equilibrium dialysis data it can still bind 6 Ca^{2+} although the binding is weaker (the affinity constant is 720 μM) than in case of the wild type TG2 (Fig. 16A). The active site mutant also showed the same ITC response as wild type (data not shown). These results demonstrate that self-crosslinking does not have a significant influence on Ca^{2+}-binding of our recombinant wild type TG2.

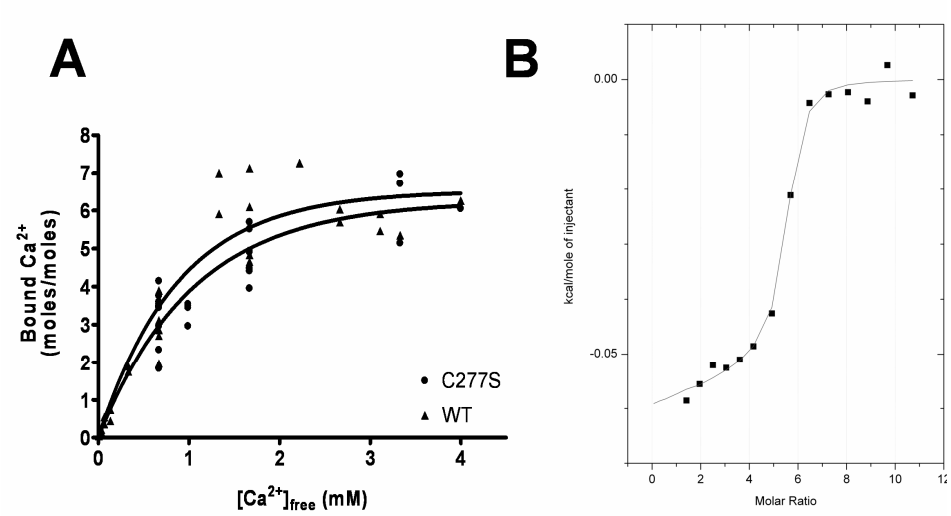


Figure 16. Ca^{2+}-binding of recombinant wild type and C277S mutant TG2. (A) The Ca^{2+}-binding curve of wild type (WT) and C277S mutant TG2 measured by equilibrium dialysis. (B) The net heat change of isothermal calorimetric titration of Ca^{2+} binding to TG2. To a solution of the enzyme a series of injections of 2, 5, 20 and 25 µl of 2mM $CaCl_2$ were added. The data shown are representative.

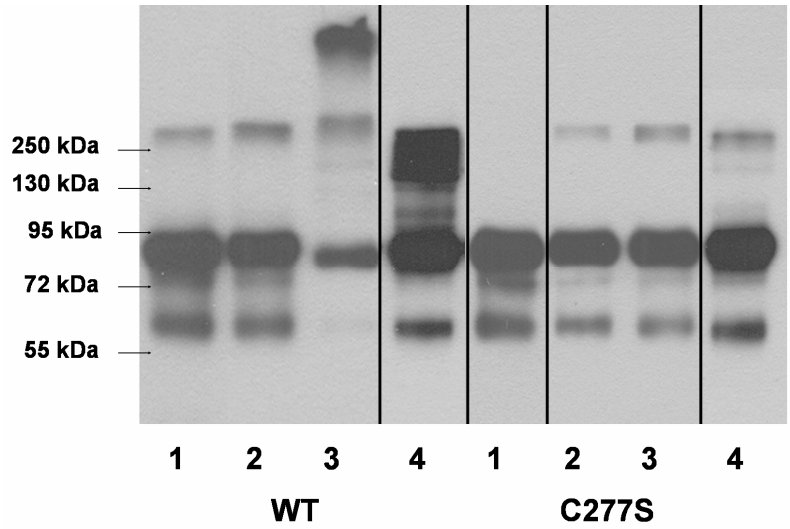
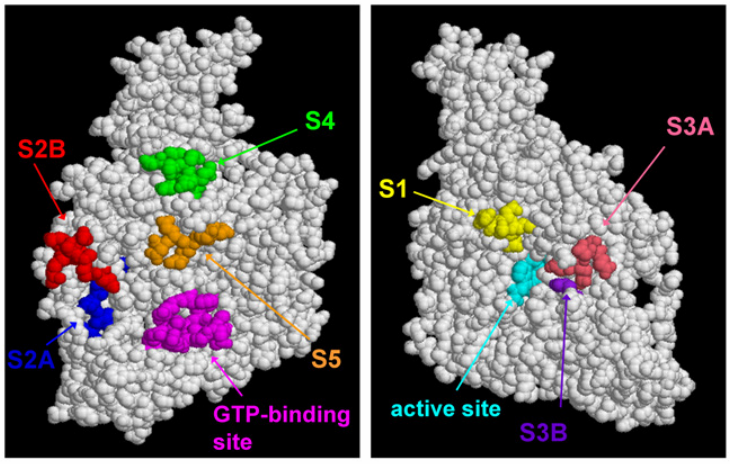


Figure 17. Self-crosslinking of recombinant wild type and C277S mutant TG2. Self-crosslinking of TG2s. Wild type (WT) or mutant TG2 (1) was incubated with 10 mM EDTA (2) or 1.7 mM calcium (3) in equilibrium dialysis buffer for 48 hours at 4°C and (4) enzyme sample after ITC experiment (2 hours incubation at 25°C during ITC measurement). SDS-PAGE sample buffer was added and samples were boiled for 5 min prior to analysis by SDS-PAGE on 10 % polyacrylamide gels. Proteins were transferred to PVDF membrane and detected by Western blot using an anti-TG2 polyclonal antibody. Dividing lines show different parts of 2 gels.

5.3.2. Design and preparation of site-specific mutants of TG2

Based on the high sequence homology shared by transglutaminases and the available X-ray structures of Factor XIIIa as well as TG3 and their identified calcium

binding sites (Fox BA *et al.* 1999; Ahvazi B *et al.*, 2002), 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 (Fig. 18).



Mutant	Original sequence	Mutant sequence
S1	228 V N C N D D Q G V	228 V S C S N N Q G V
S2A	395 A E V N A D V	395 A Q V S A N V
S2B	445 Y P E G S S E E R E A	445 Y P Q G S S Q Q R Q A
S3A	305 H D Q N S N L	305 H N Q S S S L
S3B	326 D K S E M I W N	326 D K S Q M I W N
S4	149 Y L D S E E E R Q E Y	149 Y L N S Q Q Q R Q Q Y
S5	432 G R D E R E D I T	432 G R N Q R Q N I T

Figure 18. Mutagenised site on the surface of TG2.

The site mutants S1 and S3 were chosen based on homology to TG3 Ca^{2+}-binding sites (S1 and S3, respectively). The S2 mutants were planned based on homology to Ca^{2+}-binding site of Factor XIIIa, which has strong similarities to one of the TG3 Ca^{2+}-binding sequences. In case of S2 and S3 we generated two separate mutants (S2A, S2B and S3A, S3B) since here the suspected calcium binding sites are formed by two opposing loops (Fig. 18). S4 and S5 were selected based on surface patches characterised by higher local density of negatively charged amino acids on TG2 (Ambrus A *et al.*, 2001; Nakanishi K *et al.*, 1991). Mostly conservative amino acid replacements were performed to target Ca-binding function specifically and to prevent significant conformational changes or structural disruptions. In most cases only negative charges were removed (e.g. E to Q or D to N) or the potential for Ca-complexation was decreased (e.g. N to S). According to

previous results (Ikura K *et al.*, 1995) this type of amino acid replacement did not alter the gene expression and stability of the mutant proteins.

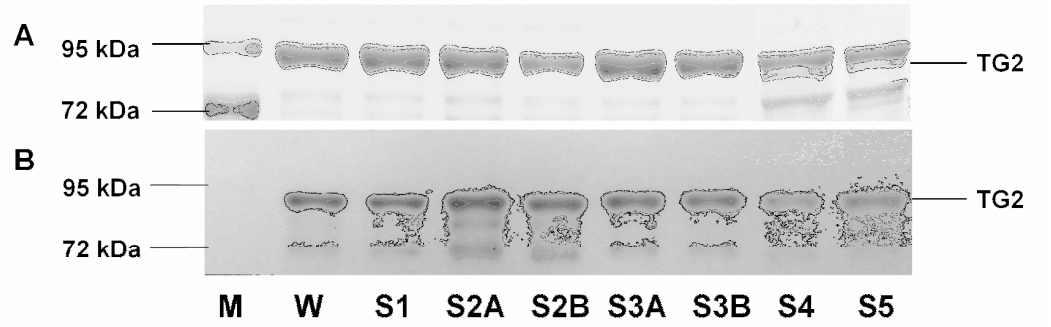


Figure 19. Purity of the expressed recombinant wild type and mutant TG2s. Purity of and binding of monoclonal TG100 antibody to the expressed recombinant wild and mutant TG2s. (A) Protein samples analyzed by 10 % SDS-PAGE and Coomassie Blue staining; 2.2 µg protein/lane. (B) Immunoblot of recombinant proteins was developed with polyclonal anti-TG2 antibody; 0.5 µg protein/lane. Data are representative of two experiments.

For normalisation of protein expression and purity (Fig. 19A and B) the binding of a monoclonal antibody (TG100; epitope: aa 447-538 based on its data sheet) to each mutant was examined using an ELISA method (Fig. 20) since antibodies are more sensitive to conformational changes and could give a good normalization (Goldberg ME., 1991).

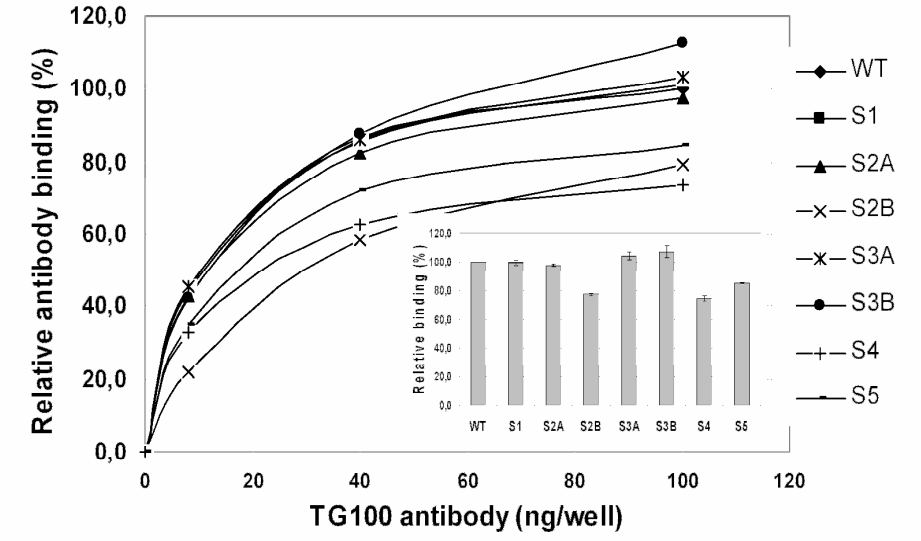


Figure 20. Binding of monoclonal TG100 antibody to the expressed recombinant wild and mutant TG2s. Binding curve of TG100 monoclonal antibody to recombinant TG2s (wild type is 100%) and the relative coefficients of the logarithmic part from the curve equation (insert). Data are representative of two experiments.

Based on the decreased binding and curve linearization, the correction numbers for S4 and S5 mutants were determined as 1.37 and 1.18, respectively. The corresponding primary data were multiplied with these two numbers. The S2B mutant also showed lower antibody binding because the mutation is at the recognition site of the TG100 antibody.

5.3.3. Study of CD spectra of mutants

The native state of the purified protein was checked by circular dichroism spectroscopy (Fig. 21).

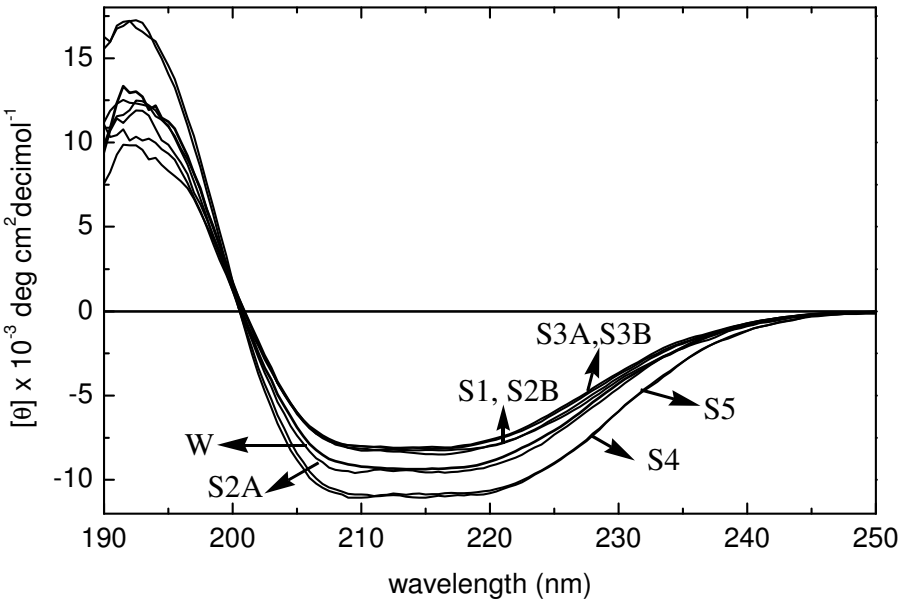


Figure 21. CD spectra of recombinant TG2 proteins.

The native state of the purified protein was checked by circular dichroism spectroscopy (Fig. 21). The CD spectra of the mutants showed distinct but slight deviation from that of the wild type TG2, which suggested that their secondary structures were not altered significantly by the mutations. The CDs of the mutants could be classified into three different groups; 1) mutant S2A gave near identical CD curve with that of wild type TG2, 2) S4 and S5 had larger negative ellipticities in the range 200-240 nm and 3) the rest of the mutants (S1, S2B, S3A and S3B) had smaller ellipticities in the range 200-240 nm than that of the wild type TG2. The CD deconvolution (Table 3.), performed with the analysis programs continll, cdsstr and selcon3 (Lobley A *et al.*, 2002; Whitmore L and Wallace BA., 2004 and 2008) were used to characterize the small CD deviations numerically between the wild type TG2 and its mutants. It showed that unordered and

turns structural segments put up about 50% contribution to the secondary structure and their values are very similar for all the studied structures.

Table 3. Results of the deconvolution of CD spectra.

Deconvolution		Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
S1	continll	0.100	0.101	0.183	0.111	0.221	0.284	1.00
	cdsstr	0.07	0.09	0.18	0.11	0.23	0.31	0.99
	selcon3	0.108	0.099	0.178	0.108	0.210	0.275	0.978
S3A	continll	0.087	0.092	0.196	0.109	0.219	0.297	1.00
	cdsstr	0.08	0.09	0.19	0.11	0.23	0.31	1.01
	selcon3	0.096	0.094	0.196	0.107	0.207	0.282	1.01
S3B	continll	0.089	0.098	0.208	0.113	0.214	0.278	1.00
	cdsstr	0.07	0.09	0.19	0.11	0.23	0.30	0.99
	selcon3	0.097	0.101	0.195	0.113	0.202	0.273	0.980
S2B	continll	0.104	0.102	0.173	0.105	0.223	0.294	1.001
	cdsstr	0.09	0.10	0.18	0.10	0.22	0.30	0.99
	selcon3	0.124	0.105	0.178	0.108	0.200	0.271	0.986
WT	continll	0.116	0.113	0.166	0.106	0.223	0.276	1.00
	cdsstr	0.09	0.10	0.17	0.11	0.22	0.30	0.99
	selcon3	0.124	0.112	0.160	0.103	0.220	0.278	0.998
S2A	continll	0.119	0.116	0.157	0.101	0.222	0.285	1.00
	cdsstr	0.10	0.11	0.17	0.10	0.22	0.30	1.00
	selcon3	0.130	0.117	0.155	0.100	0.221	0.281	1.004
S5	continll	-	-	-	-	-	-	-
	cdsstr	0.17	0.13	0.13	0.09	0.19	0.29	1.00
	selcon3	0.169	0.137	0.105	0.084	0.218	0.294	1.006
S4	continll	0.163	0.137	0.106	0.084	0.221	0.288	0.999
	cdsstr	0.18	0.13	0.13	0.08	0.19	0.28	0.99
	selcon3	0.175	0.143	0.099	0.080	0.215	0.294	1.007

The wild type TG2 and S2A had almost identical CD curves and thus very close secondary segments contributions as well. S4 and S5 had nearly identical CD curves but they differed from the wild type in their larger helix and smaller strand contribution resulting in larger negative ellipticities in the range 200-240 nm. In contrast, S1, S2B, S3A and S3B had smaller helix and larger strand contributions compared to that of the wild type resulting in smaller ellipticities. Subtle differences could be observed among the members of this group 3 as well; S3A and S3B had near identical CDs and hence secondary structures, while S1 and S2B are slightly different from them with somewhat larger helix and smaller strand segment contributions.

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

To compare the Ca^{2+}-binding of wild type 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 at other parts of the curve.

All Ca^{2+}-binding site mutant proteins bind less Ca^{2+} than wild type at 1.7 mM $[Ca^{2+}]_{free}$ (Table 4.) and the mean values are significantly different ($p<0.0001$) as calculated using ANOVA. The experimental Ca^{2+}-binding values confirmed that each of the five mutagenised sites contributes to Ca^{2+}-binding of TG2. The disruption of one site by mutation leads to weaker/loss of binding to other sites which suggest cooperative Ca^{2+}-binding properties. For instance, one site was mutated in S1 and the number of bound Ca^{2+} dropped to only two Ca^{2+}-s.

Table 4. The Ca^{2+}-binding of wild type and mutant TG2s at 1.67 mM free Ca-ion concentration measured by equilibrium dialysis. Data are presented as means from three separate experiments done in duplicate.

Mutants	WT	S1	S2A	S2B	S3A	S3B	S4	S5
Bound Ca^{2+} at 1.67 mM $[Ca^{2+}]_{free}$ moles/moles	5.6 ± 0.7	1.7 ± 0.4	4.1 ± 0.7	1.6 ± 0.3	2.3 ± 0.9	2.5 ± 0.5	3.2 ± 1.1	3.0 ± 1.4

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 (S1 binds <0.03 mol of Ca^{2+} /mol TG2) while the wild type binds 0.5 mol Ca^{2+}/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.

The mutant S2A, having almost identical CD curve with the wild recombinant TG2, could bind 4.1±0.7 Ca^{2+}, the largest value out of the studied mutants. Moreover, the mutants S4 and S5, having near identical secondary structural segments by CD deconvolution, but different from that of the wild type recombinant TG2 and mutants of group 3, gave similar values for the bound Ca^{2+}; 3.2±1.1 and 3.0±1.4, respectively. In general, group 3 mutants exhibited lower Ca^{2+} binding activity; S3A and S3B showed similar values but different from those of S1 and S2B in accordance with their calculated segment contributions.

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

Since transglutaminase activity of TG2 is calcium dependent, decreased transglutaminase activity of the mutants could be expected (Fig. 22A). 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 assay as well as by the filterpaper method (Fig. 22B). 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 show GTP-sensitivity (Fig. 23): 100 μM GTP decreased the transglutaminase activity of both wild type and S1, S2A and S2B TG2 with ~40%. These results suggested that GTP can bind to the mutants.

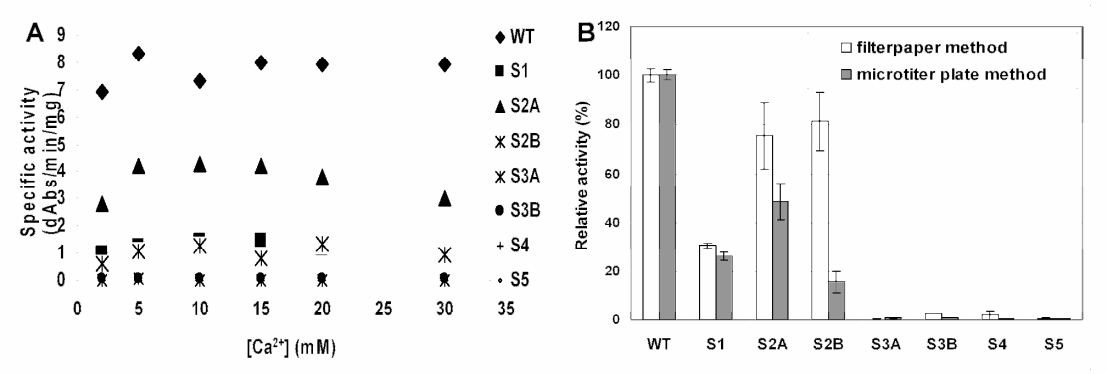


Figure 22. Ca^{2+}-dependent transglutaminase activities of wild type and mutant TG2s. (A) Calcium-dependent transglutaminase activity of recombinant TG2s as determined by using a microtiter plate method. Variation between experimental values was less than 10%. (B) Transglutaminase activity is shown as a percentage of activity of wild type TG2 (microtiter plate method: 8.4 ΔAbs405/min/mg protein, filterpaper method: 77.4 pmol putrescine/min/mg protein) in the presence of 5 mM Ca^{2+}. Data are presented as means from three separate experiments done in triplicate.

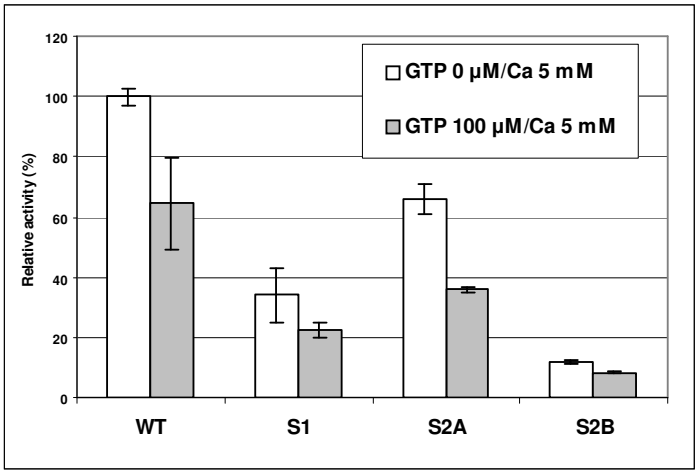


Figure 23. Inhibition of Ca^{2+}-dependent transglutaminase activities of wild and mutant TG2s by GTP. Inhibition of remaining transglutaminase activity of recombinant TG2s by GTP using microtiter plate method. Activity is shown as a percentage of the activity of wild type TG2; the Ca^{2+} concentration was 5 mM.

5.3.6. GTPase activity of mutant TG2 proteins

GTPase activity of mutant proteins was studied, since it is known that Ca^{2+}-binding influences both GTP binding and GTPase activity (Achyuthan KE and Greenberg CS., 1987). Initially, photoaffinity GTP labelling experiments were performed. As shown by autoradiography on Figure 24A, 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 that

wild type protein. Neither longer UV irradiation nor higher amount of the protein led to labelling in case of S4 and S5 (data not shown).

GTPase activity of the mutants correlated well with photoaffinity GTP labelling results except for the two mutants, S4 and S5 (Fig. 24B), which showed increased GTPase activity. Most of the mutants have lower or similar GTPase activity compared to the wild type recombinant TG2. Remarkably, S4 and S5 are characterised with a GTPase activity 1.5-2-fold higher than that of the wild type protein.

The presence of Ca^{2+} decreases binding of GTP to TG2 (Achyuthan KE and Greenberg CS., 1987). In case of the mutants we can see slight decreased GTPase activity at increasing Ca^{2+} concentration except for S4 and S5 similarly to the wild type (Fig. 24B).

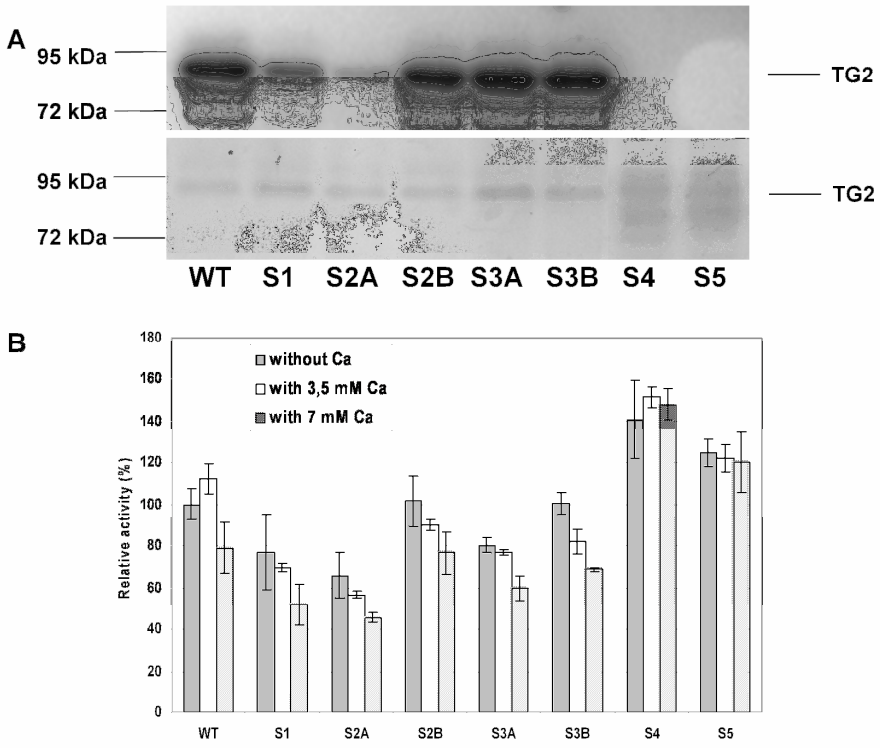


Figure 24. Photoaffinity GTP labelling and GTPase activity of recombinant TG2s. (A) Photoaffinity labelling of TG2 proteins. 2.2 μg protein/lane (upper panel). Proteins were visualised with Coomassie Blue staining (lower panel) (B) GTPase activity and effect of calcium on GTPase activity of recombinant TG2s. Data are presented as means with±SD from three separate experiments done in triplicate.

The mutants S4 and S5 were also produced in GST-fused form at higher purity. Transglutaminase activities of GST-S4 and GST-S5 mutants were measured using the two

methods described above and both failed to detect any activity (data not shown). We found slightly increased GTPase activity in case of GST-S4 mutants (130.7±3.4% compared to GST-WT as 100%) and super GTPase activity in case of GST-S5 mutant (353±38% compared to GST-WT).

5.3.7. Antigenicity of mutant TG2 forms

In coeliac disease transglutaminase 2 has been demonstrated as the main autoantigen. The epitopes are conformational (Seissler J *et al.*, 2001) and the presence of Ca^{2+} can increase the binding of coeliac autoantibodies to TG2 (Roth EB *et al.*, 2003; Sulkanen S *et al.*, 1998) – though there are some contradictory results (Nakachi K *et al.*, 2001; Korponay-Szabó I *et al.*, 2001). In attempt to dissolve this controversy our Ca^{2+}-binding site mutants were tested in an ELISA system to see whether they interact with coeliac antibodies or not (Fig. 25). The S3A, S3B and S5 mutants showed slightly decreased affinity to coeliac autoantibodies. Interestingly, S4 mutant showed significantly lower binding (11.5±8.2 % compared to wild type 100%) than other mutants.

The binding of coeliac autoantibodies to TG2 is influenced by the presence or absence of Ca^{2+} in case of guinea pig TG2 (Roth EB *et al.*, 2003). Therefore, we also examined the effect of Ca^{2+} and GDP on the binding of coeliac autoantibodies to mutant TG2s. The presence of 2 mM EDTA, 20 μM GDP or 5 mM calcium failed to alter the antigenicity of the enzymes (Fig. 25, insert). It has been recently reported (Byrne G *et al.*, 2007) 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 (data not shown).

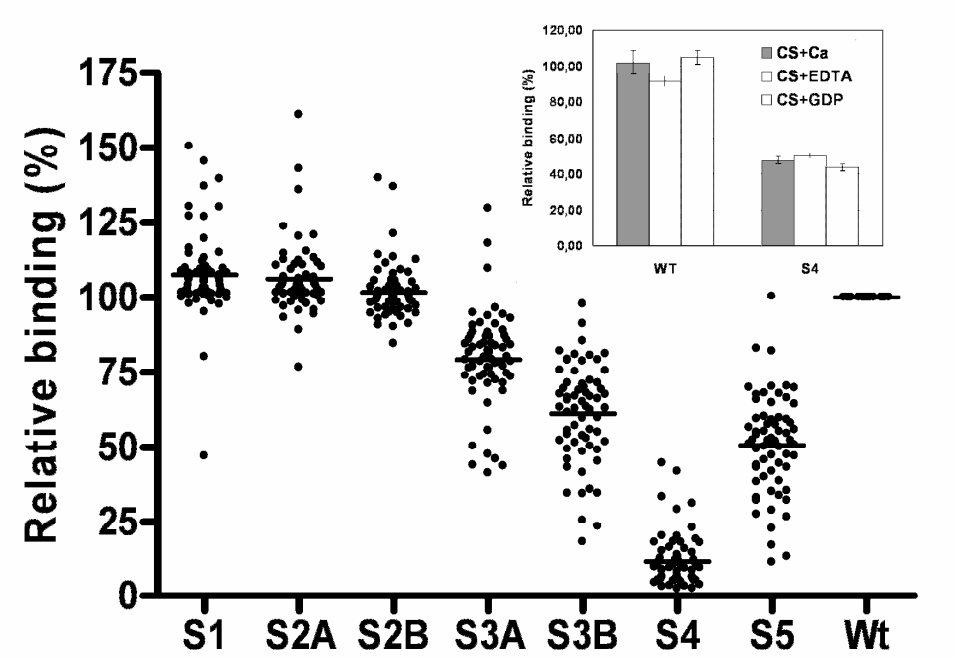


Figure 25. Binding of coeliac antibodies to wild and mutant recombinant TG2. The wild type (Wt) is 100%, sera dilution: 1:200, n=64. The effect of coating with 2 mM EDTA or 20 µM GDP compared to 5 mM calcium on the antibody binding (insert).

6. 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.

6.1. Effects of coeliac autoantibodies on TG2 activities

TG2 activity and protein have been detected at critical sites of coeliac disease, such as the intestinal brush border and subepithelial compartments (Esposito C *et al.*, 2003; Sharp G *et al.*, 1988; Hansson T *et al.*, 2002; Brachet P and Tome D., 1992). 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 (Korponay-Szabó IR *et al.*, 2004).

The potential effects of deposited antibodies are highly relevant in the progression of the disease both in the intestine and in other organs; 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 (Jones RA *et al.*, 1997).

In this study, we did not observe significant inhibition of transglutaminase activity by coeliac autoantibodies. In the opposite, we found activation of enzyme activity of both recombinant and natural TG2 under some conditions. Coeliac patient antibodies enhanced transglutaminase reaction velocity in assays with large and immobilised glutamine acceptor proteins. This type of assay is the most relevant for substrates encountered in the extracellular matrix, where the enzyme works while being anchored to fibronectin (Griffin

M *et al.*, 2002; Verderio EA *et al.*, 2003). Within one patient different antibody clones are produced against various epitopes of TG2 (Sblattero D *et al.*, 2002) and the spectrum (epitope specificity mixture) of these antibody populations may significantly vary among patients (Seissler J *et al.*, 2001). This heterogeneity is reflected in the presented data; however, when normalised antibody concentrations have been compared, the results were less heterogeneous, clearly showing activating effects. 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. In fact, conventional affinity purification may result in a skewed antibody population, as antibodies with the highest affinity can be retained on the column, and may thus be missing. It is also difficult to ensure that TG2 preserves its correct conformation during the purification and one really gets the antibodies with relevant epitope specificities.

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 (Folk JE., 1983). The first part of the reaction was accelerated by both coeliac and non-coeliac immunoglobulins as detected by the release of ammonia. Therefore, the specific activating effect of the coeliac antibodies seems to result from their influence on the second part of the reaction, i. e. the accessibility of the glutamyl-acceptor substrate.

The activating effect of coeliac antibodies on TG2 was not observed before (Esposito C *et al.*, 2002; Roth EB *et al.*, 2003; Dieterich W *et al.*, 2003). However, previous investigators used only liquid phase assays and used a much longer incubation time (up to two hours) (Dieterich W *et al.*, 2003). TG2 activity declines rapidly after 30 minutes at 25 or 37˚C, which could mask any initial activation effects. In addition, TG2 is known to incorporate also itself into its substrates (Barsigian C *et al.*, 1991; Fleckenstein B *et al.*, 2004) which are present in the cell extracts and which process could inhibit the enzyme activity. One further explanation can be that we examined young children (groups E, D and G) in whom antibodies are composed mainly of initially targeted epitope specificities.

Gain of enzymatic function upon binding of an antibody has been observed with a number of enzymes, such as elastase (Morcos M *et al.*, 1998), alkaline phosphatase (Shinozaki T *et al.*, 1998) and also with rabbit antibodies raised against the Ca^{2+}-activated form of guinea pig TG2 (Fesus L and Laki K., 1977). Tetanus toxin and ethanol are also capable to increase transglutaminase activity and decrease thermal deactivation (Facchiano F and Luini A., 1992; Sessa A *et al.*, 1997). Antibodies may stabilise conformation and may prevent inactivation by self-cross-linking or by other mechanisms, such as nitrosylation observed upon nitric oxide release (Lai TS *et al.*, 2001; Melino G *et al.*, 2000). TG2 is indeed a very sensitive enzyme with rapid loss of transamidating activity in purified form and also outside of cells (Griffin M *et al.*, 2002; Verderio EA *et al.*, 2003). Alternatively, antibodies also may facilitate access of substrates by modifying the conformation surrounding the active site. It was shown that mutagenesis of flexible residues resulted in increased enzymatic activity of microbial transglutaminase (Shimba N *et al.*, 2002). Binding of coeliac antibodies also may interfere with the regulatory function of the C-terminal part where important target epitopes were located (Sblattero D *et al.*, 2002; Seissler J *et al.*, 2001; Nakachi K *et al.*, 2004). 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 red blood cell TG2, thus also with the naturally produced, correctly folded enzyme. 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* (Esposito C *et al.*, 2003).

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 (Esposito C *et al.*, 2002); 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.

Severe malabsorption in young children aged 9-24 months is the classical textbook presentation of gluten-enteropathy (Walker-Smith JA and Murch S., 1999), but

malabsorption can develop at any time during life. Although TG2 autoantibodies are present in virtually all the coeliac cases, 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. The antibodies of young female coeliac patients were indeed shown to bind to distinct TG2 epitopes (Tiberti C *et al.*, 2003) as compared to those of adults.

TG2 activity is normally tightly regulated in tissues. Its inappropriate activation may trigger cell death and extracellular matrix deposition (Shin DM *et al.*, 2004; Gross SR *et al.*, 2003) and may counteract programmed inactivation of TG2 by nitric oxide during inflammation (Melino G *et al.*, 2000). Many coeliac subjects are clinically symptom-free despite a flat small-intestinal mucosa, but have increased cell proliferation and matrix turnover (Przemioslo R *et al.*, 1995). Sustained TG2 activity may lead to a reduced rate of matrix turnover (Gross SR *et al.*, 2003) 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. Penetration of IgA autoantibodies into subcellular compartments and into the nucleus has been described in some autoimmune diseases (Malmborg AC *et al.*, 1998). 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 (Im MJ *et al.*, 1997).

6.2. Specific anti-TG2 antibodies in coeliac disease

When autoantibodies (EmA) against TG2 were not measurable in the serum, TG2-specific gluten-dependent autoantibodies were deposited and detectable in the small bowel mucosa in all coeliac disease patients. 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 (Salmi TT *et al.*, 2006).

As KSCN is frequently used to test avidity of antigen-antibody binding (Jones CL., 1987) our results also indicate that coeliac antibodies are bound to intestinal TG2 with considerably high avidity. During a long-standing immune reaction antibodies with increasing avidity are produced, which makes it understandable 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 (Halttunen T and Mäki M., 1999), but the fact that these autoantibodies are not present in the serum of every coeliac patient contradicts this conception (Feighery *et al.*, 2003; Prasad S *et al.*, 2001). We showed that autoantibodies (equivalent to EmA) targeted against TG2 were deposited in the small bowel mucosa of even seronegative coeliac disease patients, and further, that these deposits were gluten-dependent. Moreover, we also could demonstrate that the deposited IgA is functional towards TG2, as it was able to bind also externally added recombinant human 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.

6.3. Calcium binding of TG2

We presented that specific coeliac autoantibodies 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 (Roth EB *et al.*, 2003; Sulkanen S *et al.*, 1998). The Ca^{2+}-bound X-ray structure of TG2 is not known but the exact knowledge of Ca^{2+}-binding site of the enzyme and its relation with activities of TG2 is very important. Interestingly, TG2 also has GTP-binding site and can hydrolyse GTP but does not have typical GTP-binding site (Liu S *et al.*, 2002). 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 low sequence similarities the Ca^{2+}-binding site of FXIIIa, which is homologous to S2 in TG2 and TG3, was originally considered as an EF-hand like motif. Later, the studies of real EF-hand domains and NMR data (Ambrus A *et al.*, 2001) clarified that it is not an EF-hand like motif.

Members in the transglutaminase enzyme family have some highly conserved negatively charged amino acids with high surface potential. Ikura *et al.* mutated two highly conserved anionic sites of the guinea pig TG2 (Ikura K *et al.*, 1995) which were earlier suggested by sequence comparison as putative Ca^{2+}-binding sites (Nakanishi K *et al.*, 1991); their data showed that these sites are not essential or directly involved in Ca^{2+}-binding. By sequence comparison the Ca^{2+}-binding sites studied here somewhat overlap with these negatively charged surface patches. Every mutated sites investigated by us are located on a loop or border of a loop which could allow the appropriate coordination of Ca^{2+} and, in addition, may induce a change in the structure of the protein. Without Ca^{2+}-bound X-ray structure, however, it is not possible to establish the exact participation of the different side chains in Ca^{2+}-binding and protein function.

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 (Ahvazi B *et al.*, 2003). According to our data 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 effective 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 a still good residual transglutaminase activity. The loss of S3 calcium binding 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 this activity similarly to the case of TG3. It is very likely that Glu-329 (replaced in the S3B mutant) plays a crucial role in Ca^{2+} coordination and regulation of transglutaminase activity. It is interesting to note that in the active form of TG2 (Pinkas DM *et al.*, 2007) the S3 site shows important changes just as the GTP binding site which is also built up by two or three loops. Datta *et al.* (Datta S *et al.*, 2006) studied how three Ca^{2+}-binding site mutants of TG2 influence cell survival; these sites correspond to our S1, S2 and S3A sites. They changed two amino acids to Ala at targeted sites and this resulted in decreased

transglutaminase activity but similarly to our results no change in GTPase activity and GTP binding, except for the N229A, D233A mutant (labelled S1 by us).

Each Ca^{2+}-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 (Gifford JL *et al.*, 2007) among the Ca^{2+}-binding sites of TG2. Ahvazi et al. also found indications that the S2 and the S3 sites may cooperate in TG3 (Ahvazi B *et al.*, 2003). 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 (Di Venere *et al.*, 2000)? In case of the canonical C2 domain it is a known feature that a third Ca^{2+} binds with lower affinity to the domain but in the presence of interaction partners – phospholipids in case of C2 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+} (Rizo J *et al.*, 1998). Further study is required to clarify whether substrates, other interacting partner or lipid molecules can regulate Ca^{2+} affinity of the enzyme.

Interestingly, the S1-S3 mutants and wild type showed Ca^{2+}-sensitive GTPase activity but in case of S4 and S5 this could not be observed. In the TG3 structure the Asp-324 amino acid, which coordinates the S3 analogue Ca^{2+}-binding site directly and is located on a loop forming a part of the S3B analogue site, is responsible for a switch between GTP and Ca^{2+}-binding by opening a channel for the acyl glutamine donor substrate (Ahvazi B *et al.*, 2002 and 2003). 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 regulating 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. Moreover, these two mutations resulted in an increased basal GTPase activity. The two mutated sites are sterically close to the hydrophobic pocket for GTP/GDP binding which is formed by the side chains from Phe-174, Val-479, Met-483, Leu-582 and Tyr-583 (Liu S *et al.*, 2002). They may conformationally influence GTP binding and GTPase activity of TG2 altering the position

of Phe-174 docking amino acid and of the Lys-173 which is the nucleophilic attacking group in GTP hydrolysis (Fig. 26).

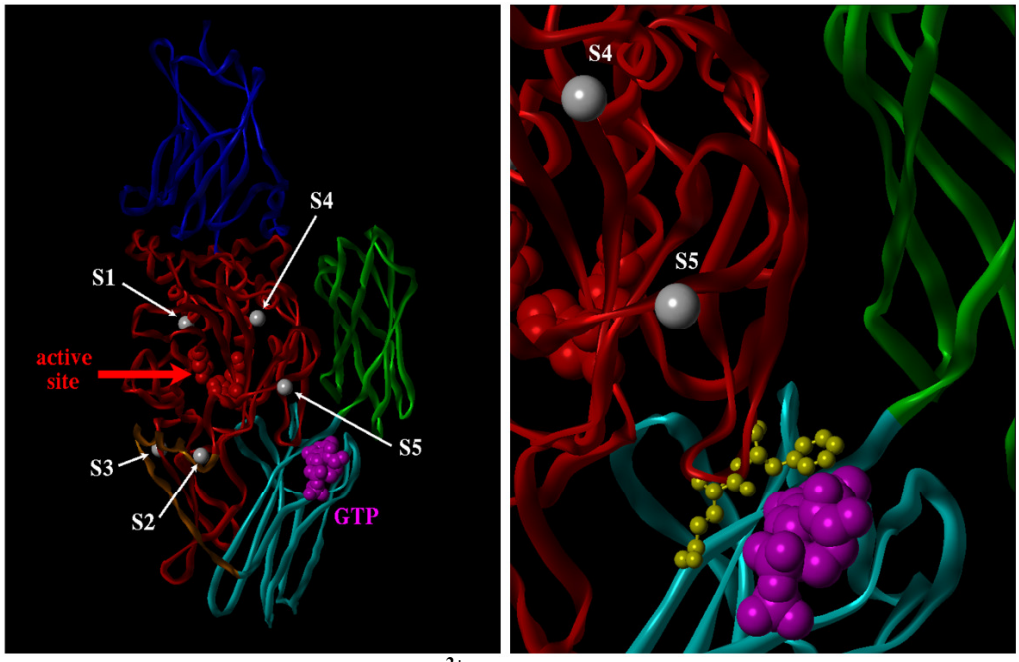


Figure 26. The presumable five Ca^{2+}-binding sites of TG2 on molecule model and circumstances of S4 and S5 which can regulate the GTP binding and GTPase activity of TG2. On the Cα backbone of TG2 the core domain is red, the first β-barrel is blue and the second β-barrel is green. The transglutaminase active site amino acids are labelled with red and GDP with purple spheres. The yellow ball and stick indicate the Lys-173 and Phe-174 while the grey spheres show the proposed location of bound Ca^{2+}-s.

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.

Mutagenesis of the S4 and S5 sites leads to decreased binding of coeliac anti-TG2 autoantibodies to the enzyme. It has been reported that mutation of the catalytic triad of TG2 at its active site for transglutamination disturbs the binding of coeliac autoantibodies to TG2 (Byrne G *et al.*, 2007). However, our C277S mutant demonstrates similarly good binding of autoantibodies as the wild type enzyme and the wild type enzyme can still work as a transglutaminase in the presence of bound coeliac antibodies. 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 (Siegel M *et al.*, 2008). 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. Previous results suggested that binding of Ca^{2+} to TG2 is needed to promote the binding of coeliac antibodies to the enzyme (Roth EB *et al.*, 2003; Sulkanen S *et al.*, 1998). The S1-S3 sites do not have a role in antibody binding because these mutants can equally well bind the antibodies as wild type. According to our data the S4 and S5 calcium binding sites may be needed to form the coeliac epitope on TG2. 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. 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.

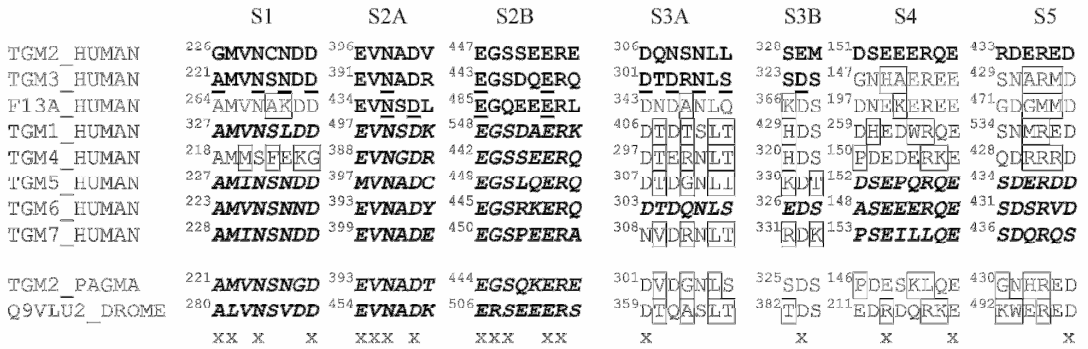


Figure 27. Multiple sequence alignment of Ca^{2+}-binding sites of human transglutaminases
Sequence alignments of Ca^{2+}-binding sites of TG2 compared to the other members of transglutaminase family using ClustalW. The bold characters mark the proven Ca^{2+}-binding sites and the underlined characters show the amino acids which coordinate Ca^{2+} in known crystal structures. Characters in bold-italics show potential Ca^{2+}-binding sites as compared to those in TG2. Squares indicate amino acids which may preclude calcium binding compared to the homologous sites in other members of transglutaminase family where Ca^{2+}-binding sites were verified. Invertebrate transglutaminase used in the alignments: Q9VLU2_DROME is the A isoform of *Drosophila melanogaster* transglutaminase. TGM2_PAGMA is red sea bream (*Pagrus major*) TG2. 'x' indicates the amino acids which are conserved in the enzyme family.

Members of the mammalian transglutaminase family have evolved through duplication of a single gene and subsequent redistribution to distinct chromosomes (Grenard P *et al.*, 2001). 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 (Fig. 27) 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 as well. 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 site: 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 (Grenard P *et al.*, 2001) 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; this perhaps explains how these transglutaminases may compensate (Szondy Z *et al.*, 2003) for the loss of TG2 in knock-out mice.

7. 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.

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10. PUBLICATION LIST

10.1. List of publications used in the thesis:

Kiraly R, Vecsei Z, Demenyi T, Korponay-Szabo IR, Fesus L. Coeliac autoantibodies can enhance transamidating and inhibit GTPase activity of tissue transglutaminase. Dependence on reaction environment and enzyme fitness. *J Autoimmun* 2006;**26:**278-287. (JCR 2006 IF: 2,154)

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Kiraly R, Csosz E, Kurtan T, Antus S, Vecsei Z, Korponay-Szabo IR, Keresztessy Z and Fesus L. Functional significance of five non-canonical Ca^{2+}-binding sites of transglutaminase 2 characterized by site directed mutagenesis (submitted manuscript)

10.2. Other publications:

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