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ORIGINAL ARTICLE

Investigation of Phosphorylation Site Responsible for CaLP (*P. fucata*) Nucleo-cytoplasmic Shuttling Triggered by Overexpression of $p21^{Cip1}$

Z. Fang · Q. Wang · W. Cao · Q. Feng · C. Li · L. Xie · R. Zhang

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Abstract Calmodulin (CaM) is a highly conserved and ubiquitous Ca^{2+}-binding protein regulating intracellular Ca^{2+} concentration by acting as a sensor of this divalent cation in eukaryotic cells. Being such a very important signal sensor, CaM is susceptible to undergo many posttranslational modifications. One of these important modifications is its phosphorylation. Our previous investigations showed that CaM and calmodulin-like protein (CaLP) cloned from *Pinctada fucata* have many different characteristics in spite of their high similarity to each other. We have narrowed down that the C-terminal domains of CaM and CaLP are responsible for their discrepant subcellular localizations and shuttling of CaLP when it is co-transfected with $p21^{Cip1}$, which is commonly considered as an important cell cycle regulating protein. In this study, we first predicted the potential phosphorylation site responsible for the shuttling and confirmed by fluorescence confocal microscopy. Together with fluorescence activated cell sorter analysis, we further investigated the releasing ability of wild type and point mutated CaLP from arrested cell cycle caused by $p21^{Cip1}$ overexpression. By performing pull-down analysis and phosphorylation status of CaLP in cytoplasm fraction of transfected COS-7 cells with CaLP alone and phosphorylation status of CaLP in nuclear fraction of co-transfected COS-7 cells with CaLP and $p21^{Cip}$, we propose that the CaLP staying in the cytoplasm is in the state of phosphorylation, but when $p21^{Cip1}$ is overexpressed in mammalian cells, some signal triggers CaLP dephosphorylation and translocation into the nucleus.

Keywords *P. fucata* · Calmodulin · Calmodulin-like protein · $p21^{Cip1}$ · Phosphorylation · Nucleo-cytoplasmic shuttling

Z. Fang · Q. Wang · W. Cao · Q. Feng · C. Li · L. Xie · R. Zhang
Institute of Marine Biotechnology,
Department of Biological Science and Biotechnology,
Tsinghua University,
Beijing, 100084, China

R. Zhang
e-mail: rqzhang@mail.tsinghua.edu.cn

L. Xie (✉) · R. Zhang
Protein Science Laboratory of the Ministry of Education,
Tsinghua University,
Beijing, 100084, China
e-mail: lpxie@mail.tsinghua.edu.cn

Introduction

As a sensor of intracellular Ca^{2+} signals (Means and Dedman 1980), calmodulin (CaM) can regulate a great variety of cellular processes, such as gene expression and protein phosphorylation (Agell et al. 1998; Means 2000), through binding to a variety kinds of CaM-binding proteins (Weinstein and Mehler 1994; Ikura 1996). Then, CaM is involved in a large number of cellular events, including cell division and differentiation (Agell et al. 2002).

We have cloned CaM and a novel calmodulin-like protein (CaLP) successfully from *Pinctada fucata* previously (Li et al. 2004, 2005). Many works had been done to investigate their characteristics in common and in particular. CaM is highly conserved in structure in different species, so it is not very surprising when CaM (*P. fucata*) shared similar characteristics with mammalian CaM such as its calcium-dependent shift and its subcellular localization throughout the cytoplasm and nucleus in our previous research (Fang et al. 2008a, b). But when compared with CaLP (*P. fucata*) which shows high identity and similarity with the CaM, a very significant discrepancy turned out in subcellular distribution and protein–protein interaction (Fang et al. 2008a, b).

In our research, we chose mammalian $p21^{Cip1}$ to test the potential differences between CaM and CaLP in protein–protein interaction. The significance of CaM to cell cycle progression has been studied for several decades in different kinds of cell types and organisms. For example, the addition of specific anti-CaM drugs to cell cultures can inhibit the progression into and through S phase (Cheung 1980), and CaM has been proven to act as a regulator of some important 'checkpoints' in the mammalian cell cycle, including early G_1, the G_1 to S phase transition, and G_2/M transition (Kahl and Means 2003) indicated in Fig. 1. In the protein–protein interaction, research between $p21^{Cip1}$ and cyclin D/Cdk4 complexes, $p21^{Cip1}$, which was described as an inhibitor of Cdk before, was also found to be responsible for the formation and nuclear translocation of cyclin D/Cdk4 complexes (Taules et al. 1998). Later investigations exhibited that its dual behavior was most probably because of its direct interaction with CaM (Taulés et al. 1999) and its different phosphorylation state (Zhou et al. 2001; Rodríguez-Vilarrupla et al. 2005; Agell et al. 2006). Their results not only implicated a relevant interaction between CaM and $p21^{Cip1}$ for the process of nuclear entry of cyclin D/Cdk4 complexes but also determined the carboxyl-terminal domain of $p21^{Cip1}$ responsible for CaM binding.

We have previously reported the different subcellular localization of *P. fucata*-originated CaM, CaLP, and the shuttling of CaLP from cytoplasm to nucleus when co-transfected with $p21^{Cip1}$, but not for CaM (Fang et al. 2008a, b). Under fluorescence confocal microscopy and using method of co-IP, different mutants of CaLP showed the importance of the C-terminal globular domain in its subcellular location and its translocation when $p21^{Cip1}$ was overexpressed.

It is well known that a very important result of protein phosphorylation is to change the subcellular localization of the phosphorylated protein itself or the target protein of the phosphorylated protein (Blenis and Resh 1993). The phosphorylation of CaM has been investigated since the 1970s (Wolff and Brostrom 1974; Brostrom et al. 1975). It can be phosphorylated by diverse protein kinases not only in vitro but also in vivo, and these diverse phosphorylated CaM produced by these enzymes present distinct biological activities when acting on multiple CaM-dependent systems. Phosphorylated CaM has been shown to have a differential biological activity compared to unphosphorylated CaM when assayed on a variety of CaM-dependent systems. There are many potential phosphorylation sites in CaM, and as described in the review of Benaim and Villalobo (2002), the tyrosine phosphorylation of CaM is a widespread phenomenon (Haring et al. 1985; Fukami et al. 1986; Graves et al. 1986; José et al. 1992; Benguría et al. 1994; Mukhin et al. 2001). Therefore, we propose that the potential phosphorylation site in CaLP which is different from CaM may lead to the shuttling of CaLP caused by the overexpression of $p21^{Cip1}$. In this study, we first used point mutation and fusion of the proteins to enhanced green fluorescent protein (EGFP) to identify which amino acid is essential for the shuttling of CaLP. Furthermore, cell cycle analysis by fluorescence activated cell sorter (FACS) and Western blot exhibited the differences between the wild-type and mutant CaLP in cell cycle check point release from G_0/G_1 phase.

Finally, we propose a model of protein–protein interaction between $p21^{Cip1}$ and phospho-/unphosphorylated CaLP using pull-down analysis on nuclear/cytoplasmic extracts with Ab-phospho-Tyr, and it is very interesting to further investigate the potential kinase to phosphorylate CaLP in the future.

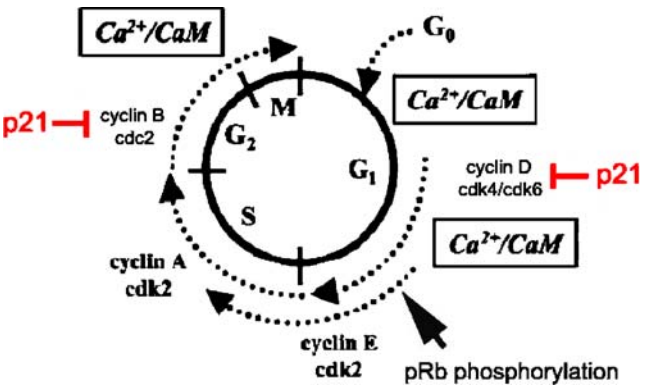

Fig. 1 Schematic diagram of CaM and $p21^{Cip1}$ in the mammalian cell cycle transitions (with some modifications of Kahl and Means 2003). Cell cycle transitions are regulated by Cdk/cyclin complexes. The CaM is required at G_1/S boundary and G_2/M transition. In this progress, $p21^{Cip1}$ acts as an inhibitor by interaction with CaM and Cdk/cyclin complexes

Materials and Methods

Plasmid Construction and Mutagenesis Putative tyrosine phosphorylation sites were predicted using Scansite 2.0 (http://scansite.mit.edu; Obenauer et al. 2003). Our previous research has narrowed down the potential influential domain into the C-terminal globular domain, so we focused on the putative phosphorylation sites in this domain. Benefited from the high similarity between CaM and CaLP, it is very lucky for us to pick up one tyrosine site of Y139 in CaLP which seems very distinct in phosphorylation potentiality (with the threshold of medium stringency; Fig. 2). Therefore, we construct mutate by point mutating this tyrosine into alanine. All of the expression plasmids for all the green fluorescent fusion proteins of wild type of CaM, CaLP, and $p21^{Cip1}$ were constructed as described in our previous research (Fang et al. 2008a, b). For GFP-CaLPY139A, the forward oligonucleotide is the same as

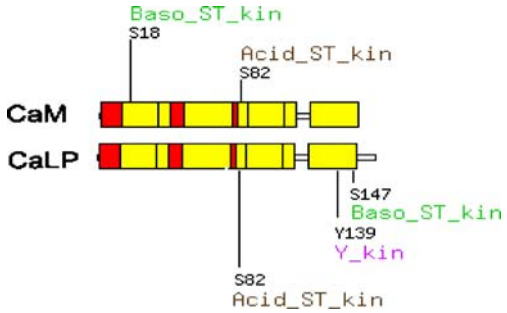

Fig. 2 Potential phosphorylation sites of CaM and CaLP predicted by Scansite (medium stringency). The site was narrowed in the Y139 which was located in the C-terminal globular domain

CaLP-WT and the reverse oligonucleotides were 5′-GGAGAAATAAATGCTGAGGAGTTTGTC-3′ and 5′-GACAAACTCCTCAGCATTTATTTCTCC-3′, and it was amplified by two rounds of polymerase chain reaction. All of the resulting constructions were verified by sequencing.

Cell Culture, Transient Transfection, and Fluorescence Microscopy 293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.5 g/l $NaHCO_3$, and 100 U penicillin/streptomycin solutions at 37°C in 5% CO_2 on 35-mm dishes. Mammalian expression vectors were transfected into cells using Vigorous transfection reagent (Vigorous Biotechnology, P.R. China) in accordance with the manufacturer's instructions. For transient transfection, 10 μg of DNA plus 4 μl transfection reagent was added to cells (>40% confluent for fluorescence confocal microscopy and >90% confluent for Western blot, respectively) in 35-mm dishes.

For intracellular localization analysis of EGFP and DsRed fusion proteins, 293T 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. The fluorescent images were captured using Olympus inverted microscope equipped with a charge-coupled camera (Olympus FV500).

Preparation of Nuclear and Cytoplasmic Fractions After 24-h transfection, subcellular fractionation of COS-7 cells was separated using a nuclear/cytoplasm extraction kit (Tianlai, Beijing, P.R. China) following the instruction 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 pellet was washed one more time and the cells 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 for 5 min at 1,000×*g*, 4°C. The pellet contains 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 pellet prepared in previous step was used to extract the nuclei protein. After removing the membrane components contaminated in cytosol extraction, 100 μl of cold nuclear extraction buffer was added into the crude nuclei pellet, vortexed, and incubated on ice for 30 min. It was centrifuged at 12,000×*g* for 5 min, 4°C. The supernatant fraction contains the proteins extracted from nuclear and is transferred to a clean tube and stored at −70°C.

Protein Expression, Purification, and Pull-Down Assays GST-p21-containing plasmids were transformed into *Escherichia coli* strain BL2 (DE3) carrying the pLysS plasmid. Expression and purification were performed by standard procedures following the manufacturer's instructions (Amersham Biosciences). Recombinant CaM and CaLP of *P. fucata* were also expressed in *E. coli* and purified as previously described (Li et al. 2004, 2006). Pull-down assays were performed as follows: 2 μg of purified GST-p21 fusion protein was incubated for 1 h at 4°C with 2 μg of either purified CaM, CaLP, or the nuclear/cytoplasmic fractions of CaLP and $p21^{Cip1}$ co-transfected cells. The pull-down buffer was composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 0.1 mM $CaCl_2$. After centrifugation, bound proteins were washed three times in the same buffer and eluted directly with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer, electrophoresed, and analyzed for the following Western blot analysis.

Immunoprecipitation of Proteins, Gel Electrophoresis, and Immunoblotting Twenty-four hours after transfection, the COS-7 cells were lysed in lysis buffer (Beyotime Institute of Biotechnology, P.R. China) containing 1 mM phenylmethylsulphonyl fluoride. The nuclear and cytoplasmic fractions prepared as mentioned previously were used during the next steps. One minute after lysis, cell extracts of different fractions were spun down at 14,000×*g* for 10 min at 4°C. The extracts were pooled into a 1.5-ml tube and were exposed to 2 μg antibodies. They were 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 washing the immunoprecipitates five times with 1 ml of lysis buffer, the protein A sepharose beads were collected and suspended in 2× SDS sample buffer with 2.5 mM Ca^{2+}, boiled for 5 min, and subjected to Western blot analysis with different antibodies. Proteins were electrophoresed on a 15% SDS/PAGE. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane for 2 h at 250 mA. Membrane was then

pre-incubated in Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% bovine serum albumin (BSA) for 1 h at room temperature and then incubated for 1 h at room temperature in TBS, 0.05% Tween 20, 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 visualized by electrochemiluminescence (Vigorous).

Cell Cycle Synchronization in G_0 Phase by Serum Starvation and Cell Cycle Analysis COS-7 cells were grown 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. Cells were synchronized in G_0 by serum starvation (0.1% FBS) on confluent cultures for 48 h. Reentry into the cell cycle of G_0-arrested cells was achieved by cultivation in 10% FBS DMEM (Tedesco et al. 2002).

Analysis of the cell cycle was carried out by flow cytometry. In three independent experiments, COS-7 cells were plated on 60-mm dishes. After a certain time required for protein expression, 1.5×10^5 cells were harvested (in collecting co-transfected cells, cells expressing both of red and green fluorescence were harvested by the sorter), pelleted, and washed with phosphate-buffered saline three times. After being fixed in 1 ml of ice-cold 70% ethanol overnight at 4°C, the cells were pelleted by centrifugation and incubated with RNAse (100 μg/ml) at 37°C for 30 min. Propidium iodide (20 μl of 1 mg/ml) was added to each cell suspension, and the cells were passed through a fluorescence activated cell sorter (FACS Calibur, BD Biosciences, San Jose, CA, USA); data were acquired using CellQuest (BD Biosciences) software. Percentages of cells in different phases were calculated directly by the software.

Results

Mutation of Y139A Changed CaLP Localization and Its Translocation As indicated in Fig. 3a–h, CaM-WT distributed ubiquitously in the cells, but CaLP-WT was sequestered in the cytoplasm (Fang et al. 2008a, b). When co-transfected with $p21^{Cip1}$, it is very interesting to find out that CaLP could translocate from cytoplasm to nucleus and co-localize with $p21^{Cip1}$, but not for CaM. As we investigated in the previous research, the C-terminal globular domain is

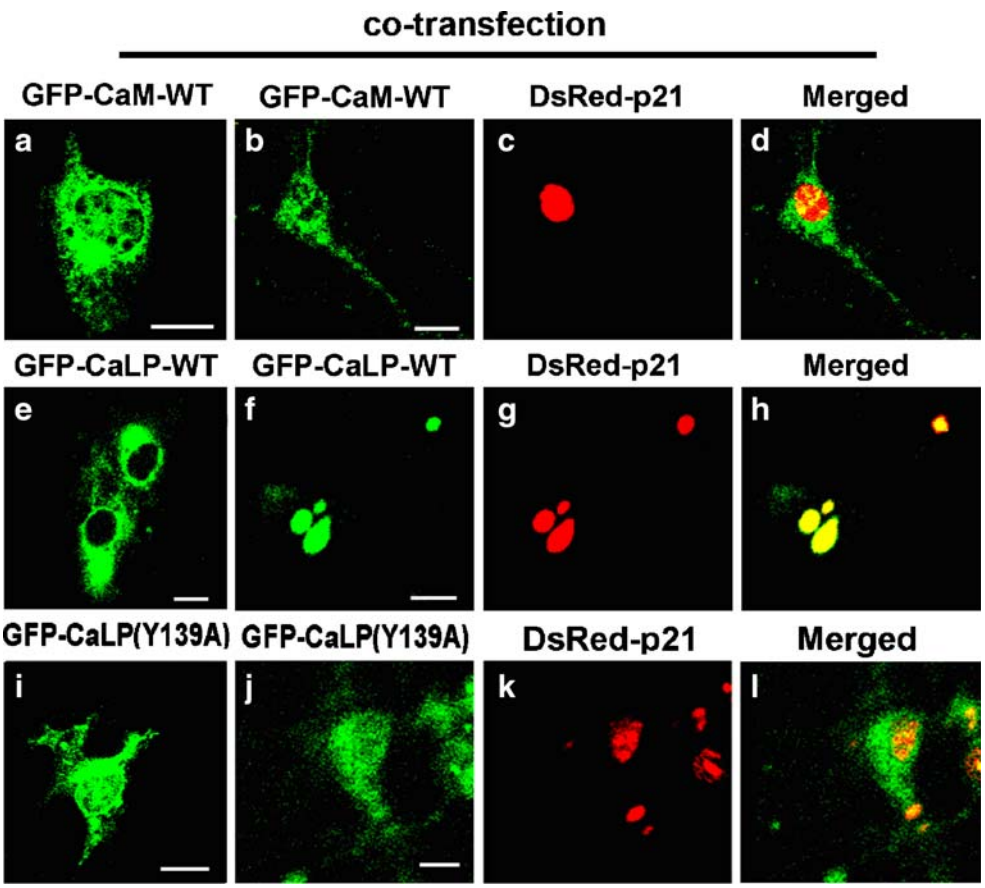

Fig. 3 Subcellular localization of wild-type CaM, wild-type CaLP, and CaLPY139A before and after co-transfection of $p21^{Cip1}$. **a**, **e**, **i** Subcellular localization of wild-type CaM, CaLP, and CaLPY139A in 293T cells. 293T cells were transfected with different recombinant plasmids and visualized by fluorescence microscopy 24 h after the transfection. **a** Distribution of wild-type CaM both in cytoplasm and nucleus. **e** Cytoplasmic localization of wild-type CaLP. **i** CaLP transfer into nucleus after Y139 was mutated to A139. **f**–**h** Subcellular co-localization of $p21^{Cip1}$ with wild-type CaLP. CaLP was stimulated by $p21^{Cip1}$ to translocate from cytoplasm to nucleus. **b**–**d**, **j**–**l** Localization of $p21^{Cip1}$ with wild-type CaM and CaLPY139A. The mutant's localization was not affected by co-transfection with $p21^{Cip1}$ just as CaM

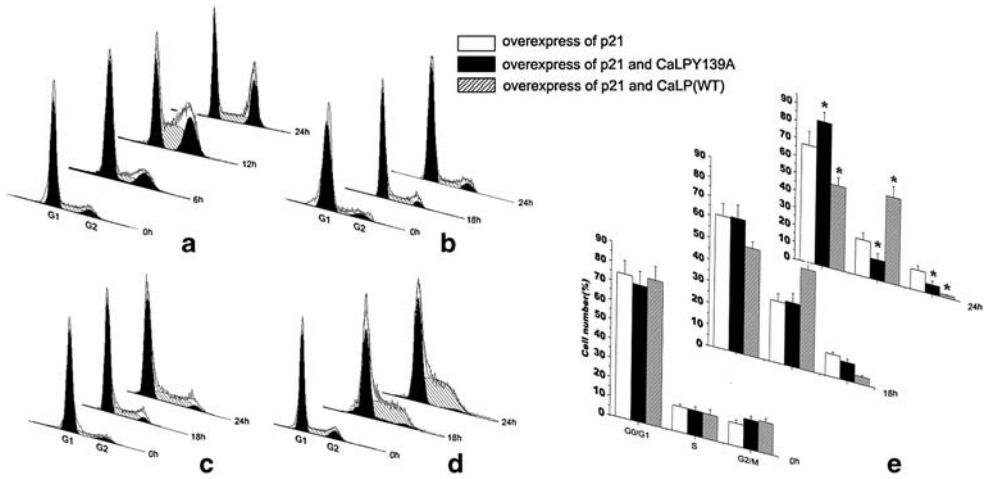

Fig. 4 Effect of Y139 mutation on cell cycle distribution examined by flow cytometry of COS-7 cells synchronized by serum starvation and then transfected by different recombinant plasmids for different time periods after reentry into the cell cycle of G_0-arrested cells was achieved by cultivation in 10% FBS DMEM. Results expressed one representative experiment performed in triplicate. **a** The typical cell cycle released from serum starvation for 0, 6, 12, and 24 h. On the 6 h, S phase cells increased and so did the G_2 phase; on the 24 h, the cells entered into G_2 normally. **b** Cell cycle distribution of $p21^{Cip1}$-transfected COS-7 cells after serum starvation. The cell cycle was stuck in G_0/G_1 phase and failed to enter into the next cell cycle phase. **c** Cell cycle distribution of $p21^{Cip1}$ and CaLPY139A co-transfected COS-7 cells after serum starvation. CaLPY139A failed to rescue the cell cycle checked by $p21^{Cip1}$ transfection. **d** Cell cycle distribution of $p21^{Cip1}$ and CaLP-WT co-transfected COS-7 cells. The FACS results showed that the transfection of CaLP-WT made the amount of S phase cells much more than the control. **e** The statistic analysis of different cell cycle phases of different time periods. *Bars* represent mean+SD of three independent experiments, each performed three times. *$P<0.001$, statistically significant differences

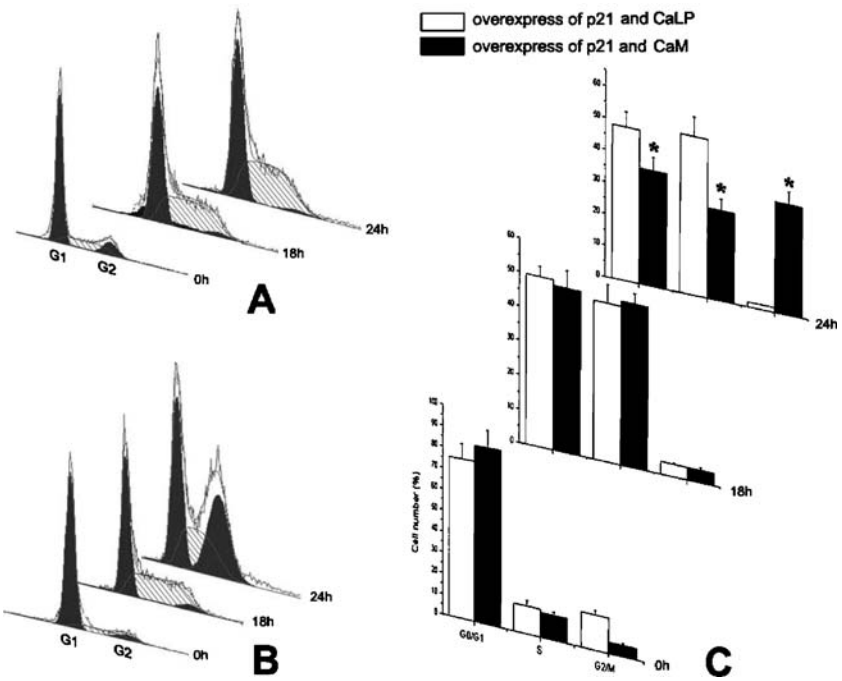

Fig. 5 Comparison between CaLP and CaM on their effect on cell cycle distribution examined by flow cytometry of COS-7 cells synchronized by serum starvation and transfected by different recombinant plasmids for different time periods. Results expressed one representative experiment performed in triplicate. **a** Cell cycle released from serum starvation for 24 h. On the 24 h, though the cells of S phase elevated greatly, the G_2 phase cells were hardly seen. **b** Compared with the CaLP, after co-transfection of $p21^{Cip1}$ and CaM, the distribution of cell cycle turned back to normal after 24-h transfection. **c** The statistic analysis of different cell cycle phases of different time periods. *Bars* represent mean+SD of three independent experiments, each performed three times. *$P<0.001$, statistically significant differences

responsible for this phenomenon (Fang et al. 2008a, b). By bioinformatics, we identified one tyrosine site predicted to be phosphorylated in CaLP (Fig. 2). To confirm that P-(Y139) of CaLP may play a very important role in its subcellular localization, especially the interesting nuclear-cytoplasm translocation, we transfected the 293T cells with CaLPY139A alone or co-transfected the cells with CaLPY139A together with $p21^{Cip1}$ and compared the results with those of CaLP-WT and CaM-WT. We observed that the mutant distributed both in nucleus and cytoplasm and the translocation phenomenon disappeared (Fig. 3i–l). These results suggest that the Y139 of CaLP is a crucial site and its phosphorylation state may be responsible for the distinct characteristics of CaLP from CaM.

CaLPY139A Failure in Releasing Cells from G_0/G_1 Arrest To further validate our observations that Y139 plays an important role in its translocation into the nucleus, we investigated the ability of CaM, CaLP, and CaLPY139A in releasing COS-7 cells from G_1 arrest caused by overexpression of $p21^{Cip1}$. The results of synchronization of COS-7 cells by serum starvation and the representative profiles of overexpression of $p21^{Cip1}$ on cell cycle were shown in Fig. 4. The left large initial peak represents cells in G_0/G_1, the intervening area represents cells in S phase, and the right tail peak represents cells in G_2/M. Figure 4a showed typical cell cycle released from serum starvation. After 24 h, the cells entered into G_2 normally. The cell cycle distribution of COS-7 cells transfected with $p21^{Cip1}$ alone after serum starvation exhibited the arrest in G_0/G_1 phase after 24-h transfection and failed to enter into the next phase. When co-transfected with $p21^{Cip1}$ and CaLP-WT after serum starvation, the FACS results showed that the percentage of cells in S phase increased much more compared with those transfected with $p21^{Cip1}$ alone (50.11±6.12% vs 21±4.1%). But when CaLPY139A and $p21^{Cip1}$ were co-transfected after serum starvation, it failed to rescue the cell cycle arrested by $p21^{Cip1}$, and the cell cycle was still arrested in the G_0 phase. The data indicate that Y139 is responsible for the releasing of cells from G_0/G_1 phase caused by overexpression of $p21^{Cip1}$. It is very interesting and noteworthy that when we compare the FACS figures of co-transfection of CaM/$p21^{Cip1}$ and CaLP/$p21^{Cip1}$, we found that the CaM overexpression cannot only release the cell cycle from G_0/G_1 arrest but also impel the cycle in the subsequent phases normally, but the CaLP overexpression can only make it possible to transit from G_1 to S but cannot make the cell cycle go to the next phase: the cells were blocked in S phase (Fig. 5).

Protein–Protein Interaction Between $p21^{Cip1}$ and CaLP Nuclear/Cytoplasm Fractions Indicated by the previous results, we hypothesize if the phosphorylation state of CaLP is different in cytoplasm and nucleus and if this different state is the reason for CaLP sequestration and translocation. To testify this hypothesis, in this part, we separated nuclear/cytoplasm fractionations of the COS-7 cells transfected with CaLP alone or co-transfected the COS-7 cells with the CaLP and $p21^{Cip1}$ together for a period of 24 h. As shown in Fig. 6a, upper panel, we did GST pull-down assays on complex of GST-p21 and purified recombined CaLP followed by positive interaction detection by usage of CaLP polyclonal antibody. In

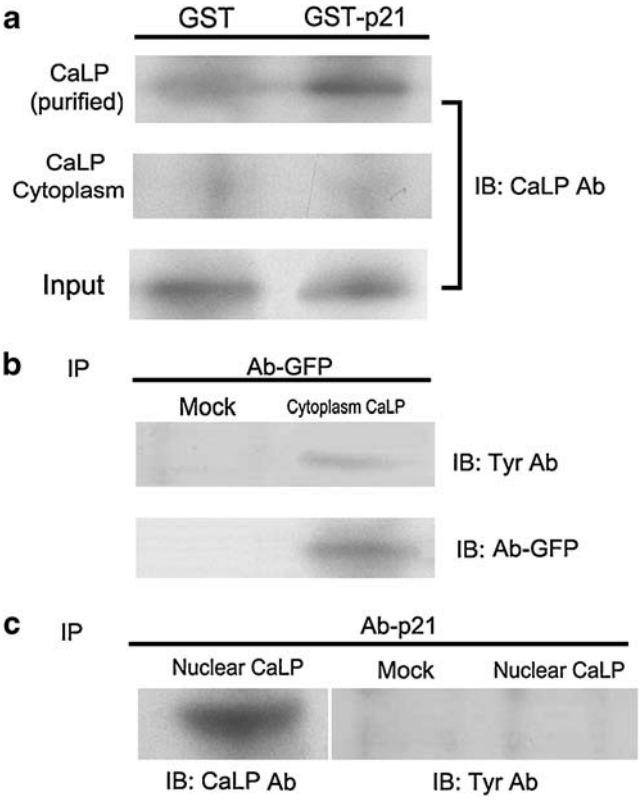

Fig. 6 Protein–protein interaction between nuclear/cytoplasmic fractions of CaLP and $p21^{Cip}$. **a** *Upper panel* is GST pull-down assay of GST-p21 and recombined CaLP expressed in *E. coli* (purified as we described before). GST-p21 was first incubated with glutathione-Sepharose beads. After sufficient elution, the beads were then incubated with recombined CaLP expressed in *E. coli*. After several rounds of sufficient elution, the complexes of proteins were boiled and the CaLP was detected by the polyclonal antibody of CaLP. The *middle panel* is GST pull-down assay of GST-p21 and cytoplasm GFP-CaLP expressed in COS-7. Glutathione-Sepharose beads without $p21^{Cip}$ acted as control and were incubated with the CaLP. Input represented CaLP amount used in the experiment by being blotted with antibody of CaLP. **b** Phosphorylation assay of cytoplasm CaLP. Total CaLP were immunoprecipitated by GFP-Ab and the state of phosphorylation was detected by the Tyr-Ab. The *lower panel* represents the band detected by Tyr-Ab the same as CaLP. Mock indicates that no CaLP plasmid was transfected. **c** Co-IP of $p21^{Cip}$ with nuclear CaLP and the phosphorylation state of the co-immunoprecipitated CaLP. After the immunoprecipitation with Ab-$p21^{Cip}$, the nuclear CaLP was then blotted with antibodies of CaLP and Tyr. Mock indicates that neither $p21^{Cip1}$ nor CaLP plasmid was transfected

Fig. 6a, middle panel, the cytoplasmic fractions of COS-7 cells transfected with CaLP alone failed to interact with GST-p21 when performed pull-down analysis. In Fig. 6b, we first immunoprecipitated the CaLP-GFP expressed in cytoplasm with antibody of GFP and then detected its phosphorylation state. The positive signal in the upper panel showed that the CaLP was phosphorylated in tyrosine. Then, we did the same experiment with CaLP in the nucleus. After immunoprecipitation with antibody of $p21^{Cip1}$ followed by the immunoblot with CaLP or tyrosine antibody, we found that though the CaLP in the nucleus can be co-immunoprecipitated with $p21^{Cip1}$, the CaLP combining with $p21^{Cip1}$ was not phosphorylated as least on the tyrosine site. These results are important evidences for our hypothesis that the CaLP phosphorylation status in the cytoplasm and nucleus are different and is crucial for its subcellular localization and translocation when co-transfected with $p21^{Cip1}$.

Discussion

Using Scansite 2.0 to predict the phosphorylation sites of CaLP and CaM cloned from *P. fucata*, we first set the threshold of prediction as "low stringency" and got the results in Table 1. Y139 is the only tyrosine site of potential phosphorylation both in CaLP and CaM. As for CaM, only Src Kinase can phosphorylate it, but as for CaLP, many other kinases such as PDGFR kinase, Lck kinase, and IRK are all potential candidates for CaLP phosphorylation. We then set the threshold of prediction as "medium stringency" and found that in the C-terminal globular domain, Y139 and S147 can still be phosphorylated, but not for CaM. We have mentioned that this domain is responsible for CaLP subcellular localization and translocation, and these two sites are just located in this domain. Together with the analysis of sequence alignment between CaLP and CaM which is illustrated in Fig. 7 (with some modifications according to Li et al. 2005), we found that although the Y139 is conserved in CaM and CaLP, the site of aa136 and aa137 is highly variable in these two proteins. From the results of point mutation and fluorescence confocal microscopy, we infer that these two amino acids may affect the Y139 phosphorylation ability of these two proteins via affecting protein's space configuration.

It is well known that signal transduction involves the inactive and active state transitions which can be achieved by different status of phosphorylation, nucleo-cytoplasmic shuttling, and other processes (Beirer and Höfer 2006). The signal of tyrosine kinase is highly related with cell's growth and differentiation. Because CaM is a very important factor controlling these two important physiological progresses, the research of tyrosine phosphorylation of CaM is very important in the study of CaM. Mammalian CaM has two Tyr phosphorylation sites besides four Ser and 12 Thr phosphorylation sites, which have been proven by strong evidences (Meggio et al. 1987; Nakajo et al. 1988; Wong et al. 1988; Sacks et al. 1992; Quadroni et al. 1994; Davis

Table 1 Prediction of the phosphorylation sites of CaLP and CaM (*P. fucata*) with "low stringency" by Scansite 2.0

Site		Kinase group		Kinase		Score	
		CaM	CaLP	CaM	CaLP	CaM	CaLP
18	Ser	Acid-ST-kin	–	CK-1	–	0.5260	–
		Baso-ST-kin		CaMKII		0.6026	
				PKC-ζ		0.5166	
27	Ser	–	Baso-ST-kin	–	PKC ε	–	0.4993
35	Thr	Baso-ST-kin	–	PKC-δ	–	0.5072	–
58	Thr	–	Acid-ST-kin	–	CK-2	–	0.5657
71	Thr	Baso-ST-kin	–	PKC-ζ	–	0.6011	–
80	Thr	Baso-ST-kin	–	PKC-ζ	–	0.4764	–
		Acid-ST-kin		CK-2		0.5457	0.5289
*82	Ser	Acid-ST-kin		CK-2		0.4729	0.4561
111	Thr	Baso-ST-kin		PKC-ζ		0.5975	0.5975
118	Thr	Acid-ST-kin	–	CK-2	–	0.5097	–
*139	Tyr	Y-kin		Src Kinase		0.5838	0.4779
		–	Y-kin	–	PDGFR kin	–	0.5046
					Lck kinase		0.6005
					IRK		0.7029
*147	Ser	–	Baso-ST-kin	–	PKC ε	–	0.4245
					PKC-α/β/γ		0.5210
151	Thr	–	Acid-ST-kin	–	CK-2	–	0.5159

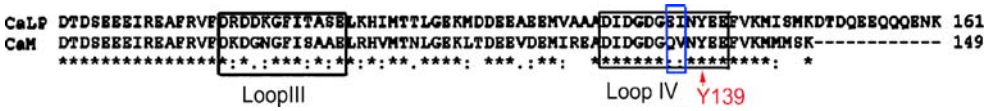

Fig. 7 Diagram of amino acid sequence alignment from 79aa to 161aa for CaLP and 79aa to 149aa for CaM. The third and fourth Ca^{2+}-binding domains were *boxed* and indicated as Loop III and IV; homologous and identical amino acids are indicated by *dots* and *stars*, respectively. Although the Y139 is conserved in sequence in CaM and CaLP, the site of aa136 and aa137 is variable in these two proteins and these two amino acids may affect the phosphorylation ability of these two proteins via affecting protein's secondary structure

et al. 1996; Joyal et al. 1996; Benaim et al. 1998). It is noteworthy that in vivo, phosphorylated and dephosphorylated CaM exists as equilibrium status and CaM is very prone to be dephosphorylated (Plancke and Lazarides 1983), although little is known on the protein phosphatases involved in the dephosphorylation of CaM. Unfortunately, the functional implications of CaM phosphorylation have been explored to a limited extent, though many enzymes and transport systems have been studied in vitro (Sacks et al. 1992; Quadroni et al. 1994; Saville and Houslay 1994; Williams et al. 1994; Corti et al. 1999; Leclerc et al. 1999; Palomo-Jiménez et al. 1999). Until now, the emphasis of the phosphorylation of CaM in different species was mainly focused in the enzyme kinetics and its affinity to other proteins, but no report of its phosphorylation state's effect on CaM subcellular localization was presented. Being highly similar to CaM, the effect of phosphorylation of CaLP (*P. fucata*) on its subcellular localization was firstly reported in this research. From the point mutation and phosphorylation prediction result, we propose that the CaM in the cell is in the dynamic and equilibrium between phosphorylation and dephosphorylation states, but the Y139 of CaLP can ensure CaLP in a state of high degree of phosphorylation so it can be sequestrated in the cytoplasm. CaLP139A destroyed the strong proneness of CaLP to phosphorylate, so its subcellular localization turned to be like that of CaM and distributed both in the cytoplasm and nucleus.

The regulational function of CaM was proven very important to cell cycle because CaM is required at two important points in the cell cycle: the G_1/S boundary and the G_2/M transition. In the progress of cell cycle, a family of serine/threonine protein kinases that are dependent upon cyclin binding for its activity (Morgan 1995, 1997; Morgan et al. 1998) acts as a crucial regulator. It is called the cyclin-dependent kinases (Cdks). The activity of Cdks is highly related with the progress of cell cycle. Its activity can be regulated via many factors, and one of the most important ways is to bind the cyclin-dependent kinase inhibitors (CKIs), which can combine with the Cdk or cyclin/Cdk complex, preventing the activation of the complex. In the CKIs, $p21^{Cip1}$ is broadly investigated. By combining with both Cdk2 and Cdk4, $p21^{Cip1}$ can regulate the progression of G_1/S and G_2/M transition (Fig. 1, with some modification from Kahl and Means 2003). In this research, the change of cell cycle distribution when co-transfected with $p21^{Cip1}$ and CaLP or CaLPY139A maybe originated from the direct interaction between the $p21^{Cip1}$ and Cdk complex which was affected by the protein interactions between $p21^{Cip1}$ and CaLP. The mutation of CaLP139A took place in the C-terminal domain of CaLP, and this domain can directly affect the interaction between $p21^{Cip1}$ and CaLP (Fang et al. 2008a, b). From the result of CaLPY139A's failing to rescue the cell cycle, we cannot only reassure the importance of this C-terminal globular domain but also propose that it is the exact reason why CaLP139A cannot rescue the cell cycle.

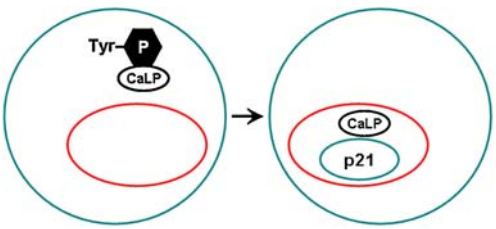

Fig. 8 A model explaining the translocation of CaLP when cotransfected with $p21^{Cip1}$. The *left circle* represents the COS-7 cells transfected with CaLP alone, and the phosphorylation in the Y139 causes the sequestering of CaLP in the cytoplasm. The *right circle* illustrates the COS-7 cells co-transfected with $p21^{Cip1}$ and the CaLP. Overexpression of $p21^{Cip1}$ triggers the dephosphorylation of CaLP, and the high affinity of unphosphorylated CaLP with $p21^{Cip1}$ makes the CaLP combine with $p21^{Cip1}$ in the nucleus

Besides, the FACS results proved that the overexpression of CaM cloned from *P. fucata* can successfully trigger the arrested cell cycle reentry into normal cycle process. This result is consistent with the research of Chafouleas et al. (1984). They use the method of radioimmunoassay followed by dot-blot hybridization and found that in CHO-K1 cells, CaM plays a very important role in the reentry of cells into the cell cycle from stationary phase blocked by the anti-calmodulin drug W13. While as we see in Fig. 5, overexpression of CaLP can only help cell cycle to get across G_0/G_1 block but cannot rescue the cell cycle completely. This phenomenon indicated that although CaLP cloned from *P. fucata* can bind to $p21^{Cip}$, the signal transduction of CaLP in mammalian cells is different from that of CaM because the overexpression of CaLP cannot substitute the function of CaM in the blocked cell cycle.

There are many examples of the phosphorylation affecting the association and dissociation of two proteins.

In the PDGF stimulation of skeletal muscle, Y521 phosphorylation of Munc18c occurred concomitant with the dissociation of the Munc18c protein from Syntaxin 4 in a time frame consistent with Glut4 translocation (Umahara et al. 2008). Another example is nucleophosmin (NPM)/B23. It is a multifunctional protein in a wide variety of cellular functions which can be phosphorylated by several different kinases such as CK2 and nuclear kinase II. It localizes to centrosomes, but when phosphorylated by Cdk2/cyclin E, it dissociates from centrosomes (Shinmura et al. 2005). Suggested by these proofs together with the results of protein–protein interaction between $p21^{Cip1}$ and CaLP in different fractions of nuclear or cytoplasm, we propose a model that can explain the interesting translocation of CaLP when co-transfected with $p21^{Cip1}$ (illustrated in the Fig. 8): in the COS-7 cells transfected with CaLP alone, the phosphorylation in the Y139 by some kinase affects the subcellular localization of CaLP and sequesters CaLP in the cytoplasm. But when co-transfected with $p21^{Cip1}$, the overexpression of $p21^{Cip1}$ triggers the dephosphorylation of CaLP, and the high affinity of unphosphorylated CaLP to $p21^{Cip1}$ makes the CaLP combine with $p21^{Cip1}$ strongly and enter into the nucleus.

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