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

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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*.

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

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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.