Fish and Shellfish Immunology 104 (2020) 83–91

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transglutaminase 1 and 2 are localized in different blood cells in the freshwater crayfish *Pacifastacus leniusculus*

Kingkamon Junkunlo[a], Kenneth Söderhäll[b], Irene Söderhäll[b,*]

[a] Department of Comparative Physiology, Uppsala University, Norbyvägen 18 A, SE 752 36, Uppsala, Sweden
[b] Science for Life Laboratory, Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36, Uppsala, Sweden

ARTICLE INFO

Keywords:
Crustacean
Hematopoiesis
Hemocyte
Transglutaminase

ABSTRACT

In the present study we show that hemocytes in the freshwater crayfish *Pacifastacus leniusculus* express two different transglutaminases. We describe the sequence of a previously unknown TGase (Pl_TGase1) and named this as Pl_TGase2 and compared this sequence with similar sequences from other crustaceans. The catalytic core domain is similar to the previously described TGase in *P. leniusculus*, but Pl_TGase2 has significant differences in the N-terminal and C-terminal domains. Further, we show conclusive evidences that these different transglutaminases are specific for different hemocyte types so that Pl_TGase1 is expressed in the hematopoietic tissue and in the cytoplasm of semigranular hemocytes, while Pl_TGase2 is expressed in vesicles in the granular hemocytes. By *in situ* hybridization we show that both Pl_TGase1 and Pl_TGase2 mRNA are present only in a subset of the respective hemocyte population. This observation indicates that there may be different subtypes of semigranular as well as granular hemocytes which may have different specific functions.

1. Introduction

Coagulation is an important defense and wound healing reaction in crustaceans as well as in other animals, and since the first discovery of a crosslinking transglutaminase in lobster plasma by Lorand et al., 1966 [1], this enzyme has been proven to be important in the clotting reaction in crustaceans among which the freshwater crayfish *Pacifastcus leniusculus* is most studied [2]. With the discovery in 1993 of the protein responsible for clot formation in crayfish plasma, a large lipo-glycoprotein named the clotting protein [3,4], and characterization of a hemocyte clotting enzyme, a transglutaminase [2], the mechanism for hemolymph coagulation in crayfish was deciphered (for review see Ref. [5]). Later transglutaminases have been isolated, cloned and characterized from a number of different decapod crustaceans, such as *Penaeus monodon* [6,7], *Marsupenaeus japonicus* [8], *Fenneropenaeus chinensis* [9], *Litopenaeus vannamei* [10], and *Macrobrachium rosenbergii* [11]. Thus, transglutaminases are well studied as clotting enzymes in crustaceans and other invertebrates such as horseshoe crab [12] and *Drosophila* [13], and its role as a clotting enzyme is due to its Ca^{2+}-dependent crosslinking activity. Apart from the crosslinking, by its transamidation activity, transglutaminases in mammals have also been shown to have GTP-binding activity, and upon GTP-binding the enzyme changes into a catalytically inactive conformation [14]. However, such activity still has to be shown for crustacean transglutaminases.

In human eight genes for transglutaminases have been identified (for review see Lorand and Graham 2003 [15]), and regardless of putative functions, the crustacean transglutaminases so far studied are most similar in structure to mammalian Factor XIIIa (plasma transglutaminase) [16], that is involved in blood coagulation. Some functional studies have indicated other functions such as binding to the 3′UTR of an astakine gene [17], and assisting nodavirus entry into hemocytes [18]. During recent years some other important functions have been assigned to crustacean transglutaminase, and in *P. leniusculus,* transglutaminase was shown to be important in regulating the hematopoietic process (for review see Söderhäll 2016) [19].

First, we showed that transglutaminase is one of the most abundant proteins in the hematopoietic tissue (HPT) of *P. leniusculus,* and that knockdown or inhibition of the enzyme in the HPT results in cells migrating out of the tissue into the peripheral circulation [20,21]. Further, the production of reactive oxygen species (ROS) is closely linked to induction of hemocyte release. In agreement with this, blocking of ROS production resulted in an increase of the extracellular activity of transglutaminase in HPT cells, and subsequent decrease in hemocyte release into the hemolymph [22]. Release of hemocytes, and particularly semigranular cells, from the HPT into the circulation is induced by the cytokine astakine1 (Pl_Ast1) [20,23], and recently, we showed that this Pl_Ast1 acts by blocking transglutaminase activity directly as a non-competitive inhibitor [20,24]. Interestingly, also the clotting protein

* Corresponding author.
E-mail address: Irene.Soderhall@ebc.uu.se (I. Söderhäll).

https://doi.org/10.1016/j.fsi.2020.05.062
Received 3 April 2020; Received in revised form 19 May 2020; Accepted 23 May 2020
Available online 30 May 2020

[4] is secreted by HPT cells and is one of the proteins present as an extracellular matrix protein in the HPT [25]. The crosslinking of this clotting protein together with collagen by transglutaminase stiffens the matrix and obstructs hemocyte release. The cross-linking role of transglutaminase for stabilizing the extracellular matrix proteins in crustaceans is therefore evident, also for example by its essential role in extracellular cement polymerization in barnacles [26].

Some of the crustacean transglutaminases are shown to be adapted to specific environmental conditions for their activity. For example, transglutaminase in the marine shrimp *Pandalus nipponensis* has higher activity in the presence of 0.5 M NaCl [27] and of interest is that *P. leniusculus* as well as the Antartic Krill transglutaminase has high activity also at low temperatures [28,29]. So far, only one transglutaminase gene has been identified in *P. leniusculus* [2], while two genes have been identified in *P. monodon* [7], *P. vannamei* [30], and *M. rosenbergii* [18]. If these different transglutaminases have different functions, and/or are involved in other reactions than clotting or extracellular matrix modifications have not been conclusively shown as pointed out in recent review articles [31,32].

Here we identified a previously undescribed transglutaminase, that we detected by searching transcriptomic data from *P. leniusculus*. The sequence of this transglutaminase is similar to some other uncharacterized crustacean transglutaminases. We further localized the two different *P. leniusculus* transglutaminases and showed the specific localization in different cell types indicating specific and different roles of these two enzymes.

2. Material and methods

2.1. Animals

Freshwater crayfish, *P. leniusculus*, were from Lake Erken, Sweden. The animals were maintained in an aquarium with aeration at 10 °C. Healthy and intermolt male crayfish were used for the experiments.

2.2. Cloning of Pl_TGase2 cDNA

In order to find out if *P. leniusculus* express more than one TGase as is shown in other crustaceans and *Drosophila*, we searched the transcriptomic data deposited by our team in BioProject PRJNA368867 (https://www.ncbi.nlm.nih.gov/bioproject/368867). The partial sequences of Pl_TGase2 was obtained from crayfish transcriptome libraries contig number CL6250Contig1 and CL4837Contig. Based on these sequences, the gene-specific primers were designed for the rapid amplification of 3′ and 5′ cDNA ends (RACE) to amplify the full-length cDNA of Pl_TGase2. Total RNA (1 μg) was extracted from HPT and hemocytes and converted into 3′ and 5′ cDNA template and RACE PCR was carried out according to SMARTer RACE 5'/3′ Kit (Takara). The resulting PCR products were purified and cloned into a TOPO vector (Invitrogen) and sequenced.

2.3. Pl_TGase2 sequence analysis

The similarity analysis of the nucleotide and amino acid sequence of Pl_TGase2 was performed by using the BLAST programs at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/). The complete ORF region of Pl_TGase2 was analyzed using the ORF finder software at (https://www.ncbi.nlm.nih.gov/orffinder/). SignalP 5.0 Server software (http://www.cbs.dtu.dk/services/SignalP-5.0/) was used for the prediction of signal peptides. Protein domains were predicted with InterProScan (http://www.ebi.ac.uk/interpro/) and SMART software (http://smart.embl-heidelberg.de/). Multiple-sequence alignment of the Pl_TGase2 with the others TGase family members from different species was performed by using the ClustalW (http://www.genome.jp/tools/clustalw/). A phylogenetic tree was constructed based on multiple-alignment of Pl_TGase2 protein sequence with TGase protein sequences from different organisms using PhyML 3.0 program (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=phyml).

2.4. Total RNA preparation, reverse transcription and quantitative PCR

For the expression of Pl_TGase1 and Pl_TGase2 in different tissue, the various tissues; HPT = Hematopoietic tissue, APC = Anterior Proliferation Center, HC = Hemocytes, B = Brain, Th-Nerve = Thoracic nerve, Ab-Nerve = Abdominal nerve, and Green gland, were isolated from 4 individual crayfish, and examined separately. To separate the brain and the thin sheath surrounding the brain (brain sheath), brains were removed from 9 to 10 individual crayfish and put into crayfish phosphate-buffered saline (CPBS; 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MnCl_2$, pH 6.8). The brain sheath was dissected by using two pairs of very fine forceps, under the dissecting microscope. The different hemocyte types, GC and SGC were separated by Percoll gradient centrifugation according to Söderhäll and Smith [33]. In brief, 1–2 ml hemolymph was collected in a 1:1 volume of anti-coagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6 [33]. Then, GC and SGC were separated using a continuous gradient of 70% Percoll in 0.15 M NaCl, after centrifugation at 3600 × *g* for 30 min at 4 °C. The resulting cell bands were collected and resuspended in 0.15 M NaCl. Total RNA was extracted by using Trizol LS reagent (Invitrogen) according to manufacturer's protocol and processed to eliminate contaminating DNA by DNase I (RNase-Free) (New England Biolabs) treatment. Further, cDNA was synthesized using SuperScript™ IV First-Strand Synthesis System (Invitrogen). The relative expression of Pl_TGase1 and Pl_TGase2 was verified by qPCR using QuantiTect SYBR green PCR kit (Qiagen). The qPCR program used was 95 °C, 15 min, followed by 45 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. The transcription of a 18S ribosomal protein was used as an internal control.

2.5. Detection of Pl_TGase1 by western blotting

The HPT, brain, and Green gland (GG) were dissected and HC, GC, SGC were collected. Each tissue or cells were homogenized in protein lysis buffer (PBS containing 1% Triton X-100, and 1X proteinase inhibitor mixture (Roche)). After centrifugation of the homogenate at 13,000 × *g* for 20 min at 4 °C, the protein concentration was determined by using Coomassie Plus Assay Kit (Thermo Scientific). A total of 5 μg (GC and SGC) and 40 μg (HPT, HC, brain, and GG) of the protein samples were dissolved in Laemmli sample buffer (62.5 mM Tris-HCL, 2% Tris-HCl, 2% SDS, 10% (v/v) glycerol, 0.1 M DTT, and 0.01% bromophenol blue (pH 6.8)) and then separated using SDS-PAGE with 12.5% acrylamide. Subsequently, the proteins were electro-transferred onto a PDVF membrane (GE Healthcare Life Science) for 2 h. The blot was blocked in 10% skim milk in PBST (0.1% Tween 20 in PBS buffer) for 1 h. Then, the membrane was incubated with rabbit anti-giant freshwater prawn transglutaminase (MrTGII-N) antibody (1:3000) kindly provided by Dr. Kallaya Sritunyalucksana (National Science and Technology Development Agency, Bangkok, Thailand), or with beta actin antibody (1:5000) (PA1-16889, Invitrogen) at 4 °C overnight. After extensive washing, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (GE Healthcare) (1:7500) for 1 h at room temperature. The detection was performed using ECL substrate (Bio-Rad) according to the manufacturer's instruction.

2.6. Immunocytochemistry of Pl_TGase1 in hemocytes

Hemocytes were collected and diluted to 1:1 volume of anti-coagulant buffer. After centrifugation at 3000 × *g* for 5 min, the plasma was

removed and the cell pellet was resuspended in 0.15 M NaCl. Hemocytes at a density of 40,000 cells/well were added into 96-well plates. After attachment, the cells were immediately fixed with 4% paraformaldehyde in PBS for 1 h. After being washed 3 times with PBS for 30 min, the fixed cells were permeabilized with 0.5% Triton X 100, 3 times for 15 min to detect intracellular proteins or without 0.5% Triton X 100 for extracellular protein detection. The cells were incubated with anti-MrTGII-N antibody (1:1000) at 4 °C overnight, and then washed 5 times for 15 min each with PBST. The cells were incubated at room temperature for 1 h with FITC-conjugated anti-rabbit IgG (1:1000) in PBST together with Hoechst 33258 dye at a concentration of 1 μg/ml to stain the nuclei. Finally, the cells were washed 5 times for 15 min each with PBST, and the Pl_TGase1 protein was observed under a fluorescence microscope.

2.7. In situ hybridization of Pl_TGase1 and Pl_TGase2

Sense and anti-sense strand probes were generated following the manufacturer's instructions of the DIG RNA Labeling Kit (SP6/T7) (Roche). A DNA template of Pl_TGase1 and Pl_TGase2 was amplified with specific primer pairs. The PCR products were then purified by using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and were used as templates for DIG-labeled RNA synthesis. The labeling efficiency of the probes was verified before use according to the instructions of the DIG RNA Labeling Kit (SP6/T7) (Roche). The separated GC and SGC were each seeded on SuperFrost Plus Microscope slides (Thermo Scientific), fixed with 4% paraformaldehyde in PBS (for 30 min at room temperature, washed 3 times with PBS-DEPC (PBS buffer treated with diethyl pyrocarbonate (DEPC) (Sigma-Aldrich)), pre-hybridized with Hybridization Mix (−) buffer (HM (−)) (50% Deionized formamide, 5X SSC, 0.1% Tween, pH 6.0 with citric acid) at 60 °C for 1 h, and incubated with Hybridization Mix (+) (HM (+)) (50% Deionized formamide, 5X SSC, 0.1% Tween, 50 μg/ml heparin, 500 μg/ml RNase-free tRNA, pH 6.0 with citric acid) containing 50 ng of specific RNA probes at 65 °C for 16 h. Post-hybridization washes were performed 2 times each at 65 °C for 5 min using a serial dilution of HM (−) in 2X SSC (75% HM (−), 50% HM (−), 25% HM (−) and 100% 2X SSC), and then the cells were washed 2 times with 0.2X SSC at 65 °C for 30 min. The slides were washed with a serial dilution of 0.2X SSC in PBST (0.1% Tween 20 in PBS buffer) and 100% PBST at room temperature for 5 min 2 times each before being incubated with blocking buffer (1x PBST, 2% horse serum, 2 mg/ml BSA) at room temperature for 1 h and incubated with blocking buffer containing 1:1000 of sheep anti digoxigenin-AP IgG (Roche) at 4 °C overnight. After being washed 6 times each at room temperature for 15 min, the slides were equilibrated with staining buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl and 0.1 Tween 20) 3 times each at room temperature for 5 min. The dark purple color was developed in the dark in staining buffer containing the NBT/BCIP substrate (Roche). Slides were washed with stop solution (1X PBS, 1 mM EDTA and 0.1% Tween 20) for 5 min 3 times and with PBS for 5 min 3 time, before they were mounted with 50% glycerol in PBS.

2.8. Detection of TGase activity and cell membrane staining in hemocytes

Localization of TGase activity was investigated by combined detection of TGase activity and internal membranes stained with CellBrite™ Cytoplasmic Membrane Labeling Kits (Biotium). Briefly, hemocytes were collected in anti-coagulant as above and resuspended in 0.15 M NaCl. Hemocytes at a density of 40,000 cells/well were added into 96-well plates. After cell attachment, 1 mM of 5-(biotinamido)-pentylamine (Pierce), a substrate for TGase, was added to the plates and the cells were incubated at 16 °C for 3 h. Then, the medium was removed, and the cells were fixed with 4% paraformaldehyde in PBS for 1 h. Next, 25 mM glycine in PBS was added to the wells, and the cells were incubated for 30 min. After washing 3 times for 15 min each with PBS, the cells were permeabilized with 0.5% Triton X 100 for 3 times for 15 min for intracellular protein detection followed by blocking with 10% BSA in PBST for 30 min. Then, streptavidin, Alexa Fluor™ 594 Conjugate (S32356, Invitrogen) was added at a 1:200 dilution with 1% BSA in PBST together with Hoechst 33258 dye at a concentration of 1 μg/ml to stain nuclei. After incubation for 1 h at room temperature the cells were washed 5 times for 15 min each with PBST, and then washed 2 times for 15 min each with PBS. Next, the cells were incubated for 1 h with CellBrite™ Green dye (1:1000) in PBS for internal membrane staining. Then, the cells were washed 5 times for 15 min each with PBS, and the localization of TGase activity with internal membrane (CellBrite™ green) was observed under a fluorescent microscope.

As a positive control for internal vesicle staining, immunohistochemistry of Pl_Ast1 in hemocytes was performed as follows. Polyclonal antibodies against the Pl_Ast1 were commercially raised in rabbits at the Biomedical Technology Research Center, Chiang Mai University, Thailand. Hemocytes were collected in a 1:1 volume of anti-coagulant buffer. After centrifugation at 3000 × *g* for 5 min, the plasma supernatant was removed and the cells were resuspended in 0.15 M NaCl, and seeded at a density of 40,000 cells/well into 96-well plates and incubated at 16 °C for 3 h. Then, the medium was removed, and the cells were fixed with 4% paraformaldehyde in PBS for 1 h. After permeabilization with 0.5% Triton X 100 for 15 min for 3 times for intracellular protein detection, the cells were incubated with anti-Pl_Ast1 antibody (1:1000) at 4 °C overnight. After washing 5 times time for 15 min each with PBST, the cells were incubated for 1 h at room temperature with FITC-conjugated anti-rabbit IgG (1:1000) in PBST together with Hoechst 33258 dye at a concentration of 1 μg/ml to stain nuclei. Then, the cells were washed 5 times for 15 min each time with PBST, and followed by 2 times for 15 min each with PBS. Next, the cells were incubated for 1 h with CellBrite™ Green dye (1:1000) in PBS, washed 5 times for 15 min each with PBS, and finally the localization of Pl_Ast1 and internal membranes (CellBrite™ green) were observed under a fluorescent microscope.

2.9. Statistical analysis

The relative mRNA expression levels are shown as a combined scatter and box plots. Four to ten individual crayfish were used in each experimental group. The statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test and Tukey's test. For comparisons between two groups, a *t*-test was used, and statistical significance was considered at $P < 0.05$.

3. Results

3.1. Sequence analysis of Pl_TGase2

The full length of Pl_TGase2 was cloned from HPT and its sequence was submitted to GenBank with the accession number MN913337. The length of the Pl_TGase2 open reading frame (ORF) is 2262 base pairs (bp) and the deduced protein sequence contains 753 amino acid residues. The theoretical molecular mass is 85.8 kD and the isoelectric point (pI) is 5.8. The protein domain structure of Pl_TGase2 contains a typical transglutaminase domain, a N- domain, a core/catalytic domain, and a C-domain. As Pl_TGase1 (GenBank accession number AF336805), Pl_TGase2 lacks a signal peptide or other typical export sequences. However, a N-myristoylation site was predicted in the N-domain of Pl_TGase2, but without a consensus signature (MGXXXS/T) (Supplementary Fig. S1). An N-myristoylated glycine is not found in Pl_TGase1 sequence. Furthermore, S-palmitoylation sites were observed in both Pl_TGase1 and Pl_TGase2 sequences (data not shown). This suggests that Pl_TGase2 is a potential substrate of N-myristoylation and might be secreted through an unconventional ER/Golgi-independent pathway as in *Drosophila* [34].

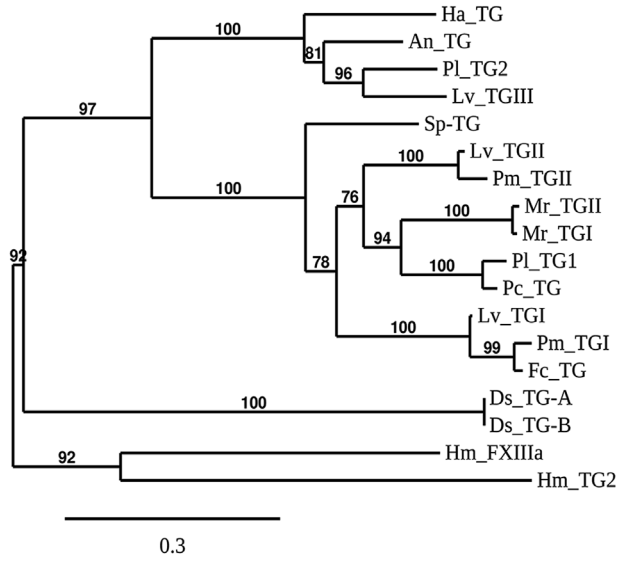

Fig. 1. Phylogram based on multiple protein alignments between Pl_TGase1, Pl_TGase2 and other transglutaminase proteins; Pl_TG1 (Pl_TGase1, transglutaminase1) *Pacifastacus leniusculus* (AAK69205.1), Pl_TG2 (Pl_TGase2, transglutaminas2) *Pacifastacus leniusculus* (MN913337), Hm_TG2 *Homo sapiens* protein-glutamine gamma-glutamyltransferase 2 (NP_001310245.1), Hm_FXIIIa *Homo sapiens* coagulation factor XIII A chain precursor (NP_000120.2), DsTgA *Drosophila melanoger* transglutaminase, *isoform A* (NP_609174.1), DsTgB *Drosophila melanogaster,* isoform B (NP_723332.1), Mr_TGAI *Macrobrachium rosenbergii* (ADX99580.1), Mr_TGAII *Macrobrachium rosenbergii* (AIN46597.1), An_TG Hemocyte protein-glutamine gamma-glutamyltransferase *Armadillidium nasatum* (KAB7503271.1), Ha_TG hypothetical protein HAZT_HAZT011482 *Hyalella azteca* (KAA0183305.1), Mr_TGI transglutaminase1 *Macrobrachium rosenbergii* (ADX99580), Mr_TGII transglutaminase2 *Macrobrachium rosenbergii* (AIN46597.1), Lv_TGI hemocyte transglutaminase type I *Litopenaeus vannamei* (ABN13875.1), Lv_TGII hemocyte transglutaminase type II *Litopenaeus vannamei* (ABX83902.1), Lv_TGIII putative hemocyte protein-glutamine gamma-glutamyltransferase type III *Litopenaeus vannamei* (ROT61750.1), Pm_TGI transglutaminase1 *Penaeus monodon* (AAL78166.1), Pm_TGII transglutaminase2 *Penaeus monodon* (AAV49005.1), Fc_TG transglutaminase *Fenneropenaeus chinensis* (ABC33914.1), Pc_TG protein-glutamine gamma-glutamyltransferase *Procambarus clarkii* (AVN99048.1), Sp-TG transglutaminase *Scylla paramamosain* (CUV66660.1). The phylogram was constructed by neighbor-joining method using PhyML 3.0. The numbers at each node represent bootstrap values in percentage. The bar represents 30% amino acid distance.

3.2. Pl_TGase2 sequence comparison

After whole amino acid sequence alignment of Pl_TGase2 with Pl_TGase1, 38% similarity was found, but Pl_TGase2 is 13 amino acid residues shorter than Pl_TGase1 (766 amino acids). The deletions were observed in the N-domain and the core/catalytic domain (Supplementary Fig. S2A and Supplementary Fig. S2B). The catalytic domains of Pl_TGase2 and Pl_TGase1 are conserved with 68% similarity, whereas the similarity for the N-domain and for the C-domain is only 19% (Supplementary Figs. S2A) and 37% (Supplementary Fig. S2C), respectively. To determine the relationship of Pl_TGase2 with other TGases from crustaceans, insects, and mammals, a phylogenetic tree was constructed (Fig. 1). We found that Pl_TGase2 exhibited the smallest genetic distance with white shrimp *Litopeneaus vannamei* TGase type 3 (Lv_TGIII) and forms a cluster together with an ancient arthropod species, *Armadillidium nasatum (*An_TG), as well as *Hyalella azteca* (Ha_TG) (Fig. 1). Pl_TGase1 on the other hand forms a sub-cluster together with other crustacean TGases in this analysis including *Macrobrachium rosenbergii, Scylla paramamosain, Penaeus monodon, Litopenaeus vannamei and Procambarus clarkii*. In addition, Pl_TGase1 has a bootstrap value of 100 with TGase from freshwater crayfish *Procambarus clarkii* and is closely related with high reliability also to both

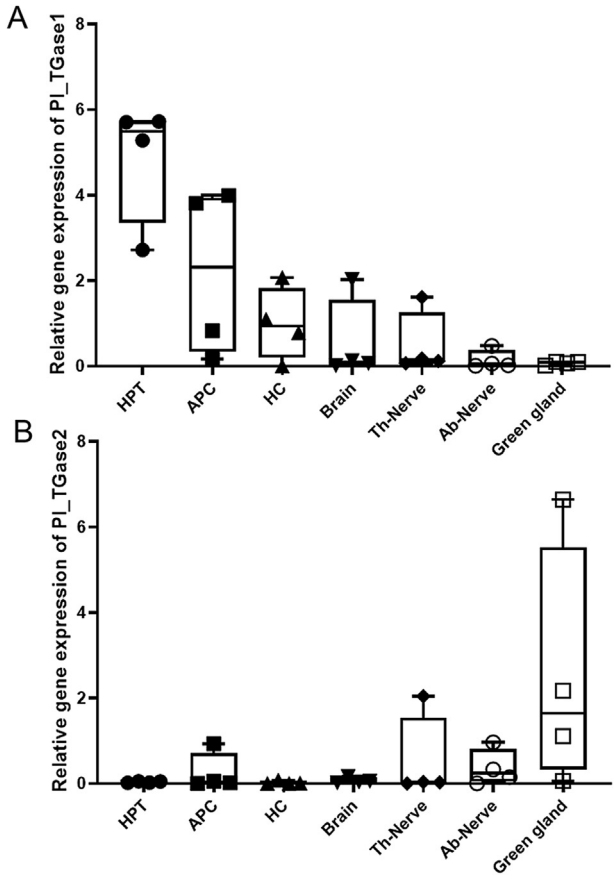

Fig. 2. Relative mRNA expression of Pl_TGase1 and Pl_TGase2 in various tissues analyzed by RT-qPCR. A) Pl_TGase1. B) Pl_TGase2. HPT = Hematopoietic tissue, APC = Anterior Proliferation Center, HC = Hemocyte, Brain (containing brain + sheaths), Th-Nerve = Thoracic nerve, Ab-Nerve = Abdominal nerve, and Green gland. Expression of 18S ribosomal gene was used as an internal control. Combined scatter and box plots represent data from 4 individual crayfish, and the line in the box represents median. The lower and upper bars show the minimum and maximum values.

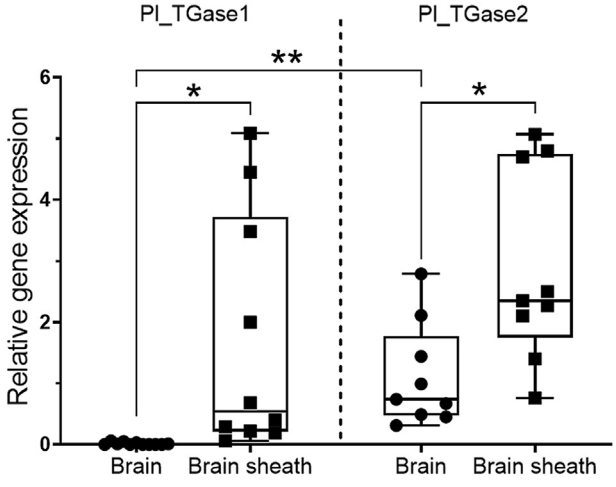

Fig. 3. Relative mRNA expression of Pl_TGase1 and Pl_TGase2 in brain and brain sheath analyzed by RT-qPCR. The combined box and scatter plots represent data from 9 to 10 individual crayfish, and the line across the box represents median. The lower and upper bars represent the minimum and maximum values. **$p < 0.001$ indicates a significant difference between samples.

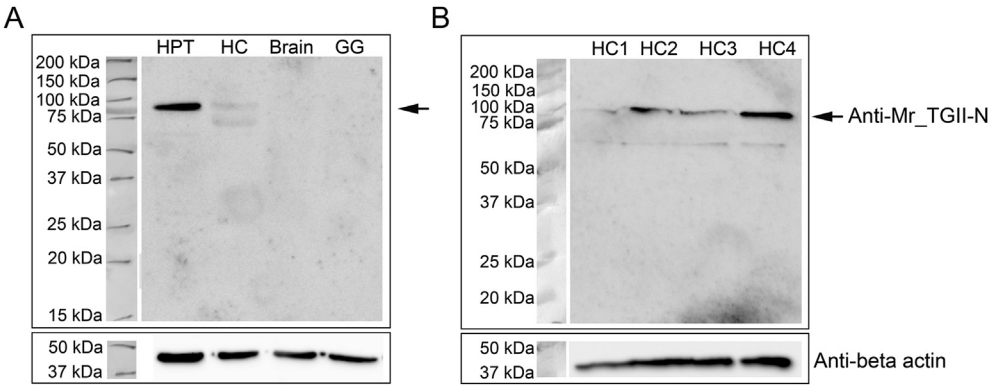

Fig. 4. Western blots of Pl_TGase1 in HPT, HC, brain (B) and green gland (GG). A) A total amount of 20 μg protein from each tissue lysate was used for Western blot by using anti-Mr_TGII-N antibody (anti-giant freshwater prawn transglutaminase2) (1:3000)) and beta actin antibody (1:10,000) was used as control. B) A total amount of 5 μg protein from total hemocyte lysate from 4 different animals was used for Western blot by using anti-Mr_TGII-N antibody (anti-giant freshwater prawn transglutaminase2) (1:3000)) and beta actin antibody (1:10,000) was used as control.

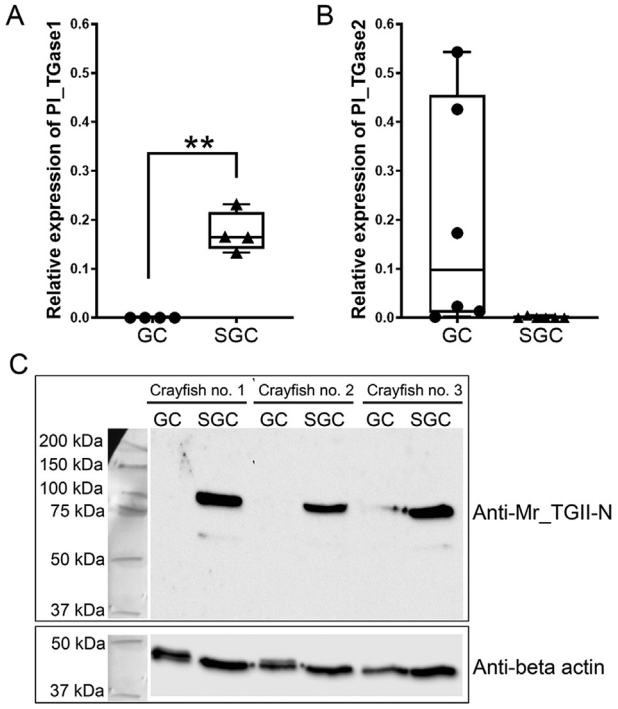

Fig. 5. Relative mRNA expression of Pl_TGase1 and Pl_TGase2 in granular (GC) and semi-granular (SGC) cells analyzed by RT-qPCR. A) Pl_TGase1. B) Pl_TGase2. The combined box and scatter plots represent data from 4 to 6 individual crayfish. The line across the box represents median and the upper and lower bars represent the minimum and maximum values. **$p < 0.001$ indicates significant differences between samples. C). Western blot showing the presence of Pl_TGase1 in SGC but not in GC. A total amount of 5 μg protein from each cell type lysate was used for Western blot by using anti-Mr_TGII-N antibody (1:3000) and anti-beta actin antibody (1:10,000) as a loading control).

TGases from the freshwater prawn *Macrobrachium rosenbergii*. The crustacean TGases form a sister group with *Drosophila melanogaster* TGases (Ds_TgA and Ds_TgB), human coagulation factor XIIIA (Hm_FXIIIa), and human TGase type 2 (Hm_TG2) (Fig. 1). The overall phylogenetic tree analysis suggests that Pl_TGase2 is closely related with a few other crustacean TGases, but distantly related with Pl_TGase1.

3.3. The expression profile of Pl_TGase1 and Pl_TGase2

We previously showed that Pl_TGase1 is an abundant protein in both the HPT and hemocytes, and the expression is especially high in semigranular cells (SGC), which is similar to the enzyme activity of TGase [20]. To examine the expression profiles of Pl_TGase1 and Pl_TGase2 respectively, the mRNA transcript was determined in dissected tissues by semi-quantitative PCR. The tissues examined were HPT, anterior proliferation center (APC), hemocytes (HC), brain, thoracic nerve (Th-Nerve), abdominal nerve (Ab-Nerve), green gland, muscle, heart, foregut, midgut, hindgut, gill, and hepatopancreas. Both transcripts were detected in the intestine, heart and hepatopancreas, but not in muscle (data not shown). We further analyzed expression of both TGases in seven of the tissues by RT-qPCR and found that the expression of Pl_TGase2 mRNA was not detected in HPT and detected at very low levels in mixed hemocytes, whereas in APC a small expression of Pl_TGase2 mRNA occurred. In contrast Pl_TGase1 was highly expressed in these tissues (Fig. 2). A high expression of Pl_TGase2 was detected in green gland, but with very high variation between animals, while Pl_TGase1 could never be detected in this tissue (Fig. 2). The tissue-specific expression suggests that Pl_TGase1 and Pl_TGase2 might have functions which are distinct in different tissues.

To gain more information about the localization of Pl_TGase1 and Pl_TGase2 in brain, localization of the mRNA expression levels was examined in brains and brain sheaths (in which hemocytes can be found). In Fig. 2, it is shown that the expression of Pl_TGase1 seems higher in brain than Pl_TGase2. However, the brain samples used in this figure do contain the intact brains with their surrounding sheaths. Since we know that hemocytes may infiltrate the space between brain and sheaths, we decided to separate these from each other and determine mRNA expression in the different parts. The results showed that while Pl_TGase2 mRNA was clearly expressed in the brain, no expression of Pl_TGase1 could be detected in this tissue (Fig. 3). In contrast, both transcripts were detected within the brain sheaths (Fig. 3). These results further support specific different roles of Pl_TGase1 and Pl_TGase2 in different cell types and in various tissues.

3.4. The expression of Pl_TGase1 and Pl_TGase2 in different hemocyte types

A protein sequence comparison of Pl_TGase1 and Mr_TG (Mr_TGI and Mr_TGII) showed 60% similarity (data not shown). Furthermore, the phylogram (Fig. 1) also reveals close relationship between Pl_TGase1 and the two transglutaminases from *M. rosenbergii*. We used an antibody raised against Mr_TGII-N in order to find out if this antibody was suitable for detection of the *P. leniusculus* TGases. We chose tissues in which the studies above had shown differential expression of Pl_TGase1 and Pl_TGase2, and performed Western blot using the Mr_TGaseII-N antibody. The Western blot experiments showed detection of a very strong band in HPT and a band in hemocytes where Pl_TGase1 is expressed, whereas this antibody did not recognize any band in brain or green gland where Pl_TGase2 is highly expressed (Fig. 4A). These results show that the antibody against Mr_TGaseII is specific to Pl_TGase1, and does not recognize Pl_TGase2 in freshwater crayfish *P. leniusculus* (Fig. 4A). The band detected in HPT and hemocytes had a molecular size of about 86 kDa, which is the size of Pl_TGase1 (Fig. 4A). The band in hemocytes is weak (Fig. 4A), but as shown in Fig. 4B is a very large variation in the amount of TGase

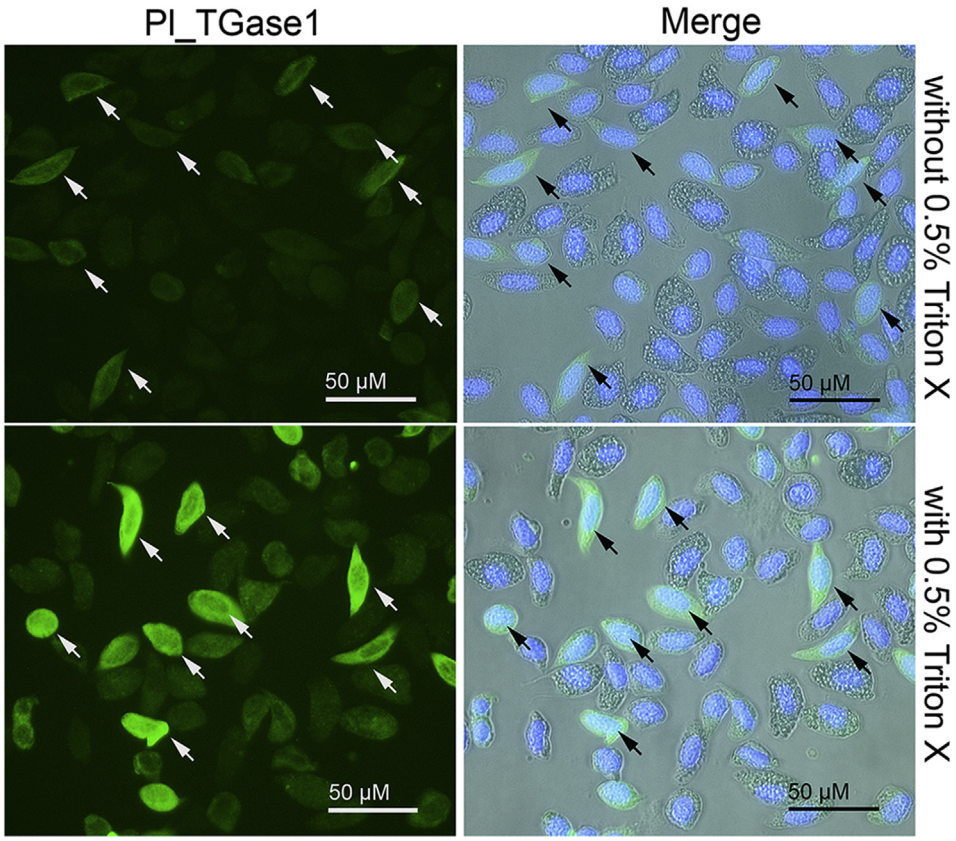

Fig. 6. Immunostaining of Pl_TGase1 in hemocytes using anti-Mr_TGII-N antibody (green) in the presence or absence of 0.5% Triton X 100 (extracellular and intracellular protein detection). The arrows indicate SGC. Hoechst 33258 (blue) was used as nuclear stain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein in hemocytes between different individual animals, which may be due to that the proportions of hemocyte types vary between animals (Fig. 4B).

We have shown in a previous report that TGase activity was very high in HPT and SGC, while it was much lower in GC [20]. We then used Western blot on separated hemocytes to analyze the presence of the Pl_TGase1 in the different hemocyte types. Due to the sequence differences between Pl_TGase1 and Pl_TGase2, as well as tissue distribution profiles, we hypothesized that these proteins may be expressed in different types of hemocytes. When using separated hemocytes, we found that the mRNA transcript of Pl_TGase1 was expressed only in SGC (Fig. 5A) while Pl_TGase2 mRNA was exclusively expressed in GC, and not in SGC (Fig. 5B). To confirm the specific expression of Pl_TGase1 in SGC and Pl_TGase2 in GC, Western blot was performed using Mr_TGII-N antibody in both SGC and GC cell lysate from several individual animals. The expected protein band (86 kDa) was detected only in SGC protein lysate (Fig. 5C), and as shown in this figure and Fig. 4A–B the variation in Pl_TGase1 amount between individuals is large.

To further confirm this specific localization of the two TGases in different hemocyte types, we performed immunostaining of Pl_TGase1 in hemocytes using the Mr_TGII-N antibody. Again, the results showed that Pl_TGase1 was localized solely in SGC (Fig. 6). The enzyme Pl_TGase1 was mainly detected in the cytoplasm of SGC cells, and not in granules, which is to be expected due to the lack of signal peptide. A low signal was also detected on the outside of some of the SGC indicating an extracellular protein (Fig. 6). Noticeably, we found that not all SGC cells expressed Pl_TGase1.

To ascertain further this apparent specific localization of the different TGases we performed *in situ* hybridization of Pl_TGase1and Pl_TGase2 using RNA probes in separated GC and SGC. The results confirmed our previous observations and showed that Pl_TGase1 was only expressed in SGC (Fig. 7A), and as expected Pl_TGase2 mRNA was detected only in GC (Fig. 7B), and again not all SGCs expressed Pl_TGase1 and not all GCs expressed Pl_TGase2. In conclusion, Pl_TGase1 and Pl_TGase2 are clearly expressed and located in different types of hemocytes, and. Pl_TGase1 is localized in SGC, while Pl_TGase2 is localized in GC.

3.5. TGase activity in hemocyte granules

TGase is a Ca^{2+}-dependent cross-linking enzyme, which has both enzymatic and non-enzymatic properties. TGase enzyme activity was high in HPT, APC, hemocytes and brain (Supplementary Fig. S3), and in our previous studies we showed that SGC has a higher enzymatic activity than GC [20]. Neither Pl_TGase1 nor Pl_TGase2 does have a signal peptide, however Pl_TGase2 contains a putative N-myristoyl site at its N-terminal sequence (Supplementary Fig. S1). In *Drosophila,* TG-A had a N-myristoyl sequence and was secreted via exosome-like structures [34], and thus, Pl_TGase2 might be transported out of the cell by the same mechanism. To localize TGase activity and the internal membrane surrounded organelles, we stained hemocytes for TGase activity together with internal membrane staining. As above, we could detect high TGase activity in the cytoplasm of SGCs (Fig. 8A, arrowheads), which corresponded to the expression of Pl_TGase1. Interestingly, TGase activity was detected inside some granules of the GCs (Fig. 8A, arrows), which indicated expression of Pl_TGase2 inside the organelles. In contrast, Pl_Ast1, was primarily detected in granules in the SGCs (Fig. 8B, arrowheads).

4. Discussion

In this study we characterized the mRNA sequence of a previously unknown TGase in *P. leniusculus*, and named it as Pl_TGase2, and then compared its sequence with similar sequences from other crustaceans. The number of genomes available for crustaceans is limited and thus we could only find three sequences in available data bases which were similar. The most similar is a TGase from *L. vannamei,* but this sequence

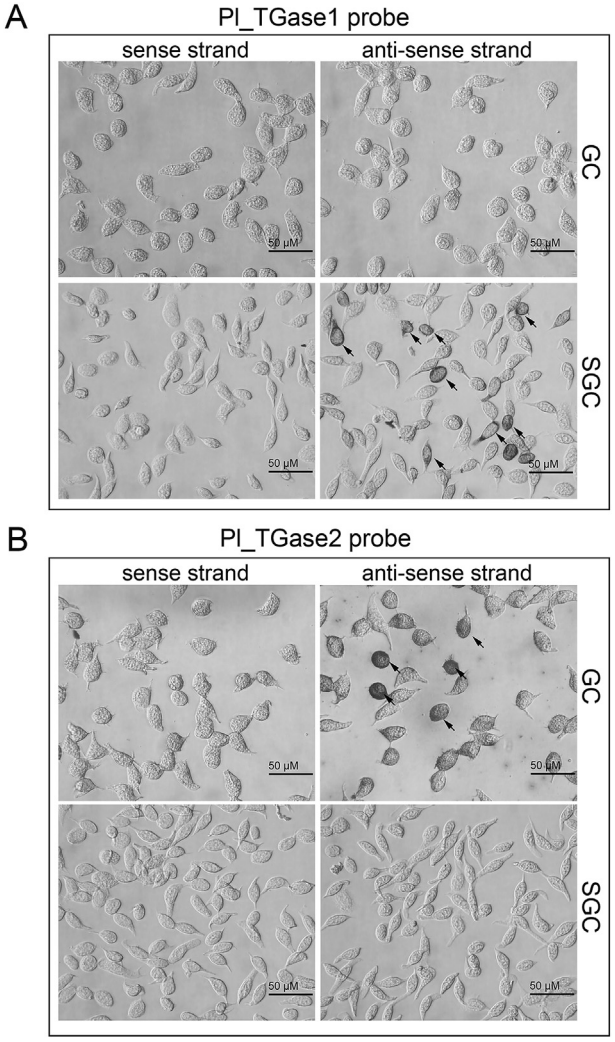

Fig. 7. *In situ* hybridization (mRNA localization) of Pl_TGase1 and Pl_TGase2 in SGC and GC, respectively, using anti-sense strand DIG-labelling RNA probes and with a sense strand probe as a negative control and developed in staining buffer containing the NBT/BCIP as substrate. Pl_TGase1 and Pl_TGase2 expression are indicated by arrows. A). Sense and anti-sense strand of Pl_TGase1 in GC and SGC cells. B). Sense and anti-sense strand of Pl_TGase2 in GC and SGC cells.

is automatically generated from genome data, and there is therefore no information so far about specific function for this shrimp TGase. In comparison with other studied crustacean TGases, the catalytic core is similar, but Pl_TGase2 has significant differences in the N-terminal and C-terminal domains compared to Pl_TGase1. These differences indicate separate functions of the two TGases, however, since there is a long list of possible substrates for TGases an understanding of the biological functions of these enzymes is a challenge and also quite difficult to examine [35,36]. We have shown in previous reports that Pl_TGase major function is in coagulation [4,5] and in addition have functions in the hematopoietic process in a crustacean mainly by regulating the stiffness of the extracellular matrix [20–22,24]. We could also show co-localization of TGase activity with the extracellular clotting protein in HPT cell cultures as well as the presence of clotting protein in the extracellular matrix of the HPT [25]. When analyzing the differential mRNA expression of Pl_TGase1 and Pl_TGase2 in this report, we can conclude that the role in regulating the extracellular matrix in the HPT, should be attributed to Pl_TGase1, since no expression of Pl_TGase2 could be detected in the HPT.

Transglutaminase activity can be blocked by the inhibitor cystamine [37], and we have recently shown that injection of this inhibitor into crayfish could severely affect movement behavior of crayfish, but this effect was only transient and the animals recovered after a few days depending of the dose of cystamine injected [21]. Interestingly this observed effect is similar to behavioral effects of cystamine shown in Sprague-Dawley rats [38]. In this study we could show that the TGase enzyme expressed in the brain seems to be the Pl_TGase2 type, which may give a clear indication that Pl_TGase2 type is responsible for this transient cystamine effect on the behavior. An interesting observation is also that brain injury caused by cutting or damaging antennae resulted in an accumulation of GCs, in the blood vessels of the brain and GCs contain exclusively the Pl_TGase2 type (data not shown).

In addition to the role in hematopoiesis, TGase is of great importance as the clotting enzyme in crustaceans [31,32], and is cross-linking the high molecular weight clotting protein in the presence of calcium [2,4]. In earlier studies we have shown that SGCs as well as GCs contain calcium-dependent TGase activity essential for the clot formation in *P. leniusculus* [2,20], but in this type of assay it is not possible to distinguish, which activity comes from which variant of TGase enzyme. Whether both Pl_TGase1 and Pl_TGase2 are active *in vivo* as clotting enzyme is not fully clarified in this study mainly because even a commercial transglutaminase (from guinea pigs) has the same activity against the crayfish clotting protein *in vitro*, and hence such an assay does not provide any actual answer to the question if both enzymes or one of them are of importance for the clotting process *in vivo*. However, in the study by Lin et al. [20], it was clearly shown that a GC lysate showed considerably lower TGase activity compared to a lysate made from SGCs, which suggests that Pl_TGase2 is not the major enzyme responsible for the clotting reaction in crustaceans. In this work we have been able to clearly show that the two different hemocyte types, SGC and GC expressed different TGases and GC expressed Pl_TGase2 and SGC expressed Pl_TGase1. In SGCs and in HPT cells, Pl_TGase1 is one of the most abundant proteins [20,39], and it is also clearly shown that the enzyme is active outside the cells and thus has to be secreted (Fig. 6) [21,22] Pl_TGase2 is probably not secreted to the outside, since it is clear from our results displayed in Fig. 8 that this enzyme is localized to vesicles in the granular hemocytes, whereas Pl_TGase1 is present in the cytoplasm of SGCs. Similar differences in localization were detected for *Drosophila* TG-A and TG-B respectively, where TG-A was detected in multi-vesicular bodies in hemocytes, and TG-B in the cytosol [34].

Interestingly *in situ* hybridization showed that both Pl_TGase1 and Pl_TGase2 mRNA are present only in a subset of the respective hemocyte populations. This observation indicates that there may be different subtypes of SGCs as well as GCs which may have different specific functions.

Funding

This work was supported by the Swedish Research Council VR to IS (2018–03669).

CRediT authorship contribution statement

Kingkamon Junkunlo: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing - original draft, Writing - review & editing. **Kenneth Söderhäll:** Conceptualization, Formal analysis, Resources, Supervision, Writing - original draft, Writing - review & editing. **Irene Söderhäll:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

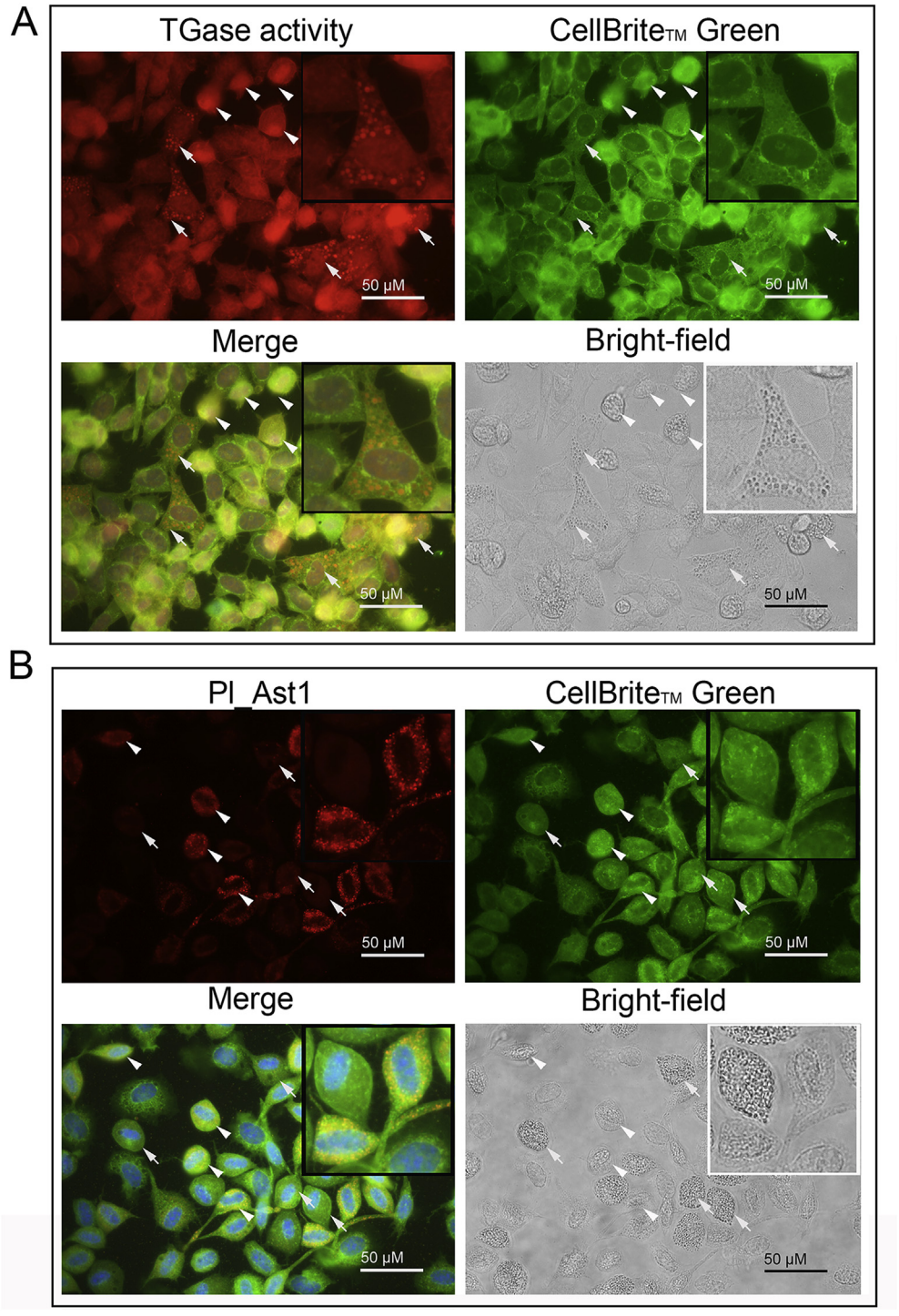

Fig. 8. Co-localization of TGase activity and internal membrane surrounded organelles staining in hemocytes. Internal membrane staining was performed using CellBrite™ Cytoplasmic Membrane Dyes (green), after incubation with TGase substrate 5-(biotinamido)-pentylamine for 3 h. The cross-linking of the substrate was visualized after Streptavidin-Alexa Flor®594 (red) addition together with Hoechst 33258 (blue) as a nuclear stain. The GCs are indicated by arrows and the SGCs are indicated by arrowheads. A). Double labelling of TGase activity and intracellular membranes. B). Double labelling of Ast1 and intracellular membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.05.062.

References

[1] J.B. Lorand, T. Urayama, L. Lorand, Transglutaminase as a blood clotting enzyme, Biochem. Biophys. Res. Commun. 23 (1966) 828–834, https://doi.org/10.1016/0006-291x(66)90562-6.

[2] R. Wang, Z. Liang, M. Hall, K. Söderhall, A transglutaminase involved in the coagulation system of the freshwater crayfish, Pacifastacus leniusculus. Tissue localisation and cDNA cloning, Fish Shellfish Immunol. 11 (2001) 623–637, https://doi.org/10.1006/fsim.2001.0341.

[3] P. Kopácek, M. Hall, K. Söderhäll, Characterization of a clotting protein, isolated from plasma of the freshwater crayfish Pacifastacus leniusculus, Eur. J. Biochem. 213 (1993) 591–597, https://doi.org/10.1111/j.1432-1033.1993.tb17798.x.

[4] M. Hall, R. Wang, R. van Antwerpen, L. Sottrup-Jensen, K. Söderhäll, The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 1965–1970.

[5] L. Cerenius, K. Söderhäll, Coagulation in invertebrates, J. Innate Immun. 3 (2011) 3–8, https://doi.org/10.1159/000322066.

[6] C.-C. Huang, K. Sritunyalucksana, K. Söderhäll, Y.-L. Song, Molecular cloning and characterization of tiger shrimp (Penaeus monodon) transglutaminase, Dev. Comp. Immunol. 28 (2004) 279–294, https://doi.org/10.1016/j.dci.2003.08.005.

[7] M.-Y. Chen, K.-Y. Hu, C.-C. Huang, Y.-L. Song, More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation, Dev. Comp. Immunol. 29 (2005) 1003–1016, https://doi.org/10.1016/j.dci.2005.03.012.

[8] M.-S. Yeh, L.-R. Kao, C.-J. Huang, I.-H. Tsai, Biochemical characterization and

cloning of transglutaminases responsible for hemolymph clotting in Penaeus monodon and Marsupenaeus japonicus, Biochim. Biophys. Acta 1764 (2006) 1167–1178, https://doi.org/10.1016/j.bbapap.2006.04.005.
[9] Y.-C. Liu, F.-H. Li, B. Wang, B. Dong, Q.-L. Zhang, W. Luan, X.-J. Zhang, J.-H. Xiang, A transglutaminase from Chinese shrimp (Fenneropenaeus chinensis), full-length cDNA cloning, tissue localization and expression profile after challenge, Fish Shellfish Immunol. 22 (2007) 576–588, https://doi.org/10.1016/j.fsi.2006.08.004.
[10] M.-S. Yeh, C.-H. Liu, C.-W. Hung, W. Cheng, cDNA cloning, identification, tissue localisation, and transcription profile of a transglutaminase from white shrimp, Litopenaeus vannamei, after infection by Vibrio alginolyticus, Fish Shellfish Immunol. 27 (2009) 748–756, https://doi.org/10.1016/j.fsi.2009.09.006.
[11] C.-H. Liu, C.-C. Chang, Y.-C. Chiu, W. Cheng, M.-S. Yeh, Identification and cloning of a transglutaminase from giant freshwater prawn, Macrobrachium rosenbergii, and its transcription during pathogen infection and moulting, Fish Shellfish Immunol. 31 (2011) 871–880, https://doi.org/10.1016/j.fsi.2011.08.001.
[12] F. Tokunaga, T. Muta, S. Iwanaga, A. Ichinose, E.W. Davie, K. Kuma, T. Miyata, Limulus hemocyte transglutaminase. cDNA cloning, amino acid sequence, and tissue localization, J. Biol. Chem. 268 (1993) 262–268.
[13] C. Karlsson, A.M. Korayem, C. Scherfer, O. Loseva, M.S. Dushay, U. Theopold, Proteomic analysis of the Drosophila larval hemolymph clot, J. Biol. Chem. 279 (2004) 52033–52041, https://doi.org/10.1074/jbc.M408220200.
[14] K.E. Achyuthan, C.S. Greenberg, Identification of a guanosine triphosphate-binding site on Guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity, J. Biol. Chem. 262 (1987) 1901–1906.
[15] L. Lorand, R.M. Graham, Transglutaminases: crosslinking enzymes with pleiotropic functions, Nat. Rev. Mol. Cell Biol. 4 (2003) 140–156, https://doi.org/10.1038/nrm1014.
[16] D. Bannerjee, R. Delaney, R.O. Morton, J.W. Hampton, Molecular structure of plasma transglutaminase precursor, Ann. N. Y. Acad. Sci. 202 (1972) 172–185, https://doi.org/10.1111/j.1749-6632.1972.tb16329.x.
[17] Y.-T. Chang, C.-Y. Lin, C.-Y. Tsai, V.S. Siva, C.-Y. Chu, H.-J. Tsai, Y.-L. Song, The new face of the old molecules: crustin Pm4 and transglutaminase type I serving as rnps down-regulate astakine-mediated hematopoiesis, PloS One 8 (2013) e72793, , https://doi.org/10.1371/journal.pone.0072793.
[18] R. Sirikharin, T. Utairungsee, J. Srisala, S. Roytrakul, S. Thitamadee, K. Sritunyalucksana, Cell surface transglutaminase required for nodavirus entry into freshwater prawn hemocytes, Fish Shellfish Immunol. 89 (2019) 108–116, https://doi.org/10.1016/j.fsi.2019.03.052.
[19] I. Söderhäll, Crustacean hematopoiesis, Dev. Comp. Immunol. 58 (2016) 129–141, https://doi.org/10.1016/j.dci.2015.12.009.
[20] X. Lin, K. Söderhäll, I. Söderhäll, Transglutaminase activity in the hematopoietic tissue of a crustacean, Pacifastacus leniusculus, importance in hemocyte homeostasis, BMC Immunol. 9 (2008) 58, https://doi.org/10.1186/1471-2172-9-58.
[21] K. Junkunlo, K. Söderhäll, I. Söderhäll, Transglutaminase inhibition stimulates hematopoiesis and reduces aggressive behavior of crayfish, Pacifastacus leniusculus, J. Biol. Chem. 294 (2019) 708–715, https://doi.org/10.1074/jbc.RA118.005489.
[22] K. Junkunlo, K. Söderhäll, I. Söderhäll, C. Noonin, Reactive oxygen species affect transglutaminase activity and regulate hematopoiesis in a Crustacean, J. Biol. Chem. 291 (2016) 17593–17601, https://doi.org/10.1074/jbc.M116.741348.
[23] I. Söderhäll, Y.-A. Kim, P. Jiravanichpaisal, S.-Y. Lee, K. Söderhäll, An ancient role for a prokineticin domain in invertebrate hematopoiesis, J. Immunol. 174 (2005) 6153–6160 Baltim. Md 1950.
[24] R. Sirikharin, K. Junkunlo, K. Söderhäll, I. Söderhäll, Role of astakine1 in regulating transglutaminase activity, Dev. Comp. Immunol. 76 (2017) 77–82, https://doi.org/10.1016/j.dci.2017.05.015.
[25] K. Junkunlo, K. Söderhäll, I. Söderhäll, Clotting protein - an extracellular matrix (ECM) protein involved in crustacean hematopoiesis, Dev. Comp. Immunol. 78 (2018) 132–140, https://doi.org/10.1016/j.dci.2017.09.017.
[26] G.H. Dickinson, I.E. Vega, K.J. Wahl, B. Orihuela, V. Beyley, E.N. Rodriguez, R.K. Everett, J. Bonaventura, D. Rittschof, Barnacle cement: a polymerization model based on evolutionary concepts, J. Exp. Biol. 212 (2009) 3499–3510, https://doi.org/10.1242/jeb.029884.
[27] H. Nozawa, S. Mamegoshi, N. Seki, Partial purification and characterization of six transglutaminases from ordinary muscles of various fishes and marine invertebrates, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 118 (1997) 313–317, https://doi.org/10.1016/s0305-0491(97)00062-x.
[28] Y. Zhang, S. He, B.K. Simpson, A cold active transglutaminase from Antarctic krill (Euphausia superba): purification, characterization and application in the modification of cold-set gelatin gel, Food Chem. 232 (2017) 155–162, https://doi.org/10.1016/j.foodchem.2017.03.135.
[29] R. Sirikharin, I. Söderhäll, K. Söderhäll, Characterization of a cold-active transglutaminase from a crayfish, Pacifastacus leniusculus, Fish Shellfish Immunol. 80 (2018) 546–549, https://doi.org/10.1016/j.fsi.2018.06.042.
[30] M.-S. Yeh, W.-L. Tsai, W. Cheng, Identification and cloning of the second type transglutaminase from Litopenaeus vannamei, and its transcription following pathogen infection and in relation to the haemolymph coagulation, Fish Shellfish Immunol. 35 (2013) 1613–1623, https://doi.org/10.1016/j.fsi.2013.09.002.
[31] M.B.B. Maningas, H. Kondo, I. Hirono, Molecular mechanisms of the shrimp clotting system, Fish Shellfish Immunol. 34 (2013) 968–972, https://doi.org/10.1016/j.fsi.2012.09.018.
[32] R. Perdomo-Morales, V. Montero-Alejo, E. Perera, The clotting system in decapod crustaceans: history, current knowledge and what we need to know beyond the models, Fish Shellfish Immunol. 84 (2019) 204–212, https://doi.org/10.1016/j.fsi.2018.09.060.
[33] K. Söderhäll, V.J. Smith, Separation of the haemocyte populations of Carcinus maenas and other marine decapods, and prophenoloxidase distribution, Dev. Comp. Immunol. 7 (1983) 229–239.
[34] T. Shibata, J. Hadano, D. Kawasaki, X. Dong, S.-I. Kawabata, Drosophila TG-A transglutaminase is secreted via an unconventional Golgi-independent mechanism involving exosomes and two types of fatty acylations, J. Biol. Chem. 292 (2017) 10723–10734, https://doi.org/10.1074/jbc.M117.779710.
[35] É. Csősz, B. Meskó, L. Fésüs, Transdab wiki: the interactive transglutaminase substrate database on web 2.0 surface, Amino Acids 36 (2009) 615–617, https://doi.org/10.1007/s00726-008-0121-y.
[36] R. Zhuang, C. Khosla, Substrates, inhibitors, and probes of mammalian transglutaminase 2, Anal. Biochem. 591 (2020) 113560, https://doi.org/10.1016/j.ab.2019.113560.
[37] L. Lorand, S.M. Conrad, Transglutaminases, Mol. Cell. Biochem. 58 (1984) 9–35, https://doi.org/10.1007/BF00240602.
[38] V. Haroutunian, R. Mantin, G.A. Campbell, G.K. Tsuboyama, K.L. Davis, Cysteamine-induced depletion of central somatostatin-like immunoactivity: effects on behavior, learning, memory and brain neurochemistry, Brain Res. 403 (1987) 234–242, https://doi.org/10.1016/0006-8993(87)90060-6.
[39] I. Söderhäll, K. Junkunlo, A comparative global proteomic analysis of the hematopoietic lineages in the crustacean Pacifastacus leniusculus, Dev. Comp. Immunol. 92 (2019) 170–178, https://doi.org/10.1016/j.dci.2018.11.016.