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ORIGINAL ARTICLE

Gluten T cell epitope targeting by TG3 and TG6; implications for dermatitis herpetiformis and gluten ataxia

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Abstract Transglutaminase 2 (TG2) is well characterized as the main autoantigen of celiac disease. The ability of TG2 to deamidate and crosslink gluten peptides is essential for the gluten-dependent production of TG2 specific autoantibodies. In patients with primarily extraintestinal manifestation of gluten sensitivity the repertoire of autoantibodies may be different. In dermatitis herpetiformis (DH), TG3 appears to be the target autoantigen whereas in gluten ataxia (GA) autoantibodies reactive with TG6 are present. A functional role for TG3 and TG6 in these diseases has yet to be described. It is also not known whether these enzymes can use gluten peptides implicated in the pathology as substrates. We here report that similar to TG2, TG3 and TG6 can specifically deamidate gluten T cell epitopes. However, the fine specificities of the enzymes were found to differ. TG2 can form covalent complexes with gluten by iso-peptide and thioester bonds. We found that both TG3 and TG6 were able to complex with gluten peptides through thioester linkage although less efficiently than TG2, whereas TG6 but not TG3 was able to form iso-peptide linked complexes. Our findings lend credence to the notion that TG3 and TG6 are involved in the gluten-induced autoimmune responses of DH and GA.

Keywords Gluten · Autoantibodies · TG3 · TG6 · Dermatitis herpetiformis · Gluten ataxia

Abbreviations

CD	Celiac disease
DH	Dermatitis herpetiformis
GA	Gluten ataxia
TGase	Transglutaminase
TG2	Transglutaminase 2
TG3	Transglutaminase 3
TG6	Transglutaminase 6

Introduction

Transglutaminases (TGases) (EC 2.3.2.13) constitute of an evolutionary conserved family of Ca^{2+}-dependent enzymes with crosslinking activity. Nine members of this family are encoded in the human genome and in eight of these, the residues constituting the catalytic triad are conserved (Grenard et al. 2001; Lorand and Graham 2003). TGases can either create iso-peptide bonds by cross-linking glutamine residues to small primary amines or protein-bound lysine residues (transamidation), or convert glutamine to glutamate (deamidation) (Folk 1983). Transglutaminase 2 (TG2) is the best characterized and also the most abundant and widely distributed member of this family in tissues. TG2 expression is responsive to acute-phase injury cytokines including IL-6 and TNFα and hence accumulates at

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sites of tissue injury and inflammation (Aeschlimann and Thomazy 2000). Besides a role in stabilization of the extracellular matrix, TG2 may modulate the immune response by regulating the availability and/or activity of critical signaling molecules including TGF-β (Szondy et al. 2003). The enzymatic activity of TG2 is implicated in several diseases including Huntington disease, Alzheimer disease, and celiac disease (CD) (Molberg et al. 1998; Nemes et al. 2001; Jeitner et al. 2001).

CD is a prevalent gluten-sensitive disease where ingestion of gluten initiates and drives a chronic inflammation in the small intestine of genetically predisposed individuals (Sollid 2002). TG2 catalyzes highly specific deamidation of gluten peptides (Molberg et al. 1998; Vader et al. 2002a). This modification improves the binding of the peptides to the disease-associated HLA-DQ2 and HLA-DQ8 molecules, and this is considered essential for the T cell mediated immune response against gluten in CD (Molberg et al. 1998). TG2 is also the primary autoantigen of CD (Dieterich et al. 1997), and the autoantibody production is dependent on the intake of dietary gluten (Sulkanen et al. 1998; Dieterich et al. 1998). This primarily IgA class anti-TG2 production is disease-specific and is used as a serological marker for CD (Fasano and Catassi 2001).

Gluten can also cause extra-intestinal disease manifestations like the skin disorder dermatitis herpetiformis (DH) and a variety of neurological conditions, the most prevalent being gluten ataxia (GA) and gluten neuropathy (Hadjivassiliou et al. 2010). These conditions can present with or without small intestinal symptoms and anti-TG2 autoantibodies (Fry 1995; Hadjivassiliou et al. 1998). DH and GA are, however, characterized by an immune response directed towards other TGase isoforms. DH patients have antibody populations primarily recognizing TG3 (also known as epidermal transglutaminase) while GA patients produce antibodies towards the newly identified isoform TG6 (Sardy et al. 2002; Hadjivassiliou et al. 2008). Antibodies rarely display cross-reactivity with different TG isoforms suggesting that independent events may be involved in their development (Hadjivassiliou et al. 2008). TG3 and TG6 are now considered to be the main autoantigens in DH and GA, respectively, and these antibody populations should prove useful for the diagnosis of these diseases. Whether extraintestinal manifestation of the disease is antibody-mediated and driven by the immune response in the gut or local inflammation remains to be shown.

TG2 can not only deamidate gluten peptides, but also form covalent enzyme-peptide complexes which are believed to act as hapten-carrier complexes fueling the production of anti-TG2 antibodies in CD (Sollid et al. 1997; Dieterich et al. 1997; Vader et al. 2002a; Piper et al. 2002; Fleckenstein et al. 2002; Fleckenstein et al. 2004). These two features are pivotal for the role of TG2 in CD. To date, no study has addressed whether TG3 and TG6 can play a functional role in gluten sensitivity with primarily extraintestinal manifestation comparable to TG2 in classical CD. A prerequisite for their involvement would be their ability to accommodate gluten peptides as substrates. We have addressed the ability of TG3 and TG6 to utilize gluten peptides as substrates and studied whether they can form covalent complexes with gluten peptides. Our findings support the notion that the enzymatic activity of TG3 and TG6 can contribute to disease development and to the autoantibody response in DH, and neurological manifestations of gluten sensitivity.

Materials and methods

Production of recombinant human TG2, TG3, and TG6

Recombinant human TGases were expressed in *E. coli* and purified as previously described (Hadjivassiliou et al. 2008).

Peptide synthesis

Synthetic peptides were prepared by solid-phase peptide synthesis on a robotic system (Syro MultiSynTech, Bochum, Germany) using Fmoc/*O*-*t*-butyl chemistry. The identity of the peptides was confirmed by MALDI-TOF mass spectrometry and purity was analyzed by reversed-phase HPLC. Some peptides were purchased from Research Genetics (Huntsville, AL, USA) and biotinylated peptides were purchased from GL BioChem (Shanghai, China). Radioactive labeling with ^{125}Iodine was performed using the Chloramine-T method (Greenwood et al. 1963). The following peptides were used in this study; peptides deriving from α-gliadin: DQ2-α-I, QLQPFPQPQLPY (Arentz-Hansen et al. 2000); DQ2-α-II, PQPQLPYPQP QLPY (Arentz-Hansen et al. 2000); 33mer, LQLQPFPQ PQLPYPQPQLP-YPQPQLPYPQPQPF (Shan et al. 2002); p31–43, PGQQQPFPPQQPY (de Ritis et al. 1988). Peptides deriving from γ-gliadin: DQ2-γ-II, GIIQPQQPAQL (Vader et al. 2002b); DQ2-γ-III, FPQQPQQPYPQQP (Arentz-Hansen et al. 2002); DQ2-γ-IV, FSQPQQQFPQPQ (Arentz-Hansen et al. 2002); DQ2-γ-VI, PQQPFPQQP QQPY (Qiao et al. 2005); 26mer, FLQPQQPFPQQPQQPY PQQPQQPFPQ (Shan et al. 2005). Finally, two peptides deriving from small heat shock protein 20 (Hsp20) were used: Hsp20 Q_{66}, ALPTAQVPTDP (Boros et al. 2006; Stamnaes et al. 2008); Hsp20 Q_{31}, GRLFDQRFGEG (Boros et al. 2006; Stamnaes et al. 2008). Glutamine

residues shown to be targeted by TG2 are underlined (Qiao et al. 2005; Stamnaes et al. 2008).

Deamidation of synthetic peptide substrates by TG2, TG3, and TG6

Prior to use, TG3 (6 μg) was activated with dispase II (0.4 μg) in a final volume of 20 μl of 50 mM Tris–HCl pH 8.0 for 30 min at 30°C. Synthetic peptides (20 or 50 μM) were incubated with 0.1 μg/μl TG2 or dispase activated TG3 in 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$ or with 0.2 μg/μl TG6 in 50 mM Tris–HCl pH 7.6, 300 mM NaCl, 5 mM $CaCl_2$. Samples were incubated at 37°C and aliquots were removed and enzymatic activity stopped with iodoacetamide (25 mM) or 1% trifluoroacetic acid (TFA) at various time points.

Quantification of deamidation by mass spectrometry

Samples were desalted by solid-phase extraction using C_{18} empore extraction disks (St. Paul, MN, USA) and spotted onto a stainless-steel target plate using 5 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile (ACN)/0.1% TFA for mass spectrometric analysis by MALDI-TOF or MALDI-TOF-TOF (Ultraflex II, Bruker Daltonics, Bremen, Germany). For analysis by tandem-MS on the ESI-Q-TOF instrument (MicroTof-q, Bruker Daltonics, Bremen, Germany), samples were either sprayed from nano-offline needles (NanoES spray capillaries, Proxeon, Denmark) at a capillary voltage of 1,100 V or mixed and run on C_{18} nano-LC (Agilent Technologies, CA, USA). Deamidation was calculated as previously described (Dorum et al. 2009).

Detection of covalent TGase-gluten peptide complexes by SDS-PAGE and Western blotting

TGases (0.05 μg/μl TG2; 0.1 μg/μl TG3; 0.2 μg/μl TG6) were incubated with a final concentration of 50 or 100 μM biotin-DQ2-α-I, biotin-DQ2-α-II, biotin-33mer or biotin-p31-43 at 37°C for 15 min, 30 min, or 1 h. The reaction was stopped by adding an equal volume of 2xSDS-PAGE sample buffer containing 5% β-mercaptoethanol, followed by boiling for 3 min. Covalent complexes were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membrane which was blocked with 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.05% Tween20 (TBS-T) and incubated with streptavidin-HRP (1:3,000 in 1% BSA/TBS-T). Biotinylated bands were visualized using ECL (GE Healthcare Life Sciences, UK) and a Kodak Image Station 1000R (Carestream Health, NY, USA). Membranes were stripped in 62.5 mM Tris/HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol for 30 min at 50°C, rinsed in TBS-T, and blocked with 5% non-fat milk in TBS before being probed with affinity purified goat antibodies to TG6 (15 μg/ml; Hadjivassiliou et al. 2008) or rabbit antibodies to TG3 (1:200; kindly provided by Dr Neil Smyth, University of Cologne, Germany; (Sardy et al. 2002)) for 90 min, followed by affinity-purified HRP-conjugated anti-goat or anti-rabbit antibodies (1:500; Dako) for 60 min. Antibodies were diluted in 5% non-fat milk/TBS as indicated, and incubation steps followed by extensive rinsing in TBS-T. Bound antibodies were revealed using the ECL™ reagent kit (Amersham).

Mini-spin column separation of TGase complexes

Covalent complexes were formed by incubation of TGases (1.25 μM) with 10, 50, 167 or 333 μM of either DQ2-α-II, 26mer, or p31-43 spiked with trace amount of radioactively labeled peptides for 30 min (TG2) or 60 min (TG3 and TG6) at 37°C. Samples were either directly separated by mini-spin column (see below) or further incubated with a final concentration of 10 mM hydroxylamine pH 7.6 for 15 min at 37°C prior to separation. Gel-filtration spin-columns were made essentially as previously described (Buus et al. 1995; Fleckenstein et al. 2004). Briefly, separation columns (200 μl pipette tips closed with siliconated glass bead) were packed with Sephadex G50 Superfine in running buffer (PBS, 0.5% Nonidet P-40, and 0.1% NaN_3) and immediately prior to use spun semi-dry ($402\times g$, 5 min). Samples were diluted in running buffer and a volume of 15 μl was gently pipetted on top of the gel matrix. The centrifugation program for separation included linear acceleration 0–3,500 rpm ($2{,}150\times g$) from 0 to 8 min followed by 2 min at 3,500 rpm. Radioactivity in the void volumes and in the columns was determined using a γ-counter (Wallac, Turku, Finland). The counts per minute were converted to an average number of gliadin peptides bound per TGase molecule as previously described (Fleckenstein et al. 2004): number of peptides/TGase = [void count/(column count + void count)] × [mol of peptide/mol of TGase]. Iodoacetamide inactivated TG2 was used as a control and no radioactivity was detected in the void volume of such control reactions. Non-specific background was calculated from samples without TGases.

Results

Gluten peptides are deamidated by TG3 and TG6

Recombinant human TG2, TG3, and TG6 were incubated with synthetic peptides harboring the most common gluten T cell epitopes in CD and with the "toxic" gliadin peptide p31–43. Deamidation was quantified at different time

points by MALDI-TOF mass spectrometry (Dorum et al. 2009). We found that both, TG3 and TG6 indeed were able to deamidate peptides harboring common gluten DQ2-α gliadin and DQ2-γ gliadin T cell epitopes from CD (Fig. 1). Comparing the total peptide deamidation for the three enzymes after 60 min indicated that the order of preference for the various peptide substrates differed between them. TG6 showed a slight preference for DQ2-α gliadin epitopes while TG3 displayed a slight preference for the tested DQ2-γ gliadin epitopes. Notably, DQ2-α gliadin epitopes frequently harbor -PQLP- motifs, whereas DQ2-γ gliadin epitopes typically carry -PQQP- motifs. We also included two peptides derived from the endogenous TG2 substrate small heat shock protein 20 (Hsp20), Hsp20 Q_{66} and Hsp20 Q_{31} (Boros et al. 2006; Stamnaes et al. 2008). The peptide Hsp20 Q_{66} is a good substrate for TG2, while Hsp20 Q_{31} is not targeted on the peptide level. Hsp20 Q_{66} was also deamidated by TG3 and TG6 (Fig. 1) while Hsp20 Q_{31} was found not to be a substrate (data not shown). Notably, the pattern of proline residues in Hsp20 Q_{66} is similar to that in gluten peptides (-QXP-), whereas no proline residues are present in Hsp20 Q_{31}. This suggests that conformational restrictions introduced by proline facilitate interaction of short peptides with the three TGases investigated.

Specific targeting of glutamine residues within gluten T cell epitopes indicates isoform dependent fine specificity

Several of the CD gluten-derived DQ2 or DQ8-restricted T cell epitopes harbor multiple glutamine residues which

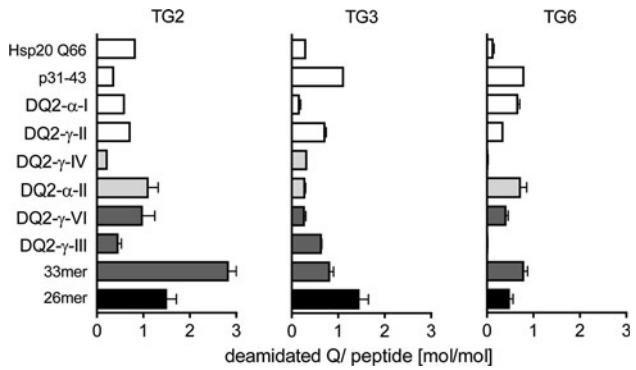

Fig. 1 Deamidation of peptide substrates by TG2, TG3, and TG6. Synthetic peptides at 20 μM were incubated with recombinant enzymes for 60 min followed by quantification of total deamidation by MALDI-TOF MS. The average number of deamidations per peptide is given on the *x*-axis and the *bar colors* represent the number of glutamine residues predicted to be targeted by TG2 in each peptide (*open bars*, 1Q; *light grey bars*, 2Qs; *dark grey bars*, 3Qs; *black bars*, 5Qs). Values are given as mean with standard deviation from two to four independent experiments. Similar results were obtained using 50 μM of peptide

can be targeted by TG2. Using tandem mass spectrometry, deamidation of individual glutamine residues can be quantified. Dorum et al. (2009) recently reported that TG2-mediated targeting of glutamine residues within gluten T cell epitopes is highly specific and sequence dependent. To address if also TG3 and TG6 display a similar specificity, we quantified and compared the deamidation of individual glutamine residues in peptides predicted to have several targeted glutamine residues. As for TG2, only glutamine residues followed by a proline residue in position +2 (-QXP-) were found to be targeted in the peptides tested, while glutamine residues directly followed by a proline residue (-QP-) were protected from targeting both by TG3 and TG6 (Fig. 2a and data not shown). For TG2 we observed a highly sequence-dependent targeting in line with previous findings (Vader et al. 2002a, b; Dorum et al. 2009). This was most notable in the tested DQ2-γ gliadin epitopes where the -PQQPQ- motif was preferred over the -PQQPY/F- motif independently of the peptide length (Fig. 2a). No similar strict, sequence-dependent tendency of targeting was observed for TG3 and TG6 with any of the peptides tested (Fig. 2a). As previously noted, TG6 showed a slight preference for DQ2-α gliadin epitopes suggesting a preference for the -PQLP- motif over the -PQQP- motif while the opposite could be denoted for TG3 (Fig. 1). However, minimal peptide length appeared to have a stronger influence on glutamine residue targeting. In some of the peptides tested, we observed a slight preference for glutamine residues either towards the peptide's N- or C-termini. To elaborate on this, we elongated the N-terminus of the peptide DQ2-γ-VI which was found to be a poor substrate for TG3 despite harboring multiple glutamine residues. This extension dramatically increased TG3-mediated deamidation of Q_1 while it had no influence on deamidation by TG2 and TG6 (Fig. 2b). Similarly, C-terminal elongation of DQ2-γ-III, originally not a substrate for TG6, allowed for targeting of Q_3 by TG6 while no difference was observed for TG3 (Fig. 2c). C-terminal elongation also increased the targeting of Q_3 by TG2 which is in line with previous findings (Dorum et al. 2009). Thus, glutamine residue targeting by TG3 and TG6 was clearly dependent on both peptide length and peptide sequence.

TG3 and TG6 deamidate a novel site in the toxic gliadin peptide p31–43

Most gluten peptides identified as TG2 substrates to date also serve as T cell epitopes in CD except for a small number of gluten B cell epitopes. In addition, much attention has been paid to the gliadin p31–43 peptide ($PGQQ_{34}QPFPPQ_{40}QPY$) which does not bind to DQ2 or

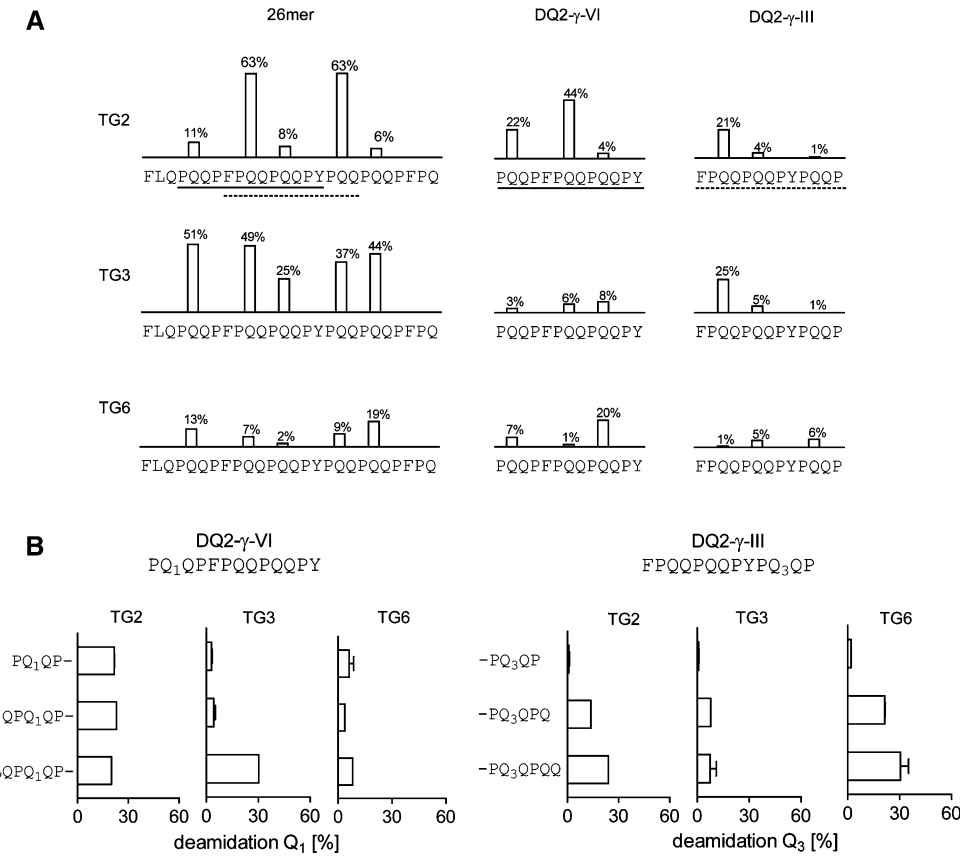

Fig. 2 Differential targeting of individual glutamine residues in gluten-derived DQ2-γ T cell epitopes. **a** Synthetic peptides at 20 μM were incubated with recombinant enzymes for 60 min. Deamidation of individual glutamine residues within peptides was quantified by MALDI-TOF-TOF MSMS and ESI-QTOF MSMS, where 100% equals complete conversion of glutamine to glutamate. The DQ2-γ-VI peptide and DQ2-γ-III peptide sequences are both found within the 26mer peptides as indicated by solid and dotted lines. **b** and **c** Glutamine targeting by TG3 and TG6 requires a minimum of flanking amino acid residues. Deamidation of individual glutamine residues was quantified in N-terminally elongated variants of DQ2-γ-VI (**b**) and C-terminally elongated variants of DQ2-γ-III (**c**). Synthetic peptides at 20 μM were incubated with recombinant enzymes for 60 min followed by quantification of glutamine residue deamidation by MALDI-TOF-TOF MSMS. The percentage of deamidation of Q_1 (**b**) and Q_3 (**c**) is given on the *x*-axis. Values are given as mean with standard deviation from two to four independent experiments

DQ8 but exerts a direct toxic effect on intestinal epithelial cells (de Ritis et al. 1988). TG2 is able to deamidate one glutamine residue within this peptide, Q_{34} (Fig. 3). In contrast, TG6 was found to only deamidate Q_{40}, which is not targeted by TG2. TG3 was able to target both Q_{34} and Q_{40} (Fig. 3). This highlights profound differences in sensitivity of the enzymes to changes in position-2 of the targeted glutamine residue.

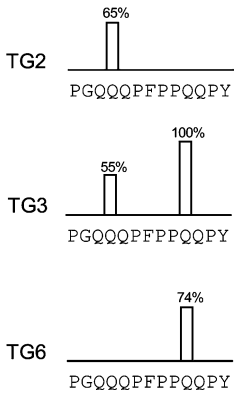

Fig. 3 TG3 and TG6 target a novel deamidation site in the toxic gliadin peptide p31–43. Deamidation of individual glutamine residues in the gluten derived peptide p31–43 was quantified by MALDI-TOF MSMS as outlined in Fig. 2, where 100% equals complete conversion of glutamine to glutamate

Complex formation of TGases with gluten peptides

TG2 has been shown to readily form covalent complexes with gluten peptides. These are believed to act as hapten-carrier complexes which will drive the anti-TG2 antibody response in CD (Sollid et al. 1997). To address if TG3 and TG6 also can form similar complexes, enzymes were incubated with N-terminally biotinylated gliadin peptides (DQ2-α-I, DQ2-α-II, 33mer or p31–43) and samples were analyzed by reducing SDS-PAGE followed by Western blotting and detection with streptavidin-HRP. Results obtained with biotin-DQ2-α-II are shown in Fig. 4. TG2 formed covalent complexes very rapidly even at low enzyme concentrations (Fig. 4, lane 1). TG3 did not form

detectable amounts of complexes with any of the peptides tested even after 60 min of incubation (Fig. 4, lane 3 and data not shown) although TG3 was able to deamidate these peptides (Fig. 1). To test if the lack of complex formation could be due to absence of solvent-exposed lysine residues, TG2 and additional peptide were added to the TG3 samples. TG2 was able to cross-link gluten peptides to TG3, demonstrating that lysine residues are available for cross-linking (Fig. 4, lane 4). TG6 was able to form complexes similar to TG2, although to a lesser extent as indicated by the need for prolonged incubation (60 min) (Fig. 4, lane 6). Similar results were obtained using biotin-DQ2-α-I, biotin-33mer and biotin-p31–43 (data not shown).

Iso-peptide and thioester bonds in gluten-TGase complexes

TG2-gluten complexes can either carry iso-peptide or thioester bonds. These complexes can be distinguished by incubation with hydroxylamine which cleaves thioester bonds but not amide bonds (Towler et al. 1988). The two types of complexes can therefore be distinguished and quantified using radioactively labeled gluten peptides in combination with gel filtration before or after hydroxylamine treatment (Fleckenstein et al. 2004). We incubated TG2, TG3, and TG6 with various amounts of gluten peptides (DQ2-α-II, 26mer or p31-43) spiked with trace amounts of ^{125}I-labeled peptides. Radioactivity included within the column matrix as well as in the flow through was measured with or without treatment with 10 mM hydroxylamine, and the number of peptides bound per enzyme molecule was calculated as previously described (Fleckenstein et al. 2004). Using 1.25 μM TG2 and 50 μM DQ2-α-II, we obtained an average incorporation of 1.4 peptide per molecule TG2 (Fig. 5) which is comparable to our previous findings (Fleckenstein et al. 2004). This value was slightly lower for the 26mer (0.8 peptide/TG2) and even lower for p31–43 (Fig. 5). Treatment with hydroxylamine significantly reduced the number of DQ2-α-II bound to TG2 indicating that a substantial amount of the TG2-DQ2-α-II complexes are thioester linked. The number of thioester linked complexes was slightly lower for the 26mer and p31–43. TG3 incorporated a much lower number of peptides per enzyme molecule under similar reaction conditions. Incubation with hydroxylamine reduced this number to background levels, suggesting that TG3 only forms thioester complexes with gluten (Fig. 5).

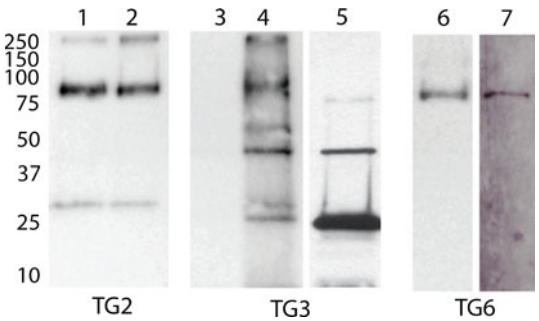

Fig. 4 Transglutaminase self-cross linking to biotinylated peptide DQ2-α-II. TG2 (final 0.05 μg/μl), dispase activated TG3 (final 0.1 μg/μl) and TG6 (final 0.2 μg/μl) were incubated with 50 μM biotin-DQ2-α-II at 37°C for 15, 30, or 60 min. Samples containing TG2 (0.5 μg) (*lane 1*, 15 min; *lane 2*, 30 min), TG3 (2.5 μg) (*lane 3*, 60 min), and TG6 (6 μg) (*lane 6*, 60 min) were separated by reducing SDS-PAGE followed by Western blotting and detection of biotinylated peptides using streptavidin-HRP. At 60 min, 50 μM fresh peptide and TG2 were added to the sample containing TG3 followed by further incubation for 30 min at 37°C (*lane 4*). In addition, the blots shown in *lane 4* and *6* were stripped and reprobed to visualize TG3 (*lane 5*) and TG6 (*lane 7*) as described in M&M. Note that TG2 and TG6 are both observed as a band at approximately 80 kDa, while dispase-activated TG3 appears as two bands of ~25 and ~50 kDa. A small amount of uncleaved TG3 is visible at ~75 kDa in *lane 5*. Migration of molecular weight standards (kDa) is indicated on the *left*

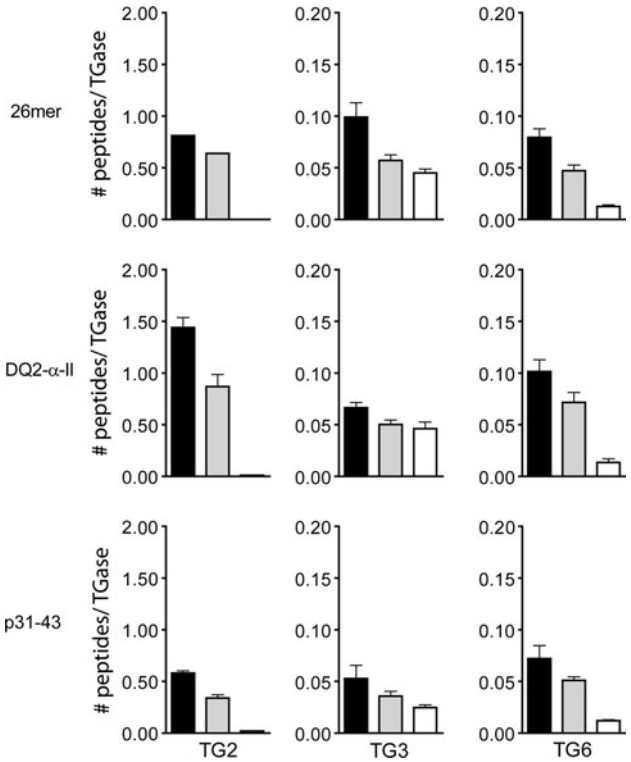

Fig. 5 Formation of thioester-linked versus iso-peptide linked TGase-gluten complexes. TG2, TG3 and TG6 (final 1.25 μM) were incubated with 50 μM 26mer, DQ2-α-II or p31–43 spiked with trace amounts of ^{125}I-labeled peptides for 30 min (TG2) or 60 min (TG3, TG6). The average number of peptides incorporated per TGase molecule (on the *y*-axis) was calculated before (*black bars*) and after (*gray bars*) treatment with 10 mM hydroxylamine (background; *white bars*). The experiment was performed in duplicates and repeated three times. Values are given as the mean from three independent experiments performed in duplicates with standard error of mean given as error bars

Similar to TG3, also TG6 incorporated a low number of peptides per enzyme molecule. These complexes did, however, not disappear after hydroxylamine treatment, indicating the presence of iso-peptide linked complexes (Fig. 5) in line with the demonstration of complexes by Western blotting (Fig. 4).

Discussion

The role of TG2 in CD is fairly well characterized, whereas no studies have addressed the catalytic activity of TG3 or TG6 which now are considered to be the main autoantigens in DH and GA. We report that also TG3 and TG6 can utilize gluten peptides as substrates and can deamidate peptides harboring the most common CD gluten T cell epitopes. We also show that TG3 and TG6 can form covalent complexes with gluten peptides. While TG2 and TG6 were able to form both iso-peptide and thioester-linked complexes, only the latter was observed for TG3. Our findings support the notion that TG3 and TG6 can be involved in the pathogenesis of DH and GA and further suggest that enzyme-substrate thioester complexes must act as hapten-carrier complexes for TG3 and TG6 to drive their own immune response in DH and GA.

TG2-mediated deamidation of gluten peptides is pivotal for the immune response towards gluten in CD. Gluten peptides are remarkably good substrates for TG2 where targeting of glutamine residues is strictly governed by the relative positioning of proline residues (Vader et al. 2002a; Piper et al. 2002; Fleckenstein et al. 2002; Sugimura et al. 2006; Keresztessy et al. 2006). While no data are available for TG6, clear differences in protein substrate processing by TG2 and TG3 were suggested by studies on cornified envelope components (Candi et al. 1995). We here demonstrate that both TG3 and TG6 can deamidate peptides harboring the most common CD gluten T cell epitopes. Similar to TG2, we found that TG3 and TG6 will not target a -QP- motif. In contrast, TG3 and TG6 were able to target the sequence motif -PPQQP- which is not a substrate sequence for TG2. Among the targeted glutamine residues of the peptides tested, substantial differences in deamidation efficiency were observed for TG2, but not for TG3 and TG6. Most of the tested peptides harbor common CD T cell epitopes and are thus already "selected" as good TG2 substrates. It is therefore possible that TG3 and TG6 will display a stronger sequence preference towards other gluten-derived peptides. An indication that different positions may be relevant comes from the observed differences towards peptide length N- or C-terminal to the reactive glutamine residue by the different enzymes. We found that both TG6 and TG2 were profoundly sensitive to the C-terminal sequence (Fig. 2b, c). This is consistent with the observation (D. Aeschlimann and K. Oertl, Zedira GmbH, Darmstadt, Germany, unpublished observation) that peptides modeled on the TG2 protein substrates osteonectin (Cbz-APQQEA) or A3-crystallin (Cbz-TVQQEL) were excellent substrates, as were the N-terminally truncated Cbz-QQEA, Cbz-QQEL whereas C-terminal truncations of these peptides (Cbz-APQQ, Cbz-PQQE and Cbz-TVQQ, Cbz-VQQE) were not. By contrast, reactivity of peptide substrates with TG3 was primarily influenced by the sequence N-terminal to the reactive glutamine residue.

In addition to TG2 reactive antibodies, which are typically found in patients with CD (Dieterich et al. 1997), DH patients have gluten-dependent production of autoantibodies recognizing TG3 while GA patients have autoantibodies which recognize TG6 (Sardy et al. 2002; Hadjivassiliou et al. 2008). Whether these antibody populations are a result of epitope spreading or if they truly are directed towards TG3 and TG6, is not clear. Notably, TG2, TG3, and TG6 are encoded on the same chromosome in humans (20q11–12). Considering the overall great homology between the human TGases, autoantibody cross-reactivity and epitope spreading would not be surprising (Grenard et al. 2001). If the immune response towards TG3 in DH and TG6 in GA on the other hand is isoform specific, this would require active involvement of both TG3 and TG6. Current data available would speak against epitope spreading as a mechanism as antibodies in most patients do not cross-react between different TGase isoforms (Hadjivassiliou et al. 2008). Further, some patients have exclusively antibodies to TG3 or TG6, respectively. Finally, as similarity between TG3 and TG6 is higher than with TG2, one might expect to see TG6 antibodies in DH, but this is not the case. Nevertheless, cross-reactivity cannot be completely excluded as current observations are based on competitive inhibition ELISAs where very low affinity TGase cross-reactivity might not be detected.

The covalent TG2-gluten complexes believed to fuel the anti-TG2 response in CD have been thoroughly characterized in vitro. TG2 can either form thioester linked complexes during catalysis, or create iso-peptide linked complexes by cross-linking of gluten peptides to several of its own lysine residues (Fleckenstein et al. 2004). When visualizing complex formation by detecting incorporation of biotinylated peptides in Western blotting, we found that also TG6 could incorporate all of the gluten peptides tested, although to a lesser extent than TG2. In contrast, TG3 did not form similar complexes despite the presence of solvent-accessible lysine residues. To clearly distinguish between covalent thioester and iso-peptide linked complexes, we quantified complexes before and after hydroxylamine treatment as previously described (Fleckenstein et al. 2004). About one-third of the DQ2-α-II-TG2-complexes were cleaved by hydroxylamine treatment

indicating that a substantial fraction was present as thioester linked complexes. Indeed, thioester-linked TG2-gluten complexes are reported to be remarkably stable (Piper et al. 2002). Moreover, deamidation remains a significant product even in the presence of excess acyl-acceptor substrate (Stamnaes et al. 2008) suggesting that acyl-enzyme complex formation is not rate limiting and hence may be biologically significant. Under similar reaction conditions, TG6 formed both thioester and iso-peptide linked complexes while TG3 was found to form only thioester complexes, in line with our Western blotting results. The fact that only thioester complexes could be observed for TG3 is in support of an important role of thioester-complexes in antibody development (Fleckenstein et al. 2004). This is consistent with the fact that the autoantibody response is so specific for TGases, whereas complexes of gluten peptides with other proteins (e.g. collagens I/III and collagen VI) also are likely to be present in vivo (Dieterich et al. 2006). Notably, both TG3 and TG6 incorporated far fewer peptides per enzyme compared with TG2. Thus, it appears that TG2 is superior at forming both iso-peptide linked and thioester-linked complexes with gluten. Nevertheless, TG3 and TG6 are both able to form complexes with gluten which could be important in an in vivo setting.

The autoantibody production in DH and GA may originate from the small intestine even though the symptoms and immune complex deposits are extra-intestinal (Sardy et al. 2002; Hadjivassiliou et al. 2008). Support for this comes from the observation that the characteristic endomysial IgA deposits associated with CD have also been demonstrated in the small intestine of GA patients (Hadjivassiliou et al. 2006). Expression of TG3 and TG6 in the small intestine could therefore argue for their active involvement in DH and GA. While TG2 is expressed in most tissues, TG3 seems less abundant and very little is known about TG6 expression (Lorand and Graham 2003). Notably, TG3 was reported to be expressed in mouse thymus, spleen, and small intestine (Hitomi et al. 2001) although another study reported the absence of TG3 at the protein level from the human gut (Sardy et al. 2002). Preliminary data indicate that also TG6 can be expressed in mouse small intestine (D. Aeschlimann, unpublished observation). However, intestinal expression of TG3 and TG6 remains to be clearly demonstrated in humans. Interestingly, immunohistochemistry of patient biopsies have indicated TG6 expression in the small intestine of some but not all GA patients (D. Aeschlimann, unpublished observation).

In conclusion, we have shown that not only TG2 but also the closely related TG3 and TG6 can accommodate gluten peptides as substrates and form either thioester or iso-peptide linked complexes with these peptides. Although TG2 seems superior at specifically targeting gluten peptides and forming gluten-enzyme complexes, the activity of TG3 and TG6 could play a role in an in vivo setting. Further studies are required to determine the expression of TG3 and TG6 in human small intestine and to identify whether intestinal expression correlates with the presence of anti-TG3 and anti-TG6 IgA in DH and GA patients. Such a correlation would strongly support the notion of an isoform-specific immune response and active involvement of TG3 and TG6 in DH and GA.

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