Identification of a specific one amino acid change in recombinant human transglutaminase 2 that regulates its activity and calcium sensitivity

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INTRODUCTION

TGs (transglutaminases) are multifunctional proteins performing post-translational protein modifications catalysing an acyl transfer reaction between peptide-bound glutamine residues and an amine donor group, that is the N-ε-amino group of a lysine residue from another protein resulting in cross-linked products or a small molecule amine resulting in mono- or poly-amination [1]. At lower pH and in the absence of amines deamidation could take place resulting in the formation of glutamic acid or the cleavage and also the UniProt database, TG2 with Gly224 is likely to have position 224 that differs from the TG2 gene in the NCBI, Ensembl, ESP (exon sequencing project) and 1KG databases, which show a valine residue at this position. On the basis of these exon data and also the UniProt database, TG2 with Gly224 is likely to have resulted from a cloning error [19]. In all human exon sequencing data available so far a valine residue is found at position 224 of TG2 and it is also highly conserved across species and all TGs presumed to work in cells (Figure 1). Further hinting at its functional importance, Val224 is located in the catalytic core domain of the enzyme [20].

We aimed to investigate the biochemical and structural characteristics of the TG2 valine residue variant (TG2 Val) and compare them with those of the TG2 glycine residue variant (TG2 Gly). To this end, we cloned and studied both valine and glycine residue variants of human TG2. In the case of TG2 Val we observed a significant increase in both the TG activities and Ca2+ sensitivity as compared with TG2 Gly. The difference in the enzymatic activities of the two variants was also observed using in situ cellular assays. The reactivity of antibodies with conformational epitopes, protein stability measurements and computational analysis consistently indicated increased protein stability and decreased metal ion mobility for TG2 Val. All these results demonstrate that Val224 has a significant role in

Human TG2 was first cloned and sequenced in 1991 [19]. This recombinant TG2, until now, has been used as a reference sequence for most of the biochemical, cellular and structural analysis by major research groups working in the TG field [5,20,21]. This sequence, however, has a glycine residue at position 224 that differs from the TG2 gene in the NCBI, Ensembl, ESP (exon sequencing project) and 1KG databases, which show a valine residue at this position. On the basis of these exon data and also the UniProt database, TG2 with Gly224 is likely to have resulted from a cloning error [19]. In all human exon sequencing data available so far a valine residue is found at position 224 of TG2 and it is also highly conserved across species and all TGs presumed to work in cells (Figure 1). Further hinting at its functional importance, Val224 is located in the catalytic core domain of the enzyme [20].

We aimed to investigate the biochemical and structural characteristics of the TG2 valine residue variant (TG2 Val) and compare them with those of the TG2 glycine residue variant (TG2 Gly). To this end, we cloned and studied both valine and glycine residue variants of human TG2. In the case of TG2 Val we observed a significant increase in both the TG activities and Ca2+ sensitivity as compared with TG2 Gly. The difference in the enzymatic activities of the two variants was also observed using in situ cellular assays. The reactivity of antibodies with conformational epitopes, protein stability measurements and computational analysis consistently indicated increased protein stability and decreased metal ion mobility for TG2 Val. All these results demonstrate that Val224 has a significant role in

Abbreviations used: AD, adenovirus; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; BODIPY FL–GTP®s, BODIPY FL–GTP®s; BPA, 5-(biotinamido)pentamine; DSCF, differential scanning fluorimetry; FBN, fibronectin; GTP®s, guanosine 5′-[γ-thio]triphosphate; HEK, human embryonic kidney; HRP, horseradish peroxidase; ITC, Isothermal titration calorimetry; Ni-NTA, Ni2+–nitrilotriacetate; RMSD, root mean square deviation; TBS, Tris-buffered saline; TG, transglutaminase; TTBS + EDTA, TBS containing 0.1 % Tween 20 and 10 mM EDTA.

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determining the Ca$^{2+}$ sensitivity of TG2 inside cells and suggest that experimental data obtained by using TG2 Gly in cellular experiments should be re-interpreted.

**MATERIALS AND METHODS**

**Cloning and expression**

TG2 Gly was cloned as described previously [22] and subcloned into a pET 30 EK/LIC vector (Novagen). The QuickChange® Site-Directed Mutagenesis kit (Stratagene) was used to generate TG2 Val. For the mammalian cell expression, both TG2 Gly and TG2 Val were subcloned from the pET 30 EK/LIC vector into a pTriEx4 Neo vector (Novagen) using the BamHI and XhoI sites of the vector and the BglII and XhoI sites of the TG2 clone in the pET vector. Clones were checked by an in-house DNA sequencing facility (ABI PRISM) and transformed into Rosetta 2 Escherichia coli cells (Novagen) using a standard protocol. The cells were induced at 18°C with 100 μM IPTG overnight. The cells were harvested by centrifugation at 5000 g for 10 min at 4°C and lysed by sonication in 50 ml of lysis buffer [binding buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol, 5 % glycerol and 5 mM imidazole) plus 1 mM PMSF and protease inhibitor cocktail tablets (Roche)]. After sonication, 1 % Triton X-100 was added and the lysate was incubated at 4°C for 30 min and then centrifuged at 20000 g for 30 min at 4°C. Clear supernatant was incubated with Ni-NTA (Ni$^{2+}$-nitrilotriacetate) resins for 1–2 h at 4°C. HisPur™ Ni-NTA metal-affinity resin (Thermo Scientific) was used for protein purification. Resins were washed several times with buffer A (binding buffer and 300 mM NaCl) and buffer B (binding buffer and 20 mM imidazole). Protein was eluted with buffer C (binding buffer and 250 mM imidazole). The purity of the protein was determined by Coomassie Blue staining of SDS polyacrylamide gels and Western blotting with the mouse monoclonal antibody CUB7402 (1:2000 dilution; Neomarkers) and the secondary antibody HRP (horseradish peroxidase)-conjugated anti-mouse (1:1000 dilution; Sigma). The bands were detected by the Chemiluminescence ECL Detection System (Millipore).

**Transamidation activity assays**

A microtitre plate assay, based on the incorporation of BPA [5-(biotinamido)pentylamine] into immobilized N,N-dimethylated casein, was used as described previously [23] with some modifications. The transamidase activity was measured at 5 mM CaCl$_2$ with 1 μg of purified TG2. The TG2 activity measurement using the radioactive putrescine method was performed as described previously [5].

For the kinetics, a spectrophotometric UV assay was used to measure the deamidation of a glutamine substrate releasing ammonia. The released ammonia was absorbed by 2-oxoglutarate in the coupled reaction generating glutamate with oxidation of NADPH to NAD $[24]$. The assay was performed as described previously [25]. For measuring in situ TG2 activity, a BPA incorporation assay was used as described by Zhang et al. [26] with some modifications. The HEK (human embryonic kidney)-293T AD (adenovirus) cells overexpressing TG2 were treated with 2 mM BPA. Next, the cells were treated with or without the active-site TG2 inhibitor ZDON (Zedira) for 1 h and then the calcium ionophore A23187 was used for 1 h to activate TG2. The cells were lysed by sonication and protein in the supernatant was quantified using the Bradford assay. Western blot analysis was performed on the cell lysate with streptavidin-conjugated HRP and HRP substrate. For quantification, 30 μg of the cell lysate was coated on to a 96-well plate and then detected as described previously [26]. The activity was calculated based on the linear part of the decreasing absorbance signal.

**Isopeptidase activity assay**

Abz-APE($\gamma$-Cad-Dnp)QEA (A102, Zedira) substrate was used to measure the isopeptidase activity of TG2. TG cleaves the isopeptide bond containing substrate releasing the cadaverine spacer-linked dark quencher (2,4-dinitrophenyl)
and, subsequently, the increase in fluorescence results from the N-terminally attached 2-Abz (2-aminobenzoyl) fluorophore was measured using Synergy 4 Biotek Microplate Reader (excitation wavelength, 313 nm and emission wavelength, 418 nm). Following the manufacturer’s instructions the assay contained 50 mM Tris/HCl, 10 mM CaCl\(_2\), 100 mM NaCl, 0.1 % PEG8000, 5 mM H-Gly-Ome-HCl, 50 \(\mu\)M A102 substrate and 2.8 mM DTT. The reaction was started by the addition of 10 \(\mu\)g of recombinant protein and DTT. For a control only DTT was added. After optimization the experiments were carried out at pH 6.8 and in the absence of H-Gly-Ome. The activity was calculated based on the linear part of the increasing fluorescence signal.

**GTP-binding studies**

The GTP analogue BOD-GTP\(\gamma\)S [BODIPY (4,4-difluoro-4-bora-3a,4a-diazas-indacene; Life Technologies) FL–GTP\(\gamma\)S (guanosine 5'-\(\gamma\)-thio)triphosphate; Sigma] was used to carry out the GTP-binding studies. The fluorescence reading was taken on a Synergy 4 (Bio-Tek) hybrid microplate reader. The excitation and emission wavelength for BODIPY fluorescence were set at 485 nm and 520 nm respectively. The assay was carried out with 500 nM TG2 in 50 mM Tris/HCl buffer (pH 7.5), 2 mM DTT and 1 mM EDTA.

**ITC (isothermal titration calorimetry)**

The Ca\(^{2+}\) affinities of TG2 Val and TG2 Gly were determined by ITC measurements using a microcalorimeter (MicroCal iTC 200, GE Healthcare). Histidine-tagged purified proteins were dialysed against ITC buffer [25 mM Tris/HCl (pH 7.5) and 1 mM 2-mercaptoethanol] with 0.5 mM EDTA overnight and then twice against ITC buffer containing Chelex 100 at 4 °C for 6 h each. Proteins were concentrated using centrifugal concentrators (Millipore). ITC measurements were performed at 25°C. TG2 Gly (40 \(\mu\)M) was placed into the sample chamber and starting from 0.5 mM CaCl\(_2\) solution (in ITC buffer) 0.4 \(\mu\)l once and 2 \(\mu\)l 19 times were injected. For TG2 Val, 50 \(\mu\)M protein was used and 0.4 \(\mu\)M once and 1 \(\mu\)l 39 times of 2 mM CaCl\(_2\) solution were injected.

**Anti-TG2 ELISA**

The ELISA measurements were performed in duplicate as described previously [using Maxisorp (Nunc) microtitre plates coated with 0.6 \(\mu\)g of TG2 in 100 \(\mu\)l of TBS (Tris-buffered saline) containing 5 mM CaCl\(_2\) (pH 7.4)] [27]. After washing the serum samples [diluted in TTBS + EDTA (TBS containing 0.1% Tween 20 and 10 mM EDTA), 1:400 dilution] and a monoclonal anti-TG2 antibody (TG100; Neomarkers) were incubated. This was followed by incubation with HRP-conjugated rabbit anti-human IgA or HRP-conjugated anti-mouse (diluted 1:5000 in TTBS + EDTA; Sigma) antibodies. The colour reaction was developed by adding 100 \(\mu\)l of 3,3',5,5'-tetramethylbenzidine substrate (Sigma) and then stopped with 50 \(\mu\)l of 1 M H\(_2\)SO\(_4\). The absorbance was read at 450 nm.

**FBN (fibronectin)–TG2 ELISA**

Microtitre plates were coated with 0.3 \(\mu\)g of human FBN (Sigma) diluted in bicarbonate buffer (pH 9.6) for 1 h at room temperature (24°C). The plates were incubated with 0.8 \(\mu\)g of TG2 in TBS containing 5 mM CaCl\(_2\) and 0.1% Tween 20 and the assay continued as above.

**DSF (differential scanning fluorimetry)**

DSF monitors the thermal unfolding of proteins in the presence of fluorescent dyes. SYPRO® Orange dye was used to monitor the thermal unfolding of TG2 variants. Real-time PCR devices (Roche, LightCycler 480\(^{\text{TM}}\) 96 system) were used to monitor protein unfolding. Protein samples (2 \(\mu\)M) in 50 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl and a 1:1000 dilution of SYPRO® Orange (Invitrogen, Life Technologies) in a reaction volume of 20–25 \(\mu\)l were incubated in 96-well PCR microplates (Qiagen) in the real-time PCR devices. The samples were heated at 1°C per min from 20°C to 75°C using the SYBR Green program (excitation wavelength, 497 nm and emission wavelength, 520 nm). The \(T_m\) value was determined from the first derivative curve obtained from the internally developed software package.

**Cell culture**

HEK-293T AD cells were grown in DMEM (Dulbeco’s modified Eagle’s medium) supplemented with 10% FBS (Sigma F7524), L-glutamine (300 mg/ml) (Sigma G7513) and penicillin/streptomycin antibiotics (Sigma, P4333) at 37 °C in an atmosphere of 5% CO\(_2\). To express TG2 in HEK-293T AD cells, histidine-tagged pTri Ex 4 neo-constrains containing TG2 Val and TG2 Gly were transfected into the cells using Lipofectamine™ (Invitrogen). Approximately 24 h post-transfection, the cells expressing TG2 were selected by using 500 \(\mu\)g/ml neomycin to generate stable cell lines. For the activity measurements the cell lysate was prepared by resuspending it in RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P40, 10% glycerol and protease inhibitor cocktail]. The cells were sonicated twice on ice and then centrifuged at 10 000 g for 10 min to obtain the whole-cell lysate.

**Stability calculations**

The impact of the G224V mutation on protein stability was computed using the FoldX program [28]. This method estimates the change in free energies based on the differences in solvation and has been tested on a large number of mutants. Stability calculations were performed using the three-dimensional crystal structures of TG2 in the ‘open’ and ‘closed’ conformation. The GDP-bound form corresponded to the closed conformation of TG2. We followed a thermodynamic cycle and mutated Leu219 to glycine and valine respectively, using the ‘closed’ structure of TG3 in the presence of the metals (PDB code 2Q3Z). Both structures had the Gly224 sequence. The Val224 side chain was engineered into these models using the FoldX rotamer libraries. No hydrogens were added for the purpose of the calculations.

We also evaluated the stability change in TG3 upon the glycine to valine mutation. In TG3 Leu219 corresponds to residue 224 in TG2. We followed a thermodynamic cycle and mutated Leu219 to glycine and valine respectively, using the ‘closed’ structure of TG3 in the presence of the metals (PDB code 1L9N). The error range of the stability calculation was 0.05 kcal/mol (based on the thermodynamic cycle).

**Flexibility analysis**

Local flexibilities were estimated based on the disorder preferences of TG2M (TG2 gene) residues. The TG2M_HUMAN sequence was derived from the UniProt database (code P21980), which corresponded to the Val224 variant. Gly224 was manually inserted into the sequence without any further modifications. Disorder calculations were carried out using the IUPred prediction program [29] using the default (“long”) window size.


Metal-binding analysis

Since none of the TG2 models contained bound metal ions, analysis of metal-binding site was performed using the crystal structure of TG3 (PDB code 1L9N). This model contained a bound Ca\(^{2+}\) and the co-ordinating residues with TG2 were conserved. Metal-binding affinities were estimated using the FoldX force field [30]. Graphical analysis was carried out using PyMOL (http://www.pymol.org).

Densitometry

Proteins were quantified by the Bradford assay along with BSA as a standard and an equal amount of protein was subjected to SDS/PAGE (12% gel). In case of any difference in protein amount, densitometry was carried by SDS/PAGE (or Western blotting in the case of lysate). After densitometry, proteins or lysates were rerun to confirm the accuracy of the calculations before carrying out the assays. ImageJ software (http://rsbweb.nih.gov/ij/) was used for densitometric calculations.

Statistical analysis

Experiments were repeated two or three times (stated in the Figure legends) with three parallels and the data reported are means ± S.E.M. from the representative experiment. Statistical significance was determined by two-tailed paired Student’s t test (parametric) by using GraphPad Prism version 5.0 and Microcal Origin 6 software. P < 0.05 was considered significant.

RESULTS

Val\(^{224}\) strongly influences the transamidation and isopeptidase activities of TG2 at low Ca\(^{2+}\) concentrations

TG2 Val showed a 20% increase in transamidase activity compared with TG2 Gly when microtitre plate or radioactive assays were used for transamidase activity measurements (Figure 2A). Since both of these assays were end-point measurements we resorted to kinetic assays to monitor real-time activities (see the Materials and methods section). Using a kinetic UV test, TG2 Val showed 10-fold higher transamidase activity as compared with TG2 Gly at low Ca\(^{2+}\) concentrations (Figure 2B) and the difference was much higher in the case of the isopeptidase activity assay (Figure 2D). The activity difference between the two variants decreased with increasing Ca\(^{2+}\) concentration, but did not disappear. This suggests that the Ca\(^{2+}\) sensitivity deviates in the two variants. Indeed, the EC\(_{50}\) values for Ca\(^{2+}\) dependence/sensitivity in the case of transamidase and isopeptidase activity were 0.36 ± 0.18 mM and 0.41 ± 0.05 mM for TG2 Val and 2 ± 0.14 and 2.4 ± 0.04 mM for TG2 Gly respectively, indicating a higher binding affinity for Ca\(^{2+}\) for TG2 Val as compared with TG2 Gly (Figures 2C and 2E).

To eliminate confounding factors owing to expression of human TG2 in bacterial cells, the activity measurements were repeated using HEK-293T AD cell lysates overexpressing TG2 variants (Figures 3A and 3B). In agreement with the results obtained by enzymes produced in E. coli cells, TG2 Val showed a higher transamidase activity than TG2 Gly.

Effect of Val\(^{224}\) on the transamidation activity of TG2 in cells

Since in vitro results showed a clear difference in the biochemical properties of the two enzyme variants, we further wanted to confirm the results via cellular assays. We used BPA, a cell-permeable amine substrate for TGs, to determine the in situ TG2 activity in HEK-293T AD cells stably transfected with the TG2 variants. Untransfected cells showed no cross-linking activity either with or without BPA (results not shown). Cells transfected with TG2 Val showed a significant increase in incorporation of BPA into intracellular proteins, whereas TG2 Gly showed a negligible increase when compared with the control (Figure 3C). On treating the samples with calcium ionophores, which had been used previously to activate TG2 in cells, both variants showed an increase in cross-linking activity, but this increase was much more pronounced in the case of TG2 Val compared with TG2 Gly (Figures 3C and 3D). The cross-linking activity could be inhibited when the cells were pre-incubated with 100 μM of the TG2 active-site inhibitor ZDON, confirming the TG2-specific reaction (Figure 3D). The expression levels of TG2 were identical in all samples, confirming that the difference in activity was not owing to different amounts of TG2 (Figure 3D). Both Western blotting and ELISA were used for visualizing the cross-linking reaction, but Western blotting was more sensitive in detecting the difference in TG2 activities, with TG2 Val-containing cells consistently showing higher transamidation activity compared with TG2 Gly-transfected cells.

TG2 Val and Gly have comparable substrate-binding affinity

α2 plasmin inhibitor was proposed to be a good substrate for both Factor XIIa and TG2 and hence was used for substrate-binding studies. [31]. The TG2 activity followed the Michalis–Menten curve on increasing substrate concentration. The curve was plotted using GraphPad Prism and the K\(_m\) value was calculated as 1.9 ± 0.41 mM and the V\(_{max}\) value as 67 ± 4.7 nmol/min for TG2 Val. TG2 Gly exhibited similar values, a K\(_m\) value of 2.1 ± 0.69 mM and V\(_{max}\) value of 75 ± 8.5 nmol/min (Figure 4A). The comparable substrate-binding affinities of the two variants corroborated that the presence of Val\(^{224}\) interfered with the catalytic mechanism of TG2 owing to a change in the metal-binding affinity.

Effect of Val\(^{224}\) on the GTP inhibition of transamidation activity

It has been reported previously that GTP acts as a negative regulator of the transamidation activity of TG2 [32,33]. The impact of GTP on the activities of the two variants was assessed at a 2 mM Ca\(^{2+}\) concentration. As anticipated, both TG2 Val and Gly showed decreasing transamidation activity with increasing GTP concentration. TG2 Gly, however, was more sensitive to GTP at lower concentrations. At a 10 μM GTP concentration for example, TG2 Gly and TG2 Val exhibited approximately 40% and 10% decreases in transamidation activity respectively (Figure 4B). The differential effect of GTP on the two variants diminished at higher GTP concentrations. The IC\(_{50}\) value calculated using the Microcal Origin 6 software was 15 ± 5 μM for TG2 Gly and 80 ± 10 μM for TG2 Val.

Val\(^{224}\) did not influence the guanine nucleotide-binding affinity of TG2

TG2 Val and TG2 Gly both showed an increase in fluorescence upon increasing BOD-GTP/S concentration, indicating that both TG2 Gly and TG2 Val bind GTP. No difference, however, could be detected in the GTP-binding affinities between the two variants (Figure 5A). As reported previously, TG2 binds GTP at a stoichiometry of 1:1 [34]. The fluorescent signal significantly
Regulation of TG2 activity and calcium affinity by Val224

Figure 2  Effect of Ca\(^{2+}\) on different types of TG activities of recombinant TG2 Gly and TG2 Val from E. coli cells

(A) TG2 transamidation activity was measured by a microtitre plate assay and the putrescine incorporation method. Recombinant TG2 purified from E. coli Rosetta 2 (DE3) cells was used. A total of 1 \(\mu\text{g}\) of protein and 5 mM CaCl\(_2\) was used. Inset: SDS/PAGE of purified proteins (4 \(\mu\text{g}; n = 2\)).
(B) TG2 transamidation activity at different Ca\(^{2+}\) concentrations measured by UV assay. EDTA was used in the blank well and was subtracted. A kinetic assay was carried out using peptide substrate (5 mM), ethyl amine (0.75 mM), 2-oxoglutarate (7.5 mM), NADPH (0.7 mM), ADP and glutamate dehydrogenase (22.5 units/ml), and 120 mM Hepes buffer containing 50 mM DTT (\(n = 2\) and \(P = 0.0027\)).
(C) Dose–response curve of transamidase activity for Ca\(^{2+}\) with values plotted on a logarithmic scale to calculate the EC\(_{50}\) values. TG2 Gly EC\(_{50}\) = 2\(+−0.14\) mM and TG2 Val EC\(_{50}\) = 0.36\(+−0.18\) mM (\(P = 0.0027\)).
(D) TG2 isopeptidase activity at different Ca\(^{2+}\) concentrations measured by a kinetic fluorescence isopeptidase assay.
(E) Dose–response curve of isopeptidase activity for Ca\(^{2+}\) with values plotted on a logarithmic scale to calculate the EC\(_{50}\) values. TG2 Gly EC\(_{50}\) = 2.4\(+−0.04\) mM and TG2 Val EC\(_{50}\) = 0.41\(+−0.05\) mM (\(P = 0.0077\) and \(n = 3\)).

Antigenicity- and FBN-binding properties of TG2 Val and TG2 Gly

TG2 is the major auto-antigen in coeliac patients [27]. Using serum samples from coeliac patients and a monoclonal antibody (TG100) the antigenicity of TG2 Gly and TG2 Val was compared. For normalization of the protein amount and purity, binding of different concentrations of TG100 antibody to both variants were decreased in the presence of a competitive inhibitor GTP\(\gamma\)S, indicating that interactions with BOD-GTP\(\gamma\)S were specific (Figure 5B).

TG2 Val had a higher Ca\(^{2+}\)-binding affinity compared with TG2 Gly

ITC measurements were also carried out to determine the Ca\(^{2+}\)-binding affinity of both variants at physiologically possible Ca\(^{2+}\) concentrations. Previous data with TG2 Gly show that it could bind six Ca\(^{2+}\), one Ca\(^{2+}\) with very high affinity and the other five with lower affinity [5]. In the present study, our main aim was to compare the Ca\(^{2+}\)-binding affinities of TG2 Val and TG2 Gly and therefore we concentrated on just one high-affinity Ca\(^{2+}\)-binding site by injecting low Ca\(^{2+}\) amounts. With TG2 Gly we could detect a single high-affinity Ca\(^{2+}\)-binding site with \(K_d\approx0.543\) \(\mu\text{M}\) (Figure 6A) in agreement with the previously published results [5]. In the case of TG2 Val we had to use a different scheme wherein we detected three Ca\(^{2+}\)-binding sites with a lower average \(K_d\) value of approximately 0.08 \(\mu\text{M}\) showing a 7-fold higher Ca\(^{2+}\)-binding affinity compared with TG2 Gly (Figure 6B). The heat changes for both the variants were approximately the same (\(\Delta H\approx−10\) kcal/mol).
always carried out (results not shown). The valine to glycine residue change did not influence the antigenicity of TG2 for the monoclonal antibody TG100. Using coeliac antibodies, which react with conformational epitopes [27], TG2 Val exhibited a ∼1.4-fold higher antigenicity than TG2 Gly in the absence of Ca$^{2+}$ (Figure 7A). An increase in binding was observed for both the variants in the presence of 5 mM calcium, but TG2 Val maintained its 1.2-fold higher reactivity when compared with TG2 Gly. An increase in antibody binding in the presence of Ca$^{2+}$ indicates that Ca$^{2+}$ binding modulates the conformation of TG2 making the formation of the conformational epitopes more favourable. This could be consistent with the higher affinity of TG2 Val for Ca$^{2+}$.

Since TG2 strongly binds and cross-links FBN [35,36], we wanted to compare the FBN-binding capacity of the two variants. However, we did not see any difference in the FBN-binding efficiency of TG2 Val and TG2 Gly most probably because Val$^{224}$ is not in close proximity to the FBN-binding domain [37] (Figure 7B).

**TG2 Gly has a decreased protein stability as compared with TG2 Val**

Both in vitro and in vivo measurements indicated that the glycine to valine residue replacement significantly increased transamidation activity. As residue 224 is part of the catalytic core [20,38], but is not the catalytic centre, we aimed to elucidate those structural changes which were induced by the presence of valine. We anticipated that insertion of a hydrophobic side chain would increase the stability of TG2. Environmentally sensitive protein dyes, such as SYPRO® Orange and ANS (8-anilinonaphthalene-1-sulfonate), are frequently used to study folding–unfolding transitions, as they bind the hydrophobic pockets of proteins [39,40]. When we analysed the dynamic fluorescence change by fluorescence scanning spectrometry upon
increasing the temperature, both TG2 Gly and TG2 Val showed increasing fluorescence in the presence of SYPRO® Orange. The change was more pronounced in the case of TG2 Gly, suggesting a drop in protein stability as compared with TG2 Val (Figure 8A). Indeed, the $T_m$ value was 45°C and 48°C for TG2 Gly and TG2 Val respectively, indicating a more extensive hydrophobic-interaction network for TG2 Val (Figure 8B).

**Impact of Val$^{224}$ on the protein dynamics of TG2**

The stability of the TG2 Gly and TG2 Val structures was also evaluated by the FoldX program [28] (Table 1). There are two conformations of TG2: the GDP-bound compact (‘closed’) [20] and the inhibitor-bound (‘open’) [38] forms. In the GDP-bound structure the catalytic site is inaccessible for the substrate. The ‘open’ structure is considered the catalytically active form [38], and thus the transition between the two forms could influence catalytic efficiency.

As we anticipated, the hydrophobic side chain increased the stability of TG2 in both conformations. The difference between TG2 Gly and TG2 Val was significant (3.98 kcal/mol) in the compact GDP-bound conformation, but a slight difference (0.67 kcal/mol) could also be observed in the extended form. This suggests that Val$^{224}$ could have an impact on the flexibility of TG2 or might interfere with the transition between the two conformations.

To analyse how hydrophobicity of residue 224 affects local structural dynamics, we quantified the preference of each residue for structural disorder using the primary sequence. Although the segment embedding Val$^{224}$ is not intrinsically disordered, the coarse-grained description of the local flexibility could be obtained by the IUPred program [29] (see the Materials and methods section). The disorder profile reflected a marked (∼30%) increase in flexibility upon changing the valine residue to glycine (Figure 8C). A considerable shift (>10%) in the disorder scores could be observed in the residue 213–234 region, which also comprises the putative metal-binding site. The increased flexibility could reflect less tighter packing, i.e.
Figure 6 Determination of Ca\(^{2+}\) -binding affinities of TG2 Gly and TG2 Val by ITC

(A) ITC of Ca\(^{2+}\) binding to TG2 Gly (40 \(\mu\)M) using 500 \(\mu\)M CaCl\(_2\). The injection scheme used was 0.4 \(\mu\)l once and 2 \(\mu\)l 19 times. The heat liberated was recorded (upper panel) and then the data were recalculated using the ORIGIN\textsuperscript{TM} software (lower panel). (B) ITC of Ca\(^{2+}\) binding to TG2 Val (40 \(\mu\)M) using 2 mM CaCl\(_2\). The injection scheme used was 0.4 \(\mu\)l once and 1 \(\mu\)l 39 times.

Figure 7 Antibody- and FBN-binding properties of TG2 Gly and TG2 Val

(A) The monoclonal antibody TG100 and the coeliac disease antibodies (S28, S30 and S40) were used to check the antigenicity of TG2 Gly and TG2 Val (0.6 \(\mu\)g) purified from E. coli Rosetta 2 cells with or without 5 mM CaCl\(_2\) (n = 2). (B) The monoclonal antibodies TG100, CUB and 4G3 were used to check the FBN (0.3 \(\mu\)g)-binding capacity of TG2 Gly and TG2 Val (0.8 \(\mu\)g) purified from E. coli Rosetta 2 cells with 5 mM CaCl\(_2\) (n = 2).

a weaker interaction network in the presence of Gly\(^{224}\). This could also influence the dynamics of the metal ion-co-ordinating residues (residues 226–233 loop), and thereby the metal-binding affinity.

The crystal structure of TG2 both in the open [38] and closed [20] conformations have a glycine residue at position 224. Recently, TG2 structures containing Val\(^{224}\) were deposited in the PDB under codes 3S3J, 3S3S and 3S3P. These newly deposited structures were bound to a novel inhibitor and were in the open conformation. Since now we have TG2 structures both with glycine (PDG code 2Q3Z) and valine (PDB code 3S3J) residues in the open conformation, we superimposed them to observe the change in structure owing to the single amino acid change. The glycine to valine residue change induced only small rearrangement in the structure of the enzyme [backbone RMSD (root mean square deviation) is 0.49 Å for full superimposition] (Figure 9A) by affecting the conformation of the loop which contains Ca\(^{2+}\) -co-ordinating residues (Figure 9B).

Computational analysis confirms the higher Ca\(^{2+}\) -binding affinity for TG2 Val

In vitro measurements showed a decrease in Ca\(^{2+}\) -binding affinity of TG2 Gly as compared with TG2 Val. The impact of Val\(^{224}\) and Gly\(^{224}\) on the metal ion-binding properties was also evaluated computationally using the FoldX program [28] (Table 1). As no Ca\(^{2+}\) -bound structure of TG2 is available, we used TG3 as a model (PDB code 1L9N) [6]. This structure superimposes well with the GDP-bound form of TG2 (PDB code 1KV3; RMSD deviation is 1.69 Å for the backbone) (Figure 9C). Residue 224 in TG2 corresponds to 219 in TG3 and is located in one of the metal ion-co-ordinating helices, but there is no direct contact with Ca\(^{2+}\) in either case (Figure 9D). The superimposition of
Regulation of TG2 activity and calcium affinity by Val\textsuperscript{224}

TG2 Val (PDB code 3S3J) on to the Ca\textsuperscript{2+}-bound TG3 structure indicates that the Ca\textsuperscript{2+}-co-ordinating loop in TG2 Val is more compatible with metal binding than in TG2 Gly (Figure 9D). Following the corresponding thermodynamic cycle we found that changing Val\textsuperscript{219} to a glycine residue in TG3 also decreases stability (by 2 kcal/mol), although to a smaller degree than in TG2 (Table 1). The difference, however, is significantly larger than the error range of FoldX calculations obtained on 1088 mutants [28].

We then computed how the altered dynamics of a metal-co-ordinating helix affects the binding affinity of Ca\textsuperscript{2+}. The corresponding free energy changes were evaluated in both the TG2 and TG3 structures using the FoldX force field [30] (Table 2). In both cases, the presence of a valine residue instead of a glycine improves metal binding by 4.4 and 2.4 kcal/mol respectively. The change in the Ca\textsuperscript{2+}-bound form is in agreement with the experimentally determined difference. This is due to decreased mobility of the metal-binding loop in the valine residue variant in both cases. Valine also creates a more hydrophobic environment around the metal ion, which results in decreased screening by water and thus stronger electrostatic interactions. Similarly to stability changes, Ca\textsuperscript{2+}-binding affinities are more affected in TG2 than in TG3 by the valine to glycine residue change.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Metal ion-binding affinities in the TG2 and TG3 structures (PDB codes 1KV3 and 1L9N respectively) as computed by the FoldX program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>$\Delta G_{\text{Val}^{224}\rightarrow \text{Gly}^{224}}$</td>
</tr>
<tr>
<td>TG2 PDB code 1KV3</td>
<td>24.10</td>
</tr>
<tr>
<td>TG3 PDB code 1L9N</td>
<td>28.49</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present study we compare the biochemical characteristics of the common human TG2, which has a valine residue at position 224 (wild-type TG2), and an artificial variant (TG2 Gly\textsuperscript{224}) used in various laboratories over the last two decades [5,20,21,41,42].

We found that the single amino acid change resulted in higher transamidase activity of TG2 both in \textit{in vitro} and \textit{in situ} transamidation assays. In addition it also showed a severalfold higher isopeptidase activity. The recombinant TG2 Val exhibited higher Ca\textsuperscript{2+} sensitivity requiring a $\sim$350 \textmu M Ca\textsuperscript{2+} concentration for the half maximum activity, whereas a
2 mM Ca\(^{2+}\) concentration was essential for TG2 Gly. These results explain why a high Ca\(^{2+}\) concentration had to be used in earlier studies for activation of TG2 [43,44]. Endogenous TG2 purified from red blood cells and platelets (TG2 Val) showed IC\(_{50}\) values for Ca\(^{2+}\) binding approximately between 100 \(\mu\)M and 500 \(\mu\)M and the results of the present study show values within this range [34,45,46]. There is a prevailing view that, under physiological conditions, TG2 cannot be active inside the cell owing to low Ca\(^{2+}\) and high GTP concentrations, which are inhibitory for the transamidation activity of TG2 [4,47]. Accordingly, in most cellular experiments calcium ionophores have been used to activate TG2 [26,48]. However, we argued in a previous review that TG2 could be activated inside cells even under physiological conditions [43]. Along these lines, we could detect the cross-linking activity of TG2 Val in the absence of calcium ionophore even in cellular experiments. The addition of A23187 increased the cross-linking activity further (Figures 3C and 3D). The IC\(_{50}\) value for GTP binding at a 2 mM Ca\(^{2+}\) concentration was significantly higher for TG2 Val than TG2 Gly. All of these results support the activity of TG2 inside the cells and call for reassessment of its potential cellular activity.

No difference was observed in the GTP- and substrate-binding affinities of TG2 Val and TG2 Gly. The cross-linking activities of the two variants differed most at low Ca\(^{2+}\) concentrations, indicating that differences in metal binding affects the catalytic efficiencies of the two variants. Indeed, a significant difference in Ca\(^{2+}\)-binding affinities has been observed, the more active TG2 Val bound metal ions more tightly than TG2 Gly. These results were confirmed by ITC measurements and computational analysis. Computational analysis demonstrated that Val\(^{224}\) increased the stability of the loop (first loop), which comprises the Ca\(^{2+}\)-co-ordinating residues. Indeed the presence of a valine residue increased the metal binding of TG2 by more than 4 kcal/mol as compared with a glycine. This is owing to decreased mobility of the loop (residues 226–232), which may result in tighter metal co-ordination. Interestingly, the analogous mutation in TG3 compromises stability and metal-binding affinity to a smaller extent (Table 2). Superimposition of TG2 open structures containing valine and glycine residues also showed a change in the conformation of the loop containing Ca\(^{2+}\)-co-ordinating residues (Figures 9A and 9B), which further confirmed our biochemical data.

ITC measurements exhibited a 7-fold higher Ca\(^{2+}\)-binding affinity for TG2 Val. TG2 Gly has only one high-affinity Ca\(^{2+}\)-binding site and its other binding sites could not be detected at low Ca\(^{2+}\) concentrations, confirming that they might have lower affinity for Ca\(^{2+}\) as described previously [5]. In contrast, on the basis of the presented ITC data, TG2 Val bound three Ca\(^{2+}\) with similarly high affinities and the computational results show that the Ca\(^{2+}\)-binding loop of TG2 Val was more rigid and that its overall structure was more stable. Taken together, these data suggest that Val\(^{224}\) is responsible for the co-operativity between the three identified strong Ca\(^{2+}\)-binding sites of TG2 and explain why it needed a higher Ca\(^{2+}\)/enzyme ratio to achieve high-affinity Ca\(^{2+}\) binding, which provided important new detailed implications for the Ca\(^{2+}\)-activation process of TG2. Since TG3 also has three strong
Ca\(^{2+}\)-binding sites [41], which are crucial in regulating the activity of the enzyme, and based on sequence alignment TG6 also might have homologous Ca\(^{2+}\)-binding sites with TG2 [17], it seems that they might have a similar Ca\(^{2+}\)-binding/activation mechanism [6].

Both fluorescence spectroscopy using SYPRO\textsuperscript{®} Orange and computational analysis indicated that the presence of Val\textsuperscript{224} increased the stability of TG2. Based on a coarse-grained description of dynamic properties, we also found that the V224G replacement introduced local changes in flexibility and mostly facilitated the dynamics of the residue 213–234 segment of TG2 (Figure 8C). This region, however, is located more than 30 Å from the GDP-binding site and thus is unlikely to have an impact on nucleotide affinity (Figure 9C), supporting our biochemical data. Another possibility is that Val\textsuperscript{224} interfered with the conformational rearrangement of TG2 between the closed and open forms.

The FBN-binding domain has been mapped on the N-terminal β-sandwich domain of TG2 [37]. In the TG2 crystal structure, Val\textsuperscript{224} is not in close proximity to the FBN-binding region of TG2, which explains why the glycine to valine residue change did not affect the FBN-binding property of the enzyme. There was a significant increase in antigenicity for coeliac antibodies in the case of TG2 Val. It is known that the coeliac antibody epitopes are conformational and TG2 should be in its native conformation for proper binding of coeliac antibodies [27]. Since TG2 Val is more stable than TG2 Gly, it is recommended that commercial assays should use recombinant TG2 Val in the laboratory diagnosis of coeliac disease to increase sensitivity and specificity.

It is interesting to note that Factor XIIIa has a glycine residue at the same position as TG2 (Figure 1, lower panel) and this glycine is conserved across all species (Supplementary Figure S1 at http://www.biochem.org/bj/455/bj4550261add.htm). Since Factor XIIIa is mostly present in the extracellular matrix and blood plasma where Ca\(^{2+}\) concentrations are much higher compared with cytosol [49], it could suggest that retaining Gly\textsuperscript{224} might be a possible regulatory mechanism to keep the activity of Factor XIIIa under control in the cells.

**Conclusion**

In summary, present studies confirm that a valine at position 224 is a critical residue, which affects the transamidation efficiency of TG2 in vitro as well as in vivo by modulating its metal-binding affinity. Since we know that transamidation activity of TG2 has several physiological and pathological significances and also TG2 Val affected the antigenicity for coeliac antibodies, this calls for reassessment of previous work done with the Gly\textsuperscript{224} form of the enzyme.

**AUTHOR CONTRIBUTION**

Kajal Kanchan, Elvan Ergulen, Zsófia Simon Vecsei and Robert Király conducted the experiments; Mónika Fuxreiter performed the computational analysis; Kajal Kanchan and Robert Király, analysed and interpreted the results and contributed to writing and revising the paper; and Mónika Fuxreiter and László Fésüs contributed to data interpretation and writing and revising of the paper.

**ACKNOWLEDGEMENTS**

We thank Professor László Muszbek (University of Debrecen, Debrecen, Hungary) for providing the glutamine donor substrate for the UV assay, Professor Ferenc Erdőd for allowing us to use the ITC instrument facility, Bécsi Ballint for performing ITC experiments and Dr Bence Kiss for the ITC data interpretation and suggestions. We are grateful to Dr Manoj Kumar for critical reading and correction of the paper prior to submission.

**REFERENCES**


**FUNDING**

This work was supported by the Hungarian Scientific Research Fund [grant number OTKA NK 105046], the New Hungary Development Plan via the TAMOP-4.2.2-A-11/1/KONV-2012-0023 VED-ELEM project, European Union Framework Programme 7 TRANSCOM IAPP 251506 and TRANSPATH ITN 289964, the European Union and the state of Hungary, co-financed by the European Social Fund in the framework of the TAMOP 4.2.4. A/2-11-1-2012-0001 ‘National Excellence Programme’ which provided personal support to R.K., and the Hungarian Academy of Sciences via the Lendület programme [grant number LP2012–41 (to M.F.)].
SUPPLEMENTARY ONLINE DATA

Identification of a specific one amino acid change in recombinant human transglutaminase 2 that regulates its activity and calcium sensitivity

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Figure S1 Alignment of Factor XIIIA from different species

Arrows marks the conserved glycine residue which corresponds to Val224 in human TG2. Identical residues are in black, whereas conserved residues are in grey. GenBank® accession numbers are: FXIIIA cow, DAA16047.1; FXIIIA human (hum), CAA00294.1; FXIIIA mouse (mou), NP_001159863.1; and FXIIIA rat, CAA73104.1

Received 25 June 2013/6 August 2013; accepted 13 August 2013
Published as BJ Immediate Publication 13 August 2013, doi:10.1042/BJ20130696

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