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Original Article

The Differential Effects of R580A Mutation on Transamidation and GTP Binding Activity of Rat and Human Type 2 Transglutaminase

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Abstract: Type 2 transglutaminase (TG2) is an acyltransferase, which also undergoes a GTP-binding/GTPase cycle, with guanine nucleotide and calcium binding reciprocally regulating its transamidation (TG) activity. TG2 is expressed ubiquitously throughout the human body and is the predominant neuronal transglutaminase. Given a postulated role for TG2 in a number of physiological and pathological processes including neurodegenerative diseases, it is of critical importance to understand how TG2 and its enzymatic activities are regulated in the cells. The various aspects of TG2 regulation are addressed by using rat and human TG2 proteins, however, despite their homologous structure, regulation of their enzymatic activities may differ, especially in the cellular context. Here, we evaluate the role of Arg580 in human TG2 and Arg579 in rat TG2 in modulating GTP binding and TG activities *in vitro* and *in situ*. We confirm the importance of Arg580 and Arg579 in TG2 for GTP binding as their mutation to Ala completely abolished GTP binding activity in both human (R580A) and rat TG2 (R579A). Next, we showed that in transfected human embryonic kidney (HEK) 293 cells, basal *in situ* TG activity of human R580A TG2 and rat R579A TG2 was significantly greater than their wild-type (WT) counterparts. However, TG activity of the mutants and WT TG2 became equivalent when the intracellular calcium concentration was maximally increased with maitotoxin. Also, *in vitro* TG activity assay revealed an intriguing difference between rat and human TG2; at a calcium concentration when their activities were maximum, the protein level of human R580A TG2 was lower than its WT counterpart, whereas rat R579A and WT TG2 protein levels were similar. Taken together, our study underscores an essential role of Arg580 in human TG2 and Arg579 in rat TG2 for their GTP binding ability and also describes for the first time that these amino acid residues differentially influence the TG activity of human or rat TG2 by calcium *in vitro* and *in situ*.

Key Words: Type 2 transglutaminase, Arg580, Arg579, GTP binding, transamidation activity, R580A, R579A

Introduction

Type 2 transglutaminase (TG2, tissue transglutaminase) is a member of a family of calcium dependent acyltransferase enzymes called transglutaminases (TGs), which catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines [1]. Eight catalytically active members of the TG family have been identified in the human genome, of these TG2 is most ubiquitously expressed and enriched in the nervous system [2]. In addition to being a transamidating enzyme, TG2 binds and hydrolyzes GTP [3, 4] and it may function as a signal transducing G protein [5, 6]. Interestingly, binding of GTP inhibits TG activity, both *in vitro* and *in situ* [3, 7]. TG2 also has protein disulphide isomerase activity [8], and recently, it has been suggested to have kinase activity [9, 10]. All of these data clearly indicate a multifunctional character of TG2 and presumably a complex mechanism for regulation of its enzymatic activities.

In the nervous system, TG2, that is mostly found in cytoplasm of neurons, has been implicated in signal transduction [11], axonal growth [12, 13], axonal regeneration [13], as well as, neuronal cell death [14-17]. However, there is a rather modest amount of data as to how TG2 and its enzymatic activities are involved in the above processes in the nervous system.

In 2002, an x-ray crystallographic model of human TG2 was published which shed significant light on the mechanism by which TG activity is modulated by calcium and GTP/GDP [18]. The Arg580 residue, that is conserved in TG2 proteins from other species, but not in other TG proteins, has been suggested to be essential for the stability of the GTP/GDP binding site [18]. This role have been demonstrated for the Arg579 residue (equivalent to human Arg580), which with Tyr516 plays an important part in the allosteric regulation of coupling the GTP binding to inhibition of TG activity [19]. The complex regulation of TG2 enzymatic activities has been further confirmed in the recent study by Pinkas et al., which also have demonstrated a very unique large conformational change of TG2 during its catalytic action [20].

Rat and human TG2 are both widely used to study the role that TG2 plays in various biological events. The importance of Arg579 in rat TG2 has been recently documented using purified recombinant rat TG2 protein [21]. Another recent study has also demonstrated the essential role of Arg580 in human TG2 protein: the R580L or R580K mutants of human TG2 have been characterized as defective in their GTP binding activity *in vitro* and *in situ* [22]. However, despite undeniable similarities between rat and human TG2 [23, 24], one can predict specific differences between them in a cellular setting.

Thus, the current study was undertaken to investigate whether rat and human TG2 are regulated in cells in a similar manner. Given the fact that results obtained from studies with rat TG2 are often extrapolated to human TG2, it is essential to determine if there are any differences in the *in situ* regulation of rat and human TG2. For example, the TG activity of human TG2 in cells is known to be modulated by post-translational modification, e.g. nitrosylation of cysteine residues by nitric oxide-releasing agents [25], and certain intracellular molecules, such as sphingosylphosphocholine increase the sensitivity of TG2 to calcium [26]. It is not known, however, whether the same type of regulation takes place in the case of rat TG2. Thus, in order to describe fully the role of TG2 in cellular processes, it is necessary to study the regulation of both human and rat TG2 *in vitro*, as well as in the cellular context.

We investigated the role of Arg580 in the function of human TG2 and Arg579 in the function of rat TG2 by mutating these sites to alanines. As expected, both R579A and R580A mutations abolished GTP binding activity in rat and in human TG2 respectively. Interestingly however, the mutations affected *in vitro* TG activity of human TG2 and rat TG2 in a different way: TG activity of human R580A TG2 was significantly lower than its wild-type (WT) counterpart, whereas the R579A mutant and rat WT TG2 exhibited comparably activities. Further, *in situ* TG activity of both R580A human and R579A rat TG2 in transiently transfected HEK293 cells was significantly greater than their WT counterparts under basal conditions. However, when the intracellular concentrations of calcium were maximally increased, this difference disappeared. Further, in both rat and human TG2, increases in intracellular calcium levels resulted in similar extent of protein degradation when compared the mutant TG2 and the corresponding WT TG2.

Taken together, these data demonstrate for the first time that even though Arg580 in human TG2 and Arg579 in its rat TG2 orthologue play the same essential role in the GTP binding of both TG2 proteins, they differentially modulate their TG activities *in vitro* and *in situ*.

Materials and methods

Constructs

The human TG2 constructs: pcDNA3.1-TG2 and pcDNA3.1-C277S-TG2 have been described previously [27]. The mutation of Arg580 to Ala580 (R580A) in human TG2 was accomplished by site-directed mutagenesis using the Stratagene QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). The rat TG2 constructs: pcDNA-TG2 [4] and pcDNA-TG2 with Arg579 mutated to Ala579 (R579A) [28] have been described

previously, and they were kindly provided to us by Dr. S. E. Iismaa and Dr. R.M. Graham.

Cell Culture

HEK293 cells were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 5% bovine growth serum (HyClone), 2 mM L-glutamine (Invitrogen, Life Technologies, Inc.), 100 µg/ml streptomycin (Invitrogen, Life Technologies, Inc.) and 100 units/ml penicillin (Invitrogen, Life Technologies, Inc.). Cells were grown in a humidified atmosphere containing 5% CO2 at 37°C. Transient transfections were carried out using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's instructions.

Immunoblotting

Cells were rinsed in ice-cold phosphate-buffered saline (PBS) and collected in lysis buffer, containing 0.5% NP-40, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 µM okadaic acid, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin. Samples were sonicated on ice and centrifuged at 16,000g for 10 min. Protein concentrations of supernatants were then determined by the bicinchoninic acid assay with bovine serum albumin (BSA) as a standard and samples were diluted to a final concentration of 1 mg/ml with 2x reducing stop buffer (0.25 M Tris-HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, 10% glycerol, and bromophenol blue as the tracking dye). Samples (5 µg of protein) were resolved on 8% SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The blots were then incubated with a mouse monoclonal TG2 primary antibody TG 100 (Lab Vision–NeoMarkers, Fremont, CA, USA; 1: 5,000 dilutions). The membranes were then washed three times with TBST and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. The membranes were rinsed three times for 30 min with TBST, followed by four quick rinses with distilled water, and developed with the enhanced chemiluminescence as described previously [29].

In vitro TG activity assay

An *in vitro* TG activity was measured in cell extracts using a procedure described previously [14]. Briefly, thirty-six hours after transfection, cells were washed with ice-cold PBS and harvested in 50 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 µg/ml concentration each of aprotinin, leupeptin, and pepstatin. Samples were briefly sonicated on ice, spun at 10,000 g and 4°C for 1 min. Protein concentration of the supernatant was determined using the bicinchoninic acid assay and samples were diluted to a final concentration of 1 mg/ml with lysis buffer. Samples containing 50 µg protein was incubated in TG assay buffer containing 0.1 M Tris-HCl (pH 7.5), 10 mM dithiothreitol, 2.5% Lubrol-PX, 0.2 mM putrescine (unlabeled), 1µCi of [1,4(n)-3H]putrescine dihydrochloride, 1.5 mg/ml N,N-dimethylcasein and 10 mM CaCl2. Reaction mixtures were incubated for 1 h at 37°C, and the reaction was terminated by addition of trichloroacetic acid to a final concentration of 10%. The samples were incubated on ice for 1 h and centrifuged at 16,000 g and 4°C for 20 min. The supernatant was removed, and the pellet was rinsed twice with 1 ml of 5% trichloroacetic acid, then 250 µl of 0.25 M NaOH was added to each tube. The samples were incubated in a boiling water bath for 10 min, cooled, and mixed vigorously. The radioactivity emitted from bound [3H]putrescine was quantitated by liquid scintillation using a Beckman LS6500 scintillation counter, and TG activity was calculated after background subtraction as nanomoles of putrescine incorporated per mg of protein per hour. Non-specific activity was measured by performing the reaction in the absence of CaCl2 and in the presence of 5 mM EGTA. For the concentration–response analysis of calcium-activated TG activity, the assay was performed as described above using 25 µg of cell lysate and the CaCl2 concentration specified.

In situ TG activity assay

An *in situ* TG activity was measured as described previously described [7]. Briefly, cells were labeled with 1 mM 5-(biotinamido)pentylamine (BAP) (Pierce) for 4 h prior to maitotoxin (MTX) treatment that is know to increase an intracellular calcium

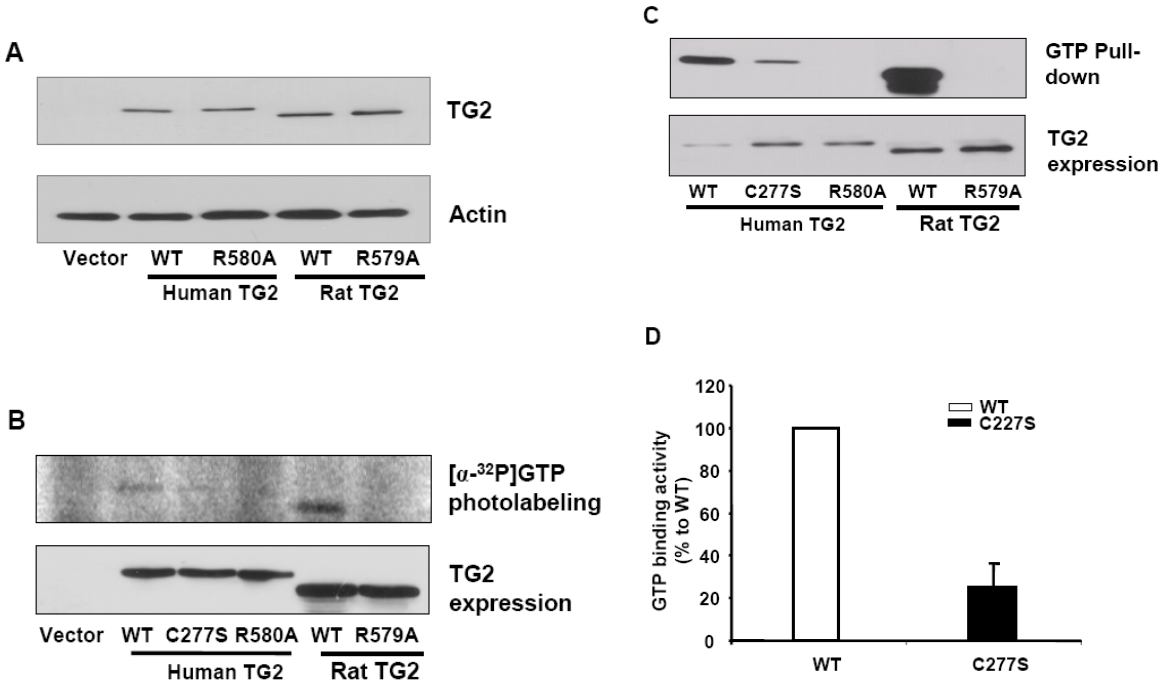

Figure 1. TG2 expression and GTP binding activity. (A) Representative immunoblot showing TG2 expression levels in HEK293 cells transiently transfected with vector, human WT TG2, human R580A TG2, rat WT TG2 or rat R579A TG2. Cells were collected and lysates were immunoblotted with the TG2 antibody TG100. The actin immunoblot showed that similar amounts of protein were loaded. (B) Representative [α-32P]GTP photolabeling of rat and human TG2. HEK293 cells were transiently transfected with vector, human WT TG2, human C277S TG2, human R580A TG2, rat WT TG2 or rat R579A TG2. Cells lysates were collected, photolabeled with [α-32P]GTP and analyzed by SDS-PAGE as described in Materials and Methods. Immunoblot of cell lysates with TG100 antibody showed similar TG2 expression levels in these cells. (C) Representative GTP binding of human or rat TG2 using GTP-agarose pull-down assay. Cells lysates of transfected cells, as above, were collected and incubated with GTP-agarose as described in Materials and Methods. Bound proteins were analyzed by SDS-PAGE and immunoblotted. Immunoblot of cell lysates with the TG 100 antibody showed similar TG2 expression levels in these cells. (D) Quantitative analysis of the GTP binding of human WT and C277S TG2 from the GTP pull-down assay. Data are expressed as a function of GTP binding by WT TG2, N=3 separate experiments.

levels [30]. Cells were collected, washed, and pelleted. Pellets were resuspended in homogenization buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA), sonicated on ice, and protein concentration was determined using the bicinchoninic acid assay. The incorporation of BAP into proteins was quantified as described previously [7].

Photoaffinity labeling of TG2

Photoaffinity labeling of TG2 was performed according to the procedure previously described [31] with our minor modifications. Briefly, 24 hours after transfection, cells were collected in lysis buffer containing 50 mM Tris-HCl (PH 7.4), 2 mM EGTA, 100 mM NaCl, 1 mM DTT, 20% Glycerol, 2 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml concentration each of aprotinin, leupeptin, and pepstatin. Samples were briefly sonicated on ice, spun at 16,000g at 4°C for 1 min. Protein concentration of the supernatant was determined using the bicinchoninic acid assay and samples were diluted to a final concentration of 1 mg/ml with lysis buffer. 50 μg of cell lysates were incubated with 5 μCi of [α-32P]GTP in 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 20% (w/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP for 10 min at room temperature. The reaction mixture were then placed in an ice bath, exposed to UV light (254 nm) for 15 min, mixed with 2x reducing stop buffer and boiled. SDS-PAGE was performed, followed by gel drying for 2 h and exposure to x-ray film for 1 day.

GTP-agarose pull-down assay

GTP-agarose pull-down assay was performed according to the procedure previously described [32]. Briefly, 24 hours after transfection, cells were rinsed in ice-cold PBS and collected in GTP-binding buffer containing 20 mM Tris–HCl (pH 7.0), 150 mM NaCl, 5 mM MgCl2, and 0.1% (v/v) Triton X-100, supplemented with 2 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml pepstatin, and 10 μg/ml aprotinin. Samples were sonicated for 15 s and centrifuged at 13,000g for 10 min at 4°C, and the supernatant was collected. The protein concentration of each supernatant was determined by the bicinchoninic acid assay. 200 μg of lysate protein were incubated with 100 μl of GTP-agarose beads (Sigma-Aldrich; equilibrated in GTP-binding buffer) in a total of 400 μl of GTP-binding buffer for 30 min at 4°C. The beads were centrifuged at 13,000g for 2 min and the supernatant was retained. Then, the beads were washed three times with 1 ml of GTP-binding buffer and the retained supernatant was incubated with the beads for another 30 min. The beads were washed again as described above and then incubated with the retained supernatant overnight at 4°C. After washing eight times with GTP-binding buffer, bound protein was eluted from the beads by boiling them in 35 μl of 2x reducing stop buffer. TG2 that had bound to the GTP-agarose beads was visualized by performing immunoblot analysis on the eluted protein, as described above, using TG 100 as the primary antibody.

In situ TG2 proteolysis assay

Thirty-six hours after transfection with the corresponding construct, HEK293 cells were transferred to serum-free media and treated with 2 nM MTX (Alexis) for 0, 0.5, 1 and 2 h at 37°C. The MTX was prepared in Me2SO, and the final concentration of Me2SO in the media was 0.1%. Control cells were treated with 0.1% Me2SO under the same conditions. Later, cells were washed with ice-cold PBS and harvested in 50 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml concentration each of aprotinin, leupeptin, and pepstatin. Samples were briefly sonicated on ice, spun at 10,000 g and 4°C for 1 min. Protein concentration of the supernatant was determined using the bicinchoninic acid assay and samples were diluted to a final concentration of 1 mg/ml with lysis buffer. Then, 20 μg samples were subjected to SDS-polyacrylamide gels and later blots were incubated with the TG 100. Experiments were repeated 3 times.

Statistics

Data were analyzed using Student's t test. Values were considered significantly different when the two-tailed p value was <0.05, unless specified. To confirm a significant difference, the groups were re-analyzed using analysis of variance (ANOVA). Results were expressed as mean ± S.E.M.

Results

R580A and R579A mutations abolish GTP binding activity of human or rat TG2 respectively

HEK293 cells were transiently transfected with DNA constructs containing either human TG2: WT, TG-inactive C277S TG2 and R580A TG2 or rat TG2: WT and R579A TG2, or with the empty vector as control. Throughout the studies, at this step, expression at of all TG2 proteins was at a comparable level, as illustrated in **Figure 1A**. To examine R580A or R579A mutations on the GTP binding ability of both human or rat TG2 proteins, cell lysates were collected from transfected HEK293 cells and subjected to a GTP binding activity assay to measure the incorporation of [α-32P]GTP into TG2. The importance of R580 to GTP binding activity of rat and human TG2 was demonstrated by the finding that R580A TG2 lacked any detectable GTP binding activity, compared to WT which showed significant GTP binding (**Figure 1B**). The endogenous levels of TG2 in HEK293 cells were very low, but they could be detected when the blots were overexposed (data not shown). As the effect of R580A on GTP binding of TG2 was of essential importance to our study, we confirmed the above finding with a GTP-agarose pull-down assay to measure GTP binding activity of human and rat TG2. These data also demonstrated that R580A or R579A mutation abolished the ability of TG2 to bind GTP (**Figure 1C**). In addition, the C277S mutation in human TG2 resulted in a significantly reduced GTP binding activity of human TG2, as indicated by both photoaffinity labeling and the GTP pull-down assay. According to the GTP pull-down data, C277S

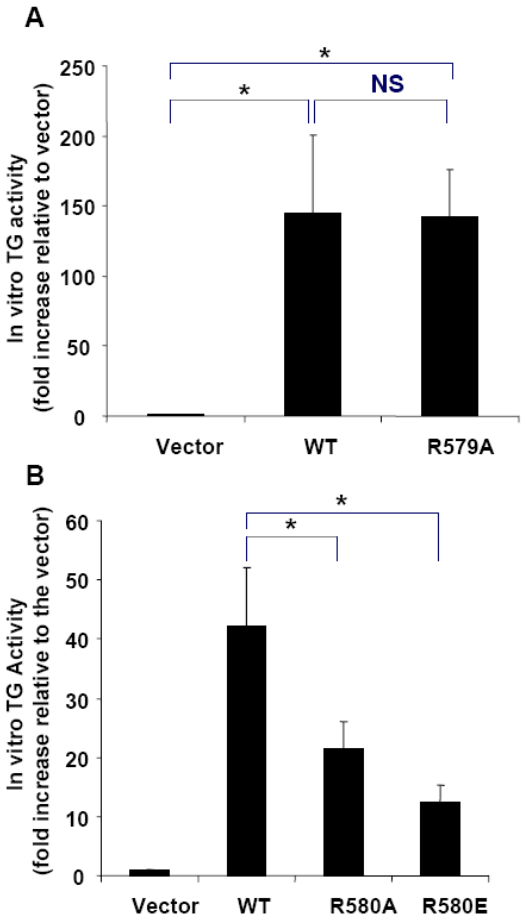

Figure 2. Quantitative analysis of in vitro TG activity in rat (A) or human (B) TG2. HEK293 cells were transiently transfected with the indicated constructs and in vitro TG activity was measured in the presence of 10 mM CaCl2 as described in Materials and Methods. Data were presented as fold increase in TG activity relative to cells transfected with vector only. N=4, *p<0.05. (NS = not significant).

TG2 has approximately 25.6% of GTP binding activity of WT human TG2 (**Figure 1D**). This finding is the first to demonstrate that the C277 residue is important for human TG2 GTP binding. Our data is consistent with the finding by Murthy et al. [28] who described C277A or C277S mutations in rat TG2 to greatly reduce its nucleotide binding. However, our finding is in contrast to previously published data with human TG2 protein [33].

R580A and R579A mutations differently affect in vitro TG activity of human or rat TG2 respectively

To determine if the R580A (or R579A) mutation effects TG activity, HEK293 cell lysates, prepared as above, were used in an *in vitro* TG activity assay which measures the incorporation of [3H]putrescine by TG2 proteins into N,N-dimethylcasein, measured at a calcium concentration that gives maximal *in vitro* TG activity [7]. These data show that Arg579 does not play an essential role in expression of TG activity of rat TG2, as *in vitro* TG activity of the R579A mutant was comparable to that of WT TG2 (**Figure 2A**). However, R580A or R580E mutations in human TG2, significantly decreased *in vitro* TG activity (**Figure 2B**), indicating that Arg580 partially contributes to TG activity in human TG2. Our study is somewhat congruent with previous findings for the R580L mutant of human TG2 that had negligible GTP binding activity and exhibited *in vitro* TG activity that was lower than that of the WT protein [22].

R580A (R579A) shifts the concentration—response curves for calcium activation of in vitro TG activity in rat and human TG2

In order to further elucidate the effect of R579A or R580A on *in vitro* TG activity in human and rat TG2, a more detailed analysis of TG enzymatic activity was carried out. A concentration–response analysis for calcium activation was performed using cell lysates from HEK293 transiently transfected with the TG2 constructs. The results were expressed as a percentage of the corresponding maximal *in vitro* TG activity. Total four independent experiments were performed for each group listed in the graph and EC50 was calculated independently for each individual experiment using SigmaPlot software (**Figure 3A, 3B**). Statistics were done with EC50 from all experiments for corresponding group using paired student t test. A determined calculated potency of calcium for calcium activation of TG activity was different between pairs of rat or human WT and theirs mutants: R579A or R580A respectively, as is illustrated by statistically different values of EC50 for pairs of wild-type TG2 proteins and their corresponding mutants. For rat TG2, WT EC50 was equal 696 ± 35.5 μM (N=4) while for R579A EC50 was 648 ± 39.8 μM (N=4) (**Figure 3A**). For human TG2, WT EC5 was equal to 713 ± 13.7 μM (N=4) compared to R580A EC50 determined as 691 ± 12.1 μM (N=4) (**Figure 3B**).

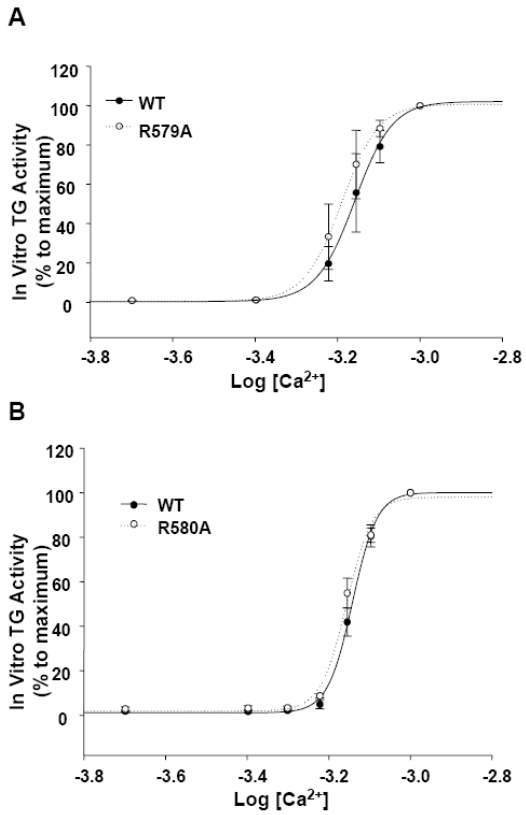

Figure 3. Concentration—response curves for calcium activation of *in vitro* TG activity of rat (A) and human (B) TG2 proteins expressed in HEK293 cells. HEK293 cells were transiently transfected with the indicated constructs and in vitro TG activity was measured in the presence of indicated concentrations of calcium as described in Materials and Methods. The results were expressed as a percentage of the corresponding maximal in vitro TG activity. The calculated potency of calcium for calcium activation of TG activity was different between pairs of rat or human WT and theirs GTP-binding defective mutants, R579A or R580A, as the EC50 values differed significantly between WT and mutant TG2 for both in rat and human. For rat TG2, EC50 values were 696 ± 35.5 μM for WT and 648 ± 39.8 μM for the R579A mutant (A). For human TG2, EC50 values were 713 ± 13.7 μM for WT and 691 ± 12.1 μM for R580A mutant. N=4, data from independent experiments with lysates obtained from separate transfection $p < 0.05$ when paired t-test was performed. Mean ± SEM.

R580A and R579A mutants exhibit significantly greater in situ TG activity than WT TG2 proteins under basal conditions

Since R580A abolishes the GTP binding activity of TG2, which could negatively modulate its TG activity *in situ* [22], it was important for us to examine the effect of this mutation on the *in situ* TG activity under basal conditions, and in response to increased intracellular calcium. An increase in intracellular calcium levels was accomplished by treatment of the transfected HEK293 cells with 2 nM MTX for 0.5, 1 and 2 h. MTX is know to produce an increase in intracellular calcium concentration in number of cell types [7, 34, 35], including HEK293 cells [30]. Further, the previous study done in our lab has demonstrated that the increases in MTX-stimulated intracellular calcium levels correlate with increases in the *in situ* TG [7]. Interestingly, *in situ* TG activity in HEK293 cells overexpressing human R580A or rat R579A TG2 was significantly greater than the activity in cells expressing their WT counterparts (**Figure 4A, 4B**). The difference was still observed between R580A and WT human TG when cells were treated with MTX for 30 min. This wasn't the case for *in situ* TG activities of R579A and rat WT TG (**Figure 4A**). However, in cells subjected to longer MTX treatment the difference in *in situ* TG activity was eliminated for both human and rat TG2 (**Figure 4A, 4B**).

Increases in intracellular calcium levels resulted in similar extent of protein degradation between R580A or R579A and their WT counterpart proteins

Although increases in intracellular calcium levels resulted in TG activation *in situ*, it was not clear whether this also led to any changes in the *in vitro* TG activity. Therefore, the effect of R580A on *in vitro* TG activity of TG2 in response to increased intracellular calcium was examined in HEK293 cells transiently transfected with corresponding TG2 constructs. After MTX treatment for the indicated times, cell lysates were collected and *in vitro* TG activity was measured. Activity from each treatment group was calculated as the percentage to the cells transfected with same construct but subjected to control condition only. In contrast to the increased *in situ* TG activity, MTX treatment resulted in a significant decrease of the *in vitro* TG activity in all transfected HEK293 cells when compared controls without MTX treatment. However, there was no significant difference in the extent of the decrease in the *in vitro* TG

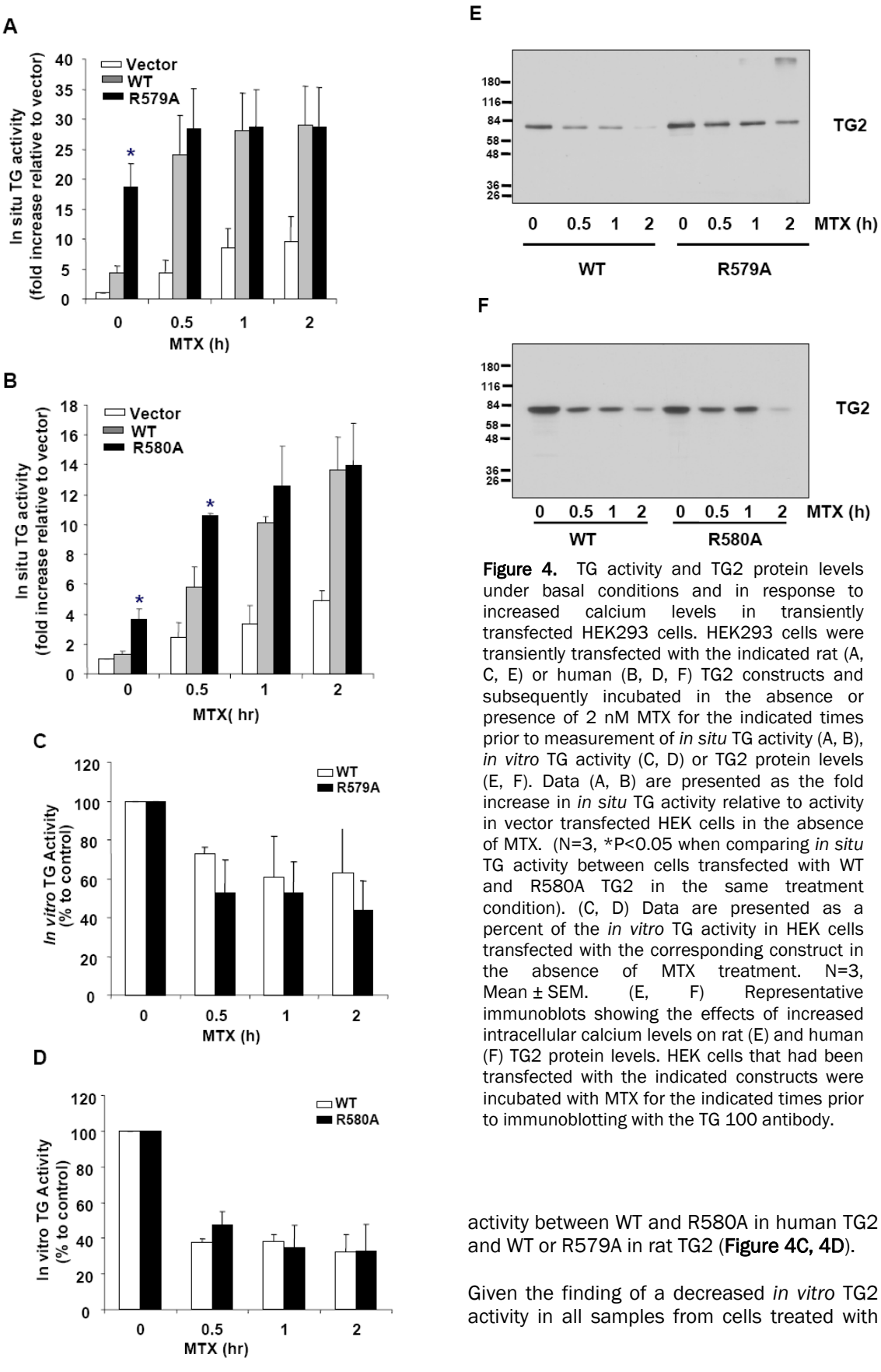

Figure 4. TG activity and TG2 protein levels under basal conditions and in response to increased calcium levels in transiently transfected HEK293 cells. HEK293 cells were transiently transfected with the indicated rat (A, C, E) or human (B, D, F) TG2 constructs and subsequently incubated in the absence or presence of 2 nM MTX for the indicated times prior to measurement of *in situ* TG activity (A, B), *in vitro* TG activity (C, D) or TG2 protein levels (E, F). Data (A, B) are presented as the fold increase in *in situ* TG activity relative to activity in vector transfected HEK cells in the absence of MTX. (N=3, *P<0.05 when comparing *in situ* TG activity between cells transfected with WT and R580A TG2 in the same treatment condition). (C, D) Data are presented as a percent of the *in vitro* TG activity in HEK cells transfected with the corresponding construct in the absence of MTX treatment. N=3, Mean ± SEM. (E, F) Representative immunoblots showing the effects of increased intracellular calcium levels on rat (E) and human (F) TG2 protein levels. HEK cells that had been transfected with the indicated constructs were incubated with MTX for the indicated times prior to immunoblotting with the TG 100 antibody.

activity between WT and R580A in human TG2 and WT or R579A in rat TG2 (**Figure 4C, 4D**).

Given the finding of a decreased *in vitro* TG2 activity in all samples from cells treated with

MTX, we examined next whether TG2 proteins were undergoing proteolysis in cells during MTX treatment. The transiently transfected cells were treated with MTX for indicated times and collected cell lysates were run on SDS-polyacrylamide gels, blotted and probed with the TG 100 antibody to detect any changes in TG2 protein levels. We discovered that MTX treatment caused a degradation of TG2 *in situ*, but there was no significant difference in the extent degradation between WT and R580A human TG2 proteins or WT and R579A rat TG2 proteins (**Figure 4E, 4F**).

Discussion

Our study provides further evidence for the essential role of Arg580 in human TG2 and its equivalent Arg579 in the rat TG2 protein for their GTP binding activities. Even though the essential role of Arg579 [21] and Arg580 [22] in GTP binding has been already demonstrated, it hasn't been clear whether these mutations would affect GTP binding and/or TG activity of TG2 proteins in a similar manner, especially in a cellular context. We demonstrate here that both mutations, in spite of the similar loss of GTP activity by TG2 proteins, influence TG activity of human TG2 (R580A) or rat TG2 (R579A) in a different way, Specifically, the rat R579A mutant showed comparable *in vitro* activity to WT TG2, whereas the R580A (or R580E) mutant in human TG2 exhibited significantly decreased *in vitro* TG activity. On the other hand, *in situ* TG activity of the R579A or R580A mutant was demonstrated to be significantly increased under basal conditions, even though an increase in the *in situ* TG activity for the R579A mutant was much more robust than for the R580A mutant when comparing to their WT counterparts. Also, an important regulatory role of nucleotide binding by TG2 in the process of activation of TG activity by calcium was demonstrated, as both mutations produced a significant increase of TG2 proteins in the potency of calcium for TG activation.

Our study suggests differences between human and rat TG2, and as to how TG activity may be increased in response to increases in intracellular calcium levels or decreases in GTP levels. A thorough understanding of the regulation of TG activity by GTP is important, given the fact that loss of GTP binding activity of TG2 has been postulated to play a major regulatory role in facilitating cell death [22, 36] or in neurological conditions such as Alzheimer's disease [37] or the spinal cord injury [38].

Among other GTP binding proteins, TG2 exhibits rather unique properties, it lacks amino acid residues with either hydroxyl or carboxyl side-chain moieties in the vicinity of the nucleotide phosphate groups [18]. Arg580, which forms two ion pairs with the α- and β-phosphates, was suggested to contribute to the stability the GTP/GDP binding site of TG2 [18] and GTP binding by GTP has been demonstrated to inhibit *in situ* TG activity of human and rat TG2 [3, 19]. Recently, a molecular mechanism of this GTP-driven inhibition has been described in details for rat TG2 [19] and it involves a GTP-mediated conformational transition of a TG2 structure to a compact TG-inactive form. Interestingly, in this model, Arg579 has been demonstrated to play a role of a destabilizing residue that is masked by GTP binding [19]. Furthermore, a similar major conformational rearrangement of TG2 structure has been recently demonstrated as a result of resolving the x-ray crystal structure of human TG2 trapped in a complex with an inhibitor that mimics inflammatory gluten substrates [20].

An underlying mechanism of differences between human and rat TG2 that we demonstrated in our study may be partially explain by considerable variations in amino acid residues between human and rat TG2. For example, Ser482 in human TG2, which has been suggested to contribute to the stability of guanine nucleotide binding by forming a hydrogen bond with the guanine moiety [18], is replaced with Gly residue in rat TG2. Next, the difference in the electrophoretic mobility on SDS-acrylamide gels between rat and human TG2 is also very intriguing. This difference can't be explained only by a slight difference in the amino acid residue length (687 amino acid residues in human TG2 and 686 in rat TG2). It is likely that this electrophoretic mobility difference may be a result of unidentified yet posttranslational modifications of TG2 protein such as previously reported [39]). Another factor that needs to be taken into consideration is that we used cell lysates in our study for *in vitro* TG activity measurements. It is possible that factors other than calcium or guanine nucleotide levels

might affect *in vitro* TG activity, thereby, contributing to the difference in activity between human and rat mutant TG2s. We conclude that differences in protein modifications or modulation of its activities, as well as in the primary amino acid sequences between human and rat TG2, may differentially affect the overall charge and structure of TG2, resulting in differences in the contribution R580A and R579A mutations to TG activity between human and rat TG2.

Our results also indicate that there are likely other intracellular factors, in addition to increases in cellular calcium levels, which may modulate TG activity of TG2. Interestingly, even at conditions of likely the maximal increase in intracellular calcium in response to MTX (approximately 750 nM [40]), which resulted in a significant increase in *in situ* TG activity, we did not observe any activation of *in vitro* TG activity. Clearly, the intracellular regulation of TG2 activity is a complex process, which is not solely dependent on the levels of calcium and guanine nucleotides. In addition, it can not be also excluded that differences between *in situ* and *in vitro* activity may be associated , to some extent, with different substrates used in these assay: BAP for the *in situ* or putrescine for the *in vitro* assay.

Another interesting observation is that increased intracellular calcium levels lead to TG activation *in situ*, despite the fact that it resulted in proteolysis of TG2 and decreases in the *in vitro* TG activity. The decrease in *in vitro* TG activity in response to increased intracellular calcium levels, which is consistent with TG2 proteolysis, also suggests that *in vitro* TG activity might not be the best indicator of the function of TG2 *in situ*. Interestingly, previous findings also have suggested the discrepancy between protein level and *in situ* TG activity of TG2 at certa*in situ*ations. For example, treatment of human neuroblastoma SH-SY5Y cells with retinoic acid resulted in a significant increase in TG2 protein levels and *in vitro* TG activity. At the same time, *in situ* TG activity did not increase concurrently with increased TG2 levels [7]. Therefore, previous conclusions with regard to TG2's involvement in disease conditions needs to be considered with caution as the protein level or *in vitro* TG activity of TG2 might not accurately reflect the role that TG2 plays in these pathological processes.

In our study, R580A mutation did not increase TG2 susceptibility to the calcium-dependent proteolysis *in situ* and *in vitro* (unpublished results, Q. Ruan, J.Tucholski, S. Gundemir, G.V.W. Johnson Voll). This was somewhat unexpected, as previously it has been shown that GTP binding protects TG2 from *in vitro* proteolysis by trypsin [3]. It was also shown that that TG2 is a substrate of calpain, a calcium-activated protease [41], and GTP also inhibits the degradation of TG2 by calpain *in situ* [40]. However, our study showed that the loss of GTP binding ability by R580A mutation did not result in any noticeable change in TG2 susceptibility to calcium-dependent proteolysis. One possible explanation is that R580A mutation may also result in certain conformational changes in addition to its effect on GTP binding ability of TG2. Since it might actually be the conformation changes induced by GTP binding that make TG2 more resistant to proteolysis, R580A TG2 may already be in a "protease resistant" conformation. This assumption is supported by a recent finding showing that R579A mutation in rat TG2 results in increased resistance to μ-calpain (but not to trypsin) compared with WT TG2 [21]. However, it is also possible that in the transiently transfected cell model we used, calpain is not the primary protease responsible for the calcium-mediated degradation of TG2 that we observe. This assumption is supported by our observation that several potent calpain inhibitors were unable to protect TG2 from the calcium-dependent proteolysis (unpublished results, Q. Ruan, J. Tucholski, S. Gundemir, G.V.W. Johnson Voll).

In conclusion, the data presented in this study clearly indicate that both Arg580 in human TG2 and Arg579 in its rat orthologue are essential for their GTP binding activity and that they significantly influence the way their TG activity is modulated by calcium *in situ*. Our findings also indicate that the loss of GTP binding activity does not affect significantly the susceptibility of either human or rat TG2 proteins to calcium-dependent proteolysis *in situ*. Further studies are required to clarify the relationship between the calcium-dependent proteolysis and TG2 role in various physiological and pathological conditions.

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