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Molecular cloning and characterization of tiger shrimp (*Penaeus monodon*) transglutaminase

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Abstract

Transglutaminases (TG) are important for blood coagulation and post-translation remodeling of proteins. Using a plaque screening assay, we isolated cDNA encoding a novel TG from a shrimp (*Penaeus monodon*) hemocyte cDNA library. The TG cDNA consists of 2988 bp with an open reading frame of 2271 bp. The deduced protein has 757 amino acid residues, a calculated molecular mass of 84,713 Da and an isoelectric point of 5.56. Neither a typical hydrophobic leader sequence nor a transmembrane domain could be identified from the deduced sequence. Thus, shrimp TG may be a typical cytoplasmic protein. The sequence of shrimp TG was similar to crayfish, other invertebrate and vertebrate TG sequences. Enzyme activity was detected in all organs tested. This is consistent with the widespread, low-level expression of TG mRNA. However, high levels of TG expression were detected in hematopoietic tissue. TG signals were stronger in mitotic cells, indicating that cell proliferation and TG synthesis are associated. Preliminary data showed that recombinant TG existed the enzyme activity but lacked coagulation activity.

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1. Introduction

Transglutaminases (TG) (EC 2.3.2.13) are known primarily for their roles in blood coagulation and post-translational protein remodeling. Enzymes in this

family use a modified double-displacement mechanism to execute a calcium-dependent acyl transfer reaction between the γ -carboxamide group of a peptide-bound glutamine residue and the ϵ -amino group of a peptide-bound lysine or the primary amino group of a polyamine. When a protein-bound lysine residue acts as an acyl acceptor, intermolecular or intramolecular ϵ -(γ -glutamyl) lysine bonds form, resulting in protein polymerization [1–4].

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In vertebrates, eight different TG gene products have been characterized based on primary structure [5]. In addition to this gene level diversity, TG undergo a number of post-translational modifications, including phosphorylation, fatty acylation, and proteolytic cleavage, that regulate their enzymatic activity and subcellular localization [6–10]. TG are widely distributed in tissues and body fluids, and specialize in cross-linking particular proteins or tissue structures for a variety of biological processes. Blood coagulation factor XIIIa catalyzes the cross-linking of fibrin monomer during blood coagulation [11]. Both TG1 (a membrane-bound form of TG_K in keratinocytes) and TG3 (TG_E occurring as a proenzyme in the epidermis) help maintain toughened skin epidermis and hair follicle epithelia by cross-linking structural proteins [12–16]. TG2 (tissue TG/TG_C distributed mainly in the cytosol of many cells and tissues) has been implicated in apoptosis, and the formation and stabilization of the extracellular matrix. In certain signal transduction pathways, TG2 acts as the GTP-binding protein, G_h. It profoundly affects cells by regulating the biological activity of signaling molecules, including transforming growth factor- β , interleukin-2 and midkine, and by modulating cell-matrix interactions [17–21]. TG4 (TG_P found in the prostate) is essential for semen coagulation [22]. Human TG X, Y and Z have been discovered recently and their function is still unknown [5,10].

In invertebrates, only one TG gene product has been identified from each species. In vertebrates and invertebrates, blood clotting reduces the blood loss following injury. A novel TG gene, whose sequence is homologous with that of factor XIIIa, was isolated from crayfish (*Pacifastacus leniusculus*) [23]. Crayfish TG plays a role in blood clotting. The presence of endogenous TG causes rapid assembly of a specific plasma clotting protein [24]. In contrast, the TG cloned from horseshoe crab (*Limulus polyphemus*) hemocytes does not recognize coagulogen or coagulin as the substrate [25,26]. However, horseshoe crabs use the TG cross-linking reaction during the final stage of coagulation [27]. Annulin is homologous to the TG expressed at limb segment boundaries in grasshopper (*Schistocerca americana*) embryos. The pattern of annulin expression within the limb and the embryo is associated with areas undergoing morphogenetic rearrangements, movements, or rapid cell division. It

may stabilize cells under mechanical stress or participate in morphogenesis in some other way [28]. A novel TG (nuclear TG, nTG) that occurs exclusively in the nucleus of embryonic starfish (*Asterina pectinifera*) cells was described recently [29]. Because nTG mRNA first appears during the early blastula stage and increases thereafter, this enzyme may be important for nuclear remodeling during early starfish embryogenesis. A TG homologue from the *Drosophila* genome, gene product CG7356, has been identified [30]. Its function is still unknown.

In this study, we isolated from tiger shrimp (*Penaeus monodon*) a cDNA that encodes a novel TG. Based on the preservation of residues critical for enzyme function and domain folding, and the extensive, overall similarity of shrimp TG to the other members of the TG family with catalytic activity, we postulate that the characterized cDNA encodes an active TG. The specific function of shrimp TG was not identified in this study. However, we found high levels of TG expression in different tissues. Thus, shrimp TG may be involved in other biological processes.

2. Materials and methods

2.1. Construction of a cDNA library and screening for transglutaminase

Live shrimp (*P. monodon*) were purchased from the local market. Shrimp hemolymph was collected using an anticoagulant, shrimp salt solution (SSS) [31], and centrifuged for 5 min at 500g and 4 °C. The hemocyte pellet was re-suspended in Trizol (Gibco BRL). The total RNA was extracted and further purified using an mRNA isolation kit (Pharmacia). An oligo-dT primed cDNA library was constructed using a UNI-ZAP XR cDNA synthesis kit (Stratagene). The cDNA was inserted into the *Eco*RI–*Xho*I site of λ phages and subjected to in vivo packaging, as described in the manufacturer's instructions.

A 392 bp-fragment, which was derived from crayfish TG (AAK69025) and contained His and Asp of the catalytic triad, was ³²P-labelled using the Megaprime™ DNA labeling system (Amersham). The labeled fragments were separated from unincorporated ³²P-labelled nucleotides (NICK™ column,

Pharmacia Biotech) and then used for the initial screening of 5,00,000 plaques from the hemocyte cDNA library. Hybridizations were carried out overnight at 63 °C in a hybridization buffer containing 5 × Denhardt's solution, 5 × SSC (0.75 M NaCl, 0.075 M Na-citrate, pH 7.0), 0.5% SDS and 1 mg/ml salmon sperm DNA. Membranes were washed at 63 °C with 2 × SSC containing 0.1% SDS and autoradiographed. Secondary screening was performed to purify positive plaques. Phagemids, obtained by *in vivo* excision using EXASSIST Helper phage with SOLR strain (Stratagene), were used for sequencing.

2.2. Rapid amplification of 5'-mRNA ends

The total RNA in shrimp hemocytes was isolated using Trizol reagent (Gibco, BRL) according to manufacturer's instructions. To synthesize cDNA by reverse transcription, 4 µg total RNA, THERMO-SCRIPT™ reverse transcriptase and 0.5 µM oligo(dT)₂₀ or 5'-TTCTTGAATCCTCCCTCCGC-3' (reverse, STG3) primer were reacted for 1 h at 50 °C in 20 µl reaction mixture containing 1 mM dNTP and 5 mM dithiothreitol in first strand buffer. The cDNA was purified using a GlassMax DNA isolation kit (Life Technologies, Inc.). To anchor the PCR product at the 5' end, cDNA was tailed for 10 min at 37 °C using 15 units of terminal deoxynucleotidyl transferase (Promega) and 200 µM dCTP. To amplify the 5'-mRNA ends of shrimp TG, we used a series of TG-specific primers as follows: 5'-CCAGATGGAGT CACGAATG-3' (reverse, STG2) and 5'-GGCCACG CGTCGACTAGTACGGGIIIGGGIIGGGIIG-3' (forward, anchor primer, AP), with nested reactions 5'-TGGAGAGTGAGATGTTGGTG-3' (reverse, STG5) and 5'-GGCCACGCGTCGACTAGTAC-3' (forward, abridged universal amplification primer, AUAP); 5'-CGAACACCCAGCACTGGCCG-3' (reverse, STG6) and AP, with nested reactions 5'-G TCCCCGCGCGCTCGGC-3' (reverse, STG7) and AUAP. PCR was performed using 1 µl reverse transcription reaction mixture, 2.5 units VioTaq DNA polymerase (Viogene) and the respective buffer supplemented with 1.5 mM MgCl₂, 0.2 mM dNTP and 0.2 µM of each primer, in a total volume of 50 µl. A robocycler ran 25 cycles. Except for the first and last cycles, each cycle included denaturation at 94 °C

for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min. The first cycle included an extended (2 min) denaturation period during which polymerase was added (hot start) and the last cycle had an extended (10 min) elongation period. The first round of PCR was carried out with AP and TG-specific primer under the conditions described above. Nested PCR was performed with the AUAP and TG-specific primer using 1 µl of the first round PCR product. Amplified products were analyzed on 1.5% agarose gel, extracted with a Gel-M™ gel extraction kit (Viogene) and sequenced.

2.3. RT-PCR analysis of TG from different tissues

Total RNA was extracted from a variety of shrimp tissues, including the eyestalk, intestine, hepatopancreas, gill, heart, lymphoid organ, sub-cuticular epithelium and hemocyte. An 806-bp fragment of shrimp TG was amplified by PCR using oligonucleotides 5'-AGGGTTCTTCAAGTCGGAC-3' (forward) and 5'-CAGTTCGTCTCCAGATTCAAG-3' (reverse). RT-PCR was performed as described above. The PCR products were sequenced to ensure that they corresponded to the expected cDNA sequence.

2.4. *In situ* hybridization of TG mRNA in tissue sections

2.4.1. Tissue preparation for histology

Juvenile shrimp were fixed in a RNA-friendly fixative containing 34.9% formalin, 40.7% ethanol and 2.2% ammonium hydroxide (pH 6.5) [32]. After dehydration, samples were embedded in Paraplast and 4 µm sections were cut, mounted on slides and stored at 4 °C until used.

2.4.2. Riboprobe preparation

A plasmid pGEM-T Easy (Promega) containing a 350-nucleotide region (1535–1884nt) of shrimp TG cDNA was used as the template for the preparation of probes. This region contained a sequence encoding the barrel 1 domain. Digoxigenin (DIG)-UTP-labeled sense and antisense riboprobes were generated from linearized cDNA plasmids (5 µg) by *in vitro* transcription RNA labeling kits T7 and SP6 RNA polymerase (Boehringer, Mannheim), respectively.

2.4.3. *In situ* hybridisation

Tissue sections were deparaffinized in xylene and hydrated through a graded ethanol series. After proteinase K digestion (20 µg/ml, 30 min at 37 °C), the sections were acetylated for 10 min at room temperature (0.25% acetic acid, 0.1 M triethanolamine, pH 8.0), dehydrated in ethanol and air-dried. Sections were incubated at 42 °C in a hybridization buffer of 1 × SSC, 50% formamide (Amresco), 1 × Denhardt's solution, 5% dextran sulfate (Sigma), 1 mg/ml yeast tRNA (Sigma), and 10 ng/slide of probe. After hybridization, the sections were washed in 1 × SSC for 30 min and in 0.1 × SSC for 15 min at 50 °C. Then the sections were digested with 20 µg/ml RNase (Sigma) for 30 min. Hybridization signals were detected with an enzyme-linked immunoassay as described in the manufacturer's manual (Nucleic Acid Detection Kit: Boehringer, Germany). The enzyme reaction was stopped with 100% methanol for 10 min. Sections were counterstained with Bismarck Brown Y (Sigma), dehydrated and made transparent with a graded ethanol and xylene series, and then mounted in GEL/MOUNT™ (BioMeda corp).

2.5. TG activity in different tissues

2.5.1. Preparation of sample

Hemolymph was collected into an anticoagulant (10 mM Tris–HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6) and centrifuged for 10 min at 500g and 4 °C to separate the hemocytes and plasma. Shrimp tissues, including the eyestalk, intestine, hepatopancreas, gill, heart, lymphoid organ and sub-cuticular epithelium, were manually homogenized. Liquid nitrogen was added continually to maintain a sufficiently low temperature. The hemocyte pellet and tissue homogenate were re-suspended in a Tris–EDTA buffer (50 mM Tris–HCl and 1 mM EDTA, pH 7.4), centrifuged to eliminate debris, and stored at –20 °C. Plasma was mixed with Tris–EDTA buffer and stored at –20 °C. Standard enzyme, a tissue-type TG from guinea pig liver (Sigma), was dissolved in Tris–EDTA buffer to an initial activity of 0.25 U/mg.

2.5.2. Assay procedure

TG activity was assayed as described by Song et al. [33]. Casein (200 µg/well), coated on the microtitre

plates overnight, and biotin-labeled casein (0.8 µg/well) were used as substrates in a TG-dependent cross-linking reaction. For the assay, each sample was serially diluted two-fold with 50 mM Tris–HCl, 1 mM EDTA, pH 7.4, and supplemented with CaCl₂ or EDTA to achieve a final concentration of 10 mM in each well. Streptavidin-labeled alkaline phosphatase (Calbiochem), followed by *p*-nitrophenyl phosphate, were added to visualize immobilized biotin. Absorbance at 405 nm was measured. Using guinea pig liver TGase, a standard curve was established for enzyme activity (unit/mg) and OD 405 nm. The protein concentration in each sample was determined by the Bradford method [34] using bovine serum albumin (Bio-Rad Protein assay Kit II) as the standard.

2.6. Analysis of nucleotide and amino acid sequences

The nucleotide and deduced amino acid sequences of shrimp TG cDNA were analyzed using a GCG (Genetic Computer Group, Inc., Madison, Wisconsin) software package. The sequences of different species were compared with the NCBI BLAST search program and NCBI Blast search with Entrez. The amino acid sequences of all cloned TG were aligned with the DAMBE (Data Analysis in Molecular Biology and Evolution, version 4.0.75) software package [35]. A multiple sequence alignment was created with Clustal W. Phylogenetic and evolutionary molecular analyses were conducted using MEGA version 2.1 [36]. The phylogenetic tree was constructed using the neighbor-joining method [37].

2.7. Expression of recombinant TG (rTG)

The *Stu*I–*Bgl*II fragment, which contained the entire coding region of shrimp TG, was inserted between the *Stu*I and *Bgl*II sites of baculovirus transfer vector pABhRpX to obtain a pABhRpX–TG transfer plasmid. Sf21 insect cells were grown at 26 °C in TNM-FH insect medium (GIBCO) containing 8% heat-inactivated fetal bovine serum (HyClone) in a monolayer flask. The pABhRpX–TG plasmid was transfected with a BaculoGold linearized Baculovirus DNA (BD) into Sf21 cells with Lipofectin (GIBCO). After plaque purification, a single clone of each recombinant virus was amplified and used for protein expression.

Harvested cells was re-suspended in Tris–EDTA buffer (50 mM Tris–HCl and 1 mM EDTA, pH 7.4), sonicated, centrifuged to eliminate debris and stored at -20°C until used in the TG activity and coagulation assay.

2.8. Coagulation assay

2.8.1. Preparation of sample

Hemolymph was collected into an anticoagulant (10 mM Tris–HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6) and centrifuged for 10 min at 500g and 4°C to separate the hemocytes and plasma. Plasma was dialyzed against Tris–EDTA buffer and stored at -20°C . The relative activity of TG was adjusted so it was the same in sample solutions from shrimp tissues and Sf21 cells transfected with rTG. The sample solutions then were used in coagulation assays.

2.8.2. Assay procedure

To assess the role of shrimp TG in coagulation, 100 μl plasma, 10 μl 200 μM CaCl_2 solution and 50 μl of each sample solution were added to a round-bottom 96-well microdilution plate (Nunc, Denmark). The solutions were mixed thoroughly and incubated at room temperature. Coagulation was evaluated by eye.

3. Results

3.1. Localization of TG activity

Tissue homogenate was extracted from several shrimp organs to determine the distribution of TG protein. TG activity was detected in the eyestalk, intestine, hepatopancreas, gill, heart, lymphoid organ, sub-cuticular epithelium and hemocytes, but no activity was detected in the plasma. In all organs tested, TG activity was blocked when 10 mM EDTA was added instead of 10 mM CaCl_2 . TG activity was greatest in the hepatopancreas, then the heart, hemocytes and other organs (Table 1). TG activity was detected in Sf21 cells transfected with rTG. No activity was detected in Sf21 cells not transfected with rTG.

Table 1

TG activity in the hemocytes, other tissues of *Penaeus monodon* and TG transfected cells

Tissue/cells ^a	TG activity (Unit/mg) ^b
Hepatopancreas	27.920
Heart	1.589
Hemocyte	1.111
Lymphoid organ	0.575
Intestine	0.322
Sub-cuticular epithelium	0.224
Eyestalk	0.118
Gill	0.042
Plasma	Undetectable
Sf21	Undetectable
TG transfected Sf21	0.195

^a All the tissues or cells were prepared in sample buffer containing 50 mM Tris–HCl and 1 mM EDTA, pH7.4.

^b There was no detectable activity in the negative control, which was supplemented with 10 mM EDTA instead of 10 mM CaCl_2 .

3.2. Determination of the cDNA and amino acid sequences of shrimp TG

Shrimp TG cDNA is comprised of 2988 bp, with an open reading frame of 2271 bp (Fig. 1). The probable initiation codon is in the sequence CAAAATGC. It exhibits limited homology with the consensus sequence identified by Kozak [38] that acts as a signal for efficient transcription in eukaryotes. However, the critical purine in position -3 is conserved. The polyadenylation signal (AATAAA) was located 626 bp downstream of the termination codon (TGA). The deduced protein is comprised of 757 amino acid residues, has a calculated molecular mass of 84,713 Da and an isoelectric point of 5.56. It does not contain a typical signal sequence or a transmembrane domain. Four potential N-glycosylation sites and an integrin-binding motif (RGD) were identified (Fig. 1).

3.3. Sequence comparison of shrimp TGase with other TG

Shrimp TG had the highest identity/similarity (55/71%) to crayfish TG (Fig. 2). Shrimp TG exhibited significant identity/similarity with other invertebrate TG and members of the vertebrate TG gene family, especially human factor XIIIa (32/50%). Detailed comparison of the sequences by individual

1	TACCTGAGGCGTCCCTTTGCTGTTCGCAAGTTGCACTGCCGTGCGCAAAATGCCACCGTGGACGCCATAAGCGACCTCGCCAAACCGCTTCAACCAAGA M P T V D A I S D L A N R F T N D	17
101	CCTCGAGCTGTGTCCCGGAGGACCGAGAATTGAGATGCCACTGACCTCAATGAGATCCCGACCGCTCTATCCCGCTCGAAAGACCCGAGATCGTG L E L C R R E D R E F E I A T E L N E I A D A L S A S K D P K I V	50
201	TCGGTGGACTTCCAGCCAAATGTACAACCGCGTCAACCCACTCGGAGTTGTACGAGCTGTGACCGCCGACCCGACTCGTGTGCTGCGTGTGCGGG S V D F Q P M Y N A V N H H C E L Y E L V D R R T D C V V V R R G	83
301	GCCTCTCTCCATGTTGTCAGCTGAAACGAGAACTGGCGCTCGCTCGGCTCAGGAGCTCAAGTTCTACTTCTCTCGGTTCTCGGCTACCTGGA G L L S M V V K L N Q N V A L A S A Q E L K F Y F S F G S R P N V E	117
401	GAAAGGACCCAGGCTCACTCGTGTGTCACGGGAAAACCAAGATTGAAAAACCCACGACGCTGGGACATAAGGCTCGTCAAGGAGCGCGGAAAGAAAG K G T Q A H L V V T G K T K I E K P H D D W D I R L V K E A G K K	150
501	GCTACCGTTGAAGTCAAATCCCGACCGCCCGCGTGGTGTGTGAAAGTGGCGCGAGGTGACCGTCCGGAACAGCGCGGAGAGAGCCACTGA A T V E V Q I P T D A P V G V W K V A A E V T L R N Q A G E R H L	183
601	GGCGTTCGACACGCTATATCCCTTCAATCCCTGGAATGAAATGATGGACACATCCTCCCGAGGAAAAATGGCGAGGATATGCTGCTTC R R F D T H V Y I L F N P W N E N D G T Y L P E E K M R Q E Y V L S	217
701	TGCCGTAGGACCGTTTTCGTGGGAGTTACCCGACACTGTGGCCGACCTGGGCTTCGGCAGTTGACGACCGCGTCTCGCCGACATGCTGCTG A V G T V F V G S Y P H T R G R H W A F G Q F D D A V L P A C M L	250
801	CTCTCGAGAGTTCGGGTCAGTCCCGAGCGCGCGGGGACCCCGTCCGAAATGTCGAGGGCCCTCTCCAAGTGGTGAACAGCAACGATGACTCCGGCC L L E R S G V S A E A R <u>G D</u> P V R M S R A L S K M V N S N D D S G	283
901	TTCTGTGGCAAGTGGGACGAGATGACGAGACCGCAGGGCGCCCGCAAGTGGCTCGGTTGATCGAGATTCTCGAGATGATCCCTCAGGACACCGCA L L V G K W D G E Y E D G R A P A K W L G S I E I L E M Y L R T R Q	317
1001	GTCCGTGAAGTACCGCAGTGTGGGTTTCGCCCTGTCTCAACACCATATGTCGGCCCTGGTTCGCCGTGCGTGTGTCGCAACTTGGCTCT S V K Y Q C W V F A A C L N T I C R A L G L P C R V V T N F A S	350
1101	GCACACGACACACATCTCACTCTCCATGACGAACTTTGACGAGGACGGGACGAGATCGATGACGCGGATCGTTACGTCACCCAGCTGGCATT A H D T N I S L S I D E Y F D E D G D Q I D A A D R Y V N P A G I	383
1201	GTGACTCCATCGAACTCGCAAGTTTGGAAAGCTGTGGATGAGCGGACCTGACCTCCCTGACCGTTTCGGAGCTGGCAGTCAATCGACCCACCGC R D S I W N F H V W N D V W M S R P D L P D G F G G W Q V I <i>D A T P</i>	417
1301	ACAGGAGACAGGATGGTCTCAACAGTCCGGCCAGCTTCCACGAGGCCATCAGGACGGCGCGTGAAGTGAAGTACGACGTCGCCCTTCGTGCTG Q E T S D G S Y Q C G P A S H E A I R Q G R V D L K Y D V P F V L	450
1401	GCCGAGTCAACGCTGATGTCGGCTGCGAGAGGACGACCGGGCGGAGGAGATTCAAGAACTGGCGACGAAACGAGCGCTGGCGCCCTGA A E V N A D V V R W Q K D D R A E G G F K K L A T Q T S S V G R L	483
1501	TCCTGACGAGAAAGCCGTCGCGTGGCCACAGGAGGTTCTTCAAGTCCGACCCGAGGACATCACTGGGAATACAGACCCCGGAGGAAACCGCGGC I L T K K A G P V A T G G F F K S D R E D I T W E Y K P P E G T R A	517
1601	CGAGGGTTCAGCATCTCAACCGCGCTGAAAGGACCGGACCGCTCGCCAGTCTTCGACATGCCAGCGGCGAAGGAGGAGTCACTTTCAGATC E R V S I L N A A R R T R T A R Q V F D M P S E A K E D V T F Q I	550
1701	GAGACCCGATCAGTCCCACTCGCGATAATTTCTCGTACGATAACCCGACGAAACACGAGCGAGGTGAGGAGATCAAGCTAGTGTGACCT E D R D Q V P I G D N F S V T I T A T N T S S E V R T I N V V M T	583
1801	GCCCAGCATGTACTACAGCGGACCAAGCCACACAACTCAAGCCCATGACCGGAGCTGCATCGAAACCCAGCAGCAAGACTATGTCTATGGA C A S M Y Y T G A K A H T I K R H D G E L H L E P S Q T K T M S M E	617
1901	AGTCTACTATCTGACTACTCGAAGCTTGTGGACCATGACCTTATCAAGGACATTGTCTGCAACGCGAATGAAACGACCCAGTCTGGCGGG V Y Y S D Y Y S K L V D H D L I K S I V I C N V N E T T Q C W G G	650
2001	GAAGACAACCTGAGGTTCAAGGCTGATCAACATTGAAGTGTGGATCGCCGTAAGGCTGTTCCTATCAAGGTTCTTCGATAACC E D N L Y E V H K P D I N I E V L G S A V E G K A V P I K V S F D N	683
2101	CTATTCCACTGCTGTGAAAGAACTGCTGTGAAGCTGGACGGCGGGGCTCATGCGTCCGAAAGACAGTCCCACTCGAAAAATCGCCCAAGGAAA P I P L L L K E C C L K L D G P G L M R P K T V P L E N I A P K G K	717
2201	GATGAACCTTGAGATGAACCTGTACCGGAAAGGCGGTGGAGGTACATTGATCGTGCAGTCAACAGTAAAGGAGCTGTGCAACCTCACTGTGCCAAG M N F E M N L Y P K K A V E G T L I V Q F N S R E L C N L T G A K	750
2301	AAGATTAGCGTCCAGTCTTGAATCTGGAGACCACTGAAGCTTACAATTCGGTGTATTATTATTGTTTTCGGTGGCCCGGGACTAAAAAAA K I S V A S A stop	757
2401	AACCTAAGCAAGAGAGAACCCCTTTCTGTTTGTGTTTACGCTTATGTGTAGCGCTATTTCGCTACTAACATGTTGAGCATAAAGTCTTCTAGAT	
2501	AAAACTTATAGGATTTAAGAACAGTATCTGCAATCGTATTTACCAAGAGCTTTGGGATAAGTATGTAATGCACTTAATGTATTTTCTTTATGT	
2601	TTGAATTAGCTATAGTAAGTTAATTAATAAAAAATGCAATAGCTTAATTTTGTGTTAAGTAAATATTTGAATAACCGTCAATGGCTGATCAACATAA	
2701	GTCTGATAACAAATCTGAAATTCAAAAATGTTATAGTATCCCAAGTAAACTATAGTATGATTAATCAAGACTAATCAAACTGGTGTATCAATTC	
2801	GAAATTCAGGAAATCACATAGAGAAATGTTGTTAGAAAGCAATTAATTTTCTTTTATCAATTTCTTTTAAATTCGAAAGTAAAGAAACATACCA	
2901	AATATGTTTATGTTGCAACGATGATGTTGATGATGACCTGCTCTTTAAATAATGATACCAAAATCAAAAAA	

Fig. 1. Nucleotide and deduced amino acid sequences of tiger shrimp TG. The number of nucleotides is shown in the right column of numbers; the number of amino acids is shown in the left column. The integrin-binding motif (RGD) and polyadenylation signal (ATTAATA) are underlined. The catalytic triad is shown with bold font and italics. Amino acid residues potentially involved with calcium binding are indicated with an asterisk '*'. Four putative glycosylation sites are indicated with dots.

tiger_shrimp_TG	-MPTVDAIS----DLANRFTNDLELCRREDR--EFEIATE----LNEIA-----	38
crayfish_TG	-MAVFERIENFFDNLSDRFRREFHDNRREEKALENEIEAT---HAETS-----	44
horseshoe_crab_TG	-MYGFGRGN-MFRNRSTRYRRRPRYRAENYHSYMLDLEN---MNEEFGRNWWGTPESH	54
drosophila_TG	-MKRAEQDGASSAVLG-----	15
starfish_TG	MVRRSTRTRSTPTRFGYTRDFEPYARKPKRETTRTEGRRYVPATP-----LTLF	49
human_factor_XIIIa	-MSETSRTAFGGRRRAVPPNNSNAEDDLPTVELQGVVPRG-----	39
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tiger_shrimp_TG	DALSASKDPKIVSVDFQPM---YNAVNHHCCELVELVDRRTDCVVVRRGGLLSMVVKLNQN	95
crayfish_TG	NAEPVTK-VKIVSVDFQAK---LNAEKHHCEKMKLVDIKEGTPITRRSGSFIVVEFNKE	100
horseshoe_crab_TG	QPDGSPSSLQVESVELYTR---DNAREHNTFMVLDVGTGKPVLIILRRGQPFSAIRFKRN	111
drosophila_TG	-----VLKVDLCLE---DNHEEHHTSHFYAAAAKE-ALVVRGPEPRLKIHFNRD	61
starfish_TG	TLKEKKTQLKVVSVDFLCVE---RNQOEKTKSKVKVDN-----LVLRRGQPFHLNVKFRD	101
human_factor_XIIIa	--VNLOEFLNVTSVHLFKERWDTNKVDHHTDKENNK-----LIVRRGQSIVQIDLSRP	92
<hr/>		
tiger_shrimp_TG	VALASAEQELKFYFSFCSRPNVEKGGQAHLVVTGKT--KIEKPHDDWDIRLVKEACKKATV	153
crayfish_TG	IDLKKQEQLTLYFSFCAGPNAQAGLTLATLVVTGKK--LFDKHHENWDRVVNQACTKATI	158
horseshoe_crab_TG	YNP-QQDRLEKLEIGFCQQPLITKGLLIMLPVSGSD--TFTKDKTQWDRRLRQHDCAVITL	168
drosophila_TG	YSP-SKDAISFIPTVADDTKPSPGHGTLNALVPHDGDYLDGDTLEWGAGIESHEGQTLTV	120
starfish_TG	FKP-STDELVLLELRMSRANVTKGRCVAPVVTSA-----PDHDDWGIKVESAKCANVIL	155
human_factor_XIIIa	YDP-RRDLFRVEYVIERYPQENKGLIYIPVIVSELQ-----SGKWGAKIVMREDRSVRL	145
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tiger_shrimp_TG	EVQIPTDAPVGVWVAAEVTLRN--QAGERHLRR-FDTHVLLFNFPWNENDGTYLPEEKM	210
crayfish_TG	EIQVSSDAPVGVWVSCSELEVRPKDETDASNRLLR-LDTLLVLLFNFPWNENDSTYMAEBER	217
horseshoe_crab_TG	EIQIPAAVAVGVWVKMIVSQTSEEQPNVSAVTHECKNKTLLFNFPWCKQDSVYMEDQW	228
drosophila_TG	LIKPPSTCPVTEWKLDIDTKLLGDGSRSYPLPLP-----LIVLFPWCPDDQVYLEDSDQ	175
starfish_TG	KVFCSEALIGYNYLYLITMSGGD-EYYESPK-----LIMLFPWCKDDVYMADEVK	209
human_factor_XIIIa	SIQSSPKCIIVGKFRMYAVWTPYGLVTRSRNPET---DTYLLFNFPWCEDDAVYLDNKE	201
<hr/>		
tiger_shrimp_TG	RQEVVLSAVGTVEVGSYPHTRGRHAFGQFDDAVLPACMLLERSG-VSAEARGDPVRMS	269
crayfish_TG	LEEYVLADVGKIWQSSYPTAKGRLVWFGQFDDVVLPAVLLMERSG-LRPEARGDPVIRMS	276
horseshoe_crab_TG	RKEYVLSDVGKIETGSEFKQPVGRRWIFGQFTDSVLPACMLLERSG-LDYTARSNPKIVV	287
drosophila_TG	RKEYVMHDTTLIWRGSYNLRPSVWIKIGQFERHVLKSLKVLGTVCRIPPAYRGPVPRVA	235
starfish_TG	RQEVVMGEVSLYFYGSYRIGSSPNYGFQEKMSLDCALYLLQKSG-MPDSSRKSPIQVMS	268
human_factor_XIIIa	REEVVLNDIGVIFYGEVNDIKTRSYGQFEDGILDTCLYVMDRAQ-MDLSGRGNPKIVS	260
<hr/>		
tiger_shrimp_TG	RALSAMVNSNDDSGLLVGGKWDGEYEDGRAPAKNLGSIETILEMYLRLTR-QSVKYGQCWVFA	328
crayfish_TG	RAISRIVNSNDDRGVVGKWDGIYDDGTSBSGWTGSIKILEEYVRKN-HPVKYGQCWVFA	335
horseshoe_crab_TG	RAISAMVNIIDDEGVLGCRWQEPYDDGVAFWMTGSSAILEKYLKTRGVVPVKYGQCWVFA	347
drosophila_TG	RALSALVNSVDDDGVLGNWSEDFSGGVAFTKWTGSVEILQQFYKTQ-KSVKFAQCWVNS	294
starfish_TG	RVLSALVNAQDDDGVLVGRWDGEYDDGISPTTWTGSAIILSOYMKTR-ESVKYGQCWVFG	327
human_factor_XIIIa	RVGSAMVNAKDDDEGVLVGSWDNIYAYGVPSAWTGSVDILLEYRSSE-NPVRYGQCWVFA	319
<hr/>		
tiger_shrimp_TG	ACLNTICRALGLPCRVTNFAHAHDNINISLIDEYFDEGDQIDAADRYVNPAGIRDSIW	388
crayfish_TG	GVVNIIVCRALGLPARVVTNLSAHDINVSLETIDEYFDEGKEIKYRSDIINPGGLNDSIW	395
horseshoe_crab_TG	GVANIVSRALGIPSRVTNYSADHDIDDTLITDKWFKNGDKIEDAT-----SDSIW	399
drosophila_TG	GVLTITARSGLGIPSRITCYSSAHDQAASLTVDFIDANNKKLDAET-----TDSIW	346
starfish_TG	SLLTGLCRSLGLPTRITTFNFAAHDIDGNLITLDYHYDENSEPLDDYD-----EDSIW	379
human_factor_XIIIa	GVFNIFLRCLGIPARTIVTNYFAHDNDANLQMDIFLEEDGNVNSKLT-----KDSIW	371

Fig. 2. Alignment of shrimp TG with crayfish TG (GenBank accession number: AAK69205), horseshoe crab TG (A45321), drosophila TG (AAF52590), starfish TG (BAB20439) and human factor XIIIa (NP_000120). Amino acid sequences start with the first methionine. Residues that are identical in all six sequences are enclosed in a black box (in web version). Residues identical in five sequences are enclosed in a dark gray box (in web version) and residues identical in four residues are enclosed in light gray (in web version). RGD motifs are underlined and amino acids in catalytic triads are shown in bold italics. Dots indicate amino acid residues that are probably involved with calcium binding. The identity and similarity of the residues of each species to shrimp TG are shown at the end of each TG sequence. The pink, orange, green, and blue bars (in web version) indicate the sandwich, core, barrel 1, and barrel 2, respectively.

tiger_shrimp_TG	NFHVWNDVWMSRPDL PDG---F G G W Q V I D A T P Q E T S D G S Y Q C G P A S H E A I R Q C R V D L K Y	444		
crayfish_TG	NFHVWNDVWMTRPDL PDG---Y G G W Q A I D A T P Q E T S D C M F Q C G P A S L E A V R R C Q M E L K Y	451		
horseshoe_crab_TG	NFHVWNDCWMPDL P T G---Y G G W Q A Y D S T P Q E T S E V V Y Q T G P A S V L A V Q R G E I G Y M F	455		
drosophila_TG	NYHVWNLWMPQRPDL G V G E H G T F D G W Q V V D A T P Q E T S D N M Y R V G P A S V A A V K N G D I L R P F	406		
starfish_TG	NFHVWNDCWMPDL E E G---Y G G W Q A V D A T P Q E T S N C V Y C M G P T S L R A I K Q C H V Y M Q Y	435		
human_factor_XIIIa	NYHCWNEAWMTRPDL P V G---F G G W Q A V D S T P Q E T S D C M Y R C G P A S V Q A I K H C H V C F Q F	427		
	H D			
tiger_shrimp_TG	DV P F V L A E V N A D V V R W Q K D D R A E G G F K K L A T Q T S S V G R L I L T K K A C P V A T G G F F K S D R E D	504		
crayfish_TG	DV P F V L A E V N A D V V R W K E D K E S Q D G F K K L F A D K S H V G R Q V L T K K P C P V D S G R F Y E K D K E E	511		
horseshoe_crab_TG	D S P F V F S E V N A D V V H W Q E D D S S E T G Y K K L K I D S Y R V G R L L L T K K I C---V D D D F G D A D A E D	513		
drosophila_TG	D G G F V F A E V N A D K L Y W R Y N G P S-Q P L K L L R K D T L A I G H L I S T K A V L-----K W E R E D	457		
starfish_TG	D T K F A F A E V N A E K V Y W K V F T K S R K A P E V I D I D S D D V G C K I S T K A V G-----K F E R E D	487		
human_factor_XIIIa	D A P F V F A E V N S D L Y I T--A K K D G T H V V E N V D A T H I G K L I V T K Q I G-----G D G M M D	477		
tiger_shrimp_TG	I T W E Y K P P E G T R A E R V S I L N A A R R T R T A R Q V F D M P S E A K E D V T T Q I E D R D Q V P T G D N E S V	564		
crayfish_TG	C T K D Y K P Q E G S R E E R V T L H S A A R R T R A R H A F R F P S E A V E D V D F S I Q D V E R V P T G D D E A I	571		
horseshoe_crab_TG	I T D Q Y K N K E G T D E E R M S V L N A A R--S S G F N Y A F N L P S P E K E D V Y E N L L D I E K I K T G Q P F H V	572		
drosophila_TG	I T D T Y K H A E R S E E R S T M L K A L K Q S R H A F S R Y L N D N F N-D I E D M E L K D D I K I G Q S E S V	516		
starfish_TG	I T E Q Y K Y K E G T E L E R I A V R E A S R H V R K A K R--I L K N L V R-D V D T D V D M A E E F P T G K D I K F	544		
human_factor_XIIIa	I T D T Y K F Q E G Q E E R L A L E T A L M Y G A K K P L N T E G V M K S R S N V D M D F E V E N-A V L G K D K L	536		
tiger_shrimp_TG	T I T A T N T S S E-V R T I N V M T C A S M Y Y T G A K A H T I K R H D G E L H L E E S Q T K T M S M E V Y Y S D Y	623		
crayfish_TG	T V K A L N T S E A-K R T V T I V M T A A S H Y Y T G A K A N D I A R A E G S F V L Q P K E N K T L A L P V T Y K Q Y	630		
horseshoe_crab_TG	T V N I E N O S S E-T R R V S A V L S A S S I Y Y T G I T G R K I K R E N G N F S L Q E H Q K E V L S I E V T P D E Y	631		
drosophila_TG	V L K V S N K S E S R T H M A T G Q I S C D A V L Y T G V G A V E V K T L G F E L E L E E K S S D Y V R M E V I F E E Y	576		
starfish_TG	T I T M V N K S Q Q T R N V F L G-V T G S T V Y Y T G V K K A V S S Y N G T L P L K A K E T R V I P V T V P A S D Y	603		
human_factor_XIIIa	S I T F R N N S H N-R Y T I T A Y L S A N I T F Y T G V P K A E F K K E T F D V T L E E L S P K K E A V L I Q A G E Y	595		
tiger_shrimp_TG	Y S K L V D H D L I K S I V I C N V N E T T Q C W G G E D N L E V H K P D I N I E V L G S A V E G-K A V P I K V S F D	682		
crayfish_TG	Y H K I V E H M I K L V A I C T V E E T S Y A W F G D D R F E V L K P R I K V E I L S T G V A G-K P L P T K F S E T	689		
horseshoe_crab_TG	L E K L V D Y M I K L Y A I A T V K E T Q Q T W S E E D D F M V E K P N L E L E I R G N L Q V G-T P F V L A I S L T	690		
drosophila_TG	Y D K L S S Q A F Q I S A A A K V K D T D Y D Y A Q D D F R V R K P D I K F Q L G E A A I V A Q K E L D V I L R L E	636		
starfish_TG	L P Q L T D Y A G V T F F I M A S V K E T K Q P F S R Q Y D A V L D K P D L E V K T-E G P I V R G K P P T A I V S L T	662		
human_factor_XIIIa	M G Q L L E Q A S L H F F V T A R I N E T R D V L A K Q K S T V L T I P E I I I K V R G T Q V V G-S D M T V T V Q F T	654		
tiger_shrimp_TG	N P I P L L K E C C L K L D G P C L M R P K T V P L E N I A P K G K M N F E M N L Y P K K A V E G T L I V Q E N S R E	742		
crayfish_TG	N P L P I P L T N S V L V D G P C L T R P K I P I S D V G P K A E M V Y D L N V Y E L K E A T C T L V A S F N S T E	749		
horseshoe_crab_TG	N P L K R V L D N C F F T I E A P G V T G A F R V T N R D I Q P E E V A V H T V R L I P Q K P G P R K I V A T F S S R Q	750		
drosophila_TG	N P L P I P L H K G V F T V E G P C I E Q P L K F K I A E I P V G G T A A T F K Y T P P Y A G R G T M L A K F T S K E	696		
starfish_TG	N P L P Y P L T D C S L L M E G S I I E G A K R V K A P H V P V N G K M A Q R V Q L T P K T A G S C D L I V S E S S P Q	722		
human_factor_XIIIa	N P L K E T L R N V W H L D G P C V T R P M K M F R E I R P N S T V Q W E E V C R E P W S G H R K L I A S M S S D S	714		
tiger_shrimp_TG	LCNLTCAKKISVASA-----	757	Identity	Similarity
crayfish_TG	LLNLTGSAPVEIAPAES-----	766	55%	71%
horseshoe_crab_TG	LIQVVGSKQVEVLD-----	764	43%	59%
drosophila_TG	LDDVDGYRHYEIEPRPEDLLQPNGSHRSSNIIRRRTDVIA	736	34%	52%
starfish_TG	LSGVKAHVTLNVKSA-----	737	36%	53%
human_factor_XIIIa	LRHVYGCELDVIQRRPSM-----	732	34%	44%

Fig. 2 (continued)

Table 2

Similarity of shrimp TG protein domains to those of other species. Domain structure is based on X-ray crystallography of the structure of human factor XIIIa-subunit dimer [43,46]. The corresponding domains of other species were inferred from the sequence alignment shown in Fig. 2. Numbers (%) indicate sequence identity and similarity (in brackets)

Origin of TG	Protein domains ^a			
	β -Sandwich	Catalytic core	β -Barrel 1	β -Barrel 2
Crayfish	40 (60)	66 (81)	51 (69)	46 (66)
Horseshoe crab	29 (44)	55 (70)	41 (58)	25 (49)
Starfish	26 (38)	46 (63)	31 (56)	34 (44)
<i>Drosophila</i>	22 (32)	43 (60)	30 (48)	28 (49)
Human factor XIIIa	23 (33)	44 (62)	25 (48)	29 (46)

^a The four individual domains are shown in Fig. 2.

TG domains revealed that the highest identity/similarity occurred between catalytic core domains, especially those of shrimp and crayfish (66/81%) (Table 2). In the aligned TG, all amino acid residues involved in the catalytic triad are perfectly conserved (shrimp: Cys324, His391, Asp414; Fig. 2). The main human factor XIIIa Ca²⁺-binding site involves the main chain oxygen atom of Ala-457, and the side chains from residues Asn-436, Asp-438, Glu-485 and Glu-490 [39]. Except for Thr-477, the other four Ca²⁺-binding residues in shrimp TG (Asn454, Asp456, Glu513, Glu518) are conserved in all aligned proteins (Fig. 2). The sequence surrounding His352, Ser350-Ala-His-Asp353, was conserved. These data, along with crystallography data for factor XIIIa [40], suggest that His352 interacts with Glu452. In contrast, residues for the putative GTP-binding region [41] in TG2 were not observed in shrimp TG.

To analyze the evolutionary relationship between shrimp TG and TG from other species, we calculated the amino acid similarity between aligned sequences. TG from different species are clearly related. All the algorithms indicated shrimp TG was most closely related to crayfish TG, then horseshoe crab TG, starfish TG, and insect TG. Among vertebrate TG, factor XIIIa and TG1 were most closely related to invertebrate TG (Fig. 3).

Shrimp TG contains the motif RGD (Arg-Gly-Asp), which is present in most ligands binding to a family of membrane receptors called integrins [42]. An RGD motif is present in the same location in crayfish TG [23], fruit fly TG [30] and human keratinocyte TG [43,44].

3.4. Distribution of TG mRNA in shrimp tissues

Based on RT-PCR analysis, low TG expression was detected in the intestine, hepatopancreas, gill, heart, lymphoid organ, sub-cuticular epithelium and hemocytes, but not the eyestalk (Fig. 4). In situ hybridization failed to detect signals in most tissues tested. However, strong TG signals were detected in young hemocytes in hematopoietic tissue (Fig. 5a). Binucleated cells and cells with more condensed cytoplasm always yielded stronger TG signals (Fig. 5b).

3.5. Coagulation assay

Only hemocyte lysate supernatant showed coagulation activity. No activity was detected in shrimp eyestalk, intestine, hepatopancreas, gill, heart, lymphoid organ or sub-cuticular epithelium cells. No coagulation activity was detected in Sf21 insect cells transfected with rTG or in untransfected Sf21 cells. TG from guinea pig liver exhibited no coagulation activity.

4. Discussion

We sequenced the full-length cDNA of a novel shrimp TG. By comparing shrimp TG to previously characterized TG, we found that the structural requirements for TG activity and Ca²⁺ binding are conserved. The TG catalytic mechanism was determined using biochemical data for several TG [2,3]

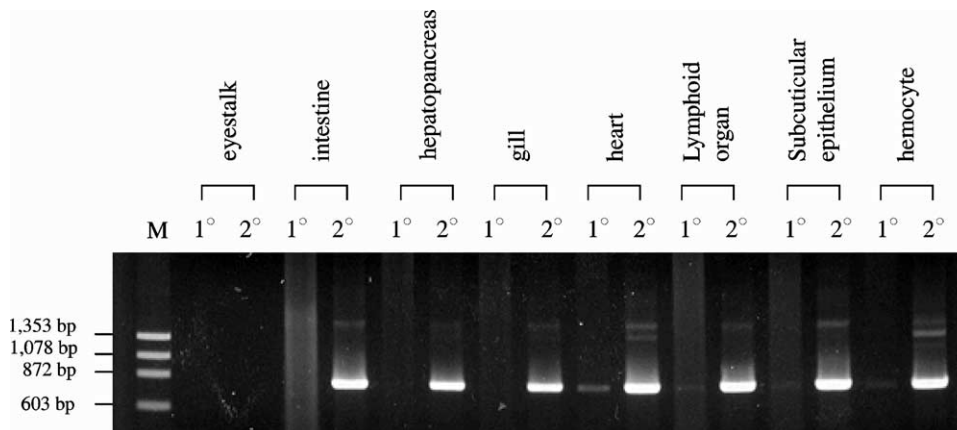


Fig. 4. RT-PCR analysis of TG expression. Total RNA was extracted from the eyestalk, intestine, hepatopancreas, gill, heart, lymphoid organ, subcuticular epithelium and hemocytes of *Penaeus monodon*. Primers specific for *Penaeus monodon* TG were designed to amplify an 806-bp fragment. RT was omitted from the control reaction to ensure that the amplified band is derived from RNA. The PCR bands were sequenced to ensure that they corresponded to the expected cDNA. M, marker; 1°, first round PCR; 2°, second round PCR.

cations, especially Ca^{2+} [1]. Shrimp TG lacks a typical Ca^{2+} -binding motif, such as the EF-hand structure that comprises the calcium binding site of calmodulin and many other proteins. The affinity of shrimp TG Ca^{2+} -binding sites is low and excess EDTA easily blocked TG catalysis. The structure of these sites probably differs from that of high affinity sites in typical Ca^{2+} -binding proteins. In TG, regions rich in negatively charged residues are potential Ca^{2+} -binding sites [48]. Except Thr-477, all the amino acids thought to be involved in calcium binding are conserved in shrimp TG.

A typical hydrophobic leader sequence and a transmembrane domain could not be identified in the deduced sequence of shrimp TG. Thus, shrimp TG may be a typical cytoplasmic protein. Glycosylation of a protein lacking a leader sequence cannot occur because, during synthesis, the cytoplasmic protein would not be translocated into lumen of the endoplasmic reticulum (ER) and Golgi apparatus where glycosylation takes place. All characterized TG lack a hydrophobic leader sequence and none are known to undergo glycosylation [1,5,10,22,26,28,29,43,44,49–51]. Expressing the factor XIIIa-subunit as a fusion protein with the preprodomain of a secretory protein in yeast yielded glycosylated variants of factor XIII that were largely retained and degraded in the ER [52]. Targeting the factor XIIIa-subunit to the conventional secretory pathway apparently causes

aberrant, post-translational modifications. Although TG are not conventionally secreted proteins and the mechanism for the release of TG from cells remains unclear, the presence of TG in extracellular space is well documented. Factor XIII circulates in blood plasma, TG4 is a component of semen and TG2 is expressed on the surface of many cells, including fibroblasts, macrophages, hepatocytes and endothelial cells [53–56]. In invertebrates, blood clotting reduces blood and body fluid loss following injury. In crayfish, an endogenous TG is involved in the rapid assembly of a specific, plasma clotting protein [24]. The mechanism by which TG induces clotting of the clotting protein has been studied in detail in crayfish. Crayfish TG cross-links the clotting protein via its free glutamine and lysine residues. This TG-mediated polymerization occurs very rapidly [57]. In addition, crayfish TG can use alpha-2-macroglobulin as a substrate. However, if the alpha-2-macroglobulin thiolester region is destroyed by methylamine treatment, TG fails to recognize it as a substrate [58]. Recently, a shrimp (*P. monodon*) hemolymph clotting protein was characterized [59]. The specific role of shrimp TG in coagulation of the clotting protein has yet to be studied.

In this study, RT-PCR analysis of TG mRNA using TG-specific primers showed that expression is widespread, except in the eyestalk. However, the lack of a detectable band in the eyestalk does not mean TG

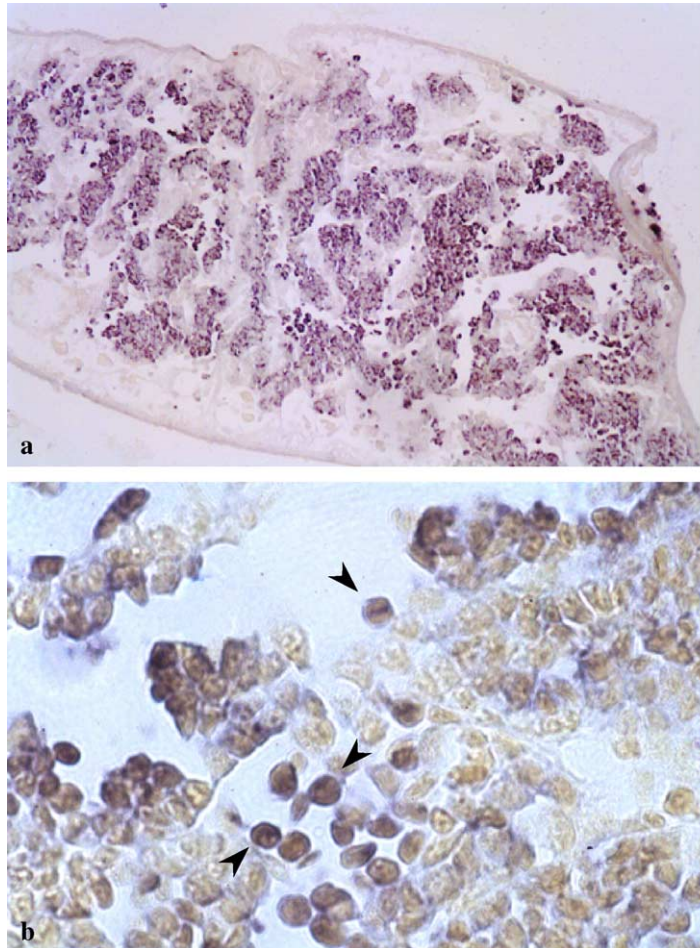


Fig. 5. In situ hybridization of shrimp TG mRNA in hematopoietic tissue. (a) In situ hybridization with an antisense shrimp TG riboprobe. The sections were slightly counterstained with Bismarck Brown Y. Positive signals were observed in hemocytes. No positive signals were observed when using a sense TG riboprobe (data not shown) ($\times 200$). (b) Binucleated cells and cells with more condensed cytoplasm (arrow heads) always contained stronger signals for TG ($\times 1000$).

mRNA was absent. PCR inhibitors are present in the eyestalk and always cause the amplification reaction to fail [60]. It usually took two rounds of PCR to visualize the amplified fragment, indicating TG gene expression was low in all organs tested or expression was restricted to a limited number of cells. In situ hybridization, failed to detect signals in these same organs, confirming that TG expression was very low. We could not determine the specific cell types that synthesize TG in these organs. Both RT-PCR and in situ hybridization indicated that the level of TG mRNA was very low in circulating hemocytes.

However, young hemocytes in hematopoietic tissue gave strong signals. Furthermore, binucleated cells and cells with more condensed cytoplasm, i.e. cells undergoing mitosis, yielded strong signals. This indicates cell proliferation and TG synthesis may be associated. Synthesis of shrimp TG may be associated with the cell cycle. Synthesis and storage of TG in young hemocytes facilitates the instant release of TG protein and blood clotting following injury. Enzyme activity was detected in all organs tested. TG activity was greatest in the hepatopancreas, then the heart, hemocytes and other organs (Table 1). However, only

hemocyte lysate supernatant showed coagulation activity. No activity was detected in other tissues. These results imply that shrimp may contain more than one type of TG. It is very difficult to distinguish different types of TG with a TG activity assay. In tissues, TG activity was not directly correlated to mRNA level. Previous research has documented the expression pattern of TG by measuring mRNA and protein levels. TG2 transcript is most abundant in the lungs, then the heart, kidneys, red blood cells, liver, spleen, and testes [6,61–63]. However, TG2 protein is most abundant in the liver, then the spleen, heart, kidney and lung [64]. These results imply that TG expression may be regulated at the transcriptional and translational levels, or by the protein turnover rate. In vertebrates, the liver and blood cells are important sites for TG expression. For example, platelets, peripheral blood monocytes and the liver may be the primary sites for plasma factor XIII synthesis [65,66]. In shrimp, the hepatopancreas and hemocytes could also be important sites for TG expression. In crayfish and horseshoe crabs, the patterns of TG expression at the mRNA and protein levels are different [23,25]. Each invertebrate TG could correspond to a specific type of TG found in vertebrates. Further research is needed to clarify the function and regulation of invertebrate TG.

TG activity was detected in Sf21 cells transfected with rTG. However, the same cells showed no coagulation activity. It is possible that the shrimp TG isolated in this study is not involved in coagulation. Another possibility is that clot formation in shrimp involves cross-linking of plasma clotting protein with components in hemocytes. In horseshoe crabs, coagulin formed coagulin gel during the early stages of coagulation [67]. During the final stage, TG cross-linked proxins, a substrate of TG, with coagulin to form a stable reticulate structure [27]. Coagulin lacks glutamine residues that function as amine acceptors, but it has several lysine residues that function as amine donors for protein cross-linking with proxins. Thus, TG does not cross-link coagulogen or coagulin, but it does cross-link proxins with coagulin [27]. Although TG acts solely on peptide-bonded glutamine residues, the specificity of different TG for different substrates, such as glutaminyl, varies [2]. Moreover, a specific TG may recognize a protein as substrate, but will have a different affinity and

specificity for different glutamine residues. For example, factor XIIIa has more stringent structural requirements for glutaminyl substrates than does TG2 [68,69]. Thus, different substrates and assay techniques may yield different results due to differences in sensitivity.

Based on amino acid similarity, vertebrate factor XIIIa and TG1 were the vertebrate TG most closely related to shrimp and other invertebrate TG. This is consistent with the finding that invertebrate TG is involved in blood clotting [24].

Based on the preservation of residues critical for enzyme function and domain folding, and the extensive, overall similarity of shrimp TG to the other members of the TG family with catalytic activity, we postulate that the cDNA characterized in this study encodes shrimp TG. Further research is needed to clarify the function and regulation of shrimp TG.

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