Molecular Cloning of Bovine Cardiac Muscle Heat-Shock Protein 70 kDa and Its Phosphorylation by cAMP-Dependent Protein Kinase in Vitro†

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ABSTRACT: The 70-kDa heat-shock protein (Hsp70) has been cloned and sequenced from bovine cardiac muscle. On the basis of sequence features, the gene corresponds to the cytoplasmic form of Hsp70. This cardiac Hsp70 cDNA clone has an open reading frame of 1926 bp coding for 641 amino acids and a predicted molecular mass of 70.25 kDa. Comparison of the amino acid sequence revealed an extensive sequence identity with other species of Hsp70. *Escherichia coli* expressed cardiac Hsp70 stimulated a 2-fold increase in calcineurin (CaN) activity. Notably, we observed that Hsp70 directly interacts with CaN using a pull-down assay. Furthermore, expressed cardiac-specific Hsp70 was phosphorylated in vitro by cAMP-dependent protein kinase. Phosphorylation resulted in the incorporation of 0.1 mol of phosphate per mol of Hsp70. The phosphorylated Hsp70 was unable to activate the phosphatase activity of CaN. This is the first demonstration that Hsp70 is phosphorylated by cAMP-dependent protein kinase and provides an on/off switch for the regulation of CaN signaling by Hsp70.

Heat-shock protein 70 kDa (Hsp70)[1] has been shown to be widely present in prokaryotic and eukaryotic cells and functions as an intracellular molecular chaperone (*1*, *2*). This protein is highly conserved among widely divergent organisms, e.g., 50% of the sequence is conserved between *Escherichia coli* and human, and some domains are 96% similar (*1*). The Hsp70 family of proteins has been implicated in a variety of processes including protein folding, the disassembly of oligomeric protein complexes, and the translocation of polypeptides across intracellular membranes (*3*). The human Hsp70 chaperone is a 640 amino acid protein composed of two major functional domains. The NH_2-terminal domain is highly conserved and contains an ATPase domain that binds ADP and ATP very tightly (in the presence of Mg^{2+} and K^+) and hydrolyzes ATP. The COOH-terminal domain on other hand is required for polypeptide binding. This latter domain is divided into functionally relevant subdomains, an 18-kDa peptide-binding domain and a 10-kDa C-terminal domain that contains the Glu−Glu−Val−Asp (EEVD) regulatory motif (*3*). Cooperation of both N-terminal and C-terminal domains is needed for protein folding (*3*).

The involvement of Hsp(s) in stabilizing the conformational transitions of newly synthesized protein is well documented (*4*). Most notable is the interaction of Hsp90 with a number of signaling proteins, including Ras, Raf, and $pp60^{v-src}$ kinase (*5*, *6*). Overexpressed Hsp70 significantly inhibited the enzymatic activities of protein kinase A and protein kinase C, but it stimulated the activity of protein serine/threonine phosphatases, protein phosphatase-1, and protein phosphatase-2A (*7*). A recent observation suggested that Hsp70 binds the dephosphorylated carboxyl terminus of mature protein kinase C, thus stabilizing the protein and allowing the rephosphorylation of the enzyme. Disruption of this interaction prevents rephosphorylation and targets the enzyme for down-regulation (*8*). Various reports demonstrated the direct interaction of Hsp(s) with calcineurin (CaN)[1] (*9*, *10*). CaN, a calmodulin (CaM)-dependent protein phosphatase, is activated by Hsp90 and Hsp70 in CaM-independent and -dependent mechanisms, respectively (*9*). Hsc82, an Hsp90 homologue in yeast, binds to the catalytic subunit of CaN and stabilizes this CaM-dependent phosphatase (*10*).

Phosphorylation and dephosphorylation of proteins are essential for cellular homeostasis. Ca^{2+}-dependent autophosphorylation in vitro has been reported for proteins in the Hsp70 family (*11*). For example, in the *E. coli* Hsp70 family of proteins, DnaK was autophosphorylated at Thr-199, and mutation of this residue abrogates its ATPase activity (*12*). Human Hsp70 can be autophosphorylated in the presence of divalent metal ions (Mg^{2+}, Ca^{2+}, and Ba^{2+}) with Ca^{2+} being the most effective (*13*). In vitro autophosphorylation was also observed for immunoglobulin binding protein, Bip (78 kDa), another member of the Hsp70 protein family (*14*). The possibility that Hsp70 family proteins may be phosphor-

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[1] Abbreviations: Hsp70, heat-shock protein 70 kDa; CaN, calcineurin; CaM, calmodulin.

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ylated by various protein kinases has not been explored, and the potential regulatory function of this protein after phosphorylation is also unclear. In this paper, we report the cloning and purification of bovine cardiac specific Hsp70 and the interaction of Hsp70 with CaN by the His-tag pull-down assay. Furthermore, in vitro phosphorylation of Hsp70 by cAMP-dependent protein kinase was demonstrated to inhibit the ability of Hsp70 to enhance CaN activity.

MATERIAL AND METHODS

Materials. Bovine brain CaN and CaM were purified as described earlier (*15*, *16*). Bovine heart cAMP-dependent protein kinase catalytic subunit was purified by the method of Demaille et al (*17*). Expression vector pQE-9 was purchased from Qiagen, Canada. Restriction endonucleases and DNA modifying enzymes were purchased from Amersham Pharmacia Biotech. Ligation Pack was purchased from New England Biolabs, USA. The monoclonal Hsp70 antibody was purchased from Sigma Canada. [8-^{14}C]ADP, [γ^{32}P]-ATP were purchased from Perkin-Elmer, USA. [8-^{14}C]ATP was purchased from Amersham Biosciences, UK. Nitrocellulose sheets were purchased from Bio-Rad Laboratories, Canada. Protein and DNA markers were purchased from Invitrogen, Canada. Nucleotides and general analytical grade laboratory chemicals were purchased from either BDH or Sigma, Canada.

Molecular Cloning of Bovine Cardiac Hsp70. General cloning techniques were carried out essentially as described by Sambrook et al. (*18*). Total RNA was prepared from bovine cardiac muscle using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription polymerase chain reaction (RT-PCR) was carried out with sense and antisense oligonucleotide primers of Hsp70. Primers were designed based on bovine skeletal muscle Hsp70 (accession number U09861). The sense and antisense oligonucleotides were (5′-**GGA TCC** ATG GCG AAA AAC ATG GCT ATC GGC AT -3′, *Bam*H1 restriction site) and (5′- **AAG CTT** CTA ATC CAC CTC CTC AAT GGT GGG GC -3′, *Hin*dIII restriction site), respectively. Amplification was carried out by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 2 min using a one-step RT-PCR kit (Qiagen) in a GeneAmp PCR system 2400 (Applied Biosystems). The amplified cDNA was purified by 0.7% agarose gel electrophoresis followed by gel extraction kit (Qiagen) and cloned into the Zero Blunt TOPO vector (Invitrogen). The complete nucleotide sequence was determined by the dideoxy chain termination method using a DNA sequencer (Applied Biosystem model 310A).

Construction of Cardiac Hsp70 into Escherichia coli Expression Vector. The bovine cardiac Hsp70 cDNA containing the entire coding region was isolated from the Zero Blunt TOPO vector by digesting with *Hin*dIII and *Bam*HI and separating the fragment by 0.7% agarose gel electrophoresis. The cDNA insert was purified using a gel extraction kit (Qiagen). The fragment was ligated to the expression vector pQE9, which had been digested with *Hin*dIII and *Bam*HI. The derivative recombinant plasmid was designated as pQE9-cardiac Hsp70. A similar method was used to generate pQE9-p85, encoding a control protein His_6-p85 containing the full-length p85 subunit of phosphatidylinositol 3-kinase.

Expression and Purification of Recombinant Bovine Cardiac Hsp70. pQE9-cardiac Hsp70 was transformed into *E. coli* M13(pREP4), grown to log phase at 37 °C in Luria-Bertani medium containing 100 μg/mL ampicillin and induced by 1 mmol/L isopropylthio-β-galactopyranoside (IPTG) for 4 h. Bacterial cells were harvested by centrifugation and resuspended in buffer A (50 mmol/L Tris-HCl, pH 8.0, 300 mmol/L NaCl, 10 mmol/L imidozole). They were then lysed by addition of lysozyme to a final concentration of 1 mg/mL on ice for 30 min. This was followed by sonication in a Sonics VibraCell sonicator, for 6 × 10 s bursts. The lysate was cleared by centrifugation (14000*g*, 30 min at 4 °C) and loaded onto a Ni-NTA agarose column, equilibrated with lysis buffer. The column was washed four times with buffer A containing 20 mmol/L imidozole, and then the bound protein was eluted from the column using buffer A containing 250 mmol/L imidozole. The purity of cardiac Hsp70 was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the purified fractions were pooled and stored at −80 °C for further analysis. Similarly, His_6-p85 fusion protein was prepared as described above.

SDS−PAGE and Western Blot Analysis. Purified cardiac Hsp70 proteins were separated on 10% SDS−PAGE according to the procedure described by Laemmli (*19*). The bacterially expressed cardiac Hsp70 was transferred to nitrocellulose membrane using the immunoblot method of Towbin et al. (*20*). The blot was incubated with the monoclonal Hsp70 antibody at 1:1000, washed and probed with an antimouse IgG horseradish peroxidase conjugate diluted 1:2000. Membranes were then incubated in chemiluminescence reagents and exposed to Kodak X-OMAT Blue XB-1 film for detection.

Pull-Down Assay. Both the *E. coli* expressed His_6-Hsp70 and control His_6-p85 fusion proteins were prepared as described above. The His_6-Hsp70 bound Ni-NTA agarose beads were incubated with purified bovine brain CaN in a buffer containing 50 mmol/L Tris-HCl, pH 7.0, 1% milk, and 0.1% Triton X-100 for 2 h at 4 °C with constant agitation. After four washes of beads with buffer containing 50 mmol/L Tris-HCl, pH 7.0, 1% NP-40, and 100 mmol/L NaCl, the bound proteins were eluted (20 μL of 1× SDS sample buffer) by boiling and resolved by SDS−PAGE. Gel transfer to nitrocellulose membrane and blocking were performed using standard procedures (*20*). The blot was incubated with the monoclonal Hsp70 or polyclonal CaN antibodies at 1:1000 dilution, washed, and probed with an antimouse or antirabbit IgG horseradish peroxidase conjugate diluted 1:2000. Membranes were then incubated in chemiluminescence reagents and exposed to Kodak X-OMAT Blue XB-1 film for detection. For phosphorylation of His_6-Hsp70 bound Ni-NTA Agarose beads, the beads were incubated with cAMP protein kinase as previously described by Kakkar and Sharma (*21*). Then, the phosphorylated of His_6-Hsp70 bound Ni-NTA agarose beads were incubated with purified bovine brain CaN, and the pull-down assay was carried out as described above. Furthermore, the addition of ATP, ADP, K^+ with His_6-Hsp70 bound Ni-NTA agarose beads were incubated with purified bovine brain CaN, and the pull-down assay was carried out as described above.

Phosphorylation of Bovine Cardiac Hsp70. Phosphorylation of cardiac Hsp70 was performed essentially as previ-

ously described by Kakkar and Sharma (*21*). The standard reaction mixture contained 100 mmol/L Tris-HCl, pH 7.0, 5 mmol/L $MgCl_2$, 5 mmol/L 2-mercaptoethanol, 0.1 mmol/L [γ-^{32}P]ATP (specific activity 150−200 cpm/pmol) and other components as described in the figure legends. An aliquot of reaction mixture was taken for determination of phosphate incorporation at different time intervals. In the remaining reaction sample, SDS sample buffer was added for the autoradiogram.

Nucleotide Binding Analysis. Characterization of ATP, ADP, and K^+ binding to the purified recombinant cardiac Hsp70 was performed by equilibrium gel penetration assay (*22*, *23*). The reaction mixture (1.0 mL) containing 100 mmol/L Tris, pH 7.0, and 5 mmol/L $MgCl_2$, 70 μg of Hsp70, 0.5−4 μM [^{14}C]ATP diluted with unlabeled ATP (to 35.1 μCi/ml), 50 units of creatine kinase (EC 2.7.3.2), and 10 mmol/L creatine phosphate for ATP binding or 0.5−4 μM-[^{14}C]ADP diluted with unlabeled ADP (to 15.3 μCi/ml), and 50 units of hexokinase (EC 2.7.1.1), and 10 mmol/L glucose for ADP binding. After the addition of Hsp70 to the reaction mixture, the mixture was equilibrated for 10 h, and then transferred to Eppendorf tubes, each containing 80 mg of dry Sepahedex G25 powder. The tubes were stored at 4 °C and gently agitated for 14 h to allow swelling of the gel and equilibration of free ligand with the solvent inside the gel. After equilibration, the tubes were centrifuged, and 10 μL aliquots of the supernatant were collected for measurements of radioactivity. A solution of Blue dextran in the same buffer was equilibrated with the gel to measure the excluded volume. The quantity of ligand bound to protein was calculated by equation described in ref *24*. Dissociation constants (K_d) were determined by the Scatchard plot method (*24*).

Other Methods. CaN activity was assayed using *p*-nitrophenyl phosphate (pNPP) as a substrate (*24*, *25*). Protein concentration was measured by the method of Bradford (*26*) using bovine serum albumin as a standard. The nucleotide and amino acid sequences were evaluated using the MacVector (version 7) computer program.

RESULTS

Cloning of Bovine Cardiac Hsp70. To clone Hsp70 from bovine cardiac muscle, total RNA from bovine cardiac muscle was reverse transcribed to cDNA using One-Step RT-PCR kit. The degenerate primers for Hsp70 were designed based on the DNA sequences of bovine skeletal muscle Hsp70 (*27*). These primers amplified an expected PCR product of ~2.0 kb. The amplified cDNA was cloned into pZeroBlunt vector and the complete nucleotide sequence was determined. The single long open reading frame (1926 bp) of bovine cardiac muscle Hsp70 specifies a protein of 641 amino acids. Sequence analysis revealed that bovine cardiac Hsp70 has 99% homology to skeletal Hsp70 at amino acid level. The N-terminal amino acids residues at positions 57 and 89 are different for cardiac Hsp70 compared to skeletal Hsp70.

To analyze the similarity of bovine cardiac Hsp70 with other Hsp70 family proteins, we compared the amino acid sequence of various species of Hsp70 using ClustralW program. The bovine cardiac Hsp70 exhibited 98% similarity with *Homo sapiens* and *Canus familiaris*, 97% with *Sus scrofa*, 96% with *Cercopithecus aethiops*, and 95% with *Rattus norvegicus* and *Mus musculus* (Figure 1). A high degree of sequence conservation was observed in NH_2-terminal sequence of different species. More sequence divergence was found in the COOH-terminal domain, which is required for polypeptide binding. The extreme COOH-terminal sequence GPTIEEVD is same for all the species tested including bovine cardiac Hsp70; this motif is characteristic for cytosolic Hsp70s.

Expression and Purification of Cardiac Hsp70. Subsequently, the cDNA of Hsp70 was subcloned into the expression vector pQE9 and transformed into *E. coli* M13 (pREP4). For the purification of recombinant cardiac Hsp70, the crude cell lysate was applied to Ni-NTA agarose column and the bound His_6-Hsp70 was eluted as described in Material and Methods. This single step purification was sufficient to produce highly purified recombinant cardiac Hsp70 as judged by Coomassie staining of samples resolved by SDS−PAGE. The molecular mass of purified cardiac Hsp70 was 70.25 kDa. Furthermore, a monoclonal antibody raised against brain Hsp70 was immunoreactive toward the recombinant cardiac Hsp70 protein.

Nucleotide Binding Analysis of Cardiac Hsp70. Hsp70 is known to bind nucleotide very tightly (*23*, *28*). Therefore, the binding between nucleotide (ATP and ADP) and cardiac recombinant Hsp70 has been studied by the equilibrium gel permeation method (*22*, *23*). The apparent K_d values of ^{14}C-labeled ATP and ADP of cardiac recombinant Hsp70 were 82 and 175 nmol/L, respectively, and are similar to those of bovine Hsp70 reported previously (*23*, *28*).

Activation of CaN by Cardiac Hsp70. Earlier, it has been reported that CaN was activated by brain Hsp70 (*9*). In our present study, we examined whether *E. coli* expressed cardiac Hsp70 can regulate purified bovine brain CaN. Ni-NTA purified cardiac Hsp70 activated brain CaN phosphatase activity 2-fold at a concentration of 70 nmol Hsp70 (Figure 2). The molar ratio of CaN to Hsp70 was 1: 4 (17.5 nmol/70 nmol). Higher concentrations of Hsp70 did not further alter CaN activity. Furthermore, no stimulation of CaN by Hsp70 was observed in the absence of calmodulin (data not shown). Binding of ATP to Hsp70 promotes conformational rearrangements in the molecule and changes its affinity for its substrates (*28*). To determine the effect of nucleotide binding on the ability of Hsp70 to stimulate CaN, the nucleotides (ATP, ADP, K^+ and K^+ with ATP) were bound to cardiac recombinant Hsp70. No change was observed in CaN stimulation by unbound and nucleotide-bound cardiac recombinant Hsp70 (Figure 3). These results indicate that there were no influences of nucleotides on the ability of Hsp70 to stimulate CaN activity in vitro.

Protein−Protein Interaction between Cardiac Hsp70 and CaN by the Pull-Down Assay. To analyze whether the activation of CaN by Hsp70 may be due to the direct interaction between Hsp70 and CaN, a His_6 pull-down assay was performed. Hsp70 was expressed as a His_6-tagged fusion protein, His_6-Hsp70. His_6-Hsp70 was immobilized on Ni-NTA and incubated with CaN (Figure 4). CaN was bound to His_6-Hsp70 (Figure 4A, lanes 2−4). Similar results were observed with monoclonal Hsp70 antibody (data not shown). However, no CaN binding was detected using an irrelevant control protein His_6-p85 immobilized similarly (Figure 4A, lanes 6−8). The His_6-p85 control protein showed no pull-

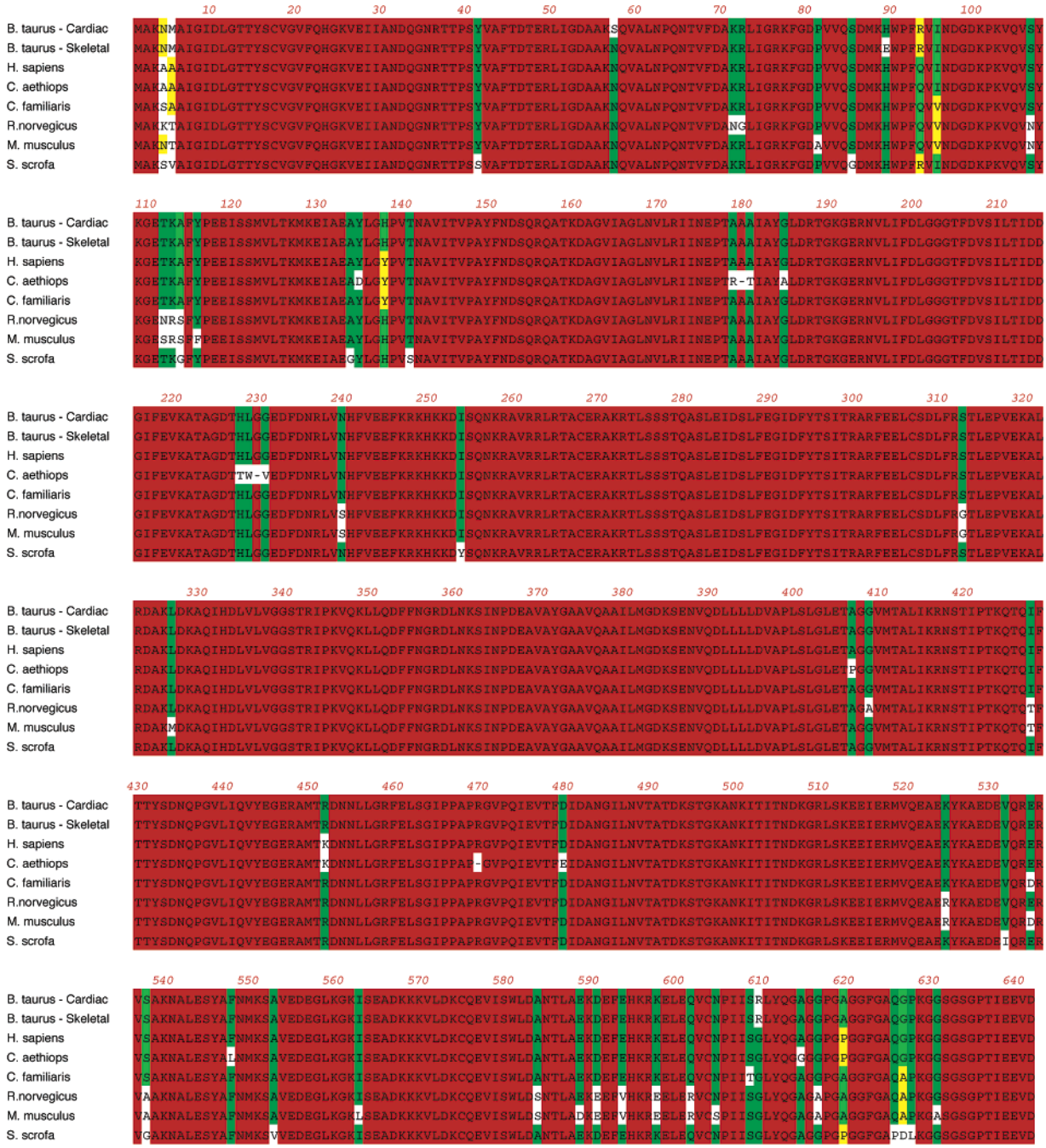

FIGURE 1: Comparison of the amino acid sequences of different species of Hsp70. The multiple sequence alignment was generated using the ClustralW program. The conservation is represented by the following colors: primary (red, 90−100%), secondary (green, 70−89%), tertiary (light green 50−69%), and quaternary (yellow, 30−49%). The deduced amino acid sequence of bovine cardiac Hsp70 (AY662497) was aligned with *B. taurus* skeletal muscle, AAA73914; *H. sapiens*, NP005337; *C. aethiops*, Q28222; *M. musculus*, AAC84169; *R. norvegicus*, CAA52328; *S. srofa*, P34930; and *C. familiaris*, BAC79353.

down signal indicating that the CaN pulled-down by the His_6-Hsp70 fusion protein was specific. This result indicates the direct protein−protein interaction between Hsp70 and CaN in vitro. Furthermore, the pull-down assay was carried out in the presence of ATP, ADP, K^+ and K^+ with ATP to analyze the effect of nucleotide(s) on this interaction. His_6-Hsp70 was immobilized on Ni-NTA and incubated with CaN in the presence of ATP, ADP, K^+ and K^+ with ATP (Figure 4B). We observed that CaN was bound to His_6-Hsp70 in the presence of ATP, ADP, K^+ and K^+ with ATP (Figure 4B, lanes 2−5). This result indicates that there was no influence of nucleotides or K^+ on the direct protein−protein interaction between Hsp70 and CaN in vitro.

Phosphorylation of Cardiac Hsp70 by cAMP-Dependent Protein Kinase. The available reports suggest that the Hsp70 family proteins are autophosphorylated in the presence of divalent metal ions (*12*−*14*). The phosphorylation of Hsp70 by other protein kinases is not yet established. To test the possibility that cardiac Hsp70 could be phosphorylated by cAMP-dependent protein kinase, recombinant His_6-cardiac Hsp70 was incubated with the catalytic subunit of cAMP-dependent protein kinase for different times, either in the presence of Ca^{2+}/CaM or EGTA. The cAMP-dependent protein kinase was found to catalyze the phosphorylation of cardiac Hsp70 with incorporation of 0.1 mol of phosphate/mol of Hsp70. Increased phosphate incorporation was not

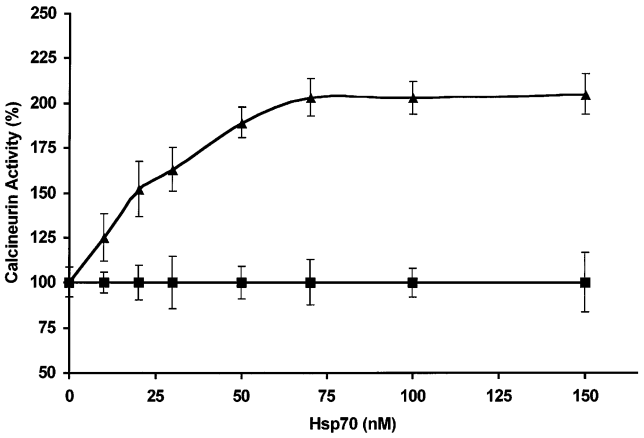

FIGURE 2: Effect of cardiac Hsp70 on CaN phosphatase activity. Different concentrations (10−150 nmol/L) of *E. coli* expressed cardiac Hsp70 were added in the CaN phosphatase assay mixture. The CaN phosphatase assay was carried out in the presence (▲) or in absence (■) of Hsp70. The data presented are representative of at least three separate experiments.

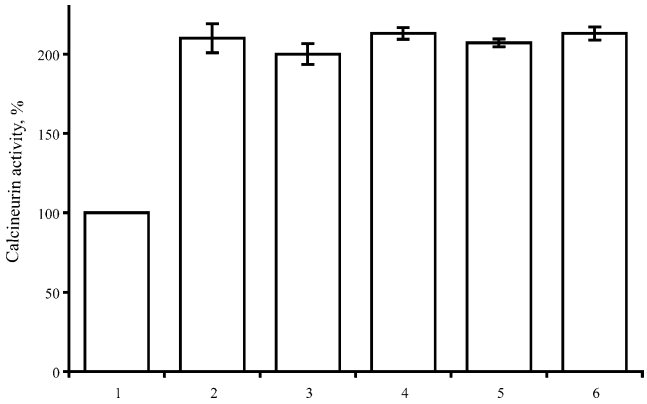

FIGURE 3: Effect of nucleotides on cardiac Hsp70 and its CaN phosphatase activity. Purified cardiac recombinant Hsp70 was incubated with 0.1 mmol/L ATP, ADP, K^+ and K^+ with ATP for 10 h at 30 °C. The nucleotide-bound cardiac recombinant Hsp70 was added to the CaN phosphatase activity as described in Materials and Methods. CaN alone (lane 1); unbound cardiac recombinant Hsp70 with CaN (lane 2); nucleotide bound cardiac recombinant Hsp70 with CaN: ATP (lane 3); ADP (lane 4); K^+ (lane 5); and K^+ with ATP (lane 6). The data presented are representative of at least three separate experiments

observed even with the use of higher concentrations of cAMP-dependent protein kinase. Furthermore, no difference in phosphate incorporation was observed in the presence of either Ca^{2+}/CaM or EGTA (Figure 5). A low level of Hsp70 autophosphorylation, measured in the absence of cAMP-dependent protein kinase, is shown for comparison (Figure 5). Phosphorylation of Hsp70 by cAMP-dependent protein kinase was further confirmed by autoradiogram studies (data not shown). These results suggest that Hsp70 could be a substrate for cAMP-dependent protein kinase. An equal amount of phosphate incorporation by Hsp70 in the presence of either Ca^{2+}/CaM or EGTA indicates that phosphorylation of cardiac Hsp70 by cAMP-dependent protein kinase is not dependent on Ca^{2+}/CaM. Furthermore, ATP/ADP bound Hsp70 was used for the phosphorylation studies. The result indicated that nucleotide additions did not alter the stoichiometry of phosphate incorporation in Hsp70.

Activation of CaN by Phosphorylated Cardiac Hsp70. To determine if the phosphorylation of Hsp70 by cAMP-dependent protein kinase could alter the ability of Hsp70 to activate CaN, CaN phosphatase assays were carried out (Figure 6). Surprisingly, the activation of CaN phosphatase activity was essentially eliminated by the phosphorylated cardiac Hsp70. However, control assays containing Hsp70 not phosphorylated by cAMP-dependent protein kinase, by omission of ATP or cAMP-dependent protein kinase, continued to show a 2-fold stimulation in CaN activity (Figure 6). Furthermore, we examined the interaction between phosphorylated Hsp70 with CaN. Pull-down analysis demonstrated that there is no interaction between phosphorylated Hsp70 with CaN (data not shown). This result suggests that cAMP-dependent protein kinase phosphorylation provides an on/off switch for the regulation of CaN by Hsp70.

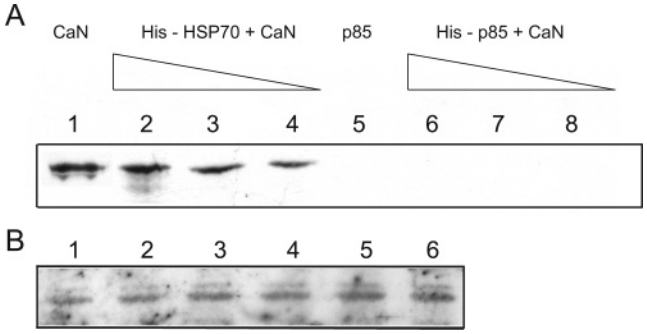

FIGURE 4: Interaction of Hsp70 with CaN in vitro. (A) Pull-down assays with His_6-Hsp70 or His_6-p85 (negative control) were performed using CaN. Purified brain CaN was incubated with 1 μg of His_6-Hsp70 immobilized in Ni-NTA beads. The beads were washed, and then subjected to SDS−PAGE. The pull-downs were analyzed by Western blotting with polyclonal antibodies against CaN. Lane 1, CaN alone (2 μg); lanes 2−4, His_6-Hsp70 Ni-NTA beads with 0.7, 0.07, 0.028 μg of CaN, respectively; lane 5, p85 alone (2 μg); lanes 6−8, His6-p85 Ni-NTA beads with 0.7, 0.07, 0.028 μg of CaN, respectively. (B) Pull-down assays with His_6-Hsp70 were performed using CaN in the presence of 10 mmol/L ATP, ADP, K^+ and K^+ with ATP. Lane 1, CaN alone (2 μg); His_6-Hsp70 Ni-NTA beads with 0.7 μg of CaN: unbound nucleotides (lane 2), 10 mmol/L ATP (lane 3), 10 mmol/L ADP (lane 4), 10 mmol/L K^+ (lane 5), 10 mmol/L K^+ with ATP (lane 6). The data presented are representative of at least three separate experiments.

DISCUSSION

Classification of various Hsp(s) in families is based on the related function, size, and the cellular compartment in which they reside. The Hsp70 multigene family consists of at least four members: Hsp70, Hsc70, Grp78 (BiP), and mitochondrial Hsp75 (mtHsp75). Hsp70, Hsc70 are found in the cytosol and nucleus, mtHsp75 in matrix of mitochondria and Grp78 in endoplasmic reticulam (*29*). Nucleotide sequence analysis demonstrated the expressed bovine cardiac Hsp70 corresponds to cytosolic form of Hsp70. This is confirmed by the extreme C-terminal end amino acid sequence GPTIEEVD. This terminal sequence is the characteristic of eukaryotic cytosolic Hsp70. In addition, higher similarity of amino acid sequence was demonstrated between cardiac Hsp70 and the Hsp70 family proteins than Hsc70. The family of Hsp70 proteins range in molecular mass between 70 and 78 kDa. The molecular mass of bovine cardiac Hsp70 was demonstrated to be 70.25 kDa by SDS−PAGE. This is in agreement with the calculated amino acid molecular weight. Hsp70 family proteins are highly conserved and demonstrate 60−70% identity among eukaryotic

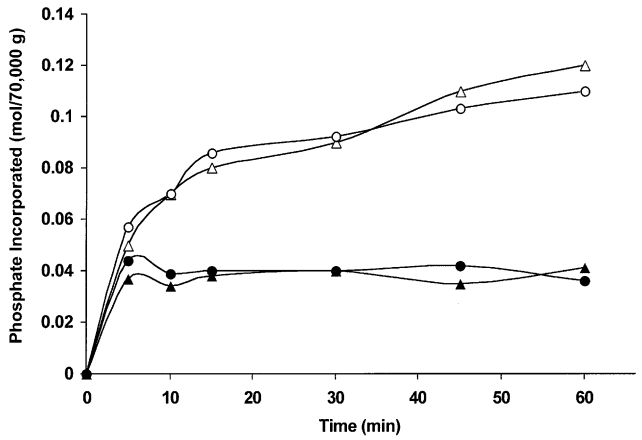

FIGURE 5: Time course of phosphorylation of cardiac Hsp70 by cAMP-dependent protein kinase. Purified cardiac Hsp70 (320 μg/mL) was phosphorylated by cAMP-dependent protein kinase (20 μg/mL) in a standard reaction mixture containing 100 μg/mL CaM in the presence of either 0.1 mmol/L Ca^{2+} (△) or 0.1 mmol/L EGTA (○). Cardiac Hsp70 without cAMP-dependent protein kinase (i.e., autophosphorylation) in the presence of Ca^{2+}/CaM (▲) or EGTA (●). The data presented are representative of at least three separate experiments.

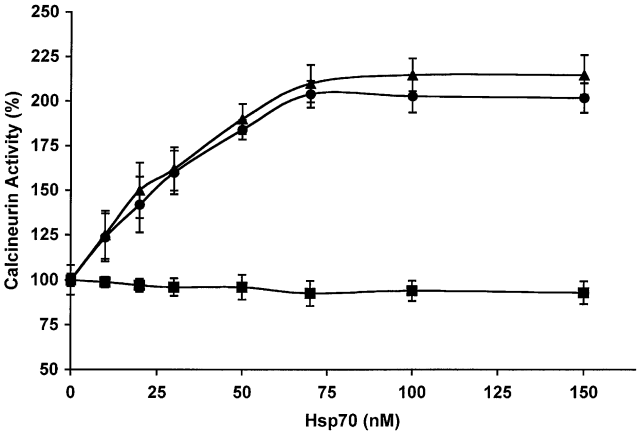

FIGURE 6: Effect of phosphorylation of cardiac Hsp70 on CaN phosphatase activity. *E. coli* expressed purified cardiac Hsp70 (320 μg/mL) was phosphorylated by cAMP-dependent protein kinase (20 μg/mL). The phosphorylated cardiac Hsp70 was dialyzed overnight with several changes against 20 mmol/L Tris-HCl, pH 7.0, 0.1 mmol/L EGTA and 10% sucrose to remove the unreacted [γ-^{32}P]ATP for CaN phosphatase activity. The basal CaN activity was taken as 100%. Different concentrations of nonphosphorylated or phosphorylated cardiac Hsp70 were added in the CaN assay mixture. Phosphorylated Hsp70 (■); nonphosphorylated Hsp70 was treated identically to the phosphorylated sample except the buffer was substituted for [γ-^{32}P]ATP (▲) or protein kinase (●). The data presented are representative of at least three separate experiments.

cells and 40−60% identity between eukaryotic Hsp70 and *E. coli* DnaK (similar to Hsp70). To date the Hsp70-II from sperm (accession number U02892) and Hsp70 from skeletal muscle (*27*) have been sequenced from bovine tissues. The sperm and skeletal muscle Hsp70 differ by two amino acid residues at positions 4 and 89 (*27*). The bovine cardiac Hsp70 encodes for Ser at position 56 and His at 89, whereas the bovine skeletal muscle encodes Asn and Glu at these respective positions. In the bovine species at least four Hsp70 genes have been identified from a genomic sperm library (*30*). Chromosomal localization of the Hsp70 gene in cattle demonstrated that Hsp70-I and Hsp70-II are tandemly localized in chromosome 23, Hsp70-III to chromosome 10, and Hsp70-IV to chromosome 3 (*31*). Alignment of the amino acid sequences of seven different species of Hsp70 revealed that the N-terminal sequence contains the ATP binding domain and is highly conserved among species. Comparison of the substrate binding domain also showed a high degree of conservation, whereas more sequence divergence was observed in the oligomerization domain.

The molecular relationships between Hsp(s) and various signaling proteins appear to be critical for the normal function of signal transduction pathways (*32*). Hsp90 and Hsp70 family proteins interact with various signaling molecules, including nuclear hormone receptors, tyrosine and serine/threonine kinases, cell cycle regulators, and cell death regulators (*33*). The influence of Hsp70 and Hsp90 in increasing CaN activity was reported by Someren et al. (*9*). They reported that maximal activation of bovine brain CaN occurred at 50 nmol/L concentration of Hsp70. In our present study, the maximum enhancement of CaN activity was observed at 70 nmol/L concentration of cardiac Hsp70. Furthermore, the His_6 pull-down assay revealed a direct interaction between Hsp70 and CaN. Substrates binding to the Hsp70 are associated with ATP binding, hydrolysis, and nucleotides exchange (*28*). In our study, 82 nmol/L ATP and 175 nmol/L ADP bound to the cardiac recombinant Hsp70, and these values were in good agreement with previously reported values for bovine Hsp70 (*23*). However, the nucleotide bound Hsp70 stimulated CaN in the same fashion as unbound cardiac recombinant Hsp70.

CaN is a heterodimer consisting of 19- and 57−59-kDa subunits referred to as CaN B and CaN A, respectively (*34*−*36*). CaN A contains catalytic (residues 70−333) and regulatory (residues 390−521) domains. The regulatory domain contains the CaN B binding helix, CaM-binding domain, and an autoinhibitory domain. Deletion analysis of CaN A revealed that deletion of the autoinhibitory domain and CaM-binding domain is essential for the induction of CaN activation, and one of its deletions is insufficient for the activation of CaN (*37*). The crystal structure analysis of the native CaN molecule showed that the structure of the COOH-terminal side from the CaM-binding domain was not visible (*38*, *39*), suggesting that this region may be structurally unstable. The interaction of CaN with a variety of proteins (perhaps Hsp70) may be important to stabilize this region of the CaN A subunit.

There are several types of modifications that may cause progressive charge shifts of Hsp70 proteins in the cell. Post-translational modifications, including methylation, (*40*) ADP-ribosylation (*41*), and phosphorylation (*42*) have been reported in Hsp70 proteins. Earlier, it was reported that there was a low level of phosphorylation of Hsp22 (5×10^{-5} mol of phosphate per mol of Hsp22) (*43*). Khan et al. (*44*) reported that the stoichiometry of phosphorylation for recombinant Hsp60 was 0.5 mol of phosphate per mole of recombinant Hsp60 when phosphorylated by protein kinase A. In our present study, the cAMP-dependent protein kinase was found to catalyze the phosphorylation of bovine cardiac Hsp70 with an incorporation of 0.1 mol of phosphate per mol of recombinant cardiac Hsp70. The low phosphorylation of recombinant cardiac Hsp70 may due to masking of the phosphorylation site during protein folding. In the presence of Ca^{2+}, in vitro autophosphorylation of Bip, a member of

Hsp70 protein family, was observed (*14*). However, in our present study, a very low level of autophosphorylation of *E. coli* expressed bovine Hsp70 was observed. Furthermore, following phosphorylation by cAMP-dependent protein kinase, we found that Hsp70 was not able to activate CaN. This is the first evidence that phosphorylation of Hsp70 by cAMP-dependent protein kinase can inhibit the ability of Hsp70 to stimulate CaN phosphatase activity. Robin et al. (*42*) reported that phosphorylation of GSTA4-4 (glutathione-*S*-transferase) by protein kinase A increases its affinity for binding to Hsp70, which helps this protein form efficient translocase complexes as well as to translocate to mitochondria. Our present work highlights the phosphorylation of Hsp70 as a mechanism to regulate CaN signaling in cardiovascular systems. Further work will be needed to identify the cAMP-dependent protein kinase phosphorylation site on Hsp70 and the location of the domain of Hsp70 responsible for the activation of CaN.

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