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Molecular cloning and localization of a PMCA P-type calcium ATPase from the coral *Stylophora pistillata*☆

Didier Zoccola[a,*], Eric Tambutté[a], Emmanuelle Kulhanek[a], Sandrine Puverel[a,b], Jean-Claude Scimeca[c], Denis Allemand[a,b], Sylvie Tambutté[a]

[a] *Centre Scientifique de Monaco, Avenue Saint Martin, MC-98000, Monaco*
[b] *UMR 1112 INRA/UNSA, Faculté des Sciences, BP 71, F-06108, Nice Cedex 02, France*
[c] *UMR 6549, CNRS/UNSA, Faculté de Médecine, F-06107, Nice Cedex 02, France*

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Abstract

Plasma-membrane calcium pumps (PMCAs) are responsible for the expulsion of Ca^{2+} from the cytosol of all eukaryotic cells and are one of the major transport systems involved in long-term regulation of resting intracellular Ca^{2+} concentration. An important feature of stony corals, one of the major groups of calcifying animals, is the continuous export of large quantities of Ca^{2+} for skeletogenesis. Here, we report the cloning and functional expression of the stpPMCA gene from the coral *Stylophora pistillata*, and whose features resemble those of the plasma-membrane Ca^{2+}-ATPase family of mammalian cells. This is the first known example of a Ca^{2+}-ATPase from the phylum Cnidaria, and thus, the most phylogenetically distant PMCA sequence in the animal kingdom described to date. We demonstrate that the localization of stpPMCA within calicoblastic cells is fully coherent with its role in calcification. We also show that the coral Ca^{2+} pump is more closely related to vertebrate PMCAs than to *Caenorhabditis elegans* PMCAs. The cloning of evolutionarily conserved genes from cnidarian species repeatedly shows that these genes encode similar functional domains. Moreover, this high level of gene conservation further validates the use of cnidarian model systems for studying processes shared by Eumetazoans.

Keywords: Ion transport; Cnidaria; Biomineralization; Calmodulin

1. Introduction

Calcium (Ca^{2+}) is an essential ion in animals, playing a crucial role in various processes from cell activation to formation and maintenance of skeletal structure. This last function is particularly important in scleractinian corals (Cnidaria, Anthozoa), one of the major groups of calcifying animals. In some corals, rates of transepithelial calcium flux are as high as 1700 nmol cm^{-2} h^{-1} [1]. At present, we have a basic understanding of the cellular mechanisms involved in this process. Ca^{2+} transport involves at least one transcellular pathway through calicoblastic cells located in the aboral tissue facing the skeleton [2–4]. A verapamil-sensitive Ca^{2+} channel is involved in Ca^{2+} entry [4]. This channel belongs to L-type α1 subunit Ca^{2+} channel [5]. Strong identities and conservative substitutions between the rabbit α1C-subunit and the *Stylophora pistillata* Ca^{2+} channel demonstrate the evolutionary conservation of ion carriers. However, mechanisms of Ca^{2+} export from the calcifying cells to the skeleton are not fully characterized. Two hypothetical mechanisms include a Na^{+}/Ca^{2+} antiport [6] and a Ca^{2+}-ATPase [7–9]. Two major classes of P-type Ca^{2+}-ATPase have been defined in vertebrates [10,11]: type IIA Ca^{2+}-ATPase, including SERCA and PMR1-type pumps that are located in intracellular membranes; and type IIB Ca^{2+}-ATPase including PMCA Ca^{2+} pumps that are

Abbreviations: DIG, digoxigenin; DMEM, dulbecco-modified eagle medium; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; PMCA, plasma membrane calcium ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}ATPase; TNN buffer, tris sodium nonidet P-40 buffer; CaM, calmodulin; SSC, standard sodium citrate; stpPMCA, *Stylophora pistillata* plasma membrane calcium ATPase

☆ The nucleotide sequences reported in this paper have been submitted to the GenBank™/EBI Data Bank with accession numbers: stpPMCA AY360080 and stpACT AY360081.

* Corresponding author. Tel.: +377-93-77-08-73; fax: +377-92-16-79-81.

E-mail address: zoccola@centrescientifique.mc (D. Zoccola).

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responsible for Ca^{2+} export from the cell, and are stimulated by interaction with calmodulin [12]. Because of its high affinity for Ca^{2+}, PMCA is believed to play a key role in Ca^{2+} homeostasis of eukaryotic cells [13].

Four genes for the PMCA pump have been found in mammals [14], and differences among these sequences are mostly in the N- and C-terminal regions of the protein [15]. Various PMCAs are now characterized in vertebrates, nematodes, protists, yeasts and plants [16]. In scleractinians, and more generally in diploblastic animals, such a protein has not yet been described.

Here, we report the cloning of a plasma membrane Ca^{2+}-ATPase gene from the stony coral, *S. pistillata*. This species has a wide distribution in tropical coral reefs and constitutes an interesting biological model for several reasons: (1) it is easy to cultivate under controlled conditions; (2) it can be grown as a clonal culture; (3) it has a high rate of calcification; (4) it can be easily manipulated for experimental purposes. Using phylogenetic construction and a calmodulin-binding assay, we show that the coral Ca^{2+} pump is closely related to the PMCA family. Moreover, the localization of this Ca^{2+}-ATPase within coral tissues, as visualized by Fluorescence in situ Hybridization, suggests that this pump plays a role in the export of Ca^{2+} from calicoblastic cells to the site of mineralization.

2. Materials and methods

2.1. Biological material

Microcolonies were propagated in the laboratory as previously described [17]. Briefly, terminal portions of branches (6–10-mm long) were cut from parent colonies, placed on a nylon net (1 × 1-mm mesh) and maintained under controlled conditions of light and temperature. After approximately 1 month, the coral fragments were covered entirely with new tissue and were used for the experiments.

2.2. Cloning procedure

Total coral RNA and poly(A)+ RNA were prepared as described previously [5]. The initial partial fragment of the calcium ATPase was obtained by degenerate oligonucleotide PCR after reverse transcription of coral mRNA. Primers were synthesized based on amino acid sequences MVTGDNINT (5′-ATGGTNACNGGNGAYAAYATH-AAYACN-3′) and QFQLTVNV (5′-NACRTTNACNGT-NARYTGRAAYTG-3′) localized in conserved regions of previously cloned Ca^{2+} pumps [18]. The PCR amplification product was purified using the CONCERT™ Rapid PCR Purification System (InVitrogen) and cloned into pGEM®-T Easy vector (Promega). After determining the sequence of this fragment, specific primers were designed for 5′ RACE (Rv1: 5′-AGGTGGCAGGATGCCACATTT-3′; Rv2: 5′-GGGCCACACTTCATCAAACTT-3′; Rv3: 5′-ATTGCGCCCCCACATAACAGC-3′) and 3′ RACE (Fd1: 5′-TTGCTTTCAAATGTGGCATCC-3′; Fd2: 5′-GTT-AAAGCTGTTATGTGGGGG-3′) using the Roche 5′/3′ RACE Kit. DNA sequencing was carried out on both strands with SP6 and T7 primer sequences using ABI Prism® BigDye terminator kit (PEBiosystems).

2.3. Phylogenetic construction

Sequence alignments were performed on the complete sequences with clustal W [19]. The phylogenetic tree was constructed using the Neighbor Joining method and bootstrapped 1000 times using Paup software [20]. The sequences used in this analysis are derived from complete open reading frame (ORF) sequences from the following organisms (accession numbers are in brackets); ACA1 ARATH: *Arabidopsis thaliana* (Q37145); ACA2 ARATH: *A. thaliana* (O81108); ACA4 ARATH: *A. thaliana* (O22218); ACA7 ARATH: *A. thaliana* (O64806); ACA8 ARATH: *A. thaliana* (Q9LF79); ACA9 ARATH: *A. thaliana* (Q9LU41); ACAA ARATH: *A. thaliana* (Q9SZR1); ACAB ARATH: *A. thaliana* (Q9M2L4); ACAC ARATH: *A. thaliana* (Q9LY77); ACAD ARATH: *A. thaliana* (Q9LIK7); ECA1 ARATH: *A. thaliana* (P92939); ECA2 ARATH: *A. thaliana* (O23087); ECA3 ARATH: *A. thaliana* (Q9SY55); ECA4 ARATH: *A. thaliana* (Q9XES1); ATC ARTSF: *Artemia franciscana* (P35316); ATC1 BOVIN: *Bos taurus* (P57709); 3549723: *Caenorhabditis elegans* (CAA09303); 3549725: *C. elegans* (CAA09308); 2826864: *C. elegans* (CAA11491)ATA2 CANFA: *Canis familiaris* (O46674); ATC1 DICDI: *Dictyostelium discoideum* (P54678); ATC1 DROME: *Drosophila melanogaster* (P22700); ATA2 FELCA: *Felis catus* (Q00779); ATA3 CHICK: *Gallus gallus* (Q9YGL9); ATA2 CHICK: *G. gallus* (Q03669); ATA1 HUMAN: *Homo sapiens* (O14983); ATA2 HUMAN: *H. sapiens* (P16615); ATA3 HUMAN: *H. sapiens* (Q93084); ATB1 HUMAN: *H. sapiens* (P20020); ATB2 HUMAN: *H. sapiens* (Q01814); ATB3 HUMAN: *H. sapiens* (Q16720); ATB4 HUMAN: *H. sapiens* (P23634); ATC1 HUMAN: *H. sapiens* (P98194); ATC4 HUMAN: *H. sapiens* I(O75185); ATA2 MOUSE: *Mus musculus* (O55143); ATA3 MOUSE: *M. musculus* (Q64518); ATB2 MOUSE: *M. musculus* (Q9R0K7); ATB2 OREMO: *Oreochromis mossa* (P58165); ATA2 RABIT: *Oryctolagus cuniculus* (P20647); ATB1 RABIT: *O. cuniculus* (Q00804); ATA1 RANES: *Rana esculenta* (Q92105); ATA3 RAT: *Rattus norvegicus* (P18596); ATB1 RAT: *R. norvegicus* (P11505); ATB2 RAT: *R. norvegicus* (P11506); ATB3 RAT: *R. norvegicus* (Q64568); ATB4 RAT: *R. norvegicus* (Q64542); ATC1 RAT: *R. norvegicus* (Q64566); ATC1 YEAST: *Saccharomyces cerevisiae* (P13586); ATC2 YEAST: *S. cerevisiae* (P38929); ATA2 PIG: *Sus scrofa* (P11607); ATB1 PIG: *S. scrofa* (P23220); ATC TRYBB: *Trypanosoma brucei brucei* (P35315); ATC1 YARLI: *Yarrowia lipolytica* (O43108).

2.4. Construction of DNA templates for expression in HEK 293 cells

PCR products were prepared from an oligo-dT RT coral cDNA and ligated to the pBAD/Thio TOPO® vector (InVitrogen). Targets extended from the penultimate base preceding the AUG codon (AUGfwd: 5′-ACCATGGCAGAACCTTCAATTAA-3′) to either the last codon preceding the stop codon (STOPrev: 5′-TACAGAGCTTTCCACACTTGCATA-3′), or before the putative calmodulin binding site (ΔCAMrev: 5′-CAACAGACGAGCCTTGCTGTCTT-3′). Two kinds of recombinant vectors were obtained. The first one (ORFHIS) contains a chimeric sequence encoding the full-length ORF of the Ca^{2+}-ATPase fused to a V5 epitope and polyhistidine region at the C terminus (Fig. 3A). The second one (ΔCAMHIS) codes for a C-terminal-deleted 83 amino acid Ca^{2+}-ATPase fused to the same V5 and 6 × His region. Further PCR experiments using AUGfwd and pBADrev (5′-CTGCGTTCTGATTTAATCTGTATC-3′) were performed on recombinant plasmids, and resulting PCR products were cloned into pCI Mammalian expression vector (Promega).

Four recombinant plasmids were obtained: ORFHIS cDNA, under control of CMV promotor (ORFHIS+); ORFHIS reverse cDNA as control (ORFHIS-); ΔCAMHIS CAMHIS cDNA under control of CMV promotor (ΔCAMHIS+); and reverse cDNA (ΔCAMHIS-) as control. After sequencing, the different plasmid clones were introduced into HEK 293 cells with the Lipofectamine transfection reagent (InVitrogen).

2.5. Calmodulin binding assay

HEK 293 cells were cultured for 48 h in DMEM for transitory expression. Cells were then lysed in TNN buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40) supplemented with Complete Mini Protease Inhibitor (Roche). Proteins were subjected to 7% SDS-PAGE and electroblotted onto a PVDF membrane. To verify expression of the proteins, blots were probed with the monoclonal mouse Anti-Histidine Tag (Serotec) at a 1:1000 dilution using the ECL Western kit (Amersham Pharmacia). Next, to test calmodulin binding, the membrane was washed twice with Ca-Tris-buffered saline (25 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, and 200 μM $CaCl_2$). The membrane was incubated for 1 h in the blocking solution (Ca-Tris-buffered saline supplemented with 3% gelatine and 0.05% Tween 20), washed twice with TBST buffer (Ca-Tris-buffered saline supplemented with 0.05% gelatine and 0.05% Tween 20), and incubated with biotinylated bovine calmodulin (Calbiochem) diluted in TBST buffer at a concentration of 10 nM for 2 h at room temperature. Finally, the blot was washed three times with TBST buffer, and biotin binding was visualized using a streptavidin-HRP (Roche) conjugate at a 1:1000 dilution as suggested by the manufacturer.

2.6. RNA in situ hybridization and detection

Coral microcolonies were fixed in 4% paraformaldehyde in S22 buffer (450 mM NaCl, 10 mM KCl, 58 mM $MgCl_2$, 100 mM Hepes, pH 7.8) overnight at 4 °C and then decalcified using EDTA 0.5 M in Ca-free S22 at 4 °C. Tissues were dehydrated in an ethanol series, cleared with toluene and embedded in Paraplast (Sherwood Medical Co.). Eight-micrometer-thick sections were attached to silane–prep slides (Sigma), deparaffinized with xylene and rehydrated in ethanol series. Sections were immersed three times for 10 min in PBS-teleostean gelatin 0.2% v/v, in H_2O_2 1% for 15 min, in HCl 0.2 N for 15 min, in PBS-collagenase 0.1% for 10 min at 37 °C, in PBS-Proteinase K 10 μg/ml for 10 min at 37 °C, in PBS–1% BSA for 5 min, and finally in PBS for 5 min.

Prehybridization was performed in moist chamber for 1 h at 37 °C (in 5 × SSC, 50% formamide, 10 mM EDTA, 25 mM NaH_2PO_4, 240 μg/ml salmon sperm). Digoxigenin (DIG)-labelled sense and antisense RNA probes were transcribed from PCR fragments using either T7 or SP6 RNA polymerase (Roche). Hybridization was carried out in moist chamber at 37 °C overnight (in prehybridization buffer plus dextran sulfate 100 μM). Slides were washed in 2 × SSC, then in 1 × SSC, each for 15 min at 50 °C. After a final 3-min wash in washing buffer (0.1 M maleic acid pH 7.5, 0.15 M NaCl, 0.3% Tween), slides were incubated for 1 h in blocking buffer and then 2 h under moister at room temperature with peroxydase conjugated anti-DIG (dilution 1/200 in blocking buffer). Amplification of the signal was performed using biotinyl tyramide (Perkin) visualized with Streptavidine Alexa Fluor 568 (Molecular Probes). Slides were mounted with Prolong Antifade (Molecular Probes) and observed by confocal laser scanning microscopy (Leica TCS 4D). A 600-nm band-pass emission filter was used for Alexa Fluor detection with the krypton–argon laser.

3. Results

3.1. Cloning a coral calcium ATPase

GenBank comparisons using BLASTX indicate that the partial sequence derived from *S. pistillata* corresponds to PMCA-type calcium pumps (for example, $p = 3.0\ e-73$ and 89% homology with rat PMCA4 [Q64542]). RACE experiments were used to obtain the full-length cDNA. The complete sequence is 3683-bp long and contains, at 78 bp from the 5′ end, a methionine in a Kozak's context [21], followed by an ORF of 3483 bp. This codes for a protein of 1161 amino acids and a calculated molecular mass of 127 kDa. BLAST analysis showed that the nucleotide sequence of *S. pistillata* Ca^{2+} pump (stpPMCA) gene is homologous only with other PMCA genes. Fig. 1 shows the predicted amino acid sequence of the stpPMCA protein and its major domains together with an alignment with the four human

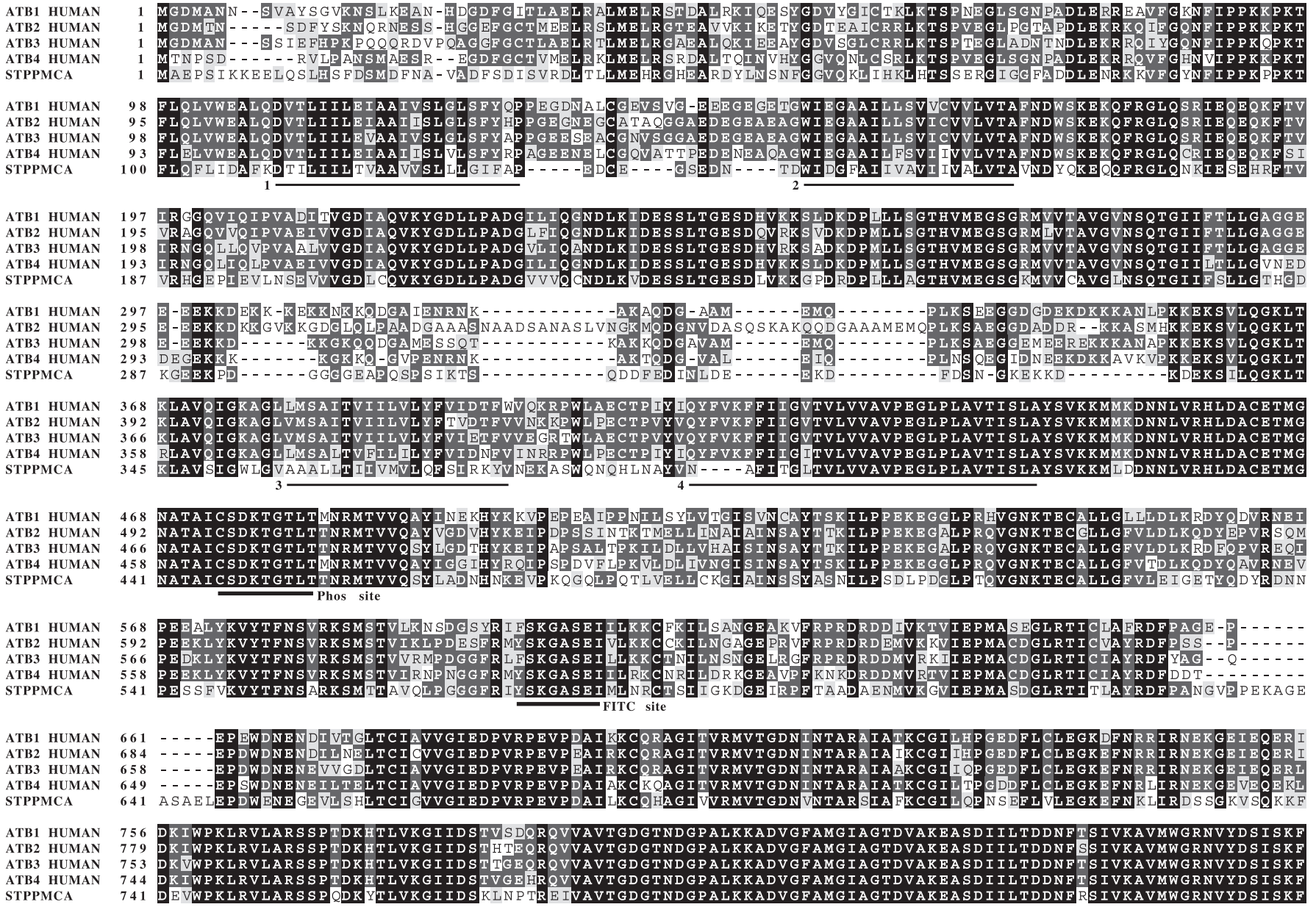

Fig. 1. Alignment of the four human PMCA gene products (ATB1 through ATB4) with the deduced amino acid sequence of stpPMCA. Black shading shows the sequences that are identical in all five proteins. Putative transmembrane domains are underlined and numbered 1 through 10. Functional sites are underlined with a thick line, i.e. phosphorylation site, FITC binding site and CaM binding site. The figure was compiled from the following SwissProt/TrEMBL entries: P20020 (ATB1), Q01814 (ATB2), Q16720 (ATB3), P23634 (ATB4).

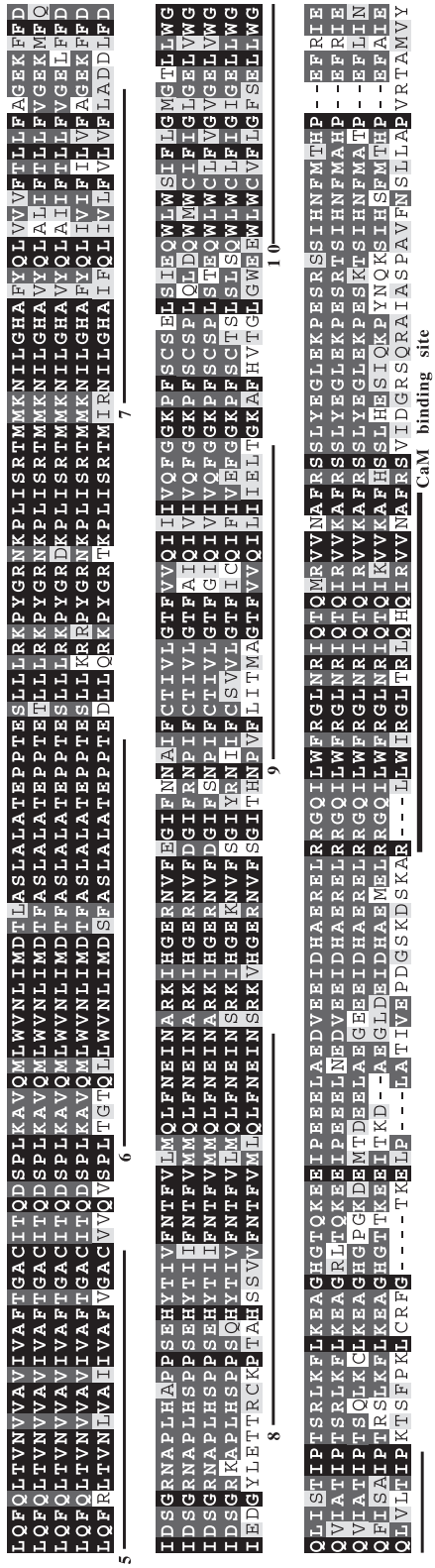

Fig. 1 (*continued*).

PMCA gene products. The best alignment of the deduced amino acid sequences of the stpPMCA protein is obtained with the human PMCA3 (ATB3) (55% in identity and 69% in conservative substitutions). Furthermore, the sequences of the phosphorylation site [22] and the consensus site of FITC binding [23] are identical in stpPMCA and the four human PMCAs. Finally, the predicted protein reveals a C-terminal extension that accounts for the molecular mass of 127 kDa, generally much larger in PMCA than in SERCA-type pumps [24]. Collectively, these observations suggest that the stpPMCA protein belongs to the PMCA family ATPases.

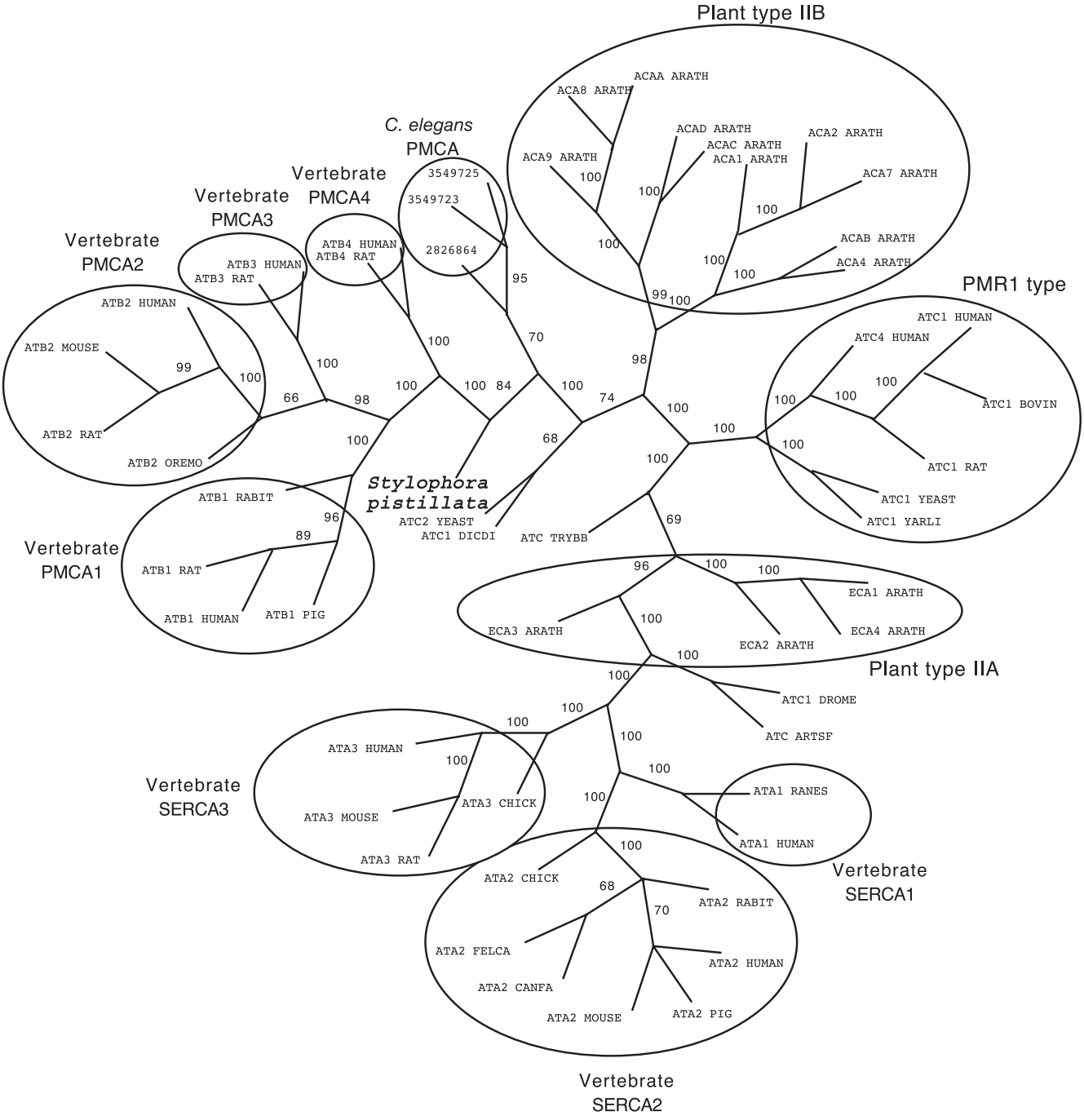

Fig. 2. Phylogenetic tree showing sequence relationships between the *S. pistillata* PMCA and other selected P-type II ATPases. Homologies between full-length amino acid sequences were calculated using PAUP software. Distances between stpPMCA protein (labelled in bold) and other ATPase amino acid sequences were calculated using a Neighbor Joining algorithm as implemented in Clustal W (ignore gaps = off; multiple substitutions = off). The trees were bootstrapped 1000 times. The numbers at the node points represent the number of times that particular node was present in the replicas. Accession number ATPase sequences found in the GenBank database (National Center for Biotechnology Information) are given in Section 2.

3.2. Phylogenetic relationship

Phylogenetic construction indicates a separate branching between the IIA and IIB subfamilies (Fig. 2) and supports inclusion of the coral Ca^{2+}-ATPase within the IIB subfamily. In this subfamily, plants form separate outgroups from other eukaryotes due to the different location of the autoinhibitory domain (N-terminal versus C-terminal, respectively). Interestingly, the phylogenetic tree shows that the coral pump is more closely related to vertebrate PMCAs than to *C. elegans* PMCAs.

3.3. stpPMCA contains calmodulin binding domain at its C terminus

Comparison with human PMCAs showed that the calmodulin-binding domain is mostly conserved. Nevertheless, due to slight differences in amino acids (Fig. 1), we performed membrane-based calmodulin binding assay. The amount and size of each different constructs (Fig. 3A) were analyzed by SDS-PAGE followed by immunoblotting, using a monoclonal anti-Histidine Tag antibody (Fig. 3B, upper panel). The full-length expressed stpPMCA protein binds bovine calmodulin at a concentration of 10 nM (Fig. 3B, lower panel). Two controls were used in this experiment. First, the expression of reverse cDNA of the stpPMCA gene (ORFHIS-) confirms the presence of endogenous streptavidin-binding proteins. Second, the expression of a calmodulin-binding domain deleted protein (ΔCAMHIS+) clearly demonstrates the specific binding of bovine calmodulin to the C-terminal domain.

3.4. Localization of stpPMCA expression

The histology of the coenosarcal tissue with the oral layers facing seawater and the aboral layers facing the skeleton is visible in light micrographs (Fig. 4A and C). Using the stpPMCA RNA antisense probe, we showed that stpPMCA is strongly expressed in the aboral calicoblastic and endodermal cells, weakly expressed in the oral endo-

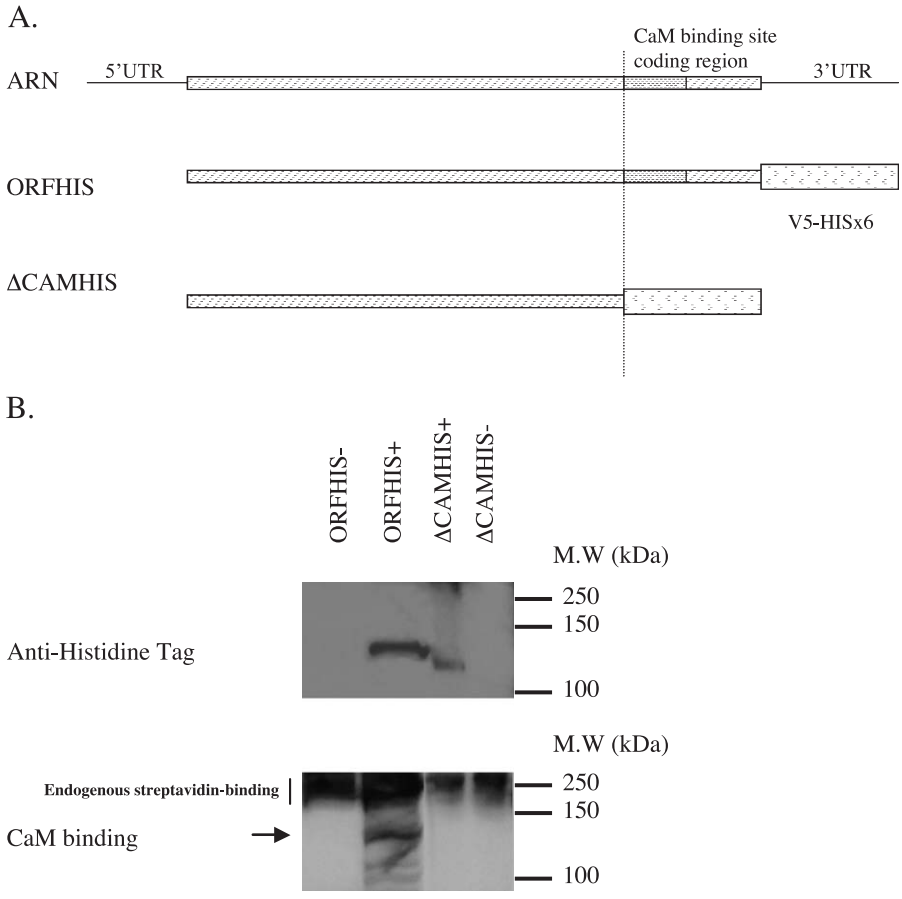

Fig. 3. Binding of bovine calmodulin to the stpPMCA protein expressed in HEK293 cells. (A) Schematic representation of construction of chimeric proteins ORFHIS and ΔCAMHIS. As described in Section 2, the coding region of stpPMCA gene was cloned in frame with the V5-Hisx6 coding sequence and it is referred as ORFHIS. Furthermore, the truncated mutant lacking the C-terminus domain (83 amino acids) was also cloned in frame with the V5-Hisx6 coding sequence and it is referred as ΔCAMHIS. These constructions were cloned in sense (+) or reverse sense (−) under the control of CMV promotor. (B) SDS-PAGE-fractionated and electroblotted proteins from HEK 293 cells transfected with ORFHIS−, ORFHIS+, ΔCAMHIS+ or ΔCAMHIS−. Upper panel shows the immunoblotting with a monoclonal anti Histidine Tag antibody. In lower panel, the membrane was incubated with 10 nM biotinylated bovine calmodulin (CaM) and 200 μM Ca_2Cl. Arrow shows the binding of CaM. Endogenous streptavidin-binding proteins are indicated.

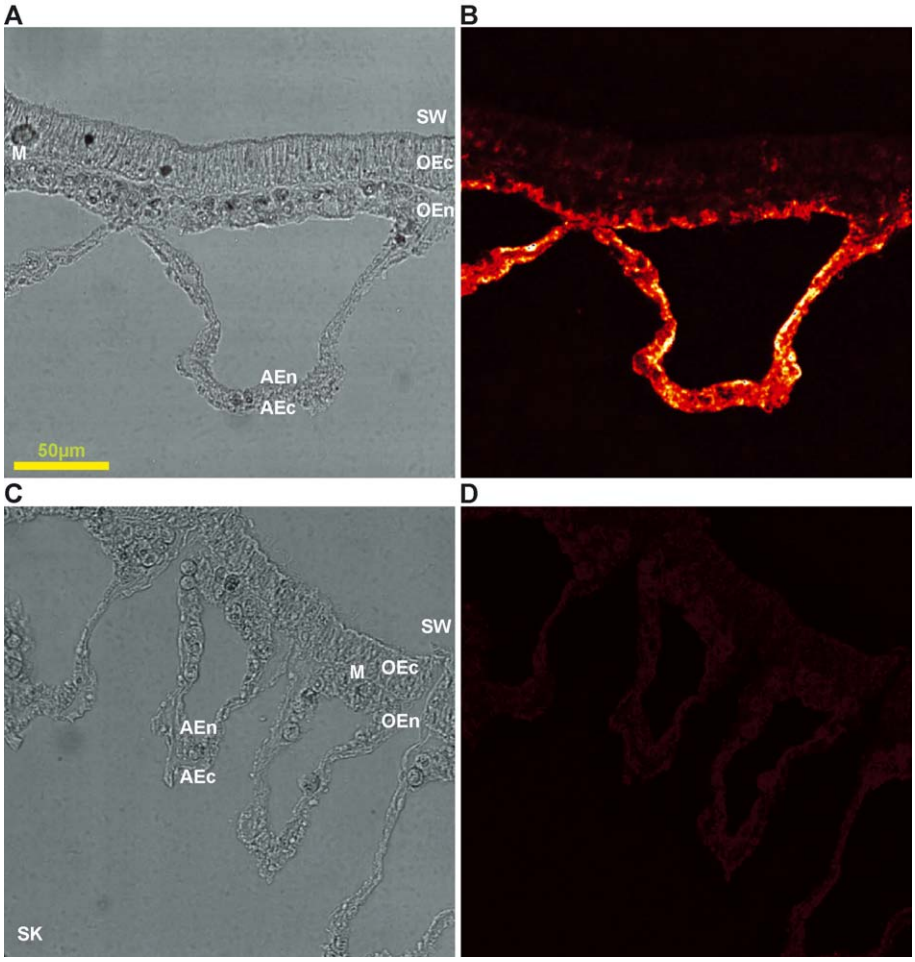

Fig. 4. In situ hybridization showing the expression pattern of stpPMCA in *S. pistillata* tissues. (A and C) Light microscopy images showing the four layers of coral tissues. OEc = oral ectoderm, OEn = oral endoderm, AEc = aboral ectoderm (calicoblastic cells), M = mesoglea, AEn = aboral endoderm, SW = localization of sea water, SK = localization of Skeleton. (B) Expression of stpPMCA is seen as fluorescent signal under observation with confocal microscope (excitation 568 nm, emission band pass 600 ± 5 nm). (D) Negative control with sense probe.

dermal cells, and not expressed in the oral ectoderm (Fig. 4B). Sense RNA probe was used as a control and showed no signal (Fig. 4D). Actin antisense RNA was used as positive control and showed signal in all layers of cells (data not shown).

4. Discussion

Here, we have isolated and characterized the first known example of a Ca^{2+}-ATPase (stpPMCA) from a diploblastic animal belonging to the phylum Cnidaria. This protein from the coral *S. pistillata* is the most phylogenetically distant calcium ATPase sequence in the animal kingdom described to date. Previous analyses of 159 P-type ATPases have revealed the existence of five monophyletic families within the P-type ATPase superfamily [18]. The Ca^{2+}-ATPases belong to the type II ATPases among which type IIA or type IIB form clearly distinct groups. The SERCA pumps are the best characterized members of the type IIA subclass and are responsible for the accumulation of Ca^{2+} into the endoplasmic or sarcoplasmic reticulum. The PMCA Ca^{2+} pumps responsible for the export of Ca^{2+} out of the cell belong to the type IIB subclass. The overall sequence similarity of our predicted protein with previous cloned Ca^{2+}-ATPases and the conservation of major domains and critical motifs of the primary sequence unequivocally place stpPMCA in the IIB subfamily of P-type Ca^{2+}-transport ATPases.

The PMCA pump protrudes into the cytoplasm and consists of four main units: (1) the first 80–90 N-terminal amino acids; (2) the phospholipid interacting site; (3) the catalytic site; (4) and several regulatory sites, among them the calmodulin-binding domain and the substrate for protein kinases (for review see Ref. [15]). All these characteristics are found in stpPMCA.

While type IIA Ca^{2+}-ATPases are inhibited by a distinct protein from the pump itself, type IIB Ca^{2+}-ATPases are inhibited by an autoinhibitor, an inhibitory sequence within the pump molecule in the C-terminus region [25]. Removal of the entire C terminus results in a constitutively active

enzyme that could not be further activated by addition of calmodulin [25,26]. We showed that stpPMCA is able to bind calmodulin. In addition, the binding region is located in the C-terminal domain as suggested by the primary sequence of the deduced protein and experiments using proteins with C-terminal deleted regions. Previous cross-linking experiments with C-terminus region synthetic peptide have revealed that the autoinhibitory domain interacts with two sites within the catalytic core of vertebrate PMCA [27]. These two internal sites are conserved in coral PMCA. However, the (D/E)EID sequence, which is thought to play an essential role by stabilizing the inhibited state of PMCAs [28], is not found. This result suggests that the activity of the stpPMCA may be independent of calmodulin binding.

Four genes for the PMCA pump have been found in mammals [15], three in *C. elegans* [16], and only one in yeast [29]. The alternative splicing variants of these isoforms differ in their affinity for calmodulin [30] and expression is dependent on tissue type. In *S. pistillata*, we have identified that stpPMCA is specifically expressed in aboral tissue and not expressed in the oral ectoderm. On global levels, corals deposit large amounts of calcium carbonate, and thus require efficient calcium transport mechanisms to carry out high rates of biomineralization. In corals, it has been shown that the limiting step in calcium transport occurs at the level of the aboral ectoderm, called calicoblastic epithelium [4]. This transcellular pathway involves L-type Ca^{2+} channel proteins [4] of which the α1 subunit has been cloned [5]. Until now, little evidence of the mechanisms of calcium buffering inside the calicoblastic cells is available. Isa et al. [7] and Ip et al. [8] have shown Ca^{2+}-ATPase activity in whole homogenates of coral tissues. Here we demonstrate that the localization of stpPMCA within the calicoblastic cells is fully consistent with its role in calcification. Moreover, since PMCAs function as obligatory Ca^{2+}/H^{+} exchangers with a probable stoichiometry of 1 to 1 [31], we validate the hypothesis of McConnaughey [9,32,33] which proposes that the H^{+} generated by $CaCO_3$ precipitation is removed by Ca^{2+}-ATPase-mediated Ca^{2+}/H^{+} exchange.

The absence of expression of stpPMCA in oral ectoderm cells is intriguing since these cells, even if they are not involved in Ca^{2+} transepithelial transport [4], also need to maintain calcium homeostasis. We propose three hypotheses to explain this paradox: (1) the presence of an isoform of the stpPMCA; (2) the presence of a Na^{+}/Ca^{2+} exchanger; or (3) the presence of both.

Finally, the coral Ca^{2+} pump is more closely related to vertebrate PMCAs than to *C. elegans* PMCAs. This is similar to results observed for the Cnidarian mesoderm specification factor Twist which is significantly closer to vertebrate than drosophila or nematode cognate sequences [34]. Furthermore, the recent cloning of evolutionarily conserved genes from cnidarian species consistently shows that these genes encode highly conserved functional domains (for review see Ref. [35]). These domains are most likely involved in gene regulation, translational control, signal transduction, apoptosis, extracellular signalling, and cell/extracellular matrix interactions. With the cloning of a Ca^{2+} channel [5] and the present molecular characterization of a Ca^{2+}-ATPase, we can extend this principle of gene conservation to ionic transport functions. Cnidarians are the simplest living real metazoans (Eumetazoans) and diverged approximately 700 millions years ago from their bilaterian sister group [36]. This high level of gene conservation further validates the use of cnidarian models for studying processes shared by Eumetazoans and establishes Cnidarians as an excellent indicator of common ancestry.

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