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Cg-TIMP, an inducible tissue inhibitor of metalloproteinase from the Pacific oyster *Crassostrea gigas* with a potential role in wound healing and defense mechanisms[1]

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Abstract We have cloned and characterized a cDNA encoding *Cg*-TIMP, the first tissue inhibitor of metalloproteinase identified in mollusks. The isolated cDNA encodes a protein of 221 residues that has a domain organization similar to that of vertebrate TIMPs including a signal sequence, and the 12 cysteines characteristic of the TIMP signature. Analysis of *Cg-TIMP* expression in adult oyster tissues, by Northern blot and in situ hybridization, indicates that *Cg-TIMP* was only expressed in hemocytes which are the key components of defense mechanisms in mollusks. We also observed that *Cg-TIMP* mRNA accumulated during shell damage and bacterial challenge. This pattern of expression suggests that *Cg*-TIMP may be an important factor in wound healing and defense mechanisms.

Key words: Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Wound healing; Mollusk immunity; Hemocyte; Oyster

1. Introduction

In vertebrates, tissue inhibitors of metalloproteinases (TIMPs) are secreted multifunctional proteins that play pivotal roles in the regulation of extracellular matrix (ECM) metabolism [1]. Their most widely recognized action is as inhibitors of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that exist in both secreted and membrane-bound forms. MMPs and their inhibitors TIMPs regulate the proteinaceous ECM homeostasis and thus play a role in a wide range of physiological processes that include embryonic development, connective tissue remodeling, wound healing, glandular morphogenesis, and angiogenesis [2]. An imbalance in MMP/TIMP expression has been shown to be implicated in various diseases such as inflammation, erosive joint disease, cardiovascular disease, and cancer [3].

In vertebrates, members of the TIMP family are divided into four groups (TIMP-1, -2, -3 and -4) and all of them share several structural features [1]. First, a leader sequence which is cleaved off upstream of the motif Cys-X-Cys (where X designates any amino acid), to produce the mature protein. Second, they all possess 12 cysteine residues in conserved regions of the molecule, forming six disulfide bonds. The bonding pattern divides the protein into two domains with each domain being folded into three loops stabilized by three disulfide bonds [4]. The N-terminal domain is responsible for metalloproteinase inhibition, whereas the C-terminal domain confers specific functions such as the ability of TIMPs to bind pro-MMP [5].

Invertebrate members of MMP have been identified in *Hydra vulgaris* [6], *Paracentrotus lividus* [7], *Caenorhabditis elegans* [8], and *Drosophila melanogaster* [9]. In all these organisms, MMPs are functionally tied to ECM remodeling taking place during development. The characterization of MMPs in invertebrates together with the observation that at least one member of the TIMP gene family is also present in flies [10,11] strongly suggest that a conserved proteolytic system of tissue remodeling can be fully reconstituted in invertebrates.

In our search for genes involved in oyster immunity we isolated a cDNA encoding a polypeptide closely related to the vertebrate TIMP family. We demonstrated that *Cg-TIMPs* are only expressed in oyster hemocytes which are the cellular components that mediate bivalve mollusk immunity. Moreover, *Cg-TIMP* mRNAs were accumulated in hemocytes of oysters subjected to shell damage or bacterial challenge, which strongly suggests the involvement of *Cg*-TIMP in wound healing as well as in defense mechanisms.

2. Materials and methods

2.1. Animals, shell damage, immune challenge and hemolymph withdrawal

Three- to four-year-old oysters (*Crassostrea gigas* Thunberg) were collected from a commercial farm (Palavas, Gulf of Lion, France) and kept in seawater at 15°C. To minimize individual variability, at least 10 oysters were used in each experimental conditions. The shell damage consisted in breaking the dorso-posterior part of the shell. After shell damage, oysters were returned to seawater tanks for 3, 6, 9, 12, or 24 h.

Oysters were challenged by filing the shell and injecting 100 μl of sterile seawater (saline) or 100 μl of a mixture of four *Vibrio* strains

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[1] GenBank accession number of *Cg*-TIMP is AF321279.

Abbreviations: *Cg*, *Crassostrea gigas*; ECM, extracellular matrix; MMP, matrix metalloproteinase; ORF, open reading frame; TIMP, tissue inhibitor of metalloproteinases; UTR, untranscribed region

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(*V. anguillarum*, *V. metshnikovii*, *V. tubiashii* and *Vibrio* S322) in the adductor muscle. The bacterial strains were grown separately overnight at 24°C in marine agar. Bacterial concentration was calculated from the optical density at 550 nm (1 unit OD_{550} corresponds to 5×10^8 bacteria/ml). The bacterial cells were collected by centrifugation (10 000×*g*, 5 min), washed twice and resuspended in saline. After injection, oysters were returned to seawater tanks for 12 h. Hemolymph from unchallenged or challenged oysters was collected and treated as mentioned below.

Then, oyster hemolymph was collected with a syringe from the pericardial cavity, through the adductor muscle, and immediately centrifuged at 1000×*g* for 10 min at 4°C. Plasma samples were frozen, while hemocyte pellets were used for RNA extraction.

2.2. RNA isolation and analysis

Hemocyte pellets were resuspended in Trizol reagent (Gibco BRL) (1 ml/10^7 cells). Oyster tissues (adductor muscle, mantle margin, mantle inner surface, heart, gills, labial palps, digestive gland, and stomach) were collected from several oysters. Tissues (100–300 mg) were washed in sterile seawater, cut into small pieces and incubated overnight at room temperature in Trizol reagent (1 ml/100 mg of tissue). Total RNAs were extracted following the manufacturer's instructions.

Total RNAs (8–10 μg) were fractionated by denaturing 1.2% agarose/formaldehyde gel electrophoresis and blotted onto Hybond-N membrane (Amersham). Size markers were simultaneously applied to the electrophoresis and visualized by staining the membrane with a methylene blue solution. Membranes were prehybridized for 2 h at 65°C in 50% formamide, 5×SSC, 8×Denhardt's, 50 mM NaH_2PO_4 pH 6.5, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridizations were performed overnight at 42°C in the same buffer containing the radiolabeled probe. Labeled DNA probes were prepared using [α-^{32}P]dCTP and random priming (Gibco BRL). After hybridization, membranes were washed twice for 15 min at room temperature in a solution of 2×SSC containing 0.1% SDS and twice for 20 min at 60°C in a solution of 1×SSC containing 0.1% SDS. Finally membranes were exposed to autoradiographic film. The obtained signal intensity was quantified using the Storm system technology from Molecular Dynamics.

2.3. Probe preparation

2.3.1. Cg-TIMP probe. The phagemid containing the 1301 bp *Cg-TIMP* cDNA was hydrolyzed by restriction enzymes *Eco*RI and *Xho*I and the insert purified from agarose gel using the Geneclean II kit (Bio 101).

2.3.2. rRNA probe. A sense oligonucleotide (5′-TGACCTCGCGGAAAGAGCGC-3′) and an antisense oligonucleotide (5′-AGGGGACGTAATCAACGCGAGC-3′) were designed from the sequence of the *Mytilus* ribosomal RNA small subunit and used in PCR experiments. One microgram of oyster genomic DNA was subjected to amplification using 40 cycles consisting of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C in 200 μM dNTP, 1×PCR buffer, 2 mM $MgCl_2$, 0.5 μM of each primer, and 1 U of Taq DNA polymerase (Promega). The PCR product (1460 bp) was purified and labeled by random priming.

2.4. In situ hybridization

In order to synthesize small probes for in situ hybridization, we designed four sense primers (1s–4s) and four antisense primers (2as–5as) along the *Cg-TIMP* open reading frame (ORF) (Table 1). These primers were combined in pairs (1s-2as, 2s-3as, 3s-4as, and 4s-5as) in four PCR reactions using *Cg-TIMP* cDNA as template (pBKCMV-timp7b). We obtained four PCR products ranging from 161 to 200 bp totally overlapping *Cg-TIMP* ORF. PCR reactions were carried out in 50 μl according to the standard conditions of the Silver-star Taq DNA polymerase (Eurogentec). After DNA denaturation at 94°C for 3 min, 30 cycles were run with an MJ-Research thermocycler as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s followed by a final 72°C elongation step of 10 min. Amplified products were analyzed electrophoretically on 1% agarose gels. Probes (sense or antisense) were synthesized by asymmetric PCR in presence of Dig-dUTP (0.5 mM) in the PCR reaction mixture containing a unique primer (sense or antisense) and 1 μl of previous PCR fragment. For in situ hybridization we used a mixture of the four sense or antisense probes.

Crassostrea gigas hearts were fixed for 3 days in 35% formaldehyde, 40% ethanol, and 2% ammonium hydroxide, embedded in paraffin wax, and serially sectioned at 5 μm. Sections were collected onto polylysine-coated slides and treated with proteinase K (100 μg/ml) in TE buffer (Tris 50 mM, EDTA 10 mM) at 37°C for 30 min. Slides were then dehydrated by immersion in an ethanol series and air-dried. The sections were prehybridized for 1 h at 42°C with 500 μl of hybridization buffer (4×SSC, 50% formamide, 1×Denhardt's solution, 250 μg/ml yeast tRNA, 10% dextran sulfate). The solution was then replaced with 100 μl of the same buffer containing 20 ng of the digoxigenin-labeled sense or antisense probes. The slides were then incubated overnight at 42°C for hybridization. The sections were washed twice for 5 min in 2×SSC at room temperature and once for 10 min in 0.4×SSC at 42°C. The detection steps were performed according to the manufacturer's instructions (Dig nucleic acid detection kit, Roche Molecular Biomedicals).

3. Results

3.1. Molecular cloning of Cg-TIMP cDNAs

To characterize genes involved in oyster immunity, we used a mRNA differential display approach (DD-RT-PCR) performed with total RNAs extracted from hemocytes of bacterially challenged or unchallenged oysters. DD-RT-PCR products were used as probes to screen an oyster hemocyte cDNA library (as previously described in [12]). We isolated and sequenced three independent cDNA clones, the sizes of which ranged from 1296 to 1301 bp.

Most of the differences between the three cDNAs occur in the 5′ untranscribed region (UTR) (11–16 bp changes) and 3′ UTR (11–32 bp changes). The three clones contain an ORF of 666 bp predicted to encode a 221-amino acid protein. Comparison of the three deduced amino acid sequences revealed that a maximum of six amino acid changes were observed in spite of the fact that up to 16 bp substitutions were detected between nucleotide sequences. The analysis of the deduced amino acid sequence by the SignalP V1.1 software revealed the presence of a 22-residue signal peptide with a predicted cleavage site located between residues 22 and 23, upstream of the Cys-Met-Cys sequence.

3.2. Homologies of Cg-TIMP with invertebrate and vertebrate TIMPs

Searches for homologies were realized with the amino acid sequence deduced from the longest cDNA cloned using the BLAST program. It appeared that the oyster amino acid sequence was homologous to the TIMP protein family. Surprisingly *Cg*-TIMP is closer to vertebrate TIMP-2 and -3 (30%

Table 1
Sequences and combinations of primers used to synthesize probes for in situ hybridization

Name	Position[a]	Sequence (5′-3′)	Name	Position[a]	Sequence (5′-3′)	PCR product size (bp)
1s	166–185	gatgaggcagtacaacttcc	2as	345–324	gaatacccattgactcgtcc	180
2s	325–344	gacgagtcaatgggtattcc	3as	524–504	cgccgtcactgacaaaacc	200
3s	505–524	aggttttgtcagtgacggcg	4as	688–669	ggtctggtccggattcaatg	184
4s	669–688	cattgaatccggaccagacc	5as	829–810	cggtagaacccctccttccc	161

[a]Position according to cDNA sequence (GenBank accession number AF321279).

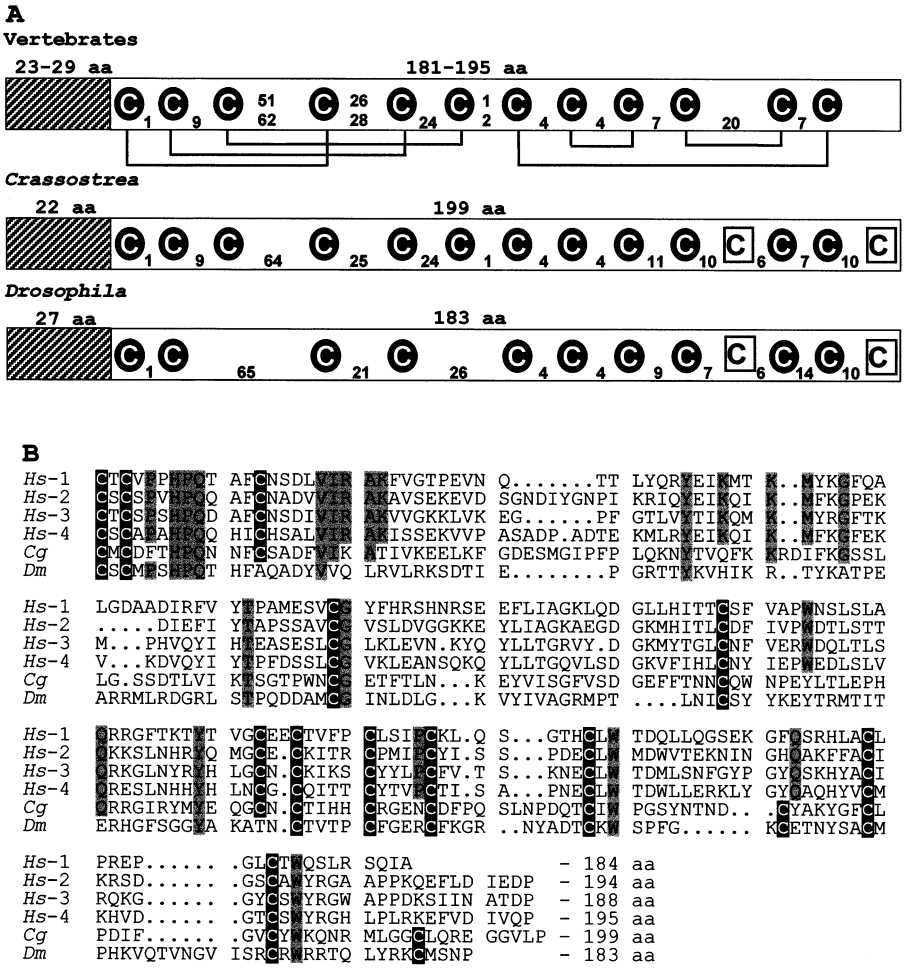

Fig. 1. Comparison between vertebrate and invertebrate TIMPs. A: Schematic representation of vertebrate, *C. gigas* and *D. melanogaster* TIMPs. Vertebrate TIMP drawing derived from the analysis of the 28 amino acid sequences available in data banks (accession numbers: NP_003245, AAB53735, AB016817, AAB30892, A33350, S67450, A26106, S96211, P49061, P30120, AAC50729, P25785, AAD28252, P16368, AAB35920, S38624, AAF21942, AF004664, AAA21815, P26652, P79121, P39876, AAD26150, CAB51854, P48032, AF042493, NP_003247, P81556). Hatched and open rectangles represent the signal peptide and the mature protein respectively. Cysteine residues represented by black ovals are conserved in all vertebrate TIMPs and recovered in *C. gigas* and *D. melanogaster* whereas boxed cysteines correspond to additional residues only present in *C. gigas* and *D. melanogaster* TIMPs. Numbers between cysteine residues indicate the distance between amino acids, if two numbers are indicated they correspond to the maximum and minimum spacing observed in the 28 vertebrate sequences analyzed. The disulfide bonding pattern is represented by the lines under the vertebrate TIMP drawing. B: Amino acid sequence comparison of the mature TIMP proteins from human (*Hs*-1–4), *C. gigas* (*Cg*) and *D. melanogaster* (*Dm*). Cysteine residues are written in white on black background. Amino acids conserved in all vertebrate TIMPs (in addition to cysteine residues) are shaded. Gaps introduced to improve the alignment are shown as dots; numbers refer to total amino acid of each protein.

identity) than to *D. melanogaster* TIMP (22% identity), the only other invertebrate TIMP described to date [10]. *Cg*-TIMP presents the organization of all TIMP family members, that is to say a signal peptide with a potential cleavage site upstream of the motif Cys-X-Cys, and a mature protein having a calculated molecular mass of 23 kDa, which is in close agreement with the molecular mass range of other TIMPs (20–23 kDa) (Fig. 1A). Moreover, 12 of the 14 cysteine residues present in the mature *Cg*-TIMP are found in the same position as in vertebrate proteins. The spacing between cysteine residues is well conserved especially in the amino-terminal part of the protein (Fig. 1A). This indicates that the six disulfide bridges may be conserved, whereas the two additional cysteines may form an additional disulfide bond in the carboxy-terminal part of the protein.

The only other TIMP gene known in invertebrates has been characterized in *D. melanogaster* [10]. The authors isolated a 1380-bp cDNA containing an ORF of 210 codons. Sequence analysis revealed that the *D. melanogaster* protein possesses a signal peptide of 27 amino acids which may be cleaved off upstream of the motif Cys-X-Cys to produce a mature protein containing 12 cysteine residues. According to the sequence alignment published by Pohar and collaborators [10], the *D. melanogaster* TIMP could be divided into two domains, like vertebrate TIMPs, except that the N-terminal domain contains only two disulfide bonds whereas the C-terminal domain contains four disulfide bonds (Fig. 1A).

Sequence alignment of the 28 TIMP proteins from vertebrates, available in protein data banks, indicates that only 35 amino acid residues are conserved among all the proteins analyzed. These 35 amino acids are represented in Fig. 2B (12 cysteines and 23 shaded amino acids). Despite this weak

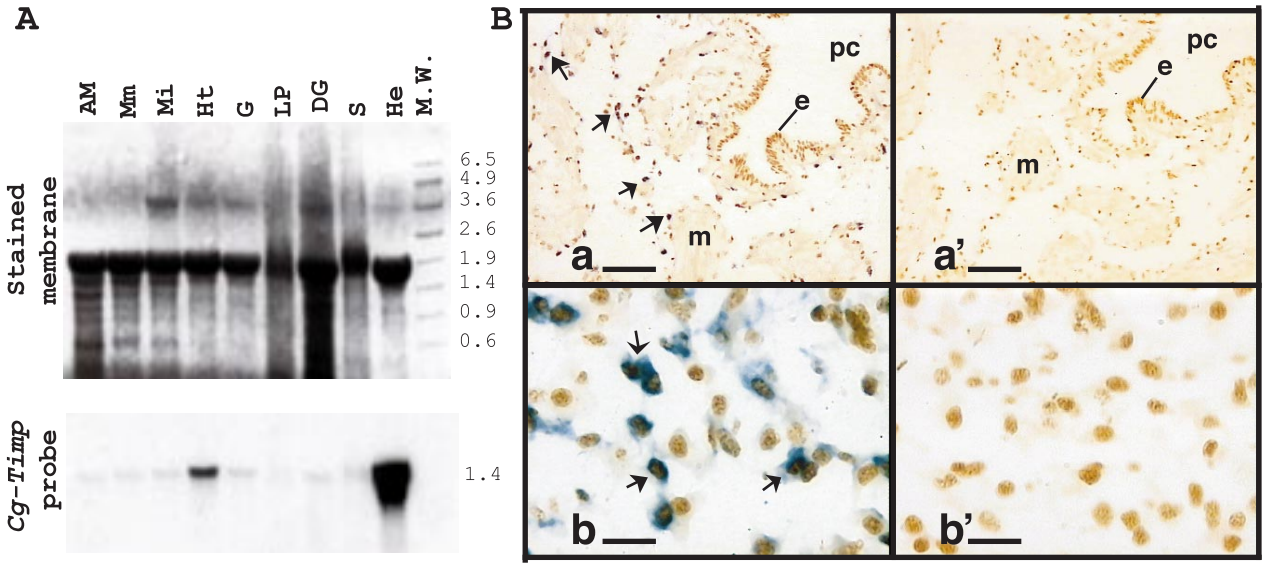

Fig. 2. *Cg-TIMP* RNAs are only expressed in oyster hemocytes. A: Northern blot analysis of *Cg-TIMP* gene expression using total cellular RNA isolated from adductor muscle (AM), mantle margin (Mm), mantle inner surface (Mi), heart (Ht), gills (G), labial palps (LP), digestive gland (DG), stomach (S) and hemocytes (He). Blot containing 10 μg of total RNA per lane was stained by methylene blue to visualize total RNA (upper panel), then decolored and hybridized with radiolabeled *Timp* probe (lower panel). B: Detection, by in situ hybridization, of *Cg-TIMP* mRNAs in hemocytes located in heart tissues. Paraffin-embedded sections of oyster hearts were hybridized with antisense (a, b) or sense (a′, b′) single strand cDNA probes labeled with digoxigenin and revealed using alkaline phosphatase-conjugated antibodies. In panel a, labeling was observed on round cells (arrows) trapped in heart sinus or attached to myocardium (m). At a higher magnitude (b), these cells were identified as hemocytes. Note that not all hemocytes are labeled. pc, pericardial coelom; e, epicardium; m, myocardium. Bars: 20 μm (a and a′); 100 μm (b and b′).

identity between the TIMP proteins, almost all of them are able to inhibit most of the MMPs described so far. This observation indicates that the inhibitor activity of TIMPs depends more on the tertiary structure of the protein than on its amino acid composition. It is interesting to note that 27 and 21 of the 35 residues conserved in vertebrate TIMPs were also conserved in *C. gigas* and *D. melanogaster* TIMPs respectively (Fig. 1B).

All these homologies strongly suggest that the cDNAs we isolated in oyster encode an inhibitor of metalloproteinases. Nevertheless *Cg*-TIMPs have at least two particularities: firstly, the presence of two additional cysteines in their carboxy-terminal part which could be a characteristic of invertebrate TIMPs as suggested by the presence of these cysteines in *D. melanogaster* TIMP; secondly, the isoelectric point of *Cg*-TIMPs was very acid (5.6) while in *D. melanogaster* or vertebrate TIMPs the isoelectric point is neutral or basic (between 6.8 and 9.7).

3.3. Tissue expression of Cg-TIMP

In vertebrates, TIMPs are expressed by a variety of cell types and are present in most tissues and body fluids. To investigate tissue expression of *Cg*-TIMP, Northern blot analyses were carried out using total RNA from adductor muscle, mantle, heart, gills, labial palps, digestive gland, stomach and hemocytes. The Northern blot showed a strong hybridization of the *Cg-TIMP* probe with hemocytes (Fig. 2A). The size of these RNAs was estimated to be 1.4 kb. Faint hybridization signals were obtained with the RNAs extracted from all the other tissues analyzed and a relatively strong signal was observed with the RNAs extracted from the heart. Since organs of bivalve mollusks are bathed in hemolymph, hemocytes are free to circulate through the body cavity. We assumed that the hybridization observed resulted in the presence of infiltrating hemocytes in tissues used for RNA extraction.

In order to verify this hypothesis we performed in situ hybridization. Heart sections of adult oysters were probed with Dig-labeled sense or antisense single strand DNA probes. No signals were observed in sections hybridized with *Cg-TIMP* sense probes (Fig. 2B, a′,b′). On the other hand, *Cg-TIMP* mRNAs were detected in round cells located in heart sinus or attached to myocardium and which have been identified as hemocytes trapped in heart sinus (Fig. 2B, a,b). Moreover, we observed that only some hemocytes were labeled, suggesting that *Cg-TIMP* was only expressed in certain types of hemocytes. *Cg-TIMP* mRNAs were also observed in some infiltrating hemocytes present in gill and digestive gland (data not shown).

3.4. Cg-TIMP expression was induced by shell damage

In vertebrates it has been shown that MMPs and TIMPs are involved in wound healing [13]. To investigate the possible role of *Cg*-TIMP in such phenomena we examined, by Northern blot, the expression pattern of *Cg-TIMP* in hemocytes extracted from oysters at different times after shell damage. We observed that the concentration of *Cg-TIMP* mRNAs started to increase in circulating hemocytes at 3–6 h after shell damage and reached a peak of accumulation at around 9 h (Fig. 3A). Then, the concentration of *Cg-TIMP* mRNAs decreased progressively to come back to the basal level 72–96 h after shell damage (data not shown). RNAs of lower molecular mass (around 1100 b) are also present but it is unclear whether these are functional mRNAs or breakdown products of the larger form.

Northern blot signals were quantified using the Molecular Dynamics Storm system for each time after shell damage and

Cg-TIMP mRNA expression was normalized according to the level of rRNAs and expressed as a function of the signal obtained for undamaged oysters (Fig. 3B). In this experiment we observed that 9 h after shell damage the concentration of *Cg-TIMP* mRNAs was increased by a factor 4.5.

3.5. *Cg-TIMP* expression was induced by bacterial challenge

In order to investigate the effect of bacterial challenge on *Cg-TIMP* RNA accumulation, Northern blot analyses were carried out using total RNAs extracted from hemocytes of challenged and unchallenged oysters. The choice of the four *Vibrio* species was justified by the pathogenicity of these strains for bivalve mollusks [14–16]. Eight batches of 10 oysters were used, three of them as controls and the others where subjected to bacterial challenge as described in Section 2. Filing the shell induced an accumulation of *Cg-TIMP* RNAs compared to untreated oysters (Fig. 4, lanes C and F). This increase was probably due to the damage done to the shell during the filing. Injections of sterile seawater into the adductor muscle did not significantly increase accumulation of *Cg-TIMP* RNAs compared to filed oysters (Fig. 4, lanes F and S). Conversely, we observed a dose-dependent accumulation of *Cg-TIMP* RNAs 12 h after bacterial injection, with a maximum when 10^8 bacteria were injected. The observation that the *Cg-TIMP* RNA accumulation was weaker for 10^{10} bacteria could be explained by the fact that most of the oysters infected with 10^{10} bacteria were moribund 12 h post infection (observed in three independent experiments).

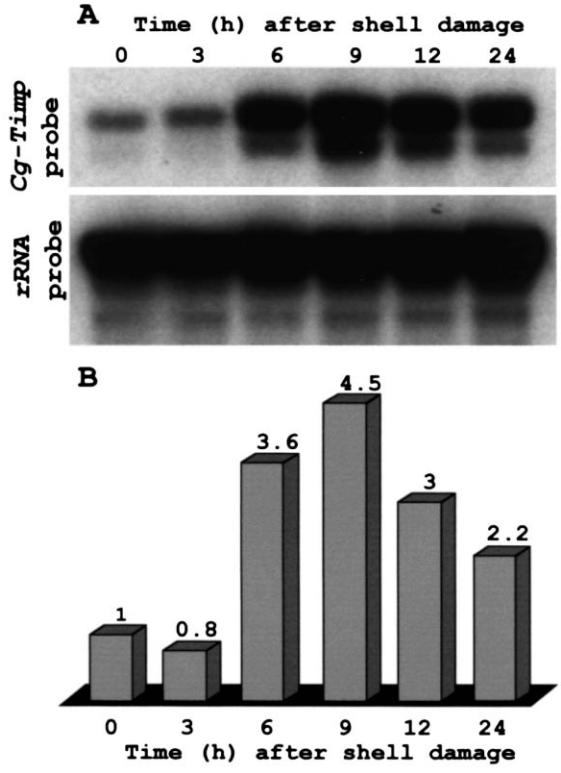

Fig. 3. Accumulation of *Cg-TIMP* transcripts was induced by shell damage. Northern blot analysis of *Cg-TIMP* gene expression using total RNA (8 μg per lane) extracted from hemocytes withdrawn from oysters subjected to shell damage. A: The membrane was hybridized with *Cg-TIMP* cDNA probe and then with a ribosomal DNA probe to check RNA loading. B: Signals obtained with the two probes were quantified using the Molecular Dynamics Storm system and *Cg-TIMP* mRNA expression was normalized according to the level of rRNAs and expressed as a function of the signal obtained for undamaged oysters.

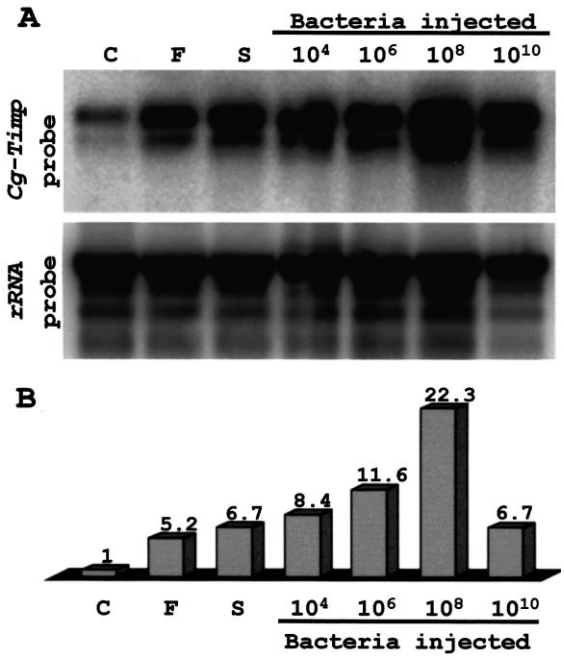

Fig. 4. Accumulation of *Cg-TIMP* transcripts was induced by bacterial challenge. Northern blot analysis of *Cg-TIMP* gene expression using total RNA (8 μg per lane) extracted from hemocytes withdrawn from oysters subjected to different treatments. Seven batches of 10 oysters were used for this experiment. Oysters from the first batch were untreated (C). For the second batch, a small notch was carved in the dorsal side of the oyster shell near the adductor muscle (F). The oysters of the five other batches were treated as the second one except that 100 μl of sterile seawater (S) or 100 μl of bacterial mixture at different concentrations (10^4, 10^6, 10^8 or 10^{10} bacteria) was injected into the adductor muscle. Twelve hours later, hemocytes were collected, RNAs extracted and analyzed by Northern blot. A: The membrane was hybridized with *Cg-TIMP* cDNA probe and then with a ribosomal DNA probe to control RNA loading. B: Signals obtained with the two probes were quantified using the Molecular Dynamics Storm system and *Cg-TIMP* mRNA expression was normalized according to the level of rRNAs and expressed as a function of the signal obtained for unchallenged oysters.

Cg-TIMP RNA accumulation presents almost the same kinetics when oyster are subjected to shell damage or to bacterial challenge. Indeed, the accumulation started 3–6 h after infection, reached a maximum around 9 h and decreased progressively to come back to the steady state 3–4 days after infection (data not shown). These results show that bacterial challenges also induced *Cg-TIMP* mRNA accumulation.

4. Discussion

We report here the isolation of oyster cDNAs encoding a 221-amino acid protein which presents the characteristics of the vertebrate TIMP protein family. That is to say, firstly, a short signal peptide which is presumably cleaved upstream of the consensus sequence Cys-X-Cys to produce the mature protein. Secondly, the location of 12 Cys residues that form, in vertebrates, intrachain disulfide bonds and fold the protein into two domains. And thirdly, the conservation of 27 of the 35 amino acid residues which are present in all the vertebrate

TIMP sequences described so far. From these homologies we suggest that *Cg*-TIMP, like vertebrate TIMPs, must be composed of two domains: an amino-terminal domain of 129 amino acids, stabilized by three disulfide bonds, which may be, as in vertebrates, sufficient for both the binding and the inhibition of MMPs [17,18], and a small carboxy-terminal domain of 70 amino acids, stabilized by four disulfide bonds, involved in specific functions. In vertebrates, it has been proposed that the C-terminal domain is involved in MMP or ECM binding [5,19].

Compared to vertebrate TIMPs, *Cg*-TIMP contains an additional pair of cysteine residues in the carboxy-terminal domain of the protein. According to Pohar and coworkers [10], it could be a common characteristic of invertebrate TIMPs. The other originality of *Cg*-TIMP is the very acid isoelectric point whereas other TIMPs from vertebrates as well as from *D. melanogaster* are neutral or basic proteins. The physiological significance of these particularities remains to be elucidated.

Using Northern blot analysis and in situ hybridization we demonstrated that *Cg-TIMP* was only expressed in hemocytes. Mollusk hemocytes are phagocytic cells involved in many functions, including wound healing, shell repair, as well as internal defense [20]. Interestingly, in vertebrates some TIMPs (1 and 2) are also expressed in mononuclear phagocytic cells [21]. Moreover, we showed that *Cg-TIMP* mRNAs were strongly accumulated in oysters subjected to shell damage or bacterial challenge. These results strongly suggest the involvement of *Cg*-TIMP in wound healing as well as in defense mechanisms.

This hypothesis is supported by studies conducted on two oyster species indicating the presence of protease inhibitors in the plasma of *Crassostrea* spp., which may have an impact upon host defense mechanisms [22]. Furthermore, studies of the eastern oyster pathogen *Perkinsus marinus* showed that this protozoan produces extracellular proteases (ECPs). It has been suggested that ECPs compromise oyster immune response [23] and enhance the protozoan's ability to propagate within the host [24].

Metalloproteinases have indeed been reported to play a predominant role in pathogen invasion. Two ways can be distinguished. In the first one, metalloproteinases are produced by the pathogen and are key factors determining the virulence of microorganisms. For instance, enhancin, a metalloproteinase associated with granulosis viruses, is reported to promote the fusion of the virus particle with epithelial cell membrane in susceptible host insects. Thus enhancin facilitates virus infection [25]. Another example comes from the identification of a zinc metalloproteinase associated with the fish pathogen *Vibrio anguillarum* invasion in host fishes [26]. The second pathway was suggested by Okamoto and coworkers [27], who showed that bacterial proteinases may participate in ECM destruction by activating the latent form of host MMP.

The most advanced work relevant to the role of inhibitors of metalloproteinases in invertebrate immune response was realized on the wax moth larva, *Galleria mellonella*. The authors characterized an inhibitor of metalloproteinase which is an inducible protein, released during the humoral immune response and able to protect the insect from exogenous metalloproteinases of pathogen origin [28]. Moreover, injection of metalloproteinase at a sublethal concentration into untreated *G. mellonella* larvae mediates acquired resistance against a subsequently injected lethal concentration of this metalloproteinase. The injection of metalloproteinase also stimulates the humoral immune response accompanied by the synthesis of an inducible metalloproteinase inhibitor released into the hemolymph [29]. These findings support the idea that a proteolytic system of tissue remodeling is conserved between vertebrates and invertebrates and further suggest that this system could be used in invertebrates, as in vertebrates [30,31], in other functions such as defense mechanisms.

To sum up, we have characterized the first mollusk TIMP, the expression of which is tissue-specific and inducible by wound healing and bacterial challenge. Current investigations should focus on the isolation, purification, and biochemical characterization of *Cg*-TIMP. To better understand involvement of *Cg*-TIMP in oyster defense mechanisms it would be interesting to follow *Cg*-TIMP expression during *C. gigas* infections as well as investigate the presence of *Cg*-TIMP in oyster species presenting different degrees of susceptibility to parasites.

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