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"Activation of Tissue Tranglutsaminase by Removal of Carboxyl-Terminal Peptides"

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ABSTRACT

Tissue transglutaminase (TGC or TG2) functions as transglutaminase (cross-linking), deamidase, kinase, and disulfide isomerase and its activities are implicated in the pathogenesis of several human diseases. Proteolytic activation of zymogens in the transglutaminase family is not unusual. Plasma transglutaminase (FXIIIa), epidermal transglutaminase (TG 3), transglutaminase-5, and microbial transglutaminase (MTG) can be subjected to proteolysis from specific proteases to generate the active functional enzyme. In the present study, calcium or GTP was essential for activation of TGC cross-linking activity by trypsin in membrane fractions from human RBC and was accompanied by the conversion of TGC (80 kDa) to a smaller TG form (55 kDa). While bacterially expressed TGC showed no activity, bacterial expression of C-terminal domain deletion constructs with carboxy-terminal ends ranging from lysine 464 (TG464) to glycine 480 (TG480) produced enzymes that were highly active in cross-linking activity. The product of a construct with a coding region ended at proline 446 (TG446), which interrupted the calcium-binding domain, exhibited weak cross-linking activity. TG480 and TG512 were characterized by about 80% and 10%, respectively, of the cross-linking activities of TG464. This may indicate that the longer the peptide after the calcium binding domain, the less the enzymatic activity expressed, possibly because the folding of such peptide which interfere with the calcium binding site or the catalytic site. Western analysis of MCF7 and T47D human breast cancer cells transfected with TGC showed TGC as a major protein and TG as a minor fragment. Incubation of lysate from transfected cells with serum resulted in the conversion of the TGC to TG, a condition that may be comparable to injury or wounds that lead to rapid enzymatic transamidation activation. J. Cell. Biochem. 112: 3469–3481, 2011.

KEY WORDS: TRANSGLUTAMINASE; CROSS-LINKING; APOPTOSIS; TG2 ACTIVATION; PROTEOLYSIS; WOUND HEALING; CANCER; CELIAC DISEASE

Tissue transglutaminase (TGC or TG2) is a multifunctional protein and its catalytic functions include cross-linking, deamidation, phosphorylation, G protein signal transfer, disulfide isomerase, and adaptor protein activities [Park et al., 2010]. The 80 kDa form of cellular or TGC is ubiquitous and is found in most mammalian tissues. We reported that purified human erythrocyte and recombinant TGC (80 kDa) have cross-linking activity [Lee et al., 1993]. In addition, we reported that the GTPase and cross-linking activities of transglutaminase are catalyzed by different active sites since conversion, by site-directed mutagenesis, of the active site cysteine 277 to serine inactivated the cross-linking activity, but did not affect the GTPase activity of TGC [Lee et al., 1993]. The TGC gene is one of nine transglutaminase genes that have been reported. Plasma transglutaminase (FXIIIa) stabilizes the fibrin clot in homeostasis, TGK (TG1), TGE (TG3), and TGX (TG5) transglutaminases are associated with the formation of the cornified cell envelope. Prostate transglutaminase (TGP, TG4) is involved in semen coagulation and protein 4.2 (P 4.2) is a structural component of the cytoskeleton. TGY (type 6) and TGZ (TG 7) are transglutaminases closely related to TGC and have been described [Grenard et al., 2001].

We reported previously two new forms of TGC mRNA from human erythroleukemia cells (HEL) induced with retinoic acid, generated by alternative splicing [Fraij et al., 1992; Fraij and Gonzales, 1996]. The encoded proteins were termed TGC or TG2 homologue (TGH) and TGH2. Further confirmation of the authenticity of these alternative transcripts was shown by the genomic data [Fraij and Gonzales, 1997]. In both transcripts, exons 6 and 10 were not spliced at the donor sites resulting in the use of alternative pseudo-exons, which were originally part of larger

Abbreviations: TGC, 80 kDa, cellular or tissue transglutaminase or TG2; TGH, 63 kDa, tissue transglutaminase homologue; TG, 55 kDa, tissue transglutaminase cross-linking active isoform; PBS, phosphate-buffered saline; HEL, human erythroleukemia cells; TCA, trichloroacetic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; LB, Luria–Bertani broth; TB, terrific broth.

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introns (5.4 and 4.6 kb) in the main TGC gene. In both alternative transcripts, the coding sequences start with the regular coding sequences for TGC. The pseudo-exon sequence completes the mature transcripts resulting in truncated coding sequences. The smaller RNA species encode for proteins with novel carboxyl termini.

TGC is regarded as the active enzyme and is often cited as the only enzyme of the transglutaminase family that does not require proteolytic activation [Greenberg et al., 1991]. Blood coagulation factor XIIIa (FXIIIa) is produced as a zymogen (80 kDa). Its digestion with thrombin or trypsin in the presence of metal ions generates a 51-kDa polypeptide that expresses partial transglutaminase activity [Mary et al., 1988]. Epidermal transglutaminase, TG 3 (77 kDa) is typically located in the cytosol and is proteolytically processed during Ca^{2+} induced terminal differentiation of stratified squamous epithelia into a functional 50/27 kDa complex [Kim et al., 1999]. Keratinocyte transglutaminase, or TG1 (106 kDa) was found mostly as membrane-bound protein of very low specific activity [Steinert et al., 1996]. During terminal differentiation of squamous epithelia, proteolytic processing activates the zymogen by converting TG1 into a 67/33/10-kDa form of very high specific activity. Microbial transglutaminase (MTG) from the genus *Streptoverticillium* is expressed as a prepro-enzyme and the secreted proenzyme is processed to the mature active form [Pasternack et al., 1998]. Recently TG5 full-length enzyme was reported to have low enzymatic activity and proteolytic activation produced active 53-kDa forms [Pietroni et al., 2008].

TGC is one of the most extensively studied of the transglutaminases because of its suspected or proven involvement in a large number of processes of medical importance. TGC was identified as a novel kinase and present as a major component of the IGFBP-3 kinase on breast cancer cell membranes [Mishra and Murphy, 2004]. Its cross-linking activities have been reported to be involved in wound healing, angiogenesis, and inhibition of tumor growth [Haroon et al., 1999]. Transglutaminase cross-linking activities mediate site-specific incorporation of polyethylene glycol (PEG) derivatives into therapeutic proteins, which could improve the extension of the in vivo circulating half-life of proteins [Sato, 2002]. Ca^{2+}dependent irreversible cross-linking of intracellular proteins represent an important biochemical event in gene-regulated cell death [Rodolfo et al., 2004; Volokhina et al., 2003]. TGC has been identified as the highly specific endomysial autoantigen in celiac disease pathogenesis mediated by deamidation of gluten peptides [Schuppan, 2000]. TGC may contribute to the pathology of neurodegenerative conditions including Alzheimer's disease [Lesort et al., 2000]. Loss of controls over the balance of GTPase and transglutaminase activities could underlie the abnormal elevation of cross-linking activity associated with neurodegenerative diseases of the central nervous system [Gill et al., 1998]. The unique presence of TGH transcripts may cause transglutaminase-mediated neurotoxic protein polymerization, which leads to neuronal death in Alzheimer's disease (AD) [Citron et al., 2001]. TGH was identified as a specific GTP-binding protein. Its GTPase activity had a K_m value several-fold lower than the reported K_m values for the native TGC [Fraij, 1996].

C-terminal deletion of TGC is reported to enhance its GTPase activity, and the C-terminus functions to inhibit the expression of TGC endogenous GTPase activity [Lai et al., 1996]. Several N-terminal homologues and fragments of TGC have been reported [Fraij et al., 1992; Fraij and Gonzales, 1996; Monsonego et al., 1997]. The TGH [Fraij, 1996] and TGC [Lee et al., 1993] were reported to bind and hydrolyze GTP. TGC was identified as a Gh protein involved in a signal transfer [Mhaouty-Kodja, 2004]. In the present study, the activitation of transglutaminase was shown to require a proteolytic process that results in TGC cleavage at region approximately 2/3 of the distance from the C- to N-terminus of the protein (between residues 464 and 480), yielding the N-terminus catalytic domain active 55 kDa enzyme (TG). Results reported here demonstrate that the 80-kDa polypeptide (TGC) can be converted in vitro and in vivo to produce active form(s) with a molecular weight of about 55 kDa (TG).

EXPERIMENTAL PROCEDURES

ANALYSIS OF PROTEOLYTIC ACTIVATION OF HUMAN TISSUE TRANSGLUTAMINASE

Human erythrocyte membranes were isolated from outdated human blood (American Red Cross, Tulsa, OK). The cells were washed three times with three volumes of phosphate-buffered saline (PBS) and then osmotically lysed with three volumes of cold water containing 10 mM $MgCl_2$. The hemolysate was centrifuged for 15 min at 25,000 × *g*, and the pellet containing membrane proteins (MP) was suspended in buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), and 5 mM EDTA. Erythroleukemia HEL cell suspentions (10^6 cells) were lysed by sonication at 4°C in 1 ml of (state the volume used here) TBS buffer (0.02 M Tris pH 7.5, 0.15 M NaCl, and 0.1% Triton X-100). After centrifugation at 2,50,000 × *g* for 15 min, the supernatant was removed and the pellet containing MP was suspended in buffer A.

MP (100 μg) were proteolyzed in 30 μl volume and using trypsin (Sigma, St. Louis, MO), at 0.01 mg/ml final concentration in buffer A, for the indicated time at 37°C and then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunodetection.

MP (100 μg) were incubated with increasing concentrations of trypsin (0, 2.5, 10, 20, and 30 μg/ml) in the presence of 5 mM $CaCl_2$ or 0.3 mM GTP for 1 min at 37°C. Control assays contained MP with 5 mm EDTA.

The proteolysis was terminated using soybean trypsin inhibitor (0.02 mg/ml). One portion of the digested proteins was assayed for transglutaminase cross-linking activity, and another was immediately transferred to SDS-loading buffer and placed in a boiling water bath for 5 min. It was subjected to SDS–PAGE and immunoblotting to assess transglutaminase cleavage. Transglutaminase activity was determined by the incorporation of 1,4-14C-putrescine into dimethylcasein as previously described [Lee et al., 1993]. Briefly, the 200 μl standard reaction mixture contained 20 mM Tris–HCl pH 7.5, 5 mM $CaCl_2$ or 5 mM EDTA, 10 mM DTT, 1 mM putrescine (1.0 μCi/mol), 2 mg/ml dimethylcasein, and 20 μl of supernatant from MP (1 μg/1 μl). The reaction mixture was incubated at 37°C for 1 h and then terminated by the addition of 2.5 ml of cold 7.5% trichloroacetic acid (TCA). The precipitate was collected on Whitman GF/A filters and was washed three times with 5 ml cold 5% TCA. The protein-bound radioactivity was determined in 10 ml EcoLume (ICN)

by liquid scintillation counting. All results include subtraction of EDTA background from $CaCl_2$ activation values for each test.

PREPARATION AND CLONING OF ACTIVE TISSUE TRANSGLUTAMINASE CONSTRUCTS

Total cellular RNA was isolated from human white blood cells as described [Lee et al., 1986]. Outdated human blood (American Red Cross) was centrifuged at $1,500 \times g$ for 15 min and the buffy coat containing white blood cells was removed and used for the total RNA preparations. The isolated total RNA was used for reverse transcription and polymerase chain reaction (RT-PCR). The first strand cDNA was synthesized from 20 μg of the isolated total RNA and 0.5 μg of primer 1 (P 1) using M-MLV reverse transcriptase. The reaction was incubated at 42°C for 50 min, heated to 70°C for 15 min, and placed on ice for 2 min. One-half of the product produced was added directly to PCR reactions with primers 1 and 2, in a final volume of 50 μl. The reaction contained 1.5 mM $MgCl_2$, 0.2 μM dNTP, and 0.8 μM of each primer. Amplifications were performed for 30 cycles with 1 min at 94°C, 2 min at 42°C, and 3 min at 72°C, and final 7-min incubation at 72°C. Five microliters of each reaction was visualized by agarose gel electrophoresis with ethidium bromide staining.

Primers (P) used were: P1: 5′-TCAGCGGGCACAGAGCAGGA-3′, P2: 5′-CAGGCGTGACGCCAGTTCTAA-3′. P1 is complementary to nucleotides 1653–1670. P2 is composed of nucleotides 3–24. (GenBank accession number M98478).

The unmodified PCR products were directly cloned into T-vectors constructed from *EcoRV*-digested pBluescript II SK (−) as described previously [Fraij et al., 1992] and the resulting plasmid designated as TG-512. DNA sequence analysis revealed the presence of two base changes for TG 512 when compared to sequences of TGC or TGH.

Constructs of C-terminal deletions were synthesized using P3 and complementary oligonucleotides with a TGA stop codon immediately following the codons, (P4), for lysine 464 (TG464), alanine, P5, 466 (TG466), glycine, (P6), 480 (TG480), and proline, (P7) 446 (TG446). TG512 was used as the DNA template for amplifications. The sequences of the specific primers were:

(1) P3: ATGGCCGAGGAGCTGGT (Met 1).
(2) P4: TCATTTGTTCAGGTGGTT (lys 464).
(3) P5: TCAGGCCAGTTTGTTCAGGT (ala 466).
(4) P6: TCAGCCCACACGGATCCGCAT (gly 480).
(5) P7: TCATGGGTATTTGTAGGTGTG (pro 446).

At the 5′-end of primers 4–7, sequences containing *NdeI* and or *XhoI* restriction sites were added for cloning purposes. All C-terminal constructs were confirmed by DNA sequencing.

EXPRESSION AND PURIFICATION OF THE TRANSGLUTAMINASE ISOFORMS

Two expression vectors were used: pET-14b (Novagen), to express complete transglutaminase sequences (native) and pRSET B (Invitrogen, Carlsbad, CA) which adds an N-terminal 4.8 kDa leader peptide containing a histidine tag. Both plasmids contain a T7 promoter and a transcriptional terminator to drive expression of the transglutaminase forms in *Escherichia coli* All amplified and cloned products were digested with *NcoI* and *XhoI* and cloned pET-14b or digested with *NcoI* and *HindIII* and cloned in pRSET B. Restriction digests identified plasmid with inserts in the correct orientation.

E. coli BL21 (Novagen) bacterial cells containing the transglutaminase-expression vectors were grown in Luria–Bertani broth (LB) or terrific broth (TB) with ampicillin or carbenicillin (50 μg/ml) at 37°C to an A_{595} of 0.6–0.7. Induction of expression was initiated by adding isopropyl β-D-thiogalactoside (IPTG), to a final concentration of 0.2 mM. Cells were harvested 1–5 h later and resuspended in 0.02 M Tris–HCl pH 7.5, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 0.5% Triton X-100. Bacterial cells were lysed by sonication at 4°C and centrifuged for 10 min at $15,000 \times g$. Supernatants were used for cross-linking activity measurements and western analysis.

PREPARATION OF IMMUNOAFFINITY REAGENT

Purified rabbit anti-TG464 antibodies prepared as described below were conjugated with activated CH Sepharose 4B gel (Pharmacia) according to the manufacturer's protocol. In brief, washed gel derived from 1 g powder was mixed with purified antibodies (1.75 mg/ml) in 0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.0 with a ratio of 1:2. The mixture was rocked for 2 h at room temperature and the gel was washed with 5 gel volumes of coupling buffer. The gel was mixed with an equal volume of 0.1 M Tris–HCl pH 8.0, and gently rocked for 1 h to block remaining active groups. The product was washed with three cycles of alternating pH consist of 0.1 M Na acetate, 0.5 M NaCl pH 4.0 followed by 0.1 M Tris–HCl, 0.5 M NaCl pH 8.0. The gel (3 ml) was washed and equilibrated with buffer B (20 mM Tris–HCl, 0.15 M KCl, 0.2 mM DTT, and 1 mM EDTA pH 7.5) and poured into a Poly-Prep Chromatography column (Bio-Rad).

CELL CULTURE AND TRANSFECTION

HEL, GM06141A (Human Mutant Repository, Camden, NJ). HEL were grown in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human breast cancer MCF7 and T47D cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (Sigma). Cells were grown in humidified atmosphere containing 5% CO_2 at 37°C. Transient transfections by using Lipofectamine 2000 (Invitrogen) were used according to the manufacturer's instructions. Transient transfections were performed the following day when the cells were 90–95% confluent. The cells were seeded at a density of 5×10^5 cells per well of a six-well plate and transfected with the full-length cDNAs encoding TGC (80 kDa), TG 955 (kDa), or pcDNA empty vector.

AFFINITY PURIFICATIONS

Extracts of human RBC or *E. coli* cells were applied to the antibody-Sepharose 4B column. The column was washed with 30 bed volumes of buffer B. Proteins adsorbed to the column were eluted with 20 mM Na carbonate, 0.4 mM DTT, 2 M KCl, and 1 mM EDTA pH 10.5. The eluted protein fractions were dialyzed against buffer A at 4°C. Pooled fractions were re-applied onto the antibody-Sepharose 4B column equilibrated in buffer B. Eluted fractions were dialyzed

again 20 mM Tris–HCl, 0.15 M NaCl, 0.2 mM DTT, and 1 mM EDTA pH 7.5. Samples were concentrated to a protein concentration of 2.0–2.5 mg/ml using centricon-30 (Amicon) columns, and stored at −80°C.

Histidine tag resin affinity purification of recombinant transglutaminase was performed as described by the manufacturer (Novagen). Briefly, cells were suspended in 4 ml cold binding buffer (5 mM imidazole, 0.5 M NaCl, 0.1% Triton X-100, and 20 mM Tris–HCl pH 7.9). Lysates were centrifuged at 15,000 × *g* for 10 min and supernatant was filtered through a 0.45 μm membrane. Supernatants were assayed for cross-linking activity and examined by western analysis before being applied to a His tag column.

Supernatant samples applied onto a column containing 3.0 ml His tag resin, equilibrated with 25 ml binding buffer, and the column was washed with 15 ml of wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl pH 7.9). Enzymes were eluted with 5 ml of 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl pH 7.9. Enzyme fractions were concentrated in 20 mM Tris–HCl, 0.4 mM DTT, 0.15 M NaCl, and 1 mM EDTA pH 7.5 and stored at −80°C.

ANTIBODY PURIFICATION

Antibody production occurred at Oklahoma State University, performed by the certified animal center and from local funding. Antibodies were prepared by mixing 5 mg of purified recombinant TG464 polypeptide with complete Freund's adjuvant. Proteins were dissolved in sterile saline and emulsified with 5 ml of complete Freund's adjuvant. Female New Zealand rabbits were injected with 1 ml of the emulsion subcutaneously. Three subsequent booster injections were given later. Serum was tested for antibodies using Western blots. A 2-cm high Ni-NTA column (Qiagen) for antibodies purification was used for the purifications. BL-21 transformed bacteria with pRSET plasmids containing TG cDNA were grown as described above. Cells were lysed in 6 M guanidine HCl, 0.1 M Na–phosphate, 0.01 M Tris–HCl pH 8.0, and 10 ml of the extract were applied to the Ni-NTA column equilibrated in the same buffer. The column was washed with buffers containing 8 M urea, 0.1 M Na–phosphate, and 0.01 M Tris–HCl pH 8.0, pH 6.3, and pH 5.9. The column was equilibrated in 0.15 M NaCl, 0.05 M Tris–HCl pH 7.4 for application of the antiserum samples. The serum (2 ml) from rabbits injected with the bacterially produced and purified recombinant forms was applied to the column. The column was washed with 5 column volumes of 150 mM NaCl equilibrating buffer, followed by 5 column volumes of 2 M NaCl, 50 mM Tris–HCl pH 7.4. The antibodies were eluted with 4 M $MgCl_2$ (no buffer). The eluted antibody was dialyzed against PBS exhaustively at 4°C and then stored at −20°C. The purified antibodies were found effective in Western blotting shown below at a dilution of 1:5,000 (v/v).

IMMUNOPRECIPITATION

Immunoprecipitation was performed as described previously (Fraij & Gonzales 1997). T47D cell suspensions (10^6 cells) in TBS buffer (0.02 M Tris pH 7.4, 0.15 M NaCl, and 0.1% Triton X-100) were sonicated 15 s three times on ice. Five hundred microliters of lysate was mixed with 50 ml of rabbit anti-human erythrocyte TGC. Gelatin was added to 0.16% total volume. The mixture was incubated overnight at 4°C, after which 100 ml of protein-A suspension (Sigma) in PBS was added. The mixture was placed on a shaker at room temperature for 2 h and then centrifuged for 2 min at 13,000 rpm. The supernatant was transferred into other tube and the pellet was washed three times with PBS containing 0.1% SDS. The washed pellet was resuspended in 50 μl of SDS–PAGE loading buffer, 40 ml from immunoprecipitation supernatant was mixed with 10 ml of 5× loading buffer, and tubes were placed in a boiling water bath for min, cooled, and centrifuged. The supernatant was removed and 10 ml used for SDS–PAGE.

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS, WESTERN BLOT

Protein mixtures were separated by SDS–polyacrylamide mini-slab gel electrophoresis using a 5% stacking gel and a 10% separating gel or as indicated in the figure legends. The gels were stained with Coomassie Blue or electroblotted onto Immobilon-P membranes and then detected with the purified antibodies to recombinant TG464 (1:5,000 dilution) or polyclonal anti-human red cells TGC (1:1,000) and goat anti-rabbit (GAR-HRP, 1:3,000, Bio-Rad) or with monoclonal antibody to guinea pig liver transglutaminase (1:1,000) and goat anti-mouse (GAM-HRP, 1:3,000, Bio-Rad). Reactivity was visualized by the HPR color development.

AMINO ACID SEQUENCE ANALYSIS

TG464 expressed in bacteria was purified by immunoaffinity column as shown above. Affinity purified TG464 enzyme was very active in cross-linking (Table I), protein samples were separated by SDS–PAGE and transferred to Immobilon-P membrane. One lane was cut for immunostaining, the visualized TG band was used as a marker to cut the remaining unstained lanes. The excised TG bands were subjected to amino acid sequence analysis on an Applied Biosystem Model 477A sequencer.

STATISTICAL ANALYSIS

Activities were analyzed with a two-tailed Student's *t*-test and results were considered significantly different when $^*P < 0.05$ (mean ± SEM, n = 3).

TABLE I. Cross-Linking Activities of Pure Transglutaminase Isoforms

Human transglutaminase isoforms	Specific activity (μmol/mg/h)
Human blood RBC isoforms	0.65
Bacterially expressed TGC (80 kDa)	0.00
Bacterially expressed TGH (63 kDa)	0.00
Bacterially PET expressed TG464 (55 kDa)	11.25
Bacterially pRSET expressed TG464 (60 kDa)	7.89

Transglutaminase activity was determined by the incorporation of 1,4-^{14}C-putrescine into dimethylcasein. The 200 μl standard reaction mixture contained 20 mM Tris–HCl pH 7.5, 5 mM $CaCl_2$, or 5 mM EDTA, 10 mM DTT, 1 mM putrescine (1.0 μCi/mol), 2 mg/ml dimethylcasein, and 60 nM of purified enzyme. A unit of enzyme activity is defined as the amount of transglutaminase which catalyzes the incorporation of 1 μmole of putrescine per hour of reaction. Human RBC isoforms, TGC, TGH, and TG464 (PET expression) were purified from the antibody affinity column and TG464 (pRSET expression) was purified from the Histidine tag affinity column.

RESULTS

TISSUE TRANSGLUTAMINASE ACTIVATION

RBC membrane and supernatant proteins were isolated from human blood as described in Experimental Procedures and incubated with proteolytic enzymes, including trypsin, pepsin, papain, cathepsin, or thrombin. Following proteolysis, membrane, and supernatant proteins samples were examined for activation of cross-linking. Trypsin is the only one of the enzymes stated that activated cross-linking with cell membrane-associated transglutaminase (data not shown). Trypsin activated cross-linking with cell membrane-associated enzyme, but not with enzyme in the soluble fraction as shown below. In the presence of calcium ions and trypsin at 37°C, there was a linear increase in transglutaminase activity in the first minute with a maximal activation of threefold compared to control (Fig. 1A). Similar activation was observed in the presence of GTP. Simultaneously with transglutaminase activation, TGC was converted to a form with a molecular weight of about 55 kDa (TG) as detected by immunoblotting (Fig. 1B). In the presence of calcium ions, proteolysis resulted in near-complete conversion of TGC to the TG polypeptide (Fig. 1B, lanes 3 and 4); proteolysis in the presence of GTP also produced similar results (Fig. 1B, lanes 6 and 7). The proteolytic activation of TGC was obtained when membranes were present in the reaction. There was no conversion in control reactions in which trypsin, calcium, or GTP was omitted (Fig. 1B, lanes 2 and 5). Transglutaminase activities increased in the presence of $CaCl_2$ or GTP to 80 nmol/mg/h compared to 25 nmol/mg/h in control samples. The extent of proteolytic cleavage of TCG was related to activation of cross-linking activity in isolated membranes from human RBC and HEL as a function of trypsin concentration. In the presence of calcium ions (Fig. 2A, inset lanes 2–5 and Fig. 2B, inset lanes 2 and 3), there was an increase in the formation of TG polypeptide band with increasing concentrations of trypsin (2.5–30 mg/ml). Also TG formation was increased as a function of trypsin concentration in samples containing GTP (Fig. 2A, inset lanes 6–9 and Fig. 2B, inset lanes 5 and 6). Formation of TG polypeptide was less in samples treated with 2.5 μg/ml trypsin (Fig. 2A, inset lanes 3 and 7 and Fig. 2B, inset lanes 2 and 5) when compared with samples treated with 20 μg/ml trypsin (Fig. 2A, inset lanes 4 and 8, and Fig. 2B, inset lanes 3 and 6). Simultaneously, there was a parallel and linear increase in cross-linking activities in the presence of calcium ions or GTP with increasing concentrations (2.5–20 mg/ml) of trypsin (Fig. 2A,B). Therefore, these results demonstrate a positive correlation between the conversion of the TGC to TG (immunoblots) and transglutaminase activity as measured by 14C-putrescine incorporation (Fig. 2). Transglutaminase had a maximal activation (3–4 folds) by trypsin (20 mg/ml) at 37°C. Treatment with trypsin (20 mg/ml, 1 min) resulted in a significant ($P < 0.026$) increase in TGC activation to 90 nmol/mg/h of control (25 nmol/mg/h).

ACTIVATION IS DEPENDENT ON MEMBRANE ASSOCIATION AND PRESENCE OF CALCIUM OR GTP

While transglutaminase cross-linking activity increased in the presence of calcium ions or GTP as shown above, in the absence of metal ions (5 mM EDTA) or GTP, trypsin digestion of MP resulted in a significant ($P < 0.011$) decrease in transglutaminase activity (Fig. 3A) which was accompanied by the degradation of TGC to several peptides (Fig. 3B). Therefore, calcium or GTP is essential for this activation, and binding of divalent metal ions to TGC in the

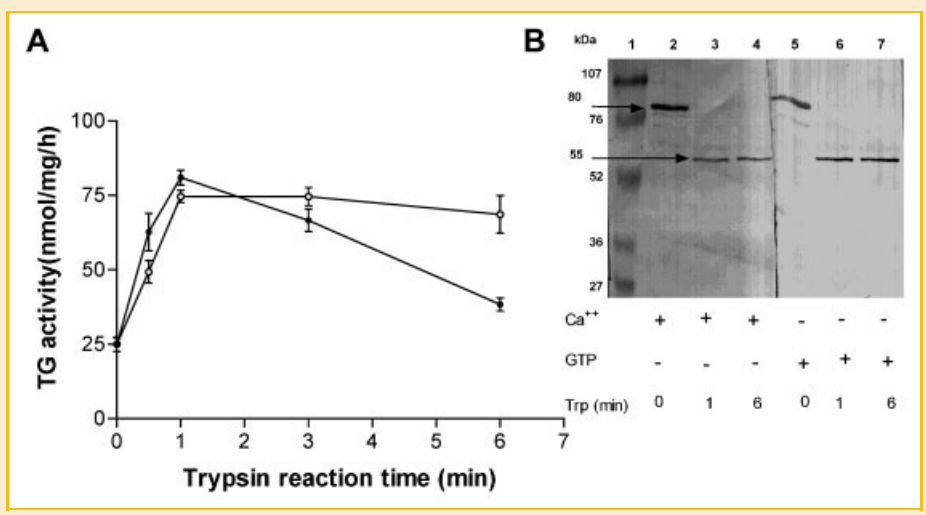

Fig. 1. Modulations of TGC activity (A) and the conversion of TGC to TG (B) by calcium, GTP, and trypsin. MP (100 μg) were proteolyzed-using trypsin at 0.01 mg/ml with 5 mM in $CaCl_2$, or 0.3 mM in GTP for the indicated time (0–6 min) at 37°C. Proteolysis was terminated using soybean trypsin inhibitor (0.02 mg/ml). One portion of the digested proteins was assayed for transglutaminase cross-linking activity, and another was immediately transferred to SDS-loading buffer and placed in a boiling water bath for 5 min. It was subjected to SDS–PAGE and immunoblotting to assess TGC cleavage. A: Transglutaminase activity determined by the incorporation of ^{14}C-putrescine into dimethylcasein for 1 h at 37°C from samples treated with $CaCl_2$ (○), or GTP (●). Individually, both calcium and GTP significantly increased TG activity by proteolysis. Statistical analysis was assessed with a two-tailed Student's *t*-test. Results were considered significant when *P*-value was <0.05 (mean ± SEM, n = 3 experiments). B: Conversion of TGC to TG immunoblot, demonstrating that treatment of membrane proteins (MP) with calcium, GTP, and trypsin (Trp) results in the conversion of TGC to TG. Arrows at the left indicate TGC and TG bands from proteolyzed RBC membranes.

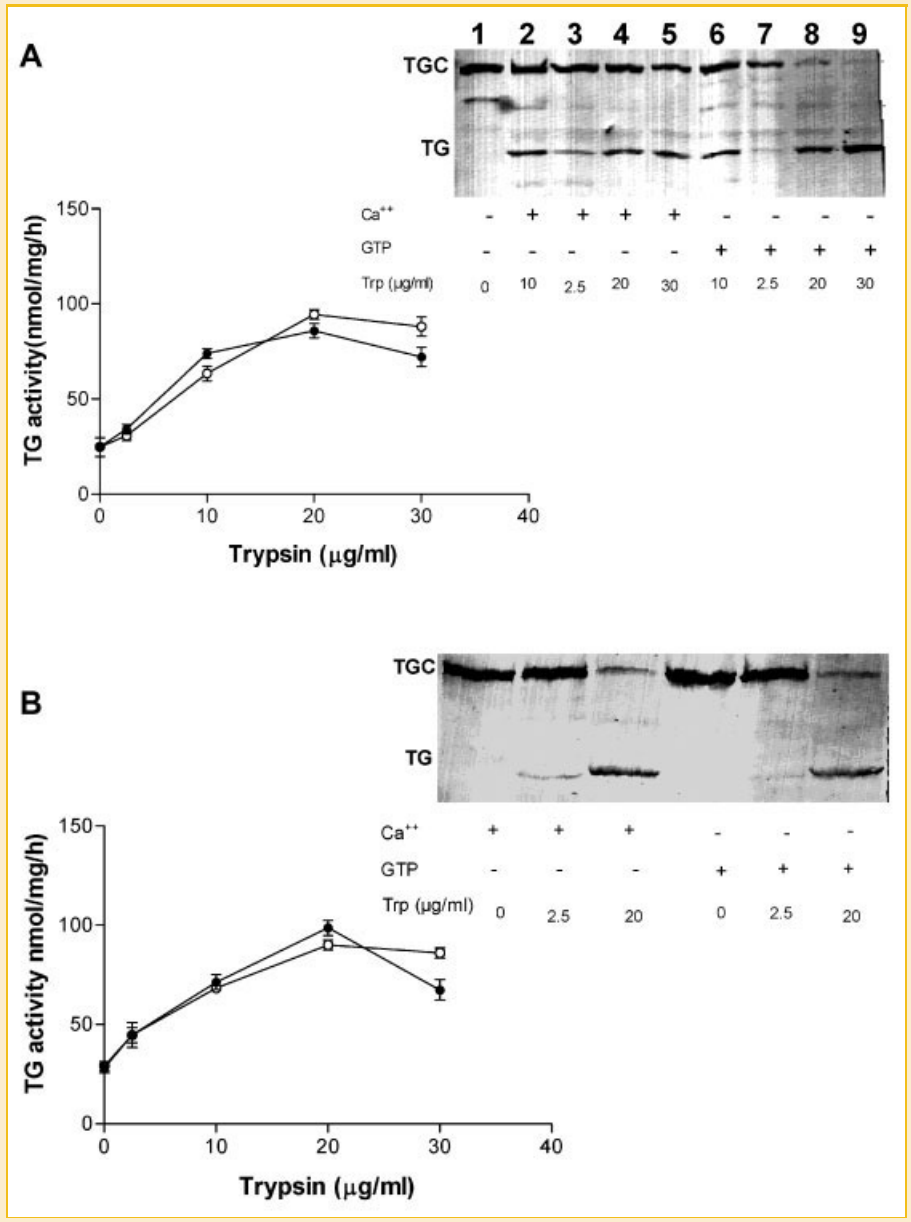

Fig. 2. TGC activation is facilitated by trypsin concentration in RBC membranes (A) and in HEL membranes (B). Membrane proteins (100 μg) were incubated with increasing concentrations of trypsin (0, 2.5, 10, 20, and 30 μg/ml) in the presence of 5 mM $CaCl_2$ or 0.3 mM GTP for 1 min at 37°C. One portion of the digested proteins was assayed for transglutaminase cross-linking activity, and another for immunoblot. A: Transglutaminase activity from isolated RBC membranes with increasing concentrations of trypsin concentrations with $CaCl_2$ (○), or GTP (●). Immunoblot insert, RBC membranes treated as indicated. B: Transglutaminase activity from isolated membranes from human erythroleukemia cells (HEL) with increasing trypsin concentrations with $CaCl_2$ (○), or GTP (●). Immunoblot insert, HEL membranes treated as indicated. Calcium and GTP significantly increased TG activity, which was accompanied, by an increase in the formation of TG polypeptide with increasing concentrations of trypsin. Activities were analyzed with a two-tailed Student's *t*-test and results were considered significantly different when *P*-value was <0.05 (mean ± SEM, n = 3).

presence of membranes may induce conformational changes in the protein that alter its susceptibility to limited proteolysis. In contrast to the response to calcium or GTP, which are essential for transglutaminase activation in MP (Figs. 1 and 2), trypsin rapidly degraded the purified TGC from human blood to low-molecular mass peptides (55-kDa and smaller) with simultaneous decrease of transglutaminase activity ($P < 0.031$) in the presence of calcium or GTP (Fig. 4A,B, lanes 1–6). Trypsin degraded TGC purified from human blood to several peptides including 55-kDa TG fragment with simultaneous decrease of transglutaminase activity in the presence of calcium or GTP. Therefore, membrane association of TGC appears to be required for conversion of TGC to an active form of TG, which may involve protection of the TGC from excessive cleavage by trypsin leading to maintenance an enzyme conformation that is active in cross-linking. Affinity purification of TGC from RBC yielded minor 55 and 25 kDa fragments that co-purified with the full-length 80-kDa enzyme (Fig. 4B, lanes 7 and 8), and activity measurement of the purified transglutaminase displayed some

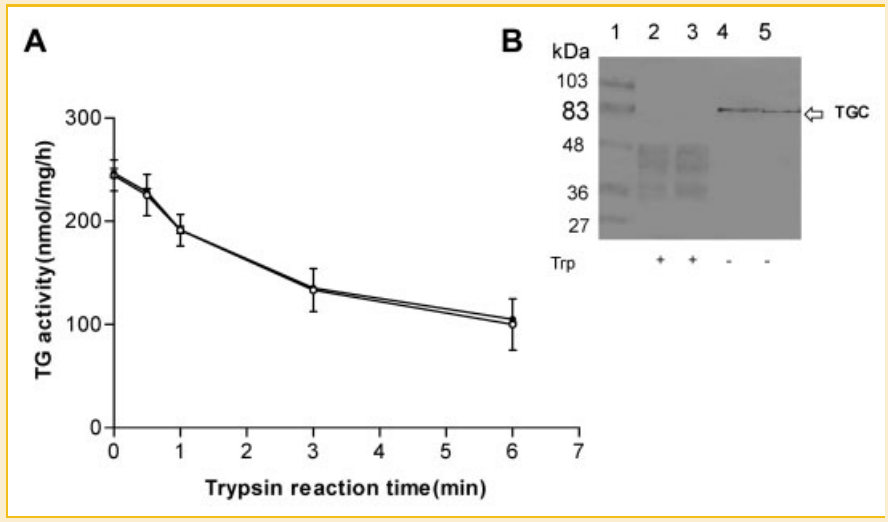

Fig. 3. Trypsin proteolysis decreases transglutaminase activity in the absence of Ca^{++} and GTP. Membrane proteins (100 μg) were treated with trypsin at 0.01 mg/ml without calcium or without GTP for 6 min at 37°C. Proteolysis was terminated using soybean trypsin inhibitor (0.02 mg/ml). One portion of the digested proteins was assayed for transglutaminase cross-linking activity, and another was used for immunoblot. A: Cross-linking transglutaminase activity reactions were assayed by the incorporation of ^{14}C-putrescine into dimethylcasein for 1 h at 37°C, samples without $CaCl_2$ (○), or without GTP (●). Individually, both the absence of calcium and GTP significantly decreased TG activity by proteolysis (mean ± SEM, n = 3; $P < 0.05$). B: Immunoblot, demonstrates the degradation of TGC from MP to low-molecular mass peptides, without Ca^{++} (lanes 2 and 4) and without GTP (lanes 3 and 5). Molecular weight markers (lane 1).

cross-linking activity as shown in Table I. This is interpreted as a consequence of a proteolytic process, which allows a protein cleavage at about 2/3 of the distance from N-terminus of the protein sequence, yielding a 25 kDa C-terminal fragment and an active 55 kDa N-terminal fragment. Human TGC has been studied extensively, and several reports, including ours [Lee et al., 1993] and those of others [Ando et al., 1987; Chica et al., 2004] have demonstrated purification of cross-linking active human TGC from RBC with a single enzyme form of about 80 kDa. The weak staining signals of the minor 55 and 25 kDa fragments makes their detection difficult in Western analysis especially when small amounts are formed (Fig. 4, lanes 7 and 8), and considered as a non specific binding product, in addition, routinely the HPR color development terminated after 1 min which normally does not allow the color development to visualize these minor fragments.

E. COLI EXPRESSION OF TG ISOFORMS AND FULL-LENGTHS TGC AND TGH

Due to the small amount of TG enzyme formed naturally in RBC (Fig. 4, lanes 7 and 8), it was not possible to isolate and obtain terminal sequences. TGC full-length is 687 amino acids. The encoded protein for the alternative spliced transglutaminase homologue TGH was 548 amino acids. C-terminal domain deletions have been used to construct nucleotide sequences to examine the expression of cross-linking active TG isoforms. Schematic illustration of the transglutaminase polypeptide forms is shown in Figure 5.The TG isoform with 55 kDa molecular mass as shown in Figures 1 and 2, and active in cross-linking, must contain the cross-linking active site cysteine located at residue 277. Therefore, cleavage of ~25 kDa peptide from the C-terminus of TGC must result in the formation of the TG enzyme. Therefore, the TG enzyme with a 55 kDa molecular mass was calculated based on an average of 115 molecular weight for an amino acid to contain about 469 amino acids. To confirm that TG is a cross-linking active species, the TGC N-terminal coding domains were amplified by the PCR. Primers were designed for amplifying the coding sequences which end at residues 446, 464, 466, and 480 as shown in experimental procedures. The amplified DNA products were cloned in expression vectors to produce the following constructs: TG446, TG464, TG466, and TG480. The resulting proteins are presented diagrammatically (Fig. 5). Several expression vectors were found to be suitable for producing the active TG including the pET and pRSET. *E. coli* BL21 cells containing the transglutaminase-expression vectors were grown in TB with carbenicillin (50 μg/ml) at 37°C to an A_{595} of 0.6–0.7. Induction of expression was initiated by adding IPTG and cells were harvested after 1–5 h.

TG EXPRESSION IN BACTERIA

Expression of TGC and TGH proteins were induced with IPTG for 5 h. After sonications and centrifugation the resulting supernatant fractions were examined by Western blot. Analysis of bacterially expressed construct isoforms from bacterial lysate supernatants migrated at their predicted sizes with estimated molecular size of 55 kDa in pET expression products and about 60 kDa in pRSET because expressed proteins contain additional N-terminal leader peptide of 4.8 kDa. pET containing inserts of TG446, TG512, TG480, TG464, TGH, and TGC (Fig. 6, lanes 1–6), and pRSET in TGC, TGH, and TG464 (Fig. 6, lanes 8–10). Full-length TGC and TGH formed TG protein with identical sizes to TG446 or TG 466 constructs in the pET expressions (Fig. 6, lanes 5 and 6), and also in pRSET expressions (Fig. 6, lanes 8 and 9). Additional minor bands reacted with the polyclonal anti-TG antibodies in the pET and pRSET preparations. These are most likely proteolytic fragments of the expressed proteins. No post-translational modifications have been reported for

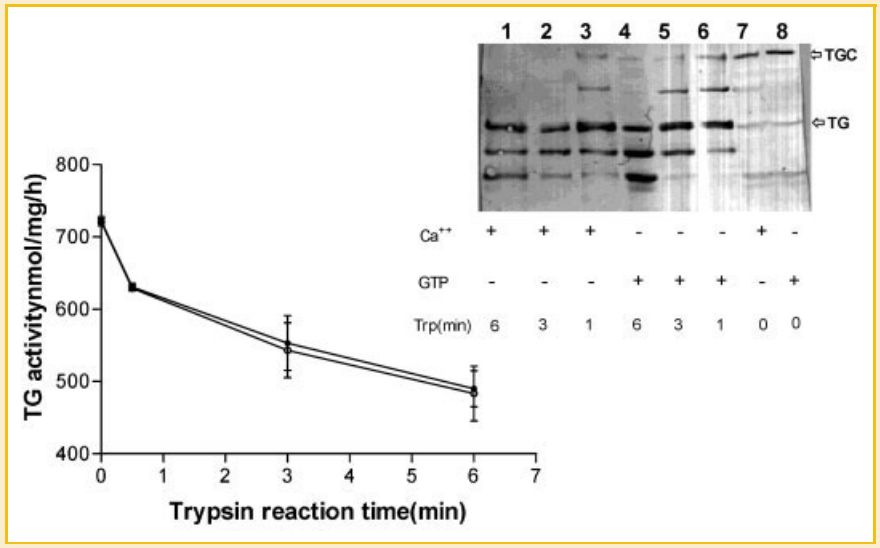

Fig. 4. Effect of time on the proteolysis of purified RBC transglutaminase. Affinity purified transglutaminase from human RBC (10 μg) was incubated with trypsin (0.01 mg/ml) for the indicated time (0–6 min) at 37°C. Proteolysis was inhibited using soybean trypsin inhibitor (0.02 mg/ml) and then one portion of the digested proteins was assayed for ^{14}C-putrescine cross-linking for 1 h at 37°C and another were subjected to SDS–PAGE and immunoblotting. A: Transglutaminase activity reactions were made 5 mM in $CaCl_2$ (○) or 0.3 mM in GTP (●). Trypsin treatment of purified TGC results in a decrease of transglutaminase activity in the presence of calcium or GTP. TG activities were analyzed with a two-tailed Student's *t*-test (mean ± SEM, n = 3; $P < 0.05$). B: Immunoblot of purified TGC digested with trypsin demonstrates that trypsin degraded the purified TGC from human blood cells to low-molecular mass peptides of 55-kDa and smaller. Arrows at the right indicate TGC and TG bands from purified RBC transglutaminase.

TGC. Post-translational modifications (e.g., glycosylation) are absent in *E. coli*. The production of highly active TG in bacterial cells together with the same molecular weight of the human cell product presented below could explain the absence of post-translational modifications in TGC. We showed earlier that the size of TGH produced by in vitro translation product was the same as the native form of HEL which ruled out glycosylation or other group modifications for TGH [Fraij and Gonzales, 1996].

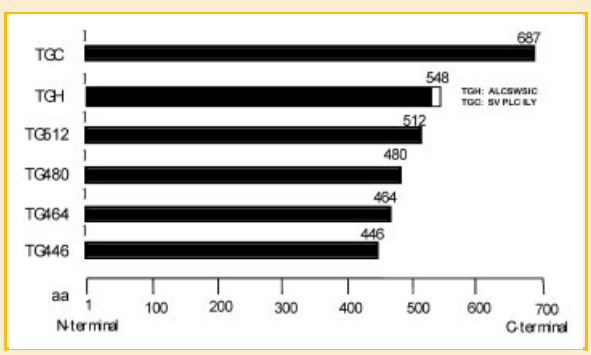

Fig. 5. Schematic representation of polypeptide length for isoforms found in cells (TGC and TGH) and constructs synthesized by PCR (others). Solid black represents shared amino acid sequences. White represents eight alternative amino acid sequences in TGH. Molecular weights of TGC and TGH are about 80 and 63 kDa, and the C-terminal deleted construct forms are about 51–58 kDa. Insert: Alternative C-terminal eight amino acids found in TGH and compared with TGC sequence. Molecular weights were calculated based on an average of 115 for amino acid.

CROSS-LINKING ACTIVITY IS CORRELATED WITH THE FORMATION OF TG ENZYME

Western analysis of these bacterial products revealed the presence of TGC and TGH proteins as major bands. Minor TG protein bands were also detected (Fig. 6). In both pRSET and pET expression systems for TGC and TGH, active TG was formed (Fig. 6, lanes 5, 6, 8, and 9). These samples exhibited low cross-linking activities when compared to TG464 or TG466 expression products (Table II). Expression of TG from pET and pRSET (Fig. 6, lanes 4 and 10) systems produced a major TG protein product with high cross-linking activity. Therefore, the higher amount of TG formed in bacterial, the higher cross-linking activities found in the supernatant fractions. The presence of the leader peptide at the N-terminal from the pRSET expression system did not lower drastically the cross-linking activity when compared with the native forms produced by the pET expression system.

BACTERIAL SUPERNATANT FRACTIONS WITH VERY ACTIVE TG ISOFORMS (53 kDa)

TG464 or TG466 (53 kDa) expression products exhibited very high cross-linking activity when compared to TGH and TGC (Table II). TG480 (55 kDa) has about 80% activity of TG464. TG 512 (58 kDa) produced a weak cross-linking activity of about 10% of TG464 (Table II). The putative Ca^{2+} binding site of the TGC or TG2 is located between residues 433–453. To determine whether the removal of carboxyl-terminal peptide at the calcium binding domain interferes with the enzyme activity, a construct coding for TG ending at proline 446 (TG446, 51 kDa) was tested and found to produce a very weak cross-linking activity of about 5% of TG464. Therefore, the removal of carboxyl end sequences beyond the calcium-binding

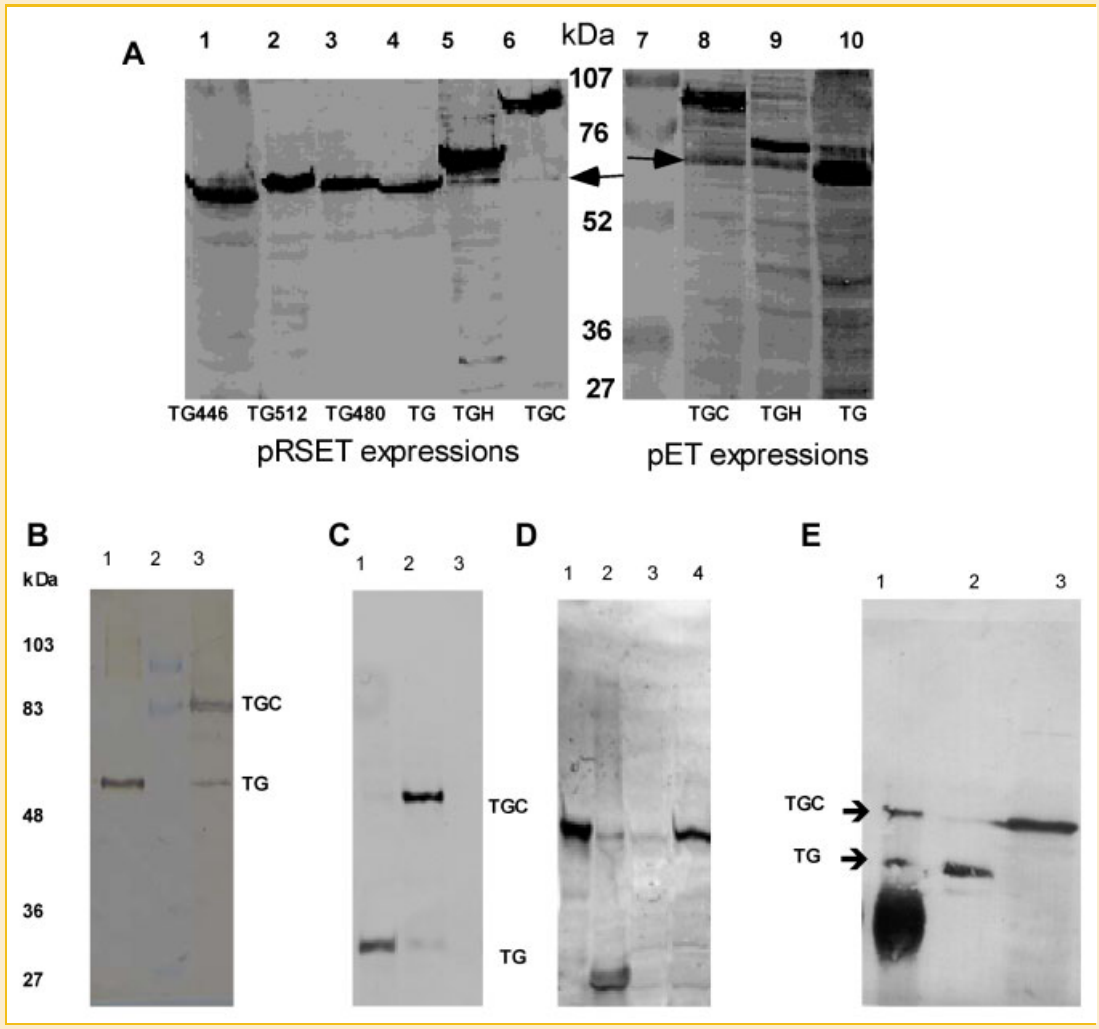

Fig. 6. Western analysis of transglutaminase expression in bacteria (A) and in human breast cancer cells (B–E). Transglutaminase construct isoforms expressed in bacteria or human breast cancer cells transfected with TGC or TG and grown for 1–3 days and then lysed. Cell lysates were separated by SDS–PAGE and processed for Western immunoblot. TG464 (TG) antibodies at a 1:5,000 dilutions were used to visualize the bands. A: pET expressions of TG446, TG512, TG480, TG464, TGH, and TGC constructs are shown in lanes 1–6, and pRSET expressions of TGC, TGH, and TG464 constructs are shown in lanes 8–10. Pre-stain size markers are shown in lane 7. Top arrow and bottom arrows indicate the TG protein formed from TGC and TGH, which demonstrates that TG protein, migrated at about 55 kDa in pET expression products and about 60 kDa in pRSET because expressed proteins contain additional N-terminal leader peptide of 4.8 kDa. B: Bacterial TG pET expression (lane 1); Pre-stain size makers (lane 2); and TGC-overexpressing MCF7 cells were grown for 3 days (lane 3). Immunoblot demonstrating that TG protein expressed in bacteria and in MCF7 cells migrated at similar molecular size levels. C: T47D cells grown for 3 days overexpressing TG (lane 1); TGC (lane 2); and control plasmid (lane 3). D: T47D cells were grown for 1 day overexpressing TGC (lane 1); and TG (lane 2). T47D cells were grown for 2 days overexpressing plasmid (lane 3); and TGC (lane 4). E: Immunoprecipitation and immunoblotting. TGC-overexpressing MCF7 cell lysates were fractionated on 15% SDS–PAGE and blot was probed with polyclonal rabbit anti-human erythrocyte transglutaminase immune sera at a 1:1,000 dilutions. Immunoprecipatation product (lane 1); immunoprecipatation supernatant (lane 2); cell lysates with no serum as control (lane 3). Top and bottom arrows indicate TGC and TG protein bands; the heavy staining band represents the heavy chains of IgG molecules.

domain seems to be required to produce an active enzyme. TG480 and TG512 produced about 80% and 10% cross-linking activities of the TG464 or TG466. This may indicate that the longer the peptide after the calcium binding domain in the protein, the less the enzymatic activity expressed, possibly because the folding of such peptide and masks the calcium binding site or the catalytic site. The cDNA sequences obtained for TG active transglutaminase cDNA constructs were compared with the previously reported transglutaminases and were determined to be nearly identical. Two base changes were found in DNA construct TG 480 resulting in two amino acid changes located at amino acid positions 250 (Ser/Arg) and 394 (Phe/Ser). In spite these changes, which resulted in a charged arginine at, position 250, the expressed transglutaminase was active enzyme. Therefore, some base changes, which do not include cysteine 277 directly, appear to be harmless.

TG EXPRESSION IN HUMAN CELLS

MCF7 overexpressing TGC for 3 days after transfection in DMEM with no serum formed TG protein with the same size of TG464 protein from bacterial expression as judged from their protein migrations (Fig. 6B, lanes 1 and 3). T47D human breast cancer cells overexpressing TGC for 3 days after transfection with no serum

TABLE II. Recombinant Transglutaminase Isoforms Cross-Linking Activities

Expressed transglutaminase isoforms (pRSET)	Specific activity (μmol/mg//h)	Percent of activity (%)
A		
TG464, TG466	0.480	100
TG480	0.385	81
TG512	0.054	11
TG446	0.025	5
TGH	0.059	12
TGC	0.085	17
Expressed transglutaminase isoforms (PET)	**Specific activity (μmol/mg/h)**	**Percent of activity (%)**
B		
TG464, TG466	0.689	100
TG480	0.545	79
TG512	0.056	9
TG446	0.034	5
TGH	0.065	9
TGC	0.043	7

Transglutaminase activity was determined by the incorporation of 1,4-^{14}C-putrescine into dimethylcasein as shown in Table I, and 20 μl of supernatant from total bacterial lysates (1 μg/1 μl) of PET and pRSET expression systems.

showed TGC as a major protein and TG was detected a minor protein (Fig. 6C, lane 2). TG protein was not detected in T47D cells overexpressing TGC for 1 day after transfection (Fig. 6D, lane 1). When these cells were grown for longer period of time (3 days) a small amount of TG protein was detected in control plasmid (Fig. 6, lane 3) and in TGC overexpressing cells (Fig. 6, lane 4), therefore, TG can be formed naturally from TGC in cells with or without overexpressing the TGC.

Immunoprecipitation of total proteins from cultured TGC overexpressing MCF7 cells for 1 day after transfection using polyclonal human erythrocyte antibody were examined by a 15% SDS–PAGE followed by immunoblotting (Fig. 6E). Immunoprecipitation of cell lysate with anti-human erythrocyte transglutaminase antibody showed TGC and TG in the immunoprecipitation pellet (Fig. 6E, lane 1). The heavy staining band of approximately 50 kDa represents the heavy chains of IgG molecules. Supernatant separated from the immunoprecipitation pellet in the above reaction shows a small amount of TGC and TG was a major protein product (Fig. 6, lane 2). It is evident that TGC was converted to TG during the incubation. A control sample from TGC-overexpressing T47D cells grown for 1 day with no serum or antibodies added was characterized by a strong TGC band and no TG could be detected (Fig. 6, lane 3). This line of evidence raises three key points. First, incubation of cellular lysate with serum resulted in the conversion of the TGC to TG (Fig. 6, lanes 2 and 3). Second, TG does not bind tightly to the TGC polyclonal antibody as shown in the presence of TG protein (Fig. 6, lane 2) in the supernatant of immunoprecipitation reaction. Third, the weak binding of TG to the TGC antibody makes its detection difficult in Western blotting especially when a small amount is formed. The TGC antibody binds to TG as shown in Western analysis in Figures 5 and 6; this is due to the excess protein amounts applied. Injury or wounds lead to the rapid TGC enzymatic transamidation activation accompanied by appearance of 55 kDa fragment accounted for 40% of total transglutaminase protein content [Haroon et al., 1999]. Formation of TG from TGC in the presence of serum (Fig. 6, E) may be comparable to injury or wounds. Sphingosylphosphocholine (lyso-SM), a lipid membrane component, protected TGC from trypsin proteolysis with the formation of about 55 kDa band and activation of cross-linking activity of transglutaminase reached maximum level in the presence of calcium ions, the 55 kDa fragment formed was not reported to be involved in cross-linking activity [Lai et al., 1997; Haroon et al., 1999]. Intracellular as well as extracellular TGC was found catalytically inactive under normal physiological conditions and physical or certain types of chemical injury lead to a rapid enzymatic transamidation activation [Siegel et al., 2008], the mechanism of TGC activation is currently not known.

PURIFICATION OF TRANSGLUTAMINASE ISOFORMS

To determine whether a short IPTG induction time can affect the formation of TG protein from the larger precursors, expression of TGC and TGH were induced for 1 h. Expression products for TGC; TGH exhibited no cross-linking activities (Table I). Western analysis of the TGC and TGH expression systems showed the presence of the TGC and TGH proteins and no TG was detected. To confirm the absence of TG in the TGC and TGH expression products, supernatants from the 1 h IPTG inductions of the pET expression systems were loaded on immunoaffinity column and purifications performed as described above. Application of excess amounts of pure proteins TGC, TGH, and TG (10 μg) on SDS–PAGE showed that these proteins migrated at their predicted molecular mass and Western blots of the purified TGC and TGH proteins showed reactivity of TGC and TGH and no TG was detected in spite the presence of 10 μg of pure proteins (Fig. 7), each protein showed no degradation products which rules out effect of degradation on enzyme activity measurements. Assay measurements of the TGC and the pure TGH shown in Figure 7 showed no cross-linking activities. Purified TG from the 1 h induction time was highly active (Table I).

TGC and TGH were also cloned in pET-24c (data not shown) in which a histidine tag was placed at the C-terminus and expressed in *E. coli* BL21 cells grown for 5 h; this allowed the TG enzyme to be eluted entirely with both TGC and TGH being retained quantitatively on the column. Affinity purified TGC and TGH showed no cross-linking activities whatsoever and no TG was detected as shown in

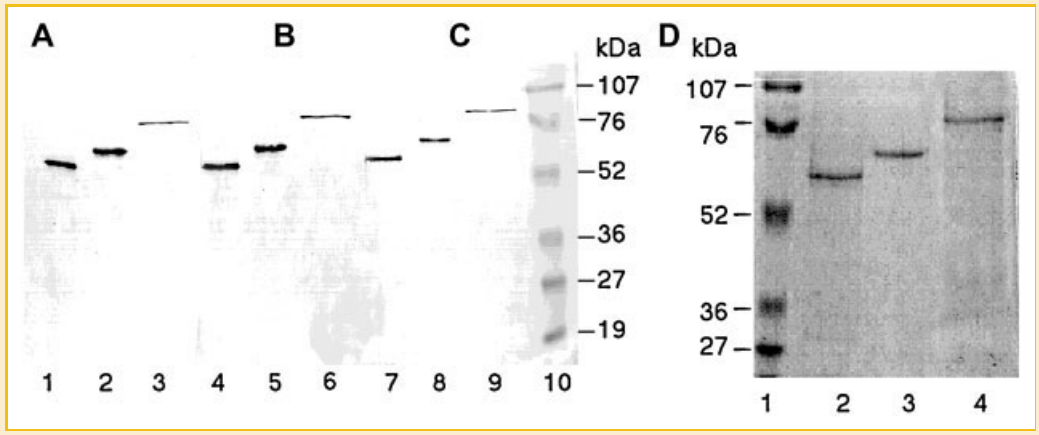

Fig. 7. Affinity pure recombinant TG, TGH, and TGC proteins detected by immunobloting (A–C) and by Coomassie blue staining (D). Purified proteins (10 μg) were subjected by SDS–PAGE and transferred to Immobilon-P membranes (A–C), or stained with Coomassie blue (D). Immunoblots were developed with a polyclonal anti-TG (A), a polyclonal anti-TGC (B), and a monoclonal anti-TGC, CUB74 (C). TG (lanes 1, 4, and 7), TGH (lanes 2, 5, and 8), TGC (lanes 3, 6, and 9), and pre-stained molecular weight markers (lane 10). D: SDS–PAGE gel stained with Coomassie blue, molecular weight markers (lane 1), TG (lane 2), TGH (lane 3), and TGC (lane 4).

Figure 7. This proves that of the three isoforms, the TG isoform is the cross-linking active species and the larger forms (TGC and TGH) are inactive as shown in Table I.

Amino acid sequencing of the immunoaffinity purified TG464 cross-linking active enzyme showed the same N-terminal amino acid sequence present in the open reading frames for TGC [Fraij et al., 1992] and TGH [Fraij and Gonzales, 1996]. For 14 cycles the amino acid sequence was AEELVLERCDLELE.

DISCUSSION

Formation of active enzyme from zymogens by proteolytic activation in the transglutaminase family is not unusual. FXIIIa, TG3, TG5, and MTG are subjected to proteolysis from specific proteases to generate the active functional enzyme. [Pietroni et al., 2008]. TGC with molecular weight of about 80 kDa converts in vitro (Fig. 1) and in vivo (Fig. 6) to TG ~55 kDa active enzyme (Table I). In the experiment in Figure 1, the sample that was not treated with trypsin exhibited moderate cross-linking activity (25 nmol/mg/h), but a several fold increase was obtained in the presence of $CaCl_2$ or GTP (80 nmol/mg/h). The moderate transglutaminase activity found in control or untreated samples most likely reflects the presence of slight TG enzyme form of translglutaminase in these samples. Staining signals of the minor TG fragments are difficult to detect in Western analysis especially when small amounts are formed (Fig. 4, lanes 7 and 8). The activation of TGC by trypsin requires the membrane associated form of the transglutaminase, calcium ions, or GTP. The removal of a carboxyl-terminal peptide is required to produce a unique size of TG active species, interpreted by the presented data as a consequence of a proteolysis process that initiates TGC cleavage at about 2/3 the distance from its N-terminus; neither the protease(s) nor mechanism(s) responsible for activation in vivo are currently known. C-terminal domain deletions have been used to construct nucleotide sequences which express an active TG enzyme. The catalytic core domain (141–471) of TGC contains a calcium-binding site (433–453) toward its C-terminal end. The carboxyl end of TGC contains two β-barrels (471–687) whose removal is required for activation. We as well as others have described that TGC from human RBC and TGC from recombinant bacterial expressions had enzyme activity [Lee et al., 1993; Liu et al., 2002]. It requires a strong antibody titers to detect TG in Western analysis especially when a small amount is formed (Fig. 4, lanes 7 and 8 and Fig. 6C). In the present study, the cell membranes obtained from human RBC were used for TGC activity assay because RBC contains large amounts of TGC and expired RBC is available and easy to obtain. Many authors described TGC as a latent enzyme in cross-linking under physiological condition, recently, intracellular and extracellular TGC was found inactive under normal physiological conditions and physical or certain types of chemical injury lead to a rapid TGC enzymatic activation [Siegel et al., 2008]. It was suggested that injury resulted in release of intracellular TGC, which rapidly assumes an active form presumably as a result of GTP dissociation and calcium binding. TGC activation may occurr as a result of stimulation of receptors, which triggers the release of extracellular TGC from cell surface integrin, which accompanied enzyme activation [Siegel et al., 2008]. In the present study TGC was found inactive even in the presence of sufficient calcium ion concentration (Table I) and trypsin activation of TGC required the presence of the membrane-associated form of TGC as well as calcium ions or GTP.

The active site region residues 274–280, residues including Trp329, Trp236, and His332 was proposed to be involved in the interactions between acyl-donor substrates and the enzyme [Iismaa et al., 2000], and the potential calcium-binding region between 433 and 453 amino acids is present in the TG active enzyme. A subunit of FXIIIa consists of four domains. The average size of the exons of TGC gene was found similar to those of FXIIIa [Greenberg et al., 1991]. The similar native size of about 80 kDa, for TGC and FXIIIa, together with the striking identity of their active site sequences indicate that the tertiary structure of these proteins are conserved [Greenberg et al., 1991]. A computer-generated model of TGC based

on the coordinates of the FXIIIa crystal structure was constructed [Iismaa et al., 1997] and illustrated a similar tertiary structure between FXIIIa and TGC: a β-sandwich at the N terminus (1–141), the catalytic core containing the transglutaminase active site cysteine 277 (141–471) and two β-barrels at the C terminus (471–584 and 584–686).

Activation of FXIIIa occurs through thrombin releasing a partial catalytically active 51 kDa fragment [Kim et al., 1999]. This site corresponds to the activation site for the TG 3 and the release of 50 kDa active isoform [Kim et al., 1990]. Proteolytic cleavage of TG5 produces highly active 53 kDa form [Pietroni et al., 2008]. The similar tertiary structure between transglutaminases leads to a conclusion that the removal of the C-terminal barrels at the end of the core domain in the TGC found in this study also corresponds to the proteolytic activation sites for FXIIIa and the TG 3. Amino acid sequence (141–471) of TGC is located in the core domain; the calcium-binding site is located toward the end of the core domain (433–453). The removal of the carboxyl end which contains two β-barrels (471–687) induces conformational changes and exposes the binding domain for calcium ions. These events are required for substrate binding and cross-linking catalytic activity. The function of the C terminus appears to be to inhibit the expression of the endogenous cross-linking activity in the native protein. Investigation of the GTP/ATPase activities for TGC showed that the hydrolytic domain is located in the amino acid residues 1–185 [Monsonego et al., 1997]. The highest nucleotide-binding site was found to be a peptide localized in a 15-amino acid segment, 159-YVLTQQG-FIYQGSVK-173 of the TGC core domain [Iismaa et al., 1997]. This region between residue 159 and 173 is found in all isoforms. In addition to the main GTP-labeling of the 15-amino acid segment, a second GTP-labeling region (residues 465–589) was found, indicating a possible additional GTP-interacting region [Iismaa et al., 2000]. The C terminus of TGC functions to inhibit the expression of the endogenous GTP/ATPase activity in the native protein [Lai et al., 1996]. These forms are different (Fig. 6). TGC isoforms lacking C-terminal sequences were reported [Fraij et al., 1992; Fraij and Gonzales, 1996; Monsonego et al., 1997]. A 36 kDa, N-terminal TGC fragment purified from rabbit liver [Singh et al., 1995] were reported to bind and hydrolyze GTP. From these reports it is clear that the N-terminus of TGC contains many important functional domains.

Several studies have reported the presence of a ~55 kDa protein fragment with no enzymatic activity. Proteolysis of purified TGC from RBC and cell surface of fibrosarcoma cells overexpressed membrane-type metalloproteinase (MT1-MMP) resulted in several cleavage products. The proteolysis of TGC by MT1-MMP resulted in four major cleavage products. Cleavage sites at histidine 461 and arginine 458 yield 53 kDa fragments that represent the N terminal parts of the TGC. Degradation of TGC by MT1-MMP resulted in the inactivation of the enzymatic activity and coreceptor functions of cell surface protein [Belkin et al., 2001]. A 55-kDa form was shown as the dominant proteolytic products of TGC from rat dermal skin wounds and from rat subcutaneous implant tumors [Haroon et al., 1999]. The isopeptide bond formed by cross-linking was increased in the extra cellular matrix after day 1 of wounding and the amount of fragmented TGC increased dramatically by day 1 where the 55 kDa form accounted for as much as 40% of total transglutaminase bands [Haroon et al., 1999]. Activation of cross-linking activity of transglutaminase reached maximum level in the presence of calcium ions in lyso-SM membrane component and protected TGC from trypsin proteolysis with the formation of about 55 kDa fragment [Lai et al., 1997].

In this study, while calcium or GTP were found essential for transglutaminase activation in MP (Figs. 1 and 2), trypsin rapidly degraded the purified TGC to low-molecular mass peptides (55-kDa and smaller) with simultaneous decrease of transglutaminase activity in the presence of calcium or GTP (Fig. 4). Thus, membrane association of transglutaminase as shown in Figures 1 and 2 protected it against rapid degradation.

The TG form reported in this study is the cross-linking active species, a function that has previously been ascribed to TGC. TGH [Fraij and Gonzales, 1996] and TGC [Lee et al., 1993] were reported to bind and hydrolyze GTP. TGH and TGH2 found to be expressed more in tumor cells acid than normal cells [Fraij and Gonzales, 1996, 1997]. Analysis of RNAs from affected AD tissues demonstrated the elevated levels of TGH and in contrast to AD brain the TGH message was not found in aged, non-demented human brain [Citron et al., 2001]. The switch to the TGH may reflect regulation of the coupled signaling pathways associated with these isoforms.

We reported earlier that sequence differences among the various transglutaminase forms might be related to differences in their function and/or specificity [Fraij et al., 1992]. Multiple soluble isoforms of the human TGC are far more complex than previously recognized. The different sizes of the isoforms with cross-linking or GTP binding and hydrolysis may perform different functions in the cell. While TG was shown to be active isoform (Table I), TGH and TGC were found inactive in cross-linking. The cross-linking activities associated with TGC or TG2 may be an important factor to determine the role of this enzyme in cell life or death processes. The present findings of the TG isoform could explain some of the reported contradictory results and the several proposed functions for TGC.

Expression of C-terminal deletions of TGC as glutathione S-transferase fusion proteins exhibited lower cross-linking activities than the full-length TGC [Lai et al., 1996]; Iismaa et al., 2000]. Less than 5% of the transglutaminase activity was detected from mutants truncated at C-terminals of amino acids 447 and 538 [Lai et al., 1996]. Activity detected (5%) from constructs truncated at C-terminals of amino acids 446 (TG446) is comparable to the reported 447 mutant, in both cases the 446 and 447 truncations interrupt calcium binding domain. While construct 512 (TG 512) showed 9% activity (Table II), less than 5% activity was reported for mutant 538, therefore, the longer the peptide present after the calcium binding domain (433–453), the less the enzymatic activity was expressed.

Recombinant TGC was reported to contain cross-linking activity [Lee et al., 1993; Liu et al., 2002]. Assay measurements of transglutaminases from bacterially expressed TGC and TGH preparations, which were induced for 5 h with IPTG (Table II) produced activities. The IPTG induction times in the bacterial expression systems can affect the amount of the conversion to the TG form and accordingly the cross-linking activities as shown in this study. These activities are dependent on the TG amount formed from

the precursors, while TG expression systems produced large amount of TG protein and exhibited very high activity, TGC and TGH expressions resulted in minor TG bands present and exhibited low cross-linking activities (Table II and Fig. 6).

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