The S18 Ribosomal Protein Is a Putative Substrate for Ca$^{2+}$/Calmodulin-activated Protein Kinase II

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The δ-isoform of Ca$^{2+}$/calmodulin-activated protein kinase II (CaMK II) is abundantly expressed in vascular smooth muscle, but relatively little is known about its regulation or its potential cellular substrates. There are few, if any, known substrates of CaMK II that are physiologically relevant in vascular smooth muscle cells. Studies presented earlier (Mishra-Gorur, K., Singer, H. A., and Castellot, J. J., Jr. (2002) Am. J. Pathol., in press) by our laboratory show an inhibitory effect of heparin on CaMK II phosphorylation and activity. During these studies we observed the specific co-immunoprecipitation of a 20-kDa protein with CaMK II. Purification and sequence analysis indicate that this protein is the S18 protein of the 40 S ribosome. S18 was found to be abundantly phosphorylated in response to serum treatment, and this effect was strongly inhibited by heparin. In addition, KN-93, a specific CaMK II inhibitor, blocks S18 phosphorylation in vascular smooth muscle cells; a concomitant 24% reduction in protein synthesis was observed. Taken together these data support the idea that S18 could be a novel substrate for CaMK II, thus providing a potential link between Ca$^{2+}$-mobilizing agents and protein translation.

S18 Ribosomal Protein Phosphorylation

S18 phosphorylation of myosin light chain kinase. Caldesmon and calponin, two other proteins involved in contraction, are also postulated to be substrates for CaMK II. Both these proteins can inhibit actin-activated myosin ATPase; however, when phosphorylated by CaMK II they lose their ability to inhibit actomyosin ATPase (7).

Despite the potentially important role of CaMK II in VSMC contraction, there is minimal information available about its regulation in vivo. Furthermore, the relative abundance of this enzyme implies a role in other cellular functions, but the lack of other physiologically relevant substrates (besides calponin and caldesmon) for CaMK II in VSMCs has blunted the analysis of other putative functions. Here we report that the S18 protein of the 40 S ribosome is a potential substrate of CaMK II. While almost nothing is known about the function of S18 in animal cells, it is a highly basic protein showing high homology to the Escherichia coli ribosomal protein rpS13. rpS13 plays important roles in both initiation and elongation during protein translation and is thought to help regulate translational efficiency. Hence, the identification of S18 as a putative substrate for CaMK II may provide a novel link between Ca$^{2+}$-mobilizing agents and protein translation control.

**EXPERIMENTAL PROCEDURES**

**Materials**—All tissue culture plasticware was obtained from Costar (Cambridge, MA) and Falcon (BD PharMingen). Fetal calf serum was purchased from Hyclone (Logan, UT). RPMI base medium, trypsin-EDTA, glutamine, and penicillin-streptomycin were purchased from Invitrogen. Heparin, obtained from Glycomed (Alameda, CA), was derived from porcine mucosa (sodium salt) (molecular mass, 12–18 kDa). Chondroitin sulfate and reagents for buffers, cell extraction, and SDS-gel electrophoresis were from Sigma. Platelet-derived growth factor was from R&D Systems (Minneapolis, MN), and lysophosphatidic acid was from Sigma. Acrylamide solution (Protogel) was from National Diagnostics (Atlanta, GA). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Invitrogen; horseradish peroxidase-conjugated sheep anti-rabbit IgG was obtained from Roche Molecular Biochemicals. Sodium orthovanadate was from Sigma; okadaic acid, calyculin, tautomycin, and KN-93 were from Calbiochem. [3H]Leucine was from PerkinElmer Life Sciences.

**Cell Culture**—Rat aortic smooth muscle cells from Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) were isolated, cultured, and characterized as described previously (8). Briefly, the abbreviated segment of the aorta was removed, and the fascia was cleaned away under a dissecting microscope. The aorta was cut longitudinally, and small pieces of the media were carefully stripped from the vessel wall. Two or three such strips were placed in 60-mm dishes. Within 1–2 weeks, VSMCs migrated from the explants; they were capable of being passaged approximately 1 week after the first appearance of cells. They were identified as smooth muscle cells by their "hill and valley" growth pattern and indirect immunofluorescence staining for VSMC-specific α-actin. VSMC cultures were maintained in RPMI 1640, 10% fetal calf serum at 37 °C in a 5% CO$_2$, 95% air incubator. Primary cultures were used at or before passage 9. Cells were counted using a Coulter Counter (Coulter Counter Corp., Hialeah, FL). Cells were routinely grown in RPMI 1640, 10% fetal calf serum (FCS). SV40 large T antigen-transformed heparin-resistant and -sensitive cells (9) were grown and maintained under similar conditions.

**Growth Arrest of Cells**—Cells were routinely plated at 5–6 × 10$^5$/100-mm dish, washed with RPMI, and placed in RPMI containing 0.2–0.4% FCS for 72 h (10). Flow microfluorometry and determination of [3H]thymidine-labeled nuclei indicated that greater than 95% of the cells were arrested in G$_0$/G$_1$. Cells were released from quiescence by replacing the low serum medium with normal growth medium (i.e. RPMI 1640 containing 10% FCS). The cells were ~40–60% confluent at the time of harvest.

**Protein Analysis**—Quiescent cells were treated with 10% FCS, RPMI 1640, or 0.2–0.4% FCS for 72 h (10). Flow microfluorometry and determination of [3H]thymidine-labeled nuclei indicated that greater than 95% of the cells were arrested in G$_0$/G$_1$. Cells were released from quiescence by replacing the low serum medium with normal growth medium (i.e. RPMI 1640 containing 10% FCS). The cells were ~40–60% confluent at the time of harvest.
Ribosomal Protein S18 Is a CaMK II Substrate

with or without the indicated concentrations of heparin or chondroitin sulfate for various time intervals. The proteins were then harvested as follows with all procedures performed at 4°C. Cells were rinsed twice with cold TBS (20 mM Tris, pH 8, 137 mM NaCl) and lysed with 100 μl of lysis buffer (TBS + 1% Nonidet P-40, 10% glycerol, 100 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium orthovanadate). The extracts were rocked for 20 min and centrifuged at 12,000 rpm (Eppendorf microcentrifuge) for 10 min to remove insoluble material. The supernatant was stored at −20°C until use. Protein estimations were done by the Pierce BCA method adapted for microtiter plates. Extracts containing 50 μg of protein were boiled with 1× SDS sample loading buffer, resolved by SDS-PAGE (11), and blotted onto nitrocellulose membrane (Schleicher & Schüll) in Towbin buffer (25 mM Tris (Tris base), 192 mM glycine, 20% methanol) at 250 mA overnight. The blots were stained with Ponceau stain to confirm equal loading and transfer of proteins to the membrane. The membranes were blocked with 5% milk in 1× TBS (20 mM Tris base, 137 mM NaCl, pH 7.6), and Western blots were performed using anti-phosphorytrosine or anti-phosphoserine antibodies (1:10,000) and horseradish peroxidase-conjugated anti-mouse IgG (1:10,000) in 1× TBST (TBS + 0.2% Tween 20). The proteins were visualized using the DuPont Renaissance Enhanced Chemiluminescence detection reagents and autoradiography as described by the manufacturer. Prestained protein standard markers (Invitrogen) were used as molecular weight markers. Densitometric analysis on the autoradiograms was performed using the Stratagene (La Jolla, CA) Eagle Eye II system and the Scanalytics (Billerica, MA) ONE D-scan software.

Protein Synthesis Measurements—Protein synthesis was measured as described previously for smooth muscle cells (12). Briefly, 2 × 10^4 VSMCs were plated in 24-well microplates. 2 days later, 2 μCi/ml [3H]leucine was added to the culture medium in the presence or absence of 30 μM KN-93, a specific inhibitor of CaMK II (1). After 2 h, cells were washed five times with ice-cold phosphate-buffered saline, and 1.0 ml of ice-cold 10% trichloroacetic acid (w/v) was added to each well. The cells were incubated on ice for 1 h and washed five times with ice-cold 10% trichloroacetic acid. 1.0 ml of 1.0 M NaOH was added to each well overnight at 37°C, pH was neutralized with 10 ml HCL, and a small portion was counted using liquid scintillation spectrophotometry. Duplicate wells within the same multiwell plate were harvested for both protein determination and cell counting (12). To ensure that intracellular and extracellular leucine pools were completely equilibrated and that the specific radioactivity of leucyl-tRNA was unchanged by KN-93, the methods outlined by Gulves and Dice were followed (13). Under the conditions used in our experiments, these parameters were found not to contribute to the changes in protein synthesis rates observed in the presence of KN-93.

Radioactive Labeling and Immunoprecipitation of CaMK II—Quiescent cells were rinsed in phosphate-free RPMI and incubated in this medium for 2 h in the presence of 200 μCi/ml [γ-32P]ATP (14). Cells were then treated with 10% FCS, RPMI + heparin or with 1 μM ionomycin, RPMI + heparin. Proteins were harvested in Buffer A (50 mM MOPS, pH 7.4, 2 mM EGTA, 100 mM NaF, 100 mM sodium pyrophosphate, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 0.4 unit/ml aprotinin) and allowed to sit on ice for 5 min, and the lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was used for immunoprecipitation with anti-CaMK II antibodies (90 min at 4°C) and protein A + G-agarose beads (Oncogene Science) (60 min at 4°C). The immunoprecipitates were resolved by SDS-PAGE and blotted onto nitrocellulose, and differential phosphorylation was analyzed after overnight exposure on a PhosphorImager.

Immunoprecipitation and Purification of p20—Quiescent VSMCs were stimulated with 10% FCS, RPMI, and the cell lysates were used for immunoprecipitation with the anti-CaMK II δ-isoform-specific antibodies as described above. The immunoprecipitates were resolved by SDS-PAGE, and the proteins were visualized by Colloidal Coomassie Blue staining. The p20 band was excised from the gel and submitted for sequence analysis to the Harvard microsequencing facility.

CaMK II Inhibitor KN-93—Quiescent VSMCs were radioactively labeled with [γ-32P]ATP as described above. 90 min into the incubation, KN-93 was added to a final concentration of 30 μM. After 30 min the cells were stimulated with 10% FCS for 5 min. The proteins were then harvested and used for immunoprecipitation as described above.

RESULTS

A 20-kDa Protein Co-immunoprecipitates with CaMK II—We have reported that heparin inhibits CaMK II phosphorylation and activation (15, 34). In these experiments, growth-arrested VSMCs were radioactively labeled with [γ-32P]ATP followed by stimulation with 10% FCS, RPMI + heparin for 5 min. The cell lysates were used for immunoprecipitation with anti-CaMK II δ-isoform-specific antibody. The immunoprecipitates were resolved by SDS-PAGE and blotted onto nitrocellulose, and phosphorylation was visualized by autoradiography. Go, quiescent VSMCs; H, heparin; Iono, ionomycin.

20 kDa

Fig. 1. A 20-kDa protein co-immunoprecipitates with CaMK II. Radioactively labeled, quiescent VSMCs were treated with 10% FCS, RPMI + heparin for 5 min. The cell lysates were used for immunoprecipitation with anti-CaMK II δ-isoform-specific antibody. The immunoprecipitates were resolved by SDS-PAGE and blotted onto nitrocellulose, and phosphorylation was visualized by autoradiography. Go, quiescent VSMCs; H, heparin; Iono, ionomycin.

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Ribosomal Protein S18 Is a CaMK II Substrate

This study provides evidence that the S18 protein of the 40 S ribosome can be phosphorylated by CaMK II, thus identifying a putative substrate for CaMK II in VSMCs. The evidence includes 1) a physical association of S18 with CaMK II as determined by co-immunoprecipitation of the two proteins and 2) pharmacologic suppression of S18 phosphorylation by two inhibitors of CaMK II, heparin and KN-93. Furthermore, inhibiting phosphorylation of S18 with KN-93 also produced a reduction in protein translation rates.

Although the initial observation of a putative CaMK II/S18 interaction was made using heparin, work in our laboratory (10, 15, 34) and by others (18–20) clearly indicates that heparin inhibits kinases other than CaMK II. We therefore used the highly specific CaMK II inhibitor KN-93 to confirm this interaction. KN-93 is a more hydrophilic analogue of the isouquinolinyl sulfonamide KN-62. Both these inhibitors prevent the activation of CaMK II by interacting with the calmodulin binding domain of the kinase (1). KN-93 has no significant effects on myosin light chain kinase, protein kinase C, or protein kinase A at the concentrations used in the current study (17). KN-93 pretreatment resulted in an attenuation of CaMK II phosphorylation upon FCS stimulation. A concomitant reduction in S18 phosphorylation as well as the reproducible co-immunoprecipitation of S18 with CaMK II may provide a link between calcium-mobilizing agents and protein translation. This hypothesis is supported by the observation that the inhibition of S18 phosphorylation is accompanied by a 24% reduction in protein synthesis.

Little is known about the eukaryotic S18 protein except for its sequence. However, its prokaryotic homolog, the E. coli rpS13, has been studied more extensively, and the available data may provide insights into the role of S18 protein in eukaryotes, especially since there are many conserved domains between the two. Both S18 and rpS13 are very basic proteins (21, 22). The E. coli rpS13 is thought to be involved in the initiation of translation as it is a surface protein at the interface of the ribosomal subunits that cross-links to all three initiation factors (23). It interacts strongly with the 20 S rRNA and has been cross-linked to the 3′ major domain of the 16 S rRNA (24–26), to ribosomal protein S19 (27), and to tRNA in both the P and A sites (25, 28). These studies indicate that rpS13 is important for both translation initiation and elongation. rpS13 can be phosphorylated by a eukaryotic protein kinase (29), and there are a number of phosphorylation sites in the eukaryotic S18 protein for phosphorylation by casein kinase II, protein kinase C, and a tyrosine kinase.

Based on the high degree of homology between S18 and rpS13, it is possible that S18 plays an important role in ribosome assembly as well as in translational efficiency. In support of this idea, a mutant of the E. coli rpS13 lacking the C-terminal 19 amino acids shows a 20–30% reduction in translation rate (30) in agreement with the 24% drop in protein translation rates observed when S18 phosphorylation is blocked in VSMCs. Interestingly the C-terminal portion is required for 16 S rRNA recognition (31), and the consensus motif for CaMK II phosphorylation is present within the C-terminal 19 residues of the protein sequence of S18.

The notion that phosphorylation of S18 regulates the rate of translation becomes even more interesting in light of the ability of heparin to inhibit both CaMK II and protein kinase C, both of which can phosphorylate the S18 protein (22), implying that...
Ribosomal Protein S18 Is a CaMK II Substrate

33540

heparin may alter translation rates. Although previous studies have indicated that heparin blocks proliferation of VSMC, this glycosaminoglycan alters the overall rate of protein synthesis in VSMCs by 20% or less (12). When one considers that growth regulatory proteins are very labile and comprise a very small percentage of the total protein in a cell while housekeeping proteins are generally much more stable, even large fluctuations in the levels of growth regulatory proteins would not be detected when measuring overall protein synthesis. In addition, mRNA encoding growth regulatory proteins has a very short half-life; thus, even a modest reduction in translation rate such as the 20–30% decrease seen in rpS13 mutants and the 23% reduction observed with underphosphorylated S18 would result in the rapid degradation/reduction of these nascent message levels in the cell.

The co-immunoprecipitation of a 20-kDa protein with CaMK II only in the presence of mitogenic stimulation has been reported earlier (32). However, further studies to identify the protein were not reported. There is one prior observation of differential phosphorylation of a ribosomal protein (33). However, this effect was independent of the cytosolic free Ca\(^{2+}\) pool and thus was not a result of calmodulin-dependent protein kinases; instead, it is a reflection of the sequestered pool of Ca\(^{2+}\) in contrast, serum treatment causes a 30% decrease seen in rpS13 mutants and –mobilizing agents and protein translation.

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