The association of CaM and Hsp70 regulates S-phase arrest and apoptosis in a spatially and temporally dependent manner in human cells

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Abstract The cell cycle is controlled by regulators functioning at the right time and at the right place. We have found that calmodulin (CaM) has specific distribution patterns during different cell-cycle stages. Here, we identify cell-cycle-specific binding proteins of CaM and examine their function during cell-cycle progression. We first applied immunoprecipitation methods to isolate CaM-binding proteins from cell lysates obtained at different cell-cycle phases and then identified these proteins using mass spectrometry methods. A total of 41 proteins were identified including zinc finger proteins, ribosomal proteins, and heat shock proteins operating in a Ca^{2+} -dependent or independent manner. Fifteen proteins were shown to interact with CaM in a cell-phase-specific manner. The association of the selected proteins and CaM were confirmed with in vitro immunoprecipitation and immu-

nostaining methods. One of the identified proteins, heat shock protein 70 (Hsp70), was further studied with respect to its cell-cycle-related function. In vivo fluorescence resonance energy transfer (FRET) analysis showed that the interaction of CaM and Hsp70 was found in the nucleus during the S phase. Overexpression of Hsp70 is shown to arrest cells at S phase and, thus, induce cell apoptosis. When we disrupted the CaM-Hsp70 association with HSP70 truncation without the CaM-binding domain, we found that S-phase arrest and apoptosis could be rescued. The results suggest that the spatial and temporal association of CaM and Hsp70 can regulate cell-cycle progression and cell apoptosis.

Keywords Calmodulin · Hsp70 · Cell cycle · Apoptosis · Spatial and temporal association

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Abbreviations

CTL	control
FRET	fluorescent resonance energy transfer
IP	immunoprecipitation
CaM	calmodulin
HSP70	heat shock protein 70
CaMBPs	CaM-binding proteins

Introduction

The cell cycle in mammalian cells is regulated in both a temporally and a spatially dependent manner. The activation and deactivation of a series of proteins that act at each stage are tightly controlled to ensure normal cell-cycle progression. For example, the successful completion of

mitosis requires a series of events occurring in a strict order: spindle assembly, metaphase–anaphase transition, chromosome segregation, spindle disassembly and chromosome packaging. These events are controlled by the dephosphorylation of Cdk substrates and the destruction of cyclins and other regulators at certain time points (Parry and O'Farrell 2001; Sullivan and Morgan 2007; Wolf et al. 2007). However, cell-cycle events are also regulated in a spatially dependent manner. Cdk1-cyclin B1 functions in the nucleus where it phosphorylates histone and laminin to trigger chromosome condensation and nuclear membrane breakdown (Miyazaki and Arai 2007).

According to our previous report, the major cytoplasmic calcium receptor, calmodulin (CaM), can regulate multiple events during the cell cycle. CaM regulates the G2/M transition, spindle structure maintenance, furrow formation, cytokinesis completion, cell morphology and migration, etc, which are dependent upon the distribution of CaM in the nucleus, furrow and central, intracellular bridge and stress fibers (Li et al. 1999; Yu et al. 2004; Shen et al. 2007; Yuan et al. 2008). CaM itself has no enzymatic activity and it is believed that the spatiotemporal interaction of CaM and its targets is essential for CaM's regulatory function (Török et al. 1998). CaM can alter the conformation of its partner when it binds to a relatively small region of the target protein with high affinity. CaM-binding proteins (CaMBPs) comprise a diverse group of proteins related solely by the fact that they interact with CaM (Rhoads and Friedberg 1997; O'Day 2003). At present, many CaM-binding proteins have been identified, including protein kinases and phosphatases, proteins involved in second-messenger generation, and proteins that regulate cytoskeletal elements (Selvin 1995; Catalano and O'Day 2008). However, the cell-cycle-related CaM-binding targets are still little known to us, especially their function during cell-cycle checkpoint transition.

Using immunoprecipitation and MALDI-TOF MS analysis, a number of known and also previously uncharacterized proteins that interact with CaM have been identified. Among these proteins, we found that Hsp70 could arrest cells at S phase and induce cell apoptosis through associating with CaM in a spatially and temporally dependent manner in the nucleus and at S phase.

Materials and methods

Plasmid construction and reagents

CaM was amplified by PCR using 5'-CCCAAGCTTC GATGGCTGACCAGCTGAC-3' and 5'-GGGGTACCT CACTTTGCAGTCATCATCT-3' as the forward and reverse primer and subcloned into the HindIII and KpnI sites

of ECFP-C1 vector (Clontech), YFP-hsp70 (hsp70-1) was constructed by PCR amplification of the hsp70 gene using forward primer 5'-CCC AAG CTT CGA TGG CCA AAG CCG CG-3' and reverse primers 5'-GGG GTA CCT CCT AAT CTA CCT CCT C-3', and then subcloned into the HindIII and KpnI sites of EYFP-C1. QuikChange XL Site-Directed Mutagenesis Kit (La Jolla, CA) was used to make a truncation of hsp70 (257–277) lacking a c-terminal calmodulin binding domain (KRAVRRLRTACER AKRTLSSS) according to manufacturer's instructions. Primers for the mutagenesis were 5'-CTCTCGCAGG CGGTCCTCACGGCTCG-3' and 5'-CGAGCCGTGAG GACCGCCTGCGAGAG-3'. All constructs were verified by DNA sequencing.

Cell cultures and cell lysate preparation

HCC7402 (human hepatic cancer cell line) and ECV304 cells (a spontaneously transformed human umbilical vein endothelial cell line), obtained from China Center for Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum and penicillin (100 IU/ml)/streptomycin (100 ng/ml) at 37°C in a water-saturated atmosphere with 5% CO₂. HCC7402 cells

Fig. 1 Silver staining for CaM-binding proteins in different portions of the cell cycle. **a** Calcium presented (*without EGTA*); **b** calcium chelated (*with EGTA*). Control proteins immunoprecipitated with a non-specific antibody. The selected proteins for further examination, Hsp90, Hsp70, Lamin B2, actin, were marked with *arrowheads*

were synchronized at M phase with nocodazole (400 ng/ml, Calbiochem, La Jolla, CA, USA) for 10 h. Synchronized mitotic cells were then detached by vigorously shaking the plate and then incubating in fresh medium. The collected

cells entered metaphase in 30–40 min and anaphase in 1–1.5 h (as determined microscopically according to metaphase plate formation) and then resumed a normal cell cycle. Cells at interphase were collected by trypsinizing

Table 1 CaM-binding proteins identified in different cell phases in a calcium-dependent or -independent manner

Proteins	Interphase		Metaphase		Anaphase	
	–EGTA	+EGTA	–EGTA	+EGTA	–EGTA	+EGTA
Heat shock proteins						
Hsp90(HSPC1)	–	–	–	–	+	–
Hsp70(HSPA1A,HSPA8)	+	–	+	–	–	–
GRP78(HSPA5)	–	–	–	+	+	+
Ribosomal proteins						
RPS9	+	+	–	–	–	–
RPL15	+	+	+	+	–	–
RPL13A	+	+	+	+	–	–
Zinc finger proteins						
Zinc finger protein 101	–	+	–	+	–	+
Ring finger protein 39	+	–	+	–	–	–
Centrosome proteins						
CEP250	+	+	+	+	+	+
CENP-F	+	+	+	+	+	+
Kendrin	+	–	+	–	+	–
Skeletal protein						
Myosin IIIA	+	+	+	+	+	+
Beta-actin	+	+	+	+	+	+
Vimentin	+	+	+	+	+	+
Afadin	+	–	+	–	+	–
Emilin-4	+	–	+	–	+	+
Formin-like protein 1	+	–	+	–	+	–
Ankyrin-3	–	–	+	–	–	–
GAPDH	–	–	+	–	+	–
Lamin-B2	+	–	+	–	+	–
Nuclear mitotic apparatus protein	+	–	+	–	+	–
Kinase						
Phosphoglucosyltransferase-2-like 1	+	–	+	–	+	–
Tyrosyl-tRNA synthetase	+	–	+	–	+	–
Alpha-enolase	–	+	–	+	–	–
ATP-dependent MRNA helicase BRIP1	+	–	+	–	+	–
ATP-dependent helicase CHD7	+	–	+	–	+	–
PRKA9	+	–	+	–	+	–
Other						
TCP-1	–	+	–	+	–	+
Coiled-coil domain containing protein 37	–	–	–	+	–	–
Kinesin-like protein KIF14	+	–	+	–	+	–
Chromosome 10 ORF 39	+	+	+	+	+	+
ZNF169 protein	+	–	+	–	+	–
P5CS	–	–	+	–	+	–
Putative protein C21orf56	–	–	+	–	+	–
PIWIL2	+	–	–	–	–	–
Tumor protein D54	+	–	+	–	+	–
SNX10	–	–	+	–	–	–
Putative calcium binding transporter	+	–	+	–	+	–
Transmembrane protein Tmp21	+	–	+	–	+	–
GCN1-like protein 1	–	+	–	+	–	+
EF-2	–	–	–	–	+	–

adherent cells. Cells were inhibited at S phase with hydroxyurea (2 mM, Calbiochem, La Jolla, CA, USA) for 24 h. The cell stage after synchronization was confirmed by flow cytometry. Cell samples were then extracted for 30 min on ice using a lysis buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 1 mmol/L Na_3VO_4 , 2 mmol/L NaF, 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 $\mu\text{g}/\text{mL}$ aprotinin. The protein concentration was quantified using the Bradford method.

Immunoprecipitation, PAGE electrophoresis, in-gel tryptic digestion and mass spectrometry

For immunoprecipitation, a total of 1 mg protein for each group was incubated with primary CaM antibody (Zymed, Invitrogen) or control IgG overnight at 4°C. Normal IgG was applied to pulldown proteins as a blank control. EGTA was also introduced into lysis buffer to check the calcium dependence of CaM-binding proteins. All the immunocomplexes were then incubated with 20 μL protein G-Agarose (Roche, Mannheim, Germany) for 2–3 h with agitation at 4°C. The beads were then washed three times with lysis buffer and boiled in Laemmli sample buffer. Eluted immunoprecipitates were resolved in 12% SDS-PAGE gels and silver-stained according to the manufacturer's instructions. All stained protein bands were excised, cut into pieces and processed according to the standard protocol of the manufacturer. The samples were finally reswollen and digested with trypsin (10 ng/ μL in 25 mM ammonium bicarbonate). MALDI-TOF analyses of trypsin digests were performed on a Bruker Biflex IV MALDI-TOF-MS (Bruker) equipped with an N_2 laser (337 nm, 3-ns pulse length) in positive ion mode at an accelerating voltage of 19 kV. Peptide data were collected in the reflectron mode. Each spectrum was the accumulation of approximately 200 laser

shots. External calibration for peptide analysis was performed using peptide calibration standards.

Immunofluorescence assay

HCC7402 cells grown on acid-cleaned glass coverslips were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min. The cells were then incubated with primary rabbit anti-CaM antibody (13–6900; 1:250 dilution, Zymed, Invitrogen), mouse anti-laminB2 (SC-56147; 1:200 dilution, Santa Cruz, CA, USA), anti-actin (SC-47778; 1:200 dilution, Santa Cruz, CA, USA), anti-Hsp70 (HSPA1A and HSPA8; SPA-810 and SPA-815; 1:200 dilution, Stressgen Biotechnologies, Inc., San Diego, CA) antibodies. After thorough washing with PBST, the cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies and rhodamine-conjugated anti-goat secondary antibodies (Calbiochem, La Jolla, CA, USA) or rhodamine phalloidin (Sigma-Aldrich Co., St. Louis, MO, USA). The fluorescence images of the cells were photographed using a cooled charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI, USA) and processed using SPOT software.

Western blotting

HCC7402 cell lysates were prepared according to standard protocols. The protein concentration was measured using the Bradford method. For western blotting, equal amounts of protein were loaded, resolved on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were then incubated with primary mouse anti-laminB2 (1:500 dilution, Santa Cruz, CA, USA), mouse anti-HSP70 (1:500 dilution, Stressgen Biotechnologies, Inc., San Diego, CA) and mouse anti-HSP90 (SC-69703; 1:500 dilution, Santa

Fig. 2 Immunoprecipitation of CaM with Hsp90, Hsp70 and Lamin B2. All selected proteins are shown to interact with CaM in vitro. **a** CaM and Lamin B2; **b** CaM and Hsp90; **c** CaM can associate with Hsp70 in both HCC7402 and ECV304 cells, and its binding is increased after recovering 2 h from heat shock at 42°C; **d**, reverse pull-down for each of the partners, using anti-laminB2, Hsp90, and Hsp70 antibodies to show recovery of calmodulin. Control group were immunoprecipitated with a non-specific antibody

Cruz, CA, USA) antibodies. Bound antibody was visualized using alkaline phosphatase-conjugated secondary antibodies (1:1,000 dilution, Santa Cruz, CA, USA).

Living cell observation and fluorescence resonance energy transfer measurement

An Olympus IX81 fluorescence microscope equipped with a temperature-controlled chamber, maintained the cells at 37°C with a humidified atmosphere of 5% CO₂. HCC7402 cells co-transfected with CFP-CaM and YFP-Hsp70 were grown in a glass-bottomed dish and mounted in the chamber. The sequence of fluorescent images was captured

with an Evolution QEi camera CCD every 5 min and the images were processed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

FRET was performed using Olympus IX81 fluorescence microscopy using a primary image splitter and CFY/YFP filter cube. FRET methodology has been described previously (Selvin 1995). For FRET measurement the standardization factors of CFP and YFP spillover were carefully calculated. The spillover factors were determined in cells expressing either CFP- or YFP-tagged proteins alone. Spillover (CFP) and Spillover (YFP) had values of 0.261 (± 0.017 , $n=30$) and 0.297 (± 0.034 , $n=40$), respectively. The final FRET ratio (FRETR and FR) value for each selected cell was then

Fig. 3 Immunofluorescence staining of CaM with Hsp70, LaminB2 or Actin to show their co-localization. **a** cytosol co-localization of CaM (red) and Hsp70 (green); **b** nuclear co-localization of CaM (red) and Hsp70 (green); **c** nuclear membrane co-localization of CaM (green) and Lamin B2 (red), **d** CaM (green) and actin (red) localized at stress fiber and cell-cell contact regions of ECV304 cells (scale bar 10 μ m)

calculated according to the following formula (Hailey et al. 2002): $FRET = I_{FRET} - C_{CFP} - C_{YFP}$, where I_{FRET} is the intensity of FRET with the FRET filter set of CFP/YFP-co-expressed cells and C_{CFP} and C_{YFP} are the contributions from CFP and YFP, respectively. Because of overlapping spectra, the final formula is $FRET_R = Ff / (A \times Af + B \times Df)$, $FR = Ff - b \times Df / a \times Af$ and $FRET^C = Ff - a \times Af - b \times Df$ (Ff is I_{FRET} , Af is C_{CFP} , Df is C_{YFP} and $a=0.297$, $b=0.261$). Pseudocolor images were displayed scaled linearly from the lowest to the highest signal within each cell where the FRET efficiency was plotted from nil (blue) to high (red).

MTT assay

One thousand five hundred cells (HCC7402) were plated in 180 μ l DMEM per well of 96-well flat-bottom plates. At the indicated times, 20 μ l of 5 mg/ml MTT solution in PBS were added to each well for 3 h at 37°C. After removal of the medium, 150 μ l of DMSO were added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). Triplicate wells were assayed for each condition and S.D. was determined.

Flow cytometry

Transfected cells (HCC7402) were harvested, washed with PBS, resuspended in 75% ethanol in PBS and kept at 4°C for 16–24 h. The fixed cells were centrifuged at 300 $\times$ g for 5 min and washed once in PBS. The cell pellets were resuspended and incubated for 30 min in propidium iodide staining solution containing 15 μ g/ml propidium iodide (Sigma) for cell-cycle-distribution analysis. The assays were performed using flow cytometry for 10,000 events (FACS Calibur, BD, San Diego, CA USA).

For the apoptosis assay, cells were rinsed with PBS, and washed three times, then centrifuged at 300 $\times$ g for 5 min. The apoptotic cells were labeled with Annexin V-FITC according to the procedures described in the Apoptosis Detection Kit I provided by BD Biosciences Pharmingen™ (San Diego, CA, USA). The proportion of Annexin V stained cells was analyzed using flow cytometry for 10,000 events (FACS Calibur, BD, San Diego, CA, USA).

Results

CaM binding of proteins during different cell phases

To explore the potential binding partners for CaM in different cell-cycle phases, we prepared cell lysates from synchronized interphase, metaphase and anaphase cells. After immunoprecipitation with indicated antibodies, pull-

down proteins were analyzed with SDS-PAGE and silver staining (Fig. 1a,b). Then MALDI-TOF mass spectrometry was applied to identify as many as 41 proteins. All proteins are shown in Table 1. Among them, 15 proteins are cell cycle involved, such as heat shock proteins, Hsp70 (HSPA8 and HSPA1A) and Hsp90 (HSPC1) (Kampinga et al. 2008); and 32 proteins are calcium-dependent (not bound with CaM when chelated with EGTA). Proteins were classified within seven groups that included heat shock proteins, ribosomal proteins, zinc finger proteins, centrosome proteins, skeletal proteins, kinase and others. The proteins are known to regulate mainly protein remodeling, mitosis events control, protein translation and transport, cell morphology and migration, etc. The proteins we chose to further examine with respect to their interaction with CaM are indicated in Fig. 1a.

CaM interacts with Hsp70, Hsp90, and laminB2 in vitro

We selected three proteins for further confirmation of association with CaM in vitro using immunoprecipitation and western blot methods. Hsp90 was identified to associate with CaM during anaphase, Hsp70 associated with CaM at interphase and metaphase, and laminB2 associated with CaM during the entire cell cycle in a calcium-dependent manner. It was also found that the interaction between hsp70 and CaM is increased upon heat shock. The results shown in Fig. 2 confirmed the data of the MALDI-TOF mass spectrum indicating that Hsp70, Hsp90, and laminB2 are able to form complexes with CaM (Fig. 2).

Fig. 4 Hsp70 associated with CaM in a temporally and spatially dependent manner. **a** IP result of Hsp70 and CaM showed that the Hsp70 and CaM complex was found to be more abundant in the nucleus in synchronized S-phase cells but not in total interphase cells. *N* nucleus, *C* cytoplasm; **b–f**, The FRET interaction of Hsp70 and CaM in living cells. CFP, YFP and FRET channel images obtained from living cells showed the fluorescence of CaM, Hsp70 and CaM-Hsp70 association. $FRET^C$ was calculated as described in “Materials and methods”, and presented as a pseudo-color image. **b** The positive control of FRET in the cell expressed the CFP-YFP fusion protein. $FRET^C = 2.2546 \pm 0.1789$, $FR = 4.4165 \pm 0.5157$; **c** the negative control of FRET in the cell expressed CFP and YFP, respectively. $FRET^C = 1.0023 \pm 0.0289$, $FR = 1.0085 \pm 0.1947$; **d** the FRET when CFP-CaM and YFP-HSP70 were co-localized in cytosole. $FRET^C = 1.216136 \pm 0.11548$, $FR = 2.2710 \pm 0.1624$; **e** the FRET when CFP-CaM was translocated into the nucleus but YFP-Hsp70 was still observed in the cytosol. $FRET^C = 1.234901 \pm 0.151903$, $FR = 2.3695 \pm 0.2231$; **f** the FRET when both CFP-CaM and YFP-Hsp70 were translocated into the nucleus. $FRET^C = 1.283909 \pm 0.10511$, $FR = 2.5782 \pm 0.1478$; **g** the FR comparing three experimental groups, positive control and negative control, $*P < 0.05$ to show the significant difference as compared with negative control. *A.l.u.f.i.* arbitrary linear units of fluorescence intensity. Bar 10 μ m

Co-localization of CaM with LaminB2, Hsp70, and Actin

Next, we performed immunofluorescent staining to check the co-localization of CaM with Hsp70, Lamin B2 and Actin which associated with CaM during the entire cell cycle in a calcium independent manner. We found that CaM and HSP70 could co-localize in the cytosol of some cells

(Fig. 3a) or in the nucleus of other cells (Fig. 3b). CaM and Lamin B2 were found in the nuclear membrane (Fig. 3c). CaM and actin were also found to co-localize in stress fiber and cell-cell contact areas (Fig. 3d). The data illustrated in Figs. 2 and 3 indicate that the protein identity we determined using IP-MALDI-TOF mass spectrometry is reliable.

HSP70 is associated with CaM in the nucleus during S phase

After finding that CaM and Hsp70 were either co-localized in the cytosol or in the nucleus, we next investigated the physiological functions induced by their association in the different compartments of the cell. First, we synchronized cells into S phase and checked the *in vitro* interaction of CaM with Hsp70. IP experiments showed that the association of CaM with Hsp70 occurred mainly in the nuclei during S phase (Fig. 4a). We then applied FRET measurement to analyze the *in vivo* interaction of Hsp70 and CaM in the living cell, which provides us not only with temporal but also spatial information. CFP labeled CaM and YFP labeled Hsp70 genes were co-transfected into 7402 cells. The images were taken from CFP, YFP, and FRET channels, and the FRET efficiency of YFP-Hsp70/CFP-CaM was calculated. The results are shown in Fig. 4b–f. During G1 phase, CaM was mainly found in the cytosol. FRET efficiency was noticeably low, although there was a significant difference as compared with control (Fig. 4d, $\text{FRETR} = 1.216136 \pm 0.11548$, $\text{FRETR} > 1.05$ is considered to be two molecules in interaction). When CaM began to translocate into the nucleus from the cytosol, the interaction of CaM and Hsp70 increased first in the cytosol around the nucleus (Fig. 4e, $\text{FRETR} = 1.234901 \pm 0.151903$). When both CaM and Hsp70 translocated into the nucleus, the FRET efficiency increased to its culmination and was observed mainly in the nucleus (Fig. 4f, $\text{FRETR} = 1.283909 \pm 0.10511$). The FR value of these groups shown in Fig. 4g also suggested that the interaction of CaM and Hsp70 in living cells is significant as compared with the CFP/YFP control. The results show that the interaction of CaM and Hsp70 occurs in a spatio-temporally dependent manner.

The time course observed in living cells indicates that the interaction of CaM and Hsp70 might be involved in cell-cycle control

Since the interaction of CaM and Hsp70 occurred mainly in the nucleus during S phase, we examined its effect on the cell cycle. First, we examined the translocation time course of CaM and Hsp70 when 7402 cells were labeled with CFP-CaM and YFP-Hsp70, respectively (Fig. 5a). According to our previous report, it was at G1 phase that CaM was distributed within the cytosol. We, therefore, began the observation at the G1 stage and recorded the behavior of CaM and Hsp70 every five minutes. We found that CaM translocated into the nucleus before Hsp70. Hsp70 entered the nucleus approximately 10 min later than CaM. After their co-localization, the cell gradually shrank and showed apoptotic morphology. In addition, the cell activity was seen to decrease upon transfection with Hsp70 (Fig. 5b).

Fig. 5 Effects of hsp70-CaM interaction on cell cycle and apoptosis. **a** Time-course observation of living cell that co-expressed CFP-CaM and YFP-Hsp70. The observation began at later G1 stage and the image was recorded every 5 min. CaM was translocated into the nucleus earlier than was Hsp70 (arrow). The cell morphology became apoptotic after CaM and Hsp70 co-localized into the nucleus at 75 min. Another cell already showing CaM Hsp70 nuclear translocation (arrowhead) is visible at the beginning of the observation showing apoptotic like morphology at 60 min; **b** MTT assay for cell growth after Hsp70 overexpression. Hsp70 can decrease cell activity; **c** FACS analysis of cell cycle and apoptosis. Overexpression of Hsp70 and CaM can arrest cells at S phase and induce apoptosis. However, disruption of Hsp70 and CaM association with truncated Hsp70 without the CaM-binding motif can rescue S-phase arrest and apoptosis. *WB* The levels of expression of CaM and Hsp70 following their transduction into cells; **d** the effect of YFP-C₁ on cell division and apoptosis. *Left histogram* calculation of division cells after overexpression YFP-C₁ and YFP-HSP70 by time course observation of living cell. *Control* no transfection any plasmid. About 77.6% of cells that expressed YFP-C₁ entered normal cell cycle and cells expressed YFP-HSP70 nearly had no division. *Right histogram* apoptosis of cells that transfected YFP-C₁ and YFP-HSP70 by flow cytometry detection

We proceeded to analyze cell-cycle progress and apoptosis after CaM and Hsp70 transfection. When CaM and Hsp70 were overexpressed, the cell cycle was arrested at S phase, resulting in an increase in apoptosis (Fig. 5c). However, upon disruption of CaM-Hsp70 association with truncated HSP70 (excluding the CaM-binding domain), S-phase arrest and apoptosis could be rescued. Simultaneously, the results of living cell observation and flow cytometry demonstrate that overexpression of YFP-C₁ has no toxicity to cells (Fig. 5d). We have also checked the effect of non-tagged Hsp70 and it has the same effect as YFP-Hsp70 (data not shown here). The results suggest that the association of CaM and Hsp70 could arrest cells at S phase and could thus induce cellular apoptosis.

Discussion

CaM works in a calcium-dependent or -independent manner by binding to and regulating the activity of target proteins called CaM-binding proteins (CaMBPs), through which Ca²⁺ sensitivity is expressed in a variety of cellular regulatory activities (Catalano and O'Day 2008). Our previous results show that the localization of CaM is controlled in a spatio-temporally specific manner (Li et al. 1999; Yu et al. 2004; Shen et al. 2007; Yuan et al. 2008), indicating that specific CaM-binding proteins may also function the same way. Through the IP-MALDI-TOF-MS strategy, we identified 41 CaMBPs and further confirmed some of them with immunoprecipitation, western blot and FRET analysis to exclude non-specifically binding proteins, among which fifteen are cell-cycle-specific in a calcium-dependent or -independent manner. Ca²⁺-independent CaMBPs typically bind CaM through conserved IQ and IQ-like motifs (Yap et al. 2000). Due to the presence of

various other binding domains, including two WW domains, these proteins may function as adaptor proteins during the CaM-mediated events of actin cytoskeleton remodeling and/or the modulation of membrane events (Nagasaki and Uyeda 2004). For example, CaM can bind with beta-actin in a Ca^{2+} independent manner. Our previous study has implied that actin interaction with CaM is essential for cell adhesion and migration (Yuan et al. 2008). Some CaMBPs that have IQ or IQ-like motifs may be required for scission of the residual cytoplasmic bridge to complete cytokinesis (Nagasaki and Uyeda 2004). Ca^{2+} -dependent CaMBPs typically bind with CaM via a CaM-binding domain consisting not of conserved sequences of amino acids but rather of sequences of hydrophobic residues located in specific positions relative to other amino acids (Chin and Means 2000; James et al. 1995; O'Neil and DeGrado 1990). Among the calcium-dependent-binding cell-cycle-specific proteins, heat shock proteins have already been shown to play an important role not only in refolding certain denatured proteins under stress conditions but also in cell-cycle regulation (Lindquist and Craig 1998).

Previous reports have shown that members of the Hsp70 family bind CaM in a calcium-dependent manner (Stevenson and Calderwood 1990). Heat shock could increase intracellular Ca^{2+} levels and promote the association CaM and Hsp70 (Rocak and Linder 2004; Drummond et al. 1986). When calcium homeostasis is reestablished, CaM and Hsp70 are then dissociated, allowing Hsp70 to bind other proteins. The reversible binding of Hsp70 with CaM allows Hsp70 to interact with other targets in response to intracellular calcium concentration changes during cell-cycle progression in a temporally dependent manner (Milarski et al. 1989). Using FRET technology, we also found that the Hsp70 and CaM association occurs not only in a temporally dependent manner but also in a spatially dependent manner. The interaction of Hsp70 and CaM was first found in the cytosol during the G1 phase. However, at S phase the strongest interaction was found in the nucleus. The interaction occurring in the nucleus indicated that CaM and Hsp70 might regulate the S-phase-related cell-cycle events.

It has been reported that continuous overexpression of Hsp70 actually stresses the cell and can inhibit cell growth (Mosser et al. 2000). We found that overexpressed Hsp70 often translocated into the nucleus. The time course observation of double labeled cells showed that the translocation of CaM from cytosol into nucleus occurred approximately 10 min earlier than that of Hsp70 at the onset of S phase. The nuclear localization of CaM is important for DNA synthesis and is favorable for cell-cycle progression (Choi et al. 2006; Agell et al. 1998). However, when overexpressed Hsp70 is translocated into the nucleus later, the association of CaM with Hsp70 might help Hsp70 to inhibit cell growth. It has been reported that CaM and

Hsp70 are related to heat shock signal transduction in wheat and to thermotolerance in *Arabidopsis* (Liu et al. 2003, 2007). Clemons and Anderson showed that overexpression of Hsp70 could enhance the extent of TRAIL-induced apoptosis through a mitochondrion dependent pathway (Clemons and Anderson 2006). The association of Hsp70 with CaM might benefit Hsp70 induced apoptosis. The CaM-binding domain found in eukaryotic Hsp70 is also the target for binding of Bag-1, an enhancer of ADP/ATP exchange activity of Hsp70s (Lüders et al. 2000). The binding of CaM to Hsp70 might compete with Bag-1 and release the apoptosis inhibition of the Bag-1-Hsp70 complex.

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