Identification of tissue transglutaminase as the autoantigen of celiac disease

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Celiac disease is characterized by small intestinal damage with loss of absorptive villi and hyperplasia of the crypts, typically leading to malabsorption[1]. In addition to nutrient deficiencies, prolonged celiac disease is associated with an increased risk for malignancy, especially intestinal T-cell lymphoma[1-3]. Celiac disease is precipitated by ingestion of the protein gliadin, a component of wheat gluten, and usually resolves on its withdrawal. Gliadin initiates mucosal damage which involves an immunological process in individuals with a genetic predisposition. However, the mechanism responsible for the small intestinal damage characteristic of celiac disease is still under debate[4-6]. Small intestinal biopsy with the demonstration of a flat mucosa which is reversed on a gluten-free diet is considered the main approach for diagnosis of classical celiac disease[7]. In addition, IgA antibodies against gliadin and endomysium, a structure of the smooth muscle connective tissue, are valuable tools for the detection of patients with celiac disease and for therapy control[7-9]. Incidence rates of childhood celiac disease range from 1:300 in Western Ireland to 1:4700 in other European countries[10-12], and subclinical cases detected by serological screening revealed prevalences of 3.3 and 4 per 1000 in Italy and the USA, respectively[13,14]. IgA antibodies to endomysium are particularly specific indicators of celiac disease[9,15], suggesting that this structure contains one or more target autoantigens that play a role in the pathogenesis of the disease[16,17]. However, the identification of the endomysial autoantigen(s) has remained elusive. We identified tissue transglutaminase as the unknown endomysial autoantigen. Interestingly, gliadin is a preferred substrate for this enzyme, giving rise to novel antigenic epitopes.

Western blotting with serum samples from active celiac disease patients containing high-titer antiendomysial IgA antibodies did not allow us to identify specific protein bands in extracts from placenta, uterus, liver or gut, possibly because of loss of antigenicity of the partially denatured protein preparations. Therefore, we used immunoprecipitation from cell cultures, a method that can be used for target antigens susceptible to denaturation.

Cell cultures were screened by immunohistochemistry for the expression of the autoantigen with high-titer sera of patients with celiac disease and the alkaline phosphatase/monoclonal anti-alkaline phosphatase (APAAP) technique[18]. This method revealed that cytoplasmic vesicles of HT1080 (human fibrosarcoma) cells, WI38 (human embryonal fibroblasts) as well as Hep1 and HepG2 (human hepatocarcinoma) cells were specifically stained by the patients' IgA antibodies. We then immunoprecipitated both supernatant and cell lysate of HT1080 cells metabolically labeled with [^{35}S]methionine, using the IgA fraction from celiac disease sera bound to Sepharose. After separation by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography, immunoprecipitation of the supernatant yielded varying amounts of a high-molecular-weight protein that was identified as fibronectin by western blotting and by the characteristic peptide pattern after protease V8 digest (data not shown). When the cell lysate was analyzed in a similar way, a single protein band with an apparent relative molecular weight (M_r) of 85,000 before and after disulfide reduction was immunoprecipitated exclusively with 25 celiac disease serum samples, but with none of 25 control serum samples (from healthy adults, patients with ulcerous colitis or Crohn's disease, or patients with Sjögren's syndrome, an autoimmune disease characterized by high-titer serum autoantibodies) (Fig. 1, *a* and *b*). As before, varying quantities of fibronectin could be precipitated in celiac disease, but also small amounts were precipitated in some control sera, suggesting that this large adhesive glycoprotein was not the primary target of the celiac disease autoimmune response. Instead, fibronectin may have precipitated nonspecifically or in association with the 85-kDa autoantigen (see below).

In order to further characterize the putative 85-kDa autoantigen, larger quantities of the protein were isolated by immunoprecipitation, SDS–PAGE and electrophoretic elution, followed by cleavage with endoproteinase Asp-N. The resultant fragments were separated in a 10% tricine gel (Fig. 2*a*) and transferred to a polyvinylidene-difluoride membrane. Three major cleavage products with M_r 10,000, 14,000 and 16,000 were excised and subjected to amino-terminal sequence analysis. All three peptides yielded sequences that could be clearly assigned to tissue transglutaminase (EC 2.3.2.13, tTG)[19] (Fig. 2*b*). An interaction of tTG with fibronectin has been described[20], which might explain the occasional coprecipitation of fibronectin with tTG in our immunoprecipitation experiments.

Tissue transglutaminase (synonymous with erythrocyte, cellular, endothelial, cytoplasmic, type II or liver TG)[21] belongs to a family of calcium-dependent enzymes that catalyze the crosslinking of proteins resulting in the formation of an ε-(γ-glutamyl)-lysine bond. Whereas several proteins can serve as acceptor substrates, only a limited number of donor substrates exists. The physiological role of the tTG has only been partly explored. Although the enzyme is normally localized in the cytoplasm, tTG can be released during wounding, where it associ-

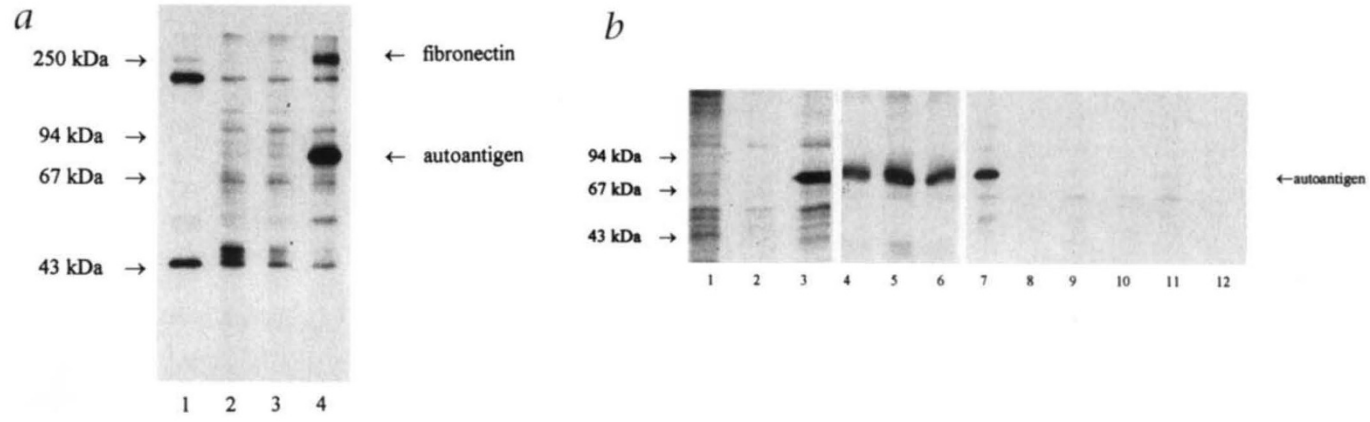
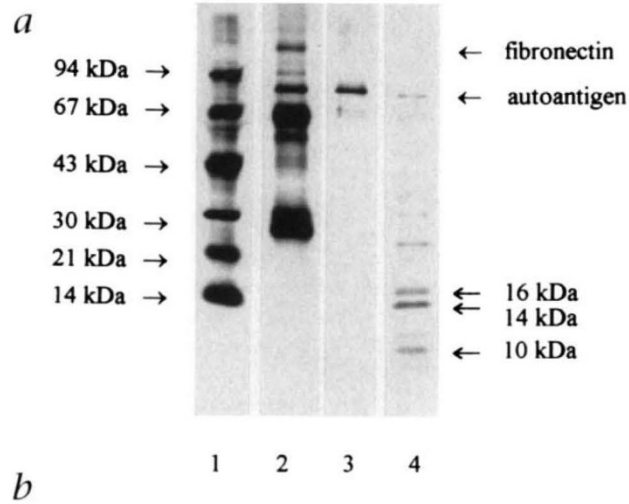


Fig. 1 IgA antibodies of celiac disease sera precipitate a characteristic protein species from cell cultures. Autoradiography of the immunoprecipitated cell lysate from HT1080 cells after separation by SDS–PAGE under reducing conditions. *a* (lane 1), Preadsorption on plain Sepharose CL-4B shows nonspecific binding of two major proteins; (lanes 2 and 3), immunoprecipitates with serum IgA from healthy controls and (lane 4), precipitation of the 85-kDa autoantigen by serum IgA from a patient with celiac disease. Note high-molecular-mass material that was identified as fibronectin, which coprecipitated from samples from patients with celiac disease as well as some with non-celiac disease. *b*, Immunoprecipitates with serum IgA from celiac disease patients and controls: (lane 1), material preadsorbed on plain Sepharose; (lane 2), healthy control; (lanes 3–7), patients with celiac disease; (lane 8), Crohn's disease; (lane 9), ulcerative colitis; (lanes 10–12), patients with Sjögren's syndrome.

ates with cell surfaces or certain extracellular matrix molecules[22]. tTG can crosslink fibronectin[20,23], osteonectin[24], collagen II[24], V and XI[25], procollagen III[26] and nidogen[27]. Thus, secreted tTG is thought to stabilize the provisional extracellular matrix in granulation tissue[22]. Furthermore, its expression is enhanced during apoptosis, leading to irreversible crosslinking of intracellular proteins, and may be deranged during growth of some tumors[28,29]. The commercially available enzyme from guinea pig liver shows a high-protein sequence identity with human tTG (>80%)[19], with a high probability of conserved antigenic epitopes, which allowed us to carry out further experiments with this preparation.

Gliadin, the dietary factor incriminated in the initial pathogenesis of celiac disease, contains numerous glutamines amounting to approximately 40% of its amino acids[4]. In order to test whether gliadin could serve as a substrate for tTG, we used an *in vitro* assay with radiolabeled putrescine as acceptor substrate[27]. Gliadin proved to be an excellent substrate and was quantitatively crosslinked by tTG, whereas the control proteins (chicken albumin, bovine serum albumin, α-lactalbumin, β-lactoglobulin) were essentially unaltered (data not shown). These results are in accord with previous reports that showed preferential incorporation of putrescine in gliadin by a transglutaminase extract from intestinal tissue[30] and also crosslinking of gliadin by a lysate of human red blood cells that contain tTG (ref. 31).

The celiac disease-specific IgA autoantibodies to endomysium are detected and semiquantified by performing indirect im-



b

tissue transglutaminase: 28′ Arg Glu Lys Leu Val Val Arg Arg Gly Gln Pro Phe Trp

10 kDa fragment: Arg Glu Lys Leu Val Val Arg Arg Gly Gln Pro Phe (Ser)

tissue transglutaminase: 581′ Asp Leu Tyr Leu Glu Asn Pro Glu Ile Lys Ile Arg Ile Leu Gly

14 kDa fragment: Asp Leu Tyr Leu Glu Asn Pro Glu Ile -X- Ile -X- Ile Leu Gly

tissue transglutaminase: 438′ Asp Ile Thr His Thr Tyr Lys Tyr Pro Glu

16 kDa fragment: Asp Ile Thr Leu Thr Tyr Gln Tyr Pro (Val)

Fig. 2 Purification and amino-terminal sequence analysis of the 85-kDa autoantigen. *a*, Separation of the unlabeled immunoprecipitated 85-kDa autoantigen by SDS–PAGE under reducing conditions; proteins revealed by silver staining. (lane 1), Molecular weight standard (as mass); (lane 2), immunoprecipitate of the 85-kDa autoantigen; additional major bands are fibronectin, and light and heavy immunoglobulin chains (25–30 kDa and 60–65 kDa, respectively); (lane 3), 85-kDa autoantigen purified by electrophoretic elution; (lane 4), fragments of the purified autoantigen after digestion with endoproteinase Asp-N; the three peptides subjected to N-terminal sequence analysis are marked. *b*, The N-terminal sequence data of the three fragments of the 85-kDa autoantigen generated by endoproteinase Asp-N were compared with the Swiss-Prot 31 data base (PC/GENE, IntelliGenetics) and found compatible with human tissue transglutaminase (EC 2.3.2.13). The identity of residue X is unknown, residues in parentheses are uncertain.

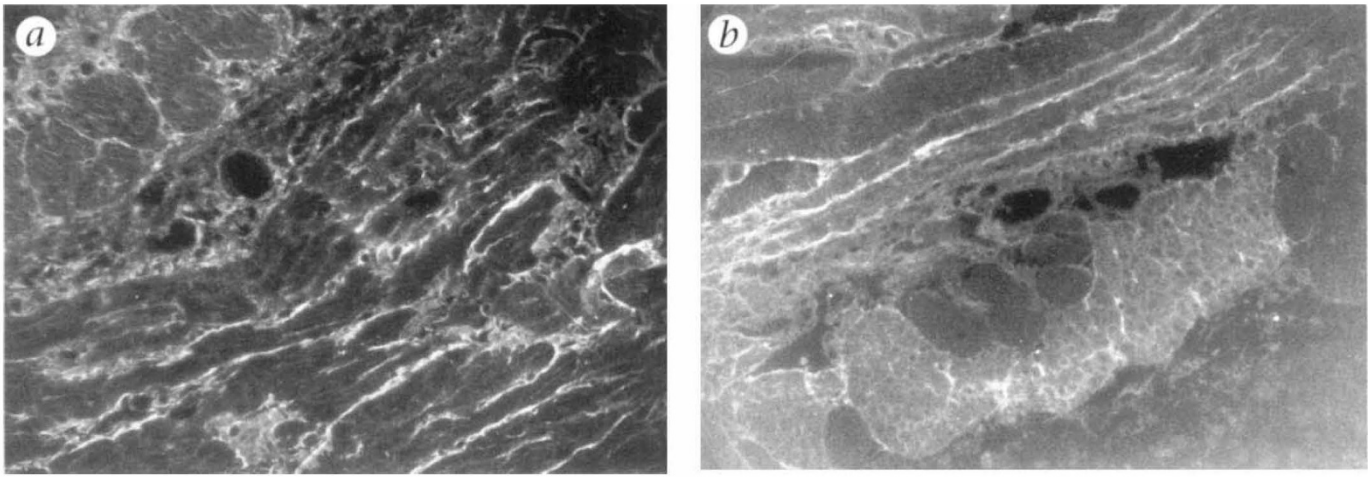


Fig. 3 Tissue transglutaminase as the prominent autoantigen of celiac disease. Indirect immunofluorescence for IgA autoantibodies on monkey esophagus (×62.5). *a*, The characteristic staining of endomysial structures by high-titer celiac disease serum (dilution 1:320). *b*, Loss of specific staining after preincubation of the same serum dilution with tTG, suggesting that the identified tTG is the prominent, if not sole, autoantigen in celiac disease.

munofluorescence on muscular sections of monkey esophagus or umbilical cord[9,15,32]. In order to further confirm tTG as the autoantigen in celiac disease, we performed indirect immunofluorescence with high-titer celiac disease serum samples on monkey esophagus with or without prior preincubation of the sera with tTG. Whereas untreated celiac disease serum samples showed the characteristic feature of endomysial labeling, pretreatment with tTG nearly completely abolished endomysial immunofluorescence (Fig. 3, *a* and *b*). This demonstrated that tTG represents the predominant, if not sole, endomysial autoantigen considered characteristic for celiac disease.

On the basis of these data, we established an enzyme-linked immunosorbent assay (ELISA) for the detection of IgA anti-tTG antibodies. Serum samples either from celiac disease patients with well-known anti-endomysium titers (as determined by immunofluorescence) or from controls were analyzed. In this ELISA only celiac disease patients displayed elevated levels of IgA anti-tTG, whereas none of the controls showed significant reactivity (Table 1). Furthermore, two of the high-titer sera were retested at higher dilutions. Even at 1:3200 these sera exhibited extinctions of $A = 1.02 \pm 0.09$ (celiac disease A) and 0.84 ± 0.12 (celiac disease C), thus demonstrating the high sensitivity of the ELISA test (data not shown). In addition, there was a good correlation between decreasing titers of anti-endomysium IgA and the IgA antibodies to tTG as measured by ELISA once patients were on a gluten-free diet (Table 2). We also measured IgG autoantibodies to tTG using the same ELISA system, in which the anti-IgA antibody was replaced by an anti-IgG antibody. Even though the high-titer celiac disease sera showed elevated anti-tTG IgG levels, this system was not comparable in sensitivity and specificity to the IgA-based ELISA (Table 2). In addition, some control sera, especially those of patients with chronic inflammatory disorders, showed raised titers of IgG anti-tTG (data not shown). The high sensitivity and specificity for celiac disease of IgA class autoantibodies to tTG is most plausibly explained by the prominent production of IgA by mucous membranes, especially those of the intestinal tract, with the IgA anti-tTG response thus reflecting the active phase of intestinal mucosal injury.

The identification of tTG as the autoantigen in celiac disease should fuel novel concepts about the insufficiently understood pathogenesis of celiac disease. The usually intracellular enzyme is released from cells during wounding[22]. One of its roles is the irreversible crosslinking of a small set of extracellular matrix and some cytoplasmic proteins, including cytoskeletal elements. This crosslinking might stabilize the wound area and protect the surrounding tissue from further damage. In addition, cytoskeletal crosslinking has been reported in apoptosis, leading to the noninflammatory elimination of potentially dangerous infected cells[28].

The finding that gliadin is a preferred substrate for the otherwise highly substrate-specific enzyme is of particular interest.

Table 1 Enzyme-linked immunosorbent assay (ELISA) based on tTG for the celiac disease (CD) autoantigen

Serum sample	EmA titer	Serum IgA anti-tTG dilution (1:400)
CD A	(1:640)	>3.500
CD B	(1:320)	>3.500
CD C	(1:320)	3.114 ± 0.024
CD D	(1:320)	1.257 ± 0.024
CD E	(1:320)	1.977 ± 0.018
CD F	(1:320)	1.525 ± 0.085
CD G	(1:160)	1.208 ± 0.019
CD H	(1:160)	0.747 ± 0.048
CD I	(1:160)	2.174 ± 0.291
CD K	(1:80)	0.667 ± 0.037
CD L	(1:80)	1.372 ± 0.093
CD M	(1:40)	0.286 ± 0.009
Ulcerative colitis	(ND)	0.119 ± 0.014
Indeterminate colitis	(ND)	0.092 ± 0.016
Crohn's disease	(ND)	0.090 ± 0.001
Sjögren's syndrome	(ND)	0.137 ± 0.004
Sjögren's syndrome	(ND)	0.082 ± 0.003
Alcoholic liver fibrosis	(ND)	0.059 ± 0.001
Healthy control	(ND)	0.070 ± 0.001

An ELISA for IgA antibodies against tTG was established. Shown are the optical densities of twelve different celiac disease serum samples with varying anti-endomysium antibody (EmA) titers as well as seven control sera. Sera were diluted 1:400 and listed according to their EmA titer (in parentheses). All celiac disease sera with high EmA-titers (>1:80) show high anti-tTG IgA titers, whereas the control sera display background levels. Celiac disease sera with low EmA-titer (<1:80) show only slightly raised ELISA titers, but are highly elevated above controls when diluted 1:100 (0.67 ± 0.02 versus 0.12 ± 0.01; celiac disease M, not shown). Values are means (±s.d.) of three parallel determinations. ND, not determined.

Table 2 IgA and IgG anti-tTG antibodies in celiac disease (CD) patients before and after a gluten-free diet

Serum sample	EmA titer	IgA anti-tTG dilution (1:400)	IgG anti-tTG dilution (1:100)
CD A	(1:640)	>3.500	1.267 ± 0.030
CD A gfd	(neg)	0.321 ± 0.013	1.055 ± 0.055
CD B	(1:320)	>3.500	3.116 ± 0.058
CD B gfd	(1:40)	0.286 ± 0.009	0.569 ± 0.009
CD C	(1:320)	3.114 ± 0.024	1.360 ± 0.009
CD C gfd	(neg.)	0.162 ± 0.002	0.959 ± 0.024
CD E	(1:320)	1.977 ± 0.018	0.827 ± 0.032
CD E gfd	(neg.)	0.186 ± 0.041	0.504 ± 0.045
CD F	(1:320)	1.525 ± 0.085	0.555 ± 0.016
CD F gfd	(1:80)	1.372 ± 0.093	3.186 ± 0.069
CD G	(1:160)	1.208 ± 0.019	1.154 ± 0.011
CD G gfd	(1:20)	0.066 ± 0.010	0.370 ± 0.008
CD H	(1:160)	0.747 ± 0.048	0.563 ± 0.014
CD H gfd	(1:80)	0.667 ± 0.037	1.632 ± 0.016

ELISA data for IgA (1:400) and IgG (1:100) class antibodies against tTG of seven different CD serum samples before and after a gluten-free diet (gfd). EmA-titers are given in parentheses. IgA anti-tTG antibodies decline in most patients kept on a gfd. Compared with IgA anti-tTG antibodies, IgG anti-tTG antibodies appear less sensitive markers for the active phase of CD; titers are means (±s.d.) of three parallel determinations.

Bruce *et al.*[30] already demonstrated that gliadin efficiently incorporates putrescine in the presence of intestinal extracts containing transglutaminase. However, these researchers did not differentiate the responsible enzyme nor implicate tTG as the endomysial autoantigen of celiac disease. Furthermore, localization of the intestinal TG within the lamina propria mucosae, with only 1% in the epithelial layer[30], is in agreement with the site of immunological damage in celiac disease.

We hypothesize that damage or hyperpermeability of the intestinal epithelium, either owing to toxic gluten fractions or to other (minor) irritants, triggers the abundant extracellular release of cytosolic tTG, mainly by lamina propria mononuclear or mesenchymal cells. Subsequent crosslinking of dietary gliadin results in gliadin–gliadin or gliadin–tTG complexes and thus creates antigenic neoepitopes. These neoepitopes could then initiate an immune response in genetically susceptible individuals, finally directed both to gliadin and tTG. In fact, a genetic predisposition has been found in celiac disease patients, who often bear the major histocompatibility complex antigens HLA-DQ(α1*0501,β1*0201)[4,33]. Further support comes from our preliminary data showing that a proportion of the IgA autoantibodies of celiac disease patients are directed to such neoepitopes (data not shown). Taken together, these data are well in line with the suggested importance of the celiac disease autoantigen in the pathogenesis of the disease as suggested by others[16,17]. Nevertheless, it must be borne in mind that celiac disease is not a classical autoimmune disease, since IgA antibodies to tTG disappear when gliadin is strictly removed from the diet, and the mucosal damage is reversed without residual fibrosis or scarring.

Our findings further suggest that the immunological detection of IgA autoantibodies to tTG, the newly discovered autoantigen of celiac disease, is a useful tool in the diagnosis and follow-up of the disease. Because of its simplicity, the ELISA for the identified autoantigen now allows an economical and rapid screening of large portions of the general population for the presence of latent or subclinical celiac disease. Therefore, the too-long intervals from early symptoms to diagnosis and finally treatment of celiac disease (median 5.4 years in Germany)[34] could be shortened decisively, leading to a decreased morbidity from this intestinal disease.

We cannot completely exclude IgA autoantibodies to tTG in diseases with mucosal lesions that are similar to celiac disease lesions, for example, tropical sprue, giardiasis, cow milk enteropathy or postenteritis syndrome. However, we think that it is quite unlikely to find these tTG autoantibodies in sera of these patients, because no IgA EmA were found in serum samples from patients with cow milk enteropathy, giardiasis or postenteritis syndrome[9]. Furthermore, no association of these diseases to the celiac disease-associated HLA-DQ genes is described.

By assuming a yet unproven potential of tTG, which is released during wound healing to serve as a further trigger for celiac disease, a novel treatment based on oral feeding of the autoantigen and induction of an oral tolerance may be envisaged[35]. tTG apparently elicits autoantibodies of the IgG class in various other chronic inflammatory conditions and might play a role in the initiation and perpetuation of certain autoimmune diseases.

Methods

Cell culture and immunoprecipitation. HT1080 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco, Eggenstein, Germany). For radioactive labeling 10^5 cells were incubated with 0.2 mCi [^{35}S]methionine (Expre^{35}S^{35}S, Du Pont-NEN, Bad Hamburg, Germany) and cultivated for further 16–20 h. For immunoprecipitation (IP) the cells were lysed in 3 ml 50 mM Tris-HCl, 150 mM NaCl, 1% nonionic detergent, protease inhibitors, pH 7.5, for 10 min at 4 °C. Cell fragments were removed by centrifugation. 1 ml lysate was incubated with 50 µl Sepharose CL-4B (Pharmacia, Freiburg, Germany) for 30 min at room temperature to remove nonspecifically binding proteins. The thus pretreated lysate was incubated with CNBr-activated Sepharose 4B (Pharmacia) preadsorbed with serum IgA either from celiac disease patients or from controls via an anti-human IgA bridging antibody from rabbit (2.4 mg antibody/ml Sepharose; Dianova, Hamburg, Germany) for 16 h at 4 °C. After several washes the precipitated proteins were dissolved in 50 µl SDS sample buffer under reducing conditions and separated by SDS–PAGE (ref. 36).

Isolation, cleavage and sequencing of the autoantigen. For isolation of the autoantigen by electrophoretic elution (Prep Cell 491, Bio-Rad, Krefeld, Germany) the discontinuous buffer system of Laemmli[36] was used. A 7.5% resolving gel (acrylamide, bisacrylamide 30:0.4, Pharmacia) was preceded by a 4% stacking gel. The immunoprecipitated sample was reduced and heated in sample buffer and then separated by a constant voltage of 225 V. Elution was performed in 25 mM Tris-HCl, 0.1 M glycin, 0.01% sodium laurylsulfate and fractions of 1.5 ml (0.8 ml/min) were collected. Fractions of interest were analyzed by tricine-SDS–PAGE (ref. 37) and further concentrated with Centriprep-50 (Amicon, Witten, Germany). Cleavage with endoproteinase Asp-N (Boehringer Mannheim, Germany) was performed in the elution buffer for 30 min at 37 °C, with an enzyme-to-substrate ratio of 1:100. Protein fragments were transferred to a polyvinylidene-difluoride membrane (Immobilon, Millipore, Eschborn, Germany) in a semi-dry-fast-blot apparatus (Fastblot B32/33 Biometra, Göttingen, Germany) and excised. Amino-terminal sequences were determined by Edman-degradation in an Applied Biosystems 477A sequencer (Foster City, CA).

Inhibition of endomysial staining. Preincubation of celiac disease sera (10 µl, diluted 1:320 in phosphate-buffered saline, pH 7.3, PBS) with 1 µg or 10 µg tTG from guinea pig liver (Sigma, Deisenhofer, Germany) or with 10 µg bovine serum albumin (Sigma) was performed for 1 h at room temperature. Monkey esophagus tissue slides (Euroimmun, Lübeck, Germany) were then incubated with the pretreated celiac disease sera, their untreated controls or non-celiac disease control sera in a humidified chamber for 1 h

at room temperature and washed 3× in PBS containing 0.2% bovine serum albumin. Bound IgA was detected with TRITC-labeled antihuman IgA from rabbit (1:50 in PBS; Dianova) for 30 min at room temperature.

Enzyme linked immunosorbent assay. For the ELISA 1 µg tTG (Sigma) in 100 µl PBS was coated per well on 96-well microtiter plates (Nunc, Wiesbaden, Germany) for 2 h at 37 °C, and unreacted sites were blocked with PBS containing 1% bovine serum albumin at 4 °C overnight. Patient and control sera were diluted in 100 µl PBS, 0.1% Tween-20 (Sigma), added to the wells and incubated for 1 h at room temperature. Three washes with PBS, 0.1% Tween-20 were followed by incubation with 100 µl peroxidase-conjugated antibody to human IgA (Dianova) diluted 1:1000 in PBS, 0.1% Tween-20, for 1 h at room temperature. Unbound antibodies were removed by three washes and color was developed by addition of 200 µl 0.1 M sodium citrate, 1 mg/ml *o*-phenylenediamine-hydrochloride, 0.06% H_2O_2, pH 4.2, for 30 min at room temperature. The absorbance was read on an ELISA reader (MRX, Dynatech Laboratories/Dynex Technologies, Denkendorf, Germany) at 450 nm.

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