Localization of calmodulin and calmodulin-like protein and their functions in biomineralization in *P. fucata*

Zi Fang a, Zhenguang Yan a, Shuo Li a, Qin Wang a, Weizhong Cao a, Guangrui Xu a, Xunhao Xiong a, Liping Xie a,b, Rongqing Zhang a,b,*

a Institute of Marine Biotechnology, Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China
b Protein Science Laboratory of the Ministry of Education, Tsinghua University, Beijing 100084, China

Received 27 August 2007; received in revised form 18 October 2007; accepted 19 November 2007

Abstract

Calmodulin (CaM) and calmodulin-like protein (CaLP) are two proteins involved in biomineralization. Their localizations in *Pinctada fucata* mantle epithelia were studied by Western blot (WB) analysis of the nuclear/cytosol fraction of primary cultured *P. fucata* mantle cells and immunogold electron microscopy. The results showed a completely different distribution of these two proteins at the subcellular level. CaM was distributed throughout both the nucleus and cytoplasm of the mantle epithelium but CaLP was distributed only in the cytoplasm. The functions of these two proteins in biomineralization were investigated by shell regeneration. During this process, the expressions of CaM and CaLP were greatly enhanced in different organelles of the mantle epithelium. Overexpression of these two proteins and a mutant of calmodulin-like protein (M-CaLP) that lacks an extra C-terminal tail in MC3T3-E1 promoted the mRNA expression of osteopontin, a biomineralization marker for osteoblasts. All of the results indicated that CaM and CaLP have completely different distributions in the mantle epithelium and affect the biomineralization process at different levels. The extra C-terminal tail of CaLP is important for its functions in biomineralization in *P. fucata*.

© 2007 National Natural Science Foundation of China and Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved.

Keywords: *Pinctada fucata*; Calmodulin; Calmodulin-like protein; Immunogold electron microscopy

1. Introduction

Biomineralization is a genetically controlled process that is directed by the formation of composite structures. This process is important for the formation and proper shaping of many hard parts such as pearl and bivalve molluscan shells, bone and teeth. The mantle is an important organ in pearl oyster biomineralization. The epithelium of the mollusca’s mantle is involved in the metabolism of bivalent cations [1], participating actively in the incorporation of calcium and some other elements from the extracellular pallial fluid into the shell [2]. Calcium, which is the most abundant mineral in this process, is involved in about half of the known biomineralized systems [3]. So the process of the metabolism of calcium, i.e., its absorption, transport, accumulation, secretion and deposition, has been studied extensively.

In the formation of mollusc shells and pearl, which are products of calcium metabolism, the deposition of calcium carbonate is a very complicated process highly controlled by many physiological and biochemical activities. It has been proven that calmodulin (CaM) constitutes at least 0.1% of the total protein in cells and is expressed at higher levels in rapidly growing cells. It mediates many basic cellular processes such as cell growth, differentiation, cyclic

---

*Corresponding author. Address: Institute of Marine Biotechnology, Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China. Tel.: +86 10 62772900; fax: +86 10 62772899. E-mail address: rqzhang@mail.tsinghua.edu.cn (R. Zhang).
nucleotide metabolism, glycogen metabolism and regulation of microtubule and synapsis formation [4–7].

Calmodulin-like protein (CaLP), a new member of the CaM superfamily, has drawn more and more attention in recent years. It acts as a multifunctional calcium sensor, and has been found in bacteria [8], nematodes [9], *Drosophila* [10], plants [11], chickens [12], rats [13] and humans [14–16]. Recent reports revealed that CaLP proteins were involved in epithelial cell differentiation [16,17], and in the regulation of Ca$^{2+}$--induced release of Ca$^{2+}$ in rat and human cell lines [18]. Sidhu and Guraya also reported that CaLP might be involved in calcium transport in buffalo sperm [19]. We also found a 161-amino acid calmodulin-like protein (CaLP) in *Pinctada fucata* that shows 67% identity and 87% similarity with the CaM protein [20]. In this study, CaM and CaLP cloned from *P. fucata* were investigated at the subcellular level and functions of these two proteins in biomineralization were investigated by shell regeneration. Together with the transient transfection of CaM, CaLP and M-CaLP, the last one is absent of an extra C-terminal tail, we found that both CaM and CaLP were involved in cell proliferation, and CaLP functioned more actively in differentiation and biomineralization than CaM. When the extra C-terminal tail of CaLP was truncated, the function of CaLP seemed to be lost, indicating that this extra C-terminal tail is crucial for the function of CaLP.

### 2. Materials and methods

#### 2.1. Antibody preparation

The polyclonal antibodies of CaM and CaLP (from *P. fucata*) were prepared and purified as described previously [21]. The antibodies against purified recombinant oyster CaM and CaLP were obtained by injection of 250 μg proteins in complete Freund's adjuvant into the rabbits, followed by two injections at a three-week interval. The whole blood was collected two weeks after the final injection and the antibodies were purified from the blood by HiTrap Protein G chromatography (Amersham Pharmacia Biotech) and stored at −80 °C.

To avoid the cross-reaction of these two proteins with a high similarity, the two antibodies were tested with the total mantle protein and the purified CaM and CaLP, respectively, as described previously [21].

#### 2.2. Localization of CaM and CaLP in primary cultured mantle cell

*Pinctada fucata* with shell lengths of 55–60 mm were collected in Beihai, Guangxi Province, China. The pearl oysters were transported to the laboratory and kept for one week at 20 °C, with constant aeration without being fed in order to reduce microbial contaminants before dissection. The primary culture of *P. fucata* was performed as previously reported [22–24]. The culture medium used in this experiment was 1 × L-15 dissolved in fresh seawater and the medium was changed every 2 days. On the 7th day after initiation of mantle cell culture, epithelial-like cells formed a monolayer on the poly-L-lysine-coated dishes and were lysed for subcellular fraction analysis using a nuclear/cytosol extraction kit (Tianlai, Beijing, China). Samples were then processed for Western blot analysis with antibodies of CaM and CaLP.

#### 2.3. Subcellular localization of CaM and CaLP in mantle epithelium

Small pieces (1 mm × 1 mm) of mantle located under the periostracum groove where biomineralization initiates [25] were fixed at 20 °C for 3 h with 1.5% glutaraldehyde and 3.4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The following steps were performed according to Caceres et al. described before [6] with some modification. Anti-rabbit serum conjugated with 12-nm diameter gold (Jackson ImmunoResearch Lab.), diluted in PBG–Tween (1:20), was used as the secondary antibody in this study and the samples were observed under the Philips CM120 Biotwin transmission electron microscope.

#### 2.4. Expression of CaM and CaLP during the shell regeneration

The rapid shell regeneration model was made as described previously [26]. The mantle was treated carefully to avoid the immune response. Newly formed shell was observed as a light brown to dark purple leather-like material and the mantles under the notched shell were taken as samples for Western blot hybridization, reverse transcription polymerase chain reaction (RT-PCR) and immunogold electron microscopy observations.

##### 2.4.1. Western blot analysis of CaM and CaLP

Western blot analysis was performed on crude protein extracts from oyster mantle samples taken at different time points of shell regeneration. Western blot analysis was conducted every day for a period of 7 days. β-Actin was used as the internal control and the ImageJ semi-quantitative image analysis software was used to analyze the comparative expression level during the course of shell regeneration.

##### 2.4.2. RT-PCR analysis of CaM and CaLP

Total RNA was prepared from mantle samples taken every day and expression levels of CaM and CaLP were determined by RT-PCR. RT-PCR conditions were referred to what had been described previously [20,21]. *GAPDH* was used as the internal control [27]. The primers used are shown in Table 1. Amplification products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. ImageJ semi-quantitative image analysis software was used to analyze the comparative mRNA level.
2.4.3. Immunogold electron microscopic observation

Mantle slices beneath the notched shell were taken as samples for immunogold electron microscopic observations. The sample's preparation was the same as that in the section of “Subcellular localization of CaM and CaLP in mantle epithelium”.

2.5. Detection of CaM, CaLP and M-CaLP overexpressed in transfected MC3T3-E1 cells

Effects of CaM, CaLP and M-CaLP on proliferation, differentiation and biomineralization were further investigated in MC3T3-E1 cells transfected with different eukaryotic expression vectors.

2.5.1. Cell culture

The mouse osteogenic cells MC3T3-E1 [28] were maintained in DMEM supplemented with L-glutamine, 10% fetal bovine serum (FBS, Hyclone), penicillin (100 IU/ml) and streptomycin (100 mg/ml) in a humidified 5% CO₂ atmosphere. Cells between passages of 6 and 10 were used for all the following experiments.

2.5.2. Construction of expression vectors and transient transfection

Expression vectors of pcDNA3.1(+)/CaM, pcDNA3.1(+)/CaLP and pcDNA3.1(+)/M-CaLP were constructed by amplifying the full-length sequences of CaM, CaLP and M-CaLP using high fidelity PCR with primers containing multiple cloning sites (MCS) of XhoI/BamHI (Table 2), which were inserted into the pcDNA3.1(+) plasmid, and were confirmed by DNA sequencing.

MC3T3-E1 cells were seeded in 24-well plates at a density of 1 × 10⁵/well the day before transfection. Transfection was carried out using Lipofectamine2000 (Invitrogen) transfection reagent according to the manufacturer’s instructions. pEGFP was cotransfected into the cells as a control. On the 1st day after transfection, designated as day 1, the media was removed and replaced with 2% FBS-containing media plus 50 mg/ml ascorbate, which would promote osteoblast differentiation [29,30]. The expressions of the three proteins were determined by RT-PCR.

2.5.3. Cell proliferation assay

The effects of CaM, CaLP and M-CaLP on the proliferation of MC3T3-E1 cells were assayed by MTT staining [31]. The assay was performed at least three times.

2.5.4. Assay for alkaline phosphatase (ALP) activity

After 48 h transfection with the recombinant expression plasmids (pcDNA3.1(+)/CaM, pcDNA3.1(+)/CaLP and pcDNA3.1(+)/M-CaLP), the MC3T3-E1 cells were harvested. ALP activity was measured by the method of Almeida et al. [32]. The assay was performed at least three times.

2.5.5. RT-PCR analysis of osteopontin after transfection

Since osteopontin is a biomineralization marker for osteoblasts, we performed RT-PCR for the effects of the three transfected plasmids on concomitant upregulation of OP gene. Primers for amplification of OP and GAPDH which acted as an internal control are indicated in Table 3. Amplification products were resolved by electrophoresis on a 1.5% agarose gel and visualized by EB staining.

2.6. Statistical analysis

The statistical analysis was performed using Student’s t-test for comparison of cell proliferation and ALP activity assays in different groups of transfected cells. The data are presented as means ± SE.

3. Results

3.1. Antibody purification and specificity test

To avoid the cross-reaction between CaM and CaLP antibodies and the proteins, the purified antibodies of CaM and CaLP were tested by Western blot analysis. The negative cross-reaction results indicated that the two antibodies were specific for CaM and CaLP, respectively.

3.2. Distribution of CaM and CaLP in primary cultured mantle cell

Forty-eight hours after initiation of the mantle cell cultures, typical fibroblast-like cells and epithelial-like cells...
were observed to migrate from the explant (Fig. 1(a)). On the 7th day after initiation of mantle cell culture, the epithelial-like cells formed a monolayer on the poly-L-lysine-coated dishes (Fig. 1(b)). Western blot on the extracts of cultured cells showed that CaM was distributed throughout the nucleus and cytoplasm of the mantle epithelium but CaLP was distributed only in the cytoplasm (Fig. 2). Immunogold electron microscopy confirmed this distribution pattern (Fig. 3).

3.3. Expression of CaM and CaLP during shell regeneration

Western blot analysis indicated that, compared with the mantle before the notching, the expressions of CaM and CaLP were activated and reached the highest level on the 3rd and 4th day (Fig. 4(a)). Moreover, results of the expression analysis by RT-PCR at mRNA level were almost the same as what we obtained by Western blot, only that the highest level was reached on the 2nd and 3rd day (Fig. 4(b)).

Localization of CaM and CaLP by immunogold electron microscopy demonstrated that during the shell regeneration, the CaM existed mainly in the nucleus, mitochondria and ER, while CaLP existed mainly in the secretory vesicles (Fig. 5).

3.4. Biological functions of CaM, CaLP and M-CaLP in transfected MC3T3-E1 cells

3.4.1. CaM, CaLP and M-CaLP promote proliferation of MC3T3-E1 cells

We examined the cell proliferation-promoting effects of CaM, CaLP and M-CaLP in transfected MC3T3-E1 cells. Cell proliferation was analyzed by MTT assay. As shown in Fig. 6, transfections of CaM, CaLP or M-CaLP, particularly CaM, significantly promoted cell proliferation when compared with cells transfected with the empty vector. Furthermore, the proliferation-promoting effect of M-CaLP was more significant than that of wild-type CaLP. These results indicated that CaM plays a more significant role than CaLP in MC3T3-E1 proliferation.

3.4.2. ALP activity in transfected MC3T3-E1 cells

Alkaline phosphatase is thought to be important to biomineralization [33,34] and often acts as a phenotypic marker for the osteoblast. Previous studies suggested that many cytokines induced MC3T3-E1 cells to develop into osteoblast-like cells in vitro and expressed alkaline phosphatase [35]. Our results showed that MC3T3-E1 cells transiently transfected by CaLP experienced a significant increase in ALP activity, but the MC3T3-E1 cells transfected by CaM and M-CaLP did not exhibit that significant increase when compared with those transfected by CaLP (Fig. 7). This difference suggested that CaLP plays a significant role in cell differentiation.

3.4.3. Expression of OP in transfected MC3T3-E1 cells

As shown by RT-PCR analysis (Fig. 8), CaM and CaLP transfected cells (especially CaLP) showed higher mRNA levels of OP than empty vector transfected cells. But the M-CaLP, which lacks the C-terminal, obviously exhibited much lower OP mRNA level compared with wild-type CaLP. These data indicated that expression of CaLP stimulated the expression of the bone matrix protein OP in MC3T3-E1 cells.

4. Discussion

Previous research on calmodulin indicated that it directly affects osteoblast differentiation by mediating the Ca²⁺ signal to a multitude of different enzyme systems [36–41]. Localization of calmodulin has been investigated in a broad range of different tissues and cultured cells,
and it is closely related with tissue types, physiological processes and developmental stages. For example, in the epidermis of the *Salmo trutta*, the calmodulin is localized in the superficial epithelial cells, indicating that it might have physiological significance in the activation of the microvillar skeleton and play an important role in controlling the permeability of the skin epithelium [42]. In mammalian tissues such as rat’s liver and cortex, calmodulin is localized...
in the cytoplasm and nucleus where it plays an important role in the nuclear process and glycogen metabolism [43]. In mitotic cells, it is in the mitotic spindle and is excluded from the nucleus in interphase cells but there is no specific enrichment in the cytoplasm [44]. The localization of calmodulin in spindle pole body of *Schizosaccharomyces pombe* indicated that it plays an essential role in chromosome segregation [45].

Preliminary studies of physiological process of shell regeneration showed that 6 days were required for a thin sheet of mineralized tissue to cover the shell notch [46] and initial mineral deposition occurred about 24–48 h after the creation of the notch in the marine snail, *Tegula* [47]. In the present study, RT-PCR analysis of CaM and CaLP confirmed the time course of the shell regeneration in *P. fucata*. Localization of CaM in the intact mantle and its obvious increase in the nucleus, mitochondria and endoplasmic reticulum (ER) after the shell regeneration indicated that

Fig. 5. Subcellular localization of CaM (a, b and c) and CaLP (d) in epithelium of mantle during shell regeneration induction. (a) Expression of CaM (arrows) increased obviously in nucleus (N); (b) and (c) expression of CaM (arrows) increased obviously in endoplasmic reticulum (ER) and mitochondria (m); (d) expression of CaLP (arrow) increased obviously in secretory vesicles (SV).

Fig. 6. Expression of CaM, CaLP and M-CaLP in MC3T3-E1 cells. The effect of CaM was the most significant and CaLP did not significantly promote cell proliferation, *p* < 0.01.

Fig. 7. ALP activity in MC3T3-E1 cells transfected with CaM, CaLP and M-CaLP. ALP activity was highest in the CaLP experimental group, *p* < 0.01.

Fig. 8. RT-PCR for *OP* which is the biomineralization-related gene after the transient transfection of CaM, CaLP and M-CaLP in MC3T3-E1 cells. Compared with the control, which was transfected with vectors, the CaM and CaLP transfected cells showed higher levels of *OP* mRNA, especially the CaLP transfected cells. But the effect of M-CaLP obviously lost the stimulating functions compared with CaLP.

Fig. 8. RT-PCR for *OP* which is the biomineralization-related gene after the transient transfection of CaM, CaLP and M-CaLP in MC3T3-E1 cells. Compared with the control, which was transfected with vectors, the CaM and CaLP transfected cells showed higher levels of *OP* mRNA, especially the CaLP transfected cells. But the effect of M-CaLP obviously lost the stimulating functions compared with CaLP.
CaM actively takes part in the shell regeneration. The transient transfection of CaM in MC3T3-E1 cells clearly confirmed its functions in cell proliferation.

Biomineralization systems such as coral, tooth, eggshell and shell of mollusca revealed that a number of small, round secretory bodies were responsible for the biomineralization process [48]. Coral calcicoblast cells contain membrane bound vesicles with homogeneously fine granular contents and these secretory vesicles may be the sites of organic matrix synthesis [49]. There are many secretory vesicles present in the newly deposited predentin matrix in teeth [50] and inside the eggshell mantle [51]. We found an obviously discrepant localization of CaLP from the CaM, especially the enrichment of CaLP in secretory vesicles after the shell regeneration, which indicated the important effects of CaLP in biomineralization, though the precise mechanism of it needs to be unveiled.

Several biomineralization markers for bone cell differentiation and mineralization have been identified such as osteopontin (OP), osteonectin (ON) and osteocalcin (OC). OP, a highly acidic phosphoprotein, is synthesized and secreted by osteoblasts. OP has been demonstrated as a multifunctional protein that can trigger signal transduction processes in osteoblasts [52], regulate fibroblast cell attachment and the calcification of extracellular matrix [53]. OP production is frequently augmented when cell attachment and the calcification of extracellular matrix [48]. OP mRNA expression of osteopontin (OP) is significantly enhanced by Ca 2+/Mg 2+. A putative prenylation site. DNA Res 1999;6(3):179–81.

Acknowledgments

This work was financially supported by the National High Technology Research and Development Program of China (2006AA09Z413, 2006AA09Z441, 2006AA10A415), and National Natural Science Foundation of China (Grant No. 30530600).

References


