Amino Acids (2012) 42:2009–2017
DOI 10.1007/s00726-011-0932-0

ORIGINAL ARTICLE

Characterization of MRNP34, a novel methionine-rich nacre protein from the pearl oysters

Benjamin Marie · Caroline Joubert · Corinne Belliard · Alexandre Tayale · Isabelle Zanella-Cléon · Frédéric Marin · Yannick Gueguen · Caroline Montagnani

Received: 19 March 2011 / Accepted: 30 April 2011 / Published online: 18 May 2011

Abstract Nacre of the *Pinctada* pearl oyster shells is composed of 98% $CaCO_3$ and 2% organic matrix. The relationship between the organic matrix and the mechanism of nacre formation currently constitutes the main focus regarding the biomineralization process. In this study, we isolated a new nacre matrix protein in *P. margaritifera* and *P. maxima*, we called *Pmarg*- and *Pmax*-MRNP34 (methionine-rich nacre protein). MRNP34 is a secreted hydrophobic protein, which is remarkably rich in methionine, and which is specifically localised in mineralizing the epithelium cells of the mantle and in the nacre matrix. The structure of this protein is drastically different from those of the other nacre proteins already described. This unusual methionine-rich protein is a new member in the growing list of low complexity domain containing proteins that are associated with biocalcifications. These observations offer new insights to the molecular mechanisms of biomineralization.

Keywords Matrix protein · Methionine-rich · Biomineralization · Mollusc · Nacre · Calcifying mantle

Abbreviations

AA	Amino-acid
AIM	Acid-insoluble matrix
ASM	Acid-soluble matrix
EST	Expressed sequence tag
ISH	In situ hybridization
MRNP	Methionine-rich nacre protein
MS	Mass spectrometry
RLCD	Repeated low complexity domain

B. Marie · C. Joubert · C. Belliard · A. Tayale · Y. Gueguen (✉) · C. Montagnani
Ifremer, LBQP, Centre Océanologique du Pacifique, Taravao, French Polynesia
e-mail: yannick.gueguen@ifremer.fr

B. Marie · F. Marin
UMR 5561, CNRS, Biogéosciences, Université de Bourgogne, Dijon, France

Present Address:
B. Marie (✉)
UMR 7245, CNRS, MCAM, Muséum National d'Histoire Naturelle, Paris, France
e-mail: benjamin.marie@u-bourgogne.fr; bijamarie@yahoo.com

I. Zanella-Cléon
UMR 5086, CNRS, IFR 128 BioSciences, IBCP, Université de Lyon 1, Lyon, France

Present Address:
C. Montagnani
UMR 5119, CNRS, Ifremer, IRD, Ecologie des Systèmes Marins Côtiers, Université Montpellier II, Montpellier, France

Introduction

Nacre, also known as mother-of-pearl, is the most extensively studied non-human organo-mineral biomaterial because of its extremely high fracture toughness (Addadi et al. 2006). Nacre constitutes the lustrous inner layer of various mollusc shells, such as the pearl oyster from the genus *Pinctada* (Bivalvia: Pteriidae). Like other mollusc shell microstructures, nacre is built extracellularly under the cellular control by the calcifying epithelium of the mantle. The nacreous layer exhibits a remarkable regular lamellar microstructure consisting of uniformly thick layers (0.5 μm) of aragonite crystals, which are densely packed with small amount of proteins, glycoproteins and polysaccharides (for review see Addadi et al. 2006). These

mantle-secreted biomacromolecules, which constitute the organic matrix, are believed to control different aspects of the mineral deposition (Lowenstam and Weiner 1989; Mann 2001). Although this organic matrix represents only 1–5% of nacre weight, according to the different species (Addadi et al. 2006), it displays key functions such as the local structuring of the 3D space in an organic framework just prior to the mineral deposition (Sudo et al. 1997; Suzuki et al. 2009), the regulation of the $CaCO_3$ precipitation (Wheeler et al. 1981), the selection of the polymorph—aragonite and/or calcite (Falini et al. 1996)—and the control of the mineral shape. Furthermore at the atomic level, some of these matrix macromolecules exert an effect on the lattice parameters of $CaCO_3$ (Pokroy et al. 2006).

Since the first description of the primary structure of a mollusc shell protein by Miyamoto et al. (1996), five and ten full-length sequences of nacre proteins have been reported from bivalve and gastropod models, respectively (for a recent review see Marin et al. 2008). These proteins are multi-domain and the domain consists of mucin-type (Marin et al. 2000), carbonic anhydrase and GN-rich type (Miyamoto et al. 1996), C- and GS-rich type (Shen et al. 1997), G- and A-rich type (Sudo et al. 1997; Marie et al. 2010a), WAP or Kunitz-like protease inhibitor types (Marie et al. 2010a), and finally, von Willebrand A, chitin-binding and D-rich type (Suzuki et al. 2009). However, although a number of shell proteins have recently been identified (Marin et al. 2008), many remain yet to be detected (Jackson et al. 2010; Joubert et al. 2010). Moreover, the relationship between the components of the organic matrix and the precise molecular mechanism of nacre formation is far from being understood and constitutes nowadays a hot topic. A deepened analysis in the mechanisms of biomineralization requires identification, characterization, and functional analysis of key matrix molecules.

The aim of the present work is to investigate the structure of the nacre matrix in two pearl oyster species that belong to the genus *Pinctada*. Recent works (Jackson et al. 2010; Joubert et al. 2010) have obtained expressed sequence tags (ESTs) from nacre-forming tissues of the pearl oysters *P. margaritifera* and *P. maxima*, two closely related species (Cunha et al. 2011). These works have notably increased the dataset of known mantle-secreted proteins. Here, we have used proteomic methods combined with transcript and protein localization to evidence a new unidentified shell matrix protein of *P. margaritifera* and *P. maxima*. These homologue forms of methionine-rich nacre proteins (MRNP), called *Pmarg*-MRNP34 and *Pmax*-MRNP34 respectively, are novel 34 kDa proteins that are specifically localized in the mineralizing tissues and exclusively in the nacre matrix of these two species.

Materials and Methods

Materials

The adult *P. margaritifera* and *P. maxima* were collected from the Vairao lagoon (Tahiti, French Polynesia) and from the western cost of Australia, respectively. These two Pterioid bivalves exhibit a bilayered shell composed of an external prismatic calcitic layer, and an aragonitic nacreous one. The two layers were separated mechanically. In addition, nacreous pearls were obtained from *P. margaritifera* after a classical 18-month grafting procedure of nucleus and the mantle tissue in the pearl pocket (Cochennec-Laureau et al. 2010). Nucleus was carefully removed from the pearl prior to proteomic analysis.

Biochemical analysis

The acid-soluble matrix (ASM) and the acid-insoluble matrix (AIM) of the nacreous and the prismatic layers were extracted with cold acetic acid (5%) as previously described (Marie et al. 2007). Shell pieces were mechanically crushed and fragments were immersed in 1% (v/v) NaOCl for 24 h to remove superficial contaminants, as well as the periostracum, prior to matrix extraction. The nacre AIM of *P. margaritifera* was denatured and partly solubilized in Leammli sample buffer before being fractionated on a preparative SDS 12% polyacrylamide gel as described by Marin et al. (2001). Eluted fractions containing the purified *Pmarg*-MRNP34 were detected with a polyclonal antibody (Marin et al. 1994), then were tested by SDS-PAGE with Coomassie brillant blue (CBB) staining, before being pooled, thoroughly dialyzed against MilliQ water and freeze-dried.

Prior to the proteomic analysis, the trypsin digestion of the matrices and the purified MRNP34 samples was performed in solution or in gel (Marie et al. 2010b). Mass spectrometry (MS) was performed using a Q-Star XL nanospray quadrupole/time-of-flight tandem mass spectrometer (nanospray-qQ-TOF–MS/MS) (Applied Biosystems, France), coupled to an online nano liquid chromatography system (Ultimate Famos Switchos from Dionex, The Netherlands). 1 µL of samples was loaded into a trap column and then separated at a flow rate of 0.300 µL min^{-1} with a linear gradient of 5–50% acetonitrile in 0.1% formic acid for 120 min. MS data were acquired automatically using Analyst QS 1.1 software (Applied Biosystems). Following a MS survey scan over 400–1,600 *m/z*, MS/MS spectra were sequentially and dynamically acquired for the three most intense peptide molecular ions over 65–2,000 *m/z*. Proteins identification was performed using the Paragon algorithm of the ProteinPilot™ software (Applied Biosystems, Foster city, CA,

USA, version 2.0.1) against *P. margaritifera* and *P. maxima* EST dataset obtained from the calcifying mantle cells (Jackson et al. 2010; Joubert et al. 2010). The different peptide hits for *Pmarg*-MRNP34 and *Pmax*-MRNP34 were manually confirmed by careful examination of the MS/MS spectra.

Sequence analysis

Protein sequence identification was attempted using BLASTp and tBLASTn analysis performed against Swiss-Prot, GenBank's nrdb and dbEST using the online tool provided by UniProt (http://www.uniprot.org) and NCBI (http://www.ncbi.nlm.nih.gov/blast.cgi). Signal peptides were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), and conserved domains were investigated using SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). A hydrophobic plot was obtained with the Kyte and Doolittle calculation (Kyte and Doolittle 1982) using ProtScale tool (http://www.expasy.org/tools/protscale.html). Following the peptide signal removal, theoretical mass and p*I* were determined with ProtParam tool (http://www.expasy.org/tools/protparam.html). The putative glycosylation and phosphorylation sites were investigated using DictyOGlyc, NetCGlyc, NetNGlyc, YinOYang and NetPhos tools from EXPASY online server (http://www.expasy.org/tools/). Putative cysteine bonds were predicted with Scratch Protein Predictor (http://www.expasy.org.tools/).

Localisation of the Pmarg-MRNP34 expression

Around 300 mg of each tissue (i.e. adductor muscle, hemocytes, gills, ungrafted and grafted pearl pocket, mantle distal and marginal zones, comprising all three mantle folds) were collected from six adult oysters *P. margaritifera* and total RNAs were extracted using the TRIzol® Reagent (Invitrogen). One µg of pooled total RNAs was reverse-transcribed using the iScript™ cDNA synthesis Kit (Bio-Rad), and cDNAs were amplified using the iQ™ Supermix kit (Bio-Rad) with *Pmarg*-MRNP34 specific primers (MRNP34_1_S GCGGTATGAGTCTA TCCAGCC/MRNP34_1_AS TCCAAAGCCTCCCATCA TAC) designed from *Pmarg-MRNP34* ORF (188 bp amplicon), on a Mastercycler (Eppendorf). Specific *EF*-1 primers (EF1_S ATGCTGCCATGGTTGATATG/EF1_AS GTGGCCTCAGCTTTCTCAAC) were used as a standard control. PCR products were analyzed on a 2% agarose gel electrophoresis.

The in situ hybridization was performed as previously described (Joubert et al. 2010). The digoxigenin-labelled sense or anti-sense probes were synthesized using the two sets of *Pmarg-MRNP34* specific primers (MRNP34_1_S/AS and MRNP34_2_S GTATGATGGGAGGCTTTGGA/MRNP34_2_AS TTGTGCGTACAGCTGAGGAG). The sections of *P. margaritifera* mantle tissues were hybridized with a pool of two probes. The detection steps were performed according to the manufacturer's instructions (Dig nucleic acid detection kit, Roche Molecular Biomedicals) and the slides were counter-stained with a solution of Bismark Brown Yellow and observed under a DM-4000B Leica microscope.

For quantitative RT-PCR analysis of *Pmarg-MRNP34* the forward and reverse primers used, were MRNP34_2_S and MRNP34_2_AS, respectively. A universal set of primers for the 18S rRNA gene sequence was used as a first reference gene, and was originally designed based on the alignment of different bivalve species (Uni1304FTTAG TTGGTGGAGCGATTT/Uni1670R TAGCGACGGGCGG TGTG) (Larsen et al. 2005). A second reference gene was used and chosen based on its ubiquitous and constitutive expression pattern (REF1S AGCCTAGTGTGGGGGTT GG/REF1AS ACAGCGATGTACCCATTTCC). First strand cDNA was synthesized from 300 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mix of poly(dT) and random hexamers primers. PCR amplifications were carried out with the Stratagene MX3000P in the presence of SYBR-Green using 400 nM of each primer and 1 µL of cDNA template. The following run protocol was used: initial denaturation at 95°C for 10 min, 95°C for 30 s, 60°C for 1 min, 72°C for 30 s with a two fluorescence measurements, melting curve program (45–95°C with a heating rate of 0.1°C s^{-1} and a continuous fluorescence measurement). The copy ratio of each analyzed cDNA was determined from three pools of ten different animals. All measures were done in duplicate. The relative expression ratio of *Pmarg-MRNP34* was calculated based on the delta–delta method for comparing the relative expression results which is defined as: ratio $= 2^{-[\Delta Ct\ \text{sample}-\Delta Ctcalibrator]} = 2^{-\Delta\Delta Ct}$ (Pfaffl 2001). Here the $\Delta Ctcalibrator$ represents the mean of ΔCt values obtained for all tissues for the target gene.

Immunolocalisation of *Pmarg*-MRNP34 protein

In order to localize *Pmarg*-MRNP34, we used a polyclonal antibody developed in previous study (Marin et al. 1994). This antibody specifically recognized a protein fraction around 34 kDa in the nacre AIM of *P. margaritifera* separated on SDS-PAGE. To assess the similarity between the recognized 34 kDa fraction and the MRNP34 protein, this antibody was tested on Western blot as previously described (Marie et al. 2008), with the ASM and AIM of both the nacreous and prismatic layers of *P. margaritifera* (1/2,000 dilution). The fraction targeted by the antibody was excised from the gel and analyzed further by proteomics.

A

Organism	Total protein scores	Matching peptides
Pinctada margaritifera		
AIM of shell prisms	0	0
AIM of shell nacre	12	6
AIM of pearl nacre	10	5
34-kDa band	16	8
Purified MRNP34	29.9	16
Pinctada maxima		
AIM of shell prisms	0	0
AIM of shell nacre	6	3

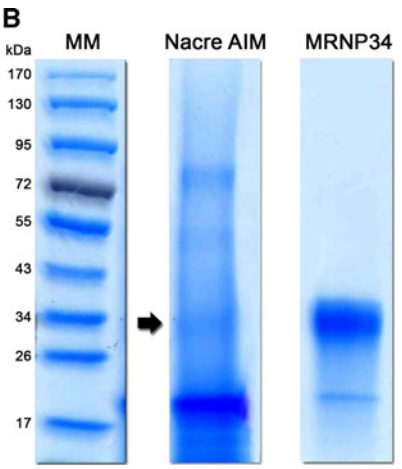

Fig. 1 Detection of MRNP34 in nacre acid-insoluble matrix of *P. margaritifera* and *P. maxima*. **a** Detection of MRNP34 by proteomics in the different matrices and the purified proteins from *P. margaritifera* and *P. maxima*. AIM fractions were analyzed on MS after trypsin cleavage, and the resulting MS/MS spectra were compared in silico to AA sequences derived from EST dataset from the mantle cells of *P. margaritifera* [14] and *P. maxima* [15] using ProteinPilot software. Total protein score indicates the sum of individual peptide scores. Respective numbers of matching peptides are indicated in the *right column*. **b** 12% SDS–PAGE stained with CBB of AIM shell nacre and purified MRNP34 from *P. margaritifera* nacre AIM. The molecular weight markers are indicated on the *left*. The *arrow* localizes the nearby 34 kDa band excised for MS analysis

For immunolocalisation, deparaffinized 5 µm sections of *P. margaritifera* mantle tissues, previously fixed for 24 h in Davidson fixative, were permeabilized for 10 min in phosphate buffered saline (PBS, pH 7.4) 0.5% triton 100×. Tissues were then incubated for 1 h in saturation medium (1% BSA, PBS) at *RT* before incubation with anti-MRNP34 (1/100 dilution) for 1 h in PBS–BSA 1% 0.5 g L^{-1} Tween 20 at *RT*. After rinsing in saturation medium, samples were incubated for 2 h at *RT* with a FITC-coupled secondary antibody (Sigma, F9887, goat anti-rabbit, 1/320 dilution). Finally, samples were briefly stained with Evans blue dye (0.5 g L^{-1} in PBS), mounted in Vectashield® medium and observed with a DM-4,000B Leica microscope. Control experiments were performed similarly without the first antibody step.

Results and discussion

Identification of a novel methionine-rich nacre protein from Pinctada spp.

In order to investigate the nacre proteins, we extracted the organic matrix from different shell and pearl layers of *P. margaritifera* and *P. maxima*. The nacre or prisms AIMs, representing 90% of the whole matrices, were analyzed by MS just after trypsin cleavage. The matching amino-acid (AA) sequences were then retrieved from the EST databases available for these organisms (Jackson et al. 2010; Joubert et al. 2010). Among the numerous protein identifications, we detected the presence of a novel putative protein that was called 34 kDa methionine-rich nacre protein (MRNP34), in the nacreous shell and pearl layers of both *P. margaritifera* and *P. maxima*, but not in prismatic layers (Fig. 1a).

The analysis of the different protein fractions from the nacre AIM of *P. margaritifera*, obtained by 1D SDS-PAGE separation or by preparative purification (Fig. 1b), indicates that *Pmarg*-MRNP34 was primarily detected in 34 kDa bands (Fig. 1a). Indeed, when 6 and 5 different peptides, corresponding to trypsic fragments of MRNP34, were observed in shell and pearl nacre AIM *P. margaritifera*, 8 and 16 MRNP34 peptides were detected in the 34 kDa SDS-PAGE band and in the fraction enriched with MRNP34, purified by preparative electrophoresis (Fig. 1a, b). It is interesting to note that we also detected in the purified fraction of *Pmarg*-MRNP34 the presence of peptide fragments of other nacre proteins, e.g. *Pmarg*-MSI60, *Pmarg*-N66 and *Pmarg*-Pif177 likely correspond to cleaved or degraded forms of the these proteins (data not shown). According to this result, the *Pmarg*-MRNP34 enriched fraction obtained from purification by preparative electrophoresis appears impure, hence are not proper for further specific biochemical characterization using in vitro tests, like those generally performed with nacre purified proteins (Marie et al. 2008). Similarly, Lao and co-workers have observed that N66 protein fractions, purified by chromatography from *P. fucata* nacre matrix, were not pure and contained fragments of different proteins (Lao et al. 2007). These observations highlight the difficulty in this

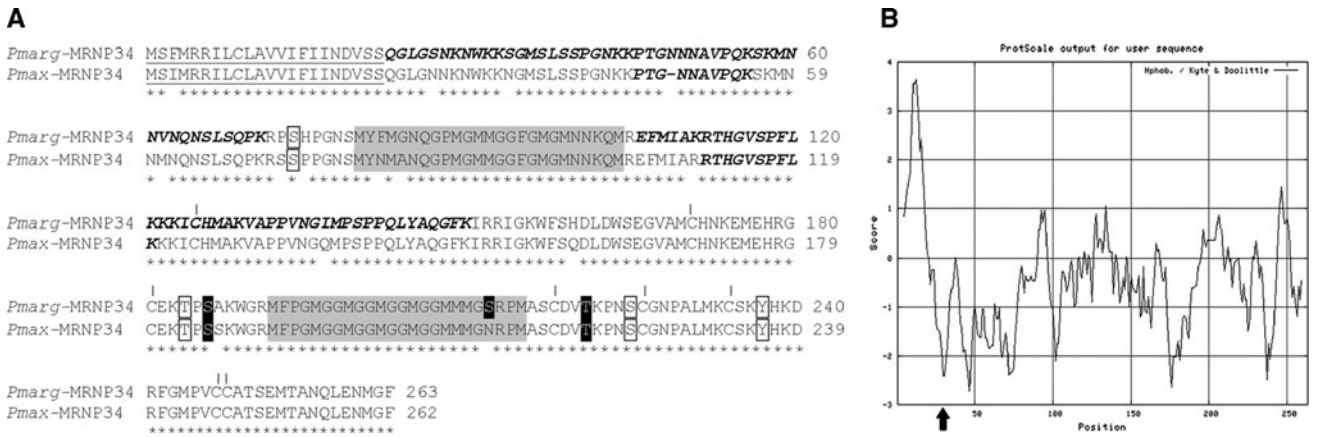

Fig. 2 Amino-acid sequence analysis of MRNP34. **a** Amino-acid (*AA*) sequence alignment of *Pmarg*-MRNP34 and *Pmax*-MRNP34 (SwissProt numbers are P86872 and P86871, respectively). Conserved AA positions are indicated by *asterisk* The putative signal peptides are *underlined*. The peptides observed by MS/MS are indicated in *italic bold face*. The two MG-rich domains are shaded in *grey*. The putative O-glycosylation sites are shaded in *black* and the putative phopshorylation sites are *boxed*. The eight cysteine residues potentially involved in disulfide bridges are indicated by *vert symbol*. **b** Hydrophobicity "Kyte and Doolittle" plot of *Pmarg*-MRNP34, performed with ProtScale tool (http://www.expasy.org/tools/protscale.html). Similar, if not identical, plot was obtained when using *Pmax*-MRNP34 (not shown)

model to extract pure protein fractions from direct separation of nacre matrix components, whatever the purification technique used.

MRNP34 structural characterization

The complete cDNA sequence of *Pmarg-MRNP34* (Genbank HQ625028) and *Pmax-MRNP34* (GenBank EZ420111) were obtained by clustering of EST sequences from calcifying mantles as previously described (Jackson et al. 2010; Joubert et al. 2010). The deduced AA sequences revealed that these cDNAs encode 263- and 262-AA-long proteins, respectively (Fig. 2a). They both exhibit signal peptides indicating that they are likely secreted proteins. Moreover, sequence alignment indicates that *Pmarg*- and *Pmax*-MRNP34 share 96% of AA sequence identity, suggesting that these are conserved proteins of the nacre. Indeed, no homolog could be found, neither in other bivalves nor in other organisms, using BLASTp and tBLASTn searches against UniProtKB, GenBank or nrdbEST. This fact is not unique, since most known biomineralization proteins do not present known homologs. Furthermore, about 62% of *P. margaritifera* and more than 70% of *P. maxima* ESTs sequences do not contain significant hits with the known protein database (Jackson et al. 2010; Joubert et al. 2010). Taken together, this suggests—but not demonstrates—that MRNP34 may be a protein, which was recently recruited among the *Pteriidae* bivalves, as the homolog was not detected in some other large genomic database of pteriomorph bivalve (Fleury et al. 2009).

Following signal sequence removal, *Pmarg*- and *Pmax*-MRNP34 are characterized by a theoretical p*I* of ten, and a theoretical molecular weight around 26 kDa. In contrast, the proteomic analysis of SDS-PAGE bands indicates that *Pmarg*-MRNP34 migrates around 34 kDa (Fig. 1). This discrepancy between the theoretical and the apparent molecular weight of *Pmarg*-MRNP34 might be explained by specific enrichment of the protein composition in some specific AA, as MG-repeats, that can quantitatively influence the binding of SDS to the protein core, then to its PAGE migration. Alternatively, it may also traduce the putative presence of post-translational modifications in *Pmarg*-MRNP34 mature protein, when it is secreted and integrated within the biomineral (Marie et al. 2007). Numerous putative glycosylation and phophorylation sites could indeed be found within the protein sequence, supporting this hypothesis (Fig. 2). Both *Pmarg*- and *Pmax*-MRNP34 protein sequences exhibit a noteworthy high amount of methionine (12%), together with an important amount of glycine (12%) and arginine (8%), but is depleted in acidic residues (2 and 3% of aspartic and glutamic acids, respectively). Mature MRNP34 proteins contain eight conserved cysteine residues that potentially form four disulfide bridges between Cys_{125}/Cys_{247}, Cys_{171}/Cys_{181}, Cys_{218}/Cys_{234}, Cys_{226}/Cys_{248}, as analyzed for *Pmarg*-MRNP34 and Cys_{124}/Cys_{170}, Cys_{180}/Cys_{246}, Cys_{217}/Cys_{233}, and Cys_{225}/Cys_{247} for *Pmax*-MRNP34 (Fig. 2a). The two methionine-rich domains present between 80–104 and 192–215 AA positions in *Pmarg*-MRNP34 are noteworthy. Although their function is unknown, they may be putatively involved in protein/protein interactions, by analogy

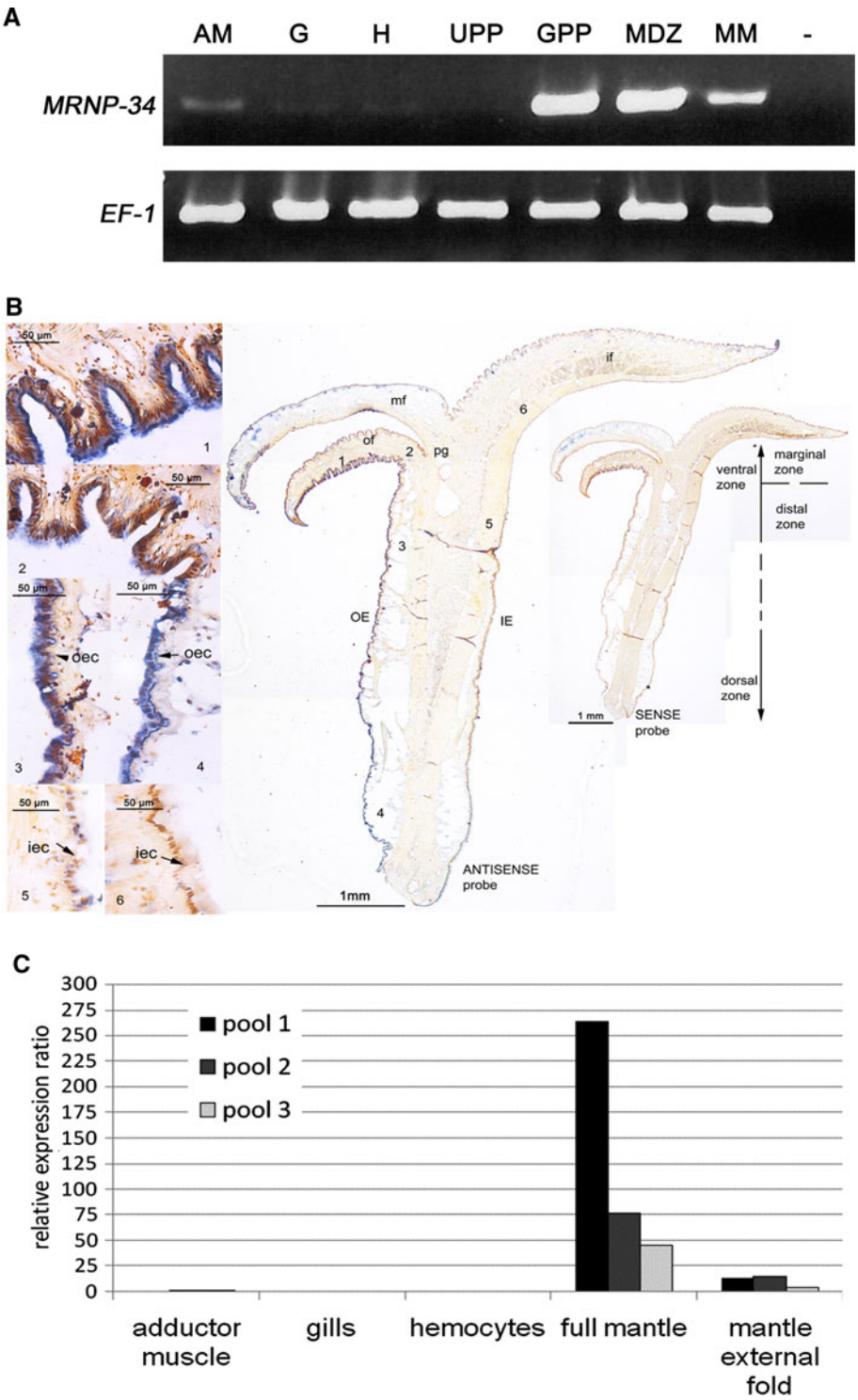

Fig. 3 *Pmarg-MRNP34* transcript localisation in oyster tissues. **a** RT-PCR analysis using RNA extracted from adductor muscle (*AM*), gills (*G*), hemocytes (*H*), ungrafted pearl pocket (*UPP*), grafted pearl pocket (*GPP*), mantle distal zone (*MDZ*), mantle margin (*MM*), transcribed and amplified with *Pmarg-MRNP34* and *EF-1* (as control) specific primers. Negative controls were performed without cDNA (−). Positive amplification control with MRNP34 cDNA was also performed (not shown). **b** Localization of *Pmarg-MRNP34* gene transcripts in *P. margaritifera* mantle tissue by in situ hybridisation. Paraffin-embedded sections of oyster tissues were hybridised with antisense or sense single stranded cDNA probes, which were labelled with digoxigenin and revealed using alkaline phosphatase-conjugated antibodies. Positive cells are stained in *dark blue*, sense probes showed no hybridisation (*inset*). Stained cells enlargements are shown in *six insets*. Results are representative of 4 independent experiments realized on 4 different tissues. *if* inner fold, *mf* middle fold, *of* outer fold, *pg* periostracal groove, *OE* outer epithelium, *IE* inner epithelium, *oec* outer epithelial cell, *iec* inner epithelial cell. **c** Real-time PCR analysis of *Pmarg-MRNP34* transcript accumulation in different oyster tissues (mantle external fold, full mantle, adductor muscle, gills, hemocytes). The values represent relative expression ratios of ten samples for three different pools of animals. All measures were done in duplicate

with other proteins, like Calmodulin (CaM), which exhibits two hydrophobic methionine-rich domains that bind to the target protein (Siivari et al. 1995). Interestingly, the nacre matrix of the gastropod *Haliotis asinina* comprises also an uncharacterized 13 kDa methionine-rich protein, previously called ML6A10 (Marie et al. 2010a), but reliable alignment can not be performed with MRNP34. The different searches for known protein domain failed to detect any already described domain. Although previous works report the presence of numerous repeated low

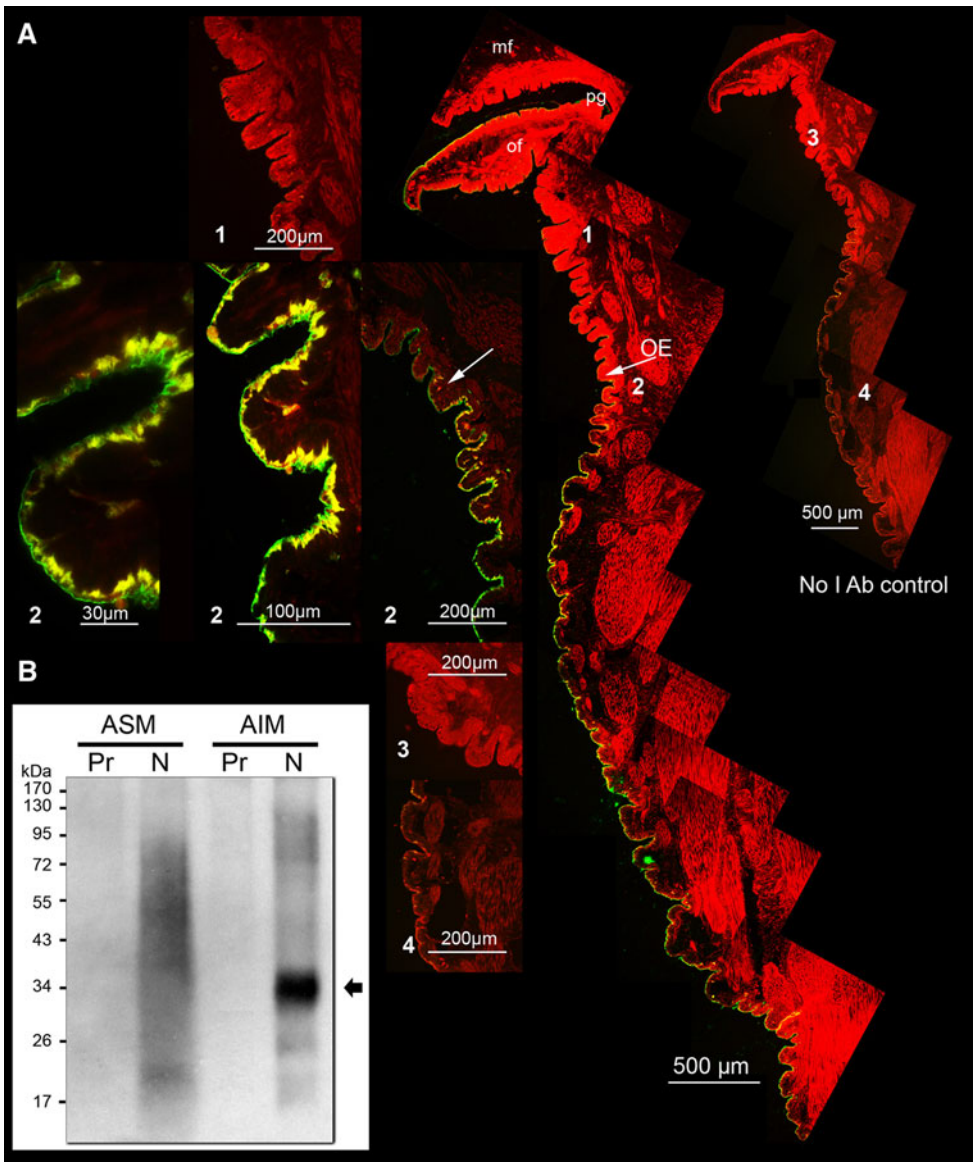

Fig. 4 Immunolocalisation of *Pmarg*-MRNP34 in the organic shell matrices and the calcifying mantle of *P. margaritifera*. **a** Immunolocalisation of *Pmarg*-MRNP34 in mantle tissue with the MRNP34 primary antibodies reacting against MRNP34-containing band, using FITC-coupled secondary antibodies. Images of the mantle outer face and details of outer epithelial zones are shown (*1, 2*). A mantle tested without primary Ab (no AbI control) showing no staining is shown (*3, 4*). *mf* middle fold, *of* outer fold, *pg* periostracal groove, *OE* outer epithelium. Results are representative of two independent experiments performed on two different tissues. **b** Western blot analysis of AIM and ASM prisms (*Pr*) and nacre (*N*) shell extracts using the primary antibodies reacting against the MRNP34-containing PAGE band. *Pmarg*-MRNP34 is indicated by an *arrow*. The molecular weight markers are indicated on the *left*

complexity domains (RLCDs) in different nacre-associated proteins (Jackson et al. 2010)—poly-Ala, poly-Asp, Poly-Ser, poly-Gly or Gly/Asn-repeats (Miyamoto et al. 1996; Shen et al. 1997; Sudo et al. 1997; Marin et al. 2008; Suzuki et al. 2009; Jackson et al. 2010; Joubert et al. 2010; Marie et al. 2010a)—the presence of such methionine-rich domain constitutes an entirely new feature of mollusc nacre matrix proteins that seems to be shared by different mollusc classes. Finally, a hydrophobic plot shows the overall hydrophobic character of *Pmarg*-MRNP34, except at four short internal regions (Fig. 2b), suggesting that this protein is predominantly hydrophobic and/or insoluble. Taken together, these results show that pearl oyster MRNP34 proteins share very close structures and further suggest that MRNP34 are insoluble proteins that interact with other matrix macromolecular components in the nacre organic framework.

The Pmarg-MRNP34 expression and translation is Specific to nacre mineralising cells

To better understand the physiological role of *Pmarg*-MRNP34 in pearl oyster, the expression pattern of its transcript has been investigated. RT-PCR analyses led on total RNAs extracted from adult main organs showed that the *Pmarg-MRNP34* gene is primarily expressed in truly calcifying tissues, i.e. the mantle distal zone (mantle tissue excluding the marginal zone), the mantle margin (mantle folds) and the grafted pearl pocket (Fig. 3a). No weak expression was detected in other tissues (adductor muscle,

hemocytes, gills, and ungrafted pearl pocket). Additionally, our in situ analysis of the mantle further revealed that the *Pmarg-MRNP34* transcript is specifically expressed in the mineralizing outer epithelial cells of the mantle. ISH observations suggest that *Pmarg-MRNP34* is preferentially expressed in the dorsal part of the mantle external epithelium (Fig. 3b), which is known to contain mineralizing cells responsible for nacre formation (Joubert et al. 2010). Indeed, quantitative expression analyses conducted on total RNAs extracted from two parts of the mantle (full mantle and mantle margin) confirmed that *Pmarg-MRNP34* gene is primarily expressed in the nacre forming zone beneath the calcifying external fold of the mantle (Fig. 3c). Those analyses also confirmed that the weak transcript expression detected using RT–PCR in other tissues were negligible (adductor muscle, hemocytes, gills). Furthermore, the protein localization revealed that *Pmarg*-MRNP34 is exclusively synthesized in the nacre secreting external epithelium of the mantle (Fig. 4a) and is detected only in nacre organic matrix, and not in the prisms (Fig. 4b).

Conclusion

Taken together, our results indicate that MRNP34 is secreted mineralization-associated protein, as it transcripts a nacre specific epithelial mantle calcifying cells. Furthermore, it is a nacre-specific protein, as it is only produced in nacre forming cells, and present only in nacre. However, because of the lack of sequence similarity with already characterized proteins, no assumption on its biological function can be proposed, and the precise contribution of MRNP34 in nacre formation remains undetermined. This study illustrates the efficiency of combine transcriptomic/proteomic approach for identifying of novel shell proteins. The challenge that now faces the field of biomineralization is the characterization of the functions in the growing list of novel biomineral associated proteins (Marin et al. 2008; Jackson et al. 2010; Joubert et al. 2010; Marie et al. 2010a) that typically do not contain sequence homology with already known proteins. Our results also give new insight into calcium carbonate biomineralization, by emphasizing the cellular control of the process. Plans to elucidate the physiology and the molecular mechanism of the nacreous formation by combining in vivo regulation of protein expression (e.g. "knock-down" approach) and in vitro crystallization experiments using recombinant protein and synthetic peptides are clearly the next step of our analysis.

Acknowledgments This study is part of a collaborative project (GDR ADEQUA) supported by the "Service de la perliculture" of French Polynesia. It is also supported by Ifremer, the University of French Polynesia, and the University of Burgundy. Authors are grateful to Nathalie Cochennec-Laureau, Marcel Le Pennec, Péva Levy, Cédrik Lo and Anne-Sandrine Talfer for helpful discussions and assistance. The work of F. Marin is financially supported by ANR funding (ACCRO-EARTH, ref. BLAN06-2_159971, coordinator Gilles Ramstein, LSCE) during the period 2007–2011.

Conflict of interest None declared.

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