

Importance of Ca^{2+} -Dependent Transamidation Activity in the Protection Afforded by Tissue Transglutaminase against Doxorubicin-Induced Apoptosis[†]

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ABSTRACT: Tissue transglutaminase II (TGase-II), which is capable of both GTP binding and transamidation activities, has been implicated in a variety of biological disorders ranging from cancer to neurodegenerative diseases. Recent studies have suggested that the transamidation activity of TGase-II is necessary for the survival of cancer cells confronted with different stresses and cellular insults. When assayed in vitro, the transamidation activity of TGase-II is Ca^{2+} -dependent. However, at present, little is known with regard to how the regulation by Ca^{2+} is manifested or if in fact it is important for the cellular functions of TGase-II. Here, we have set out to further examine the Ca^{2+} -mediated regulation of TGase-II's transamidation activity, with our goals being to identify the Ca^{2+} -regulatory sites on the protein and determine whether they are essential for TGase-II to confer survival to human breast cancer cells. On the basis of comparisons between the X-ray crystal structures of TGase-II and TGase-III, we identified three putative Ca^{2+} -regulatory sites on TGase-II. Site-directed mutagenesis was performed to individually alter key residues at each of the sites. These substitutions did not affect the ability of TGase-II to bind guanine nucleotides, nor did they cause any obvious changes in its cellular localization. While substitutions at the different Ca^{2+} -regulatory sites could either slightly enhance or markedly reduce the GTP hydrolytic activity of TGase-II, mutations at each of the three sites inhibited the Ca^{2+} -responsive transamidation activity. We further showed that the same substitutions inhibited the ability of TGase-II to protect human breast cancer cells against the apoptotic activity of doxorubicin. Overall, these findings demonstrate that the Ca^{2+} -mediated regulation of transamidation activity is essential for the ability of TGase-II to confer cell survival.

Tissue transglutaminase II (TGase-II)¹ is a member of a family of acyl transferases that catalyze the Ca^{2+} -dependent formation of an amide bond between γ -carboxamide groups of peptide-bound glutamine residues and either the ϵ -amino groups of lysine residues in proteins or the amino groups from polyamines (i.e., transamidation) (1–3). The ability of TGase-II to exhibit transamidation activity could explain its role in a number of physiological processes and disease states, including cell adhesion and migration, bone formation, apoptosis, cell survival, neurodegenerative disorders such as Alzheimer's and Huntington's diseases, Celiac's disease, and cancer (4–16). However, the ability of TGase-II to bind guanine nucleotides has also received a good deal of attention because of its potential implications for cellular signaling events. TGase-II was the first member of the transglutaminase family shown to bind and hydrolyze GTP and thus to be capable of acting as a molecular switch, along the lines

of the well-known signaling GTP-binding proteins (G proteins) (17–19). Indeed, TGase-II was identified as the G protein (originally called Gh) responsible for linking $\alpha 1$ -adrenergic receptors to the stimulation of phospholipase C δ in hepatocytes (20, 21). More recently, the GTP-binding activity of TGase-II has been suggested to play an important role in its ability to confer a survival advantage to cells treated with apoptotic agents (14).

The X-ray crystal structures of TGase-II and the highly related TGase-III have provided important insights into how these proteins are able to bind guanine nucleotides (22, 23). However, significantly less is known about the detailed catalytic mechanism underlying the acyl transferase (transamidation) activity, or the nature of the reciprocal regulation that has been observed between the binding of guanine nucleotides to TGase-II and its ability to catalyze the transamidation of substrates. The binding of guanine nucleotides to TGase-II negatively regulates its transamidation activity, although there have been conflicting suggestions about whether GTP and GDP are equipotent regulators of the enzymatic activity (18, 24, 25). An additional and potentially important regulatory feature of transamidation activity involves the interaction of Ca^{2+} with TGase-II. Millimolar levels of Ca^{2+} have been reported to be necessary for assaying the transamidation activity of TGase-II in vitro (2). Moreover, the binding of Ca^{2+} by TGase-II appears to

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¹ Abbreviations: BOD-GTP γ S, BODIPY-FL-modified form of GTP γ S; DTT, dithiothreitol; G protein, GTP-binding protein; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PARP, poly(ADP-ribose)polymerase; TGase-II, transglutaminase II.

weaken its affinity for guanine nucleotides (18), raising the possibility that the negative regulation by Ca^{2+} of guanine nucleotide-binding activity might help contribute to its ability to stimulate transamidation. Still, these requirements for Ca^{2+} are somewhat puzzling because some of the cellular functions of TGase-II likely occur at intracellular sites (e.g., the cytosol and nucleus). Thus far, a direct examination of the importance of TGase-II– Ca^{2+} interactions to the cellular function of this GTP-binding protein/acyl transferase has not been performed.

In this study, we have set out to take a closer look at the regulation exerted by Ca^{2+} on transamidation activity and to assess its importance for the ability of TGase-II to confer cell survival. Three Ca^{2+} -binding sites were identified on the basis of the X-ray crystal structure of TGase-III (23). These sites are conserved in the related TGase-II molecule, which then afforded us the opportunity to make changes at these sites and examine their impact on different aspects of TGase-II function, including its transamidation activity. We found that introducing mutations at each of the Ca^{2+} -binding sites had little effect on the guanine nucleotide-binding activity, nor did they cause obvious changes in the cellular localization of TGase-II. While the mutations exhibited differential effects on the ability of TGase-II to hydrolyze GTP (i.e., two of the mutations slightly stimulated the activity, and the other significantly inhibited it), all three clearly compromised its transamidation activity. We went on to show that inhibition of the Ca^{2+} -mediated regulation of transamidation activity affected the ability of TGase-II to confer survival to human breast cancer cells challenged with a chemotherapeutic agent.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. All TGase-II mutants were generated by using a PCR-based mutagenesis method (Quick-change mutation kit, Stratagene). For each mutant, a pair of oligonucleotide primers containing the desired mutations was used for the PCR. The parental strand was removed by DpnI digestion before transformation into *Escherichia coli* strain DH5 α . Mutations were verified by sequence analysis at the Bio-Resource Center of Cornell University.

Expression and Purification of Recombinant Wild-Type and Mutated TGase-II Proteins. The cDNAs encoding human wild-type TGase-II and the different TGase-II mutants were subcloned into the pET-28a vector (Novagen) to express these recombinant proteins with an N-terminal six-histidine (His_6) tag. Overnight cultures from colonies of *E. coli* BL21-(DE3) cells (Novagen) transformed with the expression vectors were grown at 37 °C. These were used to inoculate 1 L of medium containing 2% bactotryptone, 1.5% yeast extract, 0.2% Na_2HPO_4 , 0.1% KH_2PO_4 , 0.8% NaCl, and 0.2% glucose (all w/v). The bacterial cells were then grown at 37 °C until the cell density reached an OD_{600} reading of 0.6, at which point the temperature was reduced from 37 to 25 °C before induction with 10 μM isopropyl β -D-thiogalactoside (IPTG). The cultures were grown overnight at 25 °C, and then the cells were harvested by centrifugation at 4 °C. Cell pellets from 3 L of culture were lysed by sonication in 100 mL of lysis buffer [50 mM Na_2HPO_4 (pH 7.5), 100 mM NaCl, and 5 mM benzamidinium] with 100 μM GDP, 50 μM ATP, and 50 $\mu\text{g/mL}$ PMSF. After sonication,

cell debris were removed by high-speed centrifugation, and the supernatant was loaded onto a column containing 5 mL of Ni–NTA metal-affinity resins (Pharmacia Biotech). The column was washed with 100 mL of lysis buffer containing 100 μM GDP and then further washed with 100 mL of 20 mM Hepes (pH 7.0) and 100 mM NaCl. The His-tagged TGase-II fusion proteins were eluted with 20 mM Hepes (pH 7.0), 25 mM NaCl, and 160 mM imidazole. The eluted proteins were loaded onto a MonoQ anion-exchange column (Pharmacia Biotech) equilibrated with 50 mM Mes (pH 6.5), 25 mM NaCl, 10% (v/v) glycerol, and 2 mM DTT. After being washed with the equilibration buffer, the recombinant TGase-II proteins were eluted by using a gradient from 25 to 800 mM NaCl in the same buffer. The fractions containing TGase-II were pooled and concentrated to 10 mL by using UltraPrep filtration (Millipore, molecular weight cutoff of 30 000), then loaded onto a HiLoad 26/60 Superdex S-200 gel filtration column (Pharmacia Biotech), and eluted with 20 mM Hepes (pH 7.0), 100 mM NaCl, 10% (v/v) glycerol, and 2 mM DTT at a rate of 2.0 mL/min. Fractions containing the TGase-II proteins were pooled and concentrated.

Comparisons of the X-ray Structures of TGase-II and TGase-III. The TGase-III structure containing three Ca^{2+} ions (PDB entry 1L9N) was analyzed for this study (23). Contacts for each of the Ca^{2+} ions were calculated using the CONTACT program from the CCP4 suite of programs with a distance cutoff of 3.6 Å. Corresponding residues in TGase-II were identified by aligning its sequence with that of TGase-III. The possible contacts made by the Ca^{2+} ions with TGase-II were also determined. For this purpose, the TGase-II structure (1KV3) (22) was superimposed onto that of TGase-III.

Fluorescence Measurements. All fluorescence measurements were performed using a Varian eclipse spectrofluorimeter. The experiments were carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM DTT and 1 mM EDTA. The excitation and emission wavelengths for BO-DIPY fluorescence were set at 504 and 520 nm, respectively.

GTP Hydrolysis Assays. Wild-type TGase-II or the different TGase-II mutants were assayed for GTP hydrolytic activity using [γ - ^{32}P]GTP in a buffer containing 200 mM MOPS (Sigma) (pH 7.3), 2 mM MgCl_2 , 100 μM EDTA, and 2 mM DTT. The assays were initiated by the addition of [γ - ^{32}P]GTP (final concentration of 10 μM , specific activity of 5 Ci/mmol). Aliquots (50 μL) were removed at specific time points and added to 750 μL of 5% activated charcoal (neutralized) in 50 mM NaH_2PO_4 . The samples were centrifuged at high speed, and then aliquots of the supernatant (40 μL) were removed and counted.

Assays for Transamidation Activity. The transamidation activities of purified, wild-type TGase-II and the different TGase-II mutants were measured using a spectrophotometric assay previously developed by Day and Keillor (26). In this assay, the deamidation of a glutamine substrate was coupled to a second reaction generating glutamate from γ -ketoglutarate with the oxidation of NADH to NAD. The oxidation of NADH was monitored in a Beckman DU200 spectrophotometer at 340 nm. The standard assay contained 200 mM MOPS (pH 7.3) together with varying levels of CaCl_2 (ranging from 1 to 20 mM), 250 μM EDTA, 13 mM γ -ketoglutarate (Sigma), 260 μM NADH, 3 mM methyllysine ester, 80 enzyme units of glutamate dehydrogenase (Sigma),

and, unless otherwise indicated, 25 mM benzyloxycarbonyl-glutamyl-glycine (Z-Gln-Gly; Sigma) in a final volume of 400 μ L. The reaction was started by adding recombinant TGase-II, and transamidation activity was monitored by measuring the change in absorbance at 340 nm. The rate of the reaction was measured from the linear portion of the curve, using an extinction coefficient for NADH at 340 nm of 6220 $M^{-1} cm^{-1}$. All measurements were conducted at room temperature.

In some cases, the transamidation activities of wild-type TGase-II or the different TGase-II mutants expressed in cells were measured in cellular lysates. For these assays, whole-cell extracts were incubated with transamidation reaction buffer (20 mM NaCl and 20 mM DTT) containing 2.5 mM 5-(biotinamido)pentylamine and 20 mM $CaCl_2$ for 10 min, and then the reaction mixtures were subjected to SDS-PAGE. The proteins were transferred to PVDF transfer membranes and blocked in BBST (100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.02% Tween 20, and 80 mM NaCl) containing 10% bovine serum albumin. The proteins that incorporated 5-(biotinamido)pentylamine were detected using horseradish peroxidase-conjugated streptavidin followed by exposure to ECL (Amersham/GE Health Care).

Cell Culture. SKBR3 cells were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum and 100 units/mL penicillin. The cell line was maintained in a humidified atmosphere with 5% CO_2 at 37 °C. To express the different TGase-II proteins in SKBR3 cells, Myc-tagged pcDNA3 constructs encoding the various TGase-II mutants were generated and transfected into cells using Lipofectamine (Invitrogen). Approximately 24 h following transfection, the cells were incubated in medium containing 0.5% serum for an additional 24 h. Where indicated, vector-only transfected cells were stimulated with 100 ng/mL epidermal growth factor (EGF) (Invitrogen) and parental SKBR3 cells were exposed to various concentrations of doxorubicin (Sigma). All cell cultures were then rinsed with phosphate-buffered saline (PBS) and lysed with cell lysis buffer (10 mM Na_2HPO_4 , 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% NaF, 1 mM $NaVO_4$, 25 mM β -glycerophosphoric acid, and aprotinin and leupeptin at 1 μ g/mL each). Equal concentrations of each cell lysate were subjected to SDS-PAGE, and then the proteins were transferred to nitrocellulose. The filters were blocked with TBST [20 mM Tris, 137 mM NaCl (pH 7.4), and 0.02% Tween 20] containing 7% nonfat dry milk and then incubated with anti-Myc (Covance), anti-actin (Sigma), anti-TGase-II (Neomarkers), anti-active caspase-3 (Cell Signaling), and/or PARP (Santa Cruz) antibodies diluted in TBST. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies followed by exposure to ECL (Amersham Biosciences).

Immunofluorescence and Cell Death Assays. SKBR3 cells plated on dual-chamber microscope slides (Nalge-Nunc) were cultured in medium containing 0.5% serum and increasing concentrations of doxorubicin for 24 h and were then fixed with 3.7% formaldehyde. Cells plated on the microscope slides were also transfected with the different TGase-II expression constructs. Twenty-four hours following transfection, the cells were cultured in medium containing 0.5% serum with or without doxorubicin (0.1 μ M or 0.2 μ M) for an additional 24 h and then fixed with 3.7% formaldehyde.

The transfectants were permeabilized with PBS containing 0.2% Triton X-100, blocked with PBS containing 5% bovine serum albumin, and then probed with the anti-Myc antibody. The Myc antibody was detected with an Oregon green 488-conjugated secondary antibody (Molecular Probes). All of the slides were incubated with DAPI to stain nuclei, and the cells were then visualized by fluorescence microscopy. Cells undergoing apoptosis were identified by nuclear condensation or blebbing, and the percentage of cell death was determined by calculating the ratio of apoptotic to nonapoptotic cells for each condition.

RESULTS

Location of Ca^{2+} -Regulatory Sites within TGase-II. The transamidation activity of TGase-II has been suggested to be important for a number of its biological functions. Assays performed in vitro indicate that the transamidation activity requires Ca^{2+} , suggesting that this divalent cation may have a key regulatory role in the different cellular functions performed by TGase-II. Thus, we wanted to determine whether by perturbing the binding of Ca^{2+} to TGase-II, we could disable its transamidation activity and negate its cellular actions. However, this first required knowledge of the location of the Ca^{2+} -regulatory sites on TGase-II. Here we took advantage of the structural and functional similarities shared by TGase-II and TGase-III (22, 23), as these two members of the transglutaminase family are ~40% identical in their primary sequences, and the fact that the three major Ca^{2+} -binding sites on TGase-III have been identified.

Comparisons of the X-ray crystal structures and primary sequences of these two proteins, together with the sequences for other members of the family that exhibit Ca^{2+} -dependent transamidation activity, revealed conserved amino acid residues that seemed most likely to serve as Ca^{2+} -binding sites within TGase-II. Figure 1A shows these sequence comparisons and depicts the locations of these sites, relative to the GTP-binding domain and the active site for transamidation activity. The potential interactions between Ca^{2+} ions and the amino acid residues within the three Ca^{2+} -binding sites on TGase-II are depicted in Figure 1B. To examine the importance of these sites in the regulation of TGase-II activity, the following key residues within each of the interaction regions were mutated to alanine (these are highlighted in red in the sequence comparisons shown in Figure 1A).

(a) **Site 1.** The first of the three Ca^{2+} -binding sites in the primary sequence of TGase-II, designated site 1, consists of residues Gly226, Asn229, Asn231, and Asp233. Both Asn229 and Asp233 should be within 2.5 Å of the Ca^{2+} ion, based on an analysis of the structure for TGase-III (see Table 1). Thus, we changed each of these residues to alanine.

(b) **Site 2.** The second Ca^{2+} -binding site on TGase-II consists of residues Asp306, Asn308, Asn310, Leu312, and Glu329. We selected Asp306 and Asn310 for mutation because on the basis of the TGase-III structure, these residues should be in nearest proximity to the bound Ca^{2+} ion (Table 1).

(c) **Site 3.** The third Ca^{2+} -binding site consists of residues Asn398, Ser419, Glu447, and Glu452. Because Asn398 and Glu447 best matched our proximity criterion for binding Ca^{2+} (Table 1), we chose these residues for mutation. In addition to the corresponding residues being important for the binding

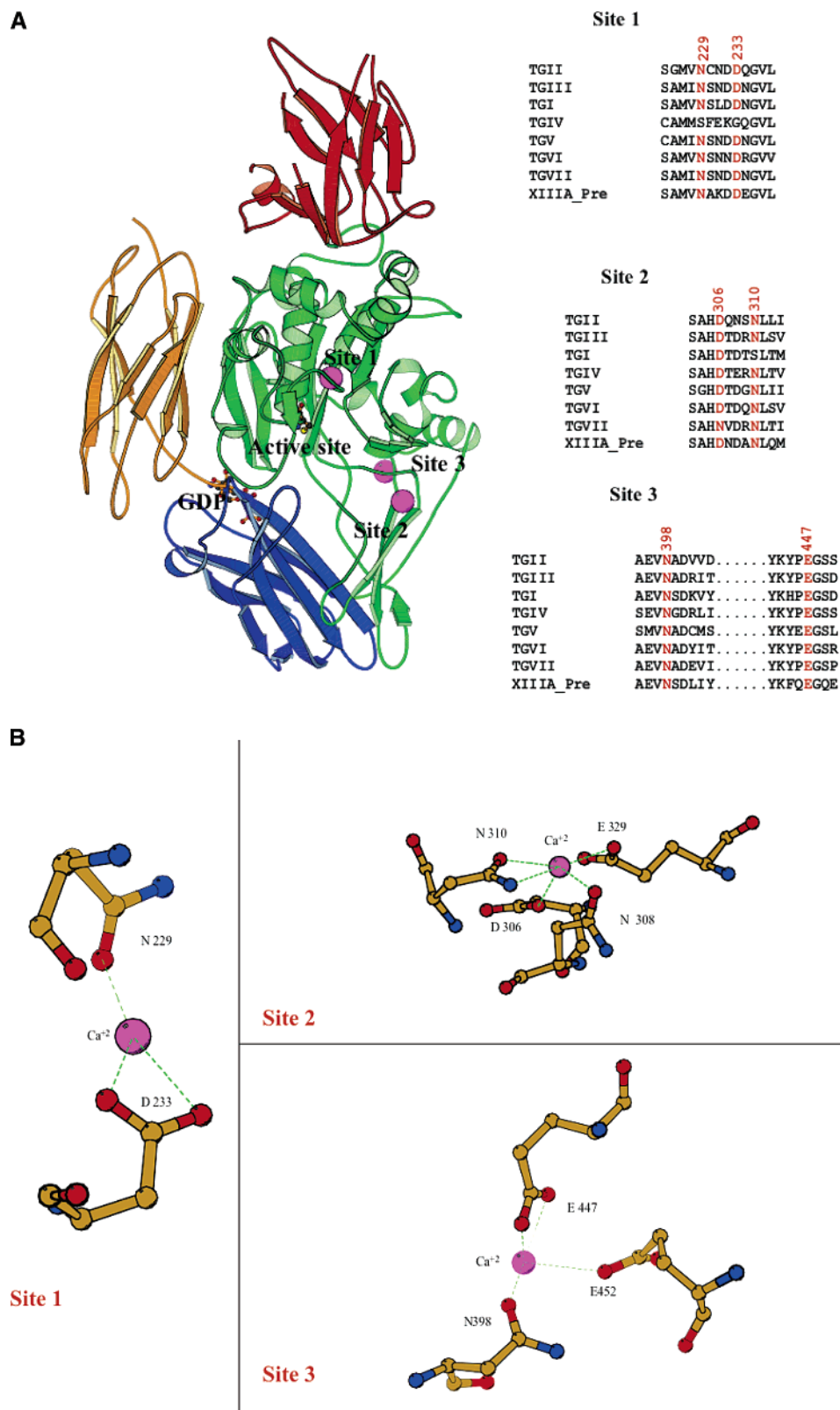


FIGURE 1: Ca^{2+} -binding sites on TGase-II. (A) Structural depiction of TGase-II showing three Ca^{2+} -binding sites, the transamidation active site, and the guanine nucleotide-binding site. The N-terminal β -sandwich domain, the catalytic core domain, and the first and second β -barrel domains are colored red, green, blue, and orange, respectively. Cysteine 277 within the active site and GDP are shown as ball-and-stick models; Ca^{2+} ions are depicted as spheres. The Ca^{2+} -binding sites were derived by superimposing the X-ray structure for TGase-II onto the structure for TGase-III. Sequence alignments using ClustalW (44) are also presented for each of the Ca^{2+} -regulatory sites of TGase-II, vs TGase-III and other members of the transglutaminase family that exhibit Ca^{2+} -dependent transamidation activity. The numbered residues in orange represent the sites mutated in TGase-II. (B) Residues proposed to interact with Ca^{2+} at sites 1–3 of TGase-II are labeled. Interactions, represented by broken lines, were derived from the TGase-III structure.

of Ca^{2+} to TGase-III, it turns out that the blood coagulation protein, Factor XIIIa, uses a similar set of residues to bind

Ca^{2+} , with substitutions at these sites reducing its transamidation activity by $\sim 80\%$ (27).

Table 1: Amino Acid Residues Comprising the Three Ca^{2+} -Regulatory Sites on TGase-II^a

Ca^{2+} atom	protein atom	distance (Å)
site 1	Asn A229 (OD1)	2.4
	Asp A233 (OD1)	2.4
	Asp A233 (OD2)	2.7
site 2	Asn A308 (OD1)	3.4
	Asn A310 (ND2)	3.5
	Asn A310 (OD1)	2.5
	Asp A306 (OD2)	2.3
	Glu A329 (OE1)	2.2
site 3	Glu A329 (OE2)	2.2
	Asn A398 (OD1)	2.2
	Glu A447 (OE1)	2.5
	Glu A447 (OE2)	2.7
	Glu A452 (OE1)	2.9

^a The different Ca^{2+} -binding sites on TGase-II were identified on the basis of their determined locations in TGase-III as described in Experimental Procedures. The estimated distances between Ca^{2+} and the side chain atoms for each residue (indicated in parentheses) are given.

Ca^{2+} -Regulatory Sites 1–3 Do Not Influence the Guanine Nucleotide-Binding Activity of TGase-II. As a first step toward examining whether substitutions at the Ca^{2+} -regulatory sites affected the ability of guanine nucleotides to bind to TGase-II, we developed a fluorescence spectroscopic read-out for assaying nucleotide binding activity. Histidine-tagged TGase-II proteins were expressed in *E. coli* and purified through a series of chromatographic steps beginning with nickel affinity chromatography and then followed by anion-exchange and size exclusion chromatography. This yielded highly purified preparations of wild-type TGase-II and each of the three TGase mutants that contained substitutions at the Ca^{2+} -regulatory sites (Figure 2A, inset). We then took advantage of the fluorescence properties of the GTP analogue, BODIPY-FL GTP γ S (BOD-GTP γ S), in monitoring the binding of guanine nucleotides to the recombinant TGase-II proteins under various conditions.

Figure 2A shows that BOD-GTP γ S was capable of a rapid binding interaction with wild-type TGase-II, as reflected by an immediate increase (~ 2 fold) in the fluorescence emission of the BODIPY moiety (dashed line). The enhanced fluorescence is due to an extension in the structure of the nucleotide analogue that occurs upon its binding to proteins (e.g., TGase-II), thus eliminating the stacking between the BODIPY moiety and the guanine base and reducing its susceptibility to intramolecular quenching (28). Upon addition of GTP γ S, the fluorescence enhancement was rapidly reversed, indicating that BOD-GTP γ S and GTP γ S freely and reversibly competed for a common guanine nucleotide-binding site on wild-type TGase-II.

The extent of the fluorescence enhancement was dependent on the percentage of recombinant TGase-II molecules that were purified without (prebound) GDP. Indeed, some of the recombinant wild-type TGase-II protein, upon isolation, existed in the GDP-bound state, based on HPLC analysis (data not shown) and as demonstrated by its X-ray crystal structure (22). Consequently, incubating wild-type TGase-II with alkaline phosphatase, which converted any bound GDP to GMP (that subsequently dissociated from the protein), resulted in a further enhancement in BOD-GTP γ S fluorescence (Figure 2A, +AP), indicating that additional TGase-II molecules became available to bind the fluorescent nucleotide analogue.

Figure 2B shows the titration of wild-type TGase-II with BOD-GTP γ S. The binding of the guanine nucleotide analogue to TGase-II was saturable and could be fit to a simple bimolecular reaction, yielding an apparent dissociation constant of 385 nM (± 12 nM; $n = 4$). The ability of guanine nucleotides to compete with BOD-GTP γ S for binding to TGase-II allowed us to use this read-out to determine the apparent dissociation constants for GDP and GTP γ S. As shown in Figure 2C, both GTP γ S and GDP caused a dose-dependent quenching of the BODIPY fluorescence, as a result of competition for a common binding site on TGase-II. The best fits to these curves yielded apparent dissociation constants of 320 nM ($n = 3$) and 1.5 μ M ($n = 3$) for GTP γ S and GDP, respectively (Table 2).

Panels A–C of Figure 3 show the corresponding titrations for the competition between GTP γ S and BOD-GTP γ S obtained with each of the TGase-II double mutants that contained substitutions at Ca^{2+} -regulatory sites 1–3, respectively. In each case, GTP γ S exhibited a potency for competing with BOD-GTP γ S similar to that observed with wild-type TGase-II (Table 2). Thus, substitutions of key residues at each of the Ca^{2+} -regulatory sites did not affect guanine nucleotide-binding activity.

Substitutions at Ca^{2+} -Regulatory Sites 1–3 Have Differential Effects on the GTP Hydrolytic Activity of TGase-II. TGase-II also exhibits GTP hydrolytic activity, similar to the more traditional G protein signaling switches (17, 19, 24). We therefore examined whether substitutions at the different Ca^{2+} -regulatory sites influenced this activity. Interestingly, we found that the three Ca^{2+} site mutants did not exhibit identical effects when we assayed their GTP hydrolytic capability. The site 1 mutant [TGase-II(N229A/D233A)] exhibited very poor GTP hydrolytic activity, as measured by $^{32}\text{P}_i$ release (Figure 4), despite its ability to fully bind GTP. On the other hand, the site 2 and site 3 mutants [i.e., TGase-II(D306A/N310A) and TGase-II(N398A/E447A), respectively] consistently exhibited slightly increased GTP hydrolytic activity compared to wild-type TGase-II. These results suggest that the site 1 substitutions cause TGase-II to adopt a different conformational state compared to those of the other two classes of substitutions. The different conformational states apparently exert specific effects on the ability of TGase-II to reach the transition state for the GTP hydrolytic activity. As we discuss below, these findings provide us with some useful insights into the potential roles of GTP binding and hydrolysis, versus transamidation activity, in the conferral of cell survival by TGase-II.

Ca^{2+} -Regulatory Sites 1–3 Are Important for the in Vitro Transamidation Activity Measured for Recombinant TGase-II. To examine the in vitro transamidation activity of the different TGase-II mutants, we used a coupled spectroscopic assay (see Experimental Procedures). In the case of wild-type TGase-II, there was no detectable transamidation activity when assayed in vitro in the absence of millimolar Ca^{2+} levels. Typically, full activity was achieved at ~ 4 mM Ca^{2+} . While it had previously been reported that the binding of guanine nucleotides to TGase-II inhibited its transamidation activity (18, 24, 25), there has been some question about whether both GTP and GDP are effective inhibitors. Figure 5A shows that the transamidation activity of wild-type TGase-II was inhibited by the nonhydrolyzable GTP ana-

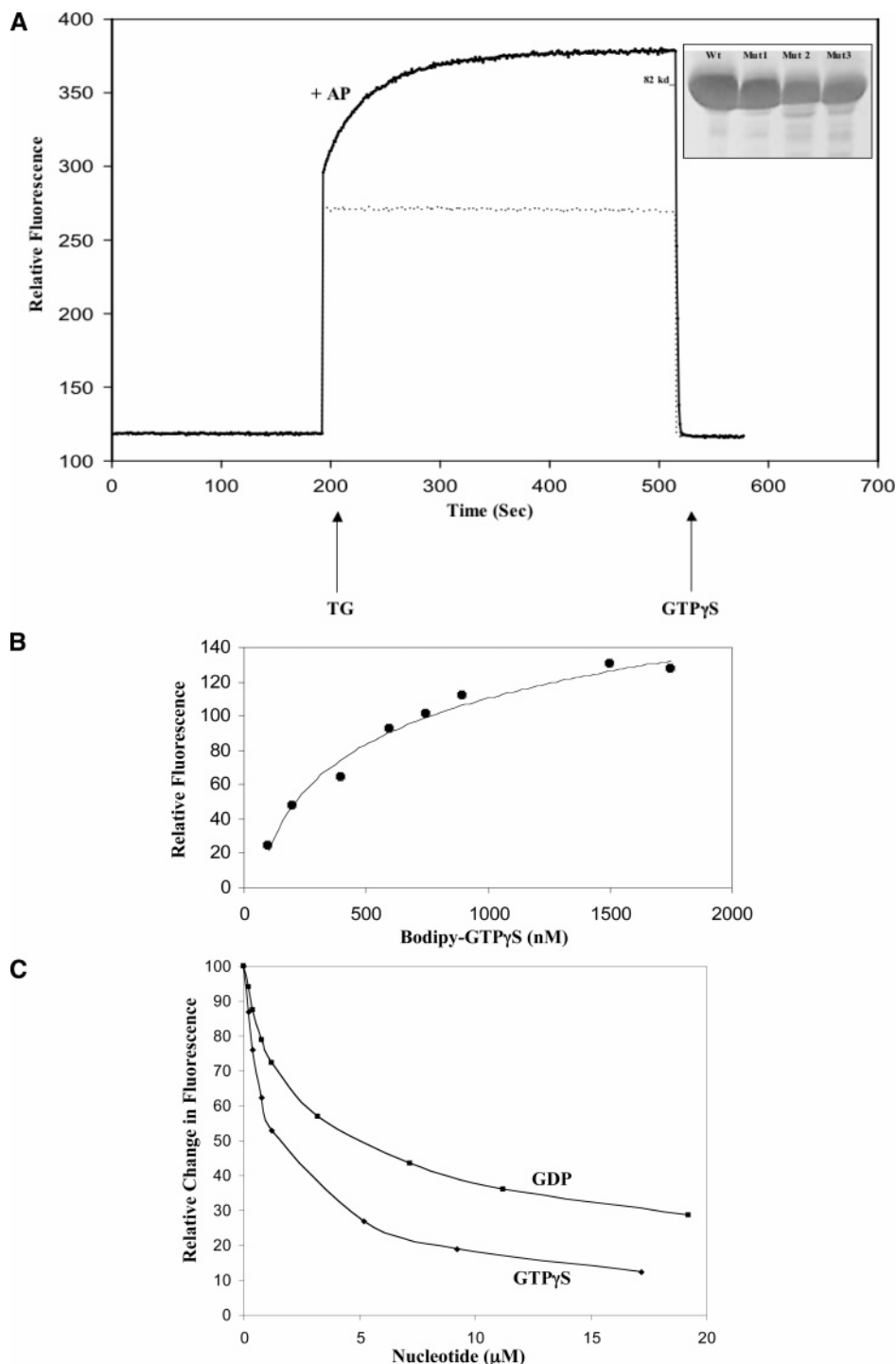


FIGURE 2: Binding of BOD-GTP γ S to wild-type TGase-II. (A) BOD-GTP γ S (380 nM), dissolved in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 mM DTT, was used to obtain a baseline fluorescence emission at 520 nm (excitation at 504 nm). After 3 min, wild-type TGase-II (190 nM) was added to the reaction cuvette. The horizontal dashed line shows the enhancement in BODIPY fluorescence that accompanied the addition of TGase-II. GTP γ S (240 μ M) was then added to compete with BOD-GTP γ S and thus decrease the fluorescence emission at 520 nm. + AP represents the increase in fluorescence emission that accompanied the binding of BOD-GTP γ S to additional nucleotide-free, wild-type TGase-II molecules generated upon treatment with alkaline phosphate. The inset shows SDS-PAGE analysis of purified *E. coli* recombinant, human wild-type His-tagged TGase-II (Wt), His-TGase-II(N229A/D233A) (Mut1), His-TGase-II(D306A/N310A) (Mut2), and His-TGase-II(N398A/E447A) (Mut3). The His-tagged TGase-II proteins were purified by a sequence of three chromatography steps: nickel affinity chromatography, anion-exchange chromatography, and size-exclusion chromatography as described in Experimental Procedures. (B) Wild-type TGase-II (270 nM) was dissolved in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM DTT, and 4 mM Ca²⁺. Increasing concentrations of BOD-GTP γ S were incubated with the protein at room temperature. The relative change in fluorescence (emission at 520 nm, excitation at 504 nm) accompanying each addition of BOD-GTP γ S was determined after 1 h. (C) Competition between BOD-GTP γ S and either GTP γ S or GDP, as read-out by changes in BODIPY fluorescence. Wild-type TGase-II (300 nM), BOD-GTP γ S (1.8 μ M), and 4 mM CaCl₂ were incubated in the Tris buffer described for panel B for 1 h. Increasing concentrations of GTP γ S and/or GDP were added over time. Each addition was followed by a 2 min equilibration, and then the corresponding change in fluorescence was recorded.

Table 2: Ability of Guanine Nucleotides To Bind to TGase-II and Inhibit Transamidation Activity^a

protein	apparent dissociation constant (nM)	
	GTP γ S	GDP
wild type	320 \pm 25	1500 \pm 17
TGase-II(N229A/D233A)	320 \pm 19	
TGase-II(D306A/N310A)	250 \pm 22	
TGase-II(N398A/E447A)	340 \pm 8	

^a The ability of varying concentrations of GTP γ S and GDP to compete with BOD-GTP γ S for binding to wild-type TGase-II and the indicated TGase-II mutants was assessed as described in the legend of Figure 2C. The apparent dissociation constants were determined from the best fits to the titration data assuming a simple competition between BODIPY-GTP γ S and either GTP γ S or GDP for TGase-II, using DYNAFIT (version 3.28.038) (45).

logue, GTP γ S, as well as by GDP. The dose-dependent inhibition by GTP γ S and GDP occurred at guanine nucleotide concentrations that were very similar to the dose-response profiles obtained with these nucleotides when their abilities to compete with BOD-GTP γ S for binding to TGase-II were assayed (Figure 2C).

Figure 5B shows the transamidation activities, measured as a function of Ca²⁺ concentration, for the different TGase-II mutants. Each of the mutants exhibited compromised Ca²⁺-dependent activity, indicating that Ca²⁺ interactions at sites 1–3 are necessary for full transamidation capability. The Ca²⁺ dose-response profiles were significantly shifted for the TGase-II(N229A/D233A) and TGase-II(N398A/E447A) mutants, suggesting that these substitutions significantly weakened Ca²⁺ binding at sites 1 and 3, respectively. However, the TGase-II(D306A/N310A) mutant was completely inactive in transamidation assays. This might be explained by structural studies (23) showing that the binding of Ca²⁺ to the comparable site on TGase-III moves a loop region (i.e., corresponding to residues 325–330 in TGase-II) and thereby opens a channel for substrates to properly access catalytic residues at the active site.

Figure 5C shows the results of in vitro transamidation assays of wild-type TGase-II and the different TGase-II mutants, when the concentration of the glutamine-containing substrate, Z-Gln-Gly, is varied at a fixed level of Ca²⁺ (10 mM). The TGase-II(N229A/D233A) mutant exhibited an apparent weaker affinity for the substrate, compared to that of wild-type TGase-II, although extrapolating to high substrate concentrations suggests that the mutant would exhibit transamidation activity comparable to that for the wild-type protein. The site 2 mutant [TGase-II(D306A/N310A)] showed little or no activity over the entire range of substrate concentrations that was examined, consistent with the interpretation that this protein is catalytically defective, whereas the site 3 mutant [TGase-II(N398A/E447A)] showed a substrate dose-response profile similar to that obtained for wild-type TGase-II. However, at saturation, the final level of transamidation activity catalyzed by the site 3 mutant was only half of that for the wild-type protein, consistent with the findings that this mutant exhibited only approximately half of the catalytic activity of the wild-type protein when assayed at 10 mM Ca²⁺ (Figure 5B).

Effects of Ca²⁺-Regulatory Site Mutations on TGase-II-Catalyzed Transamidation Activity in Whole-Cell Lysates. We then examined the abilities of Myc-tagged wild-type

TGase-II and each of the different Myc-tagged TGase-II mutants, when expressed in human breast cancer (SKBR3) cells (Figure 6A, top panel), to catalyze the transamidation of proteins in whole-cell lysates (see Experimental Procedures). As shown in Figure 6A (top panel), we were not able to detect endogenous TGase-II by Western blot analysis with anti-TGase-II antibody, except upon treatment with EGF which upregulates its expression (16). However, we suspect that some endogenous TGase-II is present even under basal conditions, as suggested by the background levels of transamidation activity that are read-out by the cross-linking of lysate proteins to biotinylated pentylamine. The expression of Myc-tagged, wild-type TGase-II resulted in a significant increase in the transamidation activity of lysate proteins, compared to the basal activity observed in control cells (in Figure 6B, bottom panel, see lanes 1 and 2; the top two panels show the relative expression of Myc-tagged, wild-type TGase-II and the different Myc-TGase-II mutants, and an actin loading control). Under the same experimental conditions, each of the TGase-II mutants was less effective at catalyzing the transamidation of lysate proteins than wild-type TGase-II (in Figure 6B, bottom panel, see lanes 4–6 vs lane 2). This was especially the case for the TGase-II-(D306A/N310A) and TGase-II(N398A/E447A) mutants, as they even reduced the basal levels of transamidation relative to what was detected in control (vector) cells (in Figure 6B, bottom panel, compare lanes 5 and 6, respectively, with lane 1). The same was true for the TGase-II(C277V) mutant (compare lane 3 with lane 1), in which the essential active site cysteine residue was changed to valine. Thus, these mutants apparently are able in some way to interfere with the binding of endogenous TGase-II to some of the cellular substrates detected under basal conditions.

Effects of Ca²⁺-Regulatory Site Mutations on TGase-II-Catalyzed Protection against Apoptotic Stimuli. Given the apparent ability of these different Ca²⁺-binding mutations to compromise TGase-II-catalyzed transamidation activity, we wanted to see what consequences these same substitutions had on the ability of TGase-II to exert its cellular functions. Previous work from our laboratory (14–16), as well as studies from other groups (29), has shown that TGase-II confers a survival advantage to cells challenged with apoptotic factors. We have recently shown that TGase-II plays an important role in EGF-mediated survival of human breast cancer cells and that it blocked the apoptotic activity accompanying the treatment of cells with doxorubicin (16). Thus, we compared the abilities of Myc-tagged wild-type TGase-II and the different TGase-II mutants to confer protection to SKBR3 cells following treatment with doxorubicin. Figure 6C shows that each of the Myc-tagged TGase-II mutants containing substitutions within the different Ca²⁺-binding sites exhibited a cytosolic distribution similar to that observed for wild-type TGase-II and the transamidation-defective TGase-II(C277V) mutant. Thus, our ability to perturb the Ca²⁺-regulatory sites of TGase-II did not significantly alter its cellular localization.

However, substitutions that impacted Ca²⁺-dependent transamidation activity did markedly influence the ability of TGase-II to promote cell survival. Figure 6D shows the dose-dependent apoptotic activity exhibited by doxorubicin in SKBR3 cells. The inset shows the corresponding effects of

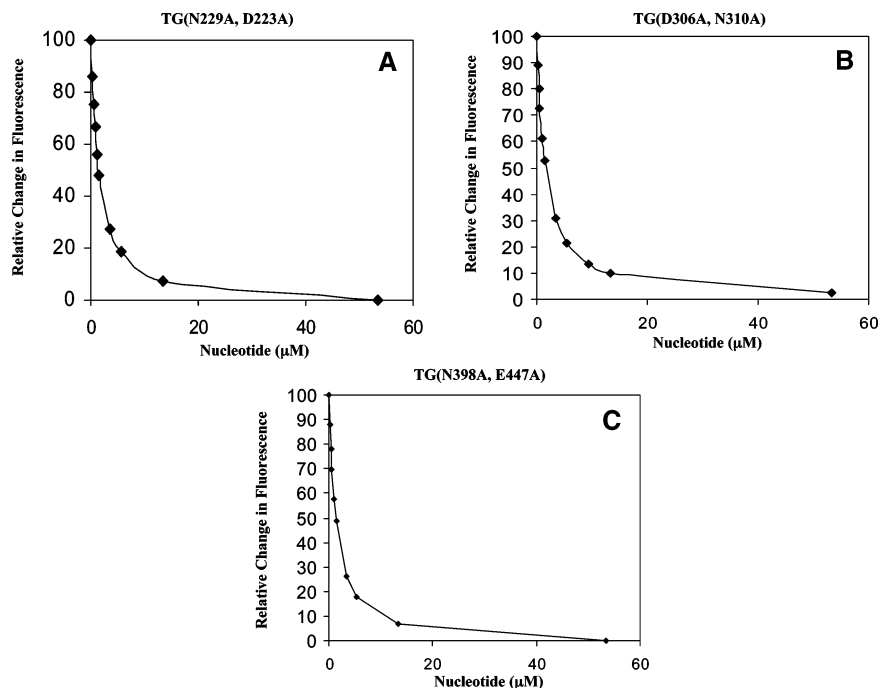


FIGURE 3: Binding of BOD-GTP γ S to the different TGase-II mutants. Competition experiments between BOD-GTP γ S and GTP γ S, like those described for Figure 2C, were performed with TGase-II(N229A/D223A) (A), TGase-II(D306A/N310A) (B), and TGase-II(N398A/E447A) (C).

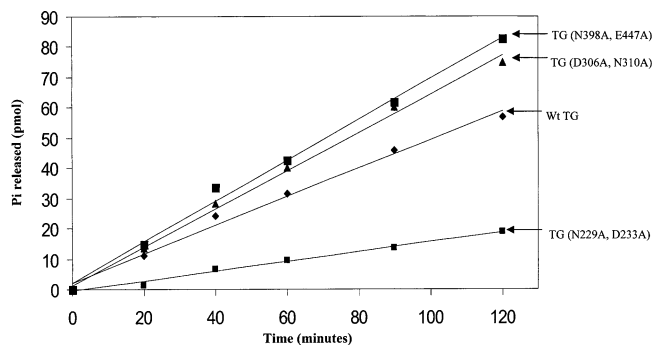


FIGURE 4: GTP hydrolytic activity of wild-type TGase-II and the different TGase-II mutants. Wild-type TGase-II (300 nM) or equivalent amounts of each of the TGase-II mutants were assayed for GTP hydrolytic activity at the indicated times in a buffer containing 200 mM MOPS (pH 7.3), 2 mM MgCl_2 , 100 μM EDTA, and 2 mM DTT. Assays were initiated by the addition of [γ - ^{32}P]-GTP, and the amount of $^{32}\text{P}_i$ (product) generated was determined as described in Experimental Procedures.

the drug on caspase-3 activation, both as read-out using a specific antibody for the activated caspase and by the degradation of the caspase substrate PARP. Two different levels of doxorubicin that caused varying degrees of apoptosis were then chosen for the examination of the protective effects of wild-type TGase-II and the different Ca^{2+} -binding site mutants (Figure 6E). A significant protection against cell death was observed in SKBR3 cells, under both conditions of doxorubicin treatment, upon overexpressing wild-type TGase-II. As previously reported (16), expression of the TGase-II mutant [TGase(C277V)] failed to provide any protection against the apoptotic activity of doxorubicin. The same was true when expressing each of the TGase-II mutants that contained substitutions at the Ca^{2+} -regulatory sites.

DISCUSSION

TGase-II is a dual-function GTP-binding/acyl transferase that has been implicated in a variety of important biological

outcomes (4–13). Perhaps foremost among these have been reports linking TGase-II to cell survival and cell death, where there have been contrasting views regarding whether TGase-II contributes to (30–32) or protects against (14–16) apoptotic challenges. Part of the confusion has likely arisen from the upregulation of TGase-II expression and activity that accompanies the exposure of cells to stress or apoptotic factors. However, a good deal of evidence now supports the idea that TGase-II is typically upregulated in response to various cellular insults as a protective measure, although in some cases, the excessive expression and/or mislocalization of TGase-II may ultimately have deleterious rather than beneficial effects. Recently, we have found that TGase-II plays a particularly important role in the survival of human breast cancer cells, with EGF treatment causing an increase in the level of TGase-II expression and activation that may underlie the oncogenic potential and chemoresistance of these cells (16).

The mechanisms by which TGase-II gives rise to cell survival are still largely unknown. Work from our laboratory showed that TGase-II protected NIH 3T3 cells from the apoptotic effects of serum deprivation and that its GTP-binding capability may be sufficient to afford these protective effects (14). This raised the question of whether TGase-II might function more in the capacity of a signaling G protein, rather than as an acyl transferase, in mediating cell survival. Moreover, there have been questions concerning whether TGase-II was able to exhibit sufficient transamidation activity in cells to account for its survival function, since the normal levels of intracellular Ca^{2+} are well below the millimolar concentrations typically used when assaying enzymatic activity in vitro (33, 34). Subsequently, we found that TGase-II mutants containing substitutions of a cysteine residue (Cys277) thought to be essential for transamidation activity were ineffective at protecting against apoptosis in fibroblasts and human breast cancer cells (14–16). However, there have

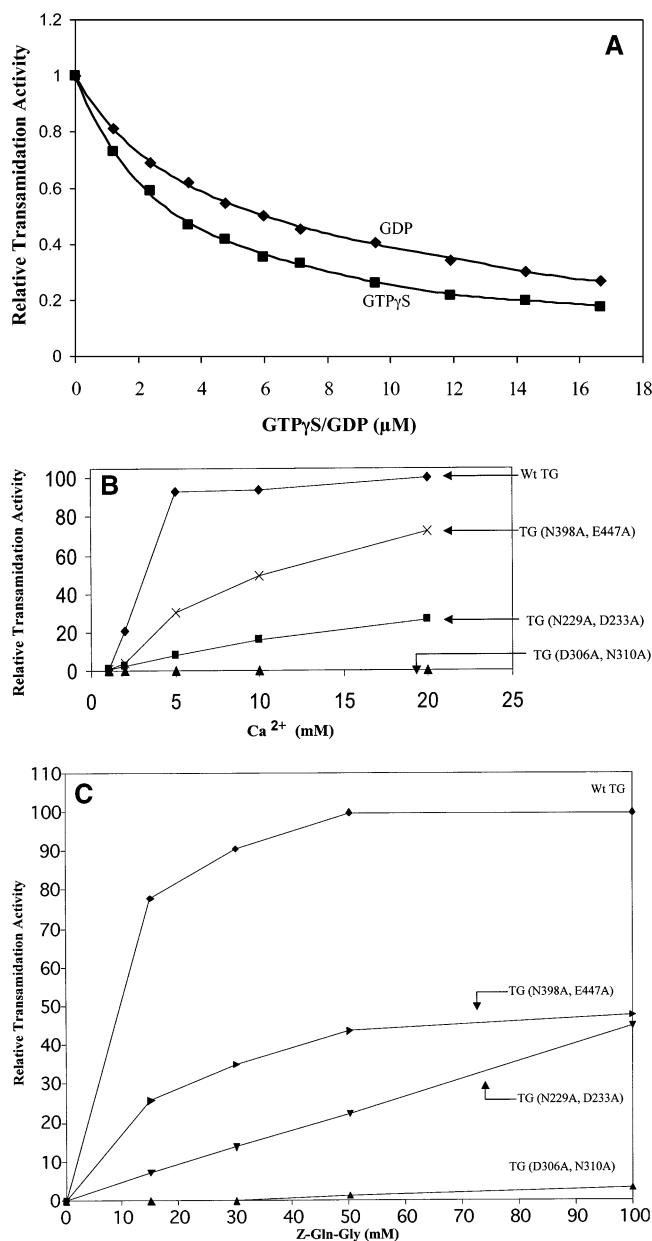


FIGURE 5: Assays of the Ca $^{2+}$ -dependent transamidation activity of wild-type TGase-II and the different TGase-II mutants. (A) Increasing concentrations of GTP γ S or GDP were incubated with wild-type TGase-II (600 nM), prior to assaying transamidation activity using a colorimetric assay as described in Experimental Procedures. The transamidation reaction generated NH $_4^+$ ions which were used by glutamate dehydrogenase to oxidize NADH to NAD, as followed by measuring the change in absorbance at 340 nm. The initial velocity of the transamidation reaction was calculated from the slope of the linear portion of the curve. Initial velocities were then plotted as relative activities vs increasing concentrations of GTP γ S or GDP. (B) The transamidation activities for wild-type TGase-II and the different TGase-II mutants were determined at the indicated Ca $^{2+}$ concentrations, as described for panel A and in Experimental Procedures. (C) The transamidation activities for wild-type TGase-II and the different mutants were assayed as a function of the indicated amounts of the glutamine-containing substrate Z-Gln-Gly and at a fixed concentration of Ca $^{2+}$ (10 mM).

been indications that mutation of this essential cysteine residue also negatively impacts GTP binding activity (14), therefore making it difficult to use substitutions at this site to arrive at an unambiguous conclusion regarding the importance of transamidation activity in the survival functions of TGase-II. Likewise, while cystamine (as well as

related compounds) has been used as a competitive inhibitor of the transamidation activity of TGase-II, questions have arisen regarding its specificity (35–37).

In this study, we set out to take a closer look at the role of transamidation in the ability of TGase-II to confer cell survival activity to human breast cancer cells. In particular, we were interested in seeing whether mutations that perturbed the Ca $^{2+}$ -dependent regulation of transamidation activity of TGase-II influenced its ability to protect breast cancer cells against apoptotic challenges. We had previously proposed a possible location for a major Ca $^{2+}$ -binding site on TGase-II (22), based on the X-ray crystal structure of Factor XIIIa bound to Ca $^{2+}$ (27). The X-ray crystal structure of Ca $^{2+}$ bound to TGase-III (23), which is more similar to TGase-II, unambiguously identified the original site that we had suggested (now designated as site 3 on TGase-II), as well as two other Ca $^{2+}$ -binding sites (see panels A and B of Figure 1 and Table 1). We therefore mutated residues at each site and examined the effects of these substitutions on transamidation activity. Substitutions at site 2 had the most severe effects, resulting in no detectable transamidation activity at all Ca $^{2+}$ and substrate concentrations that were examined, whereas mutations at sites 1 and 3 significantly shifted the dose–response curves for Ca $^{2+}$ in our in vitro assays. When assaying the transamidation of lysate proteins, we found that the site 2 mutant reduced the basal levels of activity that arise from the endogenous enzyme. The same was true for the TGase-II(C227V) mutant which is thought to be catalytically dead. Apparently, these mutants can prevent endogenous TGase-II (or perhaps another transglutaminase present in the lysates) from acting on at least some of its cellular substrates. The site 3 mutant [TGase-II(N398A/E447A)] also reduced the basal levels of transamidation activity measured in the lysates, which was somewhat unexpected, because while this mutant is catalytically compromised, it still shows measurable in vitro activity when assayed using a spectrophotometric read-out. Thus, site 3 substitutions apparently have a more significant effect on the Ca $^{2+}$ -responsive transamidation activity of TGase-II toward cellular substrates than what would be predicted from the results of our in vitro assays. The site 1 mutant does not reduce the basal levels of transamidation activity, perhaps because it exhibits a significantly weakened affinity for substrates and therefore has little capability for blocking the interactions of endogenous enzymes with their cellular targets.

None of the substitutions at sites 1–3 negatively affected GTP-binding activity, based on our fluorescence measurements using BOD-GTP γ S (Table 2). Likewise, each of the TGase-II mutants exhibited an overall cellular localization similar to that observed for wild-type TGase-II. Interestingly, each of the Ca $^{2+}$ -binding site substitutions did affect GTP hydrolytic activity, although the effects were relatively minor for the site 2 and site 3 substitutions. These two mutations exhibited a rate of GTP hydrolysis slightly higher than that of wild-type TGase-II. On the other hand, substitutions at site 1 had a more dramatic effect, resulting in a significantly reduced hydrolytic activity. This suggests that Ca $^{2+}$ interactions at site 1 may be necessary for inducing a conformational change that facilitates the development of the transition state for GTP hydrolysis. A larger fraction of the cellular pool of TGase-II molecules with site 1 substitutions would therefore be expected to exist in the GTP-bound state. When analogous

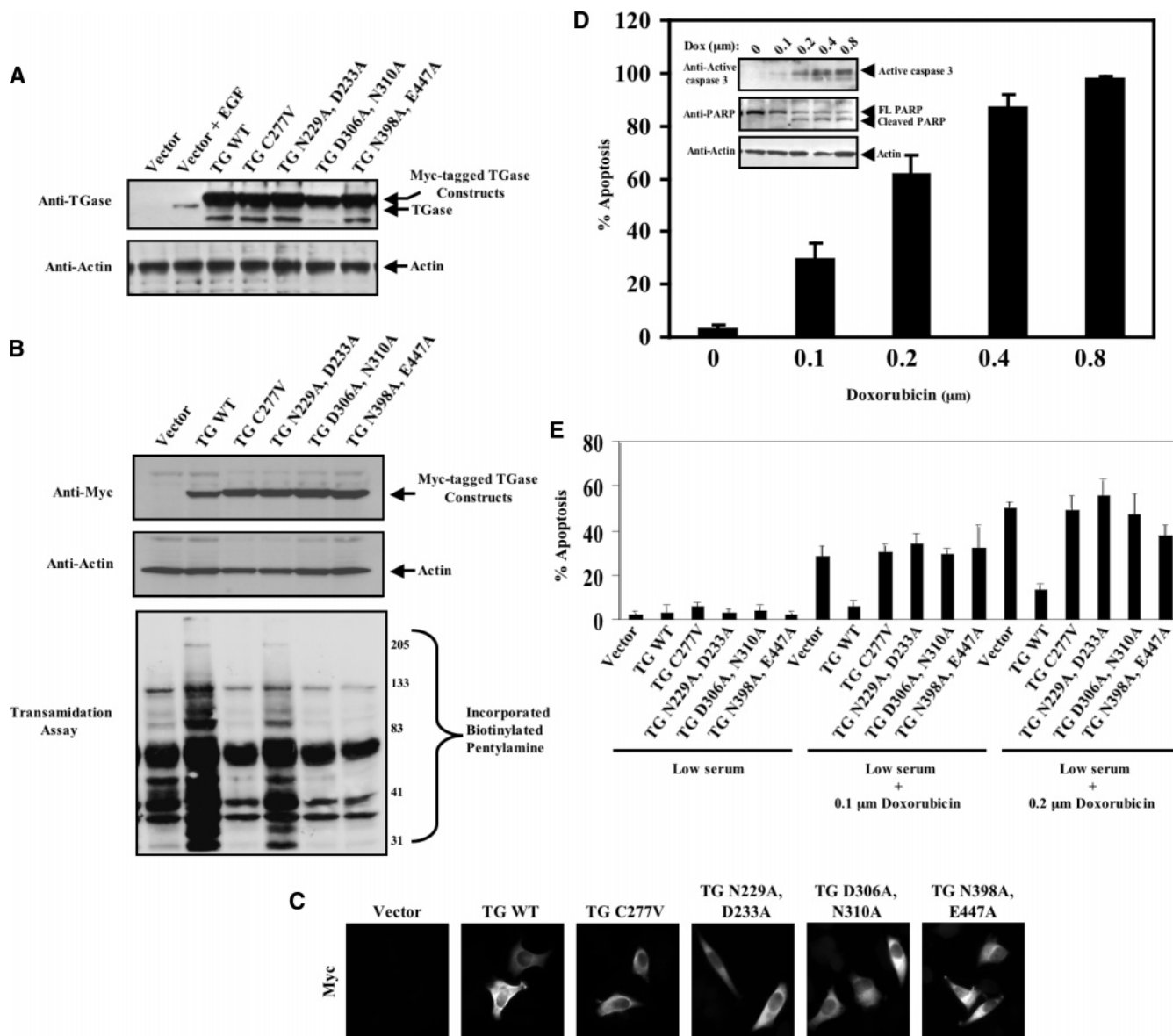


FIGURE 6: Ca^{2+} -binding defective mutants of TGase-II are compromised in their ability to protect SKBR3 cells from doxorubicin-induced apoptosis. SKBR3 cells overexpressing Myc-tagged forms of wild-type TGase-II (TG WT), a TGase-II construct in which cysteine 277 was changed to valine (TG C277V), and the different Ca^{2+} -binding mutants of TGase-II (TG N229A, D233A; TG D306A, N310A; and TG N398A, E447A) were either lysed or fixed. (A) Lysates from vector control cells treated with EGF (100 ng/mL) or untreated or lysates from cells expressing the different Myc-TGase-II constructs were subjected to Western blot analysis with the anti-TGase-II antibody to determine the relative expression levels of endogenous TGase-II and each of the TGase-II constructs (top panel). The bottom panel shows a loading control using actin. (B) Lysates from vector control cells or from cells expressing the different Myc-TGase-II constructs were assayed for transamidation activity. The top panel shows the relative level of expression of the different Myc-TGase-II proteins based on Western blot analysis with the anti-Myc antibody. The second panel from the top shows a loading control using actin. The bottom panel shows the results of transamidation assays, read-out by the incorporation of biotinylated pentylamine into proteins from whole-cell lysates prepared from the different transfectants (see Experimental Procedures). Numbers on the right side of the transamidation assay blot indicate positions of molecular mass markers (kilodaltons). (C) Immunofluorescence with an anti-Myc antibody was performed on the fixed cells to compare the cellular localization of wild-type Myc-TGase-II and the different Myc-tagged TGase-II mutants. (D) SKBR3 cells were incubated with the indicated levels of doxorubicin for 24 h and then were scored for apoptosis as identified by nuclear condensation or blebbing. The inset shows the caspase-3 activity under the different conditions of drug treatment, as assayed using an antibody that detects activated caspase-3 and by the proteolytic degradation of PARP. (E) SKBR3 cells overexpressing wild-type TGase-II or the different TGase-II mutants were incubated with either 0.1 or 0.2 μM doxorubicin for 24 h and then scored for apoptosis. The apoptotic assays were conducted at least three times, and the average percentage of cell death was plotted. The error bars indicate the standard error.

mutations are made in members of either the large or small G protein family, this gives rise to enhanced cellular signaling activities. However, the site 1 mutant behaves like the other TGase-II mutants in its inability to protect human breast cancer cells that have been challenged with doxorubicin, thereby suggesting that the GTP-binding status of TGase-II is not important for this survival response.

Overall, these findings lead us to make two important points. First, they verify that transamidation activity is in fact necessary for the protective effects conferred by TGase-II against apoptotic challenges to human breast cancer cells. This further supports the idea that the design of small molecule inhibitors of TGase-II's transamidation activity may represent a potentially useful strategy for intervention against

certain human cancers (38–40). Second, our findings show that the Ca^{2+} -mediated regulation of TGase-II is essential for its cellular function. The latter suggests that TGase-II may respond to micromolar levels of Ca^{2+} in a manner that generates sufficient transamidation activity to confer protective effects against apoptotic challenges. How this might occur is still not understood, although one interesting possibility is that other cellular proteins (i.e., binding partners for TGase-II), or even some cellular substrates, may increase the affinity of TGase-II for Ca^{2+} . However, at present, we also cannot rule out the possibility that the ability to confer a survival advantage requires that TGase-II be secreted from cells (41, 42), enabling it to have access to higher (i.e., millimolar) levels of Ca^{2+} .

We also are still left with the puzzle of how Ca^{2+} impacts the binding of guanine nucleotides to TGase-II. We find that millimolar levels of Ca^{2+} weaken the binding of guanine nucleotides to recombinant TGase-II by ~5-fold, as determined from our fluorescent read-out using BOD-GTP γ S (data not shown). However, we have not been able to attribute the Ca^{2+} -mediated negative regulation of guanine nucleotide-binding activity to any of the Ca^{2+} -binding sites that have thus far been identified, which implies the existence of another site. This site may have a more general capability for binding divalent cations, as we have found that the affinity of TGase-II for guanine nucleotides can be enhanced by Mg^{2+} , similar to what has been observed with some of the more traditional signaling G proteins, and that increasing (millimolar) levels of Ca^{2+} can negate the stimulatory effects of Mg^{2+} when assaying GTP-binding activity in vitro (S. Datta, unpublished data). It may be that under some conditions, Ca^{2+} can compete with Mg^{2+} for this regulatory site and antagonize, rather than promote, guanine nucleotide-binding interactions.

Finally, one of the more interesting questions for the future concerns the identification of the transamidation substrate(s) that is essential for conferring cell survival and protection against apoptosis. One intriguing possibility is caspase-3, as it has recently been reported that TGase-II can catalyze the transamidation of this protease and inhibit its activity (43). We had earlier found that the caspase-3-catalyzed degradation of the retinoblastoma (Rb) gene product accompanies HPR-induced apoptosis in HL60 cells (15). Thus, future studies will be directed toward determining whether the prevention of caspase-catalyzed degradation of Rb represents a critical component in the ability of TGase-II to protect human breast cancer cells against chemotherapeutic challenges.

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