The Core Domain of the Tissue Transglutaminase G_h Hydrolyzes GTP and ATP†

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ABSTRACT: Tissue transglutaminase (TGase II) catalyzes the posttranslational modification of proteins by transamidation of available glutamine residues and is also a guanosinetriphosphatase (GTPase) and adenosinetriphosphatase (ATPase). Based on its homology with factor XIIIA, an extracellular transglutaminase, the structure of TGase II is likely composed of an N-terminal β-sandwich domain, an α/β catalytic core, and two C-terminally located β-barrels. Here we used a domain-deletion approach to identify the GTP and ATP hydrolytic domains of TGase II. Full-length TGase II and two domain-deletion mutants, one retaining the N-terminal β-sandwich and core domains (βSCore) and the other retaining only the core domain, were expressed as glutathione *S*-transferase (GST) fusion proteins and purified. GST-Full and GST-βSCore exhibited calcium-dependent TGase activity, whereas GST-Core had no detectable TGase activity, indicating the β-sandwich domain is required for TGase activity but the C-terminal β-barrels are not. All three GST−TGase II fusion proteins were photoaffinity-labeled with [α-^{32}P]-8-azidoGTP and were able to bind GTP−agarose. The GTPase activity of GST-βSCore was equivalent to that of GST-Full, whereas the ATPase activity was ∼40% higher than GST-Full. GST-Core had ∼50% higher GTPase activity and ∼75% higher ATPase activity than GST-Full. The GTPase and ATPase activities of each of the GST−TGase II fusion proteins were inhibited in a dose-dependent manner by both GTPγS and ATPγS. These results demonstrate that the GTP and ATP hydrolysis sites are localized within the core domain of TGase II and that neither the N-terminal β-sandwich domain nor the C-terminal β-barrels are required for either GTP or ATP hydrolysis. Taken together with previous work [Singh, U. S., Erickson, J. W., & Cerione, R. A. (1995) *Biochemistry 34*, 15863−15871; Lai, T.-S., Slaughter, T. F., Koropchak, C. M., Haroon, Z. A., & Greenberg, C. S. (1996) *J. Biol. Chem. 271*, 31191−31195] the results of this study indicate that the GTP and ATP hydrolysis sites are localized to a 5.5 kDa (47 amino acid) region at the start of the core domain.

The GTP-binding protein (G-protein),[1] G_h, is a dual function guanosinetriphosphatase (GTPase)/transglutaminase. G_h was identified as a novel 74 kDa protein that couples to the α_1-adrenergic receptor in rat liver plasma membranes and mediates α_1-transmembrane signaling by stimulating a membrane-bound phospholipase C (Im & Graham, 1990; Im *et al*., 1990, 1992). Similar coupling of G_h with α_1-adrenergic receptors has been demonstrated in rat, dog, rabbit, bovine, and human heart preparations (Baek *et al*., 1993; Braun & Walsh, 1993). The molecular mass of the G_h family varies slightly in different species, ranging from M_r's of 74 to 80 kDa (Baek *et al*., 1993; Braun & Walsh, 1993). G_h has been shown to mediate phospholipase C activation by α_{1B}- and α_{1D}- but not α_{1A}-adrenergic receptors (Nakaoka *et al*., 1994; Chen *et al*., 1996) and appears to couple also to oxytocin receptors (Baek *et al*., 1996). Partial amino acid sequence analysis and immunological characterization (Nakaoka *et al*., 1994) have identified G_h as a tissue transglutaminase type II (TGase II, R-glutaminyl peptide:amine γ-glutamyltransferase, EC 2.3.2.13). A GTP-binding protein in rabbit liver nuclear membranes has also recently been identified as TGase II (Singh *et al*., 1995).

TGases are Ca^{2+}-dependent acyl transferases that catalyze the formation of an amide bond between the γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups in various compounds, including the ϵ-amino group of lysines in certain proteins (Folk, 1980). The TGases are a family of closely related thiol enzymes that are derived from a common ancestor (Aeschlimann & Paulsson, 1994). Five enzymatically active TGases have been identified [see Greenberg *et al*. (1991) and Aeschlimann and Paulsson (1994) for reviews]. These include keratinocyte TGase (TGase I), which is primarily membrane-associated and plays a major role in the formation of the cornified cell envelope of the epidermis; tissue TGase (TGase II, G_h), which is ubiquitously expressed in mammalian tissues, both in membrane and in cytosolic fractions, and has been implicated in apoptosis, cell adhesion, and signal transduction (Nakaoka *et al*., 1994); epidermal TGase (TGase III), a soluble proenzyme involved in differentiating epidermal and hair follicle cells that requires proteolytic activation; prostate TGase (TGase IV), which in rodents is involved in the formation of copulatory plugs in the female genital tract after

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; G-protein, GTP-binding protein; GST, glutathione *S*-transferase; GST-Full, GST fused to full-length TGase II; GST-βSCore, β-sandwich and core domains of TGase II fused to GST; GST-Core, core domain of TGase II fused to GST; GTP, guanosine 5′-triphosphate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TGase II, tissue transglutaminase type II.

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coitus; and the catalytic A subunit of factor XIII (fXIIIA) that cross-links fibrin monomers and is involved in blood coagulation. In plasma, fXIII is a heterotetrameric zymogen composed of two A subunits (80 kDa) and two B subunits (75 kDa), whereas in platelets, placenta, and other tissues, fXIII is an A_2 homodimer (Folk & Finlayson, 1977).

The three-dimensional structure of human recombinant dimeric (A_2) fXIII has been solved at 2.8 Å resolution by X-ray crystallography (Yee *et al*., 1994) and refined to 2.2 Å resolution (Yee *et al*., unpublished results). The fXIIIA monomer is folded into four distinct and sequential domains: N-terminal β-sandwich, core, barrel 1, and barrel 2 (Yee *et al*., 1994). Amino acid sequence alignments of members of the TGase family show highest identity between the core domains. Major sequence differences occur at the amino and carboxyl termini. Nevertheless, conservation of amino acid properties between the various TGases indicates the tertiary structure of these domains is conserved. The active site for TGase activity is located in the core domain and is comprised of a Cys-His-Asp catalytic triad (Cys^{314}-His^{373}-Asp^{396} in fXIIIA). A calcium-binding site is located toward the end of the core domain (Asp^{438}, Ala^{457}, Glu^{485}, Glu^{490} in fXIIIA). A 10 kDa extension of TGase I N-terminal to the β-sandwich is required for membrane association of this protein (Rice *et al*., 1990). A 4 kDa leader sequence at the N-terminus of the zymogen form of fXIII masks the active site pocket, and thrombin cleavage of this sequence leads to activation of the enzyme (Schwartz *et al*., 1973; Credo *et al*., 1978). A second thrombin cleavage site in fXIIIA, at the end of the core domain, releases a catalytically active 51 kDa fibrin-binding fragment and a 25 kDa carboxyl-terminal fragment (Takahashi *et al*., 1986; Greenberg *et al*., 1988). This site corresponds to the activation site for TGase III (Kim *et al*., 1990).

Unlike other TGases, TGase II binds guanine nucleotides in a 1:1 ratio and hydrolyzes GTP (Achyuthan & Greenberg, 1987; Lee *et al*., 1989; Im *et al*., 1990). GTP binding by TGase II inhibits calcium binding and thus TGase activity, whereas calcium binding inhibits GTP binding (Achyuthan & Greenberg, 1987). Moreover, α_{1B}-adrenergic receptor activation inhibits TGase activity (Nakaoka *et al*., 1994) and enhances GTPase activity of TGase II (Im *et al*., 1990). GTP-bound TGase II appears to activate phospholipase C by lowering the calcium requirement of phospholipase C for substrate hydrolysis (Im *et al*., 1992; Das *et al*., 1993). The binding of GTP is therefore crucial for signal transduction by TGase II.

Protein database searches and GTP-binding motif searches have revealed no significant homology between TGase II and the "classical" signal-transducing heterotrimeric G-proteins or other GTP-binding proteins such as the low molecular weight GTP-binding proteins, elongation factors, and dynamin. A 36 kDa N-terminal proteolytic fragment of TGase II has been purified from rabbit liver nuclear membranes and shown to incorporate radiolabeled GTP (Singh *et al*., 1995). More recently, a 21 kDa N-terminal fragment of human TGase II, including the β-sandwich and the first 47 amino acids of the core domain, was expressed in *Escherichia coli* as a GST-fusion protein and shown to have GTPase and ATPase activity (Lai *et al*., 1996). In this study, we adopted a domain-deletion approach to identify the GTP-binding domain of TGase II. Our results demonstrate that the core domain alone has GTPase and ATPase activity. The N-terminal β-sandwich domain is not required for GTP or ATP hydrolysis. Taken together with the results of Singh *et al*. (1995) and Lai *et al*. (1996), this work indicates the GTP- and ATP-binding and hydrolysis sites of TGase II are located within a 5.5 kDa (47 amino acid) region at the start of the core domain.

EXPERIMENTAL PROCEDURES

Materials. [1,4(n)-^{3}H]Putrescine dihydrochloride (14.4 Ci/mmol) was purchased from Amersham, [α-^{32}P]-8-azidoguanosine 5′-triphosphate (7 Ci/mmol) was purchased from ICN Pharmaceuticals, and [α-^{32}P]GTP (3000 Ci/mmol), [γ-^{32}P]-GTP (30 Ci/mmol), and [γ-^{32}P]ATP (30 Ci/mmol) were purchased from Du Pont-New England Nuclear. Activated charcoal (Norit PN.5, formerly OL) was purchased from BDH. The rat TGase II (G_h) cDNA (Nakaoka *et al*., 1994) used was modified by site-directed mutagenesis (Deng & Nickoloff, 1992) to introduce two restriction sites (*Xho*I at position 642 and *Spe*I at position 1257) and to remove one restriction site (*Nco*I at position 752) without altering the amino acid sequence encoded. Guinea pig liver TGase II was obtained from Sigma. Monoclonal anti-TGase II antibody (CUB7401; NeoMarkers, CA) recognizes the region between amino acids 331 and 478 of the guinea pig sequence.

Preparation of Polyclonal Anti-TGase II Antibody. New Zealand White rabbits were injected subcutaneously with SDS−polyacrylamide gel-eluted guinea pig liver TGase II (100 μg) in complete Freund's adjuvant, followed by four booster injections (100 μg) in incomplete Freund's adjuvant after 4, 5, 9, and 13 weeks. Sera from immunized rabbits were checked by dot blotting and Western blotting. Antibodies were purified over a protein A−Sepharose column (Harlow & Lane, 1988).

Plasmid Constructs. Polymerase chain reaction was used to amplify various regions of the TGase II cDNA for subcloning as GST fusions into the *E. coli* expression vector pGEX-2T (Amrad Pharmacia Biotech). Full-length TGase II cDNA was amplified using cloned *Pfu* DNA polymerase (Stratagene), oligonucleotide primer 1, 5′GAATTCGAATTCCCACCATGGCAGAGGAGCTGG3′, and primer 2, 5′GAATTCGAATTCGCGGCCGCAAGCTTAGGCGGGGCCGATGATAACG3′. Two truncated cDNA products, one encoding the N-terminal β sandwich and core domain of TGase II (positions 1−1413) and the other encoding only the core domain of TGase II (positions 415−1413), were amplified with primer pairs 1 and 3 (5′GAATTCGAATTCGCGGCCGCAAGCTTATGTCTCCTCTTTCTCTGCCAGTTTG3′) and primer pairs 4 (5′GAATTCGAATTCCCACCATGGCATTCAATGCCTGGTGCCCAGCGG3′) and 3, respectively. All 5′ primers contained the sequence 5′GAATTCGAATTCCCACCATGGCA3′, which has two adjacent *Eco*RI restriction sites for subcloning into either pGEX-2T or the eukaryotic expression vector pMT3, a Kozak sequence (CCACC) and ATG codon for subsequent expression in mammalian cells, and an overlapping *Nco*I restriction site for subcloning into the expression vector pQE60. All 3′ primers contained the sequence 5′GAATTCGAATTCGCGGCCGCAAGCTTA3′, which has two adjacent *Eco*RI restriction sites for subcloning into pGEX-2T, a *Not*I restriction site for subcloning into pMT3, a *Hin*dIII restriction site for subcloning into pQE60, and an overlapping translation termination codon (TAA). All

amplification products were digested with *Eco*RI and cloned in-frame with GST into the *Eco*RI site of pGEX-2T. Restriction digests identified inserts in the correct orientation, and DNA sequence analysis verified that no errors had been introduced during the PCR reaction. Recombinants were designated pGEX2T-Full, pGEX2T-βSCore, and pGEX2T-Core, respectively.

Expression and Purification of GST-Fusion Proteins. *E. coli* strain M15 containing pREP4 (Qiagen) and pGEX-2T, pGEX2T-Full, pGEX2T-βSCore, or pGEX2T-Core was grown in 1 L of 2YT medium supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin sulfate to an A_{600} of 0.6−0.7. Expression of GST-fusion proteins was induced with 50 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG) (final concentration) over a 2 h incubation at 30 °C. Cell pellets were sonicated in 25 mL of buffer A [20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% (v/v) glycerol] containing 1% (v/v) Triton X-100. After centrifugation (32500*g*, 30 min, 4 °C), the supernatant fraction was mixed at room temperature with 1 mL of glutathione−Sepharose 4B equilibrated with 20 mM Tris-HCl, pH 7.2, and 150 mM NaCl. The matrix was packed into a 1 × 10 cm column and washed with 30 bed volumes of buffer A. GST-fusion proteins were eluted with buffer A containing 10 mM reduced glutathione and stored in aliquots at −80 °C. Removal of glutathione by dialysis had no effect on the TGase or GTPase activities, photoaffinity labeling, or binding of GTP−agarose by GST-fusion proteins. Protein concentration was determined by a modification of the Bradford method (Bradford, 1976) using the commercial Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as the standard. Reproducible fusion-protein yields and enzyme activities were not achieved unless freshly transformed bacteria were used consistently at the start of each round of expression and purification. The GST tag was not removed because previous work from many groups has indicated this is unnecessary [e.g., Lai *et al*. (1996) and Zwerschke *et al*. (1994)]. The integrity of the GST portion of the GST-fusion proteins was monitored by determining the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione at A_{340} in a UV spectrophotometer over 5 min at room temperature in a 500 μL reaction containing 30 pmol of GST-fusion protein, 100 mM KPO_4, pH 6.5, 10 mM CDNB (in ethanol), and 10 mM reduced glutathione, against a reaction blank containing no added enzyme. There was no significant difference in GST activity between the GST-fusion proteins and GST alone (data not shown).

SDS−PAGE, Native PAGE, and Western Blotting Analysis. Proteins were analyzed by 10% and 15% SDS−PAGE as described previously (Chen *et al*., 1996) and stained with Coomassie Blue or electroblotted onto Immobilon-P membranes and detected with goat polyclonal antibody to GST (1:500 dilution, Amrad Pharmacia Biotech) and horseradish peroxidase (HRP) conjugated anti-goat IgG (1:1000 Zymed), rabbit polyclonal antibody to guinea pig TGase II (1:3000) and HRP-conjugated anti-rabbit IgG (1:2000, Amersham) or the monoclonal antibody to guinea pig TGase II, CUB7401 [1:1000 (Birkbichler *et al*., 1985)] and HRP-conjugated anti-mouse IgG (1:2000, Amersham). Reactivity was visualized by the ECL chemiluminescence system (Amersham). For native PAGE, precast (80 × 30 × 1 mm) 5−40% gradient gels (Gradipore) were used as described elsewhere (Woodlee *et al*., 1993).

Photoaffinity Labeling of GST-Fusion Proteins. Purified GST and GST-fusion proteins (5−10 μL) were incubated with 5 μCi of [α-^{32}P]GTP or 1 μCi of [α-^{32}P]-8-azidoGTP in 20 mM HEPES, pH 7.5, 2.5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, and 10% (v/v) glycerol, in a final volume of 20 μL, for 10 min at 30 °C, then placed in an ice−water bath, and irradiated with UV light (254 nm) for 5 min. After irradiation, samples were mixed with Laemmli sample buffer (Laemmli, 1970) and incubated at room temperature for 60 min before analysis by SDS−PAGE. Autoradiographs were exposed for 6 days.

Binding of GST-Fusion Proteins to GTP−Agarose. Purified GST-fusion proteins (50 μg) were diluted to 450 μL with binding buffer [20 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM $MgCl_2$, and 0.1% (v/v) Triton X-100] and incubated at 4 °C for 30 min with 50 μL of GTP−agarose equilibrated with binding buffer. Beads were pelleted by centrifugation (13 000 rpm, 2 min), and the supernatant containing unbound protein was retained. The beads were washed three times with 1 mL of ice-cold binding buffer before both a second 30 min incubation and a final overnight incubation with the supernatant. Following another three washes with ice-cold binding buffer, bound protein was eluted by boiling in Laemmli sample buffer (Laemmli, 1970) and analyzed by SDS−PAGE.

Transglutaminase Activity. TGase activity was determined by measuring the incorporation of [^{3}H]putrescine into *N*,*N*′-dimethylcasein (Achyuthan & Greenberg, 1987) over a 60 min incubation at 37 °C in 50 μL reactions containing 2 pmol of purified GST, GST-fusion protein, or guinea pig liver TGase II, 40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 20 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM EGTA, 0.4% (w/v) *N*,*N*′-dimethylcasein (Slaughter *et al*., 1992), 1 μCi of [1,4(n)-^{3}H]putrescine dihydrochloride, 20% (v/v) glycerol, and 2 mM $CaCl_2$. Reaction blanks contained no added calcium. Reactions were stopped with the addition of ice-cold 500 mM EGTA (final concentration), and incorporated radioactivity was collected by filtration through GF/C glass fiber filters (Whatman) wet with ice-cold 50% (w/v) TCA and successively washed with 3 × 5 mL of ice-cold 10% (w/v) TCA, 5 mL of acetone−ethanol (1:1 v/v), and 5 mL of acetone. Filters were dried and solubilized in 5 mL of Ready Protein$^+$ scintillation fluid (DuPont).

GTPase and ATPase Activity. GTPase and ATPase activities were determined by the charcoal method as described (Lee *et al*., 1989) with some modifications. A 50 μL reaction containing 10 pmol of purified GST or GST-fusion protein, 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% (v/v) glycerol, 7.2 μM GTP (or ATP), and 0.8 μM [γ-^{32}P]GTP (or [γ-^{32}P]ATP) was incubated at 37 °C for 30 min. Reaction blanks contained no added enzyme. Reactions were terminated by the addition of 750 μL of ice-cold 5% (w/v) activated charcoal in 50 mM NaH_2PO_4, pH 7.4, and centrifuged (12 000 rpm, 3 min, 4 °C), and the amount of [^{32}P]P_i released was determined by scintillation counting of 400 μL of the supernatant. For inhibition assays, GST-fusion proteins or guinea pig liver TGase II were preincubated with or without GTPγS and ATPγS for 30 min on ice before addition of substrate to start the reaction.

Statistical Analyses. All comparisons were done using unpaired Student's *t*-test.

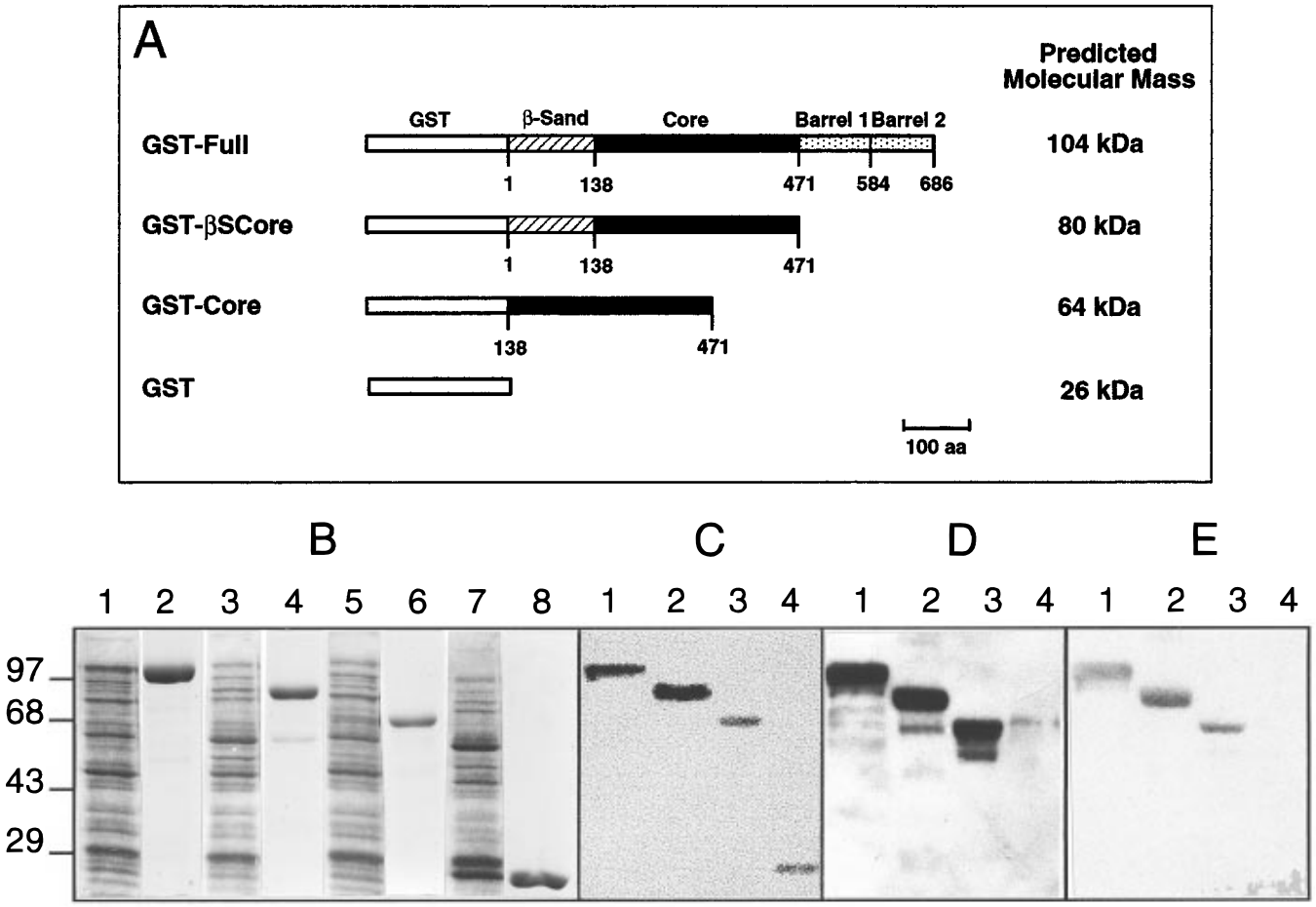


FIGURE 1: Purification and immunoreactivity of GST-fusion proteins expressed in *E. coli*. (A) Diagrammatic representation of the GST−TGase II full-length and deletion mutant fusions. Plasmid construction and protein expression in *E. coli* are detailed in Experimental Procedures. Abbreviations: GST, glutathione *S*-transferase; Full, full-length TGase II; βS, β-sandwich domain; Core, core domain of TGase II. The residue numbers at the domain boundaries of rat TGase II and the predicted molecular mass are indicated for each construct. (B) Protein samples of GST-Full (lanes 1 and 2), GST-βSCore (lanes 3 and 4), GST-Core (lanes 5 and 6), and GST alone (lanes 7 and 8) from different stages of purification were analyzed by 15% SDS−PAGE and Coomassie Blue staining. The starting material after sonication and centrifugation (lanes 1, 3, 5, and 7) was loaded onto glutathione−Sepharose 4B columns. Columns were washed with 30 bed volumes of wash buffer and GST-fusion proteins were eluted with 10 mM reduced glutathione (lanes 2, 4, 6, and 8). Molecular mass markers (kDa) are indicated. (C−E) Purified GST-Full (2.9 μg, lane 1), GST-βSCore (2.1 μg, lane 2), GST-Core (0.6 μg, lane 3), and GST alone (0.4 μg, lane 4) were subjected to 15% SDS−PAGE and transferred to Immobilon-P. Immunoblots were developed with a polyclonal anti-GST antibody (C), a polyclonal anti-TGase II antibody (D), and a monoclonal anti-TGase II antibody, CUB7401 (E).

Molecular Modeling. The three-dimensional model of the $fXIIIA_2$ zymogen, as determined experimentally by single-crystal X-ray diffraction methods, was used as a template for the modeling of the TGase II structure. The original fXIII structure was determined to 2.8 Å resolution. A more reliable molecular model has been refined against 10.0 to 2.2 Å resolution data to give a crystallographic *R*-factor of 20.0% using the computer program X-PLOR (Brunger *et al.*, 1987). The geometry for this higher resolution fXIII structure is good (rms deviation from ideality is 0.012 Å for bond lengths, 1.8° for bond angles, 25.6° for torsion angles, and 1.5° for improper torsion angles). Individually refined atomic temperature factors yield an average value of 32.5 $Å^2$. The coordinates for the original 2.8 Å resolution wild-type structure are available from the Protein Data Bank (identifying code: 1ggt); those for the most recent 2.2 Å resolution structure will be deposited shortly. A multiple alignment of 19 transglutaminase sequences along with the fXIII crystal structure was the basis for generating a homology model of TGase II using the Biosym InsightII version 2.3.0 software package (Biosym Technologies). Adjustments of the TGase II model were carried out using the computer program O (Jones *et al.*, 1991).

RESULTS

E. coli Expression and Purification of Full-Length and Domain-Deletion Mutants of TGase II. The domains of TGase II were assigned on the basis of sequence alignment with the structural domains of fXIIIA. Full-length TGase II and two domain-deletion fragments (one encoding the N-terminal β-sandwich and core domains and the other encoding only the core domain of TGase II) were amplified by the polymerase chain reaction and cloned in-frame with GST to produce the three constructs, pGEX2T-Full, pGEX2T-βSCore, and pGEX2T-Core, respectively. The resulting GST-fusion proteins, GST-Full, GST-βSCore, and GST-Core, are represented diagrammatically in Figure 1A.

Expression of the GST-fusion proteins was induced with IPTG. After sonication and centrifugation, the resulting supernatant fractions (Figure 1B, lanes 1, 3, 5, and 7) were loaded on glutathione−Sepharose 4B columns. After 30 bed volumes of column washing, GST-fusion proteins were

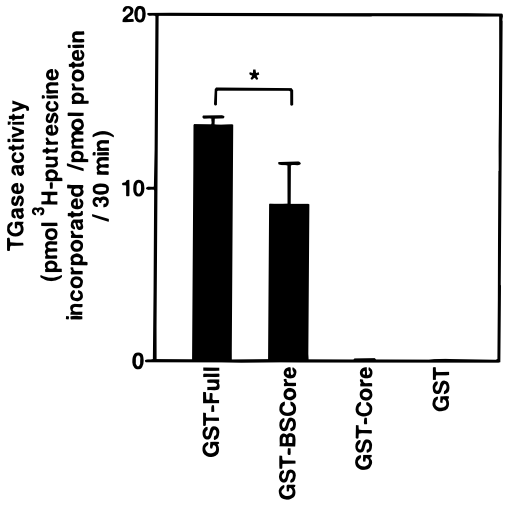


FIGURE 2: Transglutaminase activity of GST-fusion proteins. The TGase activity of 2 pmol of GST−TGase II fusion proteins or GST was determined as detailed in Experimental Procedures. Data are the means ± 1 SD (bar) of a representative experiment performed in triplicate. The asterisk indicates significant difference, $p < 0.05$.

eluted with reduced glutathione (Figure 1B, lanes 2, 4, 6, and 8). All fusion proteins migrated at their predicted molecular mass on SDS−PAGE. Average yields per liter of culture medium were 380 μg of 104 kDa GST-Full, 220 μg of 80 kDa GST-βSCore, 60 μg of 64 kDa GST-Core, and 3.8 mg of 26 kDa GST. As expected, Western blot analysis using polyclonal anti-GST antibody (Figure 1C) showed reactivity of all proteins. Both polyclonal anti-TGase II antibody (Figure 1D) and the monoclonal anti-TGase II antibody CUB7401 (Figure 1E) recognized the GST−TGase II fusion proteins but failed to recognize GST alone. Additional minor bands reacted with the polyclonal anti-TGase II antibody in the GST-βSCore and GST-Core preparations. These are most likely proteolytic fragments.

TGase Activity of GST−TGase II Fusion Proteins. Both GST-Full and GST-βSCore exhibited calcium-dependent TGase activity (Figure 2), which was comparable to that of commercially available guinea pig liver TGase II (data not shown). GST-Core and GST alone had no detectable TGase activity. The activity of GST-βSCore relative to GST-Full was consistently ~70% ($p < 0.05$).

Photoaffinity Labeling of GST−TGase II Fusion Proteins. To determine whether the GST−TGase II fusion proteins specifically bind GTP, they were covalently labeled with GTP analogues by photoactivation with ultraviolet light. GST-Full (Figure 3A, lanes 1 and 2), like guinea pig liver TGase (lanes 7 and 8), was photolabeled by [α-^{32}P]GTP in the absence and presence of 500 μM App(NH)p, indicating incorporation into a specific GTP-binding site. This photolabeling was inhibited by 500 μM GTP (lanes 3 and 9), further demonstrating specificity of binding. Since photolabeling of GST-Core was not detectable with [α-^{32}P]GTP (lanes 4−6), labeling was then evaluated with [α-^{32}P]-8-azidoGTP which we have shown previously enhances covalent modification of G_h (Im & Graham, 1990). All three fusion proteins, GST-Full (Figure 3B, lane 1), GST-βSCore (lane 3), and GST-Core (lane 5), but not GST alone (data not shown) were photolabeled in the presence of App(NH)p. The addition of GTP inhibited photolabeling (lanes 2, 4, and 6). This demonstrates not only specificity of binding but also the absence of any light-generated, long-lived chemical

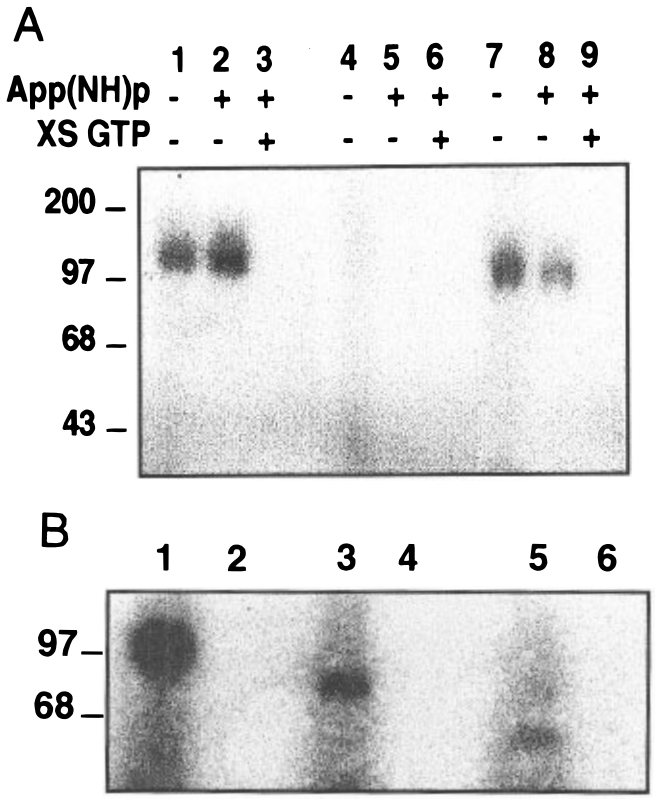


FIGURE 3: Photoaffinity labeling of GST-fusion proteins. (A) Proteins (GST-Full, 5 μg, lanes 1−3; GST-Core, 5 μg, lanes 4−6; guinea pig liver TGase II, 5 μg, lanes 7−9) were photolabeled with [α-^{32}P]GTP in the absence of excess unlabeled nucleotide (lanes 1, 4, and 7), in the presence of 500 μM App(NH)p (lanes 2, 5, and 8), or in the presence of 500 μM App(NH)p and 500 μM GTP (lanes 3, 6, and 9) as described in Experimental Procedures and analyzed by SDS−PAGE, and autoradiographs were exposed for 5−6 days. (B) GST-fusion proteins (GST-Full, 3 μg, lanes 1 and 2; GST-βSCore, 2 μg, lanes 3 and 4; GST-Core, 0.6 μg, lanes 5 and 6) were photolabeled with [α-^{32}P]-8-azidoGTP in the presence of 500 μM App(NH)p without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) 500 μM unlabeled GTP as described above. Molecular mass markers (kDa) are indicated.

intermediates which might cause pseudoaffinity labeling outside of the GTP-binding site. These results indicate that the GTP-binding site is localized within the core domain of TGase II and that neither the N-terminal β-sandwich nor the C-terminal β-barrels are essential for GTP binding.

Binding of GST−TGase II Fusion Proteins to GTP−Agarose. To further verify GTP binding to the TGase II fusion proteins, GTPγS filter-binding assays were performed with GST-Full and guinea pig liver TGase II; however, these results were variable and inconsistent. As an alternative, the binding of GST−TGase II fusion proteins to GTP−agarose was evaluated by Western analysis using an anti-TGase II antibody (Figure 4). Both GST-Full (lane 1) and GST-βSCore (lane 2) bound to GTP−agarose well, whereas binding of GST-Core was low, but detectable (lane 3). The anti-TGase II antibody reacted with additional minor bands in the GST-βSCore preparation. These are likely to have been generated by limited proteolysis. Western analysis using an anti-GST antibody detected GST-Full, GST-βSCore, and GST-Core, but not GST alone (data not shown), indicating specific GTP binding by the GST−TGase II fusion proteins.

To explain the apparent low efficiency of GTP binding by GST-Core, native gel analysis of the GST-fusion proteins

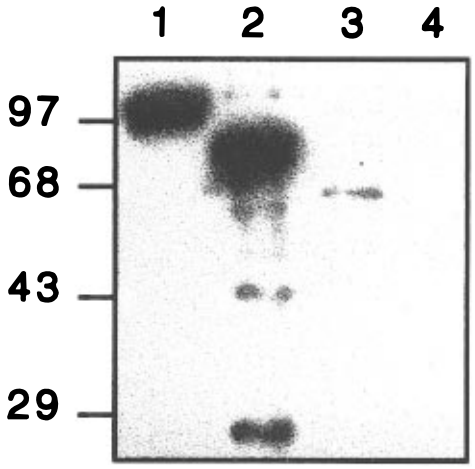


FIGURE 4: Binding of GST-fusion proteins to GTP−agarose. Fifty micrograms of GST−TGase II fusion proteins (GST-Full, lane 1; GST-βSCore, lane 2; GST-Core, lane 3; GST, lane 4) was incubated with GTP−agarose on ice as described in Experimental Procedures. Bound protein was analyzed by SDS−PAGE and immunoblotting with monoclonal anti-TGase II antibody CUB7401.

was performed (data not shown). The majority (~90%) of the GST-Core protein was present as a high molecular weight oligomer (~600 kDa) with only ~10% migrating as a monomer. GST-Full resolved as a monomer (~50%) and a higher molecular weight oligomer of ~400 kDa (~50%). GST alone ran as a dimer of ~50 kDa (100%). These data indicate that GST-Core is aggregated, and this may explain the low efficiency of binding by GST-Core to the GTP−agarose.

GTPase and ATPase Activity of GST−TGase II Fusion Proteins. Because TGase II has recently been reported to hydrolyze ATP in addition to GTP (Takeuchi *et al*., 1994), both GTPase and ATPase activities were assayed. All three GST−TGase II fusion proteins exhibited GTPase and ATPase activity comparable to that observed with guinea pig liver TGase II (Figure 5). The GTPase activity of GST-βSCore was equivalent to GST-Full, whereas the ATPase activity was consistently ~40% higher than GST-Full ($p < 0.001$). GST-Core consistently had ~50% higher GTPase activity than GST-Full and GST-βSCore ($p < 0.001$). The ATPase activity of GST-Core was ~75% higher than GST-Full ($p < 0.001$) and 30% higher than GST-βSCore ($p < 0.01$). Activities were linear over a time course of at least 35 min (data not shown). GST alone had little or no detectable GTPase and ATPase activity.

Both the GTPase and ATPase activities of the GST−TGase II fusion proteins were inhibited in a dose-dependent manner by both GTPγS and ATPγS (Figure 6). The IC_{50} values for GTPγS and ATPγS inhibition of GTPase and ATPase activity were comparable for each of the GST−TGase II fusion proteins and also for guinea pig liver TGase II, indicating that the *E. coli*-expressed proteins and the guinea pig liver-derived protein behave similarly under the conditions of this experiment.

DISCUSSION

A domain-deletion approach has been used in this study to identify the GTP/ATP binding and hydrolytic domain of TGase II. Because exons do not necessarily correspond with aspects of protein structure (Stoltzfus *et al*., 1994), a domain-deletion approach (as opposed to exon deletion or random

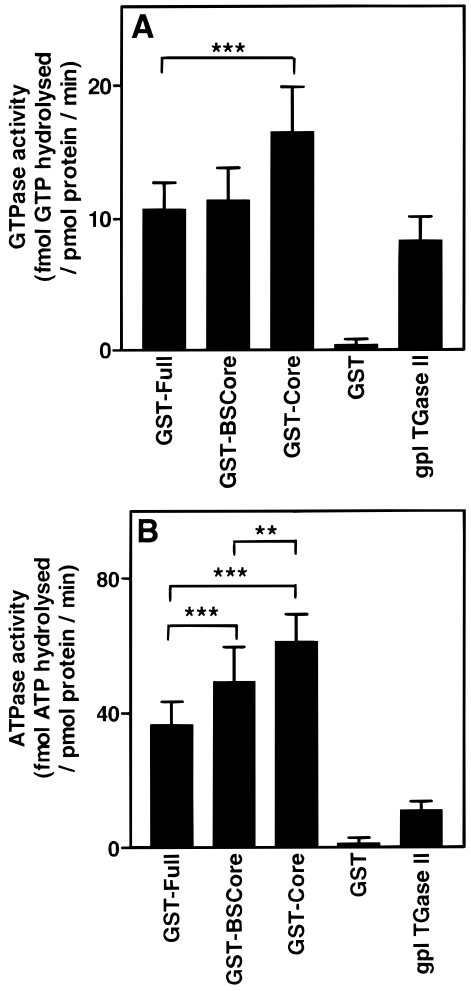


FIGURE 5: GTPase and ATPase activities of GST-fusion proteins. The GTPase (A) and ATPase (B) activities of GST−TGase II fusion proteins (10 pmol), GST (10 pmol), or guinea pig liver (gpl) TGase II (0.7 μg) were determined as detailed in Experimental Procedures. Data are the means ± 1 SD (bar) of 4−6 independent experiments performed in triplicate. Asterisks indicate significant differences: ***, $p < 0.001$; **, $p < 0.01$.

deletion of the N- or C-termini) was chosen in an effort to minimize disruption to the tertiary structure of the protein.

The GST-βSCore protein exhibited ~70% of the TGase activity of GST-Full (Figure 2), indicating the C-terminal β-barrels are not essential for TGase activity. GST-Core had no detectable TGase activity (Figure 2), indicating the β-sandwich domain is required for TGase activity. This is consistent with data from N- and/or C-terminal deletions of TGase I (Kim *et al*., 1994) and fXIIIA (Lai *et al*., 1994), which indicate that the minimal structural requirement for TGase activity is comprised of the equivalent of both the β-sandwich and core domains of TGase II. The N-terminal β-sandwich may be essential for TGase substrate binding, since the N-terminal seven residues of TGase II have been shown to be required for binding of the substrate fibronectin (Jeong *et al*., 1995). The end of the core domain of TGase II corresponds to the site of proteolytic activation of TGase III (Kim *et al*., 1990) and the second thrombin cleavage site of fXIIIA, which results in the release of the TGase catalytically active, 51 kDa fibrin-binding fragment (Takahashi *et al*., 1986; Greenberg *et al*., 1988). Recent work by Lai *et al*. (1996) indicates deletion of barrel 2, and half of barrel 1 results in a mutant TGase II that retains only 5% of the TGase catalytic activity observed for the full-length

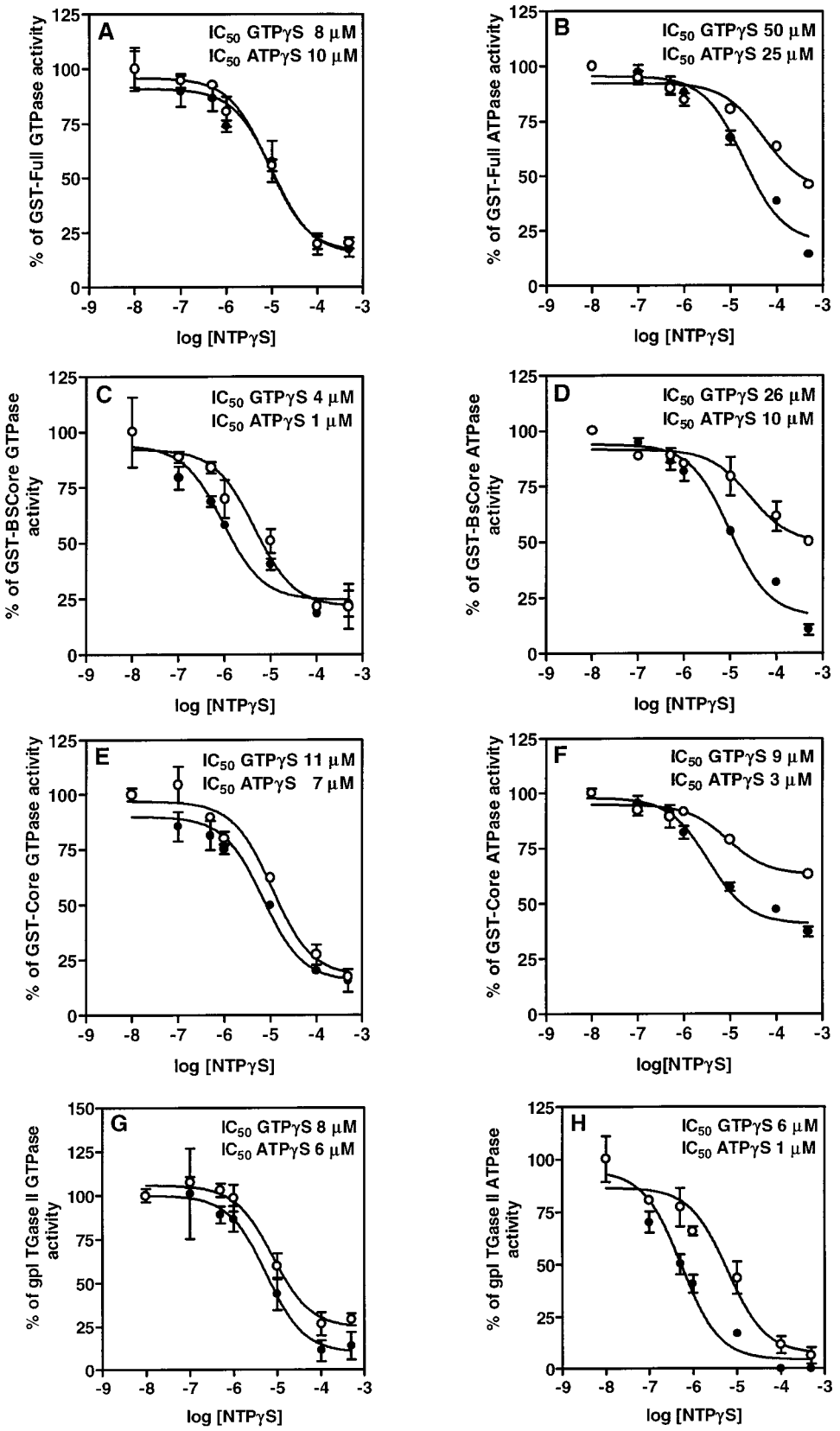
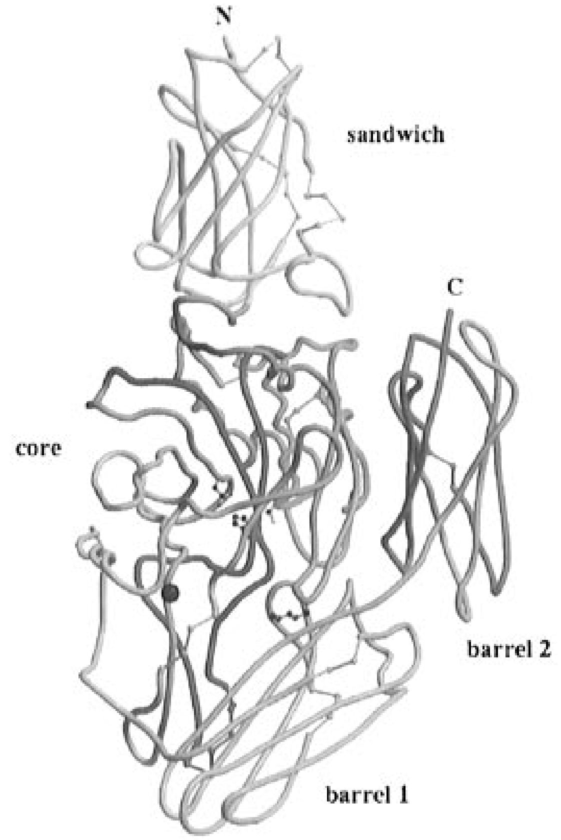


FIGURE 6: Inhibition of GTPase and ATPase activities by GTPγS and ATPγS. The GTPase (A, C, E, and G) and ATPase (B, D, F, and H) activities of 10 pmol of GST-Full (A and B), GST-βSCore (C and D), and GST-Core (E and F) or 1.4 μg of guinea pig liver (gpl) TGase II (G and H) were assayed after a 30 min preincubation on ice with GTPγS (open circles) or ATPγS (closed circles). Data are the means $\pm$ 1 SD (bar) of 1−2 independent experiments performed in triplicate. GTPase activities [fmol of GTP hydrolyzed (pmol of protein)$^{-1}$ min^{-1} $\pm$ 1 SD] were 11 $\pm$ 1 for GST-Full, 12 $\pm$ 2 for GST-βSCore, 20 $\pm$ 1 for GST-core, and 8 $\pm$ 0.5 for guinea pig liver TGase II. ATPase activities [fmol of ATP hydrolyzed (pmol of protein)$^{-1}$ min^{-1} $\pm$ 1 SD] were 33 $\pm$ 1 for GST-Full, 45 $\pm$ 0.5 for GST-βSCore, 65 $\pm$ 1 for GST-Core, and 17 $\pm$ 2 for guinea pig liver TGase II.

protein. This contrasts with the findings of the present study that demonstrate little change in TGase activity, despite deletion of both β-barrels 1 and 2. Similarly, Kim *et al.* (1994) demonstrated that TGase activity was markedly



human TGase II	139	F	N	A	W	C	P	A	D	A	V	Y	L	D	S	E	E	E	R	Q	E	158
bovine TGase II	139	F	N	S	W	C	P	A	D	A	V	Y	L	D	S	D	E	E	R	Q	E	158
rat TGase II	139	F	N	A	W	C	P	A	D	D	V	Y	L	D	S	E	A	E	R	R	E	158
mouse TGase II	139	Y	N	A	W	C	P	A	D	D	V	Y	L	D	S	E	E	E	R	R	E	158
guinea pig TGase II	139	Y	N	P	R	C	P	A	D	A	V	Y	M	D	S	D	Q	E	R	Q	E	158
chick TGase II	139	F	N	A	W	H	P	E	D	A	V	Y	L	K	E	E	D	E	R	R	E	158
Factor XIIIa	185	F	N	P	W	C	E	D	D	A	V	Y	L	D	N	E	K	E	R	E	E	204
human TGase I	243	F	N	P	W	C	P	E	D	I	V	Y	V	D	H	E	D	W	R	Q	E	262
human TGase III	134	F	N	P	W	L	N	V	D	S	V	F	M	G	N	H	A	E	R	E	E	153
rat TGase IV	128	L	N	P	W	C	S	D	D	S	V	F	M	A	S	E	E	D	R	A	E	147

								G	Φ	Ali	Aro	X	G	S	X	K												
human TGase II	159	Y	V	L	T	Q	Q	G	F	I	Y	Q	G	S	A	K	F	I	K	N	I	P	W	N	F	G	Q	F	185
bovine TGase II	159	Y	V	L	T	Q	Q	G	F	I	Y	Q	G	S	A	K	F	I	K	N	I	P	W	N	F	G	Q	F	185
rat TGase II	159	Y	V	L	T	Q	Q	G	F	I	Y	Q	G	S	V	K	F	I	K	S	V	P	W	N	F	G	Q	F	185
mouse TGase II	159	Y	V	L	T	Q	Q	G	F	I	Y	Q	G	S	V	K	F	I	K	S	V	P	W	N	F	G	Q	F	185
guinea pig TGase II	159	Y	V	L	T	Q	Q	G	F	I	Y	Q	G	S	A	K	F	I	N	G	I	P	W	N	F	G	Q	F	185
chick TGase II	159	Y	V	L	S	Q	Q	G	L	I	Y	M	G	S	R	D	Y	I	T	S	T	P	W	N	F	G	Q	F	185
Factor XIIIa	204	Y	V	L	N	D	I	G	V	I	F	Y	G	E	V	N	D	I	K	T	R	S	W	S	Y	G	Q	F	230
human TGase I	263	Y	V	L	N	E	S	G	R	I	Y	Y	G	T	E	A	Q	I	G	E	R	T	W	N	Y	G	Q	F	289
human TGase III	154	Y	V	Q	E	D	A	G	I	I	F	V	G	S	T	N	R	I	G	M	I	G	W	N	F	G	Q	F	180
rat TGase IV	148	Y	V	L	N	D	T	G	Y	M	Y	M	G	F	A	K	Q	I	K	E	K	P	W	T	F	G	R	L	174

FIGURE 7: Putative GTP- and ATP-binding region in TGase II. (A, top) A computer-generated model of TGase II was constructed as described in Experimental Procedures and drawn here as a peptide coil. The N-terminal β-sandwich of TGase II is colored yellow. The 47 amino acid region containing the putative GTP and ATP hydrolysis sites is colored orange. TGase II active site residues (Cys^{277}, His^{335}, and Asp^{358}) and Lys^{173} discussed in the text are represented as ball-and-stick side chain groups. A calcium ion, represented as the dark blue sphere, is positioned at the TGase II equivalent of the fXIIIA calcium-binding site. The rest of the molecule is colored to aid visualization of the various end points of the TGase II C-terminal deletion mutants (Lai *et al*., 1996) and the shorter TGase II isoform (Monsonego *et al*., 1997) discussed in the text: residues 186−290 are in cyan, residues 291−345 are in pink, residues 346−626 are in gray, and residues 627−686 are in green. The regions of insertions/deletions in TGase II relative to fXIIIA are indicated as bonds connecting the α-carbon atoms. This figure was generated using MOLSCRIPT (Kraulis, 1991) and Raster3D Version 2.0 (Merritt & Murphy, 1994) . (B, bottom) A comparison of the putative GTP- and ATP- binding regions of TGases II [human (Gentile *et al*., 1991); bovine (Nakanishi *et al*., 1991); rat (Nakaoka *et al*., 1994); mouse (Gentile *et al*., 1991); guinea pig (Ikura *et al*., 1988); chick (Weraarchakul-Boonmark *et al*., 1992)] with the equivalent regions in other TGases [fXIIIA (Ichinose *et al*., 1986); human TGase I (Kim *et al*., 1991); human TGase III (Kim *et al*., 1993); rat TGase IV (Ho *et al*., 1992)] is shown using the single-letter amino acid code. Shaded boxes indicate identical amino acids. A G-Φ-Ali-Aro-X-G-S-X-K motif, where Φ represents hydrophobic, Ali represents aliphatic, and Aro represents aromatic amino acids, is conserved in all TGases II (except chick) and is shown above the sequence alignment.

reduced with deletion of only part of β-barrel 1 of TGase I but was restored with complete deletion of this domain. Thus, loss of TGase activity observed by Lai *et al*. (1996) upon incomplete removal of the barrel 1 domain from TGase II most likely reflects disruption of the tertiary structure of the protein.

GST-Full, GST-βSCore, and GST-Core were specifically photolabeled with [α-^{32}P]-8-azidoGTP (Figure 3) and were able to bind GTP−agarose, although the ability of GST-Core to bind to GTP−agarose was very low (Figure 4), presumably because of the high tendency of GST-Core to aggregate when expressed alone in the absence of its surrounding domains. Despite aggregation, the GST-Core protein exhibited greater GTPase activity than GST-Full and GST-βSCore (Figure 5A). These results demonstrate that the TGase II core domain alone binds and hydrolyzes GTP. GST-Core also had greater ATPase activity than GST-Full and GST-βSCore (Figure 5B), demonstrating that neither the N-terminal β-sandwich domain nor the C-terminal β-barrels are required for either GTP or ATP hydrolysis by the core domain. The GTPase activities of GST-Full, GST-βSCore, GST-Core, and guinea pig liver TGase II were inhibited by GTPγS and ATPγS in a dose-dependent manner with similar IC_{50} values (Figure 6). However, the ATPase activity of these proteins was inhibited more potently by ATPγS than by GTPγS. Taken together with the finding that the GST−TGase II fusion proteins could be specifically photolabeled with [α-^{32}P]-8-azidoGTP, despite the presence of a high concentration of App(NH)p, this indicates that TGase II has distinct binding sites for guanine and adenine nucleotides, albeit that their binding pockets may interact or involve some residues that are common to both nucleotides. Complete resolution of this issue, however, will require more precise delineation of the GTP- and ATP-binding and hydrolytic determinants.

Previous work has shown that a 36 kDa N-terminal proteolytic fragment of TGase II, purified from rabbit liver nuclear membranes, can be photolabeled with [α-^{32}P]GTP (Singh *et al*., 1995). More recently, a 21 kDa N-terminal fragment of human TGase II (that retains the N-terminal β-sandwich and 47 amino acids of the core domain) was expressed in *E. coli* as a GST-fusion protein and demonstrated to have both GTPase and ATPase activity (Lai *et al*., 1996). Taken together with our results, this localizes the GTP and ATP hydrolysis sites to the first 47 amino acids of the core domain. Figure 7A shows a computer-generated model of TGase II based on the coordinates of the fXIIIA crystal structure. The 47 amino acid region containing the putative GTP- and ATP-binding sites is colored orange. A lysine residue is located at the base of the long loop. This may be the lysine residue that has been implicated in the GTP-binding site of erythrocyte TGase II based on covalent modification of the protein by dialdehyde−GTP (Bergamini & Signorini, 1993). A typical glycine-rich GTP-binding site consensus sequence is not present; however, a ^{165}G-Φ-Ali-Aro-X-G-S-X-K^{173} motif, where Φ represents hydrophobic, Ali represents aliphatic, and Aro represents aromatic amino acids, is conserved in all TGases II isolated to date (with the exception of chick TGase II; Figure 7B). This region of the protein interacts with part of the TGase active site main chain scaffold which contains the catalytic residues Cys^{277} and His^{335} (Figure 7A). According to the model, a conformational change induced by GTP binding could be transmitted to the TGase active site and affect TGase substrate binding and/or TGase catalytic activity.

The 36 kDa TGase II fragment purified from rabbit liver (Singh *et al*., 1995) is approximately equivalent in size to one of the C-terminal deletion mutants of human TGase II (P345) described by Lai *et al*. (1996). P345 had 34-fold higher GTP and ATP hydrolytic activity than GST-Full. Modeling studies indicate that residues 291−303 and 333−345 of human TGase II (colored pink in Figure 7A) lie close to the putative GTP-binding loop. The absence of the remaining residues of TGase II (346−687 colored gray and green) may further expose the loop for GTP and ATP binding or help to stabilize the conformation of the loop for increased GTP and ATP hydrolysis. Further deletion to C290 (Lai *et al*., 1996) results in an 8-fold drop in nucleotide hydrolytic activity relative to P345.

The TGase activity of a shorter isoform of TGase II, which lacks half of barrel 2 resulting from alternate splicing of the TGase II transcript (Monsonego *et al*., 1997), has recently been shown to be unaffected by GTP. Modeling studies indicate that the residues that are absent from the shorter form (colored green, Figure 7A) are adjacent to the putative 47 amino acid GTP- and ATP-binding domain of full-length TGase II. This suggests that tertiary structure disruption resulting from the absence of half of barrel 2 either abolishes GTP binding or prevents the GTP-induced conformational change in TGase II responsible for inhibition of its TGase activity. However, since the GTPase activity of this isoform was not investigated, it is unclear which of these mechanisms is operative.

The results of the experiments reported here, taken together with the recent results of Lai *et al*. (1996), indicate that a 5.5 kDa stretch of the core domain, involving 47 residues at the start of this domain, contains the GTP and ATP hydrolysis sites. In view of the unexplained superactivity of the truncation proteins reported in Lai *et al*. (1996), this conclusion should remain a working hypothesis until further experiments confirm its veracity.

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