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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

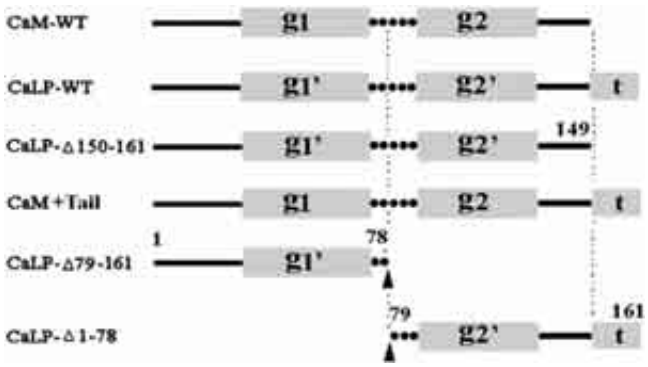

Fig. 1. Schematic drawing showing the domains and sequence of different GFP proteins. (g_1) N-terminal globular domain of CaM-WT; (g_2) C-terminal globular domain of CaM-WT; (g_1') N-terminal globular domain of CaLP-WT; (g_2') C-terminal globular domain of CaLP-WT; (t) extra tail of CaLP-WT compared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

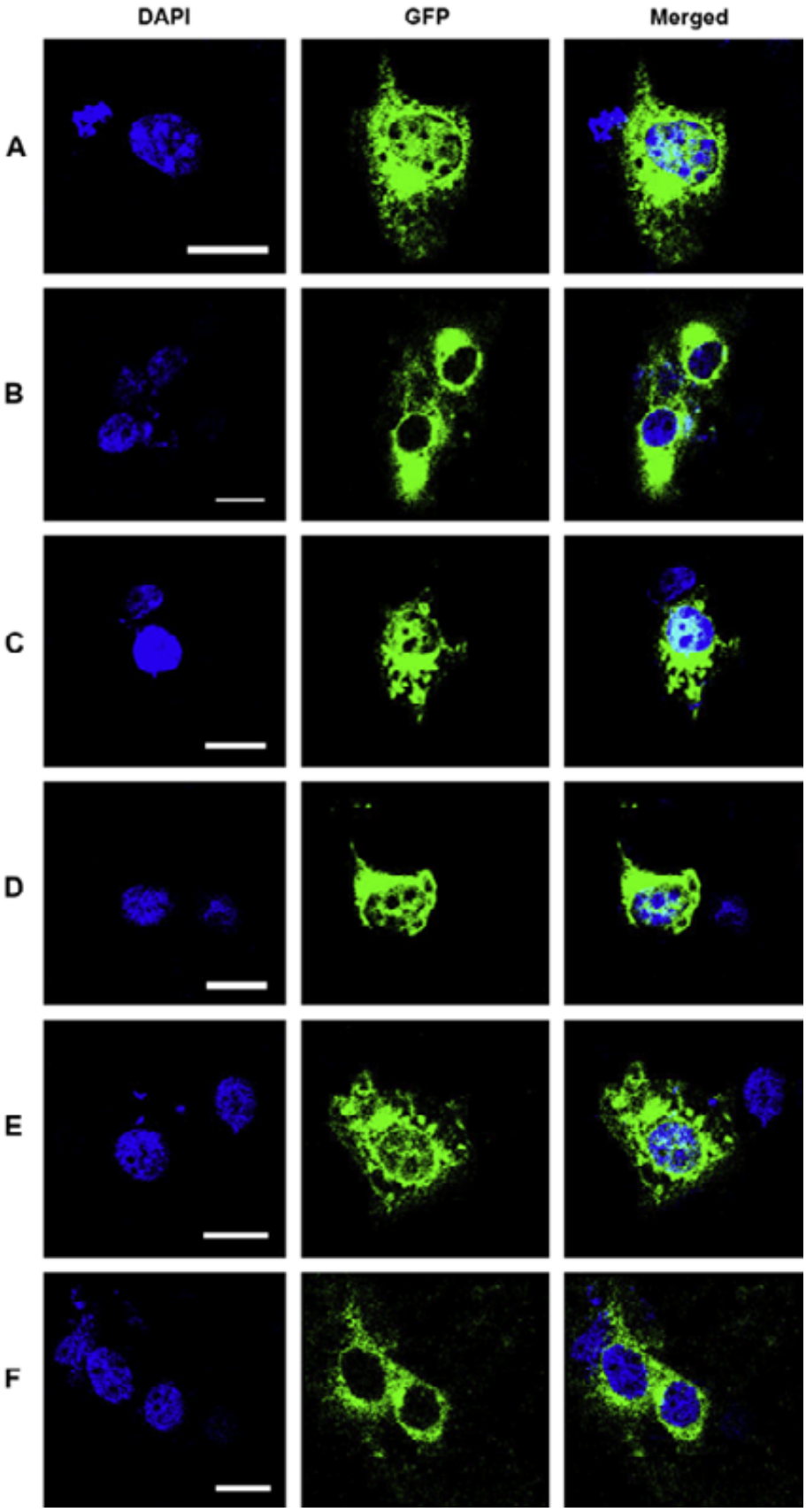

Fig. 2. Subcellular localization of wild type CaM, CaLP and different mutants. (A) Subcellular localization of wild type CaM. HEK-293T cells were transfected with pEGFP-CaM-WT and were visualized by fluorescence microscopy 24 h after the transfection. (B) Cytoplasmic localization of wild type CaLP. (C−F) Localization of expressed mutant CaLP fusion proteins was analyzed by confocal microscopy. (C) Reconstitution of CaLP-Δ 150−161 distribution. (D) Subcellular localization of CaM was not affected by the adding of the tail of CaLP. (E) CaLP transfer into nucleus after truncation of the C-terminal globular domain and extra tail. (F) C-terminal globular domain and extra tail of CaLP ensure its staying in the cytoplasm. Nuclei were counterstained with DAPI (blue); scale bar: 10 μm.

dishes. Mammalian expression vectors were transfected into 293T cells using Vigorous transfection reagent (Vigorous Biotechnology, PR China) in accordance with the manufacturer's instructions. For transient transfection, 10 μg of DNA plus 4 μl transfection reagent were added to 293T cells (>40% confluent for fluorescence confocal microscopy and >90% confluent for co-immunoprecipitation, respectively) in 35 mm dishes.

2.3. Fluorescence confocal microscopy

For intracellular localization analysis of EGFP and DsRed fusion proteins, cells were grown on coverslips and fixed in a mixture of cold acetone and methanol (1:1) for 5 min at −20 °C after 24 h transfection. Images were visualized with an Olympus inverted microscope equipped with a charge-coupled camera (Olympus FV500).

2.4. Preparation of nuclear and cytoplasmic fractions

After 24 h transfection, subcellular fractionation of 293T cells was separated by using a nuclear/cytosol extraction kit (Tianlai, Beijing, PR China) following the instructions of the manufacturer. In brief, after transfection of different expression plasmids for 48 h, the cells were scraped into a 1.5 ml centrifuge tube and centrifuged for 5 min at 800*g*. The pellets were washed one more time and the cells were collected by centrifugation. The pelleted cells were resuspended in 500 μl of Cytosol Extraction Buffer A (CEB-A) and incubated on ice for 20 min. The cells were then vortexed for 10 s every 5 min and centrifuged at 1000*g*for 5 min at 4 °C. The pellets contained crude nuclei. The supernatant was transferred to a new tube and further centrifuged at 12,000*g* for 5 min at 4 °C. The supernatant was cytosol extracted, and was transferred to a new tube. Glycerol was added to 20% and stored at −70 °C until use.

The crude nuclei pellets prepared in the previous step were used to extract the nuclei protein. After the membrane components contaminated in the cytosol extraction had been removed, 100 μl of cold nuclear extraction buffer (NEB) was added into the crude nuclei pellet, vortexed and incubated on ice for 30 min, followed by centrifugation at 12,000*g* for 5 min at 4 °C. The supernatant fraction containing the proteins extracted from the nucleus was transferred to a clean tube and stored at −70 °C.

2.5. Co-immunoprecipitation

Forty-eight hours after transfection, the cells were lysed in lysis buffer (Beyotime Institute of Biotechnology, PR China) containing 1 mM PMSF. One minute after lysis, cell extracts were spun down at 14,000*g* for 10 min at 4 °C. The extracts from each transfection were pooled into a 1.5 ml tube. For co-immunoprecipitation, the extracts were exposed to 2 μg antibody of EGFP and incubated with end-over-end mixing for 2 h at 4 °C. Protein A sepharose was then added and mixed for 1 h at 4 °C. After the immunoprecipitates had been washed five times with 1 ml of lysis buffer, the protein A sepharose beads were collected and suspended in 2 × SDS sample buffer, boiled for 5 min and subjected to Western blotting analysis with antibody against $p21^{Cip1}$.

2.6. Gel electrophoresis and immunoblotting

Proteins were electrophoresed on 15% SDS–PAGE in the presence of 2.5 mM Ca^{2+}. The sample buffer was added with 2.5 mM Ca^{2+}. After electrophoresis, the proteins were transferred to PVDF membrane and kept for 2 h at 250 mA. The membrane was then preincubated in TBS containing 0.05% Tween 20 and 3% BSA for 1 h at room temperature and then incubated for 1 h at room temperature in TBS, 0.05% Tween 20 and 0.5% BSA containing specific antibodies (Santa Cruz). After washing in TBS, 0.05% Tween 20 (three times, 10 min each), the membrane sheets were incubated with peroxidase-coupled secondary antibody (Santa Cruz) for 1 h at room temperature. After incubation, the membrane sheets were washed three times in TBS and the reaction was visualized by ECL (Vigorous).

3. Results

3.1. C-terminal of CaLP ensures the sequestration of protein in the cytoplasm

To gain insight into the biological difference between CaM and CaLP, we analyzed the subcellular localization of these two proteins. Fluorescent imaging of GFP-CaM and GFP-CaLP in HEK 293T cells indicated that CaM was distributed ubiquitously in the cells and CaLP was primarily a cytosolic protein (Fig. 2), implying that CaM and CaLP may play different roles in cells. In our previous study of CaM and CaLP cloned from *P. fucata,* CaLP showed 67% identity and 87% similarity with CaM. But the gene expression analysis of *in situ* hybridization, Ca^{2+}-dependent affinity chromatography by affinity columns and circular dichroism spectroscopy in

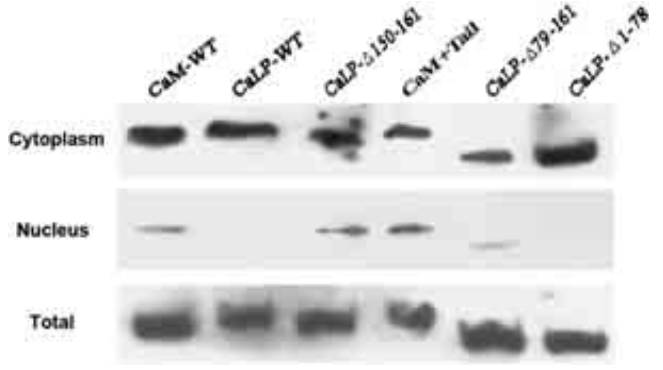

Fig. 3. Localization of CaM, CaLP and the mutants in cytoplasm and nuclear cell fractions in 293T cells. Proteins within various fractions of 293T cells were analyzed by SDS–PAGE and immunoblotting. Upper lane: cytoplasm fractions for fusion proteins. Middle lane: nucleus fractions for fusion proteins. Lower lane: total expression of exogenous GFP protein. The antibody for GFP was used for the Western immunoblots and the figure shown is representative for three experiments.

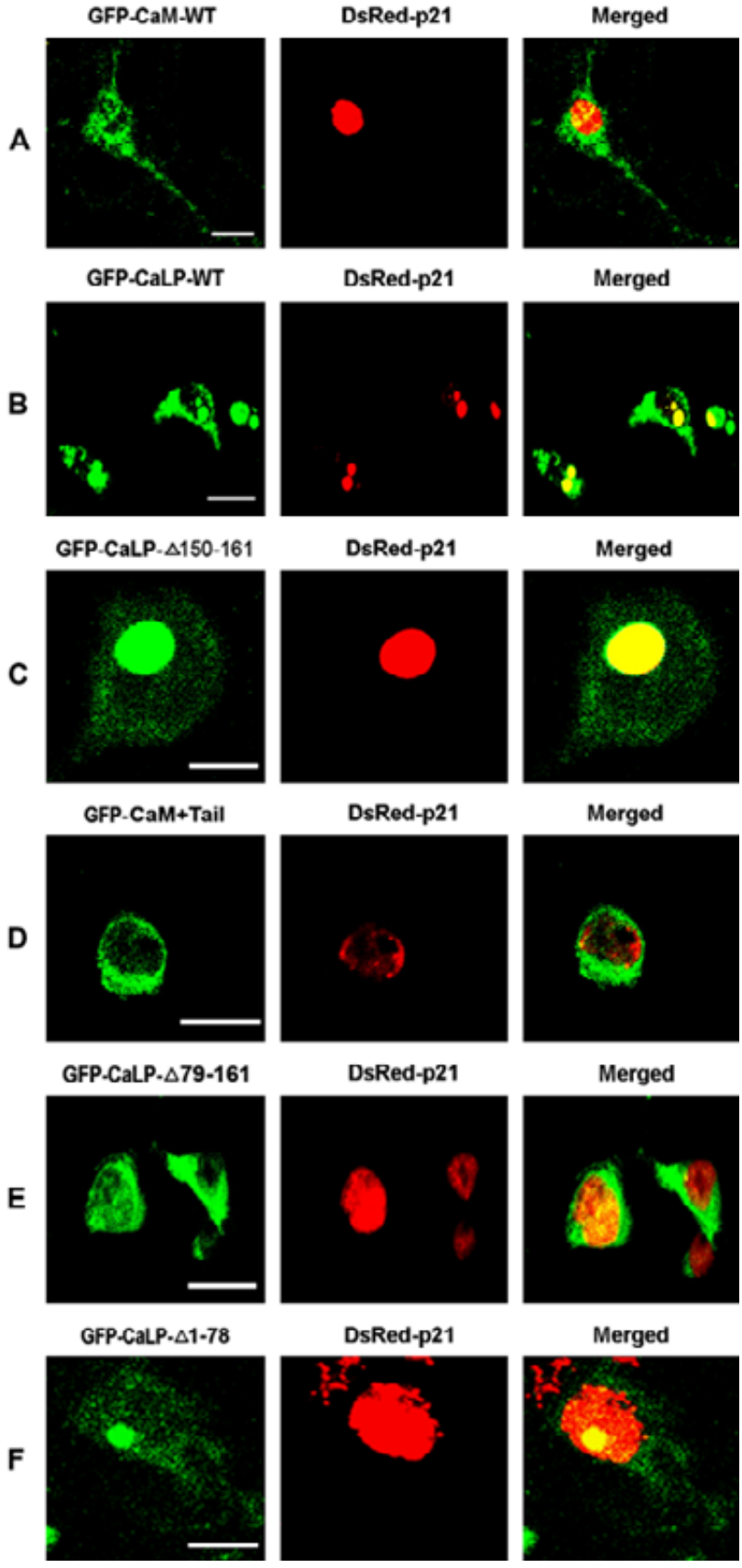

Fig. 4. Subcellular localization of $p21^{Cip1}$ with CaM, CaLP and their mutants. (A, D, E) Subcellular localization of CaM, CaM + tail and CaLP-Δ 79–161 was not affected by co-transfection with $p21^{Cip1}$. (B, C, F) CaLP-WT, CaLP-Δ 150–161 and CaLP-Δ 1–78 were stimulated by $p21^{Cip1}$ to translocate from the cytoplasm to the nucleus. Scale bar: 10 μm.

Ca^{2+}/Mg^{2+}-induced conformational changes showed the differences between these two proteins *in vitro* (Li et al., 2006). It was not very surprising to discover the ubiquitous distribution of CaM-WT because of the high conservation and extensive occurrence of CaM localization in many species. What interested us most was the cytoplasmic localization of CaLP-WT. To locate the domain responsible for the localization of CaLP, different mutants were constructed to identify the domain responsible for its sequestration in cytoplasm. It was observed that the CaLP-Δ 150–161 deletion mutant, which was truncated in the tail, lost the ability to stay in the cytoplasm. To determine the effect of the molecular weight of the extra tail of CaLP-WT, which may prevent the translocation of CaLP-WT into the nucleus through the nuclear pore complexes (NPCs), CaM + tail, which had the extra tail of CaLP added to the C-terminal of CaM, was constructed and the distribution of CaM + tail was the same as that of CaM. Previous NMR experiment showed that the two globular domains of CaM are connected by a flexible central nonhelical domain (Ikura et al., 1991). So in our research, we separated the CaM (*P. fucata*) into two segments from 79aa as indicated in research of Ikura et al. (1991). The CaLP-Δ 1–78 mutant with the truncated N-terminal globular domain of CaLP-WT, which still had the C-terminal globular domain and the extra tail, stayed in the cytoplasm; however, the CaLP-Δ 79–161 mutant with the truncated C-terminal globular domain and extra tail of CaLP-WT was distributed throughout the cytoplasm and nucleus. The results of immunoprecipitation of nuclear and cytoplasmic fractions of the wild type and these mutants further confirmed the significance of the C-terminal globular domain of CaLP together with the extra tail in the localization of protein (Fig. 3). From the results of the fluorescence confocal microscopy, it is proposed that the extra tail together with the C-terminal globular domain act to sequester CaLP in the cytoplasm.

3.2. Overexpression of $p21^{Cip1}$ stimulates CaLP translocation from cytoplasm to nucleus

It was reported that $p21^{Cip1}$ binds with CaM directly in mammalian cells (Taulés et al., 1999; Rodríguez-Vilarrupla et al., 2002, 2005). To investigate if the CaM-WT cloned from *P. fucata* also has the characteristic of binding with $p21^{Cip1}$, subcellular co-localization of the overexpression of $p21^{Cip1}$ with CaM in HEK 293T was performed by fluorescence confocal microscopy and co-immunoprecipitation. The results showed that the wild type CaM cloned from *P. fucata* can also bind with $p21^{Cip1}$ *in vivo*. When co-transfected with $p21^{Cip1}$, it was very interesting to find that CaLP could translocate from the cytoplasm to nucleus and co-localize with $p21^{Cip1}$ (Fig. 4). To further investigate the different domains of CaLP responsible for the translocation, different pEGFP mutants as mentioned above were co-transfected with $p21^{Cip1}$. As shown in Fig. 4, when co-transfected with $p21^{Cip1}$, (GFP)-CaLP-Δ 150–161 could be translocated into the nucleus and co-localize with $p21^{Cip1}$; the same phenomenon happened with CaLP-Δ 1–78. But when co-transfected with CaLP-Δ 79–161, which was missing the C-terminal globular domain, the recombinant protein lost the ability to stay in the nucleus and could not co-localize with $p21^{Cip1}$. CaM + tail, which was constructed by adding the tail of CaLP (aa150–161) to the C-terminal of CaM, remained throughout the cytoplasm and nucleus and was not affected by the transfection of $p21^{Cip1}$.

Co-immunoprecipitation was also performed to confirm the interaction between the domains of CaLP and $p21^{Cip1}$. As shown in Fig. 5, CaM-WT, CaLP-WT, CaLP-Δ 150–161 and CaLP-Δ 1–78 could be co-immunoprecipitated with $p21^{Cip1}$ but CaLP-Δ 79–161 could not interact with $p21^{Cip1}$ *in vivo*. It is very interesting to notice that though CaM-WT could be co-immunoprecipitated with $p21^{Cip1}$, when the tail of CaLP was added onto CaM, it could not be interacted with p21. In this case, we propose that the tail of CaLP may have some interaction with CaM and then affected its interaction with p21, although proof of this is required.

Calmodulin (CaM) can upregulate the Ras/Raf/MEK/ERK pathway (Agell et al., 2002). We therefore tested the degree of phosphorylation of ERK1/2 in 293T cells of co-transfected (GFP)-CaM-WT, CaLP-WT, CaLP-Δ 150–161, CaLP-Δ 79–161, CaLP-Δ 1–78 and CaM + tail together with (Red)-$p21^{Cip1}$ to delineate the functional connection between different domains of CaLP, CaM and $p21^{Cip1}$. The cells transfected with $p21^{Cip1}$ exhibited a reduced expression level of p-ERK1/2 but when co-transfected with CaM-WT, CaLP-WT, CaLP-Δ 150–161 or CaLP-Δ 1–78, the p-ERK1/2 was rescued, while CaLP-Δ 79–161 and CaM + tail had no effect on the expression level of p-ERK1/2 (Fig. 6). Collectively, these experiments strongly indicate that the C-terminal globular domain of CaLP was probably responsible for the interaction with $p21^{Cip1}$ and the translocation of CaLP from the cytoplasm to the nucleus.

4. Discussion

In eukaryotic cells, nuclear pore complexes (NPCs) residing in the nuclear envelope regulate the trafficking of many nucleocytoplasmic-shuttling proteins. The NPC is a large, multiprotein structure with a molecular mass of about 125 MDa. In the center of the NPC is a 10 nm-diameter diffusion

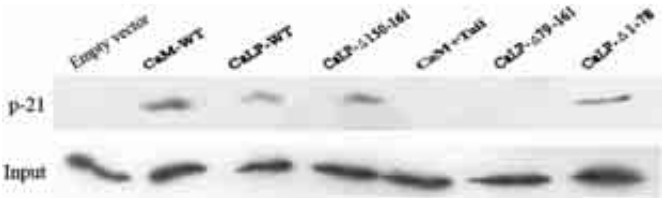

Fig. 5. Analysis of the p21-binding domain of CaLP. 293T cells were lysed and immunoprecipitated with anti-GFP antibody. After washing, immunoprecipitated proteins were eluted with SDS-containing buffer and the presence of co-immunoprecipitated $p21^{Cip1}$ was analyzed by WB. Immunoprecipitation of empty vector of EGFP co-transfected with p21 was acted as control and same amount of EGFP proteins were loaded in co-immunoprecipitation which is indicated in the lower panel. The figure shown is representative for three experiments.

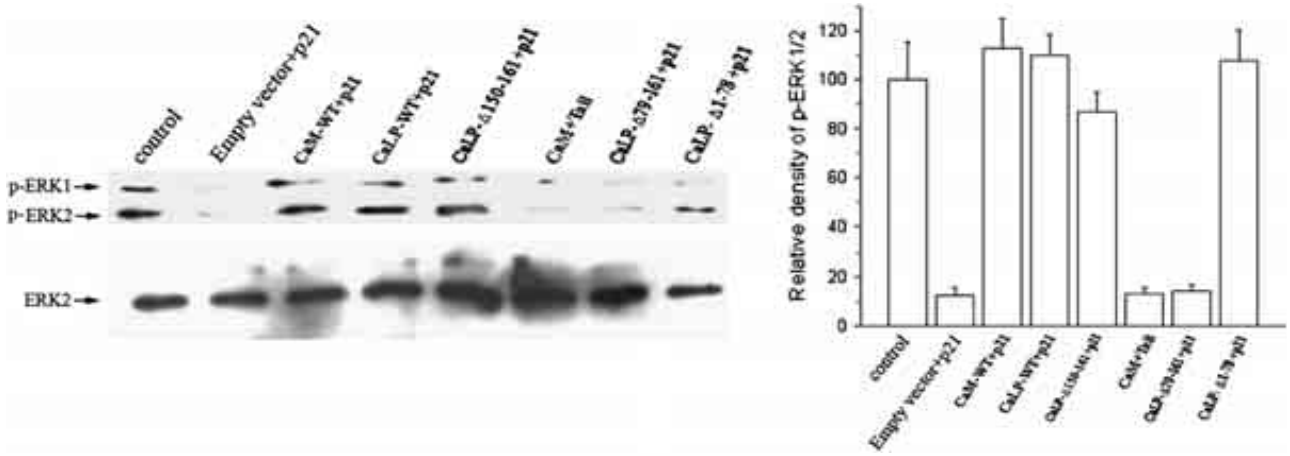

Fig. 6. Phosphorylation of ERKs after co-transfection with $p21^{Cip}$ and different mutants to analyze the functional interaction between the p21 and the different domains. ERK2 was acted as inner control and the relative density of ERK was quantified by ImageJ semi-quantitative image analysis software. The figure shown is representative for three experiments.

channel allowing free passage of molecules smaller than 60 kDa (Greber and Gerace, 1995; Doy and Hurt, 1997). Nucleocytoplasmic trafficking of molecules larger than this cutoff requires signals of trafficking embedded in the primary structure of the molecule and requires energy. Two of the best characterized import signals are the nuclear localization signal (NLS) and the M9 signal (Michael et al., 1995; Siomi and Dreyfuss, 1995; Weighardt et al., 1995; Macara, 2001). Signal-mediated import requires the assistance of cytosolic factors, such as the M9 binding proteins and the activity of Ran GTPase (Görlich, 1997; Nakielny and Dreyfuss, 1997). CaM has no identifiable import signals and is small enough to pass through the diffusion channel. Previous research supported by fluorescence imaging and fluorescence recovery after photobleaching studies of fluoresceinated CaM microinjection into intact cells indicated that CaM is diffused freely through NPCs, and that nuclear CaM-binding proteins act as a sink for Ca^{2+}–CaM. The steady state level of CaM in the nucleus depends on both the intracellular concentration of free Ca^{2+} and the relative concentration and affinity of CaM-binding proteins in the nucleus and cytoplasm.

From our fluorescence confocal microscopy results, the extra 12aa tail of CaLP is obviously not large enough to sequester it in the cytoplasm because the CaM + tail cannot stay in the cytoplasm. DNA sequence analysis revealed that CaLP has no identifiable import signals either. But the CaLP-Δ 1–78 with the deleted globular domain N-terminal exhibited full functions of staying in the cytoplasm. This result, along with the ubiquitous distribution of CaLP-Δ 79–161 in cells, indicated that the C-terminal together with the 12aa tail ensures the sequestration of CaLP in the cytoplasm.

Many previous biophysical studies of CaM have revealed the mechanism of Ca^{2+}–CaM binding to its target proteins and how calmodulin functions (Brini and Carafoli, 2000; Chin and Means, 2000). CaM has four Ca^{2+}-binding sites with two in a globular N-terminal domain separated by a flexible a-helix from a C-terminal globular domain containing the other two Ca^{2+}-binding sites. It is considered that in the presence of Ca^{2+}, each domain adopts an open conformation and exposes a hydrophobic pocket that allows CaM to bind to target proteins. In the process of the interaction between CaM and target protein, the C-terminal hydrophobic pocket of CaM first interacts with the hydrophobic residues in the target N-terminal sequence followed by interactions between the N-terminal globular domain and the C-terminal sequence of the CaM-binding domain. CaM thus collapses and wraps around the peptide, resulting in the formation of a high affinity complex. Fluorescence confocal microscopy assay of co-transfection and co-immunoprecipitation of different mutants of CaLP with $p21^{Cip1}$ revealed that the overexpression of $p21^{Cip1}$ can stimulate the translocation of CaLP-WT, CaLP-Δ 150–161 and CaLP-Δ 1–78 and it was deduced that the C-terminal globular domain of CaLP is responsible for its translocation. The nucleocytoplasmic trafficking of many proteins including CaM were because of the phosphorylation of protein by casein kinase II (Sacks et al., 1992), insulin receptor tyrosine kinase (Joyal et al., 1996) and epidermal-growth-factor-receptor tyrosine kinase (Benguría et al., 1994). Therefore, future work should be conducted to determine if CaLP is directly phosphorylated or if CaLP is released from the interacting protein in the cytoplasm and diffused into the nucleus as a result of $p21^{Cip1}$ transfection. According to the results of this research, the phosphorylated site or interacting site with target proteins most probably resides in the C-terminal globular domain of CaLP.

Acknowledgements

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