FEBS Letters 580 (2006) 1846–1852

The chitin synthase involved in marine bivalve mollusk shell formation contains a myosin domain

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Received 20 January 2006; revised 16 February 2006; accepted 16 February 2006

Available online 24 February 2006

Edited by Felix Wieland

Abstract Chitin is a key component in mollusk nacre formation. However, the enzyme complex responsible for chitin deposition in the mollusk shell remained unknown. We cloned and characterized the chitin synthase of the marine bivalve mollusk *Atrina rigida*. We present here the first chitin synthase sequence from invertebrates containing an unconventional myosin motor head domain. We further show that a homologous gene for chitin synthase is expressed in the shell forming tissue of larval *Mytilus galloprovincialis* even in early embryonic stages. The new data presented here are the first clear-cut indication for a functional role of cytoskeletal forces in the precisely controlled mineral deposition process of mollusk shell biogenesis.

Keywords: Biomineralization; Mollusk shell; Chitin biosynthesis; Mollusk larvae in situ hybridization

1. Introduction

The mollusk shell and nacre are among the most fascinating biomaterials [1]. Recently, new concepts have arisen for understanding the mechanisms of their formation and the interaction of organic macromolecular components with the various inorganic phases [2–4]. Chitin is well-known to be a key component in mollusk shell and nacre formation [5–10]. Chitin forms the framework for other macromolecular components that obviously guide the mineralization process, even in the regime of crystal polymorphism [11]. It has been shown that certain crystallographic axes of calcium carbonate or apatite are aligned with chitin fibers in extracellular composite biominerals [12–14]. Despite the importance of the chitin, no attention was payed so far to the enzyme complex responsible for chitin formation in the mollusk shell. Chitin synthesis has been primarily studied in fungi, nematodes, and arthropods [15–19]. The first enzymatic synthesis of chitin was performed using cell-free extracts of *Neurospora crassa* [20]. Only recently, light was shed on the complexity of chitin synthesis and its regulatory network based on the yeast genome data [21]. The first chitin synthases from invertebrates were discovered in arthropods [22], the major source for commercially produced chitin. No relationship with motor proteins, like in the case of cellulose synthesis [23–25], was found. A functional role for cytoskeletal interaction and chitin deposition was found for the first time in fungi [26–29].

Here, we present the complete cDNA of the first chitin synthase (CS, CHS) sequence from invertebrates (the calcium carbonate crystallizing *Atrina rigida*) containing a myosin motor head domain. The CS domain of this enzyme (*Ar*-CS) shows highest similarity to *Tc*CHS1 and *Tc*CHS2 of the insect *Tribolium castaneum* [30], especially with respect to transmembrane architecture. We further show that a homologous gene (*Mg*-CS1) is specifically expressed in the shell forming tissue of larval *Mytilus galloprovincialis* as early as in the veliger stage. Therefore, we propose that the mollusk chitin synthase participates in the complex network of regulation of mollusk shell formation, which likely includes interactions of the unconventional myosin head domain with cytoskeletal components.

2. Materials and methods

All procedures were performed according to established protocols of molecular cloning [31], or according to the manufacturers recommendations. Polymerase chain reaction (PCR) products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) prior to sequencing (MWG, Ebersberg, Germany). The following computer resources were used: BCM [32]; BioEdit (Tom Hall, NCSU Raleigh, NC, USA); Boxshade (www.ch.EMBnet.org); CDD [33] ; ExPASy [34]; GCG (WI, USA); NCBI-BLAST [35]; NetNGlyc (www.cbs.dtu.dk); pDraw (AcaClone Software); TMHMM [36].

2.1. cDNA library

The cDNA library made from mantle tissue of *A. rigida* in the adult stage was a generous gift of Weiner, Tuross, and Addadi, and is described in detail elsewhere [37]. The cDNA was cloned into a λZAPII vector system (Stratagene, Amsterdam, The Netherlands) with an insert size of >0.4 kb, yielding 12.3×10^6 primary plaques. *Escherichia coli* XL1-Blue MRF' cells were used for processing of the *Ar*-λZAPII library.

2.2. PCR screening using degenerate primers

Purified *Ar*-λZAPII DNA (100 ng/μl) was used for PCR screening of the library with the Expand High Fidelity system (Roche, Mannheim, Germany) in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using standard PCR programs (4 min 94 °C, 30× [1 min 94 °C, 2 min $T_A \pm 5$ °C, 3 min 72 °C], 7 min 72 °C). T_A represents the optimum annealing temperature of the degenerate primers (pf0724_1, 5′-AATTTRGGIGCIGCITGTGGIAGRATWCATCC; pr0724_5, 5′-

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Abbreviations: CS,CHS, chitin synthase; WISH, whole mount fluorescence in situ hybridization; ORF, open reading frame; PBT, 0.1% Tween 20 in PBS; PCR, polymerase chain reaction; T_A, annealing temperature; *Ar*, *Atrina rigida*; *En*, *Aspergillus* (*Emericella*) *nidulans*; *Lc*, *Lucilia cuprina*; *Mg*, *Mytilus galloprovincialis*; *Po*, *Pyricularia oryzae*; *Tc*, *Tribolium castaneum*

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.044

CAICGRTCTTCICCTTGITCRTAYTG). Two independent overlapping clones yielded a 257 bp fragment of the *Ar*-CS1 containing the GHWLQKA motif of *Lc*-CS1 [22].

2.3. cDNA library screening

The 257 bp fragment of *Ar*-CS1 was used for PCR labeling of a DIG-dUTP probe for screening of the cDNA library with chemoluminescent CSPD signal detection (Roche). Two overlapping clones yielded 6119 bp of the complete *Ar*-CS1 cDNA (7882 bp).

2.4. Nested PCR for 5′-terminal sequence determination

Ar-λZAPII mass excision DNA (100 ng/μl) was used for nested PCR screening (2 min 94 °C, 35× [15 s 94 °C, 15 s $T_A \pm 4$ °C, 1–4 min 72 °C], 7 min 72 °C). Both, the first reverse primer and the respective nested primer were combined with temperature-adjusted T7 or T3 primers. Cloned PCR products were screened by colony PCR using an *Ar*-CS fragment specific forward primer and the nested reverse primer. Sequence overlaps of particular *Ar*-CS fragments were >220 nucleotides. 15 different overlapping clones yielded 2574 bp of the complete *Ar*-CS1 cDNA (7882 bp).

2.5. Genomic DNA isolation

Adult *M. galloprovincialis* were maintained as described [38]. Genomic DNA (gDNA) was isolated from homogenized, liquid nitrogen frozen tissue [39].

2.6. Cloning of Mg-gDNA sequence fragments

Partially degenerate primers were designed based on the *Ar*-CS1 cDNA sequence. Gradient hot-start (75 °C) touchdown PCR reactions (2 min 94 °C, 29× [15 s 94 °C, 15 s $T_A \pm 4$ °C – 0.4 °C/cycle, 1 min 72 °C], 5× [15 s 94 °C, 15 s $T_A \pm 4$ °C – 11.6 °C, 1 min 72 °C], 7 min 72 °C) were performed using 1–5 μg of *Mg*-gDNA as a template. Specific primers were used to generate PCR fragments of the partial *Mg*-CS1 of desirable length for in vitro transcription. A *Mg*-actin cDNA fragment of comparable length was cloned and used as a control.

2.7. FITC-labeled RNA probes

The *Spe*I or *Nco*I digested pGEM-T Easy plasmids that contained exon fragments of *Mg*-CS1 and *Mg*-actin were used as templates for the in vitro transcription of FITC-labeled RNA probes [40]. Reactions were performed in the presence of RNAguard (Amersham, Freiburg, Germany), using the Fluorescein RNA labeling mix (Roche) and T7 or Sp6 RNA polymerases, respectively.

2.8. Whole mount in situ hybridization of M. galloprovincialis larvae

It was not possible to obtain larvae of *Atrina rigida* for functional studies. Larvae of the closely related species *M. galloprovincialis* were obtained as described elsewhere [38]. The procedure for whole mount in situ hybridization (WISH) of marine bivalve larvae of various age was developed based on *Drosophila* protocols (S. Schneuwly, Universität Regensburg, personal communication). As recommended by B. Ruthensteiner (Zoologische Staatssammlung München, personal communication), larvae were concentrated in a nylon mesh, washed several times with filtered artificial seawater and transferred into precooled embryo dishes for slow reduction of motility. Artificial sea water was replaced by 3.57% $MgCl_2$ solution in order to avoid the closure of the valves during the fixation procedure [41]. Main fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3) was added in portions, and the solution was exchanged quickly several times. The fixed larvae were processed immediately or stored for several days at 4 °C in main fixative. All samples were irradiated with UV-light (350–400 nm) for 1 h prior to probe hybridization in order to reduce autofluorescence of the fixed tissue. After washing (0.1% Tween 20 in PBS, PBT), dehydration (100% methanol), and rehydration (methanol/PBT), a 4 min proteinaseK digest was performed. Specimens were transferred into hybridization solution (50% formamide, 100 μg/ml fish sperm DNA, 150 μg/ml tRNA, 1 mg/ml heparin, 5× PBS, 40 U/ml RNAse inhibitor) and preincubated for 1 h at 50 °C prior to hybridization with 3 ng/μl RNA probe for 48 h at 50 °C. Specimens were washed several times with hybridization solution and/or PBT at 55 °C, and subjected to confocal laser scanning microscopy. The cell nuclei of the specimens were stained using 2.5 μg/ml propidium iodide in PBT for 10 min at 20 °C and subsequent washing with PBT for 20 min. Signal detection was performed using a LSM510 Confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3Oil Ph3 objective. FITC fluorescence (excitation 488, Ar laser) was detected in single channel PMT mode using a band-pass 505–530 nm emission filter. Propidium iodide fluorescence was detected in a separate channel using a 560–615 nm band-pass filter. Imaging parameters were optimized for working in the range of significant signals. Data were routinely collected in various focal planes. The Zeiss LSM5 imaging software was used for image processing.

3. Results

The full-length cDNA sequence of the chitin synthase of *A. rigida*, a marine bivalve model organism for the biomineralization of nacre, was cloned (*Ar*-CS1, GenBank Accession No. DQ081727) from an *Ar*-cDNA library using a 257 bp degenerate primer PCR fragment coding for the GHWLQKA motif characteristic for invertebrate metazoan chitin synthases [22]. A 6119 bp fragment corresponding to 1754 amino acids and 857 bp 3′-UTR of the *Ar*-CS1 was obtained by conventional screening. A nested primer PCR approach was chosen in order to verify the start codon of the obtained sequence fragment. It was deduced from 15 independent overlapping clones that a continuation of another 1763 bp occured in the 5′ direction of the cDNA sequence. The final sequence revealed a start codon at position 168. The N-terminus (732 amino acid residues) of the translated 6858 bp open reading frame (ORF) sequence corresponding to 2286 amino acid residues (Fig. 1) showed significant similarity (up to 39% identical amino acid residues, Expect = e^{-137}) to conserved N-terminal domains including the ATP binding sites of the myosin protein family [33], whereas the C-terminus (1554 amino acid residues) of the *Ar*-CS1 was homologous (up to 36% identical amino acid residues, Expect = 0.0) to insect chitin synthases [19,22,30]. Multiple sequence alignments (Fig. 2) showed that apart from two conserved motifs, there was no overall homology to *En*-CsmA or *Po*-Csm1, two fungal chitin synthases with myosin domains [26,27]. According to sequence alignments, the presence of a myosin motor head domain with all sequence features necessary for its function as a force transducing element is therefore currently a unique finding among all the chitin synthases with complex transmembrane architectures.

The molecular weight of the *Ar*-CS1 protein was predicted to be 264 kDa, including a 84 kDa myosin head. A low complexity region was identified to separate the myosin head and the CS domain of the *Ar*-CS1. The transmembrane architecture of the CS domain of *Ar*-CS1 showed very high similarity with the *Lc*-CS [22], as calculated by TMHMM [36]. Fig. 3 shows a preliminary model of the *Ar*-CS1 protein with sequence features highlighted as in Fig. 1. According to this model, the myosin head domain is located intracellularly. The characteristic NQRRRW motif of the *Ar*-CS1 active site is also located intracellularly. Three potential glycosylation sites (marked by an asterisk in Fig. 1) indicated extracellular posttranslational modifications. Three low complexity-regions were located in two C-terminal, extracellular domains of 20.4 and 22.9 kDa, respectively. The 22.9 kDa domain did not

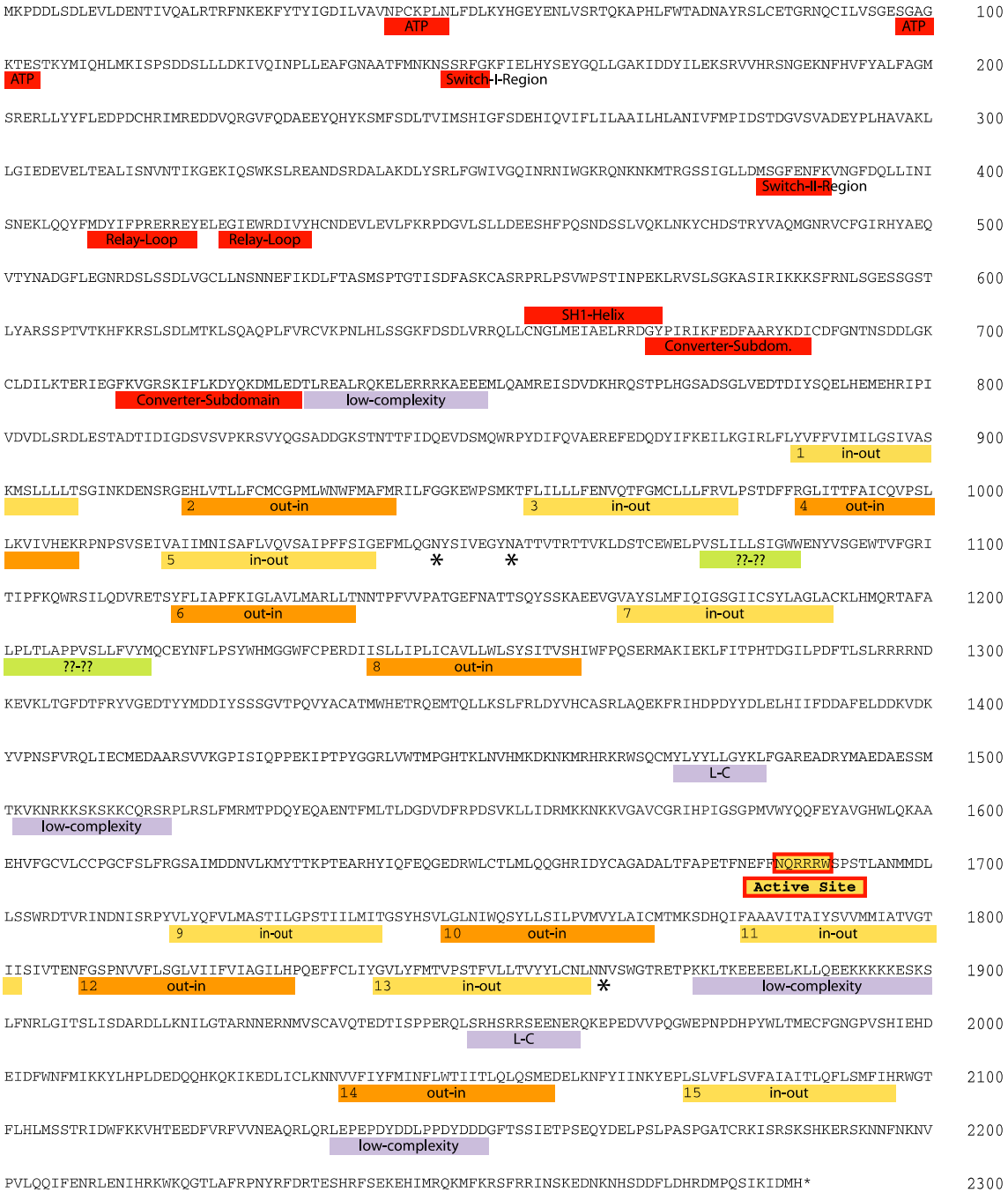

Fig. 1. Primary structure of the translated *Ar*-CS1 ORF. Conserved motifs of the myosin head domain, the orientation of predicted transmembrane helices, the catalytic site (NQRRRW) of the chitin synthase domain, and low-complexity regions are highlighted. Regions indicated by question marks refer to predicted transmembrane helices beyond the significance threshold. (*) Putative glycosylation sites.

show any significant similarity to current protein database entries.

A 1626 bp fragment of a gene homologous to the *Ar*-CS1 was identified and cloned from *M. galloprovincialis* gDNA (*Mg*-CS1, GenBank Accession No. DQ358105). Fig. 4 shows a pairwise amino acid alignment of the CS domain of *Ar*-CS1 with the partial ORF deduced from the *Mg*-CS1 fragment. The chitin synthases of these two bivalve species are almost completely conserved with respect to the catalytic domain and certain highly charged, extracellular C-terminal low complexity-motifs.

We studied the expression of the *Mg*-CS1 gene in *M. galloprovincialis* larvae by WISH using RNA probes. The confocal images presented in Fig. 5 show that mRNA for *Mg*-CS1 is present in the early, and as well in late veliger larval stages. The probe was located only in cells that were in close contact to the larval shell. In contrast, the *Mg*-actin probe displayed signals of significant intensity in all cells that contained a propidium iodide stained cell nucleus (data not shown). False positive signals were ruled out by performing the procedure with non-complementary sense strand probes.

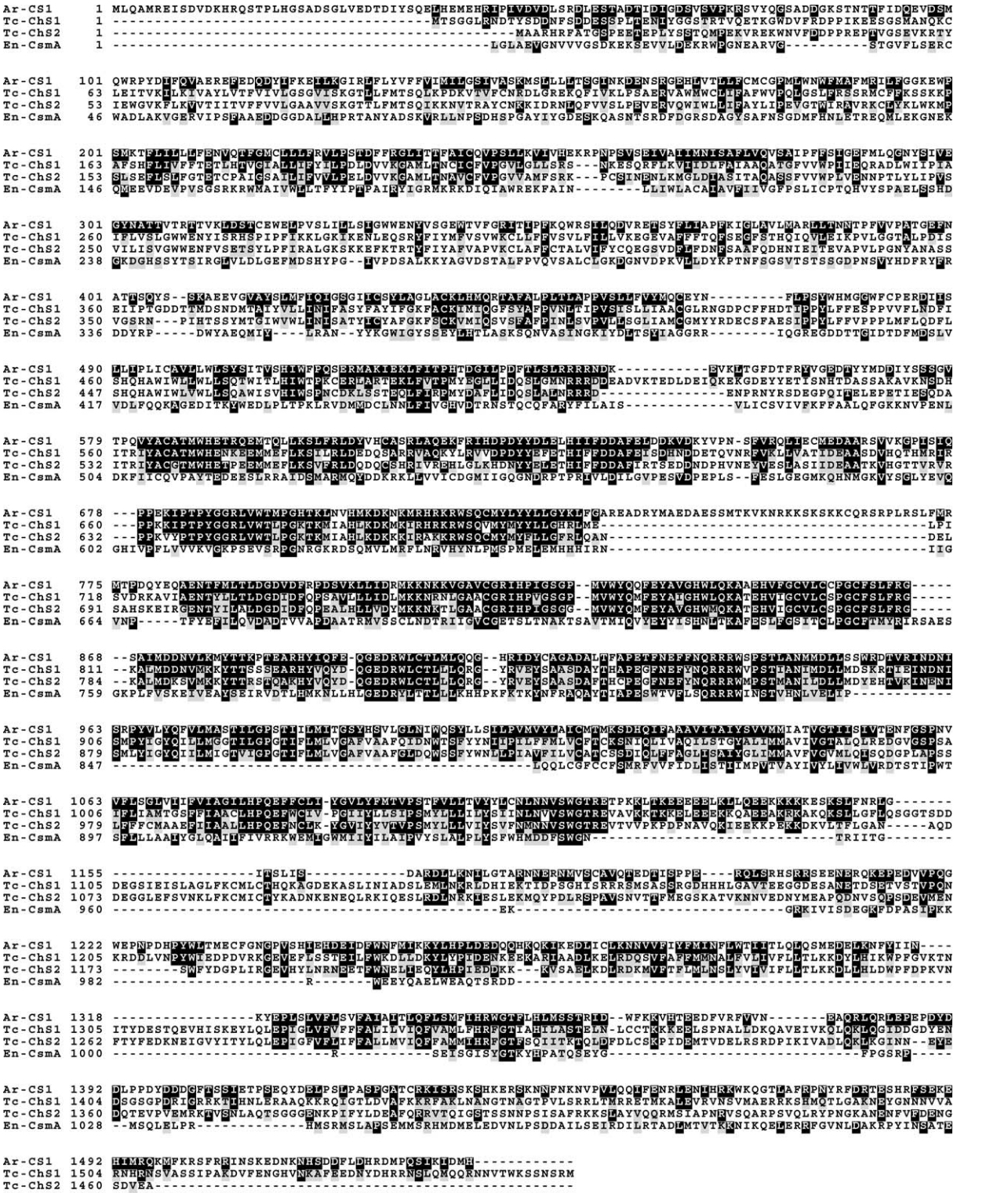

Fig. 2. Alignment of the chitin synthase domain of *Ar*-CS1 with *Tc*-CHS1, *Tc*-CHS2, and *En*-csmA. Note that the homology between the invertebrate chitin synthases and the fungal *En*-csmA is restricted to the PGCF, GEDR, QRRRW, and SWG motifs.

4. Discussion

Chitin is proposed to play a major role in calcium carbonate biomineral formation of adult and larval mollusks [4,38]. We cloned the chitin synthase of the bivalve model organism *A. rigida* to generate the molecular base for understanding chitin formation in the context of mollusk shell biomineralization.

Chitin synthases belong to the group of glycosyltransferases and comprise a transmembrane protein family with a minimum

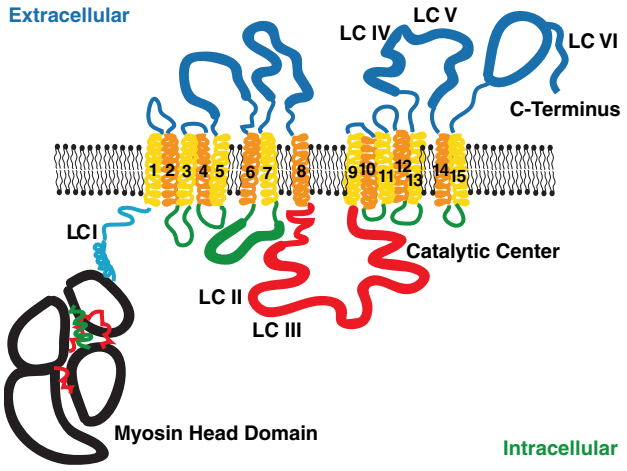

Fig. 3. Current model of the *Ar*-CS1 based on predicted structural elements as highlighted in Fig. 1.

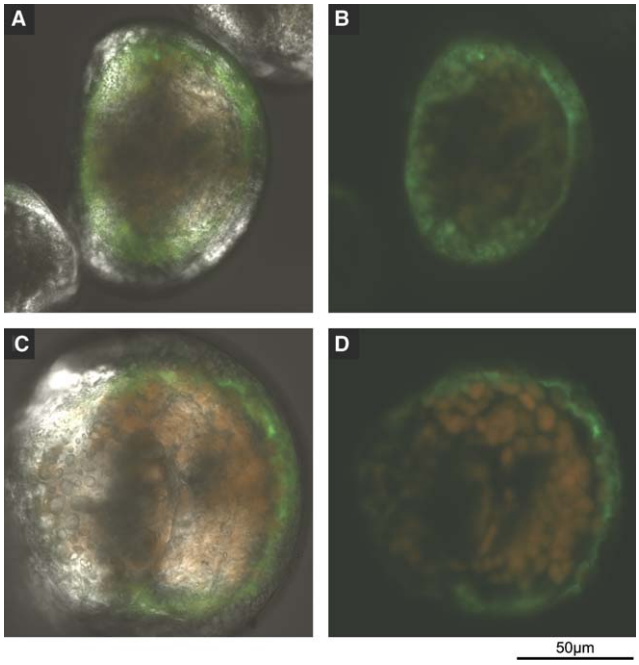

Fig. 5. WISH of *Mytilus galloprovincialis* larvae with *Mg*-CS1 specific RNA probes. Note that FITC signals (green) are located only in tissue sections close to the shell, whereas cell nuclei staining (red) indicates a well-preserved organism. The focal planes shown here were adjusted close to the shell edges, but are representative for all focal planes in each specimen. (A) DIC and fluorescence 3-channel-overlay image of a 6 day old specimen. (B) Fluorescence 2-channel-overlay image of A. (C) DIC and fluorescence 3-channel-overlay image of a 15 day old specimen. (D) Fluorescence 2-channel-overlay image of C.

of three transmembrane helices. Only the intracellular catalytic site is highly conserved, whereas N- and C-terminal regions might vary even in isoforms of one species. The mechanism of chitin translocation through the cytoplasmic membrane is still not fully understood.

Characteristic extracellular domains of the mollusk chitin synthases, *Ar*-CS1 and *Mg*-CS1, contain low complexity-motifs with a high number of acidic or basic amino acid residues. This feature is characteristic for various mollusk shell proteins [42]. The motifs indeed raise questions on their functionality in the interplay between chitin synthesis and protein mineral-interaction. The fact that chitin synthase gene expression takes place in shell forming tissue sections of the larvae indeed supports our previous hypothesis that chitin plays a major role in larval shell formation [38].

In terms of conserved motifs and transmembrane architecture, the CS domain of *Ar*-CS1 does not differ significantly from the insect chitin synthases [19,22,30]. A major difference is the presence of a myosin motor head domain at the intracellular N-terminus that contains all conserved motifs necessary for a myosin head to act as a force transducer. The motor domain of *Ar*-CS1 is a strong hint for a regulatory function of the actin cytoskeleton in chitin deposition. Among the possible functions are transport, localization, activation, and regulated pattern formation of CS complexes within the cytoplasmic membranes. The tightly packed microvilli surface of mollusk mantle epithelial cells [43,44] is supportive for such a scenario. This in turn might not only guide the assembly process of chitin in all dimensions, but furthermore suggests that the mineralization process might be actively influenced by the mantle epithelial cells, for example by creating force fields via the cytoskeleton.

The structural organization of the chitin synthesizing complex in mollusks, with its direct link to the cytoskeleton as presented here, opens the stage for understanding the mantle–shell relationship as a dynamic transmembrane regulation – in addition to a pure secretory function of the mantle epithelium prior to self-assembly of the calcifying extracellular matrix. There-

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Ar    1 AEHVFGCVLCCPGCFSLFRGSAIMDDNVLKMYTTKPTEARHYIQFEQGEDRWLCTLMLQQGHRIDYCAGADALTFAPETF
Mg    1 AEHVFGCVLCCPGCFSLFRGSAVMDDNVLKMYTTPPTEARHYIQFEQGEDRWLCTLMLQQGHRIDYCAGSDALTFAPETF

Ar   81 NEFFNQRRRWSPSTLANMMDLLSSWRDTVRINDNISRPYVLYQFVLMASTILGPSTIILMITGSYHSVLGLNIWQSYLLS
Mg   81 NEFFNQRRRWSPSTLANMMDLLASWRDTVRINDNISRPYMLYQFVLMASTIIAPSTVILMITGSYHSVFKLSIFESYLLS

Ar  161 ILPVMVYLAICMTMKSDHQIFAAAVITAIYSVVMMIATVGTIISIVTENFGSPNVVFLSGLVIIFVIAGILHPQEFFCLI
Mg  161 LLPVVIYLAICLTMKSDIQILAAAVITALYAVIMMIATVGTVISIVTENFGSPNVVFLTGLTTIFLIAGILHPQEFFCLV

Ar  241 YGVLYFMTVPSTFVLLTVYYLCNLNNVSWGTRETPKKLTKEEEEELKLLQEEKKKKKESKSLFNRLGITSLISDARDLLK
Mg  241 YGALYFMVPSTFILLTIFYLCNLNNVSWGTRETPKKLTKEEEEEQERVNEDKKKKKESKSFLNRLGITNLIRDLRDLIK

Ar  321 NILGTARNNERNMVSCAVQTEDTISPPERQLSRHSRRSEENERQKEPEDVVPQGWE
Mg  321 NFLGSRATIEKVSASCQTDENNCLARTVSRNSRNSVQSKDTSMNMNVEDVVPPGRE
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Fig. 4. Pairwise alignment of *Ar*-CS1 and *Mg*-CS1 ORF fragments. The two mollusk chitin synthases are nearly identical with respect to the catalytic domain including a highly charged poly-E/poly-K motif. Note that there is no overall homology towards the C-terminus.

fore, the interfacial cytoplasmic membranes might play a crucial role in mollusk shell biogenesis.

Acknowledgments: We thank M. Felle for assistance with library screening. This work was financially supported by the DFG (WE2629/2-1, I.M.W.) and the Universität Regensburg (M.S.).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.02.044.

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