Mutation of a Critical Arginine in the GTP-binding Site of Transglutaminase 2 Disinhibits Intracellular Cross-linking Activity

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Transglutaminase type 2 (TG2), also known as Glu, is a multifunctional protein involved in diverse cellular processes. It has two well characterized enzyme activities: receptor-stimulated signaling that requires GTP binding and calcium-activated transamidation or cross-linking that is inhibited by GTP. In addition to the GDP-binding residues identified from the human TG2 crystal structure (Liu, S., Cerione, R. A., and Clardy, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2743–2747), we have previously implicated Ser171 in GTP binding. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: TG, transglutaminase; WT, wild type; DTT, dithiothreitol.

including apoptosis, bone ossification, wound repair, cell adhesion, and signal transduction and in the pathophysiology of various diseases, including gluten-induced enteropathy (celiac disease), neurodegenerative disorders, tumor growth, and diabetes (1). It is constitutively expressed by fibroblasts and endothelial and smooth muscle cells as well as a number of organ-specific cell types (2). At the subcellular level, it is found in the cytosol in association with plasma and nuclear membranes and is also found attached to the extracellular matrix (1).

TG2 has at least five distinct biological activities. It is a member of the transglutaminase family of cross-linking enzymes that catalyze post-translational covalent linkages, the best studied of which is transamidation. Transamidation results in either a protein cross-link between a glutamine and lysine residue to form an Ne-(γ-glutamyl) isopeptide bond, incorporation of an amine into a glutamine residue, or acylation of a lysine residue. TG2 also binds and hydrolyzes GTP (3, 4), thereby mediating signaling by various G-protein-coupled receptors, including α1B and α1D-adrenergic (5–7), thromboxane A2 (8, 9), and oxytocin (10) receptors. Additionally, TG2 can act as an adaptor protein that facilitates extracellular interaction between fibronectin and β1/3-integrins (11), as a protein disulfide isomerase (12), and as a kinase for insulin growth factor-binding protein-3 (13), p53 (14), and histones (15).

The functional switch between the transamidation (TG) and GTP binding activities of TG2 is intricately regulated by calcium and GTP. Calcium activates TG activity, and GTP inhibits TG activity at sub saturating calcium concentrations (3, 16). In the presence of physiological intracellular concentrations of calcium and GTP, it has been suggested that TG2 exists largely as a latent transamidase (16–18) but as an active G-protein involved in receptor signaling.

The sensitivity of TG activity to the inhibitory effect of GTP is increased by post-translational modification, e.g. nitrosylation of cysteine residues by nitric oxide-releasing agents (19). In contrast, sphingosylphosphocholine, a relatively minor membrane phospholipid component that is increased in cells undergoing apoptosis, increases the sensitivity of TG2 to calcium (20). In pathological states, when cells become leaky to calcium and GTP generation is impaired, the TG activity of TG2 contributes to programmed cell death events, such as apoptotic body stabilization (17, 21). Disregulation of the activities of TG2, therefore, could potentially have catastrophic cellular consequences due, for example, to unrestrained protein cross-linking and activation of pro-apoptotic pathways.

Of the nine distinct, but closely related, members of the TG family (TG 1–7, Factor XIIIa and Band 4.2), only TG2, 3, (22), and 5 (23) have been shown to bind guanine nucleotides. Recent crystallographic studies have revealed the tertiary structure of the inactive form of human TG2 bound to GDP (24), which, like all other members of the TG family, is comprised of four domains: an N-terminal sandwich domain, a core
domain that contains the TG active site catalytic triad (Cys277, His335, and Asp386) and transition state stabilizing residue (Trp241) (25), and two β-barrel domains (Fig. 1A). The GDP-binding site of TG2 is located in a hydrophobic pocket between the core and β-barrel 1, on the opposite face to the proposed glutamyl substrate-binding site (24). It has a unique architecture that, although conserved in TG3 (22), is entirely distinct from that of other GTP-binding proteins and involves mainly residues from β-barrel 1 (amino acids 476–482 and 580–583) and Phe174 from the core. This is in agreement with our previous work, which localized GTP binding to residues 159–173 of the core and residues 465–589 of β-barrel 1 and additionally showed impaired GTP binding and hydrolysis by site-directed mutants of Lys126 (26). Although not contained within the GDP binding pocket delineated by the crystal structure, our previous studies also implicated Ser171 in GTP binding, because such binding was lost with the substitution of this residue by a glutamate, the homologous residue in a non-GTP binding member of the mammalian TG family, xIII (26).

The present study shows that several key residues of TG2 implicated in GDP binding in the crystal structure are indeed important for guanine nucleotide binding, because mutation reduces or abolishes GTP binding as well as GTPγS inhibition of TG activity in vitro. Ser171 does not interact directly with GTP, because nucleotide binding is unaltered with alanine substitution. Among the GTP binding residues, we have identified a particularly important residue, Arg179 in rat TG2 (equivalent to Arg180 in human TG2). Mutation of this residue to alanine not only renders it resistant to cleavage by the intracellular calcium-activated protease, μ-calpain, but also abolishes GTP regulation of TG activity in intact cells, resulting in dysregulated intracellular TG activity. This study, therefore, has provided the first physiological confirmation that the intracellular TG activity of TG2 is regulated by GTP.

**EXPERIMENTAL PROCEDURES**

**Constructs, Cell Culture, and Transfection of Cells**—Site-directed mutants were constructed in rat TG2 cDNA as described (27) using glutathione S-transferase-TG2/pGEX2T (28) as the template. Wild-type (WT) and mutant rat TG2 cDNAs were subcloned into the EcoRI-NotI sites of pcDNA3.1 and stably transfected into human neuroblastoma SH-SY5Y cells as described (29). Vector (pcDNA3.1) DNA was transfected as a control. After transfection, cells were maintained in RPMI medium containing 10% dialysed fetal bovine serum, 2 mM l-glutamine, and 100 µg/ml G-418.

**Purification of Recombinant TG2 Proteins**—WT and mutant rat proteins were expressed as thrombin-cleavable glutathione S-transferase fusion proteins (28). Protein expression was induced overnight (30 °C, 100 mM NaCl, 0.5 mM EDTA, 2 mM MgCl2, 2 mM DTT) for 16 h at room temperature, followed by exhaustive dialysis at 4 °C against isothermal titration calorimetry (ITC) buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM MgCl2, 0.5 mM DTT). GTPγS (Roche Applied Science) was dissolved in ITC dialysis buffer, and the concentration was checked by absorbance at 256 nm (ε = 1.241 × 104 M−1 cm−1). ITC experiments were carried out at 25 °C on a Microcal VP-ITC. Solutions were de-gassed for 5 min using a vacuum degasser immediately prior to experiments. GTPγS was injected into TG2 samples at the following concentrations: 100 µM GTPγS into 13.5 µM WT TG2, 90 µM GTPγS into 22.3 µM F174A TG2, 500 µM GTPγS into 17.9 µM R579A TG2. The injection sequence consisted of an initial injection of 1 µl followed by 20–30 sequential injections of 7–8 µl. Blank runs were also performed in which TG2 was omitted. Base-line-subtracted data were fitted by a single-site model using Origin ITC Analysis software (Microcal Software, Northampton, MA). Data for R579A TG2 were weaker and fitted with fixed stoichiometries (n = 0.3–1), and an average estimated Kd from these fits is presented.

**GTP Photolabeling**—Recombinant WT or mutant TG2 (1.3 µM) was incubated with 67 nM [α-32P]GTP (30 °C, 10 min, final volume 20 µl) in 20 mM Na-HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, 0.5 mM 5′-adenyl-β,γ-imidodiphosphate, 5% glycerol, photolabeled by UV irradiation (254 nm, 2 min, ice water bath), incubated with SDS sample buffer (100 °C, 5 min), and analyzed by SDS-PAGE. Gels were visualized by Coomassie Blue staining followed by autoradiography.

**Immunoblotting**—TG2 expression level was evaluated by Western blotting, using either anti-TG2 monoclonal antibody CUB 7402 (Neomarkers; 1:500 dilution in Tris-buffered saline/5% skim milk for 1 h at room temperature) or anti-human TG2 polyclonal antibody (generated in-house).

**Transamidase Assays**—Purified proteins (10 nM WT or mutant TG2) or stable transfectant lysates (15 µg) were assayed for TG activity (26). TG activity in intact cells was determined via incorporation of biotinylated amine (5-biotinamidopentylamine) into intracellular proteins. Stably transfected SH-SY5Y cells were cultured for 72 h on 10-cm plates in RPMI medium containing 3% fetal bovine serum, 2 mM l-glutamine, and 100 µg/ml G-418. Plates were washed once with serum-free RPMI, preincubated for 2 h at 37 °C/5% in 3 ml of RPMI containing 1% serum, glutamine, G-418, 2 mM 5-biotinamidopentylamine (prepared in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and stimulated for 90 min with 0, 0.1, or 0.25 mM carbocarbo or for 60 min with 0, 0.1, or 0.25 mM A23187. Cells were harvested and sonicated. Crude cell lysates (5 µg) were quantitated for TG activity by enzyme-linked immunosorbent assay (18) and TG expression by immunoblotting.

**Measurement of Intracellular Calcium**—Cell monolayers were washed once with Krebs/HEPES buffer (KHB, 118 mM NaCl, 4.7 mM KCl, 5 mM NaHCO3, 1.2 mM MgSO4, 1.3 mM CaCl2, 10 mM glucose, 10 mM HEPES, pH 7.4), harvested in 25 ml KHB, and centrifuged (3 min, 180 × g). Cells were resuspended in 5 ml of KHB with 1 mM FURA 2-AM, incubated at 37 °C for 30 min, centrifuged (3 min, 180 × g), washed twice in 5 ml of KHB, and resuspended to 1 × 106 cells/ml in KHB. A 2-ml aliquot of cells was placed in a cuvette, and carbachol was...
added to 0.25 mM. Fura 2 fluorescence was monitored at 505 nm after excitation at 340 nm. Calibration of Ca²⁺/H₁₁₀₀₁ was performed after cell lysis (0.04% (v/v) Triton X-100) by determining minimal and maximal fluorescence in the presence of EGTA (10 mM) or CaCl₂ (7.6 mM), respectively. Base-line, peak, and plateau values for [Ca²⁺/H₁₁₀₀₁] were calculated as described (31).

Data Analysis—Results shown are the means ± S.E. Statistical differences were determined by analysis of variance followed by Bonferroni’s post-test, with p ≤ 0.05 being considered significant.

RESULTS

Mutation of Proposed GTP-binding Site Residues Affects GTP Binding—We mutated some of the key GDP-binding site residues (Phe₁⁷⁴, Arg₄₇₆, Arg₅₇₉, and Arg₅₈₀) as well as Ser⁻¹¹ in rat TG2 (Fig. 1A) and tested their ability to bind GTP. Phe¹⁷⁴ in the core domain forms part of a hydrophobic pocket around the guanine base, where it is thought to stabilize one side of the guanine ring through an aromatic stacking interaction (24). In agreement with this interpretation, removal of the phenylalanine side chain by substitution with alanine abolished [-³²P]GTP photolabeling, a qualitative measure of GTP binding, whereas tryptophan substitution retained wild-type binding (Fig. 1B). In β-barrel 1, Arg⁵₈₀, one of four positively charged residues surrounding the phosphate groups of GDP in the crystal structure, forms two ion pairs with the α- and β-phosphates of GDP, as well as interacting with the guanine base (24). Mutation of Arg⁵₇⁹ of rat TG2 (equivalent to human Arg⁵₈₀) to alanine abolished [α-³²P]GTP photolabeling, as did a more conservative mutation to lysine (the homologous residue in the GTP binding TG member, TG5 (23)), which retains the charge and much of the bulk of

FIGURE 1. Mutation of proposed GTP-binding site residues affects GTP binding. A, left, space-filling representation of human TG2 bound to GDP (24) (Protein Data Bank code 1KV3) showing the four domains, N-terminal β-sandwich (blue), core (magenta), β-barrel 1 (dark green), and β-barrel 2 (light green). GDP is shown in red. Right, the GDP-binding site of human TG2. Core domain backbone, magenta; β-barrel 1 domain, green; carbon atoms of mutated residues, gray, and those of GDP, light blue. Functional groups include oxygen, red; amino groups, deep blue; phosphorous, magenta. Hydrogen bonds and ion pair interactions are shown as dashed lines. Arg⁵₈₀ in human TG2 is equivalent to Arg⁵₇₉ in rat TG2. B, photoaffinity labeling of WT or mutant TG2 with GTP was carried out as described under “Experimental Procedures.” Gels were subjected to autoradiography to discern radiolabeling (top panel) and to Coomassie Blue staining to visualize the proteins directly (bottom panel). C, isothermal titration calorimetry of GTP·S binding to TG2. GTP·S was titrated into solutions of WT, F174A, and R579A as described under “Experimental Procedures.” Raw data from injections is shown in the upper panels. Peaks were integrated to give the heat change associated with each injection, and buffer control was subtracted to give the data plotted in the lower panels. The stoichiometry (N) for WT and F174A TG2 was 0.3–0.35. The R579A data could not be fitted while floating the N value, but values from 0.3–1 gave similar Kᵣ values (140–160 μM).
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the arginine side chain. This confirms the importance of the arginine residue for nucleotide binding. In contrast, substitution of alanine for Arg478, which interacts with the β-phosphate, reduced but did not abolish [α-32P]GTP photolabeling. Similarly, alanine substitution of Arg476 (Fig. 1B) or leucine substitution of Lys173 (26), residues postulated to stabilize the γ-phosphate of GTP during binding and hydrolysis (24), caused only a minor reduction in GTP photolabeling. The double mutant R476A/R478A photolabeled similarly to R478A alone. Although glutamate substitution of Ser171 prevents GTP binding (26), photolabeling, indicating Ser171 does not interact directly with GTP. Taken together, these results indicate that residues interacting only with phosphate groups contribute less to guanine-nucleotide binding than residues interacting with the guanine base.

Isothermal titration calorimetry was used to quantitate the GTP binding affinities of the F174A and R579A mutants relative to that of WT TG2, as both of the mutants showed significant loss of GTP binding by photolabeling. Although it has been shown that TG2 binds GTP with a 1:1 stoichiometry (32), the best fit to our initial calorimetry data gave a stoichiometry value of around 0.1 for GTP binding to both WT and F174A, indicating that only ~10% of the protein was available to bind nucleotide. Native gel electrophoresis showed that almost all of the samples migrated in the faster GTP-bound form, as previously demonstrated (27), indicating that the proteins were competent to bind GTP and that most of the protein was already nucleotide bound. Attempts to strip nucleotide from the proteins by dialysis against 2.5 M KCl (33) resulted in the appearance of high molecular weight bands on native gels indicating aggregation, and less than 5% of the protein was available to bind GTP. As we have shown previously that TG2 binds less GTP in the presence of 5 mM MgCl2 (26), samples were dialysed against a buffer containing 5 mM MgCl2 prior to exchange into ITC buffer (as described under "Experimental Procedures"), resulting in ~30% of the protein being available to bind GTP, and better quality data were obtained. WT TG2 bound GTPyS with a Kd of 1.6 μM, whereas the affinities of F174A (Kd 2.9 μM) and R579A (Kd 150 μM) for GTPyS were around 2- and 100-fold weaker, respectively (Fig. 1C). These dissociation constants were very similar to those obtained initially without magnesium pretreatment.

In Vitro Inhibition of TG Activity by GTP Correlates with GTP Binding Affinity—All mutants exhibited dose-dependent Ca2+-stimulated TG activity comparable with WT (Fig. 2A and Table 1), indicating that the proteins were correctly folded and that their TG activity was not compromised by the mutation(s). GTP inhibition of TG2 activity is inversely proportional to Ca2+ activation and is greatest under conditions where TG activity is minimal (26). GTPyS inhibition of TG activity was evaluated in the presence of 75–125 μM free Ca2+ (which stimulates ~40% of maximal activity; Fig. 2, B and C). Under these conditions, GTPyS inhibited the TG activity of WT TG2 with an IC50 of 27 μM. The TG activity of R476A and R478A was less sensitive to GTPyS inhibition than WT (Fig. 2B), yielding IC50 values >350 μM (R476A) and 450 μM (R478A). The TG activity of R476A/R478A was not measurably inhibited by GTPyS (Fig. 2B; IC50 >500 μM), indicating that mutation of both arginines further reduced GTP binding. The TG activities of the S171E (26), F174A, and R579A mutants were insensitive to GTPyS inhibition (Fig. 2C). Thus, loss of GTP inhibition of TG activity correlated with the degree of [α-32P]GTP photolabeling of the mutants (Fig. 1B).

Resistance of GTP Binding Mutants to Trypsin and Calpain Cleavage—As demonstrated previously (3) and as shown in Fig. 3A, resistance of WT TG2 to proteolytic digestion by trypsin is increased with increasing concentrations of GTPyS. Consistent with little or no GTP binding, the sensitivity of the S171E and R579A mutants to trypsin digestion was unaltered even at 1 mM GTPyS (Fig. 3A), whereas F174A, which has

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2-fold lower affinity for GTP than WT (Fig. 1C), was resistant to digestion at GTPγS concentrations greater than 50 μM.

Similarly, GTPγS prevented cleavage of WT TG2 by μ-calpain (34) (Fig. 3B). Again consistent with reduced GTP binding, the increased resistance to μ-calpain digestion in the presence of GTPγS was moderate for F174A and negligible for S171E and R579A (Fig. 3B). Interestingly, and in contrast to trypsin digestion, R579A demonstrated some inherent resistance to μ-calpain digestion. In the absence of GTPγS, R579A was more resistant to calpain digestion than WT, and in the presence of GTPγS R579A was as resistant as F174A. This is likely due to the alanine substitution of Arg579 resulting in a local conformational change that renders the resulting protein more resistant to calpain, but not trypsin, cleavage.

R579A TG2 Has Dysregulated TG Activity in Intact Cells—To determine the effect of the R579A mutation on intracellular TG activity, SH-SY5Y human neuroblastoma cells were stably transfected with either pcDNA3.1 (vector control) or pcDNA3.1 containing WT or R579A TG2 cDNA. Two independent clonal cell lines of each type were analyzed. Lysates from clones transfected with WT or R579A TG2 were incubated with trypsin (0.3 μg) and incubated at 37 °C for 1 h, then samples were transferred to nitrocellulose and detected by Western blot using anti-TG2 polyclonal antibody. Arrows indicate full-length protein.

FIGURE 3. Resistance of WT and mutant TG2 to cleavage by trypsin or calpain. A, WT or mutant TG2 (3 μg) was incubated with trypsin (0.3 μg) for 1 h at 37 °C in a reaction buffer comprising 100 mM Tris-HCl, pH 8.5, 0.2 mM EDTA, and 0–1000 μM GTPγS (final reaction volume 10 μl). Proteolysis was stopped by placing samples on ice, SDS-PAGE sample buffer was added, and samples were boiled for 5 min prior to analysis by SDS-PAGE on 10% acrylamide gels. B, WT or mutant TG2 (0.3 μg) was incubated with μ-calpain (1.6 μg) at 37 °C in a reaction buffer of 40 mM HEPES, pH 7.5, 2 mM DTT without (upper panels) or with (lower panels) addition of 100 μM MgCl₂ and 100 μM GTPγS (final reaction volume 200 μl). Proteolysis was started by addition of CaCl₂ to 100 μM. Aliquots (20 μl) were removed at indicated times and placed on ice, SDS-PAGE sample buffer was added, and samples were boiled for 5 min prior to analysis by SDS-PAGE on 10% acrylamide gels. Proteins were transferred to nitrocellulose and detected by Western blot using anti-TG2 polyclonal antibody. Arrows indicate full-length protein.

DISCUSSION

Alanine substitution of individual β-barrel 1 domain residues Arg⁴⁷⁶, Arg⁴⁷⁹, and Arg⁵⁷⁹ and the core domain loop residue Phe¹⁷⁴ impaired GTP binding by TG2 (Figs. 1–3). Residues binding the guanine ring (Arg⁵⁷⁹ and Phe¹⁷⁴) are individually more important for GTP binding than vector-transfected clones because of the increased amount of TG2 protein in these cells, neither vector- nor WT-transfected clones showed significantly increased TG activity in response to low dose carbachol stimulation (Fig. 4C). R579A-transfected clones, on the other hand, displayed slightly increased basal TG activity compared with WT-transfected clones, as well as significantly increased TG activity upon low dose carbachol stimulation (Fig. 4C).

Western blot analysis indicated an absence of TG2 protein degradation in WT- or R579A-transfected cells during the course of carbachol stimulation (Fig. 4D). Intracellular calcium concentrations were assessed using Fura-2 and found not to be statistically significantly different at base line or at the plateau after peak stimulation, although WT-transfected clones generally showed a higher calcium spike than R579A-transfected clones (Fig. 4E). Chronic treatment with low doses of calcium ionophore A23187 (36), which transports calcium across the cell membrane, activated the TG activity of both WT- and R579A-transfected clones (Fig. 4F). Taken together, these results demonstrate that the loss of GTP binding by R579A TG2 renders the TG activity of R579A-transfected clones more sensitive than that of WT-transfected clones to small increases in intracellular calcium such as those induced by low dose carbachol treatment. This loss of GTP inhibition of R579A TG activity thus results in dysregulated TG activity in vivo.

Stably transfected clones were stimulated with the muscarinic cholinergic receptor agonist carbachol (18), which acts on endogenous M3 receptors to stimulate calcium release from the endoplasmic reticulum via phospholipase C-mediated production of inositol 1,4,5-trisphosphate. Although both WT- and R579A-transfected clones had higher basal TG activity than vector-transfected clones because of the increased amount of TG2 protein in these cells, neither vector- nor WT-transfected clones showed significantly increased TG activity in response to low dose carbachol stimulation (Fig. 4C). R579A-transfected clones, on the other hand, displayed slightly increased basal TG activity compared with WT-transfected clones, as well as significantly increased TG activity upon low dose carbachol stimulation (Fig. 4C).
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FIGURE 4. *In vivo* TG activity of SH-SY5Y cells stably transfected with R579A TG2 is dysregulated. A and B, lysates from two independent SH-SY5Y clonal cell lines, each stably transfected with either pcDNA3.1 (vector control) or pcDNA3.1 plus WT or R579A rat TG2 cDNA, were centrifuged (10 min, 10,000 × g, 4 °C). A, supernatants (2.5 μg) were transferred to a Western blot with purified recombinant human (h), rat (r), and goat (g) secondary antibodies. The blot was developed using anti-TG2 monoclonal antibody CUB 7402. B, supernatants (15 μg) were assayed for TG activity, calculated as nanomoles of putrescine incorporated/mg of protein/h. Data for vector clones 3 and 8, WT clones 2 and 41, and R579A clones 3 and 6 are means ± S.E. of five, four, one, three, or four experiments, respectively, performed in triplicate. C, stably transfected SH-SY5Y cells were treated for 90 min with 0.05, 0.1, 0.25 mM carbachol, and TG activity of intact cells was quantitated by enzyme-linked immunosorbent assay as described under "Experimental Procedures" using 1 μg of crude cell lysates. Data are means ± S.E. of one experiment performed in triplicate and are representative of three, nine, nine, five, six, and nine experiments performed in triplicate using vector clones 3 and 8, WT clones 2 and 41, and R579A clones 3 and 6, respectively. Statistically significant differences, *p < 0.05; **p < 0.01; ***p < 0.001, are indicated by asterisks above the bars (vector versus WT or R579A) or brackets (ns, not significant). D, crude cell lysates (5 μg) from panel C were transferred to the same Western blot, and the blot was developed with an anti-TG2 polyclonal antibody. Immunoblot shown are from the same autoradiograph and have been separated to allow alignment with activity measurements (vector versus WT or R579A) or (vector versus vector 8) or (vector versus WT or vector 8) or (vector versus R579A). Consistent with ITC results for F174A, which showed ~2-fold weaker affinity for GTP*Y*S than WT (Fig. 1C), F174A exhibited some resistance to protease cleavage in the presence of GTP*Y*S (Fig. 3). However, F174A did not photolabel (Fig. 1B), and its TG activity was insensitive to GTP*Y*S inhibition (Fig. 2C). This apparent discrepancy is most likely because of the different assay conditions and may be explained by the documented inhibitory effect of Mg*2+* on GTP binding in the standard photolabeling assay and the inverse relationship between Ca*2+* activation and GTP inhibition of TG activity (26).

Ser*171* in the core domain does not appear to interact directly with GTP, as S171A was unaffected in GTP binding (Fig. 1B). The dramatic loss of GTP binding by S171E (26) (Fig. 2C) is thus likely due to an indirect conformational effect on the positioning of Lys*273* and Phe*274*, which are C-terminal to Ser*171* on the same β-strand.

Unlike the rat isoform, human S171E is poorly expressed and/or lacks TG activity (37, 38), which has led to the hypothesis that TG activity is stabilized by, and critically dependent on, GTP binding (38). This is clearly not the case for rat TG2, as indicated by both our *in vitro* and intact cell (Fig. 4F) findings, which demonstrate TG activity comparable with that of the WT protein not only for S171E but for all GTP binding-deficient mutants examined. This is also not the general case for human TG2, as the R580A mutant (equivalent of rat R579A) has robust TG activity in intact cells. This thus, the reduced TG activity expressed of human S171E may be peculiar to this particular mutant but in any case is unrelated to loss of GTP binding.

3 G. Johnson, personal communication.
Although both S171E and R579A bind little or no GTP (26) (Fig. 1C), R579A is inherently more resistant to digestion by its endogenous protease, calpain (Fig. 3). Proteases generally cleave their substrates in sterically accessible and flexible regions; however, this is particularly important for calpain, which recognizes secondary structural elements in addition to sequence determinants and cleaves in disordered regions of proteins (39). The relative resistance to proteolytic cleavage of the R579A mutant is likely due to an alteration in its accessibility and/or flexibility in the region of the calpain cleavage site. As a result, we cannot definitely exclude the possibility that failure of Arg<sup>579</sup>-substituted mutants to bind GTP is due to the substitution causing a conformational change that indirectly inhibits GTP binding rather than to direct involvement of Arg<sup>579</sup> in nucleotide binding. However, this is unlikely, given that the crystallographic data indicate coordination of Arg<sup>579</sup> with multiple guanine-nucleotide moieties. Our findings are thus consistent with the geometry of the arginine side chain and its bidentate charge distribution being absolutely critical in establishing the multiple inter- 

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REFERENCES

1. Lorand, L., and Graham, R. M. (2003) Nat. Rev. Mol. Cell. Biol. 4, 140–157
2. Thomaz, V., and Félix, L. (1989) Cell Tissue Res. 255, 215–224
3. Achuthan, K. E., and Greenberg, C. S. (1987) J. Biol. Chem. 262, 1901–1906
4. Im, M.-J., Riek, R. P., and Graham, R. M. (1990) J. Biol. Chem. 265, 18925–18960
5. Nakaoka, H., Perez, D. M., Bak, K. J., Das, T., Hussain, A., Misono, K., Im, M.-J., and Graham, R. M. (1994) Science 265, 1593–1596
6. Chen, S. H., Lin, F., Ismaa, S., Lee, K. N., Birckbichler, P. J., and Graham, R. M. (1996) J. Biol. Chem. 271, 22858–22861
7. Dupuis, M., Levy, A., and Mhouty-Kodja, S. (2004) J. Biol. Chem. 279, 19257–19263
8. Veza, R., Habib, A., and FitzGerald, G. A. (1999) J. Biol. Chem. 274, 12774–12779
9. Zhang, Z., Veza, R., Plappert, T., McNamara, P., Lawson, J. A., Austin, S., Pratico, D., Sutton, M. S.-J., and FitzGerald, G. A. (2003) Circ. Res. 92, 1153–1161
10. Park, E. S., Won, J. H., Han, K. J., Suh, P. G., Ryu, S. H., Lee, H. S., Yoon, H. Y., Kwon, N. S., and Baek, K. J. (1998) Biochem. J. 331, 283–289
11. Tsukrov, S. S., and Belkin, A. M. (2001) J. Cell. Sci. 114, 2989–3000
12. Hasegawa, G., Suwa, M., Ichikawa, Y., Obutsuka, T., Kugami, S., Kikuchi, M., Sato, Y., and Saito, Y. (2003) Biochem. J. 373, 793–803
13. Mishra, S., and Murphy, L. J. (2004) J. Biol. Chem. 279, 23863–23868
14. Mishra, S., and Murphy, L. J. (2006) Biochem. Biophys. Res. Commun. 339, 726–730
15. Mishra, S., Saleh, A., Espino, P. S., Dave, J. R., and Murphy, L. J. (2006) J. Biol. Chem.
16. Bergamini, C. M. (1988) FEBS Lett. 239, 255–258
17. Smethurst, P. A., and Griffin, M. (1996) Biochem. J. 313, 803–808
18. Zhang, J., Lesort, M., Guttman, R. P., and Johnson, G. V. W. (1998) J. Biol. Chem. 273, 2286–2295
19. Lai, T. S., Hausladen, A., Slaughter, T. F., Eu, J. P., Stamler, J. S., and Greenberg, C. S. (2001) Biochemistry 40, 4904–4910
20. Lai, T. S., Bialecka, A., Peoples, K. A., Hannum, Y., and Greenberg, C. S. (1997) J. Biol. Chem. 272, 16295–16300
21. Nanda, N., Ismaa, S. E., Owens, W. A., Hussain, A., Mackay, F., and Graham R. M. 2001) J. Biol. Chem. 276, 20673–20678
22. Álvarez, B., Boeshans, K. M., Idler, W., Baxa, U., Steinert, P. M., and Rastinejad, F. (2004) J. Biol. Chem. 279, 7180–7192
23. Candi, E., Paradisi, A., Terrironi, A., Pietroni, V., Oddi, S., Cadot, B., Jogi, V., Meiappan, M., Claridy, J., Finazzi-Agro, A., and Melino, G. (2004) Biochem. J. 381, 313–319
24. Liu, S., Cerione, R. A., and Clarély, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7347–7352
25. Ismaa, S., Holman, S., Wouters, M. A., Lorand, L., Graham, R. M., and Husain, A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12636–12641
26. Ismaa, S. E., Wu, M.-J., Nanda, N., Church, W. B., and Graham, R. M. (2000) J. Biol. Chem. 275, 18259–18265
27. Murthy, S. N., Ismaa, S. E., Beeg, G., Freymann, D. M., Graham, R. M., and Lorand, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2738–2742
28. Ismaa, S. E., Chung, L., Wu, M.-J., Teller, D. C., Yee, V. C., and Graham, R. M. (1997) Biochemistry 36, 11655–11664
29. Zhang, J., Tucholski, J., Lesort, M., Jope, R. S., and Johnson, G. W. V. (1999) Biochem. J. 343, 541–549
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
31. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
32. Bergamini, C. M., and Sogniorini, M. (1993) Biochem. J. 291, 37–39
33. Murthy, S. N., Lomanay, J. W., Lik, E. C., and Lorand, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11815–11819
34. Zhang, J., Guttman, R. P., and Johnson, G. W. V. (1998) J. Neurochem. 71, 240–247
35. Baek, K. J., Das, T., Gray, C., Antar, S., Murugesan, G., and Im, M.-J. (1993) J. Biol. Chem. 268, 27390–27397
36. Hartigan, J. A., and Johnson, G. W. V. (1999) J. Biol. Chem. 274, 21395–21401
37. Antonys, M. A., Singh, U. S., Lee, D. A., Boehm, I. E., Combs, C., Zgola, M. M., Page, R. L., and Cerione, R. A. (2001) J. Biol. Chem. 276, 33582–33587
38. Jeon, J.-H., Cho, S.-Y., Kim, C.-W., Shin, D.-M., Kweon, J.-C., Choi, K.-H., Park, S.-C., and Kim, I.-G. (2002) Biochem. Biophys. Res. Commun. 294, 818–822
39. Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilágyi, A., Bánoczi, Z., Hudecz, F., and Friedrich, P. (2004) J. Biol. Chem. 279, 20775–20785