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Calcyclin Binding Protein Promotes DNA Synthesis and Differentiation in Rat Neonatal Cardiomyocytes

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Abstract During cardiac muscle development, most cardiomyocytes permanently withdraw from the cell cycle. Previously, by suppressive subtractive hybridization, we identified calcyclin-binding protein/Siah-interacting protein (CacyBP/SIP) as one of the candidates being upregulated in the hyperplastic to hypertrophic switch, suggesting an important role of CacyBP/SIP in cardiac development. To show the importance of CacyBP/SIP during myoblast differentiation, we report here that CacyBP/SIP is developmentally regulated in postnatal rat hearts. The overexpression of CacyBP/SIP promotes the differentiation and DNA synthesis of H9C2 cells and primary rat cardiomyocytes, as well as downregulates the expression of β-catenin. Besides, CacyBP/SIP promotes the formation of myotubes and multinucleation upon differentiation. To investigate the cardioprotective role of CacyBP/SIP in cardiomyocytes, a hypoxia/reoxygenation model was employed. We found that CacyBP/SIP was upregulated during myocardial infarction (MI) and hypoxia/reoxygenation. As a conclusion, CacyBP/SIP may play a role in cardiomyogenic differentiation and possibly protection of cardiomyocytes during hypoxia/reoxygenation injury. J. Cell. Biochem. 98: 555–566, 2006.

Key words: CacyBP/SIP; DNA synthesis; differentiation; H9C2; cardiomyocytes

INTRODUCTION

Cardiomyocytes in mammals undergo a switch from hyperplasia to hypertrophy during neonatal heart development shortly after birth [Li et al., 1996]. In rats, most cardiomyocytes gradually cease to undergo DNA replication, which is a prerequisite for cell proliferation, after the first 2 weeks of birth [Clubb and Bishop, 1984]. During myocardial infarction (MI), the loss of cardiomyocytes is only replaced by fibroblasts to form scar tissues [Olson and Srivastava, 1996]. This eventually leads to contractile dysfunction. In spite of this, whether cardiomyocytes undergo terminal differentiation and permanently withdraw from the cell cycle is controversial. However, clinically significant myocardial regeneration has not been observed after cardiac injury [Anversa and Kajstura, 1998].

Calcyclin (S100A6) is a calcium-binding protein that belongs to the S100 family [Zimmer et al., 1995]. It has been suggested that calcyclin plays an important role in cell proliferation, differentiation, and secretion [Breen and Tang, 2003]. Other evidence suggests that its expression is cell cycle related with maximum

Abbreviations used: CacyBP/SIP, Calcyclin binding protein/Siah interacting protein; EAT, Ehrlich ascites tumor; APC, adenomatours polyposis coli; Siah, homologs of *Drosophila* sina; SD, Sprague–Dawley; HBS, HEPES bovine serum; PCNA, proliferating cell nuclear antigen; ITSG, insulin-transferrin-selenium-G supplement; BrdU, bromodeoxyuridine; APS, adenylyl sulfate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl floride; NaF, sodium floride; TEMED, N,N,N′,N′-tetramethylenediamine; EDTA, ethylenediaminetetraacetic acid; SSC, 3M NaCl, 300 mM sodium citrate; DAPI, 4′,6-diamidino-2-phenylindole; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; PI, propidium iodide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green florescent protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; CK, creatine kinase; MI, myocardial infarction; MLC2, myosin light chain 2; ROS, reactive oxidative species.

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expression in G_0 and S phases [Calabretta et al., 1986]. Calcyclin-binding protein (CacyBP) was discovered as one of the protein interacting partners of calcyclin [Filipek and Kuznicki, 1998] and found in cytosolic fraction of EAT cells, mouse brain, and spleen [Nowotny et al., 2000]. Moreover, it is highly expressed in brain, particularly in neurons of the cerebellum, hippocampus, and cortex [Jastrzebska et al., 2000]. Changes in localization of CacyBP were observed upon induction of differentiation in BT325 cells [Liu et al., 2002] and elevation of intracellular calcium concentration in cortical neurons [Filipek et al., 2000].

Recently, a protein called SIP (Siah-1 interacting protein) was identified as a human homolog of CacyBP (hereafter we use CacyBP/SIP as the abbreviation of this protein). CacyBP/SIP acts as a component of the β-catenin degradation pathway. In the ubiquitin ligase complex, the *N*-terminal and C-terminal regions of CacyBP/SIP interact with Siah-1 and Skp1, respectively. Skp1 in turn binds to Ebi, which recognizes β-catenin for ubiquitination and degradation [Matsuzawa and Reed, 2001]. This Siah-1-CacyBP/SIP-Skp1 complex requires adenomatours polyposis coli (APC) as a scaffold protein for binding β-catenin, which is able to regulate the activity of Tcf/Lef-family transcription factors and promotes cell proliferation by the induction of cyclin D1, c-myc, and other transcription factors [Shtutman et al., 1999]

Here, we report the developmental regulation of CacyBP/SIP in postnatal rat hearts. Overexpression of CacyBP/SIP promotes differentiation and DNA synthesis of H9C2 cells and primary rat cardiomyocytes. H9C2 cells were used as a cell model in this project because they can differentiate into myotubes and express cardiac specific genes. The upregulation of CacyBP/SIP during MI suggests that CacyBP/SIP may protect cardiomyocytes from injury triggered by hypoxia/reoxygenation.

MATERIALS AND METHODS

Experimental Animals

Postnatal male Sprague–Dawley rats hearts were carefully dissected from decapitated animals and were rinsed in phosphate-buffered saline. Only the lower two-thirds of the myocardium, which contained mostly ventricular cells, was used in subsequent experiments.

Culture of Primary Neonatal Rat Cardiomyocytes

Ventricular heart tissues from 1-day-old or 7-day-old SD rats were dissected and washed in 15 ml ice-cold HBS with 40 U/ml heparin. Afterwards, the tissues were minced with scissors in 1 ml of HBS. The minced heart tissues were washed with 15 ml ice-cold HBS to remove blood cells and trypsinized at 4°C for 20 h. Afterwards, the tissues were centrifuged at 200*g* for 3 min at 20°C and then transferred to 10 ml of pre-warmed collagenase digestion solution. After incubating at 37°C for 15 min, the cell suspension was transferred to a new tube and centrifuged. The pellet was resuspended and washed with 20 ml HBS with $CaCl_2$ and $MgCl_2$. After another centrifugation at 200*g* for 5 min at 20°C, the supernatant was removed and the pellet was resuspended in 20 ml of plating medium. To selectively enrich cardiomyocytes, the cell suspension was poured through a sieve with a screen of 200 mesh (Sigma) into 140 mm culture dishes and preplated at 37°C in a humidified incubator with 5% CO_2 for 1 h. Next, the cell suspension was plated in 24-well plates (2.6×10^5 cells/well) with Dulbecco's modified Eagle Medium/F12 (DMED/F-12) containing 0.1% insulin-transferrin-selenium-G supplement (ITSG, Invitrogen), 5% horse serum (HS), 0.75% penicillin–streptomycin (PS, Invitrogen), 0.1 mM ascorbic acid, and 0.1 mM bromodeoxyuridine (BrdU, Sigma). After culturing at 37°C for 48 h in a humidified incubator with 5% CO_2, the culture medium was replaced with low serum medium and cells were further cultured for 4 days during which prewarmed low serum medium was refreshed on alternate days. Highly enriched preparations of cardiomyocytes (over 90%) with little fibroblast contamination were routinely obtained by this preplating and selective cultivation procedure. The purity of cardiomyocytes was estimated by staining the cells with eosin and then identifying and counting the number of cardiomyocytes based on their morphology.

Cell Line and Culture Conditions

H9C2 rat cardiac myoblasts (ATCC, Cat. No.: CRL 1446) were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% PS under a humidified atmosphere containing 5% CO_2. To induce the differentiation of myoblasts, the

medium was changed to DMEM supplemented with 1% HS. During cell differentiation, the medium was changed every day.

Cloning of the GFP-tagged CacyBP/SIP Plasmid

The recombinant construct pEGFP-C1-CacyBP/SIP was prepared by cloning the coding region of CacyBP/SIP (Forward: 5′-TAG GGC GAA TTC TAT GGC TTC CGC TTT GGA GGA G-3′, Reverse: 5′-TAG GGC GGA TCC TCA GAA TTC TGT GTC TTC CCT G-3′) into the pEGFP-C1 vector (BD Biosciences). DNA sequencing was used to confirm the success of cloning and the absence of mutations in the recombinant construct.

Antibody Production

To produce specific anti-CacyBP/SIP antibody, antisera were prepared by immunizing a rabbit with a synthetic peptide corresponding to the *N*-terminus of the CacyBP/SIP: TTTTT-GYTVKISNYGGWDQ. One hundred micrograms of the synthetic peptide was injected into the rabbit every month and blood was collected five times after each injection. Afterwards, the blood samples were allowed to clot and centrifuged at 3,000 rpm for 10 min. Finally, the clot was discarded and the antiserum was aliquoted and stored at -20°C until use.

Total RNA Isolation

Total RNA was extracted from different days of rat ventricular myocardium using TRIZOL reagent (Invitrogen), according to the manufacturer's protocol. Integrity of the RNA was examined on a 1% agarose-formaldehyde gel and the purity of the RNA was examined by the ratio of absorbance at 260 nm and 280 nm. The RNA was then treated with DNase I (Invitrogen) at 37°C for 20 min.

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Ten micrograms of total RNA was reverse transcribed into first-strand cDNA using the Thermoscript™ RT-PCR system (Invitrogen) in the presence of 50 ng of oligo $(dT)_{25}V$ according to the manufacturer's protocol. One-tenth of the product was then subjected to PCR amplification with 10X PCR buffer (Amersham), 2 mM $MgCl_2$, 2 mM dNTP, and 5 U of *Taq* DNA polymerase (Amersham), 10 μM of CacyBP/SIP forward (5′-ATC AGT AAT TAT GGA TGG GAT CAG-3′), and 10 μM of CacyBP/SIP reverse (5′-ATT AAT GGT TCG CTT CAT ATC ATC-3′) primers in a final volume of 50 μl. To normalize the total RNA input, the same amount of first-strand product was subjected to another PCR that targeted the glyceraldehydes-3-phosphate dehydrogenase (G3PDH), a housekeeping gene transcript, under the same conditions except 10 μM of G3PDH forward (5′-CCA CAG TCC ATG CCA TCA CTG CCA-3′) and 10 μM of G3PDH reverse (5′-GGC CAT GAG GTC CAC CAC CCT GTT-3′) primers were used instead. The thermal profile of each cycle was denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. Five microliters of each sample was withdrawn at different number of cycle and resolved on a 1% TAE agarose gel. Finally, the results of gel electrophoresis were analyzed by the UVIPhoto gel documentation system (UVITec).

Tritiated Thymidine Incorporation Assay

Cells were grown on 24-well plates to 80% confluence. After transient transfection with the pEGFP-C1-CacyBP/SIP plasmid or the pEGFP-C1 vector into cells for 36 h, ^{3}H-thymidine (1 mCi/ml, Amersham) was added to each well and labeled for 8 h. Cells were then washed and fixed with 15% TCA for 1 h. Next, cells were lysed and the radioactivity of the cell lysate was measured by a scintillation counter. Statistical significance of the difference between controls and CacyBP/SIP overexpressing cells was evaluated by the paired Student's *t*-test. A probability of $P < 0.01$ was considered to represent a significant difference.

Western Blot Analysis

Total protein was extracted from cells using lysis buffer containing 50 mM Tris at pH 8.0, 0.3 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 mM NaF, 0.2 mM PMSF, and protease inhibitors. Equal amounts of proteins were normalized by Bradford Assay (Bio-Rad) and were loaded with 5× loading buffer, 1 mM DTT, and denatured at 100°C for 5 min. Proteins were then electrophoresed on a 12% SDS–polyacrylamide gel in 1× Tris-glycine buffer at a constant voltage. The gel was then immersed in Tris-glycine buffer at pH 8.3 for 5 min and proteins were transferred onto a PVDF membrane (Millipore), which was pre-inactivated by methanol for 20 s before transfer, in a semi-dry electroblotter. Non-specific binding sites on the membrane were blocked with 5%

non-fat milk powder in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20) for 2 h. The membrane was hybridized with 1:100 anti-CacyBP/SIP antibody or 1:1,000 of other antibodies at 4°C overnight. The unbound antibody was washed away with TBST three times for 10 min at room temperature. Then, suitable secondary antibody (1:1,000) labeled with horseradish peroxidase was added to the membrane for another hour. Unbound antibody was washed with TBST at room temperature. Finally, signals were detected by Enhanced Chemiluminescence (ECL) detection kit (Santa Cruz) and autoradiographic films.

Determination of the Subcellular Localization

Cells on a 1 well chamber were transfected with 1–2 μg of the recombinant plasmid using LIPOFECTAMINE PLUS™ Reagent (Invitrogen). At 36 h after transfection, cells were washed with phosphate buffered saline (PBS) three times, fixed for 30 min at 4°C with 3.7% paraformaldehyde in PBS, and then stained with 300 nM DAPI solution, 0.1% Triton X-100, 2 mM EDTA, 8 μg/ml DNase-free RNase at room temperature for 30 min. Cells were mounted with coverslip by molwiol mounting medium (40× magnification) and viewed under a florescence microscope (Nikon).

Cell Cycle Analysis

Cells were grown to 80% confluence. After transient transfection with the pEGFP-C1 or pEGFP-C1-CacyBP/SIP plasmid for 36 h, cells were washed with PBS, detached with trypsin-EDTA, and fixed with 1.1% paraformaldehyde for 1 h. Samples were then fixed with 70% ethanol for 1 h. Later, cells were spun down and stained by propidium iodide (PI) solution (Molecular Probe) with 20 μg/ml PI, 2 mM EDTA, 0.1% Triton X 100, and 8 μg/ml DNase free RNase at 37°C for at least 30 min. Cell cycle pattern was analyzed by florescence channel 2 (FL2) with a wavelength of 490 nm in flow cytometer FAC Sorter (BD Biosciences). Finally, the results were analyzed by software WinMDI version 2.8.

Differentiation Assay

Cells were grown to 80% confluence. After transient transfection with the pEGFP-C1-CacyBP/SIP plasmid for 24 h, culture medium was changed from 10% FBS with 1% PS into 1% HS with 0.1% PS. At different time points of differentiation, cells were collected, and then treated by TRIZOL reagent for the preparation of RNA. RT-PCR was performed as previously described.

Creatine Kinase Activity Assay

Cardiomyocytes were washed with Ca^{2+}- and Mg^{2+}-free PBS and homogenized in 0.1 M sodium phosphate buffer (pH 7.0) supplemented with 0.1% Triton X-100. Total creatine kinase activity of the cell lysate was determined by a coupled enzymes assay method using the Creatine Phosphokinase Assay Kit (Sigma). In each determination, the enzyme activity for each sample was normalized with the protein concentration determined by the Bradford assay.

Induction of Myocardial Infarction in SD Rats

MI was induced in male SD rats (250–350 g) by isoproterenol. The rats were caged in wire-bottomed cages to allow good ventilation and deprived of drinking water for 1 h. Isoproterenol (300 mg/kg) was then administered subcutaneously to the rats. Drinking water was then withheld for 3 h. Then, the rats were orally administered with water in a single injection of 5 ml/kg followed by resumption of their free access to the drinking bottle. MI was evident at 10 h by the observation of the characteristic ECG pattern. This was further confirmed by subsequent histological examination of the excised heart at 30 h. Total RNA from the hearts of five isoproterenol-treated rats and that from five saline-treated controls were extracted 10 h after isoproterenol injection.

Simulated Ischemia/Reperfusion of Cardiomyocytes

To mimic the ischemia/reperfusion in vitro, cultured cardiomyocytes were incubated in 0.3 ml of stimulated ischemia buffer: DMEM Base (Sigma) at pH 6.2, deprived of glucose and serum for 9 h, with a constant stream of water-saturated 5% CO_2–95% N_2 (<1% O_2). After 9 h of exposure to the stimulated ischemia buffer, cells were subjected to reoxygenation in a water-saturated atmosphere of 5% CO_2–95% air for 2–3 h. The ischemia buffer was replaced with 0.3 ml reoxygenation medium. Afterwards, the activity of the cytoplasmic lactate dehydrogenase (LDH) in the culture media was determined. The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm.

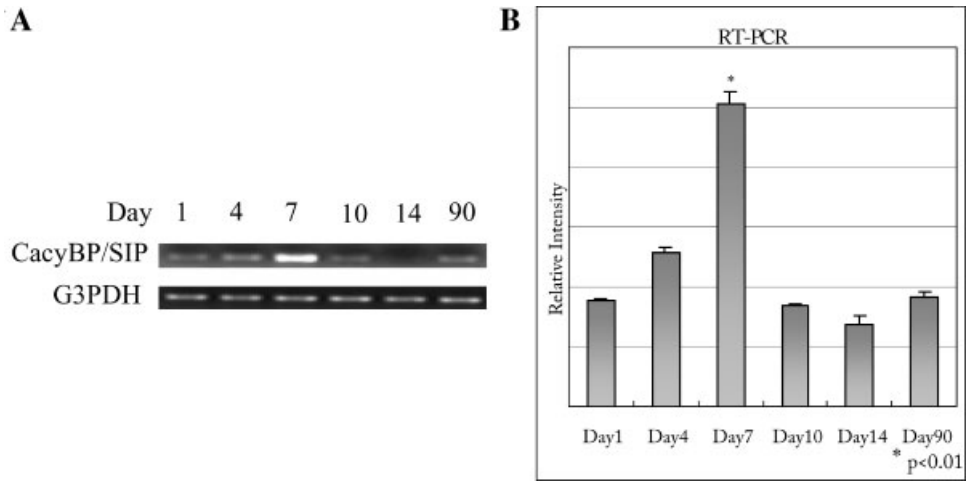


Fig. 1. Expression of CacyBP/SIP in rat heart on different postnatal days. **A**: Developmental regulation of CacyBP/SIP in the postnatal rat heart. RNA from day 1, 4, 7, 10, 14, and 90 rat hearts was used as template for RT-PCR. Not less than five rat hearts were pooled from the indicated days. **B**: Graphical representation of the relative expression of CacyBP/SIP at the indicated days. The data are the average of ±SD from three independent experiments.

RESULTS

Increase in Expression Level of CacyBP/SIP in Postnatal Rat Heart Development

Results of RT-PCR showed that CacyBP/SIP was upregulated from day 1 to day 4 and peaked at day 7 (Fig. 1A). This time interval coincides with the critical switch from hyperplastic to hypertrophic growth in cardiomyocytes. At day 10, the expression of CacyBP/SIP returned to control levels.

Differential Expression of CacyBP/SIP and β-Catenin During Cardiomyogenic Differentiation

Western blot analysis indicated that CacyBP/SIP expression remained at low levels from day 0 to day 7 differentiating H9C2 myoblasts, and gradually increased from day 9 to day 14 after differentiation (Fig. 2). Interestingly, the expression level of the β-catenin remained high during the first 10 days of differentiation but gradually dropped when the CacyBP/SIP is upregulated from day 10 to day 14, indicated that CacyBP/SIP and β-catenin may be reciprocally regulated.

Induction of DNA Synthesis in CacyBP/SIP-Overexpressing Cells

To elucidate the role of CacyBP/SIP in myocardial development, we overexpressed CacyBP/SIP in primary rat cardiomyocytes and H9C2 cells, followed by DNA synthesis assessment using [^{3}H]-thymidine incorporation assay after 36 h of transfection. We found that the DNA synthesis was increased by 36% and 15% in CacyBP/SIP-overexpressing primary rat cardiomyocytes and H9C2 cells, respectively (Table I), when compared with control cells transfected with the empty vector. We further checked the cell cycle status in CacyBP/SIP-overexpressing cells by flow cytometry at 36 h post transfection. From our results, there was an increase in the number of S-phase H9C2 differentiated myoblasts in CacyBP/SIP-overexpressing cells. The increase in S-phase cells was 10% in undifferentiated H9C2 cells, whereas the increase in S-phase cells was only 6% in day 3 differentiated H9C2 cells (Fig. 3).

Promotion of Myotube Formation and Multinucleation by Overexpression of CacyBP/SIP in Differentiation Medium

The morphology of CacyBP/SIP-overexpressing cells was examined under a florescence microscope. After 36 h of transfection, H9C2 cells transfected with either control or CacyBP/SIP did not result in any significant changes in the morphology (compare Fig. 4A and F). On the first day, with the replacement of differentiation medium, both groups of cells did not result in any significant changes in morphology (compare Fig. 4B and G). However, on the third day of differentiation, there were cell elongations with the formation of myotubes in CacyBP/SIP-overexpressing cells, whereas there were no myotubes observed in control cells (compare Fig. 4C and H). On the fifth day of differentiation, CacyBP/SIP-overexpressing cells became

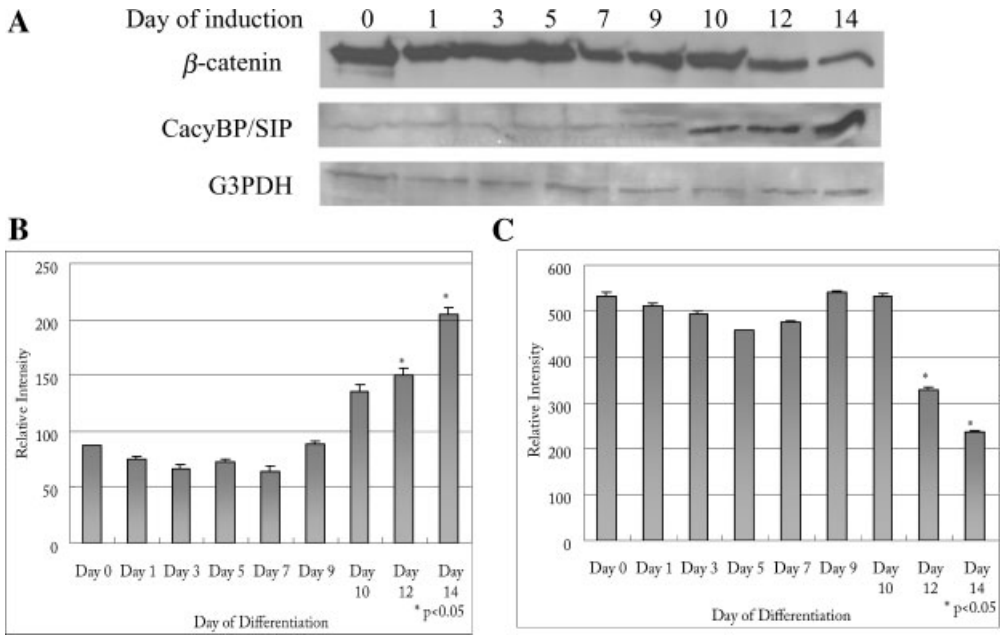


Fig. 2. **A**: Western blot analysis of CacyBP/SIP expression in H9C2 cells during serum deprivation-induced differentiation. Protein levels of G3PDH were used as normalization control. **B**: Graphical representation of the relative expression of CacyBP/SIP in the differentiation process. The data are the average of ±SD from three independent experiments. **C**: Graphical representation of the relative expression of β-catenin in the differentiation process. The data are the average of ±SD from three independent experiments.

multinucleated. Yet, there were no multinucleated cells observed in control (compare Fig. 4D and I). On the eighth day of differentiation, multinucleation was observed in both CacyBP/SIP-overexpressing and control cells (compare Fig. 4E and J). Thus, CacyBP/SIP may probably accelerate cellular differentiation and myotube formation.

Promotion of Cardiomyogenic Differentiation in CacyBP/SIP-Overexpressing Cells

To elucidate the role of CacyBP/SIP in cardiomyogenic differentiation, we compared the expression levels of myosin light chain 2 (MLC2) in CacyBP/SIP-overexpressing cells with control cells. We found that the mRNA level of MLC2 was significantly upregulated in CacyBP/SIP-overexpressing cells (Fig. 5A), suggesting that CacyBP/SIP is able to induce the differentiation of cardiomyocytes. In addition, the cellular creatine kinase (CK) activity was measured to verify the positive effect of CacyBP/SIP on the differentiation of cardiomyocytes. As shown in Figure 6, there was a significantly higher CK activity in CacyBP/SIP-overexpressing day 3 differentiated cells than cells in the GFP control, implying that a higher myogenic activity was induced by the overexpression of CacyBP/SIP.

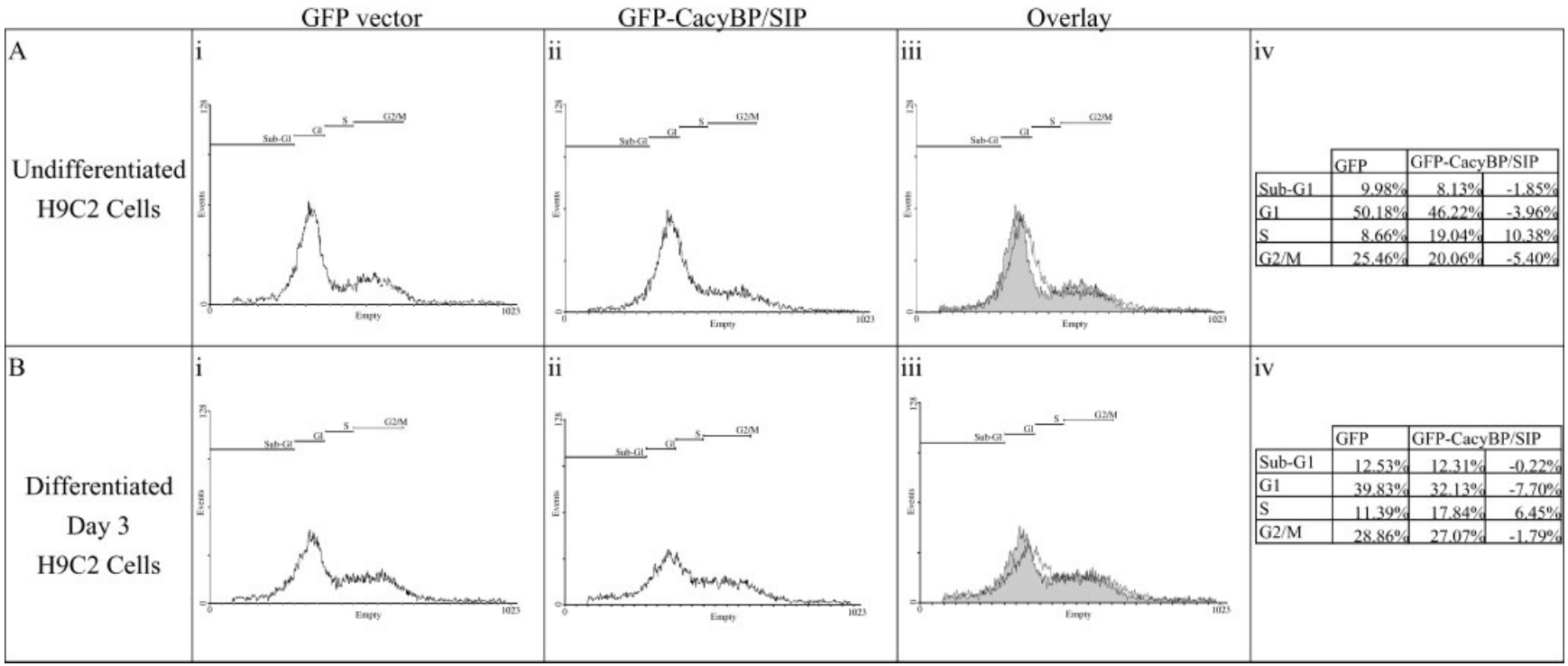
Downregulation of β-Catenin in CacyBP/SIP-Overexpressing H9C2 Cells

To explore the role of CacyBP/SIP in the regulation of β-catenin, the effect of overexpression of CacyBP/SIP on the protein level of β-catenin in H9C2 cells was examined. As shown

TABLE I. CacyBP/SIP Promotes DNA Synthesis

	Rate of DNA synthesis in GFP-CacyBP/SIP overexpressing cells			
	Mock transfection (PBS)	GFP vector	GFP-CacyBP/SIP	*P**
DNA synthesis (%)				
Primary cardiomyocytes	100.77 ± 2.03	100.00 ± 2.77	136.43 ± 1.55	<0.005
H9C2 Cells	95.57 ± 3.26	100.00 ± 1.2	115.31 ± 2.17	<0.002

DNA synthesis in primary cardiomyocytes and H9C2 cells were determined as [^{3}H] thymidine incorporation at 36 h after transfection with the CacyBP/SIP recombinant construct. Each sample measurement was performed in triplicates, and the data are expressed as mean ± SD from three independent experiments.
*Statistical significance assessed by Student's *t*-test.



A	GFP	GFP-CacyBP/SIP	
Sub-G1	9.98%	8.13%	-1.85%
G1	50.18%	46.22%	-3.96%
S	8.66%	19.04%	10.38%
G2/M	25.46%	20.06%	-5.40%

B	GFP	GFP-CacyBP/SIP	
Sub-G1	12.53%	12.31%	-0.22%
G1	39.83%	32.13%	-7.70%
S	11.39%	17.84%	6.45%
G2/M	28.86%	27.07%	-1.79%

Fig. 3. Cell cycle patterns of CacyBP/SIP in both undifferentiated (**A**) and differentiated day 3 (**B**) H9C2 cells. Cells were harvested at 36 h after transfection with the CacyBP/SIP recombinant construct. Cells were stained with propidium iodide and analyzed by flow cytometry. Cell transfected with (i) GFP vector only; (ii) GFP-CacyBP/SIP; (iii) Overlay of GFP vector and GFP-CacyBP/SIP; (iv) Table showed the percentage of each region in the histograms. There was an increase in S-phase cells by 10% and 6% in undifferentiated (A) and differentiated (B) CacyBP/SIP overexpressing cells, respectively, compared with cells transfected with GFP vector only.

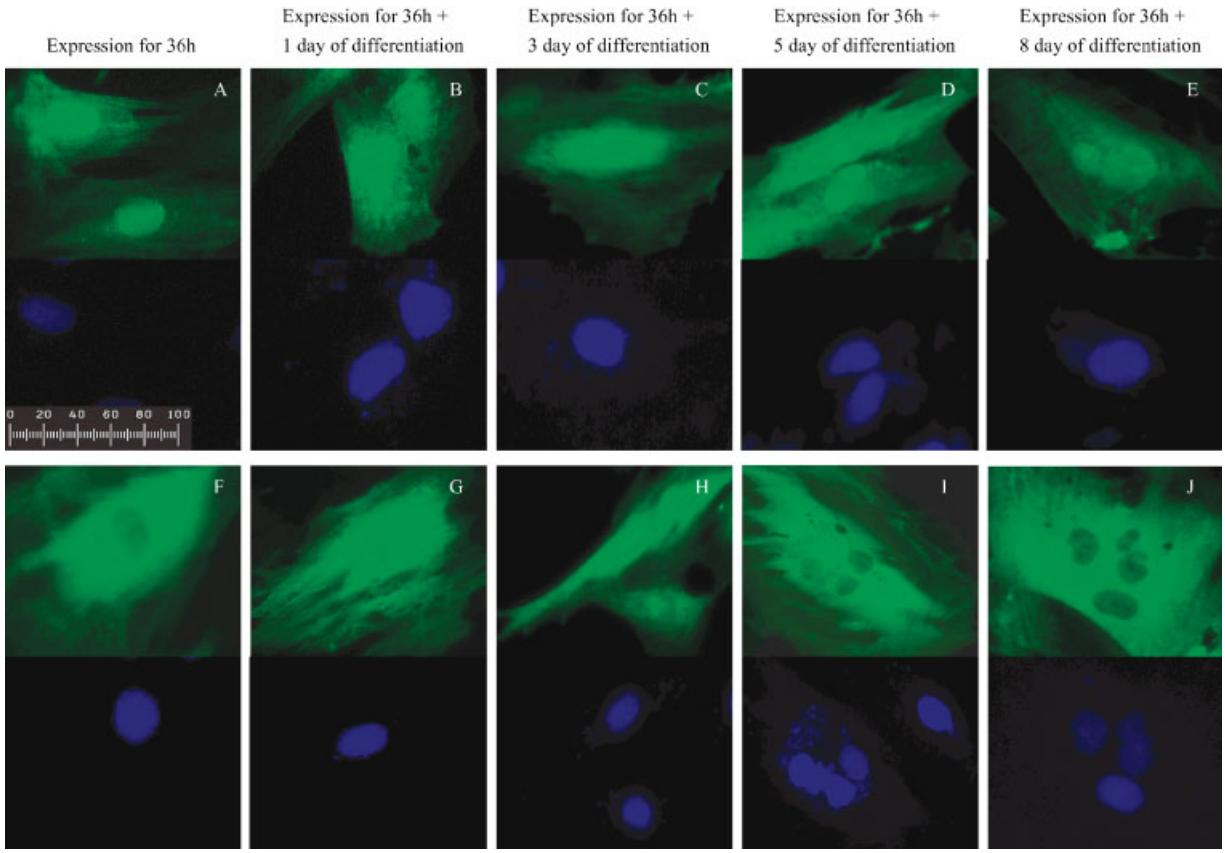


Fig. 4. Florescent images comparing the effect of transiently expressed GFP vector only or CacyBP/SIP-GFP hybrid constructs in H9C2 cells in different days of induced differentiation. H9C2 cells transfected with GFP vector alone for 36 h (**A**), 36 h followed by 1 day of DM (**B**), 36 h followed by 3 days of DM (**C**), 36 h followed by 5 days of DM (**D**), 36 h followed by 8 days of DM (**E**), and GFP-CacyBP/SIP for 36 h (**F**), 36 h followed by 1 day of DM (**G**), 36 h followed by 3 days of DM (**H**), 36 h followed by 5 days of DM (**I**), 36 h followed by 8 days of DM (**J**). DAPI stain showed the nucleus of the corresponding images. Elongation of GFP-CacyBP/SIP overexpressing cells was observed after replacement of DM for 3 days. Multinucleation was observed in GFP-CacyBP/SIP overexpressing cells after 5 days of DM under florescent microscope. Multinucleation can only observed in control vector after 8 days of differentiation.

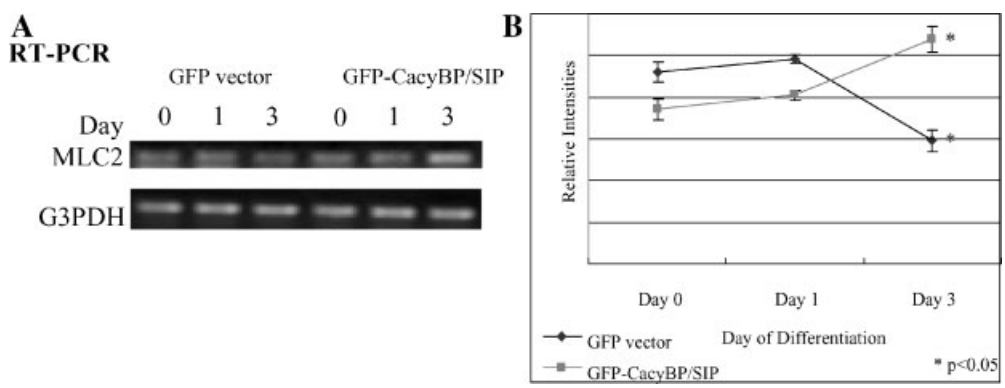


Fig. 5. **A**: The expression profiles of endogenous CacyBP/SIP and the differentiation marker MLC2 in different days of H9C2 cell differentiation as indicated by RT-PCR analysis. The results were normalized by the expression of G3PDH. **B**: The expression of MLC2 was increased in GFP-CacyBP/SIP overexpressing H9C2 cells as indicated by RT-PCR results. The data are the average of ±SD from three independent experiments.

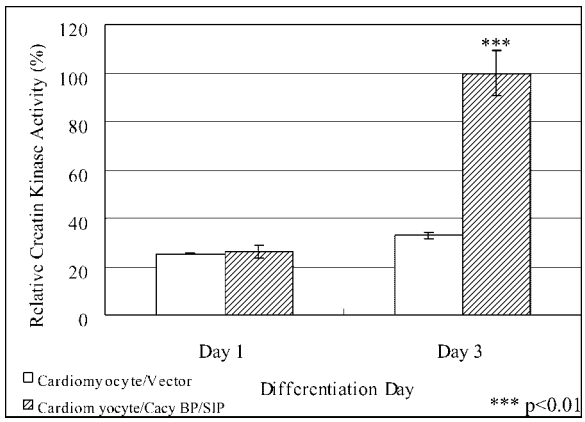


Fig. 6. The creatine kinase (CK) activities of cardiomyocytes transfected by GFP-CacyBP/SIP and GFP vector were determined. Results are expressed as the relative CK activity (%) between cardiomyocyte/GFP-CacyBP/SIP and control GFP cells. CK activity of cardiomyocyte/CacyBP/SIP in day 3 was arbitrarily set at 100%. The error bar means the maximum variation of data from five independent experiments.

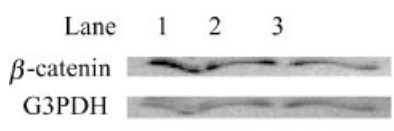
in the Western blot, the expression of β-catenin was significantly downregulated after transient transfection with the CacyBP/SIP recombinant construct (Fig. 7).

Cardioprotective Effects of CacyBP/SIP in Hypoxia-Reoxygenation Injury in Cardiomyocytes

Beside the role in differentiation, we also investigated the possible role of CacyBP/SIP in response to hypoxia-reperfusion stress. The expression levels of CacyBP/SIP were compared between MI and control rats by RT-PCR. We demonstrated an upregulation of endogenous CacyBP/SIP in MI hearts when compared with controls (Fig. 8A). To mimic the ischemia/reperfusion in the rat heart, the primary cardiomyocytes were subjected to hypoxia/reoxygenation injury. We found that the expression level of CacyBP/SIP remained relatively constant during the first 3 h of hypoxia, and then the level dropped at 5.5 h of hypoxia but significantly upregulated after reoxygenation for 2 and 3 h (Fig. 8B). To further elucidate the cardioprotective effect of CacyBP/SIP, we examined the LDH release in CacyBP/SIP-overexpressing primary rat cardiomyocytes subjected to hypoxia and reoxygenation stress. During hypoxia, the LDH release in both CacyBP/SIP-overexpressing cells and control cells were expectedly low. We found that overexpression of CacyBP/SIP resulted in a reduction of LDH release in cardiomyocytes subjected to reoxygenation when compared with control (Fig. 8C). These findings suggest that CacyBP/SIP may play a possible cardioprotective role in IR injuries.

DISCUSSION

We previously found that CacyBP/SIP is one of the putative differentially expressed genes during neonatal heart development of rats [Chim et al., 2000]. Here, using H9C2 rat cardiac myoblasts and primary cardiomyocytes as cell models, we further clarify the role of CacyBP/SIP in cardiomyogenic differentiation. First of all, the differential expression of CacyBP/SIP was studied by RT-PCR. We found that the expression of CacyBP/SIP is significantly upregulated from day 1 to day 7 but abruptly downregulated afterwards. Interestingly, this



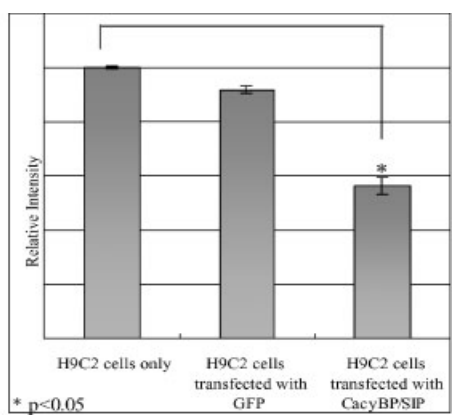


Fig. 7. Expression levels of β-catenin in untreated, GFP vector-transfected, and CacyBP/SIP-transfected H9C2 cells. **A**: Western blotting analysis of the expression of β-catenin in CacyBP/SIP-transfected H9C2 cells as compared with controls. **B**: Graphical representation of the relative expression of β-catenin in different conditions. The data are the average of two independent experiments.

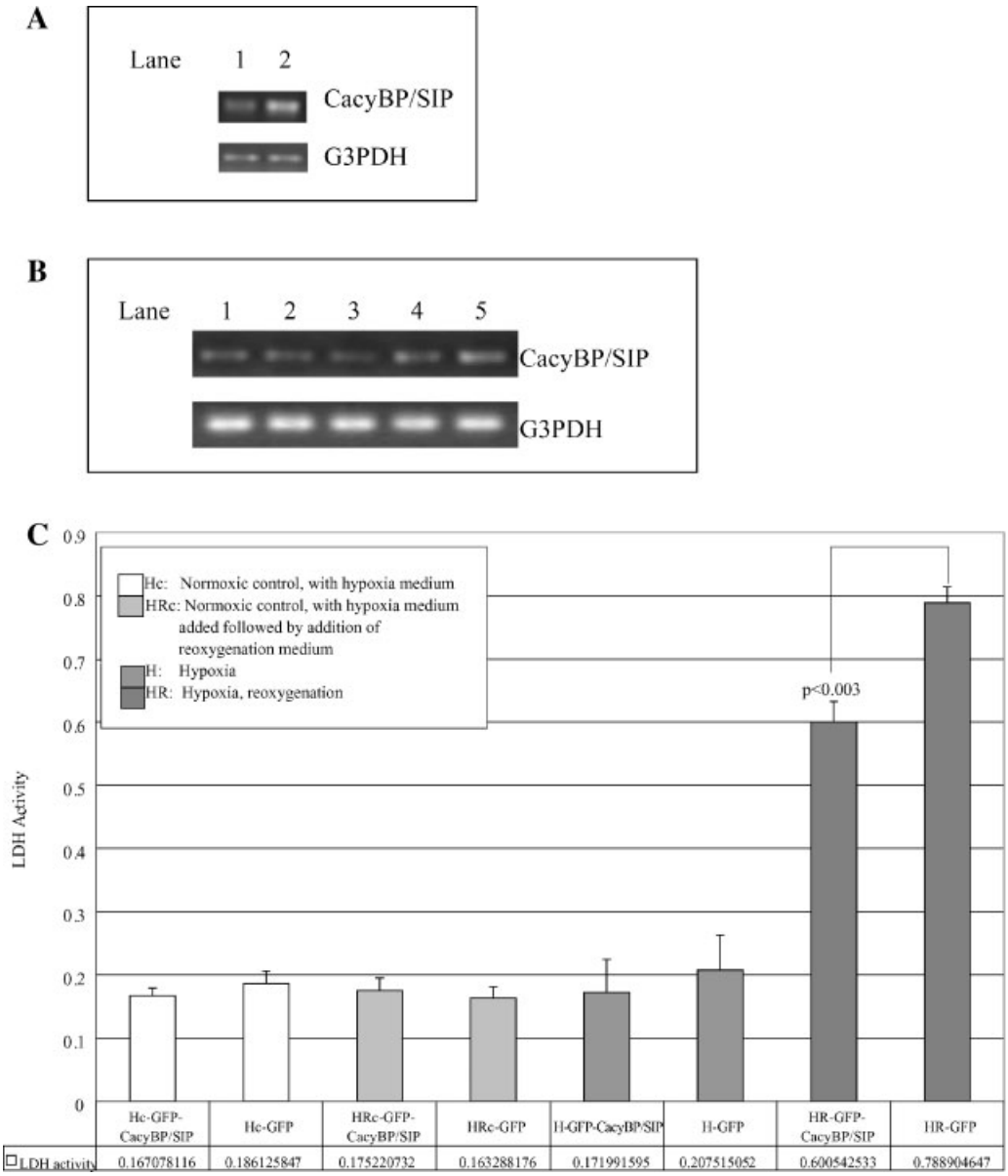


Fig. 8. **A**: Expression of CacyBP/SIP was upregulated in myocardial infarction (MI) rat heart. **Lane 1**: Control normal hearts from five rats. **Lane 2**: Ischemic hearts from another five rats. **B**: Upregulation of endogenous CacyBP/SIP during reoxygenation in primary cardiomyocytes. Lane 1: normal cell control, Lane 2: hypoxia for 3 h, **Lane 3**: hypoxia for 5.5 h, **Lane 4**: hypoxia for 5.5 h followed by reperfusion for 2 h, and **Lane 5**: hypoxia for 5.5 h followed by reoxygenation for 3 h. **C**: Cardioprotective effect of CacyBP/SIP overexpressing cardiomyocytes in hypoxia/reoxygenation. Hc, normoxic control, with hypoxic medium; HRc, normoxic control, with hypoxia medium added followed by addition of reoxygenation medium; H, hypoxia; and HR, hypoxia, reoxygenation.

peculiar differential expression of CacyBP/SIP coincides with the hyperplastic to hypertrophic switch of the rat neonatal heart. Li et al. [1996] reported that the cell number of rat neonatal cardiomyocytes increases by 68% from day 1 to day 3, and remains constant thereafter. On the other hand, the cardiomyocyte volume remains constant from day 1 to day 3 and then a significant change was detected at day 4. After that, it increases 2.5-fold during day 3 to day 12. The percentage of binucleated cardiomyocytes also begins to increase at day 4, and reaches 90% of the adult level at day 12. Another study found that the relative cyclin-dependent kinase 2 and cyclin-dependent kinase 4 activities of day 7 rat cardiomyocytes plummeted, respectively, to 18.2% and 22.2% of day 1 [Kang and Koh, 1997]. Notably, in this report we found that

CacyBP/SIP is also upregulated during the differentiation of H9C2 cells (Fig. 2). Therefore, we initiated the study on the involvement of CacyBP/SIP in the proliferation and differentiation of cardiac myocytes.

It has been shown that CacyBP/SIP is one of the components of the ubiquitin ligase complex, which interacts with Siah-1 and Skp1 [Matsuzawa and Reed, 2001]. Siah-1 regulates the degradation of β-catenin, which has a well-documented role in heart development [Rezvani and Liew, 2000; Hurlstone et al., 2003; Liebner et al., 2004]. On the other hand, Skp1 is a central component of Skp1-Cullin-F box complexes. Multinucleation and altered proliferation can be found in cells of knockout mice with the downregulation of β-TrCP1 and Skp2, which are F-box proteins [Nakayama et al., 2000; Nakayama et al., 2003].

It is generally believed that the decrease in DNA synthesis is concomitant with the differentiation of cardiomyocytes. Interestingly, although obvious signs of differentiation could be observed in the CacyBP/SIP-overexpressing cells (Figs. 4 and 6), there was also a significant increase in the rate of DNA synthesis, which was supported by an increase in the thymidine uptake (Table I) and the increase in the number of S phase cells revealed by the flow cytometry analysis (Fig. 3). This suggests that during the neonatal rat myocardial development, CacyBP/SIP may promote cardiomyocyte differentiation and induce the cardiomyocytes to enter the S phase and replicate the DNA for the multinucleation process. Moreover, we also found that β-catenin was downregulated during the differentiation of the H9C2 cells (Fig. 2) and in CacyBP/SIP overexpressing cells (Fig. 7), suggesting that the action of CacyBP/SIP on cardiomyogenic differentiation is possibly mediated through the APC/β-catenin pathway.

Previous studies reported that expressions of APC and β-catenin are upregulated in rat hearts during MI. The expression levels are especially high in endothelial cells and cardiomyocytes of the infarct region [Bruzzoni-Giovanelli et al., 1999]. On the other hand, calcyclin, the interacting partner of CacyBP/SIP, is induced in rat kidney during the recovery process after acute ischemic injury [Lewington et al., 1997]. Therefore, we hypothesize that CacyBP/SIP may also play a role in the MI or the recovery from ischemic damage in the rat heart. In this report, we have shown that CacyBP/SIP was upregulated during MI. However, the exact role of CacyBP/SIP in MI remains to be elucidated. Then, we speculate that the upregulation of CacyBP/SIP may be a response in the rat heart to increase the tolerance to ischemic injury and oxidant stress. Using the hypoxia/reoxygenation model in rat cardiomyocytes, we demonstrated the cardioprotective effect of CacyBP/SIP against the damage induced by the reoxygenation. During reperfusion of the rat heart, reactive oxidative species (ROS) are generated. This can result in the impairment of ventricular systolic function, the increase in myocardial stiffness and myocardial edema [Matheis et al., 1993]. It is still unknown whether CacyBP/SIP acts on the ROS directly or induces other proteins to scavenge the ROS.

We conclude that CacyBP/SIP is developmentally regulated in postnatal rat heart. The overexpression of CacyBP/SIP promotes the differentiation and DNA synthesis in H9C2 cells and primary rat cardiomyocytes. Being a protein upregulated during MI, CacyBP/SIP may have a protective effect on the cardiomyocytes against hypoxia/reoxygenation. The exact functional role of CacyBP/SIP in the differentiation and hypoxia/reoxygenation injury of cardiomyocytes needs to be clarified by future studies.

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