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Significance of the C-terminal globular domain and the extra tail of the calmodulin-like protein (*Pinctada fucata*) in subcellular localization and protein–protein interaction

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Abstract

Calmodulin (CaM) plays a very important role in many physiological processes and is highly conserved in different species. In a previous study, we successfully cloned CaM and a novel calmodulin-like protein (CaLP) with an extra C-terminal sequence from the pearl oyster *Pinctada fucata* and *then expressed in Escherichia coli*. In this research, we used fluorescence confocal microscopy to analyze the protein–protein interaction between CaM/CaLP and $p21^{Cip1}$, which is cloned from mammalian cells, to show the different characteristics of these two proteins *in vivo*. The fluorescence confocal microscopy showed that the C-terminal globular domain together with the extra tail of CaLP is very important in CaLP's sequestration in cytoplasm. The most interesting phenomenon is that transfection of $p21^{Cip1}$ can stimulate translocation of CaLP from the cytoplasm to the nucleus, but this is not the case for CaM. Fluorescence confocal microscopy and co-immunoprecipitation on different mutants of CaLP with $p21^{Cip1}$ indicated that the C-terminal globular domain of CaLP is responsible for the trafficking of CaLP from cytoplasm to nucleus.

Keywords: *Pinctada fucata*; Calmodulin; Calmodulin-like protein; $p21^{Cip1}$; Nucleocytoplasmic trafficking; ERK

1. Introduction

Calcium has been shown to be necessary for many physiological processes such as muscle contraction, the cell cycle and cyclic nucleotide metabolism (Ringer, 1883; Janis and Triggle, 1983; Means, 1994; Zufall et al., 1994). Research over the past 50 years has revealed that intracellular Ca^{2+} mediates a large number of cellular responses with the high affinity and specificity required for a regulatory second messenger. Calmodulin (CaM) is a Ca^{2+}-binding protein that acts as a transducer of intracellular Ca^{2+} signals (Chin and Means, 2000; Means and Dedman, 1980). When bound to Ca^{2+}, CaM is able to bind to CaM-binding proteins (CaMBPs), directly regulating their activities (Klee and Vanaman, 1982; Weinstein and Mehler, 1994; Ikura, 1996). Through the action of these CaMBPs, such as CaMK, calcineurin, hnRNP, cyclic nucleotide phosphodiesterase, adenylate cyclase, Ca^{2+}-ATPase and others, CaM regulates a great variety of cellular processes, such as gene expression, protein translation and protein phosphorylation (Reddy et al., 1996; Xia and Storm, 1997; Agell et al., 1998; Klee et al., 1998; Kakkar et al., 1999; Soderling, 1999; Means, 2000; Villalonga et al., 2001).

In recent years, more and more target proteins of CaM have been found, including caldesmon, β-arrestin, $p21^{Cip1}$ and EGFR (Smith et al., 1987; Crivici and Ikura, 1995; Martín-Nieto and Villalobo, 1998; Taulés et al., 1999; Wu et al., 2006). The cyclin-dependent kinase (cdk) inhibitor $p21^{Cip1}$ is

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a protein with important roles in cell proliferation, differentiation and apoptosis (Sherr and Roberts, 1999; Gartel and Tyner, 2002; Christina and Means, 2003). Although it does not have catalytic activity, it interacts with a broad range of other proteins, thereby regulating their activities (Dotto, 2000). As for the function of CaM in mediating the cell cycle and in cell differentiation, it has been found that the binding of CaM to the C-terminal of $p21^{Cip1}$ specifically (Rodríguez-Vilarrupla et al., 2005) may be essential to regulate nuclear translocation of Cdk4 and cyclin D (Taulés et al., 1998); thus CaM is responsible for the phosphorylation of pRb (Takuwa et al., 1993; Taulés et al., 1998). During these physiological processes, ERK activation determines the direction of proliferation or differentiation, and an inhibitor of CaM was found to induce sustained activation of ERK and expression of $p21^{Cip1}$ (Bosch et al., 1998).

Localization of CaM has been investigated in a broad range of different kinds of tissues and cultured cells (Harper et al., 1980; Zaccone et al., 1989). Although most CaMBPs are identified as originating in the cytoplasm, CaM has very important functions in the nucleus. A relatively high concentration of CaM has been found in the nuclei of all cell types (Bachs et al., 1992) and several lines of evidence have shown that many transcription factors are regulated by CaM, suggesting its role in gene expression (Szymanski et al., 1996; Onions et al., 1997).

In *Pinctada fucata*, we previously successfully cloned CaM and a novel calmodulin-like protein (CaLP) that shows 67% identity and 87% similarity with the CaM protein. Some comparisons between CaM and CaLP have been done *in vitro* and the results showed that the extra tail of CaLP can significantly decrease the exposure of the hydrophobic patches in CaLP. CD results demonstrated that the target binding proteins of CaLP were greatly influenced by this extra tail (Li et al., 2005, 2006). We constructed different expression constructs of CaM, CaLP and their mutants. After these proteins and mutants were transfected into HEK 293T cells, different subcellular localizations were analyzed. The most interesting phenomenon was that, when co-transfected with $p21^{Cip1}$, CaLP was stimulated from cytoplasm to nucleus, but this was not the case for CaM. After fluorescence confocal microscopy investigation of different mutants and co-immunoprecipitation of $p21^{Cip1}$ with mutants of CaLP, it was shown that the extra tail of CaLP together with the C-terminal of CaLP is responsible for its sequestration in the cytoplasm and the C-terminal globular domain of CaLP is responsible in the trafficking of CaLP from cytoplasm to nucleus. The trafficking mechanism may be phosphorylation of CaLP affected by the overexpression of $p21^{Cip1}$ or its interaction with other binding proteins; this is still waiting to be investigated.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Expression plasmids for all the green fluorescent fusion proteins were constructed by PCR from cDNA of *P. fucata*. The forward and reverse oligonucleotides for (GFP)-CaM-WT (wild type) are 5′-CCgCTCgAgCTATggCCgATCAgCTgACAgAg-3′ and 5′-CggggTACCTCATTTCgACATCATCATTTT-3′; for (GFP)-CaLP-WT (wild type) amplification the forward and reverse oligonucleotides are 5′-CCgCTCgAgCTATggCggAAgATCTCACAgAA-3′ and 5′-CggggTACCTCATTTATTTTCTTgTTgCTg-3′. The CaLP-Δ 150−161 deletion mutant of wild type CaLP was amplified using the same forward primer of (GFP)-CaLP-WT; the terminal reverse oligonucleotide of 5′-CggggTACCTCATTTCATTgAAATCATTTTgAC-3′ was used to amplify the C-terminal. The other deletion mutant CaLP-Δ 79−161 of wild type CaLP was amplified using the forward primer of 5′-CCgCTCgAgCTATggCggAAgATCTCACAgAA-3′ and the reverse primer of 5′-CggggTACCTCATTTCATTTTCTTAgCCATCAT-3′. The deletion mutant, named CaLP-Δ 1−78, was amplified by using the same reverse oligonucleotide as CaLP-WT to amplify the C-terminal and 5′-CCgCTCgAgCTATggACACCgACTCggAAgAggAA-3′ to amplify the N-terminal. Recombinant mutant CaM + tail was constructed by adding the tail of CaLP (aa150−161) to the C-terminal of CaM. Amplification was performed by two-step PCR using the same forward primer as (GFP)-CaM-WT and the two reverse primers of 5′-TTCTTCTTgATCggTgTCTTTCgACATCATCATTTTT-3′ and 5′-ggggTACCTCATTATTTTCTTgTTgCTgTTCTTCTTgATCggT-3′.

Expression plasmid for red fluorescent protein (Red) − $p21^{Cip1}$ fusion protein was constructed by PCR from human cDNA. The forward and reverse primers are 5′-CCgCTCgAgATgTCAgAACCggCTggggAT-3′ and 5′-CggggTACCgTgggCTTCCTCTTggAgAAgAT-3′. All of the primers containing the MCS of XhoI and KpnI allowed the insertion of amplified fragments into the MCS of pEGFP-C_1 and DsRed-N_1 vectors (Invitrogen). The wild type and mutants are illustrated in Fig. 1.

2.2. Cell culture and transfection

HEK 293T cells were maintained in DMEM supplemented with 10% FBS, 1.5 g/l $NaHCO_3$ and 100 U penicillin/streptomycin solution at 37 °C in 5% CO_2 and 95% air on 35 mm

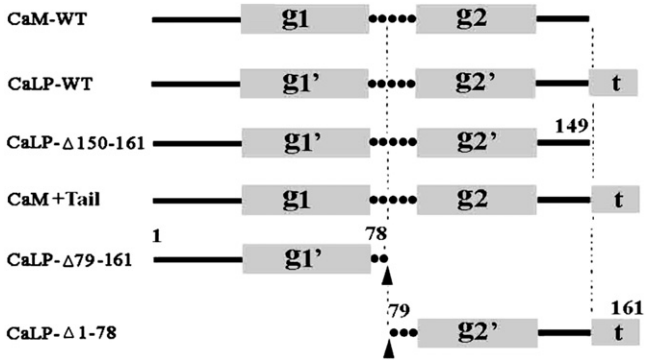

Fig. 1. Schematic drawing showing the domains and sequence of different GFP proteins. (g_1) N-terminal globular domain of CaM-WT; (g_2) C-terminal globular domain of CaM-WT; (g_1') N-terminal globular domain of CaLP-WT; (g_2') C-terminal globular domain of CaLP-WT; (t) extra tail of CaLP-WT compared with CaM-WT.