Functional characterization of naturally occurring transglutaminase 2 mutants implicated in early-onset type 2 diabetes

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Abstract

Transglutaminase 2 (TG2) is an enzyme with diverse biological functions. TG2 catalyzes transamidation reactions, has intrinsic kinase activity, and acts as a G-protein in intracellular signaling. *TG2* (*Tgm2*)-null mice are glucose intolerant and have impaired glucose-stimulated insulin secretion (GSIS). Furthermore, three naturally occurring missense mutations in the human *TGM2* gene, corresponding to amino acid substitutions of Met330Arg, Ile331Asn, and Asn333Ser in the TG2 protein, have been reported and found to be associated with early-onset type 2 diabetes. However, their effect on TG2 function is not fully understood. To determine this, we have reproduced naturally occurring mutations in *TG2* using site-directed mutagenesis. Overexpression of Myc-TG2 mutants in INS-1E cells resulted in a reduction of GSIS in comparison with cells overexpressing wild-type Myc-TG2 (WT-TG2). The maximum reduction was found in cells overexpressing Ile331Asn-TG2 (32%) followed by Met330Arg-TG2 (20%), and the least in Asn333Ser-TG2 (7%). Enzymatic analysis revealed that TG2 mutants have impaired transamidation and kinase activities in comparison with WT-TG2. GTP-binding assays showed that TG2 mutants also have altered GTP-binding ability, which is found to be modulated in response to glucose stimulation. Collectively, these data suggest that naturally occurring mutations in *TG2* affect transamidation, kinase, and GTP-binding functions of TG2. While reduced insulin secretion, as a result of naturally occurring mutations in *TG2*, is due to the impairment of more than one biological function of TG2, it is the transamidation function that appears to be impaired during the first phase, whereas the GTP-binding function affects the second phase of insulin secretion.

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Introduction

Transglutaminase 2 (TG2) is a multi-functional protein-modifying enzyme (EC 2.3.2.13) that has been mapped to the locus 20q12 on human chromosome 20 (Gentile *et al.* 1994). The locus 20q12 has been identified as a susceptibility locus for non-insulin-dependent type 2 diabetes in linkage studies and is believed to contain more than one susceptibility gene (Ji *et al.* 1997, Zouali *et al.* 1997, Ghosh *et al.* 1999). One such gene is hepatocyte nuclear factor 4 α (*HNF4A*). Mutations in the *HNF4A* gene have been reported to be involved in young-onset type 2 diabetes (Johnson 2007). In addition to *HNF4A*, mutations in the *TG2* (*TGM2*) gene have also been implicated in young-onset type 2 diabetes (Bernassola *et al.* 2002, Porzio *et al.* 2007). Three heterozygous missense mutations in the *TG2* gene, which lead to amino acid changes in the TG2 protein at positions 330, 331, and 333 from methionine to arginine (Met330Arg), isoleucine to asparagine (Ile331Asn), and asparagine to serine

(Asn333Ser) respectively, have been identified in patients with young-onset type 2 diabetes (Bernassola *et al.* 2002, Porzio *et al.* 2007). Furthermore, mice lacking *TG2* (*TG2*^{-/-}) are glucose intolerant and have impaired glucose-stimulated insulin secretion (GSIS) and exhibit a phenotype similar to maturity onset diabetes of the young (MODY), a group of autosomal dominant, single-gene disorders that cause diabetes in young adults or children (Bernassola *et al.* 2002). The genetic evidences together suggest an important physiological role for TG2 in pancreatic β -cell function, though the actual mechanism involved is not understood.

A well-known function of TG2 is calcium-dependent transamidation, which is also known as a 'protein crosslinking' function, due to the formation of a γ -glutamyl- ϵ -lysine bond between the side chain of lysine and glutamine residues of two protein molecules (Fesus & Piacentini 2002). Recently, serotonylation (covalent coupling of serotonin to protein) of small G-proteins in pancreatic β -cells mediated by the transamidation function of TG2 has been implicated

in GSIS (Paulmann *et al.* 2009). In this paper, the enzyme TG2 placed serotonin on glutamine residue(s) of small GTPases to form a glutamyl amide bond (serotonylation), resulting in the activation of G-proteins (Paulmann *et al.* 2009). Small GTPases have been known to be involved in insulin secretion from β -cells (Kajio *et al.* 2001, Kasai *et al.* 2005). For example, a small GTPase, Rab3a, has been shown to regulate replenishment of the readily releasable pool of β -granules at early stages of vesicle transport (Kajio *et al.* 2001). Furthermore, Rab27a has been reported to act directly in the targeting of β -granules from the resting pool to the readily releasable pool in the plasma membrane (Kasai *et al.* 2005).

In addition, we have reported that TG2 has intrinsic kinase activity and phosphorylates a number of proteins including insulin-like growth factor binding protein-3 (IGFBP3; Mishra & Murphy 2004, Mishra *et al.* 2006, 2007*a,b*). We have recently found that IGFBP3 has a role in insulin secretion (Nguyen *et al.* 2011). Moreover, we have shown that ATP and Ca^{2+} reciprocally regulate transamidation and kinase activities of TG2 (Mishra & Murphy 2004). ATP facilitates kinase activity and has an inhibitory effect on transamidation activity, whereas Ca^{2+} has an opposite effect (Mishra & Murphy 2004, Mishra *et al.* 2007*a,b*). Furthermore, TG2 binds to GTP and functions as a G-protein in cell signaling processes (Baek *et al.* 2001), and the GTP-binding and transamidation functions of TG2 are inversely associated with each other (Murthy *et al.* 2002, Begg *et al.* 2006). Although the transamidation catalytic triad (Cys²⁷⁷, His³³⁵, and Asp³⁵⁸) and putative GTP/ATP (¹⁶⁹Gln-Gly-Ser-Ala-Lys¹⁷³ and ⁵⁸⁰Arg-Asp-Leu-Tyr⁵⁸³) binding loops are located distantly from each other in the primary structure of TG2, they are very close to each other in the secondary structure, as revealed by the crystal structure of TG2 (Murthy *et al.* 2002, Begg *et al.* 2006). However, the functional consequences of naturally occurring TG2 mutations on TG2 enzymatic activities and on GTP-binding function have not been characterized yet. Although a role for the transamidation function of TG2 in GSIS has been proposed (Porzio *et al.* 2007), there is no unequivocal evidence to support this hypothesis. Moreover, the possibility of the involvement of TG2 functions other than the transamidation function was not addressed before. It is possible that these mutations in TG2 affect both transamidation and kinase activities of TG2 in a mutually exclusive or in an inclusive manner. Moreover, it may also affect the GTP-binding ability of TG2 and its function as a G-protein. In this study, we investigated the functional impact of naturally occurring TG2 mutations on TG2 enzymatic activities as well as on the GTP-binding function and their relationship with GSIS in clonal INS-1E pancreatic β -cells.

Materials and methods

Reagents

Cell culture reagents were obtained from American Type Cell Collection (ATCC, Danvers, MA, USA), and heat-inactivated fetal bovine serum and anti-rabbit Alexa Fluor 488 from Invitrogen. Anti-Myc antibody was obtained from Cell Signaling Technology (Danvers, MA, USA), and anti-serotonin antibody and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. ProFound c-Myc Tag IP/Co-IP Kit and 5-(biotinamido)pentylamine (BPNH₂) were purchased from Pierce (Rockford, IL, USA) and Ultrasensitive Insulin ELISA kits (mouse and rat) were obtained from Chrystal Chem (Downers Grove, IL, USA). Enhanced chemiluminescence (ECL) reagents and sequencing grade trypsin were purchased from Promega. ATP- and GTP-agarose resins were obtained from Innova Biosciences (Cambridge, UK). Other reagents were purchased from Sigma-Aldrich or as otherwise stated.

Animals

TG2 knockout C57BL/6 mice were generously provided by Dr Nikolaos Frangogiannis (Baylor College of Medicine, Houston, TX, USA) with permission from Dr Gerry Melino (University of Leicester, UK). Wild-type (WT) and TG2^{-/-} C57BL/6 mice were housed at the University of Manitoba Central Animal Care Facility. Mice were housed under controlled temperature (21 °C) and light conditions (12 h light:12 h darkness cycle) with *ad libitum* access to water and a standard diet. The experiments involving mice were performed as approved by the Animal Care Committee of the University of Manitoba.

Isolation of pancreatic islets

Islets were isolated by collagenase (Roche) digestion of the mouse pancreas as described (Lacy & Kostianovsky 1967). Once isolated, the islets were cultured in RPMI-1640 medium containing 11 mM glucose, 10% heat-inactivated fetal bovine serum, and 100 $\mu\text{g}/\text{ml}$ penicillin–streptomycin for 24 h (Nguyen *et al.* 2011).

Batch incubations

Batches of four islets were pre-incubated in Krebs–Ringer bicarbonate (KRB) buffer solution (118.4 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , and 25 mM NaHCO_3 (equilibrated with 5% CO_2 –95% O_2 , pH 7.4), 0.2% BSA, and 10 mM HEPES) containing 3.3 mM glucose for 30 min. The islets were then incubated for 30 min in KRB buffer

containing 16·7 mM glucose. The buffer samples were collected for the insulin assay by ELISA according to the manufacturer's instructions.

Cell culture

The rat insulinoma cell line INS-1E was a generous gift from Dr Michael Wheeler (University of Toronto, Toronto, ON, Canada) with permission from Prof. Claes Wollheim (University of Geneva, Geneva Switzerland). INS-1E cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 100 μ g/ml penicillin–streptomycin (Gauthier *et al.* 2009, Lee *et al.* 2010).

TG2 constructs and cell transfections

The pCMV6 vector containing the Myc-tagged human TG2 (Myc-TG2) clone was obtained from Origene Technologies (Rockville, MD, USA). Naturally occurring TG2 mutants and the transamidation-activity dead Cys277Ala-TG2 mutant (as a negative control for transamidation function of TG2) were made using a site-directed mutagenesis kit (Ande *et al.* 2009a,b) using two complementary oligonucleotide primers containing the desired mutation and Myc-TG2 as the template. The primers used for generating various TG2 mutants are shown in Table 1. The parental strand was removed by DpnI digestion before transformation into *Escherichia coli* strain DH5 α . Authenticity of all constructs was confirmed by DNA sequencing. Transfections with various constructs were performed using FuGENE HD transfection reagent (Roche) according to the manufacturer's instructions (Ande *et al.* 2009a,b). A consistent transfection efficiency of 40–45% was obtained using 2 μ g plasmid DNA/3 μ l transfection reagent.

Immunoprecipitation

The ProFound c-Myc Tag IP/Co-IP Kit was used, according to the manufacturer's instructions, to

immunoprecipitate the Myc-tagged WT and TG2 mutants. For the samples to be used in kinase and transamidation activity assays, rather than subjecting to elution, the protein–bead complexes were resuspended in 50 mM Tris buffer (pH 7·4) to preserve enzyme activity.

Western blotting

Cells were rinsed in PBS and collected in lysis buffer (Sigma–Aldrich), containing protease inhibitor cocktail (Ande *et al.* 2009a,b). The samples were incubated on ice with intermittent vortexing for 30 min, followed by centrifugation at 13 000 *g* for 10 min. Protein concentrations of the supernatants were determined by the Bradford protein assay with BSA as the standard. SDS–PAGE sample buffer was added to the samples, and the samples were then boiled for 5 min. Proteins were resolved on 10–12% SDS–PAGE, according to target protein size, and transferred onto nitrocellulose membranes. The membranes were blocked in 5% milk for 1 h at room temperature and then incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. Membranes were washed three times with TBST wash buffer (10 mM Tris, 150 mM NaCl, 0·05% Tween 20, pH 8·0), and then incubated with either HRP-conjugated secondary antibodies or streptavidin–HRP conjugate for 1 h at room temperature. Membrane washing steps with TBST were repeated, followed by the addition of ECL reagent. Membranes were exposed to Kodak Biomax MR Film (Sigma) and protein bands were analyzed by densitometry using Scion Image software (Frederick, MD, USA).

In vitro kinase assay

An *in vitro* kinase assay was performed in kinase buffer (50 mM Tris–HCl, pH 7·5, 10 mM magnesium chloride, [γ - 32 P]ATP (25 μ Ci/ml)) for 30 min at 30 °C as described previously (Mishra & Murphy 2004, Mishra *et al.* 2006). The reaction was stopped by the addition of SDS–PAGE sample buffer and the samples were boiled for 5 min. The samples were resolved on SDS–PAGE,

Table 1 Primer sequences used to develop TG2 mutants by site-directed mutagenesis

TG2 mutants	Primer sequences
Met330Arg	Forward: 5'-GTGACAAGAGCGAGAGGATCTTGAACCTCCAC-3' Reverse: 5'-GTGGAAGTTCCAGATCCTCTCGCTCTTGTCACC-3'
Ile331Asn	Forward: 5'-GACAAGAGCGAGATGAACTGGAACCTCCACTGC-3' Reverse: 5'-GCAGTGGAAGTTCCAGTTCATCTCGCTCTTGTC-3'
Asn333Ser	Forward: 5'-GCGAGATGATCTGGAGCTTCCACTGCTGGGT-3' Reverse: 5'-ACCCAGCAGTGGAAGCTCCAGATCATCTCGC-3'
Cys277Ala	Forward: 5'-GTCAAGTATGGCCAGGCTGGGTCTTCGCCGC-3' Reverse: 5'-GCGGCGAAGACCCAGGCTGGCCATACTTGAC-3'

TG2, transglutaminase 2; Met, methionine; Arg, arginine; Ile, isoleucine; Asn, asparagine; Ser, serine; Cys, cysteine; Ala, alanine.

transferred onto nitrocellulose membranes, and analyzed by autoradiography. TG2 protein content in the samples was determined by probing nitrocellulose membranes with a rabbit anti-Myc antibody, which was detected with HRP-conjugated anti-rabbit IgG followed by exposure to ECL.

Transamidation activity measurement

The Transglutaminase Assay Kit (Sigma) was used, according to the manufacturer's instructions, to evaluate the transamidation activity of WT- and mutant TG2. TG2 protein samples used in the assay were purified by Myc immunoprecipitation (IP) from the cell lysates prepared from INS-1E cells transfected with WT- or mutant TG2 (Brymora *et al.* 2001).

In situ transamidation activity assay

INS-1E cells were cultured in 60 mm tissue culture plates at 60% confluency. They were then transfected with vectors expressing Myc-tagged WT- and mutant TG2 and further cultured for 48 h. Cells were pre-incubated for 1 h with 1 mM BPNH₂ in standard culture medium (Antonyak *et al.* 2006). They were then rinsed in PBS and sensitized to glucose for 30 min in glucose-free standard medium. Cells were rinsed again in PBS, and incubated in medium containing either 3.3 or 16.7 mM glucose for 30 min. They were then lysed and the supernatant was collected for further analysis by western blot.

Insulin secretion assays

INS-1E cells were cultured and transfected with various TG2 constructs as described above. After 48 h post-transfection, cells were rinsed with PBS and sensitized to glucose for 30 min in glucose-free KRB buffer solution. Cells were rinsed again with PBS, and incubated with KRB buffer containing either 3.3 or 16.7 mM glucose for 30 min. KRB buffer was collected and analyzed for insulin content with the Ultra Sensitive Rat Insulin ELISA kit, according to the manufacturer's instructions. Lysates of cells were collected and total protein content was determined. The insulin content of KRB buffer was normalized to the total protein content. Protein content was determined by the Bradford assay (BioRad).

Perfusion of INS-1E spheroidal clusters

INS-1E cells were cultured in 60 mm cell culture plates at 60% confluency and transfected with various TG2 constructs. At 2 days post-transfection, cells were trypsinized and seeded in non-adherent Petri dishes, allowing the formation of INS-1E spheroids as

described by Merglen *et al.* (2004). At 48 h in culture, 50 spheroids were collected from each dish and expression levels of WT- and mutant TG2 were confirmed by western blot. At 72 h, 500 spheroids were collected from each dish, washed in 3.3 mM glucose/KRB buffer, and were loaded into perfusion chambers. Spheroids were perfused for 10 min with 3.3 mM glucose/KRB buffer prior to initiating the collection of the effluent. Spheroids were then perfused at 0.3 ml/min with basal 3.3 mM glucose for 24 min and 16.7 mM for 30 min, followed by 3.3 mM for 10 min, while the effluent was collected every 2 min, as described for the isolated islets (Nguyen *et al.* 2011). The collected samples were measured for insulin content as described above.

Fluorescence microscopy

INS-1E cells were grown on cell culture slides (BD Biosciences, Mississauga, ON, Canada) at 60% confluency and transfected with various TG2 constructs. At 48 h post-transfection, cells were washed with PBST (PBS containing 0.05% Tween 20) and fixed with 10% formalin for 20 min at room temperature. After washing with PBST (2 × 5 min), cells were blocked with 1% BSA for 2 h at room temperature. Subsequently, the cells were incubated with an anti-Myc antibody (1:200 in 1% BSA) for 2 h at room temperature or overnight at 4 °C. The cells were washed three times with PBST, and incubated for 1 h at room temperature in a solution containing anti-rabbit Alexa Fluor 488 (1:300) in 1% BSA. The cells were then washed with PBST, mounted, and observed under a fluorescence microscope (Ande & Mishra 2009).

In-gel digestion of proteins and mass spectrometry

Coomassie Blue-stained bands (matching serotonylated bands on the overlay) were excised from the gel and cut into 1 mm³ cubes. In-gel trypsin digestion was performed as described previously (Mishra *et al.* 2007a,b). In brief, gel pieces were washed in 100 mM NH₄HCO₃ for 10 min, centrifuged, and the supernatant was discarded. Next, they were washed in 40% acetonitrile (ACN) in 100 mM NH₄HCO₃ for 10 min, and subsequently in ACN 5X gel volume for 5 min. These washing steps were repeated until all visible dye in the gel disappeared. The pieces were vacuum-dried briefly, then bathed in 3X gel volume of 10 mM dithiothreitol in 100 mM NH₄HCO₃ for 45 min at 55 °C, centrifuged, and the liquid was discarded. The difference between the volume of the liquid added and discarded was the volume of trypsin used for in-gel digestion. Then, 5 ng/μl of trypsin in 50 mM NH₄HCO₃ containing 5 mM CaCl₂ were added to the gel fragments and incubated at 37 °C

overnight. Peptides were extracted through liquid collection using 0.01% trifluoroacetic acid in 50% ACN and absolute CAN, and processed for mass spectrometry (Mishra *et al.* 2007a,b, Fofana *et al.* 2010).

ATP/GTP-binding assays

Cell culture and transfection were carried out as described above. At 48 h post-transfection, cells were rinsed in PBS and sensitized to glucose for 30 min in glucose-free standard medium. The cells were rinsed again in PBS and incubated in medium containing either 3.3 or 16.7 mM glucose for 7 and 30 min. After lysis, the supernatant was collected and 100 µg of lysates (total protein) were incubated with 20 µl of either GTP-agarose resin (50% (v/v) slurry in 10 mM Tris/300 mM NaCl/1 mM EDTA, pH 8.0) or ATP-agarose resin diluted in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl) to a total volume of 300 µl for 2 h at 4 °C. The resin was subjected to five cycles of washing and centrifugation using ice-cold TBST before the agarose beads were resuspended in 50 mM Tris-HCl buffer (pH 7.5). The pulled-down protein was analyzed by western blot utilizing an anti-Myc antibody to determine the quantity of Myc-TG2 bound to the ATP/GTP-agarose beads.

MTT assay

In living cells, yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple formazan by mitochondrial reductases. INS-1E cells were cultured in 96-well plates at 60% confluency and transfected, as applicable, with vectors expressing Myc-tagged TG2 WT and TG2 mutants and then further cultured for 48 h. The culture media were replaced with media containing 0.5 mg/ml MTT, and the INS-1E cells were further incubated for 3 h at 37 °C. The media were then carefully removed and replaced with DMSO to dissolve formazan. The INS-1E cells were incubated with DMSO for 30 min in the dark at room temperature. The production of formazan was quantified by measuring absorbance at 540 and 630 nm using a FLUOstar OPTIMA plate reader (BMG Labtechnologies GmbH, Ortenberg, Germany) and was expressed as a percentage of the positive control (Lee *et al.* 2010).

Reactive oxygen species assay

Cellular reactive oxygen species (ROS) generation in INS-1E cells was determined using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) as described before (Lee *et al.* 2010). CM-H₂DCFDA passes freely through cellular membranes and is deacetylated by intracellular esterases. This deacetylation results in the

non-fluorescent compound 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H₂DCF), which is rapidly oxidized into the highly fluorescent compound CM-DCF. The INS-1E cells were cultured in 96-well plates at 60% confluency, transfected with various TG2 constructs, and were incubated for 48 h. The culture media were replaced with 16.7 mM glucose/KRB buffer containing 5 µM CM-H₂DCFDA and the cells were incubated at 37 °C for 1 h. KRB buffer was replaced with PBS after washing, to remove extracellular dye. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 505 nm using the FLUOstar OPTIMA plate reader and was expressed as a percentage of the positive control.

Statistical analysis

Results are presented as means ± S.E.M. One-way ANOVA with Dunnett's test was used for multiple comparisons. *P* values < 0.05 were considered significantly different.

Results

Reduced insulin secretion from pancreatic islets isolated from TG2-null (TG2^{-/-}) mice

TG2^{-/-} mice have impaired glucose tolerance as a result of reduced insulin secretion from the pancreatic islets (Bernassola *et al.* 2002). To confirm that the reduced insulin secretion in TG2^{-/-} mice is a result of the loss of TG2 function in the pancreatic islets and not due to other peripheral factors, we measured GSIS from the pancreatic islets isolated from the WT and TG2^{-/-} mice. No difference in insulin secretion was observed between the pancreatic islets from the WT and TG2^{-/-} mice under basal glucose (3.3 mM) levels (Fig. 1A). However, in response to glucose stimulation (16.7 mM), a significant (*P* < 0.01) reduction in insulin secretion from the pancreatic islets of TG2^{-/-} mice was observed in comparison with WT mice (Fig. 1A). These results confirm that the lack of TG2 reduces GSIS from the pancreatic islets.

Reduced insulin secretion from INS-1E cells overexpressing Myc-TG2 mutants

Reduced insulin secretion from the pancreatic islets of TG2^{-/-} mice confirms the importance of WT-TG2 for β-cell function (Bernassola *et al.* 2002). To further establish the role of TG2 in β-cell function, we sought to demonstrate whether naturally occurring mutations also affect TG2 activity and its function on β-cell function. INS-1E cells were transfected with WT

Figure 1 Glucose-stimulated insulin secretion from the pancreatic islets and INS-1E cells. (A) Batches of four islets were pre-incubated in KRB buffer containing 3.3 mM glucose for 30 min. The pre-incubation buffer was collected and the islets were subsequently incubated for 30 min in KRB buffer containing 16.7 mM glucose. At the end of the incubation, the buffer was collected and insulin was measured by a mouse insulin ELISA kit (mean $\pm$ S.E.M., $n=12$; $**P<0.01$). (B) INS-1E cells were cultured in 60 mm tissue culture plates at 60% confluency and then transfected with various *TG2* constructs. After 48 h, the cells were rinsed in PBS, sensitized to glucose in glucose-free KRB buffer and, finally, incubated in KRB buffer containing either 3.3 or 16.7 mM glucose for 30 min. The buffer was then collected and analyzed for insulin concentration using a rat insulin ELISA kit as described in the Materials and methods section. Insulin concentration was normalized to the total cellular protein content. (Mean $\pm$ S.E.M., $n=5$; $*P<0.05$; $**P<0.01$). V, vector; WT, wild-type; 277, 330, 331 and 333 represent *TG2* mutants.

and naturally occurring mutant-*TG2* constructs. Post-transfection, INS-1E cells were batch-challenged with glucose and insulin secretion was measured in the conditioned media. A significant increase in GSIS was found in cells transfected with WT in comparison with the vector-only control group (Fig. 1B). However, GSIS was consistently found to be lower in INS-1E cells transfected with *TG2* mutants in comparison with cells transfected with WT-*TG2* (Fig. 1B). This indicates that *TG2* function(s) involved in insulin secretion is impaired in the naturally occurring *TG2* mutants. The maximum reduction was found in Ile331Asn-*TG2* (32%) followed by Met330Arg-*TG2* (20%) and the least (7%) in Asn333Ser-*TG2* (Fig. 1B). A reduction in GSIS (22.75%) was also found in INS-1E cells transfected with the Cys277Ala-*TG2* mutant lacking transamidation function, suggesting a role for the transamidation function of *TG2* in GSIS (Fig. 1B). Similar results were found with RINm5F β -cells (data not shown).

To study the effect of *TG2* mutants on insulin secretory phases, insulin secretion was determined during the perfusion of INS-1E spheroidal clusters (Merglen *et al.* 2004). Both the first and second phases of GSIS were upregulated in spheroids overexpressing WT-*TG2* in comparison with vector-transfected spheroids (Fig. 2). Both GSIS phases were reduced in spheroids expressing Ile331Asn-*TG2* and the transamidation-null Cys277Ala-*TG2* in comparison with WT-*TG2* (Fig. 2). However, Cys277Ala-*TG2*-expressing INS-1E cells secreted more insulin in comparison with the vector-only control group (Fig. 2). Together, these data suggest that *TG2* contributes to both phases of insulin secretion.

Cellular localization and expression of *TG2* mutants

To verify that the cellular localization, expression and stability of *TG2* mutants are not altered and are similar to WT-*TG2*, INS-1E cells were transfected with various *TG2* constructs and processed for fluorescence microscopy and immunoblotting. Intracellular localization and expression levels of *TG2* mutants were found to be similar to WT-*TG2*, suggesting that naturally occurring mutations in *TG2* have no effect on cellular localization, stability and expression of *TG2* (Fig. 3).

TG2 mutants have no effect on the viability of INS-1E cells

TG2 has been associated with cell viability and cell death (Fesus & Piacentini 2002). To determine whether reduced insulin secretion from INS-1E cells overexpressing *TG2* mutants was due to reduced cell viability, we performed the MTT assay. No difference in cell viability

Figure 2 Effect of WT and mutant *TG2* overexpression on GSIS from spheroidal clusters composed of INS-1E cells. (A) INS-1E spheroids expressing WT and mutant *TG2* were perfused at 0.3 ml/min with basal 3.3 mM glucose prior to stimulation with 16.7 mM glucose. Data points show insulin released expressed as mean $\pm$ s.e.m. ng/ml per 500 spheroids ($n=3$). (B) Representative photomicrographs of spheroidal clusters of INS-1E cells used for the perfusion experiments.

was observed among the different groups, except that Ile331Asn-TG2-overexpressing cells had a non-significant reduction in cell viability in comparison with the vector and WT *TG2*-transfected control groups (Fig. 4A).

Reduced ROS levels in INS-1E cells overexpressing Myc-TG2 mutants

In addition to the detrimental effect of ROS on β -cell function, emerging evidence suggests that ROS signaling plays a role in GSIS (Szypowska & Burgering 2011). To investigate whether the overexpression of *TG2* mutants alters intracellular ROS production, we measured ROS levels in INS-1E cells transfected with various *TG2* mutants. A decrease in ROS level was observed in INS-1E cells overexpressing *TG2* mutants, including the transamidation-null Cys277Ala-TG2 (Fig. 4B). These results are consistent with an earlier report showing decreased ROS production in cancer cells as a result of the downregulation of *TG2* (Bae *et al.* 2006).

TG2 mutants have altered enzymatic activities

To determine which function of *TG2* is affected in the naturally occurring *TG2* mutants, INS-1E cells were transfected with WT and mutant Myc-TG2 constructs. Subsequently, Myc-TG2 was affinity purified using a Myc-IP kit and used for the measurement of *TG2* transamidation and kinase activities by *in vitro* assays. A reduction in both transamidation and kinase activities was found in *TG2* mutants in comparison with the WT control (Fig. 5). A maximum reduction in

Figure 3 Expression patterns of *TG2* and *TG2* mutants in INS-1E cells. INS-1E cells were transfected with various Myc-TG2 constructs at 60% confluency. At 48 h post-transfection, the cells were processed for fluorescence microscopy and immunoblotting. (A) For fluorescence microscopy, the cells grown in chambered slides were first incubated with a rabbit anti-Myc primary antibody (1:200 in 1% BSA) and then with an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:300 in 1% BSA). After mounting, the cells were visualized by fluorescence microscopy. WT-TG2-transfected cells without primary antibody exposure were used as a negative control. (B) For immunoblot analysis, the cells were grown in 60 mm culture discs. Equal amounts of proteins (30 μ g) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were sequentially incubated with a rabbit anti-Myc primary antibody (1:1000) and then with a HRP-conjugated secondary antibody (1:3000) as described in the Materials and methods section, and visualized by enhanced chemiluminescence. Empty vector-transfected cells were included as a negative control and β -actin blot as a loading control.

Figure 4 Effect of WT and mutant *TG2* overexpression on cell viability and ROS generation in INS-1E cells. INS-1E cells were transfected with WT and mutant *TG2*. Cell viability was determined by the MTT assay (A) and ROS levels were measured using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (B) as described in the Materials and methods section. One-way ANOVA ($n=4$). * $P<0.05$.

TG2 transamidation activity was observed in Asn333Ser-*TG2* ($P<0.01$) followed by Ile331Asn-*TG2* ($P<0.05$) and the least in Met330Arg-*TG2* (Fig. 5A). In addition, we examined the *in situ* transamidation activity of *TG2* mutants in INS-1E cells using biotinylated BPNH₂ as an amine substrate. A similar pattern of transamidation activity in *TG2* mutants was found as that of the *in vitro* transamidation assay, especially in the lower half of the blots where the transamidated protein bands were relatively distinct from each other (Fig. 5B). In the case of kinase activity, a reduction in the range of 15–23% was found in all *TG2* mutants including transamidation activity-dead mutant-*TG2* (Fig. 5C). However, no difference in kinase activity was observed when the

assay was performed at the 7-min time point in response to glucose stimulation (Fig. 5C). Collectively, these data suggest that the naturally occurring *TG2* mutants have impaired transamidation and kinase activities. However, the effect on transamidation activity was found to be more pronounced than the effect on kinase activity.

Naturally occurring mutations in *TG2* differentially affect GTP-binding function

To determine the effect of the naturally occurring *TG2* mutations on its GTP-binding function, cell lysates prepared from INS-1E cells transfected with various Myc-*TG2* constructs were processed for the pull-down assay using GTP-agarose affinity resin. GTP-agarose resin comprises GTP attached to the agarose beads via its γ -phosphate and is resistant to phosphatases present in crude cell/tissue extracts (Iismaa *et al.* 1997). GTP resin-bound *TG2* was analyzed by immunoblotting using an anti-Myc antibody followed by densitometric analysis. Under the basal glucose concentration (3.3 mM), WT-*TG2* was found to have a low GTP-binding ability, whereas Ile331Asn-*TG2* was unable to bind GTP and Met330Arg had a very weak GTP-binding ability (Fig. 6A and B). Surprisingly, Asn333Ser-*TG2* was found to have elevated GTP-binding ability (Fig. 6A and B). Most importantly, the GTP-binding ability of WT- and Met330Arg-*TG2* was found to be increased and that of Asn333Ser-*TG2* was decreased in response to glucose stimulation (Fig. 6A and B). GTP binding to Ile331Asn-*TG2* was found to be low under both conditions (Fig. 6A and B). A similar binding pattern was found with ATP for Ile331Asn- and Asn333Ser-*TG2* (Fig. 6A and B). However, unlike GTP binding, WT- and Met330Arg-*TG2* were found to have maximum ATP-binding ability (Fig. 6A and B). Furthermore, no apparent change in ATP binding was observed under the basal and high glucose concentrations (Fig. 6A and B). Similar to kinase activity, no difference in the GTP-binding ability of *TG2* was observed in response to glucose stimulation when the assay was performed at the 7-min time point (Fig. 6C). Thus, the GTP-binding function was found to be increased in WT-*TG2* by glucose stimulation only at the 30-min time point and its loss more closely correlated with significantly reduced insulin secretion from INS-1E cells expressing Ile331Asn-*TG2*. Taken together, these data suggest an important role for the GTP-binding function of *TG2* in GSIS from the pancreatic β -cells, which is most likely involved during the second phase of insulin secretion.

Protein serotonylation in INS-1E cells overexpressing WT- and mutant-*TG2*

Recently, activation of small GTPases through serotonylation by *TG2* in response to glucose has been

Figure 5 Impact of the naturally occurring mutations in *TG2* on its enzymatic activities. *Myc*-tagged WT and mutant *TG2* were affinity purified as described in the Materials and methods section. (A) Histogram showing the relative transamidation activity of WT and mutant *TG2* as determined by *in vitro* transamidation (mean $\pm$ S.E.M., $n=6$). * $P<0.05$; ** $P<0.01$ vs WT control. (B) Western blot showing *in situ* transamidation activity using 5-(biotinamido)pentylamine (BPNH₂) as a substrate. Cell culture and transfection were performed as described in Fig. 1. Subsequently, INS-1E cells were pre-incubated for 1 h with 1 mM BPNH₂ in standard culture medium. The cells were rinsed with PBS, and sensitized to glucose for 30 min in glucose-free standard medium and subsequently incubated in medium containing either 3.3 or 16.7 mM glucose for 30 min. The cells were then harvested and 30 μ g of proteins were analyzed by SDS-PAGE and western blot using streptavidin-HRP conjugate and ECL. (C) Autoradiographs showing the kinase activity of WT and mutant *TG2* at the 7- and 30-min time points in response to glucose stimulation as determined by an *in vitro* kinase assay. The *in vitro* kinase assay was performed in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, [γ -³²P]ATP (25 μ Ci/ml)) containing IGFBP3 (2 μ g) in a final volume of 25 μ l at 30 °C. Subsequently, the mixture was resolved on SDS-PAGE and processed for autoradiography. Representative autoradiograph of three different experiments is shown. Histograms show relative changes in kinase activity as determined by the densitometric analysis of phosphorylated IGFBP3 (mean $\pm$ S.E.M., $n=3$). * $P<0.05$.

reported to play a role in the regulation of insulin secretion from pancreatic β -cells (Paulmann *et al.* 2009). Our findings of reduced transamidation activity in *TG2* mutants prompted us to examine protein serotonylation in INS-1E cells expressing various *TG2* constructs. The overexpression of WT-*TG2* was found to be sufficient to upregulate protein serotonylation in β -cells even under the basal glucose

concentration in comparison with the vector-only control group (Fig. 7A). On the other hand, the overexpression of Cys277Ala-*TG2* resulted in the down-regulation of protein serotonylation (Fig. 7A). A significant upregulation of protein serotonylation was observed in cells expressing Asn333Ser-*TG2* at the basal glucose concentration (Fig. 7A). Interestingly, out of the three major serotonylated bands, two were

Figure 6 Impact of the naturally occurring mutations in *TG2* on its GTP/ATP binding function. (A) INS-1E cells were transfected with various Myc-*TG2* constructs. At 48 h post-transfection, the cells were incubated with basal (3.3 mM) and high (16.7 mM) glucose concentrations for 30 min. Equal amounts of total proteins (100 µg) of each sample were incubated with 20 µl GTP- or ATP-agarose in 10 mM Tris buffer (pH 7.5) for 2 h at 4 °C on a rotating device. After washing, 30 µl of loading buffer was added to each tube, boiled in a water bath for 10 min and analyzed by SDS-PAGE and immunoblotting using an anti-Myc antibody as described in Fig. 2. (B) Histograms showing relative changes in GTP and ATP binding in WT and mutant *TG2* as shown in (A). (C) Anti-Myc immunoblots showing GTP-binding ability of WT and mutant *TG2* in response to glucose stimulation (for 7 min). The experiment was performed as described in (A).

downregulated in response to glucose stimulation, whereas one remained unchanged in cells expressing Asn333Ser-*TG2* (Fig. 7A). In contrast, all three bands were consistently upregulated in cells overexpressing WT and Met330Arg-*TG2* under the high glucose concentration. Furthermore, a unique serotonylated band appeared in response to glucose stimulation in cells expressing Ile331Asn-*TG2*, most likely due to proteolytic cleavage of the 72 kDa band (Fig. 7A). Subsequently, we analyzed protein serotonylation in the pancreatic islets from the WT and *TG2*^{-/-} mice in response to glucose stimulation. A similar band pattern of serotonylated proteins was found in both cases as in INS-1E cells; however, a difference in protein serotonylation was apparent between the WT and *TG2*^{-/-} mice (Fig. 7B). Unexpectedly, out of the three prominently serotonylated bands, two were upregulated in the

pancreatic islets from the *TG2*^{-/-} mice, whereas a decrease in serotonylation was found in the 72 kDa band (Fig. 7B). This may be a result of a compensatory response through an upregulation of other members of the TG family of proteins (Porzio *et al.* 2007). However, the inability to compensate the serotonylation of the 72 kDa band may be associated with reduced insulin secretion in *TG2*^{-/-} mice, which is consistent with the data obtained in INS-1E cells expressing the naturally occurring *TG2* mutants, especially Ile331Asn-*TG2*. To reveal the identity of this uniquely serotonylated 72 kDa protein band, the band was excised from an identical Coomassie Blue-stained gel and processed for mass spectrometry. A number of GTP-binding proteins were identified (Table 2). These data are consistent with a recent report on an important role of serotonylation of GTPases in insulin secretion from β-cells.

Figure 7 Protein serotonylation in INS-1E cells overexpressing *TG2* and *TG2* mutants. (A) Cell culture, transfection and treatment were performed as described in Figs 1, 2 and 3. Subsequently, the cells were harvested and equal amounts of proteins were analyzed by SDS–PAGE and immunoblotting using an anti-serotonin antibody (1:500). β-Actin immunoblots are shown as a loading control. The 72 kDa serotonylated protein is shown with an arrow (←) and a unique serotonylated band in the case of Ile331Asn-*TG2* is indicated by an asterisk (*). (B) Anti-serotonin immunoblot showing protein serotonylation in the pancreatic islets in response to glucose stimulation from the WT and *TG2*-null (*TG2*^{−/−}) mice. Coomassie Blue-stained gel is shown as a loading control.

Discussion

In this study, we analyzed the effect of naturally occurring mutations in *TG2* on its transamidation, kinase, and GTP-binding functions and their association with GSIS. Our results indicate that all three reported mutations in *TG2* differentially affect *TG2* biological functions. This is consistent with the location of these mutations in close proximity of the transamidation catalytic triad (Cys²⁷⁷, His³³⁵, and Asp³⁵⁸) and in the proximity of the residues involved

in GTP/ATP binding (Fig. 8). In fact, the Met³³⁰, Ile³³¹, and Asn³³³ mutation sites in *TG2* are sandwiched between the transamidation catalytic triad and GTP/ATP binding residues in *TG2* (Fig. 8).

TG2 activity is regulated by ATP, GTP, and Ca²⁺. Protein transamidation by *TG2* is inhibited by both ATP and GTP and activated by Ca²⁺, whereas *TG2* kinase activity is inhibited by Ca²⁺ and promoted by ATP (Mishra & Murphy 2004, Mishra *et al.* 2007*a,b*). As ATP and Ca²⁺ modulate both *TG2* transamidation and kinase activities and GSIS requires the upregulation of ATP and Ca²⁺ in a sequential manner within β-cells (Yada 1994, Pinkas *et al.* 2007), both *TG2* activities may potentially contribute to insulin secretion. A reduction in the transamidation activity of *TG2* mutants and a consistent decrease in insulin secretion from INS-1E cells overexpressing these mutants suggest a role for transamidation activity in insulin secretion. These results are in line with earlier reports on the reduction of insulin secretion from rat β-cells treated with inhibitors of *TG2* transamidation activity (Bungay *et al.* 1986, Driscoll *et al.* 1997). However, the increase in Ca²⁺ level that leads to insulin secretion in β-cells in response to glucose is much lower (~150–250 nM) than the level of Ca²⁺ (~150–500 μM) required for *TG2* transamidation activity (Lai *et al.* 1997, Parkash *et al.* 2002). In addition, intracellular levels of GTP, which is known to inhibit transamidation activity, are many fold higher (~100 μM) than Ca²⁺ levels (Siegel & Khosla 2007). It is possible that ATP/GTP binding or other changes in *TG2* (e.g. phosphorylation) alter the Ca²⁺ sensitivity of *TG2*, resulting in the activation of the transamidation function. However, the relatively lower reduction in insulin secretion from cells overexpressing the transamidation activity-dead Cys277Ala-*TG2* mutant and the Asn333Ser-*TG2* natural mutant with the greatest loss of transamidation activity, compared with cells overexpressing the Ile331Asn-*TG2* mutant, suggests that *TG2* transamidation activity *per se* is not sufficient in this process.

We then analyzed serotonylated proteins as a specific instance of transamidation, and we found that an approximately 72 kDa serotonylated protein band was more dynamically altered especially in the pancreatic islets from the *TG2*^{−/−} mice and in INS-1E cells expressing the Ile331Asn-*TG2* natural mutant, which have the greatest reduction in insulin secretion. This suggests that the 72 kDa protein band may be an important target for serotonylation (transamidation) associated with insulin secretion. Analysis of the ~72 kDa serotonylated band by mass spectrometry identified a number of Rab GTPases. It should be noted that the molecular masses of proteins identified by mass spectrometry are lower than the molecular mass of the overlay serotonylated band excised from the Coomassie Blue-stained reducing gel. This would mean that these

Table 2 List of proteins identified by mass spectrometry in the 72 kDa serotonylated band

Uniprot/SWISSPROT or Ref Seq_peptide no.	Probability log(e) score ^a	No. of unique peptides	Mr	Protein
P48036	−168.2	18	35.6	ANXA5, annexin-A5
P55260	−115.1	11	35.7	ANXA4, annexin-A4 (36 kDa zymogen granule membrane-associated protein)
Q9Z2L0	−113.3	12	32.2	VDAC1, voltage-dependent anion channel 1
P63245	−80	9	35.4	GNB2/1, guanine nucleotide-binding protein subunit beta-2-like 1
Q09073	−54.7	8	32.9	SLC25A5, ADP/ATP translocase 2 (solute carrier family 25)
P81115	−53.3	7	31.8	VDAC2, voltage-dependent anion channel 2
Q6NYB7	−45.5	7	22.4	RAB1, Ras-related protein Rab-1A
Q05962	−44.4	3	32.9	SLC25A4, ADP/ATP translocase 1 (solute carrier family 25)
Q9R1Z0	−41.4	4	30.9	VDAC3, voltage-dependent anion channel 3
Q9Z269	−40.3	5	26.9	VAMP-associated protein B, Vesicle-associated membrane protein-associated protein B
P09527	−37.9	4	23.5	RAB7A, Ras-related protein Rab-7a
P53994	−29.3	4	23.5	RAB2A, Ras-related protein Rab-2A
NP_596909	−26.6	4	31.4	SLC25A10 (solute carrier family 25)
P10536	−22	1	22.2	RAB1B, Ras-related protein Rab-1B
Q9Z270	−21	2	27.8	VAMP-associated protein A, Vesicle-associated membrane protein-associated protein A
NP_001099310	−14.3	2	25.2	RAB5C, Ras-related protein Rab-5C
P61107	−13.5	2	23.9	RAB14, Ras-related protein Rab-14
Q4KM74	−11.8	2	24.7	Vesicle-trafficking protein SEC22b
P97700	−11	2	34.1	SLC25A11 (solute carrier family 25 member 11)
O35509	−9.5	2	24.5	RAB11B, Ras-related protein Rab-11B
NP_001100389	−6.7	2	34.4	Actin-related protein 2/3
NP_00103405	−5.1	1	23.7	RAB5B, Ras-related protein Rab-5B
XP_343460	−3.2	1	23.4	RAB6B, Ras-related protein Rab-6B
P16036	−1.9	1	39.6	Phosphate carrier protein (solute carrier family 25 member 3)
P62747	−1.7	1	22.1	Rho-related GTP-binding protein RhoB
Q62636	−1.6	1	20.8	GTP-binding protein smg p21B
P63012	−1.5	1	25	RAB3A, Ras-related protein Rab-3A
NP_001100085	−1.3	1	19.5	Actin-related protein 2/3 complex, subunit 4, subunit 4

^aBase −10 log of expectation that this assignment is stochastic. Low expect score (< −3.0) corresponds to a confident identification. Bold – proteins with a log expect score > −3.

proteins are not only serotonylated, but also cross-linked. A unique serotonylated band that appeared only in the case of Ile331Asn-TG2 in response to glucose stimulation may be due to the perturbed crosslinking of serotonylated proteins rather than the cleavage of the 72 kDa serotonylated band. As observed for transamidation, there was no correlation between insulin secretion and serotonylation levels of the ~72 kDa band in the vector and Cys277Ala-TG2 control groups, suggesting that protein serotonylation *per se* is not sufficient to explain the insulin secretory phenotype of INS-1E cells overexpressing TG2 mutants. In addition to Rab GTPases, a number of actin, tubulin, and vesicle membrane network proteins were also identified by mass spectrometry in the 72 kDa serotonylated protein band. As Rab GTPases regulate many steps of membrane traffic, including vesicle formation, vesicle movement along with actin and tubulin networks, and membrane fusion, it is possible that serotonylation of these cytoskeletal and vesicle membrane network proteins is involved in GSIS from β -cells.

Moreover, our results suggest that naturally occurring mutations in TG2 differentially affect GTP- and ATP-binding function of TG2. Most importantly, GTP-binding ability was found to be increased in WT-TG2 in response to glucose stimulation only at 30 min (i.e. corresponding to the second phase of insulin secretion) and its loss was more closely correlated in the Ile331Asn-TG2 mutant with the greatest reduction in insulin secretion. A lack of the correlation between GTP/ATP-binding ability and the reduction in insulin secretion in the case of the Asn333Ser-TG2 mutant may be due to the compensatory effect of ATP on GTP binding, which is not the case with the Ile331Asn-TG2 mutant. However, a lack of the correlation between GTP/ATP binding and decreased insulin secretion in Met330Arg-TG2 again suggests the involvement of more than one function of TG2 in insulin secretion. Furthermore, our findings of the impaired GTP-binding function of naturally occurring TG2 mutants are consistent with an earlier report by Murthy *et al.* (2002) in which the substitution of a conserved

Figure 8 Naturally occurring mutations in *TG2* are flanked between the transamidation catalytic triad and GTP-binding residues. Relative position of the transamidation catalytic triad (Cys²⁷⁷, His³³⁵, and Asp³⁵⁸), naturally occurring mutations (Met³³⁰, Ile³³¹, and Asn³³³), and GTP/ATP-binding loops (169 Gln-Gly-Ser-Ala-Lys¹⁷³ and 580 Arg-Asp-Leu-Tyr⁵⁸³) in *TG2* are shown in a ball-and-stick model (upper panel) and in a space-filled model (lower panel).

tryptophan residue at position 332 (which is flanked between two naturally occurring mutations in *TG2*) has been found to significantly reduce GTP binding.

In summary, the data presented here demonstrate that naturally occurring mutations in *TG2* differentially affect transamidation, kinase, and G-protein functions of *TG2*. This would imply that transamidation, kinase, and G-protein functions of *TG2* are associated with each other and regulated in a coordinated manner. Furthermore, our data indicate that various functions of *TG2* are activated in a sequential manner during the different phases of GSIS. Reduced insulin secretion as a result of naturally occurring mutations in *TG2* is most likely due to the impairment of more than one biological function of *TG2*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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