A new transglutaminase-like from the ascidian *Ciona intestinalis*

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Abstract A cDNA clone encoding a transglutaminase (TGase) was isolated from a cDNA library prepared from the larval stage of *Ciona intestinalis*. The cDNA sequence has an open reading frame encoding a protein of 696 amino acids and is about 36% identical to 11 other TGase sequences. In addition, the critical residues thought to form the catalytic center are conserved. The *Ciona* TGase (CiTGase) has an extension of 39 amino acids in the NH_2-terminal region similar to that reported for keratinocyte TGases. A phylogenetic analysis among other types of TGases demonstrated that CiTGase represents a new type of the enzyme.

Key words: Transglutaminase; *Ciona intestinalis*; cDNA cloning; Mesenchyme cell; Muscle cell

1. Introduction

Transglutaminases (TGases) catalyze the post-translational modification of proteins. This process, known as the R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction, leads to the formation of an isopeptide bond either within or between polypeptide chains, and the covalent incorporation of polyamines into proteins through glutamine side chains. This calcium-dependent reaction, which results in a stable, insoluble macromolecular structure, is widespread throughout the plant and animal kingdoms [1–4]. In humans TGases are distributed in various organs, tissues and body fluids [5]. Enzymes with a similar function to vertebrate TGases [4] have been found in fish, invertebrates, slime mold, plants and bacteria [6–14]. The characterization of the primary structure of several TGases together with the discovery of the primary structure of enzymes from plants and microorganisms revealed that these enzymes evolved as a separate lineage from the eucaryotic TGases [4]. There are three classes of TGases: extracellular and two intracellular or tissue TGases. Only in few cases has the physiological significance of protein cross-linking been established [15–19]. No definite function has been attributed to the cellular or cytosolic TGase, although evidence suggests they play a physiological role [for reviews, see refs. [2,4]]. TGases have also been implicated in activation during spontaneous differentiation [7,20], cytokinesis [21–23], apoptosis [24–30] and chromatin remodelling [31], and have been associated with a variety of phenomena in a wide range of cell types [32–36]. The finding that tissue TGase modulates levels of intracellular messengers reinforces the physiological significance of these enzymes [37–39].

To learn more about invertebrate TGases, their physiological role, and their evolutionary relationship to the other members of the TGase family we examined the cDNA sequence of *Ciona* TGase (CiTGase) mRNA and messenger expression during development. The ascidian *Ciona intestinalis* is important for phylogenetic studies. Ascidians are one of the simplest chordates: the tadpole larva contains only a few thousand cells and about six different types of tissue. The ascidian genome is small for a chordate: only 1.8×10^8 nucleotide pairs. Ascidian embryos are highly mosaic, i.e. early cleavage cells are committed to a defined cell lineage.

Here we report the cloning and sequencing of CiTGase cDNA. We found that the enzyme is coded for by an mRNA species of 2627 bp constituted by a 5′-untranslated region of 49 bases, a coding segment of 2088 bases, a stop codon, and a large 3′-untranslated region of 487 bases that includes a poly(A)tail. The deduced protein has 696 amino acids and shares sequence similarities with the other members of the TGase family. Northern blot analysis and reverse transcription (RT)-PCR analysis of mRNA demonstrated CiTGase mRNA in several stages of development with a maximum at larval stage. Whole-mount in situ hybridization showed CiTGase mRNA in mesenchyme cells at gastrula stage and in primordial muscle cells from neurula to late tailbud stage.

2. Materials and methods

2.1. Animals and embryos

Adult specimens of *C. intestinalis* were collected in the Bay of Naples. *Ciona intestinalis* is a self-sterile hermaphrodite. Eggs were fertilized with suspensions of sperm from another individual and cultured at 18°C. When the embryos reached the appropriate stage, they were packed by hand centrifugation, frozen at −20°C for RNA preparation or fixed for in situ hybridization.

2.2. RNA isolation

Total RNA was extracted from ovaries, eggs and embryos at various stages of development by the guanidine hydrochloride method [40]. Poly(A)$^+$RNA was isolated by the oligo(dT) cellulose batch binding method according to Sambrook et al. [41].

2.3. Probe preparation

Enzymes were purchased from Promega Biotech or New England Biolabs unless stated otherwise. A recombinant genomic library constructed into the EMBL3 SP6/T7 vector (Clontech) with *C. intestinalis* muscle DNA partially digested with *Mbo*I was screened with a 128-fold degenerate oligonucleotide (5′-GTSMMVTAYGGMCAGTGC-TGGGT, where M = A or C; R = A or G; S = C or G; V = A or C or G; Y = C or T; TGase1), coding for the conserved amino acid sequence of the TGase active site. Phage lifts were hybridized as described by Woods et al. [42]. Recombinant phage DNA was extracted according to [43]. About 6 μg of phage DNA was digested with *Eco*RI–*Sal*I and 1 μg was run on 1% agarose gel. The gel was blotted

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Abbreviations: TGase, transglutaminase; CiTGase, *Ciona intestinalis* transglutaminase

Data deposition: The sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank (accession number: Y10212).

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onto nylon membranes (Hybond-N, Amersham) and hybridized with TGase1 to identify the fragment containing the CiTGase active site. The remaining 5 μg were run in the same conditions and the fragment hybridizing to TGase1 was purified, subcloned in pBluescript SK(-) vector and sequenced on both strands by the dideoxynucleotide procedure [44]. The CiTGase cDNA probe was obtained by PCR amplification of the coding region of the active site performed using 50 ng of the positive fragment in the presence of 0.2 mM of each dNTP, 1 μM primers and 2.5 U of *Taq* polymerase (Perkin Elmer). Thermocycling conditions were: 5 min at 95°C, 1 min at 46°C and 1 min at 72°C for 30 cycles followed by 10 min at 72°C. Amplified products were size-fractionated on a 1.2% agarose gel, blotted onto nylon membranes and hybridized with TGase1. The 175-bp reaction product contained the sequence coding for the TGase active site. 5′-GTCAACTGTAACGATGAC (sense) and 5′-GTAGTAAGCAACCCGGAG (antisense) primers were used for amplification.

2.4. cDNA library construction and screening

cDNA was synthesized with 4 μg of larval stage poly(A)$^+$ RNA. After the addition of *Eco*RI adapters, the cDNA was inserted into the vector arms of λZAP II. This ligated DNA was encapsidated using Gigapack II Gold packaging extract according to the manufacturer's instructions (Stratagene), and was used to infect the *E. coli* strain XL-1 blue, thus constructing a cDNA library. Approximately 5×10^5 recombinant λ phages were screened by plaque hybridization using the CiTGase cDNA probe labeled by random priming (Multiprime DNA Labelling System, Amersham). Hybridization was carried out at 50°C for 16 h in Church buffer (1 mM EDTA/0.5 M NaHPO4/7% SDS) [45]. Subsequently, the filters were washed 3 times for 10 min each at room temperature in 2× standard saline citrate (SSC)/0.1% SDS and twice for 15 min at 60°C in 2× SSC/0.1% SDS.

The cDNA inserts from positive clones were rescued as pBluescript SK(-) by helper phage-mediated in vivo excision as described by the manufacturer (Stratagene).

2.5. DNA sequencing and analyses

Positive cDNA clones were isolated and sequenced on both strands by the dideoxynucleotide procedure [44]. The sequence was extended by a primer-directed sequencing approach with sequence-specific oligonucleotide primers. The complete cDNA was derived from sequencing both strands of overlapping cDNA clones. The DNA and amino acid sequences were analyzed using the GCG computer program (Wisconsin Sequence Analysis Package).

2.6. Cloning of the 5′-end of CiTGase cDNA

The 5′-RACE System (GIBCO/BRL) was used with 1 μg of larval stage poly(A$^+$) RNA and a CiTGase-specific primer: 5′-TAGCAGATAACAGCCTGCTACAATAGGCAG (complementary to nucleotide 912–941). Poly(dC)-tailed cDNA was subjected to 35 PCR cycles using an annealing temperature of 55°C in a 50 μl reaction containing 25 mM Tris-HCl, pH 8.4/50 mM KCl/1.5 mM $MgCl^2$/40 μM of a nested CiTGase-specific primer: 5′-CTTCAACGCGATGTCTAATATTCCTTCCTC (complementary to nucleotide 818–847)/40 μM of a poly[d(GI)]-anchor primer/10 U of *Taq* polymerase (Perkin Elmer). The PCR product was isolated and cloned into the pMOS*Blue* T-vector (pMOS*Blue* T-vector kit, Amersham) and sequenced.

Another RACE analysis was performed using the thermostable r Tth reverse transcriptase RNA (Perkin Elmer) at 70°C on CiTGase poly(A)$^+$ RNA. Transcribed cDNA was used for the 5′-RACE System with a CiTGase-specific primer: 5′-TCTGCAGAACCTCAGTCGGGGCGGGCCCGC (complementary to nucleotide 234–263) designed according to the clone of the previous RACE analysis. Poly(dC)-tailed cDNA was subjected to 35 PCR cycles using an annealing temperature of 57°C in a 50 μl reaction containing 25 mM Tris-HCl, pH 8.4/50 mM KCl/1.5 mM of a nested CiTGase-specific primer: 5′-CGCTAACTTTGTCCAACCACTTTTGTATAG (complementary to nucleotide 207–236)/40 μM of a poly[d(GI)]-anchor primer/10 U of *Taq* polymerase (Perkin Elmer). The PCR product was isolated and cloned into the pMOS*Blue* T-vector (pMOS*Blue* T-vector kit, Amersham) and sequenced.

2.7. Reverse RT-PCR amplification

Total RNAs from ovaries, eggs and embryos at various stages were reverse transcribed using the SuperScript Preamplification System for First Strand cDNA Synthesis (GIBCO/BRL) and then amplified directly using PCR, according to the manufacturer's instructions. The following CiTGase-specific primer were used for amplification: 5′-TAAGTTTGTTTTGGAGGATGAA (nucleotide 1678–1699, sense) and 5′-AAGGACATGGGGAAATCCATCT (nucleotide 2460–2481, antisense). The PCR products were subjected to electrophoresis on 1% agarose gels, transferred to nylon membranes (Hybond-N) by blotting, and hybridized with the radiolabeled oligonucleotide 5′-AGTGCGATGACGTCAGCTG (nucleotide 2131–2149).

2.8. Northern blot analysis

Poly(A)$^+$ RNAs from eggs and embryos at various stages were electrophoretically separated on 1.3% agarose–3% formaldehyde denaturing gel and blotted onto nylon membranes (Hybond-N). The nylon filters were hybridized overnight with labelled CiTGase cDNA probe in a solution containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, 6× SSPE and 100 μg/ml denatured salmon sperm DNA at 65°C for 16 h. Filters were washed twice in 2× SSC and 0.1% SDS at room temperature for 10 min, twice in 2× SSC/0.1% SDS at 60°C for 15 min before autoradiography.

2.9. In situ hybridization procedures

Whole-mount in situ hybridization was performed using digoxigenin (DIG)-labeled RNA probes as described previously with minor modifications [46]. In brief, embryos at various stages of development were fixed in 4% paraformaldehyde in (3-[*N*-morpholino] propanesulfonic acid (MOPS) buffer (pH 7.5), and 0.5 M NaCl for 90 min at room temperature. After a thorough wash with phosphate-buffered saline (PBS), embryos were deprived of their envelopes by needles, washed with PBT (PBS containing 0.1% Tween 20), treated for 30 min at 37°C with 2 μg/ml proteinase K (Sigma) in PBT and then post-fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. After 1 h of prehybridization at 42°C, embryos were allowed to hybridize with the digoxigenin-labeled antisense or sense probe for at least 16 h at 42°C. After hybridization, specimens were washed and treated with RNase A (Sigma), then washed again with PBT. Samples were then incubated overnight with 1:2000 alkaline-phosphate-conjugated anti-DIG (Boehringer Mannheim) and treated for development of color according to the manufacturer's instructions. Probes were synthesized as indicated by the supplier of the kit (Dig RNA Labeling kit, Boehringer Mannheim). The gene expression reported here was examined with probes from the full-length mRNA.

3. Results and discussion

We screened a *Ciona* recombinant genomic library in EMBL3 SP6/T7 with a synthetic 128-fold degenerate oligonucleotide DNA fragment encoding the active site of TGases as probe. One positive genomic clone was isolated and subcloned into pBluescript II SK (-). This 2032-bp clone was sequenced and was found to encode a region of the *CiTGase* gene that includes the exon coding for the active site.

Four positive clones were isolated out of the 5.0×10^5 clones screened and sequenced. The longest insert (≈2.5 kb) included the cDNA regions from the other three clones. This insert was completely sequenced and contained a single large open reading frame of 1850 bp encoding from the middle region to the 3′-encoding portion of CiTGase, which includes the sequence similar to the active site region of other TGases [47]. To obtain a cDNA clone encoding the 5′-portion of CiTGase we carried out a 5′-RACE analysis. Sequence analysis of the RACE product extended the sequence of the CiTGase clone by about 600 bp in the 5′-region. Because this clone did not reach the first ATG codon we carried out a further 5′-RACE analysis using the thermostable r Tth reverse transcriptase RNA with new primers. The RACE product yielded a sequence of 170 bp which included 49 bp of the 5′-noncoding region and the first ATG codon. The complete CiTGase cDNA consisted of 2137 nucleotides, followed by a TAG termination codon and a 3′-noncoding region consisting

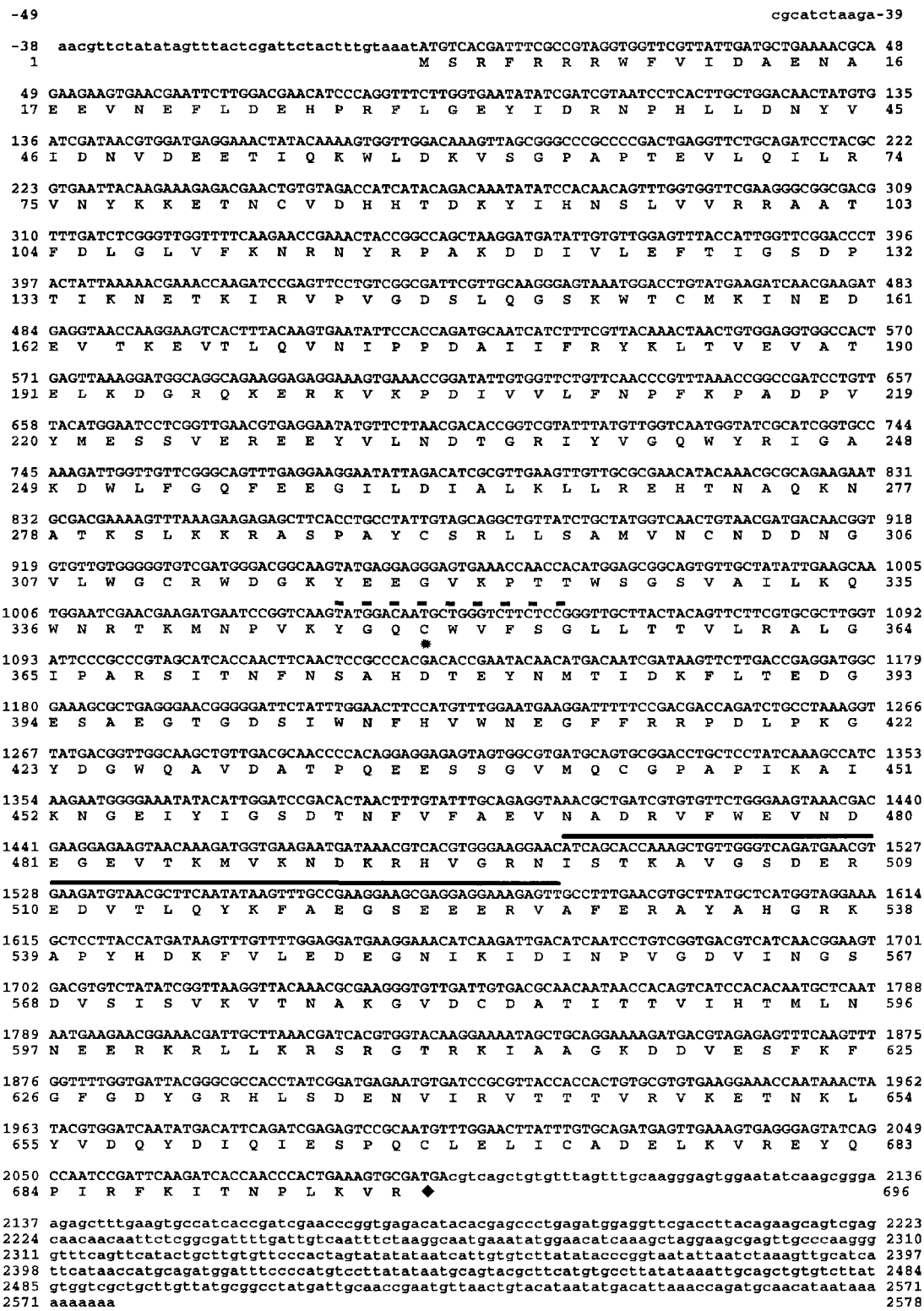

Fig. 1. Nucleotide sequence of CiTGase and the deduced amino acid sequence. The diamond indicates the termination codon. The broken line indicates the putative active site for TGase function; the star indicates the cysteine residue that is critical for activity. The continuous line shows the CiTGase region that is homologous to a proposed calcium-binding site for TGases.

of 487 bp (Fig. 1). The deduced amino acid sequence of the open reading frame corresponded to a protein of 696 amino acids (79615.40 Da) and included the conserved pentapeptide (Tyr–Gly–Gln–Cys–Trp) containing the TGase Cys active site [47].

The amino acid sequence of CiTGase was 34–38% identical to that of the 11 other TGases examined: limulus hemocyte TGase [10]; the human factor XIIIa subunit [48,49]; human fibroblast TGase K [50]; human keratinocyte TGase [51]; rat keratinocyte TGase [51]; human endothelial TGase [52]; bo-

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                          349                                377
                           ▼                                  ▼
CiTGase          KMNPVKYGQCWVFSGLLTTVLRALGIPARSITNFNSAHDTEYNM
hKTGase          TGYSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDTDTSL
rKTGase          TGYSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDTDTSL
hFTGase          TGYSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDTDTSL
hFXIIIa          SENPVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDNDANL
IHTGase          RGVPVKYGQCWVFAGVANTVSRALGIPSRTVTNYDSAHDTDDTL
annulin          KK.PVKYGQCWVFAGVLTTVCRALGLPARTVTTYSAAHDTQNSL
bETGase          GCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQNSNL
hETGase          GCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQNSNL
mMTGase          GCQQVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQNSNL
gpLTGase         GCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNFNSAHDQNSNL
fLTGase          GVRPVKYGQCWVFAAVACTVLRCLGIPTRPITNFASAHDVDGNL
                     * *******     *  * **      *    ***

                      407                          430          468
                       ▼                            ▼            ▼
CiTGase  WNFHVWNEGFFRRPDLPKG....YDGWQAVDATPQE.DTNFVFAEVNAD
hKTGase  WNFHVWNDCWMKRPDLPSG....FDGWQVVDATPQE.DTPFIFAEVNSD
rKTGase  WNFHVWNDCWMKRPDLPSG....FDGWQVVDATPQE.DTPFIFAEVNSD
hFTGase  WNFHVWNDCWMKRPDLPSG....FDGWQVVDATPQE.DTPFIFAEVNSD
hFXIIIa  WNYHCWNEAWMTRPDLPVG....FGGWQAVDSTPQE.DAPFVFAEVNSD
IHTGase  WNFHVWNDCWMARPDLPTG....YGGWQAYDSTPQE.DSPFVFSEVNAD
annulin  WNFHVWTEVWMERPDLMPGDGAHYGGWQAVDSTPQE.DSAYVFAEVNAD
bETGase  WNFHCWVESWMTRPDLQPG....YEGWQALDPTPQE.DAPFVFAEVNAD
hETGase  WNFHCWVESWMTRPDLQPG....YEGWQALDPTPQE.DAPFVFAEVNAD
mMTGase  WNFHCWVESWMTRPDLQPG....YEGWQALDPTPQE.DAPFVFAEVNAD
gpLTGase WNFHCWVESWMTRPDLEPG....YEGWQALDPTPQE.DAPFVFAEVNAD
fLTGase  WNFHCWVESWMSREDLPEG....NDGWQVLDPTPQE.DAPFVFAEVNAD
         **** *       * **  *      ***  * **** *    * *****
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Fig. 2. Alignment of the active-site region and of a part of the middle region of CiTGase with the other 11 TGases sequenced so far. The numbers above the sequences represent the positions of the amino acids in CiTGase critical for TGase catalytic activity. Residues identical in all 12 sequences are indicated by asterisks. IHTGase, limulus hemocyte TGase; hFXIIIa, human factor XIIIa subunit; hFTGaseK, human fibroblast TGase K; hKTGase, human keratinocyte TGase; rKTGase, rat keratinocyte TGase; hETGase, human endothelial TGase; bETGase, bovine endothelial TGase; mMTGase, mouse macrophage TGase; gpLTGase, guinea pig liver TGase, fLTGase, red sea bream liver TGase.

vine endothelial TGase [53]; mouse macrophage TGase [52]; guinea pig liver TGase [54]; red sea bream liver TGase [6]; and annulin [9]. Although the overall sequence identity is not very high, the middle region of the molecule is highly conserved. Indeed, the Val^{344}–Glu^{530} region was 51–56% identical to the corresponding regions of the 11 TGases mentioned above. In particular, CiTGase retained 12 residues out of the 25 amino acids of the active site. In addition, the residues surrounding His^{377}, which may interact with Glu^{468} by analogy with the crystallography data on factor XIIIa [55,56], were conserved in CiTGase. Two critical residues, His^{407} and Asp^{430}, which are thought to be essential for TGase catalytic activity [55,57], were also situated in the highly homologous region of CiTGase (Fig. 2).

A putative calcium-binding site, located between residues 499 and 527, which is common to all known TGase sequences, was also found in CiTGase (Fig. 1). CiTGase also shares some extended stretches of identity found in the other members of the TGase family, suggesting that these highly conserved regions or residues may be critical determinants of

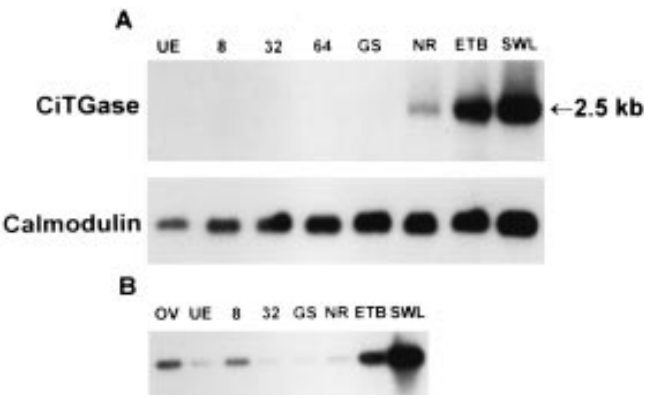

Fig. 3. Northern blot analysis of the expression of CiGTase in ascidian eggs and embryos at various stages. Poly(A)$^+$ RNA was prepared from unfertilized eggs (UE), 8-cell stage embryos (8), 32-cell stage embryos (32), 64-cell stage embryos (64), gastrula stage embryos (GS), neurula stage embryos (NR), early tailbud stage embryos (ETB) and swimming larval stage embryos (SWL), and hybridized with CiTGase cDNA labeled probe. Transcript of CiTGase is evident from the neurula stage. As a control, all stages were also hybridized with labeled calmodulin probe. The numbers indicate the size in kilobases (A). Quantitative RT-PCR assays of RNAs prepared from ascidian ovary (OV), unfertilized eggs (UE), 8-cell stage embryos (8), 32-cell stage embryos (32), gastrula stage embryos (GS), neurula stage embryos (NR), early tailbud stage embryos (ETB) and swimming larval stage embryos (SWL). Transcript of CiTGase is evident in all stages, but reaches the maximum at swimming larval stage (B).

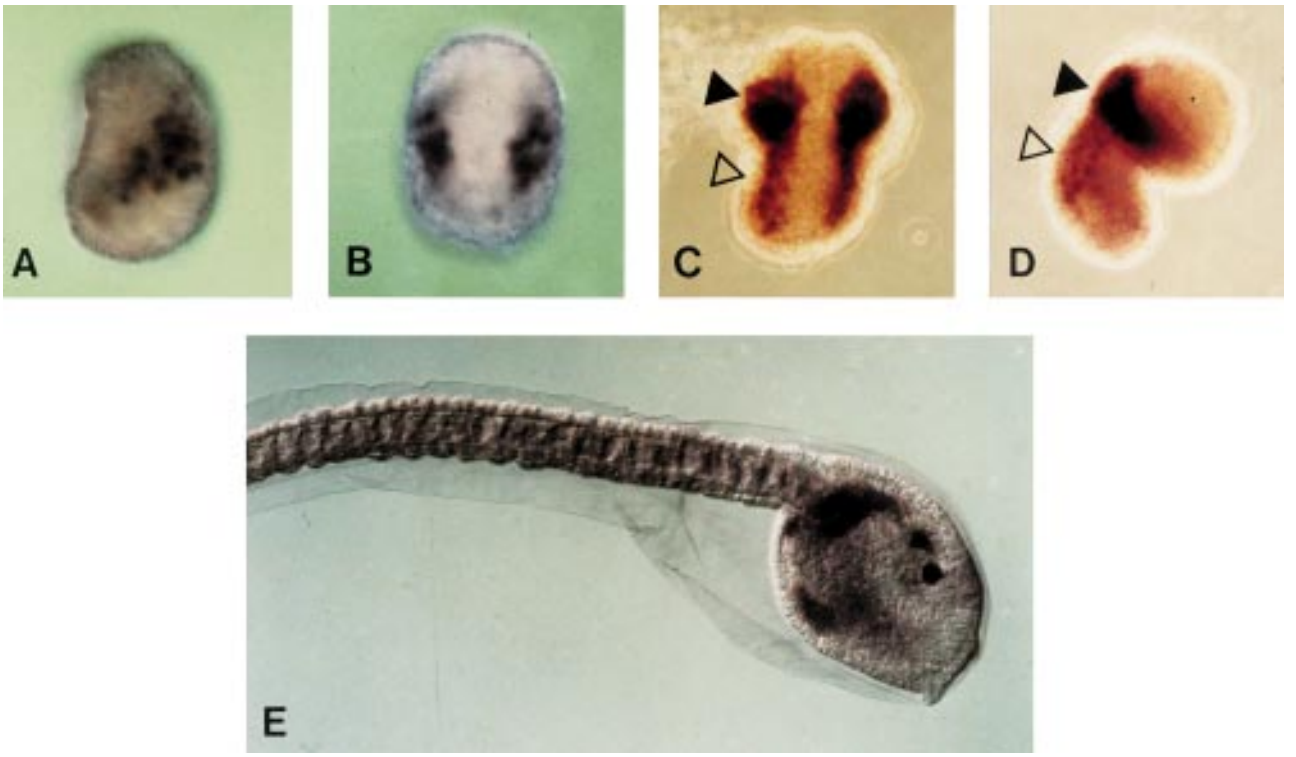

Fig. 4. Expression of CiTGase in ascidian embryos, as revealed by whole-mount in situ hybridization with a digoxigenin-labeled antisense probe. A,B: Embryo at gastrula stage, vegetal and lateral view, respectively. C: Embryo at neurula stage. D: Embryo at early tailbud stage. E: Embryo at swimming larval stage. Hybridization signals are evident in mesenchyme cells (closed arrowheads) and in muscle cells (open arrowheads).

enzyme activity. Thus, CiTGase appears to be a functional TGase. CiTGase contains a 5′-extension similar to that reported for keratinocyte TGase: both contain a large percentage of arginine residues in proximity to a serine residue that is thought to be a phosphorylation site [51]; however, the CiTGase 5′-extension is shorter than keratinocyte TGase ($\approx$39 vs. 105 amino acids). A short extension of the NH_2-terminal region, together with the lack of Cys residues in the first amino acid positions suggests that CiTGase is not anchored to the cell membrane [58].

Northern blot analysis of poly(A)$^+$ RNA prepared from eggs and from *Ciona* embryos yielded a single band of approximately 2.5 kb (Fig. 3A) in embryos from the early tailbud stage to the larval stage where it reached a maximum. This finding coincides with the results of RT-PCR analysis which showed a low level of the transcript starting from the egg (Fig. 3B). The messenger length correlates well with the length of the total clone of cDNA and indicates that probably a single transcript arises from the genomic locus that corresponds to CiTGase. Genomic southern blot analysis carried out under low-stringency conditions confirmed this observation (data not shown).

CiTGase transcripts can first be detected using whole-mount in situ hybridization of gastrula stage material. At this stage the staining was restricted to the cells of the posterior vegetal region of the embryo (Fig. 4A,B). As development proceeded to the neural plate and early tailbud stage, it became evident that cells with distinct signals were mesenchyme cells (Fig. 4C,D). At these two stages, another distinct signal appeared in the presumptive muscle cells. The muscle cell staining became less intense at the late tailbud stage (data not shown) and disappeared at swimming larval stage, while the mesenchyme cell expression remained intense. This observations coincides with results obtained in limulus [10], where TGase is expressed in hemocytes; in *Ciona*, mesenchyme cells

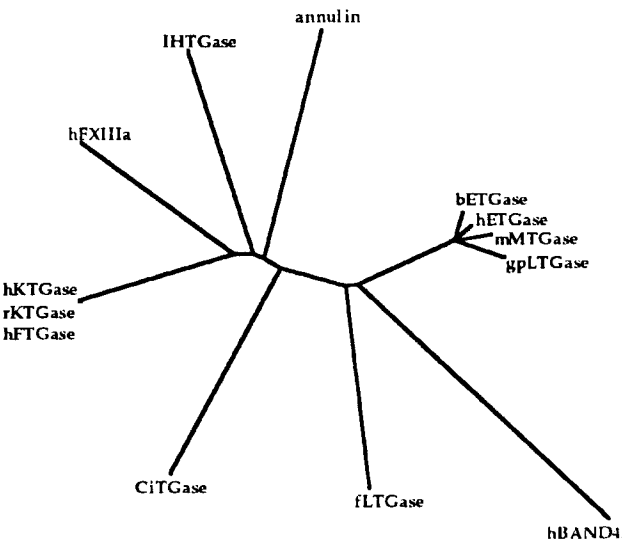

Fig. 5. Phylogenetic tree of the TGase family. This tree was inferred by the neighbor-joining method [60]. Branch lengths are proportional to the numbers of accumulated amino acids substitutions. IHTGase, limulus hemocyte TGase; hFXIIIa, human factor XIIIa subunit; hFTGaseK, human fibroblast TGase K; hKTGase, human keratinocyte TGase; rKTGase, rat keratinocyte TGase; hETGase, human endothelial TGase; bETGase, bovine endothelial TGase; mMTGase, mouse macrophage TGase; gpLTGase, guinea pig liver TGase, fLTGase, red sea bream liver TGase.

are thought to form various mesodermal tissue including blood cells [59].

A phylogenetic tree representing evolutionary relationships among the members of the TGase family was inferred by the neighbor-joining method [60], in which the sequence of CiTGase was aligned with those of the 11 other TGase sequences in the region between residue 119 and residue 781 (Fig. 5). The inferred phylogenetic tree revealed four distinct clusters: type I, the keratinocyte TGase group; type II, the endothelial/macrophage/liver TGase group, together with the erythrocyte 4.2 band [61]; type III, the factor XIIIa subunit group; and type IV, the invertebrate TGase group, which includes annulin [49]. CiTGase does not seems to belong to any of these four groups.

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