

Tissue Transglutaminase Has Intrinsic Kinase Activity

IDENTIFICATION OF TRANSGLUTAMINASE 2 AS AN INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-3 KINASE*

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Tissue transglutaminase (TG2) is a ubiquitous enzyme that cross-links glutamine residues with lysine residues, resulting in protein polymerization, cross-linking of dissimilar proteins, and incorporation of diamines and polyamines into proteins. It has not previously been known to have kinase activity. Recently, insulin-like growth factor-binding protein-3 (IGFBP-3) has been reported to be phosphorylated by breast cancer cell membranes. We purified the IGFBP-3 kinase activity from solubilized T47D breast cancer cell membranes using gel filtration, ion-exchange chromatography, and IGFBP-3 affinity chromatography. The fractions containing kinase activity were further purified by high pressure liquid chromatography and analyzed by tandem mass spectroscopy. TG2 was detected in fractions containing kinase activity. Antisera to TG2 and protein A-Sepharose were used to immunoprecipitate TG2 from membrane fractions. The immunoprecipitates retained IGFBP-3 kinase, whereas immunoprecipitation deleted kinase activity in the membrane supernatant. The inhibitors of TG2, cystamine and monodansyl cadaverine, abolished the ability of the T47D cell membrane preparation to phosphorylate IGFBP-3. Both TG2 purified from guinea pig liver and recombinant human TG2 expressed in insect cells were able to phosphorylate IGFBP-3. TG2 kinase activity was inhibited in a concentration-dependent fashion by calcium, which has previously been shown to be important for the cross-linking activity of TG2. These data provide compelling evidence that TG2 has intrinsic kinase activity, a function that has not previously been ascribed to TG2. Furthermore, we provide evidence that TG2 is a major component of the IGFBP-3 kinase activity present on breast cancer cell membranes.

IGFBP-3 can both enhance and inhibit the effects of IGF-I *in vivo* and *in vitro* depending upon experimental conditions (2–6). Enhancement of IGF-I action may result from enhanced delivery of IGF-I to its plasma membrane receptor, whereas inhibition may be a consequence of competition between IGFBP-3 and the type 1 IGF receptor for binding of IGF-I (1, 2).

In addition to these IGF-dependent effects, emerging evidence suggests that IGFBP-3 also functions directly to stimulate apoptosis and inhibit cellular proliferation of various cell lines, including human breast cancer cells (4). However, these IGF-independent effects are only apparent under conditions where the IGF-I-dependent effects are not observed, such as studies with mutant IGFBP-3 and IGFBP-3 fragments that have minimal affinity for IGF-I (5, 6) and with cell lines devoid of IGF-I receptors (3).

To further understand the mechanisms that allow for these opposing effects of IGFBP-3, we have investigated the interaction of IGFBP-3 with breast cancer cell membranes. In addition to proteolysis we have recently reported that IGFBP-3 is phosphorylated by breast cancer cells by a process that occurs on the cell membranes, does not require internalization, and is inhibited by IGF-I (7). Phosphorylation of IGFBP-3 by this membrane-associated kinase enhanced the binding affinity of IGFBP-3 for IGF-I (7). Thus, phosphorylation of IGFBP-3 at the membrane favors the interaction of IGF-I with IGFBP-3 rather than the IGF-I receptor. Furthermore, because formation of IGF-I-IGFBP-3 complexes inhibits binding of IGFBP-3 to the cell membrane, phosphorylation of IGFBP-3 may modulate its pro-apoptotic anti-proliferative effects. To further understand the role of this kinase in physiological regulation of IGFBP-3 action, we purified this kinase activity from T47D breast cancer cells. Here we report that this kinase activity is attributable to TG2.

TG2 is a widely expressed enzyme that is involved in polymerization and aggregation of proteins via cross-linking glutamine residues. Although it contains a GTP binding domain and can hydrolyze both GTP and ATP (8), it has not previously been reported to have kinase activity.

EXPERIMENTAL PROCEDURES

Materials and Reagents—T47D and MCF-7 cells were obtained from the American Type Tissue Collection (Manassas, VA). Cell culture reagents were from Invitrogen. Glycosylated and non-glycosylated IGFBP-3 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant TG2 was purchased from Roboscreen. Guinea pig liver TG, the inhibitors cystamine and monodansyl cadaverine (MDC), biotinylated phospho-specific monoclonal antibodies, streptavidin-horseradish peroxidase conjugate and all other reagents, unless otherwise stated, were obtained from Sigma-Aldrich.

Biotinylation of IGFBP-3—Non-glycosylated *Escherichia coli*-derived IGFBP-3 was biotinylated using *p*-biotinoyl-aminocaproic acid-*N*-hydroxy-succinamide ester (Roche Applied Science) as previously described (7).

Purification of IGFBP-3 Kinase Activity—Solubilized T47D cell mem-

Insulin-like growth factor-binding protein-3 (IGFBP-3)¹ is the most abundant of the IGFBPs in the circulation. It is a multifunctional protein that not only transports insulin-like growth factors (IGF)-I and -II and modulates the actions of these growth factors but also has IGF-independent anti-proliferative and proapoptotic effects (1).

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¹ The abbreviations used are: IGFBP-3, insulin-like growth factor-binding protein-3; MDC, monodansyl cadaverine.

branes were prepared using a membrane preparation kit (Pierce, Rockford, IL) according to the manufacturer's instructions in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 mM aprotinin, and 10 μ g/ml leupeptin). 3 ml of solubilized membranes was filtered through a 0.22- μ m filter and loaded on a 16/60 Sephacryl S-100 gel filtration column that had been equilibrated with 20 mM Tris, 0.02% NaN_3 , pH 7.5. The eluate was monitored for absorbance at 280 nm through a Pharmacia UV-1 single path monitor. 1-ml fractions were collected at a flow rate of 1 ml/min and stored at -70°C . The activity was consistently found in fractions within molecular mass range of 65–85 kDa. A 20- μ l aliquot of each fraction was assayed for IGFBP-3 kinase activities. Active fractions were pooled and concentrated with an Amicon Centricon 30 filter. A buffer-exchanged sample was passed through a High Q anion exchange column (Bio-Rad) that had been equilibrated with 50 mM Tris, pH 8.0, containing 0.05 M NaCl, 0.02% NaN_3 . Separation was performed in a linear gradient from 0.05–0.5 M NaCl over 50 min at flow rate of 1 ml/min, and 1-ml fractions were collected. Fractions containing kinase activity were concentrated using an Amicon Centricon filter and desalted. The buffer was exchanged using Micro Bio-Spin chromatography columns (Bio-Rad) and loaded onto an IGFBP-3-Sepharose 4B affinity column (2-ml bed volume). Bound proteins were first eluted with 0.05 M sodium phosphate containing 0.15 M NaCl, pH 7.2, followed by 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0. Eluted fractions were desalted, concentrated, and used for IGFBP-3 kinase assay. A fraction with IGFBP-3 kinase activity was processed for liquid chromatography mass spectroscopy.

For liquid chromatography mass spectroscopy analysis, 100 μ l of desalted affinity fraction was digested with sequencing grade trypsin. The peptide mixture was lyophilized and resuspended in 10 μ l of 0.05% trifluoroacetic acid and used for μ HPLC-matrix-assisted laser desorption ionization quadrupole time-of-flight analysis. Chromatographic separation was performed using an Agilent 1100 Series system. Samples (5 μ l) were injected into a 150 $\mu\text{m} \times 150$ mm column (5 μ , Vydac 218 TP C18) and eluted with 1–80% acetonitrile (0.1% trifluoroacetic acid) in 60 min. Major ion peaks of the total ion chromatogram were analyzed by mass spectrometry in a Manitoba/Sciex prototype quadrupole time-of-flight mass spectrometer. In this instrument, ions are produced by irradiation of the target using proton pulses from a 20-Hz nitrogen laser; the mass accuracy is within a few mDa in time-of-flight spectra. Identification of the tryptic peptides was done by data base searching against the peptide fingerprints using the Mascot search engine (www.matrixscience.com).

Phosphorylation of IGFBP-3—Polystyrene tubes were coated with streptavidin and blocked with bovine serum albumin, washed in saline, and stored at -20°C until used. Biotinylated IGFBP-3 (500 ng) was added in streptavidin-coated tubes for 2 h on ice. At the end of incubation, excess unbound IGFBP-3 was removed. Tubes were placed on ice, and a phosphorylation reaction mixture containing 20 mM Tris-buffered saline, pH 7.5, 10 mM Mg/ATP, 60 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP was added. Reaction was initiated by the addition of a membrane fraction and allowed to proceed for 30 min at 30°C . Reaction was stopped by the addition of SDS-PAGE sample buffer, boiled for 7–10 min, and analyzed on 11% gel. Subsequently, gels were dried and processed for autoradiography. In some cases two microunits of guinea pig liver TG2 or histidine-tagged full-length human TG2 expressed using the baculovirus expression system in insect cells and purified by nickel(II)-nitroacetate-agarose chromatography was used to phosphorylate IGFBP-3 in the presence or absence of TG2-specific inhibitors. To determine the stoichiometry of IGFBP-3 phosphorylation, the reaction was carried out in a final volume of 500 μ l containing 10 μ g of substrate and 5 μ g of transglutaminase. Aliquots were removed at various time points, mixed with sample buffer, and boiled for 5 min. Phosphorylated proteins were separated on SDS-PAGE, and gels were stained with Coomassie Blue. Bands corresponding to phosphorylated IGFBP-3 were excised and their radioactivity measured. Kinetic parameters were determined by linear regression analysis. For the Lineweaver-Burk plot, phosphate was incorporated into IGFBP-3 incubated at various concentrations in the presence of 0.25 μ g of transglutaminase for 15 min. In experiments where cell monolayers were used to phosphorylate IGFBP-3, cells were grown in 24-well culture plates to near confluence and washed with phosphate-buffered saline to remove residual medium and serum. Phosphorylation was then performed in 100 μ l of reaction mixture as above, containing 1 μ g of IGFBP-3 for 10 min at 37°C . At the end of incubation, the reaction mixture was aspirated, and the reaction was stopped by the addition of sample buffer and analyzed on 11% gel. In some experiments cell monolayers were treated with TG2 inhibitor for 30 min prior to the phosphorylation reaction.

Investigation of Phosphorylation Sites in IGFBP-3—The potential phosphorylation sites in IGFBP-3 were identified by the NetPhos 2.0

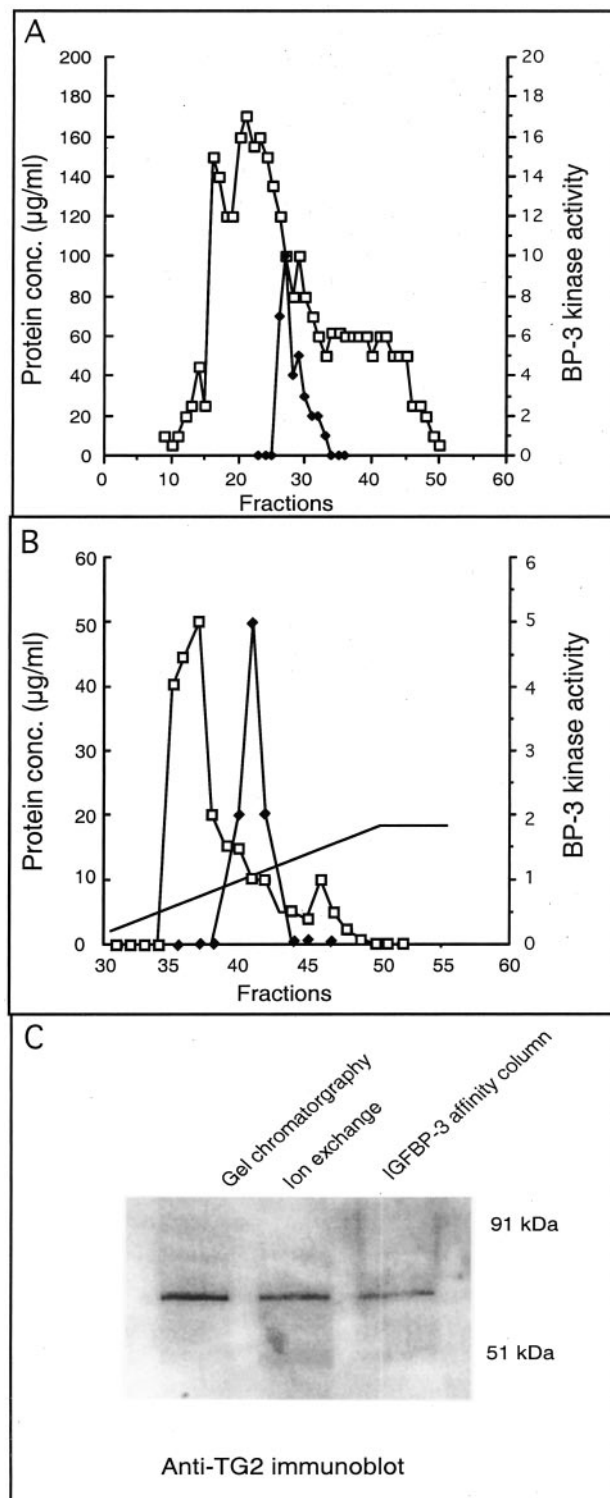


FIG. 1. Purification of IGFBP-3 kinase from T47D cells and identification as transglutaminase. Solubilized membranes were chromatographed on Sephacryl S-100 (A), and fractions containing kinase activity (filled symbols) were pooled and analyzed on a High Q anion exchange column (B). The active fractions (38–43) were pooled and applied to an IGFBP-3 affinity column. The fractions from each purification step were analyzed by immunoblot using TG2 antiserum (C).

program (www.cds.dtu.dk/Services/NetPhos) under low stringency conditions and by the Scansite program (scansite.mit.edu) using medium stringency conditions. For determination of serine-, threonine-, and tyrosine-specific phosphorylation within the IGFBP-3 molecule, intact IGFBP-3 and IGFBP-3 synthetic peptides (107–138 and 152–181) were phosphorylated using recombinant TG2. Phosphorylated proteins and

TABLE I
Proteins whose tryptic peptide fragments were identified by HPLC/tandem mass spectroscopy in IGFBP-3 affinity column fraction containing kinase activity

Protein	Score ^a	Accession number
Transglutaminase	50	P21980
ROCK2	48	NP-004841
WW domain containing adaptor isoform 1	37	NP-057712
Glutamate receptor	36	AAD15616
G protein-coupled receptor GPR 44	35	AAD21055
KIAA0322	33	BAA20780

^a Score determined as $-10\log(P)$, where P is the probability that the observed match is a random event. A score >40 indicates identity or extensive homology, $p < 0.05$.

TABLE II
Transglutaminase peptide fragment identified by tandem mass spectroscopy in IGFBP-3 affinity column fraction containing kinase activity

TG2 residues	Sequence
31–35	LVVRR
223–240	VVSGMVNCNDDQGVLLGR
422–433	VGLKISTKSVGR
553–562	DCLTESNLIK

peptides were separated on 14% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with biotinylated-phospho-specific monoclonal antibodies (dilution 1:8000; Sigma-Aldrich) overnight at 4 °C. Subsequently, membranes were incubated with streptavidin-horseradish peroxidase conjugate (dilution 1:40,000) for 1 h at room temperature and detected using the ECL detection system (Amersham Biosciences).

Immunoprecipitation—10 μ l of anti-TG2 goat polyclonal antiserum (Upstate Biotechnology) was added to 200 μ l of solubilized membranes from T47D or MCF-7 cells and incubated for 1 h at 4 °C. 20 μ l of Protein A-agarose (Pierce) was added and further incubated on a rotating device overnight at 4 °C. At the end of incubation the pellet was washed four times in ice-cold phosphate-buffered saline. The supernatant was discarded, and the pellet was resuspended in 50 μ l of kinase buffer. 10 μ l of the resuspended sample was used for phosphorylation of 500 ng of IGFBP-3. The samples were analyzed by SDS-PAGE, autoradiography, and immunoblotting with TG2 antiserum.

Western Blotting—Various column fractions that had IGFBP-3 kinase activity were analyzed on 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk, incubated with TG2 antiserum diluted to 1:1000, washed three times (5 min each) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0), and incubated with horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology) secondary antibody (1:3000 dilution) for 1 h at room temperature. Membranes were washed three times in TBST and subsequently analyzed with ECL.

RESULTS

Purification of IGFBP-3 Kinase Activity from T47D Cell Membranes—The IGFBP-3 kinase activity was purified from solubilized T47D cell membranes using immobilized biotinylated IGFBP-3 as a substrate. A three-step procedure was used involving gel permeation, ion exchange, and IGFBP-3 affinity chromatography (Fig. 1). Fractions eluted from the IGFBP-3 affinity column under acidic conditions that contained IGFBP-3 kinase activity were further analyzed by high pressure liquid chromatography and tandem mass spectroscopy. Using the Mascot search engine a variety of proteins were identified that had significant scores (Table I). Of these, TG2 had the highest score, and there was wide coverage over the entire TG2 molecule with peptides from various regions of the molecule identified. Tandem mass spectroscopy was used to confirm the sequence of various peptide fragments (Table II).

Fractions containing peak IGFBP-3 kinase activity from the various purification steps were analyzed by immunoblot using TG2 antiserum. Immunoreactive TG2 was present in all three

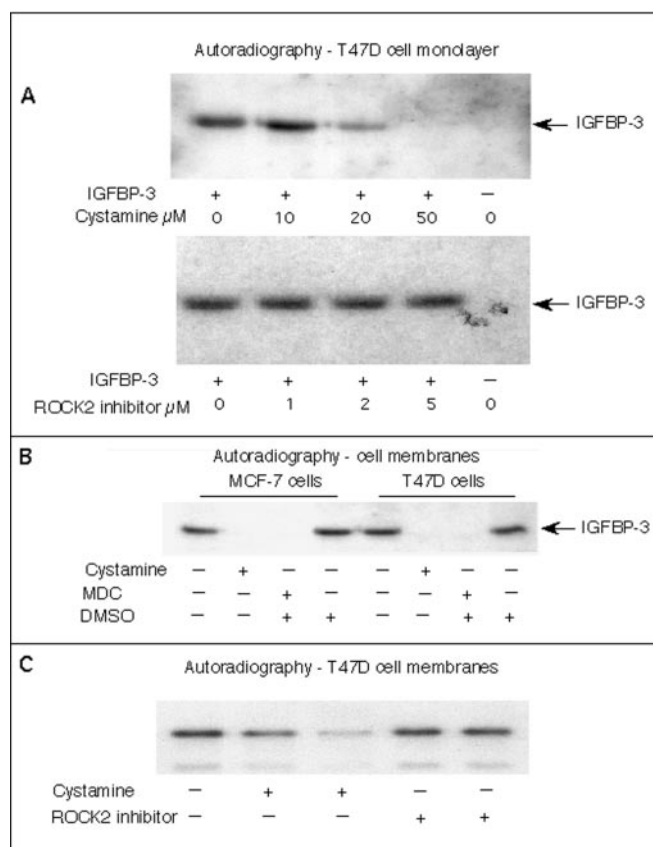


FIG. 2. The effect of TG2 and ROCK2 inhibitors on phosphorylation of IGFBP-3 by breast cancer cell monolayers (A) or cell membranes (B and C). A, various concentrations of cystamine or the ROCK2 inhibitor R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride were preincubated with T47D cell monolayers prior to determining the ability of the cell monolayer to phosphorylate IGFBP-3. B, cystamine (20 μ M), monodansyl cadaverine (MDC, 200 μ M), or the vehicle Me₂SO (DMSO) (0.005%) was added to MCF-7 and T47D cell membranes, and the ability of these membranes to phosphorylate IGFBP-3 was determined. C, the ability of cystamine (10 and 20 μ M) and the ROCK2 inhibitor R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride (2 and 5 μ M) to inhibit phosphorylation of IGFBP-3 by affinity-purified T47D cell membranes was compared.

samples (Fig. 1C). ROCK2 was also detectable in T47D cell membrane fractions.

We assessed the effect of the TG2 and ROCK2 inhibitors on phosphorylation of IGFBP-3 by cell monolayers. We have previously shown that intact washed cells were able to phosphorylate IGFBP-3 immobilized on polystyrene tubes (7). T47D cell monolayers were incubated for 30 min in the presence of various concentrations of cystamine, an inhibitor of TG2. The washed cell monolayers were incubated with IGFBP-3 in the presence of [γ -³²P]ATP for 10 min at 37 °C. After termination of the incubation, the IGFBP-3 was analyzed by SDS-PAGE and autoradiography (Fig. 2A). Inhibition was seen with as little as 20 μ M, and complete inhibition was apparent with 50 μ M of cystamine. Because TG2 has been found in association with ROCK2, a Rho-kinase (9), and ROCK2 was present in the affinity-purified cell membrane fractions, we also investigated the effect of the ROCK2 inhibitor, R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride. The ROCK2 inhibitor had no effect on IGFBP-3 phosphorylation. Similar experiments were undertaken utilizing membrane preparations from both T47D and MCF-7 cells. As reported previously, both T47D and MCF-7 cells were able to phosphorylate IGFBP-3. This process was inhibited by cystamine and MDC in both cells lines (Fig. 2B). Me₂SO, the vehicle in which

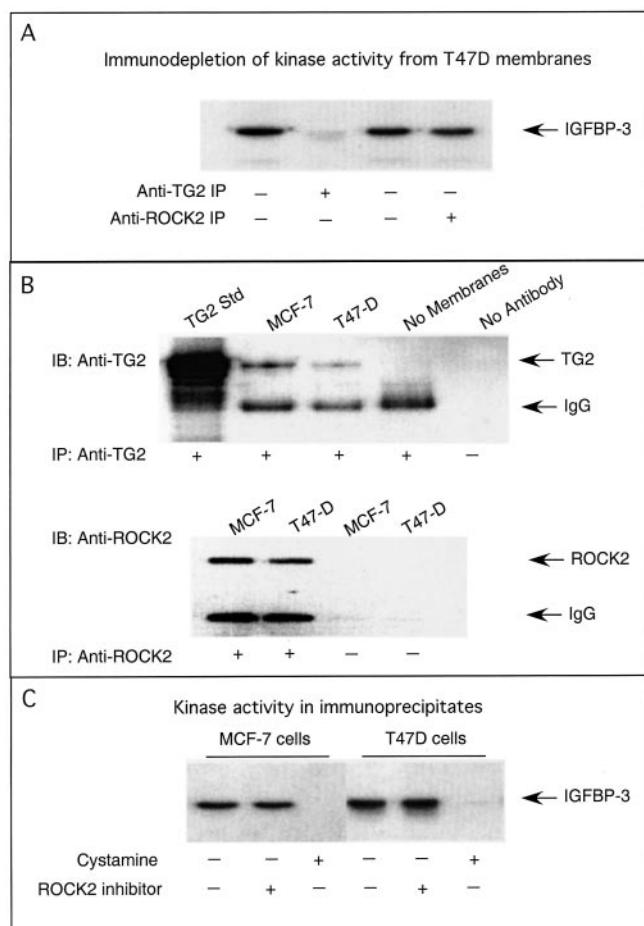


FIG. 3. Immunodepletion and immunoprecipitation of IGFBP-3 kinase activity from T47D solubilized membranes. *A*, solubilized T47D cell membranes were incubated with antibodies to TG2 or ROCK2. After the immunoprecipitates were pelleted using protein A-agarose, the supernatants were tested for the ability to phosphorylate IGFBP-3. *B*, TG2 and ROCK2 were immunoprecipitated from solubilized MCF-7 or T47D cell membranes and analyzed on SDS-PAGE. To demonstrate the presence of these two proteins in the immunoprecipitates, the membrane was immunoblotted with anti-TG2 or anti-ROCK2 antibodies. *C*, the presence of IGFBP-3 kinase activity in the anti-TG2 immunoprecipitates was demonstrated, and ability of cystamine (20 μ M) and the ROCK2 inhibitor (5 μ M) to inhibit this kinase activity was assessed.

MDC was dissolved, had no effect. The ROCK2 inhibitor had no effect on IGFBP-3 phosphorylation (Fig. 2C).

We next examined the effect of TG2 and ROCK2 antisera on IGFBP-3 kinase activity present in cell membrane preparations. Antiserum against TG2, but not ROCK2 antiserum, was able to immunodeplete IGFBP-3 kinase activity from membrane preparations (Fig. 3A). When the immunoprecipitates were analyzed for kinase activity, the precipitates obtained with TG2 antiserum, but not those obtained with ROCK2 antiserum, had kinase activity. Furthermore, the IGFBP-3 kinase activity present in the immunoprecipitates was inhibited by cystamine but not by the ROCK2 inhibitor (Fig. 3C).

Purified guinea pig liver TG2 and human recombinant TG2 both were able to phosphorylate IGFBP-3 (Fig. 4A). This process was inhibited by cystamine and MDC. The related binding protein IGFBP-5 was also phosphorylated by TG2, whereas IGFBP-1 was not phosphorylated by TG2 (Fig. 4B). Consistent with a previous report (10), an increase in the molecular mass of IGFBP-1 was observed, suggesting that TG2 can polymerase IGFBP-1 (data not shown). Fibronectin, another reported substrate for TG2 (11), was not phosphorylated by this enzyme

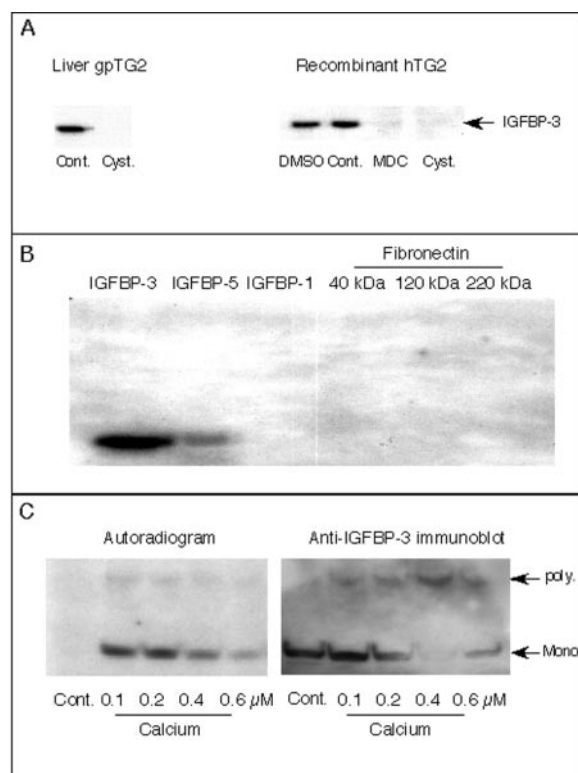


FIG. 4. Purified guinea pig (gp) liver TG2 and recombinant human TG2 have IGFBP-3 kinase activity. *A*, the effect of cystamine (cyst, 20 μ M) and MDC (200 μ M) on the IGFBP-3 kinase activity of guinea pig and recombinant human TG2 was examined and compared with controls (cont.) or vehicle only (Me₂SO, DMSO, 0.005%). *B*, the ability of recombinant human TG2 to phosphorylate IGFBP-1, IGFBP-5, fibronectin, and fibronectin fragments was investigated. *C*, the effect of increasing calcium concentration on human recombinant TG2 kinase activity and cross-linking activity was investigated. An autoradiogram demonstrating the effect of increasing calcium concentration on TG2 kinase activity is shown (left panel). In the control lane (cont.), IGFBP-3 in the absence of TG2 and [γ -³²P]ATP was loaded. In the right panel the same blot has been immunoblotted with anti-IGFBP-3 antibody. The arrows indicate the positions of polymerized (poly) and monomeric (mono) IGFBP-3.

under the conditions we used to phosphorylate IGFBP-3 (Fig. 4B).

Because calcium is necessary for the cross-linking activity of TG2 (12), we examined the effect of increasing concentrations of calcium on the kinase activity of TG2. As the calcium concentration was increased we observed a decrease in the ability of TG2 to phosphorylate IGFBP-3 (Fig. 4C, left panel). Concomitantly, there was an increase in polymerization of IGFBP-3 observed (Fig. 4C, right panel). Polymerized IGFBP-3 was only lightly phosphorylated compared with monomer IGFBP-3 (Fig. 4C, left panel).

The stoichiometry of phosphorylation of IGFBP-3 by TG2 was next examined (Fig. 5). Under maximal conditions \sim 2.5 pmols of ³²P was incorporated per pmol of IGFBP-3, indicating the presence of at least three potential TG2 phosphorylation sites in IGFBP-3. The K_m and V_{max} for the TG2 phosphorylation reaction was $13.2 \pm 1.2 \mu$ M and 5.2 pmol/min/ μ g of IGFBP-3, respectively.

Immunoblotting of TG2-phosphorylated IGFBP-3 with anti-phospho-amino acid-specific antibodies yields a strong reaction with anti-phosphoserine antibody (Fig. 6), a weak reaction with anti-phosphothreonine, and no reaction with anti-phosphotyrosine (data not shown). Using NetPhos 2.0 under low stringency a total of 15 serine and 5 threonine residues were identified (Table III). Of these, 13 serine and 2 threonine resided in

FIG. 5. The stoichiometry and kinetics of phosphorylation of IGFBP-3 by TG2. TG2 phosphorylation of IGFBP-3 was carried out as described under "Experimental Procedures." Aliquots were removed at various time points. For the Lineweaver-Burk plot, phosphate was incorporated into IGFBP-3 incubated at various concentrations in the presence of 0.25 μ g of transglutaminase for 15 min.

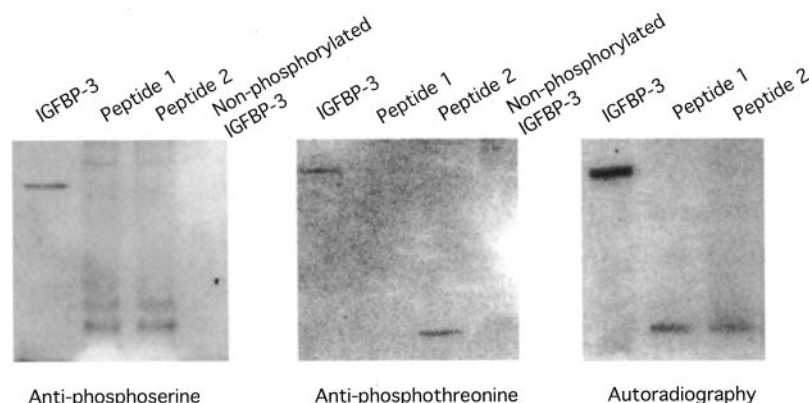
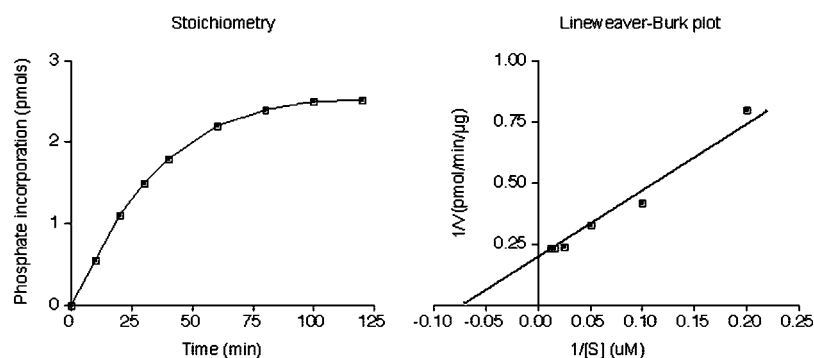


FIG. 6. Phosphorylation of IGFBP-3 and IGFBP-3 peptides by TG2. Equimolar amounts of IGFBP-3 or IGFBP-3 peptides 1 and 2 were phosphorylated with TG2 and analyzed on SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes and immunoblotted with either anti-phosphoserine or anti-phosphothreonine antibodies. Non-phosphorylated IGFBP-3 was included as a control. In some reactions [γ - 32 P]ATP was included, and phosphorylation was detected by autoradiography.

TABLE III
Identification of potential phosphorylation sites in human IGFBP-3

Position	Context	Score ^a	Prediction	Included in peptide ^b
70	RCQSPDEA	0.991	S	
111	PGNAESEEE	0.988	S	Peptide 1
113	NAESEEDR	0.997	S	Peptide 1
118	EEDRSAGSV	0.866	S	Peptide 1
121	RSAGSVESP	0.994	S	Peptide 1
124	GSVESPSVS	0.935	S	Peptide 1
126	VESPSVSST	0.985	S	Peptide 1
129	PSVSSTHRV	0.760	S	Peptide 1
130	SVSSTHRVS	0.689	T	Peptide 1
134	THRVSDFPK	0.958	S	Peptide 1
156	HAKDSQRYK	0.854	S	Peptide 2
165	VDYESQSTD	0.840	S	Peptide 2
174	TQNFSESSEK	0.955	S	Peptide 2
175	QNFSESSEK	0.767	S	Peptide 2
177	FSSESSEK	0.996	S	Peptide 2
181	SKRETEYGP	0.910	T	Peptide 2
193	EMEDTLNHL	0.582	T	
227	QCRPSKGRK	0.996	S	
249	LPGYTTKGG	0.653	T	
250	PGYTTKGGK	0.904	T	

^a Score derived from NetPhos 2.0 program (www.cbs.dtu.dk/services/NetPhos/). The closer the score is to 1, the more likely the site represents an actual phosphorylation site.

^b Sequences contained within peptides 1 or 2.

the central domain, residues 107–181. Using Scansite software at medium stringency only four potential phosphorylation sites were identified, serine 121, serine 156, threonine 170, and threonine 181. Two peptides were generated corresponding to residues 107–138 (peptide 1) and residues 152–181 (peptide 2) and covering the two clusters of potential phosphorylation sites. These two peptides included all potential phosphorylation sites with the exception of residues serine 70, threonine 181 (blocked in peptide 2), threonine 193, serine 227, and threonine 249 and 250. Both peptides were phosphorylated by TG2, and densitometric analysis of autoradiographs indicated that peptide 1 accounted for $44.2 \pm 1.17\%$ of the phosphorylation of IGFBP-3, whereas peptide 2 accounted for $40.3 \pm 3.3\%$. Both

peptides were recognized by an anti-phosphoserine-specific antibody (Fig. 6). Peptide 2, corresponding to residues 152 to 181, was also recognized by an anti-phosphothreonine antibody. Because threonine 181 is blocked and threonine 130 in peptide 1 is apparently not phosphorylated, most likely threonine 170, predicted by Scansite software, is phosphorylated.

DISCUSSION

Analysis of purified fractions from breast cancer cells containing IGFBP-3 kinase activity identified a number of potentially interesting proteins. Of these only ROCK2 was previously known to have kinase activity, and we assumed that this was responsible for phosphorylation of IGFBP-3. However, a specific inhibitor of ROCK2 kinase activity had no effect on the IGFBP-3 kinase activity of breast cancer cell monolayers or purified membrane preparations. Furthermore, immunoprecipitation of ROCK2 from membrane preparation did not deplete the IGFBP-3 kinase activity, whereas this activity could be completely removed by immunoprecipitation with TG2 antiserum and blocked by TG2 inhibitors. These data, together with the demonstration that both guinea pig liver TG2 and recombinant human TG2 could phosphorylate IGFBP-3, provided convincing evidence that TG2 can function as an ectokinase in breast cancer cells. Furthermore, it appears to account for virtually all the IGFBP-3 kinase activity present on the membrane of these cells because very little residual activity was apparent after immunoprecipitation of TG2 from breast cancer membrane preparations. We have previously shown that IGFBP-3 can also be phosphorylated by an ectokinase present on COS cells (7) and human umbilical vein endothelial cells.² The latter is particularly relevant because endothelial cells are known to express high levels of TG2 on their plasma membranes (13).

Phosphorylation of IGFBP-3 by TG2 was predominantly at the multiple serine residues in the central domain of the molecule. This domain shares sequence similarity with IGFBP-5,

² S. Mishra and L. J. Murphy, unpublished observations.

which is also phosphorylated by TG2. In contrast, the central domain of IGFBP-1 is quite distinct from IGFBP-3, and this probably accounts for the inability of TG2 to phosphorylate IGFBP-1. Hoeck and Mukku (14) report that serine to alanine double mutations at residues 111 and 113 resulted in an 80% reduction of phosphorylated IGFBP-3 secreted by transfected Chinese hamster ovary cells. However, the kinase responsible was not identified, and it is not clear what effect, if any, such mutations would have on availability of other serine residues for phosphorylation. It is possible that serine 111 and serine 113 are the sites of TG2 phosphorylation in peptide 1. However, peptide 1 only accounted for ~44% of the phosphorylation of IGFBP-3 by TG2. Other serine residues further upstream in peptide 2 were also phosphorylated by TG2. In addition to serine phosphorylation, we provide evidence that TG2 also results in phosphorylation at a threonine residue, most probably threonine 170.

TG2 is a ubiquitous enzyme that has been implicated in a variety of biological processes. It is important in post-translational protein modification and protein-protein interactions. It functions as a calcium-dependent transamidating acyltransferase that cross-links glutamine residues with lysine residues in the same proteins, resulting in polymerization, or with lysine residues in other proteins, resulting in protein cross-linking (13). In addition to adding diamines and polyamines to proteins, it can also deamidate glutamine residues to glutamic acid, which introduces a negative charge and changes the pI of the protein. Recently it has been reported to also function as a protein disulfide isomerase (15). However, this latter function, unlike other functions described for TG2, was not calcium-dependent and was not inhibited by GTP. TG2 has also been reported to function as novel G protein-coupled membrane receptor (16) and has been shown to have a role in transmitting signals from classical seven-transmembrane helix G-coupled receptors such as the α_{1B} -adrenergic receptor (17). Here we report that TG2 has another novel enzymatic function, namely kinase activity. Although we have as yet only identified IGFBP-3 and the closely related protein IGFBP-5 as substrates for this kinase activity, it is likely to phosphorylate a variety of other proteins.

Interestingly, the kinase activity of TG2 was inhibited by increasing calcium and, consistent with previous reports (12), increasing the calcium concentration enhanced the cross-linking activity of TG2. In the case of TG2 activity directed against IGFBP-3, calcium appeared to act as a switch, inhibiting kinase activity and enhancing cross-linking activity. It is not clear, however, from our studies whether high calcium concentrations inhibit TG2 kinase activity directly or rather simply enhance TG2 cross-linking of activity and thereby prevent phosphorylation by reducing the availability of phosphorylation sites in IGFBP-3. The latter explanation appears likely because polymerized IGFBP-3 is only lightly phosphorylated compared with monomeric IGFBP-3 (Fig. 4C).

TG2 has been implicated in a variety of processes where phosphorylation is important. These include activation of RhoA and mitogen-activated protein kinase pathways (18), activation of cAMP-response element-binding protein (19), and activation of phospholipase C (16). It is unclear whether the kinase activity of TG2 is important in these situations.

In most cell types TG2 is predominantly localized in the cytoplasm and the nucleus (13), but it is also localized to the cell membrane (20). It can be released from various cell types under certain circumstances such as inflammation and during

apoptotic cell death (21). In the latter case it appears to be important in the later stages of the process and may function to prepare dying cells for phagocytosis by macrophages (21). However, TG2 gene expression is activated early in apoptosis, particularly morphogenic apoptosis in developing embryonic limbs (22) and retinoid-induced apoptosis (23).

Interestingly, IGFBP-3 has been shown to be pro-apoptotic in a variety of cell lines (4, 6, 24). This process is thought to be an IGF-independent effect of IGFBP-3 mediated by binding of IGFBP-3 to a surface receptor (25). The presence of IGF-I inhibits the interaction of IGFBP-3 with binding sites present on breast cancer cells (26) and thus would potentially inhibit the pro-apoptotic IGF-independent effects of IGFBP-3. We have previously shown that phosphorylation of IGFBP-3 by TG2 enhances the affinity of this binding protein for IGF-I. Thus, phosphorylation of IGFBP-3 by TG2 could serve to attenuate the pro-apoptotic effects of IGFBP-3 and the proliferative effect of IGF-I by enhancing formations of IGFBP-3-IGF-I binary complexes and reducing the interaction of IGF-I and IGFBP-3 with their cognate membrane binding sites.

In summary, we have identified a novel kinase function for TG2. We provide compelling evidence that TG2 is the major IGFBP-3 kinase present on breast cancer cell membranes. The observation that TG2 has kinase activity should serve as a stimulus to re-examine the role of TG2 kinase activity in other biological processes where TG2 kinase activity could be important.

REFERENCES

- Jones, J. I. & Clemmons, D. R. (1995) *Endocr. Rev.* **16**, 3–34
- DeMellow, J. S. M. & Baxter, R. C. (1988) *Biochem. Biophys. Res. Commun.* **156**, 199–204
- Valentinis, B., Bhala, A., DeAngelis, T., Baserga, R. & Cohen, P. (1996) *Mol. Endocrinol.* **9**, 361–367
- Oh, Y., Gucev, Z., Ng, L., Muller, H. L. & Rosenfeld, R. G. (1995) *Prog. Growth Factor Res.* **6**, 205–212
- Lalou, C., Lassarre, C. & Binoux, M. A. (1996) *Endocrinology* **137**, 3206–3212
- Hong, J., Zhang, G., Dong, F. & Rechler, M. M. (2002) *J. Biol. Chem.* **277**, 10489–10497
- Mishra, S. & Murphy, L. J. (2003) *Endocrinology* **144**, 4042–4050
- Lai, T. S., Slaughter, T. F., Peoples, K. A., Hettasch, J. M. & Greenberg, C. S. (1998) *J. Biol. Chem.* **273**, 1776–1781
- Singh, U. S., Kumar, M. T., Kao, Y.-L. & Baker, K. M. (2001) *EMBO J.* **20**, 2413–2423
- Sakai, K., Busby, W. H., Jr., Clarke, J. B. & Clemmons, D. R. (2001) *J. Biol. Chem.* **276**, 8740–8745
- Akimov, S. S. & Belkin, A. M. (2001) *J. Cell Sci.* **114**, 2989–3000
- Kang, S. K., Kim, D. K., Damron, D. S., Baek, K. J. & Im, M. J. (2002) *Biochem. Biophys. Res. Commun.* **293**, 383–390
- Fesus, L. & Piacentini, M. (2002) *Trends Biochem. Sci.* **27**, 534–539
- Hoeck, W. G. & Mukku, V. R. (1994) *J. Cell. Biochem.* **56**, 262–273
- Hassegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y. & Saito, Y. (2003) *Biochem. J.* **373**, 793–803
- Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J. & Graham, R. M. (1994) *Science* **264**, 1593–1596
- Chen, S., Lin, F., Iismaa, S., Lee, K. N., Birckbichler, P. J. & Graham, R. M. (1996) *J. Biol. Chem.* **271**, 32385–32391
- Singh, U. S., Pan, J., Kao, Y. L., Joshi, S., Young, K. L. & Baker, K. M. (2003) *J. Biol. Chem.* **278**, 391–399
- Tucholski, J. & Johnson, G. V. (2003) *J. Biol. Chem.* **278**, 26838–26843
- Gaudry, C. A., Verderio, E., Aeschlimann, D., Cox, A., Smith, C. & Griffin, M. (1999) *J. Biol. Chem.* **274**, 30707–30714
- Griffin, M. & Verderio, E. (2000) in *Tissue Transglutaminase in Cell Death in Programmed Cell Death in Animals and Plants* (Bryant, J. A., Hughes, S. G. & Garland, J. M., eds) pp. 223–240, BIOS Scientific Publishers Ltd., Oxford
- Thomazy, V. A. & Davies, P. J. (1999) *Cell Death Differ.* **6**, 146–154
- Kochhar, D. M., Jiang, H., Harnish, D. C. & Soprano, D. R. (1993) *Prog. Clin. Biol. Res.* **383B**, 815–825
- Longobardi, L., Torello, M., Buckway, C., O'Rear, L., Horton, W. A., Hwa, V., Roberts, C. T. Jr., Chiarelli, F., Rosenfeld, R. G. & Spagnoli, A. (2003) *Endocrinology* **144**, 1695–1702
- Oh, Y., Muller, H. L., Pham, H. & Rosenfeld, R. G. (1993) *J. Biol. Chem.* **268**, 26045–26048
- Yamanaka, Y., Fowlkes, J. L., Wilson, E. M., Rosenfeld, R. G. & Oh, Y. (1999) *Endocrinology* **140**, 1319–1328