Differential Activation of p70 and p85 S6 Kinase Isoforms during Cardiac Hypertrophy in the Adult Mammal*

(Received for publication, March 19, 1998, and in revised form, June 15, 1998)

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An adult feline right ventricular pressure overload (RVPO) model was used to examine the two S6 kinase (S6K) isoforms, p70S6K and p85S6K, that are involved in translational and transcriptional activation. Biochemical and confocal microscopy analyses at the level of the cardiocyte revealed that p70S6K is present predominantly in the cytosol, substantially activated in 1-h RVPO (>12 fold), and phosphorylated in the pseudosubstrate domain at the Ser-411, Thr-421, and Ser-424 sites. p85S6K, which was localized exclusively in the nucleus, showed activation subsequent to p70S6K, with a sustained increase in phosphorylation for up to 48 h of RVPO at equivalent sites of p70S6K, Thr-421 and Ser-424, but not at Ser-411. Neither isoform translocated between the cytosol and the nucleus. Further studies to determine potential upstream elements of S6K activation revealed: (i) similar time course of activation for protein kinase C isoforms (α, γ, and ε) and c-Raf, (ii) absence of accompanying phosphatidylinositol 3-kinase activation, (iii) activation of c-Src subsequent to p70S6K, and (iv) similar changes in adult cardiocytes after treatment with 12-O-tetradecanoylphorbol-13-acetate. Thus, these studies suggest that a protein kinase C-mediated pathway couples pressure overload to growth induction via differential activation of S6K isoforms in cardiac hypertrophy.

Hypertrophic cardiac growth occurs by an increase in cellular mass via a relatively coordinate increase in cellular protein content (1). In the terminally differentiated adult cardiac muscle cell, translational mechanisms have a key role in regulating the rate of protein synthesis during hypertrophic growth. Phosphorylation of the carboxyl terminus of 40 S ribosomal S6 protein (S6 protein) has been shown to regulate protein synthesis rate by mediating protein translational initiation and elongation (2), and a growing number of studies show strong correlation between translational efficiency and S6 protein phosphorylation (3–5). Two distinct families of S6 kinases, p90Rsk (ribosomal S6 kinase) and p70S6 kinase (S6K),

1 This work was supported by Program Project Grant HL-48788 from the NHLBI, National Institutes of Health, by research funds from the Department of Veterans Affairs, and by a grant from the Deutsche Forschungsgemeinschaft (to M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: S6K, p70 and p85 S6 kinase isoforms; LV, left ventricle; RV, right ventricle; RVPO, right ventricular pressure overload; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; DTT, dithiothreitol; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C.

24610 This paper is available on line at http://www.jbc.org
substantial activation of S6K in pressure overloaded myocardium in vivo. This activation is accompanied by a distinct pattern and time course of phosphorylation of the cytoplasmic and the nuclear isoforms of S6K occurring at the level of the cardiomyocytes of pressure overloaded myocardium. Our studies also indicate that this mechanically induced signaling pathway is mediated by PKC and is independent of PI 3-kinase.

EXPERIMENTAL PROCEDURES

Experimental Animal Models—Right ventricular pressure overload (RVPO) hypertrophy was induced by partial occlusion of the pulmonary artery in adult cats of either sex, weighing 2.6–3.9 kg, either by external banding (for 48–h and long term RVPO: Ref. 24) or by a balloon-tipped catheter (for 1- or 4-h RVPO: Ref. 25). In both models, pressure in the right ventricle (RV) was more than doubled, whereas systemic arterial pressure remained unaltered. Controls, as appropriately specified, consisted of sham-operated cats submitted to thoracotomy and pericardiotomy without hemodynamic intervention, or the normally loaded left ventricle (LV) from each cat. At least three animals were used for each group for subsequent studies. After removing the heart from the surgically anesthetized cats, the aorta was cannulated, flushed retrograde with ice-cold phosphate-buffered saline, and the heart was either immediately processed or flash-frozen in liquid nitrogen for later use. Cardiomyocytes were isolated from normal adult cat hearts as described previously (26).

Chemicals—E-64 (trans-epoxysucinyl-l-leucylamilide-(4-guanidino) butane), aprotonin, phenylmethylsulfonyl fluoride, and 1,4-dithiothreitol (DTT) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Triton X-100, sodium orthovanadate, leupeptin, pepstatin, p-aminobenzamidine, EGTA, phosphatidylinositol, phosphatidylinositol-3-phosphate, β-glycerophosphate, and insulin were from Sigma; dithiobisuccinimidylpropionate from Pierce; TPA and okadaic acid from Calbiochem (La Jolla, CA), and S6 substrate peptide obtained from Du Pont. Sheep anti-rabbit IgG coated dynabeads (M-280) were purchased from Dynal (Lake Success, NY).

Preparation of Cell and Tissue Lysates—Triton X-100-lysed subfractions, namely Triton X-100-soluble high spin supernatant, Triton X-100-insoluble low spin pellet (cytoskeleton), and high spin pellet (membrane skeleton) from ventricular tissue samples were prepared as described previously (27, 28). Cytosolic and membrane fractions from ventricles were prepared as described previously with minor modifications (29). Briefly, 100 mg of tissue was homogenized in a Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) in 2 ml of homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 1 m M DTT, 10 mM EGTA, and protease inhibitors (0.5 m M phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 10 μg/ml aprotonin, 2 μg/ml pepstatin, 2 μM E-64, and 200 μg/ml p-aminobenzamidine) and phosphatase inhibitors (1 μM okadaic acid, 10 μM β-glycerophosphate, and 1 mM sodium orthovanadate). The homogenate was transferred into a Dounce homogenizer (Wheaton Co., Millville, NJ) and further processed with 40 strokes on ice. To remove unbroken cells and debris, the homogenate was centrifuged at 3,000 × g for 10 min at 4 °C. The supernatant was transferred to a fresh set of tubes and further centrifuged at 30,000 × g for 30 min. The pellet, which contains predominantly the membrane fraction, was resuspended in 1 ml of SDS sample buffer (30) and boiled for 5 min. The supernatant, referred to as the cytosolic fraction, was used either for kinase assays or for Western blot analysis after boiling with an equal volume of SDS sample buffer.

Western Blot Analysis—Immunoblotting was performed as described previously (27), and the following antibodies were commercially obtained: monoclonal antibodies: anti-c-Raf from Transduction Laboratories Inc., Lexington, KY; anti-c-Src from Upstate Biotechnologies Inc. Polyclonal antibodies: anti-COXH-terminal S6K (C-18) from Santa Cruz Inc., Santa Cruz, CA; anti-phospho-Ser-411 S6K (P-Ser-411), anti-phospho-Thr-421/Tyr-422/Ser-424 S6K (P-Thr-421) and anti-phospho-Ser-473 Akt from New England Biolabs Inc., Beverly, MA; isofrom-specific anti-PKC and anti-phosphotyrosine from Transduction Laboratories Inc., Lexington, KY; and anti-Pi-3-kinase from Upstate Biotechnologies Inc. Polyclonal antibody specific for the p85 isoform of S6K (C-2) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-phospho-Ser-411 S6K (P-Ser-411) from Transduction Laboratories Inc., Lexington, KY; and anti-Pi-3-kinase from Upstate Biotechnologies Inc. Polyclonal antibody specific for the p56 isoform of S6K (C-2) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-phospho-Ser-473 Akt from New England Biolabs Inc., Beverly, MA.

Immunoprecipitation and Kinase Assays—p70S6K activity was measured after immunoprecipitation with C-18 antibody using cytosolic samples obtained from control and pressure overloaded ventricles. For this, 100 μl of the cytosolic fraction was added to 0.9 ml of dilution buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, and protease and phosphatase inhibitors as described above). The samples were then mixed with 1 μg of antibody or non-immune IgG (controls), and incubated overnight at 4 °C. Pre-washed protein A beads (10 μl, Pierce) were then added, and the incubation was continued for an additional 1 h. The immune complexes were washed three times with 1 ml of wash buffer containing 0.1% Nonidet P-40, 1 mM DTT, 0.5 mM EGTA, 10 mM Tris, pH 7.4, 10 mM β-glycerophosphate, 1 μM DTT, and 1 μM EGTA. The immune complexes were then used for measuring kinase activity as defined by Kuppuswamy et al. (29) in the presence or absence of 2 mM synthetic peptide substrate (31), homologous to a region in the S6 protein. In order to measure the p70S6K activity in TPA-treated cells, samples (4 × 105 cells) were cultured on 100-mm laminated plates. After 12 h, the cardiomyocytes were treated with 100 nm TPA for various time periods, washed with phosphate-buffered saline, and used for preparing cytosol as described for the tissue samples except that the homogenization was performed by sonication for 1 min on ice. p70S6K was immunoprecipitated with C-18 antibody and assayed for kinase activity. For measuring and comparing p70S6K activity with p85S6K in pressure overloaded myocardium, respective antibodies, C-18 and C-3 (specific for p85S6K), were conjugated with secondary antibody-coated magnetic beads (Dynabeads), as specified by the manufacturer and used for immunoprecipitation with total extracts obtained with 2% Triton X-100 buffer as described previously (27) and assayed for kinase activity.

Measurement of PI 3-Kinase Activity—PI 3-kinase activity was measured similar to the protocol described by Auger et al. (32). Briefly, 50 μg of tissue or 4 × 105 cultured cardiomyocytes were homogenized in 1 ml of extraction buffer containing 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM DTT, and protease and phosphatase inhibitors as described above. The supernatant after centrifugation at 30,000 × g was diluted to 1 ml with extraction buffer (final protein concentration at 0.5 mg/ml), mixed with 1 μg of antibody raised against the p85 subunit of PI 3-kinase (Upstate Biotechnology, Inc.; polyclonal), and incubated overnight at 4 °C. The immune complex was precipitated with protein-A agarose beads, and sequentially washed as follows: three times with the extraction buffer; three times with 0.1 mM Tris, pH 7.4 containing 5 mM LiCl and 0.1 mM sodium orthovanadate; and twice with extraction buffer containing 0.5% Triton X-100. The PI 3-kinase activity was measured using phosphatidylinositol (PI) as a substrate. The organic phase, containing the lipid products, was used for TLC separation in chloroform/methanol/ water/ammonia (60:47:11:3.2, v/v/v). Unlabeled phosphatidylinositol 3-phosphate was run in parallel to determine the position of phosphorylated PI.

Immunofluorescence Staining and Confocal Microscopy—Fresh frozen ventricular tissue sections (7 μm) were fixed and immunolabeled as follows. Sections were cross-linked with 1 mM dithiobisuccinimidylpropionate in Hanks’ balanced salt solution at 37 °C, extracted with 0.5% Triton X-100 prepared in Hanks’ balanced salt solution, and fixed in 4% paraformaldehyde (33). After blocking the sections with 10% goat serum in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% bovine serum albumin, they were incubated with primary antibodies overnight. After washing with Tris-buffered saline, the sections were further incubated with fluorescence-labeled secondary antibodies (Jackson Laboratories Inc., West Grove, PA), as well as TR-phalloidin and YoYo-1 (Molecular Probes Inc., Eugene, OR). Imaging was done on a Bio-Rad MRC-1000 laser scanning confocal microscope. The pictures were processed using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA).

Statistical Analysis—The ratios of the nuclear phosphorylation signal (P-Thr-421 and P-Ser-424) from RV and LV were calculated as follows. Sections were cross-linked with 1 mM dithiobisuccinimidylpropionate in Hanks’ balanced salt solution at 37 °C, extracted with 0.5% Triton X-100 prepared in Hanks’ balanced salt solution, and fixed in 4% paraformaldehyde (33). After blocking the sections with 10% goat serum in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% bovine serum albumin, they were incubated with primary antibodies overnight. After washing with Tris-buffered saline, the sections were further incubated with fluorescence-labeled secondary antibodies (Jackson Laboratories Inc., West Grove, PA), as well as TR-phalloidin and YoYo-1 (Molecular Probes Inc., Eugene, OR). Imaging was done on a Bio-Rad MRC-1000 laser scanning confocal microscope. The pictures were processed using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA).

RESULTS

Pressure Overload Induces Phosphorylation and Activation of the Cytoplasmic p70S6K—The focus of the present study was to determine whether pressure overload-induced hypertrophic cardiac growth is accompanied by the activation of one or both of the isoforms of S6K. In order to determine the complete activation profile, both short term (1–4 h) and long term (48 h or longer) pressure overload feline models were used. Three dif-
To demonstrate whether S6K isoforms are activated in pressure-overloaded myocardium, pressure-overloaded right ventricles (RV) and normally loaded same-animal control left ventricles (LV or RV) were used to obtain total lysates by extracting with Triton X-100 buffer as described under “Experimental Procedures” and used for Western blot analysis. Among the four known phosphorylation sites of p70S6K and p85S6K are indicated by their respective molecular sizes. In the Western blot with C-18 antibody, the ECL development for the detection of p85S6K was doubled compared with the 2-min detection time of p70S6K, which has been observed as early as 1 h RVPO, persisted up to 4 h, and returned to the control level in 1-week pressure-overloading RV. The detection of p85S6K (Fig. 1A), which also showed a doublet in control ventricles, revealed distinct differences in pressure-overloaded ventricles, when compared with p70S6K. Although retention of mobility was similar at 1 and 4 h RVPO, p85S6K did not decline appreciably at 48 h RVPO (i.e., the lower band for p85S6K was still absent in contrast to the reappearance of the lower band for p70S6K) and was back to control levels only at the later time point of 1-week RVPO. Furthermore, the exposure time for p85S6K during ECL development of the Western blots needed to be doubled. This relatively low level of detection could be due to either an isoform-specific change in the antibody binding epitope or low levels of the p85S6K isoform in the heart. We confirmed these changes at each time point of pressure overloading with at least three independent experimental cats. In summary, data clearly indicate that both isoforms of S6K exhibit mobility band shift on SDS-PAGE in response to pressure overloading with observable differences in the time course.

Although mobility change on SDS-PAGE has been reported to be indicative of kinase activation due to phosphorylation of the rapamycin-sensitive sites (35, 36), it does not represent phosphorylation of the pseudosubstrate domain. To address the possibility that the pseudosubstrate domain of S6K also undergoes phosphorylation in response to pressure overloading, phosphospecific antibodies were employed in the Western blot experiments. Among the four known phosphorylation sites of the pseudosubstrate domain, only Ser-411 has been shown to be sensitive to inhibition by rapamycin (15). When P-Ser-411 antibody was used in the Western blot analysis, Ser-411 phosphorylated p70S6K was detected exclusively in the pressure-overloaded myocardium, whereas it was almost completely absent in all the normally loaded ventricular samples (Fig. 1B). The time course of Ser-411 phosphorylation, which was seen maximally between 1 and 4 h of RVPO and declined sharply at 48 h, matches the time course of load-induced p70S6K band shifting shown in the previous experiment (Fig. 1A). These experiments, therefore, suggest that p70S6K is phosphorylated on the Ser-411 residue in addition to other sites of S6K (as multiple species were observed in Fig. 1A) in response to pressure overloading. Interestingly, the P-Ser-411 antibody did not detect the p85S6K isoform either in control or pressure-overloaded ventricular samples, indicating that this isoform is not phosphorylated at the Ser-411 residue in response to pressure overload. This was true even after increasing either the sample volume for SDS-PAGE (twice the concentration, 40 μg of protein) and/or a longer time period of ECL detection in the Western blot analysis (data not shown). All these studies indicate that the kinase(s) responsible for the phosphorylation of the Ser-411 site of p85S6K is either absent or inactive in the nucleus, whereas it is activated in the cytoplasm in response to pressure overloading.

To determine whether the changes in the phosphorylation status of S6K are associated with kinase activation, we analyzed the activity of p70S6K using a synthetic peptide substrate homologous to the S6 protein after immunoprecipitating the kinase from cytosolic samples of control and pressure-overloaded ventricles with the regular S6K antibody (C-18). Western blot analysis with C-18 antibody did not detect p85S6K in the cytosolic samples (data not shown). Therefore, when the immune complexes are prepared with the cytosolic samples, their activities represent mostly that of p70S6K. As shown in Fig. 2, a more than 12-fold activation of S6K (most of it due to p70S6K) was observed following 1-h pressure overloading, slightly decreased at the 4-h time point, markedly declined when RVPO was extended for 48 h, and reached the basal condition by 1 week of RVPO. We confirmed the time course of

Fig. 1. Western blot showing the time course of S6K phosphorylation during RVPO. LVs and RVs obtained from sham-operated control feline hearts (Cont) and from 1-h, 4-h, 48-h, and 1-week RVPO feline hearts were extracted with Triton X-100 buffer as described under "Experimental Procedures" and used for Western blot analysis with non-selective anti-S6K antibody, C-18 (A) or with P-Ser-411-selective anti-S6K antibody (B). The bands corresponding to p70S6K and p85S6K are indicated by their respective molecular sizes. In the Western blot with C-18 antibody, the ECL development for the detection of p85S6K was doubled compared with the 2-min detection time of p70S6K, hr, hour; wk, week.
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Fig. 2. Time course of p70<sub>S6K</sub> activation during RVPO. Cytosolic fractions were prepared from a control feline heart (Cont) and from feline hearts after RVPO for 1 h, 4 h, 48 h, and 1 week. The cytosolic samples were used for immunoprecipitation with C-18 antibody, and the kinase activity was measured in duplicates with S6 peptide as substrate. Activities in terms of RV/LV ratio were calculated for each experimental feline heart and plotted against the time period of RVPO. In a typical 4-h RVPO experiment, the activity (in cpm) was 206,618 ± 5,543 for RV and 29,529 ± 5,414 for LV. hr, hour; wk, week.

Fig. 3. Immunofluorescence confocal microscopy showing no translocation of p70<sub>S6K</sub> to the nucleus. LVs and RVs from control (Cont) and 4-h RVPO cats were used to prepare fresh-frozen tissue sections and processed for confocal microscopy as described under "Experimental Procedures." In the figure, S6K is decorated in red with C-18 antibody, the nucleus is decorated in green with YoYo-1 dye, and actin is decorated in blue with TR-phalloidin. Bar, 25 μm. hr, hour.

this kinase activation in two other independent experimental cats for each time point. Therefore, both Western blot experiments and immune complex kinase assays show that the cytosolic p70<sub>S6K</sub> is activated substantially in pressure-overloaded myocardium as early as 1 h of RVPO.

Next, to analyze whether the activated p70<sub>S6K</sub> translocates to the nucleus during pressure overloading, we used confocal microscopy of fresh-frozen tissue sections. Although P-Ser-411 antibody detected exclusively p70<sub>S6K</sub> (Fig. 1B), this antibody was not effective for immunostaining. Therefore, we took advantage of the regular C-18 antibody, which was found to be strongly reactive with the p70<sub>S6K</sub> (cytoplasmic) compared with the p85<sub>S6K</sub> (nuclear) isoform of S6K (Fig. 1A). When this antibody was used for confocal microscopy, it detected only the cytoplasmic S6K (corresponding to the p70<sub>S6K</sub> isoform) as anticipated, i.e., the signal is distributed evenly in the sarcoplasm (decorated in red) of both control and 4 h pressure-overloaded ventricular cardiocytes (Fig. 3). No S6K signal was found in the nuclei (labeled in green) when analyzed either in the normally loaded control ventricles or in the 4-h pressure-overloaded right ventricles. These data suggest that p70<sub>S6K</sub>, following load-induced phosphorylation and activation, did not translocate to the nucleus.

p70<sub>S6K</sub> and p85<sub>S6K</sub> Undergo Differential Phosphorylation and Activation during RVPO—As phosphorylation of the Ser-411 residue of the pseudosubstrate domain was observed exclusively for the cytoplasmic isoform (p70<sub>S6K</sub>) during pressure overloading (Fig. 1B), we employed another phosphospecific antibody (P-Thr-421) to determine phosphorylation at other known sites in the pseudosubstrate domain of S6K isoforms. This antibody detects S6K when both Thr-421 and Ser-424 residues in the pseudosubstrate domain are phosphorylated simultaneously. Interestingly, Western blot analysis with this antibody revealed phosphorylation not only of p70<sub>S6K</sub> but also of p85<sub>S6K</sub> in response to pressure overload (Fig. 4A). Neither isoform was phosphorylated in normally loaded control ventricles (LV or RV). Phosphorylation of Thr-421/Ser-424 residues of both the isoforms could be detected as early as 1 h of RVPO; however, although phosphorylation of these residues for the p70<sub>S6K</sub> isoform declined sharply after pressure overloading for 48 h, phosphorylation for the p85<sub>S6K</sub> isoform was sustained in the 48-h pressure-overloaded myocardium and required a longer time period for the phosphorylation to decline, as the basal level was found to be reached only when pressure overloading was extended for 1 week. This time course of phosphorylation was confirmed for each time point with two other independent groups of experimental cat samples. These data imply that both nuclear and cytosolic isoforms undergo pressure overload-induced phosphorylation at Thr-421 and Ser-424 sites; however, the phosphorylation of p70<sub>S6K</sub> is more transient than the phosphorylation of the nuclear p85<sub>S6K</sub>. Overall, these experiments show that, in addition to a difference in phosphorylation at the Ser-411 site (Fig. 1B), these two S6K isoforms exhibit a difference in the time course of load-induced Thr-421/Ser-424 phosphorylation.

In order to confirm that the differential phosphorylation of S6K isoforms occurs at the level of individual cardiocytes, analysis of fresh-frozen tissue sections by confocal microscopy was performed with the P-Thr-421 antibody using the same ventricular tissue samples used for the Western blot analysis (Fig. 4A). In the confocal pictures shown in Fig. 4B, sarcomeric actin is decorated in blue (TR-phalloidin), nuclei in green (YoYo-1 dye), and the Thr-421/Ser-424 phosphorylated S6K in red. Control ventricular tissue sections (both LV and RV) show nuclear and sarcomeric staining, but show no signal for the phosphorylated S6K isoforms (Fig. 4B). Interestingly, upon pressure overloading for 1 or 4 h, diffuse red staining could be seen throughout the sarcoplasm, suggesting phosphorylation of the cytoplasmic p70<sub>S6K</sub>. In the 1-h, 4-h, and 48-h pressure-overloaded RV, staining of the phosphorylated p85<sub>S6K</sub> (nuclear isoform) could be seen in yellow/orange due to the merger of the p85<sub>S6K</sub> (red) signal with the nuclear (green) signal. Therefore, phosphorylation of p85<sub>S6K</sub> at Thr-421/Ser424 sites persists longer when compared with that of p70<sub>S6K</sub>, and the data precisely match the Western blot data shown in Fig. 4A. The quantification of phosphorylation from the confocal microscopy pictures (Fig. 4C) confirms this finding that pressure overloading resulted in an increase in the level of phosphorylation of nuclear S6K (p85<sub>S6K</sub>), reaching a statistically significant maximal level after 48-h RVPO, when compared with 4-h RVPO and control. Furthermore, based on the results obtained with phospho-Ser-411 (Fig. 1B) and phospho-Thr-421 (Fig. 4, A and B) antibodies, phosphorylation in the pseudosubstrate domain of p85<sub>S6K</sub> appears to occur preferentially at Thr-421/Ser-424 sites when compared with Ser-411 phosphorylation, and the load-induced differential activation/deactivation of S6K isoforms occurs at the level of individual cardiocytes.

To further confirm that there is a different time course of p85<sub>S6K</sub> activation relative to p70<sub>S6K</sub> activation, as well as to demonstrate that the p85<sub>S6K</sub> is indeed catalytically active in pressure-overloaded myocardium, we measured and compared the kinase activities between 1 and 4 h in pressure-overloaded cat ventricles (Fig. 5). For this, an antibody specific for the
The p85S6K isoform (C-3) was used to immunoprecipitate the kinase from Triton X-100 tissue extracts and compared with the previously used C-18 antibody. As mentioned earlier, C-18 antibody detects mostly the p70S6K isoform. Therefore, when the immune complex kinase assays were performed with this antibody, there was a remarkable kinase activation following 1 h pressure overloading (14-fold) relative to normally loaded LV control, which decreases substantially (7-fold) when pressure overloading is extended for 4 h. This activation profile is very similar to the activation profile of p70S6K when the cytosolic samples were used (Fig. 2). Interestingly, when the assays were performed for p85S6K, there was a clear difference in the activation profile, i.e. there was almost no activation of p85S6K at 1 h of pressure overloading (1.5-fold), whereas there was a significant increase in the kinase activity after 4 h of RVPO (6.2-fold). These experiments reveal two major points. First, similar to p70S6K, pressure overloading results in the catalytic activation of p85S6K; and second, p85S6K activation occurs subsequent to the activation of p70S6K.

Finally, as the P-Thr-421 antibody detected the phosphorylated nuclear isoform (p85S6K) in pressure-overloaded myocardium, we used this antibody to examine whether this activated kinase shifts to the cytoplasm during load-induced activation. Total lysates prepared with Triton X-100 extraction buffer and samples prepared with detergent-free buffers (cytosol) were obtained from a 4-h RVPO cat ventricle and used for Western blotting with P-Thr-421 antibody. Interestingly, in the pressure-overloaded RV, whereas the Thr-421/Ser-424 phosphorylated p85S6K could be readily detected in the Triton X-100-solubilized total tissue lysates, it was absent in the cytosolic preparation, although phosphorylated p70S6K could be detected in both types of tissue samples (Fig. 6). These studies clearly suggest that during pressure overloading the Thr-421/Ser-424-phosphorylated p85S6K did not translocate from the nucleus to the cytoplasm.

**S6K Activation Is Accompanied by PKC-mediated Signaling Independent of PI 3-Kinase**—S6K has been shown to be activated by both PKC-dependent and -independent pathways (6, 20). In the case of the PKC-independent pathway, tyrosine kinases are known to play an important role in S6K activation (21, 37), with the possible involvement of PI 3-kinase (37). Therefore, to determine if PI 3-kinase was activated during pressure overloading, we immunoprecipitated PI 3-kinase from either 1-h or 4-h RVPO ventricular lysates and examined both for tyrosine phosphorylation and kinase activity (32, 38, 39). As a positive control, insulin-treated feline adult cardiocytes were used as a positive control. The results shown in A and B were confirmed in two other independent cats for each time point of RVPO. Bar represents 25 μm. C, the nuclear phospho-p85S6K signal (red signal appearing as yellow/orange) was quantitated, and mean ratios of the density (RV/LV) ± S.E. were plotted against the time course of RVPO. Statistically significant differences (p < 0.016) were observed for control versus 48 h RVPO (*) and for 4 h versus 48 h RVPO (‡).
used in these studies. Measurement of PI 3-kinase activity showed no appreciable load-induced increase in the basal activity, i.e. there were no observable differences between RV and LV of either control or 1- or 4-h RVPO cat samples (Fig. 7A). As shown previously, activation of both the isoforms of S6K was observed between 1 and 4 h of pressure-overloaded myocardium (Fig. 5). Furthermore, our observation showing absence of PI 3-kinase activation could not be due to any experimental difficulties, since, as observed in other cell types (21, 23), a substantial amount of stimulated activity could be observed when cultured adult cardiocytes were treated with insulin for 30 min (Fig. 7A, right panel), and this activity was accompanied by a retarded mobility of both S6K isoforms on SDS-PAGE (data not shown). Therefore, these studies clearly indicate that load-induced activation of S6K is not accompanied by PI 3-kinase activation. As studies show PI 3-kinase activation via c-Src-mediated tyrosine kinase pathways (40), as a next step, c-Src activation was analyzed in pressure-overloaded ventricular samples. We have recently shown that p60c-Src, a non-receptor tyrosine kinase, associates with the cytoskeleton of pressure-overloaded myocardium and becomes substantially tyrosine-phosphorylated for kinase activation (27). In order to determine whether c-Src plays an upstream role for S6K activation, we analyzed Triton X-100-lysed ventricular subfractions, namely Triton-soluble, cytoskeleton, and membrane skeleton (27). Similar to what we have shown before, c-Src movement to the cytoskeleton was present in significant amounts in the 48-h pressure-overloaded ventricles and was absent in all normally loaded ventricles (Fig. 7B). Such analysis in the 4-h pressure-overloaded ventricles showed very low levels of cytoskeleton-bound c-Src, and was completely absent in the 1-h pressure-overloaded ventricles. In addition, we did not observe any differences in the levels of tyrosine-phosphorylated proteins when 1-h RVPO samples were used for Western blots with anti-phosphotyrosine antibody, whereas in 48-h RVPO samples several cytoskeleton-bound proteins including c-Src became tyrosine-phosphorylated (Ref. 27 and data not shown). In contrast, the maximal activation of p70S6K and S6K in these tissue samples was seen at 1 h of pressure overload (Fig. 2). Therefore, c-Src association and activation at the cytoskeleton occurs subsequent to p70S6K activation. However, as shown in the previous experiments (Figs. 4 and 5), p85S6K activation was observed at 4 h of RVPO, whereas phosphorylation at Thr-421/Ser-424 sites occurred as early as 1 h of RVPO. Therefore, whereas these studies clearly ruled out the possible involvement of c-Src in p70S6K activation, it is not clear whether c-Src is linked to p85S6K activation.

As PKC is a well known activator of S6K (20, 22), we analyzed whether members of the PKC family are activated with a time course similar to S6K activation. Our initial screening showed the presence of the following PKC isoforms in the feline heart sample: α, β, γ, ε, μ, ζ, ι, λ, and very low expression of the θ and δ isoforms. Of these isoforms, we were able to detect a
shift in PKC α, PKC γ, and PKC ε to the membrane between 1 and 4 h of pressure overloading (Fig. 8A), which persisted thereafter for a long time period (1 week). Such membrane translocation of PKC has been shown to be indicative of kinase activation (41). Furthermore, we analyzed their immediate downstream effector kinase, c-Raf, for load-induced changes in the membrane preparations used for PKC analysis. As an unexpected finding, c-Raf was found to be membrane-associated in both normal and pressure-overloaded ventricles without any appreciable difference (data not shown). However, when we used Triton X-100-lysed subfractions (27), the following load-induced changes in c-Raf, measured in terms of both translocation and mobility band shift on SDS-PAGE separation, were observed (Fig. 8B). First, c-Raf was present in all of the Triton X-100-lysed subfractions of 1–4-h pressure-overloaded ventricles and exhibited retarded mobility on SDS-PAGE separation, indicative of phosphorylation and activation as reported previously (42). Second, we show for the first time that pressure overload results in the association of substantial amounts of c-Raf with the cytoskeletal fraction, which was found to be completely absent in all of the normally loaded control ventricular fractions. Third, a significant amount of c-Raf was present in the Triton X-100-insoluble membrane skeletal fraction of both normal and pressure-overloaded ventricles, even after the detergent extraction. Most of these changes observed between 1 and 4 h of RVPO returned to the basal level after long term pressure overloading (1 week), except for the membrane skeleton-bound c-Raf, which was present at high levels even up to 1 week of RVPO, as was observed for PKC isoforms (Fig. 8A).

**Load-induced Changes in S6K Activation Can Be Mimicked in TPA-stimulated Adult Feline Cardiocytes—**To demonstrate that PKC stimulation could result in the activation of S6K in cardiocytes, we treated cultured adult cardiocytes for various time points with 100 nM TPA, and total extract was prepared. Extracts prepared from control and TPA-treated cells were Western blotted (Fig. 9A) with C-18 antibody (top panel), P-Ser-411 (middle panel), and P-Thr-421 antibody (lower panel). Treatment of adult cardiocytes with TPA for 1, 4, and 24 h resulted in a mobility shift of both p70S6K as well as p85S6K on SDS-PAGE separation (top panel), although the latter isoform required double the exposure time during ECL reaction similar to the previous observation with tissue samples (Fig. 1A). This mobility shift decreased to almost control levels after 48 h of treatment. Furthermore, Western blot experiments with P-Ser-411 and P-Thr-421 antibodies (Fig. 9A, middle and lower panels, respectively) using detergent extracts of TPA-treated cells revealed the following. First, Ser-411 phosphorylation of p70S6K was seen early as 1 h after TPA treatment, continued to be present up to 24 h, and then decreased to undetectable levels after 48 h of treatment; second, similar to the observation seen in pressure-overloaded ventricular samples, Ser-411 phosphorylated p85S6K was not present in detectable amounts in control and TPA-treated cell extracts; and third, both iso-

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**Fig. 8. Redistribution of PKC isoforms and c-Raf in pressure-overloaded myocardium.** A, translocation of PKC isoforms to the membrane. LVs and RVs from control (Cont) and RVPO cats at 1 h, 4 h, 48 h, and 1 week were processed to obtain cytosol and membrane fractions (Memb.) as described under "Experimental Procedures." The samples were processed for Western blotting with isoforrm-specific PKC antibodies. B, cytoskeletal association and activation of c-Raf. LVs and RVs from control (Cont) and RVPO cats at 1 h, 4 h, 48 h, and 1 week were processed to obtain Triton X-100-lysed subfractions, namely soluble (Sol), cytoskeleton (CSK), and membrane-skeleton (MSK) as described previously (27). The samples were processed for Western blotting with c-Raf monoclonal antibody. Data were confirmed in at least two independent experiments. hr, hour; wk, week.

**Fig. 9. TPA treatment of cultured adult cardiocytes mimics load-induced in vivo changes in S6K and c-Raf.** Isolated adult cardiocytes were cultured with serum-free medium on laminin-coated plates for 12 h and treated with 100 nM TPA for 1, 4, 24, or 48 h, or treated with vehicle alone (Cont). For experiments shown in A and B, Triton X-100-soluble fractions were prepared and Western blotted with C-18, P-Ser-411, and P-Thr-421 antibodies (A) or c-Raf monoclonal antibody (B). hr, hour.
forms of S6K were found to be phosphorylated at Thr-421/Ser-424 sites in 1–24-h TPA-treated cells and returned to the control level after 48 h of TPA treatment. All of these observations obtained with TPA treatment reflect the changes seen in the pressure-overloaded myocardium. One noticeable difference was that the p85<sub>S6K</sub> (nuclear isoform) phosphorylation at Thr-421/Ser-424 did not persist longer than that of p70<sub>S6K</sub> as observed with pressure overloading experiments (compare experiments shown in Fig. 9A with Fig. 4A).

In order to determine whether the band shift and pseudosubstrate phosphorylation of p70<sub>S6K</sub> in TPA-treated cells (Fig. 9A) represent enhanced kinase activity, cytosolic samples from control and TPA-treated cardiocytes were immunoprecipitated with the C-18 antibody and the associated kinase activity measured (as described in Figs. 2 and 5). TPA treatment for 1 h resulted in a 13.8-fold stimulation ($82,469 \pm 5,920$ cpm when compared with control cells $5,964 \pm 355$ cpm). Kinase activity fell to 61% of the 1 h stimulated activity after 4 h of treatment ($50,432 \pm 1,855$ cpm), remained at 45% of the 1-h stimulated activity after 24 h of treatment ($36,705 \pm 4,126$ cpm), and returned to the basal condition after 48 h of TPA treatment. This profile of TPA-stimulated p70<sub>S6K</sub> activation was confirmed in two other independent experiments. Therefore, both the band shifting and pseudosubstrate phosphorylation of the p70<sub>S6K</sub> in TPA-treated cardiocytes correlate with its kinase activation, and the time course of this activation was similar to the in vivo observations with pressure-overloaded myocardium.

Next, as in pressure-overloaded myocardium many of the changes seen with S6K isoforms were accompanied by activation of c-Raf, we also examined whether such changes could be seen in TPA-treated adult cardiocytes. Therefore, control and TPA-treated adult cardiocytes were processed to obtain the Triton X-100-soluble fraction, as performed for tissue samples, and Western blotted with anti-c-Raf antibody. As shown in Fig. 9B, c-Raf exhibited a mobility band shift in 1-, 4-, and 24-h TPA-treated cells, which returned to the control status by 48 h of TPA treatment. The time course of these changes associated with c-Raf are very similar to the time course of changes seen with S6K isoforms (Fig. 9A).

**DISCUSSION**

Quantitative and qualitative changes, as a result of transcriptional and translational activation, are two major events associated with hypertrophic growth. The present study was designed to determine whether the two isoforms of S6K, p70<sub>S6K</sub> and p85<sub>S6K</sub>, which are known to regulate both transcriptional and translational activation (20, 43), are activated in response to pressure overloading of the heart. Furthermore, in a finding having general rather than cardiac-restricted implications, this study distinguishes for the first time the activation profiles of the two isoforms of S6K. Previous studies have demonstrated p70<sub>S6K</sub> activation in several cell types after either mitogenic stimulation (for review, see Ref. 20), mechanical stretch (34), or p70<sub>S6K</sub> activation in several cell types after either mitogenic activation, and translational activation (20, 43), are activated in response to pressure overloading; third, our immune complex kinase assay performed in 1- and 4-h pressure-overloaded myocardium clearly show activation at 4 h of RVPO, which occurs as an event subsequent to p70<sub>S6K</sub> activation. One possible role of activated p85<sub>S6K</sub> in the nucleus is to phosphorylate and activate transcriptional factors such as the cyclic AMP-responsive element-binding protein (CREB) and the cyclic AMP responsive element-binding protein modulator (CREM) (13). Activation of these factors has been shown to regulate the transcriptional activation of immediate early and delayed response genes (48–50).

Both the pattern and time course of phosphorylation in the pseudosubstrate regions are significantly different for the two S6K isoforms, suggesting that distinct regulatory mechanisms control their phosphorylation during pressure overload. First, the phosphorylation of p70<sub>S6K</sub> is more transient, peaks between 1 and 4 h of RVPO, and returns almost to the basal level at 48 h of RVPO, whereas p85<sub>S6K</sub> phosphorylation persists longer by peaking between 4 and 48 h of pressure overload and returns to the basal condition at 1 week of RVPO. This suggests that the kinase(s) and/or phosphatase(s) that control phosphorylation of these residues for the two isoforms are differently regulated. Second, as mentioned earlier, whereas in pressure-overloaded myocardium p70<sub>S6K</sub> is phosphorylated at Ser-411, Thr-421, and Ser-424 sites, phosphorylation of p85<sub>S6K</sub> is only observed at Thr-421 and Ser-424 but not at the Ser-411 site. This was also true for the TPA-treated cardiocytes. All of these studies indicate that the kinase that phosphorylates Thr-421/Ser-424 sites of both the S6K isoforms might be different from the one that phosphorylates Ser-411, although all of these phosphorylation sites in the pseudosubstrate domain are flanked by a COOH-terminal proline. Previous studies show that the phosphorylation of Ser-411, but not Thr-421 or Ser-424, could be prevented by treatment with rapamycin (15), suggesting that a rapamycin-sensitive pathway is responsible for Ser-411 phosphorylation. The mammalian target of rapamycin (mTOR) has been identified recently as a kinase responsible for the phosphorylation of PHAS-I (eIF-4E binding protein) at Ser/Thr residues flanked by a COOH-terminal proline (51–53). As Ser-411 phosphorylation is sensitive to rapamycin and is followed by a COOH-terminal proline, the same kinase that phosphorylates PHAS-I could be responsible for the phosphorylation of Ser-411. The kinase(s) responsible for the phosphorylation of Thr-421 and Ser-424 sites are largely unknown at the present time. We suspect cyclin-dependent kinases as possible candidates, as they phosphorylate not only proteins containing the “Ser/Thr-Pro” motif but also are present both in the nucleus and in the cytoplasm, and are activated in response to growth factors (29).

Our efforts to identify upstream pathways of S6K activation...
indicate that the PKC-independent PI 3-kinase pathway is not the primary mechanism for load-induced activation of both of the S6K isoforms for the following reasons: first, in 1–4 h pressure-overloaded myocardium where both isoforms of S6K are active, neither was the 85-kDa subunit of PI 3-kinase found to be tyrosine-phosphorylated (data not shown), indicative of kinase activation (38, 39), nor was its overall kinase activity increased (Fig. 7A). Second, analysis of the phosphorylation status of Akt/protein kinase B, an immediate kinase that functions downstream of PI 3-kinase (54), using phosphospecific antibody reveals the absence of phosphorylation at all time points of S6K activation (data not shown). Taken together, these data strongly suggest that a PI 3-kinase-mediated pathway is not the mechanism for load-induced activation of the S6K isoforms. However, our studies do not exclude the possibility of PI 3-kinase activation occurring prior to 1 h of RVPO. Unfortunately, such early time points are difficult to examine by surgical interventions in the acute pressure overload model. In addition, our studies indicate the absence of any changes in the pattern of tyrosine-phosphorylated proteins present in 1-h RVPO tissue samples (Ref. 27 and data not shown). Also, similar to our earlier observation (27), cytoskeletal association and activation of c-Src loading (Fig. 7B and Ref. 27). Taken together, these data suggest that the cytoskeletal association and activation of c-Src that were observed between 4 and 48 h of RVPO and the resulting tyrosine phosphorylation may not contribute to the activation of p70S6K that was maximally observed at 1 h of RVPO.

We favor the idea that a PKC-dependent pathway is responsible for load-induced S6K activation. PKC, which has been shown to activate c-Raf (55) as well as S6K (22, 44), is known to undergo activation in pressure-overloaded myocardium (41, 56). Our present studies also show membrane translocation of classical PKC isoforms α and γ as well as the novel isoform PKC-ε in the same tissue samples wherein the S6K isoforms were found to be activated. Previous studies have shown PKC-mediated S6K activation in the absence of PI 3-kinase activity (21, 23), which supports the idea that load-induced S6K activation could be partly due to PKC-mediated signaling. Furthermore, our studies also demonstrate that c-Raf, which is immediately downstream of PKC, showed retarded mobility on SDS-PAGE, indicating kinase activation (42), as well as cytoskeletal association in response to RVPO. As a recent study (57) indicates that constitutively active c-Raf could activate S6K, it is possible that PKC-mediated c-Raf activation might contribute to the activation of S6K in the pressure-overloaded myocardium. The functional role of cytoskeleton-bound c-Raf is largely unknown at the present time, but might be linked to the subsequent recruitment of c-Src to the cytoskeleton for kinase activation. Importantly, most of the load-induced in vivo changes of the S6K isoforms can be mimicked in TPA-stimulated cardiocytes. Overall, these data from TPA-treated cardiocytes support our view that activation of S6K is mediated by a PKC-dependent pathway, and c-Raf could be an intermediate player. However, it is possible that additional pathways might regulate S6K phosphorylation in pressure-overloaded myocardium as opposed to the defined PKC-mediated pathway after TPA stimulation.

In summary, we have demonstrated for the first time that acute pressure overloading of the heart results in substantial activation of both isoforms of S6K, with changes that are distinctly different for the two isoforms. Our attempts to identify potential upstream pathways that are responsible for the phosphorylation and activation of both S6K isoforms suggest that a PKC-mediated pathway independent of PI 3-kinase is responsible for the load-induced kinase activation. In addition, our study extends the importance of S6K, wherein the activation of specific isoforms might regulate both translational and transcriptional events that are critical for the cardiac hypertrophic response.

Acknowledgments—We thank Dr. George Thomas for the polyclonal pSer240/244 antibody (C-5), Mary Barnes for excellent technical assistance, Dr. Donald R. Menick and Dr. Paul J. McDermott for careful reading of the manuscript, and Dr. Robert Thompson and Dr. Robert Gourdie for support whereas performing the confocal microscopy studies.

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