An Alliance between Ras GTPase-activating Protein, Filamin C, and Ras GTPase-activating Protein SH3 Domain-binding Protein Regulates Myocyte Growth*

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We have previously reported that Ras GTPase-activating protein (RasGAP) is involved in a pathway that regulates total cellular mRNA and protein synthesis in cardiac myocytes. A yeast two-hybrid screen resulted in identification of filamin C (FLN-C) as one of its targets. Knockdown of RasGAP or FLN-C, or severing their interaction, resulted in down-regulation of the RNA polymerase II kinase, cyclin-dependent kinase 7 (Cdk7). This appeared to be provoked by the release of cdk7 mRNA from RasGAP SH3 domain-binding protein, G3BP, and its subsequent degradation. In parallel, myocyte growth was also inhibited. On the other hand, overexpression of RasGAP induced a Cdk7- and FLN-C-dependent growth. Thus, we propose that the physical interaction between RasGAP and FLN-C facilitates an interaction between G3BP and cdk7 mRNA. This results in stabilization of cdk7 mRNA, an increase in its protein, which is required for cell growth.

Cell growth or hypertrophy is defined as an increase in cell size and mass. Some organs, such as the heart, grow after birth by a process of cellular hypertrophy, whereas others expand by multiplication of cells, but even then cell growth is a prerequisite to division. The mechanism of growth involves an increase in the synthesis of total cellular protein and other macromolecules. Many studies have focused on identifying the signaling pathways that result in this effect. One main pathway is the phosphatidylinositol 3-kinase-Akt-target of rapamycin (TOR) that results in phosphorylation of ribosomal S6 kinase (S6K) and initiation factor 4E-binding protein (4E-BP), which enhances ribosome biosynthesis (reviewed in Ref. 1). Another potential pathway that is coupled to proliferation includes Cyclin D1 and Cdk4 (2), but the mechanism is not yet fully understood. It should be remembered, however, that mRNA is a necessary template for protein synthesis, and, thus, its own synthesis is a prerequisite for this process. But studies pertaining to pathways that regulate total mRNA synthesis are limited. Our previous data implicate RasGAP in a pathway that appears to regulate both mRNA and protein synthesis. Overexpression of RasGAP* or a mutant lacking the GTPase-activating domain results in enhanced global mRNA and protein synthesis that is accompanied by up-regulation of Cdk7 and hypo- and hyperphosphorylated RNA polymerase II (3). The present study attempts to identify other members of this pathway.

RasGAP through its catalytic domain interacts with Ras-GTP (4), enhancing its GTPase activity (5) and resulting in its inactivation. On the other hand, the N-terminal two-thirds of RasGAP (nGAP) consist of multiple domains, including hydrophobic SH3-binding, Src-homology 2 and 3 (SH2 and SH3), and pleckstrin homology domains. In the presence of apoptotic insults, the C-terminal catalytic domain is cleaved off. The remainder of the molecule (nGAP) confers protection to the cells from apoptosis (6). We subsequently discovered that RasGAP interacts with and activates Akt (7). Thus, it is clear that RasGAP has a myriad of functions that are independent of its catalytic domain and its negative regulatory effect on Ras. Effectors of RasGAP remain largely unknown, although they could potentially be ascribed to any of the known partners of RasGAP. For example, in addition to its recruitment to the platelet-derived growth factor receptor (8–10), RasGAP interacts with tyrosine-phosphorylated p62SH3 (11), p56 (12), and p190 (11) proteins through its SH2 domains. p62SH3 inhibits the catalytic activity of RasGAP (13), whereas p190 is also a RhoGTPase-activating protein (14). RasGAP binds G3BP through its SH3 sequence (15). This protein has an endoribonuclease activity and thus implicates RasGAP in the regulation of RNA stability (16). RasGAP also binds the Src-related family of kinases, including Src, Hck, Fyn, and Lck, through its N-terminal proline-rich domain, serving as a substrate for these kinases (17). It binds Rb and stimulates its activity (18), whereas, it co-precipitates with p85 phosphatidylinositol 3-kinase, which inhibits its activity (19). It binds annexin VI through its C2, Ca²⁺-dependent, lipid-binding domain (20), the significance of which is still unknown. Interestingly, RasGAP also associates with the transcriptional suppressor of cytokine signaling (SOCS-3) (21), which is a negative regulator of STAT-3, also a mediator of cardiac hypertrophy (22). SOCS-3 blocks the function of RasGAP through interacting with its SH2 domain, thus, prolonging the activity of Ras, but a reciprocal relationship is unknown.

Filamin, on the other hand, is mainly a structural protein that belongs to an extended family of actin-binding proteins; Cdk7, cyclin-dependent kinase 7; Cdk9, cyclin-dependent kinase 9; FLN-C, filamin C; nGAP, N-terminal domain of RasGAP (amino acids 1–666); shRNA, short hairpin RNA; 3FLN, deletion mutant of FLN-C (repeats 15–17); G3BP, Ras GTPase-activating protein SH3 domain-binding protein; m.o.i., multiplicity of infection; TUNEL, terminal deoxynucleotidyld transferase-mediated dUTP nick end labeling; ET-1, endothelin-1; FBS, fetal bovine serum; CTD, C-terminal domain.

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§ The abbreviations used are: RasGAP, Ras GTPase-activating protein; Cdk7, cyclin-dependent kinase 7; Cdk9, cyclin-dependent kinase 9; FLN-C, filamin C; nGAP, N-terminal domain of RasGAP (amino acids 1–666); shRNA, short hairpin RNA; 3FLN, deletion mutant of FLN-C (repeats 15–17); G3BP, Ras GTPase-activating protein SH3 domain-binding protein; m.o.i., multiplicity of infection; TUNEL, terminal deoxynucleotidyld transferase-mediated dUTP nick end labeling; ET-1, endothelin-1; FBS, fetal bovine serum; CTD, C-terminal domain.

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Filamin-A and -B are ubiquitous, whereas filamin-C (FLN-C) is a muscle-restricted isoform. These three forms of filamin have 24 repeats, ~96 amino acids each. Both FLN-A and FLN-B have a hinge between repeats 15 and 16, whereas all three isoforms have a hinge between repeats 23 and 24. FLN-C also has an additional 81 amino acids in repeat 20 (23). All three have a highly conserved N-terminal actin-binding domain and a C-terminal dimerization domain. The main known function of filamin is to cross-link actin filaments. This is achieved by the binding of a filamin dimer to two actin filaments through its N-terminal actin-binding domain, leading to the formation of a hinge between the two filaments. It has been reported that the angle between the two filaments is inversely proportional to the concentration of filamin (24).

But, as the list of signaling molecules and receptors that bind to the C-terminal region of filamin has been growing, it is now becoming clear that filamin is not merely a cytoskeletal support protein, but a bridge between cell signaling and architecture. For example, the C-terminal half of filamin A seems to be a docking site for many intracellular signaling molecules and membrane receptors. It binds RaIa, Rac, Rho, and Cdc42 between amino acids 2554 and 2647 (25), Trio, a Rho guanine nucleotide-exchange factor, between amino acids 2354 and 2647, which is necessary for Rho guanine nucleotide-exchange factor-induced membrane ruffling (26). It binds SEK-1 between amino acids 2282 and 2554, an association necessary for activation of the kinase in response to tumor necrosis factor-α (27). Interestingly, tumor necrosis factor-receptor-associated factor 2 also binds to filamin between amino acids 1644 and 2118 (28).

The latter suggests that recruitment of molecules to the C-terminal region of filamin may facilitate their functional communication. FLN-C also binds some transmembrane proteins such as sarcoglycan (23), presenilin (29), caveolin-1 (30), and N-cadherin (31).

In this study we describe the interaction between RasGAP and filamin. To determine the functional significance of this interaction we used RNA interference to knockdown each protein independently. Our results show that the two phenotypes have common features. Those include reduction in Cdk7 and ribosomal S6 proteins and failure to develop hypertrophy. Competing peptides disrupting the interaction between RasGAP and filamin had similar effects. In addition, RasGAP-induced growth was inhibited in the absence of Cdk7 or filamin. In an attempt to determine the underlying mechanism for these effects, we discovered that the RasGAP-binding protein, G3BP, interacts with Cdk7 and influences its stabilization in a filamin-dependent manner. Therefore, we conclude that the interaction between RasGAP and filamin is necessary for regulating Cdk7 and S6 ribosomal levels and cell growth. In addition, although neither protein seemed essential for cell survival, their knockdown proves that they do enhance resistance to apoptotic insults.

MATERIALS AND METHODS
cDNA Library Construction and Screening Using the Yeast Two-hybrid System—Messenger RNA from 1- to 2-day-old neonatal rat hearts was extracted using Qiagen’s Oligotex mRNA isolation kit. The mRNA was reverse transcribed and subcloned into the EcoRI site of the pGAD10 vector, upstream of the activation domain of GAL4. Methods and ingredients used were as provided with the Yeast Two-hybrid Library Construction Kit from BD Biosciences. The library consisted of ~2 × 10^7 independent clones, with cDNA inserts that ranged in size from 0.5 to 4 kb.

nGAP (amino acids 1–666 of RasGAP) was subcloned downstream of, and in-frame with, GAL4 DNA binding domain in the pAS2.1 vector. This construct was transfected into the yeast strain Y190 (MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4Δ, gal80Δ, cyh2, LYS2::GAL1URA3::HIS3, HIS3, URA3::GAL1GAL1GAL1TATA::LacZ), using BD Biosciences Plasmid Transfection Kit. This was followed by transfection of the library cDNA and plating of the transformants on tryptophan-, leucine-, and histidine-deficient plates plus 3-aminotriazol, for selection of positive interactions between the “bait” and its targets. Histidine-positive clones were also tested for LacZ expression, using a colorimetric filter assay, as described in the BD Biosciences handbook Yeast Two-hybrid Matchmaker Manual. The library cDNA plasmid was isolated then reintroduced into the yeast with the bait or control constructs to reconfirm the interaction before sequencing the insert.

Generation of Filamin-C-specific Antibody—An affinity-purified, rabbit polyclonal, FLN-C-specific antibody was custom made by ProSci Inc. The peptide sequence (LATEEPVVPFPMSMES) used for immunization falls within the RasGAP-binding domain that was isolated by the yeast two-hybrid screen and corresponds to amino acids 1733–1748 of human filamin (accession number CAB46442) and 1827–1841 of rat FLN-C (accession number XP_342654, expect for the first amino acid). The epitope bears no significant homology to FLN-A or -B, because a hinge region, which is lacking in FLN-C, disrupts the corresponding sequence in these isoforms. The antibody detects a single band of ~280 kDa in the heart and skeletal muscles and was displaced by the immunizing peptide.

Culturing Cardiac Myocyte and Adenovirus Infection—Cardiac myocytes were prepared as previously described (32). Briefly, hearts were isolated from 1- to 2-day-old Sprague-Dawley rats. The cells were subjected to Percoll gradient centrifugation followed by differential pre-plating to enrich for cardiac myocytes and deplete non-myocytes. Cells were then plated in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 10% fetal calf serum, at a density of 0.5–1 × 10^5 cells/cm^2. Twenty-four hours after plating, serum was removed, and the cells were infected with recombinant adenoviruses at a multiplicity of infection (m.o.i.) of 5–20 particles/cell. These cells were analyzed for viral effects 20–24 h after infection, unless otherwise indicated. For cells infected with shRNA-expressing virus, the cells were harvested after 96 h to allow enough time for depletion of endogenous proteins.

Construction of Adenoviruses—Recombinant adenoviruses were constructed, propagated, and titered as previously described by Dr. Frank Graham (33). Briefly, pBHGlloxE1ΔCre (Microbi)R, including the E1 deletion, was transcribed with the pDC shuttle vector containing the gene of interest, into 293 cells using CaCl2 banding followed by dialysis against 20 mM Tris-buffered saline with 2% glycerol. Titering was performed on 293 cells overlaid with Dulbecco’s modified Eagle’s medium plus 5% equine serum and 0.5% agarose.

Construction of shRNA Adenoviral Expression Vectors—shLencer 1.0-5/6 expression vector was purchased from Ambion. The U6 RNA polymerase III-dependent promoter and the polycoding region were subcloned into the adenoviral shuttle vector pDC311 (Microbi). The hairpin forming oligonucleotides corresponding to bases 2315–2336 (5’-AAAGGATAACAGCCTTTGCTTCCACTACACCGAGAGAGAGGATCTTGGTGTGGCAGTTGTTTTTT), and bases 281–300 of mouse RasGAP SH3-binding protein (NM_013135) (5’-CAAGCCTGCCACCTCACAATTTTCACAAGAAATGTGAAGGAGGTGCAGTTGTTTTTT), and bases 372–392 (5’-AAAGGATAACAGCCTTTGCTTCCACTACACCGAGAGAGAGGATCTTGGTGTGGCAGTTGTTTTTT) of the rat RasGAP cDNA (accession number NM_013135), bases 489–509 (5’-CAGAGATATCTGAGGAGGAGGATCTTGGTGTGGCAGTTGTTTTTT) of rat filamin C cDNA fragment identified in the yeast two-hybrid system, bases 372–392 (5’-AAAGGATAACAGCCTTTGCTTCCACTACACCGAGAGAGAGGATCTTGGTGTGGCAGTTGTTTTTT), and bases 281–300 of mouse RasGAP SH3-binding protein (accession number NM_013135) (5’-CAGAGATATCTGAGGAGGAGGATCTTGGTGTGGCAGTTGTTTTTT), and their anti-sense with ApaI- and HindIII-compatible overlaps, were synthesized, annealed, and subcloned distal to the U6 promoter. The loop sequence is an internal deletion of the E1-deleted adenoviral genome. The viruses were propagated on 293 cells and purified using CaCl2 banding followed by dialysis against 20 mM Tris-buffered saline with 2% glycerol. Titering was performed on 293 cells overlaid with Dulbecco’s modified Eagle’s medium plus 5% equine serum and 0.5% agarose.

Cell Fractionation, Immunoprecipitation, and Western Blotting—Cells were fractionated into cytosol, membranes, nuclei, and cytoskeleton using Subcellular ProteoExtract kit from Calbiochem according to the manufacturer’s directions.

For immunoprecipitation studies, a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 was used to lyse the cells. The sample was processing of either 100 μg of total cellular protein or 0.1 μg of recombinant protein were then incubated with the indicated antibody in the presence of protein A/agarose, for 2 h at 4 °C, rocking. The beads were then washed three times with the above buffer, and the proteins were released using 50 μl of 2% sample buffer at 95 °C. Twenty microliters of the final sample was analyzed on a 4–15% gradient SDS-PAGE followed by blotting on a nitrocellulose membrane. The primary and secondary antibody incubation
buffer consisted of 20 mM Tris-HCl (pH 7.5), 138 mM NaCl, 0.1% Tween-20, 5% bovine serum albumin.

The antibodies used include: polyclonal and monoclonal anti-RasGAP (Oncogene Sciences); anti-FLN-C (custom made by ProSci Inc.); anti-sarcomeric actin (Biomedica); anti-pS6 ribosomal, anti-Cdk7, anti-Cdk9, anti-Cdk4, anti-Akt, and anti-phospho-Akt-Ser473 (Santa Cruz Biotechnology); anti-ribosomal S6 (Cell Signalning); anti-RNA polymerase II MMS-126R (BabCO).

Detection of G3BP-bound mRNA—Cells were lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and G3BP was immunoprecipitated using a mouse monoclonal antibody against amino acids 210–323, excluding the RNA-binding domains (BD Biosciences). RNA was extracted from the immunoprecipitated complex using RNeasy (Qiagen). The RNA was reverse transcribed and used for semi-quantitative PCR detection of cdk7, cdk9, and β-actin mRNA. Primers used encompass nucleotides 476–585 of mouse cdk7 cDNA and nucleotides 348–498 of mouse cdk9 cDNA.

Immunocytochemistry and TUNEL Assay—The cells were fixed in 3% paraformaldehyde/0.3% Tween in phosphate-buffered saline at 25 °C for 5 min followed by 3% paraformaldehyde in phosphate-buffered saline at 25 °C for 20 min. The cells were then incubated with anti-myosin heavy chain (MF-20) or anti-RasGAP at 1:100 in Tris-buffered saline with 1% bovine serum albumin. After an overnight incubation the cells were washed and the secondary antibody-Alexa-488 were added to the cells (Molecular Probes). After washing, the slides were either mounted, as described below, or the TUNEL reaction was performed using a kit from BD Biosciences according to the recommended protocol. The reaction was terminated by 2×SSC followed by mounting of the slides using Prolong Gold anti-fade with 4′,6-diamidino-2-phenylindole (Molecular Probes).

Caspase-3 Assay—Caspase-3 activity was measured using a colorimetric assay kit from BioSource International, as described by the manufacturer.

Transverse Aortic Constriction—Mice were anesthetized (intraperitoneally) with a mixture of ketamine (0.066 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g). The animals were then ventilated via tracheal intubation connected to a rodent ventilator (Harvard Apparatus) with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths per minute. The left chest was opened at the second intercostal space, and the thymus glands were superiorly reflected. The transverse thoracic aorta between the innominate artery and left common carotid space, and the thymus glands were superiorly reflected. The transverse aortic constriction resulted in similar reductions, 70% and 65%, respectively, of nuclear Cdk7, but not Cdk9 or Cdk4, after 5 days. In addition, we discovered that both knockdowns were disrupted by the competing shFLN peptide isolated in the YTH screen.

FLN-C and RasGAP Knockdown, or Disruption of Their Interaction, Results in Reduced Cdk7 and Ribosomal S6 Levels—To determine the functional significance of the interaction between RasGAP and actin, we generated recombinant adenoviruses expressing short hairpin RNA (shRNA)-forming sequences, specific for either molecule. The viruses were used to infect neonatal cultured myocytes. Five days later, we confirmed >90 ± 6% reduction in the proteins, each with its respective shRNA only, as detected by Western blotting (Fig. 2). The FLN-C knockdown showed normal levels and general distribution of RasGAP and Akt proteins (Fig. 2a). The cells responded normally to acute serum stimulation by phosphorylation of Akt and Erk1/2. There was no decrease or redistribution of RhoCE, another filamin-binding protein and regulator of cytoskeletal organization. Similarly, Ras protein was unaffected. FLN-C, but not RasGAP knockdown, was associated, but with a decline in sarcomeric actin protein, possibly due to loss of actin bundling as will be seen later (Fig. 2a). On the other hand, RasGAP knockdown, as we recently reported (7), was associated with loss of Akt, but not Erk1/2, phosphorylation in response to serum.

We have previously reported that RasGAP overexpression in cardiac cells induces up-regulation of Cdk7 and its substrate, RNA polymerase II, and enhanced RNA and protein synthesis, aspects that are critical for the development of hypertrophy (3). We therefore asked whether the knockdown of RasGAP had the inverse effect and whether filamin was an accessory. Concur- ringly, the results show that both RasGAP (Fig. 2b) and filamin (Fig. 2a) resulted in similar reductions, 70 ± 10% and 55 ± 10%, respectively, of nuclear Cdk7, but not Cdk9 or Cdk4, after 5 days. In addition, we discovered that both knockdowns were accompanied by a reduction in S6 ribosomal protein, 75 ± 15% and 83 ± 10%, respectively. Another important change was a 60–70% reduction in RNA polymerase II levels in both phenotypes. The reductions in these proteins appeared to correlate well with the level of filamin, as confirmed by another shRNA construct with less interference (not shown).

These results suggest, but do not prove, that the changes in protein levels associated with both knockdowns are regulated by the interaction between both molecules. To investigate this further we generated a recombinant adenovirus harboring the FLN-C deletion mutant consisting of repeats 15–17 (ΔFLN), isolated from the YTH screen, or the hydropho
FIG. 1. **RasGAP binds to FLN-C.** a, yeast cells were co-transformed with the yeast two-hybrid bait, pAS.nGAP, and deletion mutants of the FLN-C target, repeats 15–17. As a negative control, the cells were transfected with the inserted free target vector, pAct2. The different deletion mutants are as shown in the diagram below. The transformed yeast was then streaked on leucine- and tryptophan-deficient medium (-Leu, -Trp), for the selection of the transformed plasmids, or on leucine-, tryptophan-, and histidine-deficient medium (-Leu, -Trp, -His), for selection of interactions between the transformed constructs. A filter lift β-galactosidase (Lacz) assay was performed on the former yeast plate. b, yeast cells were transformed with the target pAct. FLN-C (15–17) plus the nGAP bait, its distal SH2 domain (SH2), SH3 domain, a pleckstrin homology domain deletion mutant (PH), a pleckstrin homology and hydrophobic domains deletion mutant (PH HD), or the inserted free bait vector, pAS2.1. Transformants were plated on selective (-Leu, -Trp, -His) medium for positive interaction, upper panel, or on -Leu, -Trp plates for a Lacz filter assay, bottom panel. c, brain, lung, skeletal muscle (Sk ms), large intestine (Lg In), and heart tissues were isolated from an adult mouse. Ten μg of total protein extract was analyzed by Western blotting to test a custom made anti-FLN-C antibody. d, heart and Sk ms tissues were fractionated into cytosol (C), membrane (M), nuclei (N), and cytoskeletal (S) fractions. 10 μg of total protein extract was analyzed by Western blotting to test localization of the anti-FLN-C target protein. e, 100 μg of total protein extract from cultured cardiac myocytes infected with a adenoviruses expressing control Lacz, ΔFLN, as indicated, or a His-tagged RasGAP mutant lacking the hydrophobic domain (ΔHD-His, right panels) were incubated with no antibody (no Ab), rabbit polyclonal RasGAP, or rabbit polyclonal anti-His antibodies, as indicated. The immunocomplex was precipitated with agarose-linked protein A and analyzed by Western blotting using anti-FLN-C (top panels), monoclonal anti-RasGAP (bottom left panel), or polyclonal anti-His antibody (bottom right panel).
bic domain only of RasGAP (ΔRasGAP), which encompass the RasGAP- and FLN-C-interacting domains, respectively. The assumption is that overexpression of either of these mutants will compete with the endogenous proteins’ binding sites. Three days after delivery of the constructs to cardiac myocytes, we observed 85±5% reduction in Cdk7 and ribosomal S6 with ΔFLN (Fig. 3a) and 81±9 with ΔRasGAP (Fig. 3b). These effects are in contrast to overexpression of wild type RasGAP (Fig. 3c), which was reversed upon deletion of its N-terminal hydrophobic filamin-interacting domain (Fig. 3d). The inhibitory effect seen with the latter mutant may be due to its squelching of other necessary RasGAP-binding proteins, such as G3BP, as will be seen later. Thus, we conclude that the interaction between RasGAP and FLN-C regulates Cdk7 and ribosomal S6 protein levels.

**FLN-C and RasGAP Knockdown, or Disruption of Their Interaction, Inhibits Hypertrophic Growth—**Cdk7 is a major kinase for the C-terminal domain of RNA polymerase II, whereas ribosomal S6 regulates translation of mRNAs with 5′-oligopyrimidine tracts, mainly constituting those of the translational machinery. Thus, it may be predicted that RasGAP and FLN-C, through modulating the concentration of these two molecules, regulate cell growth. Cultured neonatal cardiac myocytes plated on a suitable extracellular matrix protein, such as fibronectin, will exhibit maximal growth, similar to that induced by serum or growth factors. The cells in this case are also characterized by parallel organization of their sarcomeres, as visualized by phalloidin (Fig. 4a). When the FLN-shRNA was delivered to these cells, the organized bundling of actin filaments within the sarcomeric structure was completely disrupted and was replaced by a loose network. This phenotype confirms for the first time a role for FLN-C in actin bundling in muscle. In addition, it supports its role in assembly of the thin actin filaments at the z-lines of the sarcomeres. Except for minimal loss of parallel bundling of filaments, these effects were not seen with RasGAP-shRNA. But in both cases there was a 30–50% reduction in cell size (Fig. 4a).

To test whether the reduction in cell size is a result of inhibition of protein synthesis or an increase in degradation, we plated the cells on a glass surface, which in contrast to fibronectin results in minimal growth of the cells, unless supplemented by growth factors. This culturing condition allowed...
us to test the effect of the shRNAs on serum- or endothelin-1 (ET-1)-induced growth of the cardiac cells. The data in Fig. 4b show that either RasGAP or FLN-C knockdown completely inhibited serum as well as ET-1-stimulated growth of these cells (also see the graph in Fig. 4d). In addition, disruption of their interaction also resulted in inhibition of growth, albeit partial (50–60%). This may be a result of an increase in RasGAP and filamin levels induced by hypertrophic stimuli (see Fig. 7 below) that partially neutralizes the effect of the FLN mutant. In corroboration ET-1-induced 31 ± 12% increase in phenylalanine incorporation, was completely inhibited by FLN and RasGAP knockdowns, and was reduced to a 11 ± 3% increase by ΔFLN. Thus, these data would suggest that RasGAP and FLN-C, and their association, are required for cardiac hypertrophic growth possibly through regulating protein synthesis.

We show in Fig. 3c that RasGAP overexpression induces an increase in Cdk7 and ribosomal S6. Fig. 4c shows that this is also accompanied by an increase in cell size and reorganization of the sarcomeres, a phenotype similar to that seen with serum-induced growth. This effect was inhibited by FLN-C knockdown, indicating a necessary role for FLN-C in a RasGAP-mediated growth pathway.

Depolymerization of Actin Filaments Does Not Inhibit Myocyte Growth—Cultured myocyte hypertrophy is accompanied by cytoskeletal reorganization. However, it isn’t known whether this is a cause or an effect of growth. Thus, we don’t know if the RasGAP-FLN pathway regulates cell growth via regulating cytoskeletal reorganization. To indirectly address this, we induced actin depolymerization in the cardiac cells using cytoskeleton D, to test its consequence on growth and Cdk7 and S6 expression. Although the treatment resulted in dramatic reduction in cell size (50–70%) under serum-free conditions, as expected, it curiously did not inhibit the ability of the cells to hypertrophy in response to serum (2.8 ± 1-fold, Fig. 5a). In agreement, serum-stimulated phenylalanine incorporation was not diminished relative to control (29 ± 8%). Obviously, though, hypertrophy here was not accompanied by cytoskeletal assembly. Importantly, Cdk7 and ribosomal S6 were unchanged by cytochalasin D treatment under basal (Fig. 5b) or FBS-induced hypertrophy (Fig. 5c). Note that, in cytochalasin D-treated cells, FLN-C was absent in the cytoskeletal fraction, confirming complete actin depolymerization. This suggests that the effects of filamin or RasGAP on Cdk7, ribosomal S6, and cell growth are not due to cytoskeletal reorganization.

The RasGAP-binding Protein G3BP Binds cdk7 and cdk9 mRNA through a Filamin-dependent Mechanism—RasGAP binds to another protein, G3BP, through its SH3 domain (15). Intriguingly, G3BP has two RNA-binding domains that interact with select mRNA species and has been reported to be involved in either their stabilization or degradation. Therefore, we questioned the possibility that RasGAP and filamin might regulate gene expression through regulating the interaction between G3BP and select mRNA, especially the report that 70% of mRNA is associated with the cytoskeleton and co-localizes with filamin, α-actinin, or EF-1α (34). Thus, to test whether G3BP binds cdk7 mRNA directly and whether disruption of RasGAP-filamin interferes with this interaction, we performed a G3BP immunoprecipitation followed by RNA extraction and semi-quantitative reverse transcription-PCR for detection of bound cdk7 or β-actin mRNA from control cells or treated with ΔFLN (encompassing the RasGAP-binding domain) for 24 h only, to avoid the dramatic reductions in total cdk7 mRNA. The results show that cdk7 mRNA co-immunoprecipitated with G3BP and was released upon dissociating RasGAP and FLN by the ΔFLN-competing peptide (Fig. 6, a and b). In contrast, β-actin mRNA did not co-precipitate with G3BP, in agreement with previous reports (35). Total cdk7 and β-actin mRNA are shown for comparison. Western blot analysis of the immunoprecipitated complex shows that ΔFLN did not disrupt the interaction between RasGAP and G3BP (Fig. 6c). Thus, the data show that G3BP binds cdk7 mRNA, which may be enhanced by the binding of RasGAP to filamin.

While Cdk7 protein was reduced by the knockdown of filamin, RasGAP, or disrupting their association, we observed a consistent increase in Cdk9 (~1.8×). Similar to cdk7, cdk9 mRNA also co-precipitated with G3BP and was disrupted by ΔFLN overexpression (Fig. 6, a and b). It is important to mention that, unlike cdk7, cdk9 mRNA contains the G3BP-binding SELEX (systemic evolution of ligands by exponential enrichment) consensus sequence (ACCC(A/C)(U/ C)/(A/C)/(C/G)GC(C/A)/(G/C)) that is a target for cleavage at the CA sites by the intrinsic endonucleolytic activity of G3BP (36). Therefore, although G3BP may bind to and stabilize cdk7, its binding to cdk9 mRNA would result in its degradation. To confirm the role of G3BP on cdk7 versus cdk9 expression, we knocked down the protein by shRNA. As seen in Fig. 6d, G3BP was effectively down-regulated in the cardiac myocytes and was accompanied by ~70% reduction of Cdk7, but not RasGAP or filamin. In contrast, Cdk9 was increased ~60%. Thus, it appears that G3BP has dichotomous effects on cdk7 versus cdk9. This may be dictated by the presence of the cleavable consensus sequence.

Cdk7 Knockdown Inhibits Serum and RasGAP-induced Myocyte Growth—Although Cdk7 is established as an RNA polymerase II kinase, it does not seem to be required for transcription of all promoters (37). Therefore, it remained necessary to
address its role in myocyte growth. Using shRNA we were able to specifically, albeit partially (75%\%), knockdown basal levels of Cdk7, but not Cdk9, RasGAP, or filamin (Fig. 7a). To determine its role in the expression of some growth-related genes, we treated knockdown cells with serum for 24 h, where specified. The cultures were then fixed and stained with anti-MHC (red, all insets) and phalloidin (green, all panels). Cells were then imaged with an epifluorescence microscope at a 60× magnification. Relative two-dimensional cell dimensions were estimated from the number of pixels forming the cell image, which was outlined by the “lasso” tool in Adobe Photoshop. Lacz control, FLN-shRNA, RasGAP-shRNA, or FLN modified cells were untreated (basal, white bars), or treated with 10% FBS (gray bars), 1 μM ET-1 (black bars), or RasGAP (black bars), as indicated. The y-axis represents cell size in pixels (×10²) calculated from the same magnification and on-screen image size for all panels. Bars represent the average size of 20 cells, and error bars represent their S.D. *, p < 0.05 versus basal, calculated by unpaired Student's t test.
RasGAP knockdown cells after 6 days in culture, but that these levels were $-2 \times$ greater than control when induced by staurosporine (Fig. 8). From this we concluded that FLN-C or RasGAP are not required for cell survival, but rather their regulation of mRNA and protein synthesis may be necessary to help neutralize apoptotic signals. This also suggested that the inhibitory effect of the knockdowns on cell growth is not due to ensue of apoptosis in these cells.

**DISCUSSION**

In our previous studies we have shown that overexpression of RasGAP enhances total cellular mRNA and protein synthesis. We also found that this effect was associated with an increase in Cdk7 and hypo- and hyperphosphorylated RNA polymerase II (3). In this study we attempt to identify partners of RasGAP that participate in this pathway. Using the yeast two-hybrid system we identified FLN-C as a RasGAP-binding molecules. Our results confirm that these partners participate in a growth pathway upstream of Cdk7 and ribosomal S6 protein. This is possibly achieved through regulating mRNA metabolism via the RasGAP-binding protein, G3BP.

There are a couple of factors that link filamin and RasGAP to mRNA localization and stabilization. First, a study by Bassell et al. (34) has shown that 72% of poly(A) mRNA localizes with networks of F-actin filaments. In particular, they show that it co-localized with filamin, α-actinin, and EF-1a, until now it isn’t known how this is achieved. It is also thought that mRNA localization at these sites is a prerequisite for its translation. Intriguingly, the RasGAP-binding protein, G3BP, has two RNA-binding domains (15) that have been shown to interact with select mRNA species, including c-myc (36) and Tau (35). Thus, our data provide a potential explanation of how poly(A) mRNA co-localizes with filamin. Namely, RasGAP, which binds filamin via its hydrophobic domain and the RNA-binding protein G3BP via it SH3 domain, forms a link between mRNA and filamin. We propose that the binding of RasGAP to filamin recruits G3BP to the vicinity of mRNA concentrations, thus facilitating its interaction with select mRNA species.

With that in mind, we found that actin depolymerization results in a reduction in cell size, but does not reduce to any extent basal or serum-induced Cdk7, S6 ribosomal, or -fold increase in myocyte hypertrophy. Thus, it appears that the extent of cytoskeletal support may dictate the final cell size but has no impact on protein or RNA synthesis required for hypertrophic growth. This also suggests that mRNA stabilization and translation are probably regulated by its co-localization with the actin-binding proteins indicated above, rather than the actin cytoskeleton per se. Thus, by extrapolaion, inhibition of hypertrophy by FLN or RasGAP knockdown is not a consequence of disruption of the cytoskeleton.

G3BP has been shown to bind c-Myc and tau mRNA, inducing either degradation or stabilization, respectively. It appears that G3BP induces degradation through an endogenous endonuclease activity. Our study shows that cdk7 and cdk9 mRNA also bind G3BP. When the interaction between RasGAP and filamin is interrupted, both cdk7 and cdk9 mRNA are released from G3BP. As a consequence, Cdk7 protein is down-regulated while Cdk9 is up-regulated. We do not understand the reason for this dichotomous effect, but what c-myc and cdk9 have in common is a consensus sequence (ACCC(A/C)(U/C)(A/C/G)GC(C/A)(G/C)) that has been predicted to bind to G3BP and is cleaved at CA sites, thus, initiating mRNA degradation (36). It is thus our prediction that G3BP will bind to various mRNA species and either degrade or stabilize as dictated by the bound sequences. In this case cdk7 is stabilized, and cdk9 mRNA is degraded upon binding to G3BP. The significance of this inverse regulation of the two RNA polymerase II kinases is not clear. It is thought that Cdk7 is involved in early while Cdk9 is involved in late mRNA elongational activity of RNA
polymerase II. One thought is that this mode of regulation might be necessary to synchronize global mRNA synthesis. Cdk7 is a component of TFIIH involved in phosphorylation of the CTD of RNA polymerase II (40, 41), but the requirement of the kinase for the transcription of some genes has been controversial (37). Since then many studies have emerged trying to determine the requirement of Cdk7 in transcription. Those reports show that, at least in yeast (42) and Drosophila (42), it proves necessary. In higher eukaryotes in vivo studies have been more limited, leaving the requirement for Cdk7 debatable. In this study we show that knockdown of Cdk7 was detrimental to cell growth, resulting in a reduction of cell size, but it did not seem to affect basal or induced levels of Cdk9, filamin, or ribosomal S6 protein within the 5 days of culturing. On the other hand, growth-induced RasGAP concentration was reduced commensurate with the reduction in Cdk7. From these results we conclude that Cdk7 is required for transcription of RasGAP, but until we determine the half-life of Cdk9, filamin, or S6 mRNA and whether they are growth-regulated at the transcriptional level, we cannot conclude that Cdk7 is not

FIG. 6. G3BP interacts with cdk7 and cdk9 mRNA through a RasGAP-filamin-dependent association. Cultured cells were infected with 20 m.o.i. of adenoviruses expressing Lacz or ΔFLN for 24 h. Total cell lysate was then immunoprecipitated with anti-G3BP. a, RNA was extracted from the immunocomplex and analyzed by reverse transcription-PCR for 23, 27, 31, and 35 cycles with cdk7, cdk9, or β-actin primers, as indicated. b, the results of 6 data points at 27 cycles of amplifications were scanned and quantitated. The bars represents the relative amount of cdk7 or cdk9 mRNA bound to G3BP in the absence (open bars) or presence (solid bars) of ΔFLN ± S.E., after adjusting the value of cdk7 mRNA to 1. c, the immunocomplex analyzed by Western blotting using anti-G3BP, anti-RasGAP, and anti-FLN, as indicated. d, cardiac myocytes were infected with 20 m.o.i. of adenoviruses expressing G3BP-shRNA or a lacz-shRNA control for 4 d in serum-free medium and then treated, or not, with 10% FBS for an additional 24 h. After that, protein was extracted fractionated into cytosol (cyto), membranes (mem), nuclei (nuc), and cytoskeleton (cytoSk), and 10 μg was analyzed by Western blotting using antibodies for the proteins indicated to the left of each panel.* p < 0.01 versus basal, calculated by unpaired Student’s t test.
required for their expression as well. Therefore, at minimum Cdk7 knockdown proves that the kinase is required for the expression of some growth-related genes. Regulation of molecules that mediate transcription or translation may occur through post-translation modifications or through changes in their effective concentration. While the former has been extensively studied, as for example the regulation of protein synthesis by the TOR-S6K pathway, the latter changes have received little attention. Because secondary modifications are often associated with transient signaling events, it is plausible that more chronic signals eventually induce changes in a molecule’s concentration. Cdk7 activity has been shown to be regulated by phosphorylation (43), but the kinase has not been identified. This phosphorylation event of Thr-170 within the T-loop appears to be necessary for association of Cdk7 with its cyclin H partner and in turn, full CAK activity. Alternatively, a third partner, MAT1, has been shown to stabilize the assembly of Cdk7 and cyclin H (44–46). The presence of MAT1 in this complex and its incorporation into TFIIH shifts the substrate specificity of Cdk7 from Cdk2 to CTD (47). On the other hand, our results show that Cdk7 abundance is modulated by growth signals through a RasGAP/FLN-C-dependent pathway. This suggests that limiting the availability of a molecule may serve as an alternative mechanism for regulating its function. Our previous study shows that cyclin H levels were unchanged, but we have not yet determined whether MAT1 fluctuates during growth. RNA polymerase II, the substrate of Cdk7, was increased during cardiac hypertrophic growth (~7-fold), both the hypo- and hyperphosphorylated forms equally (3), an effect that is also regulated by the RasGAP-FLN-C pathway. It should be noted that Cdk7 alone has the capacity to equally generate hypo- and hyperphosphorylated forms of the full-length CTD, unlike other known CTD kinases, which predominantly generate the hypophosphorylated form (48). Thus, given the established role of Cdk7 and RNA polymerase II in regulating RNA transcription, it is plausible that their increase is a determinant of cell growth.

Cdk9 is another RNA polymerase II kinase that plays a role in its phosphorylation and activation. Although Cdk7 is thought important for phosphorylation of Ser-5, Cdk9 predominately phosphorylates Ser-2, within the heptad repeats of the CTD (reviewed in Ref. 49). This implicates Cdk7 in the earlier stages of transcription, versus Cdk9. Cdk9 is also up-regulated during hypertrophic growth (50), but our data show that, al-

**FIG. 7.** Cdk7 is necessary for RasGAP-dependent cell growth. a, cultured cardiac myocytes were infected with 20 m.o.i. of adenoviruses expressing Cdk7-shRNA or a lacz-shRNA control in serum-free medium. Four days later cells were treated, or not, with 10% FBS for an additional 24 h. Following that, protein was extracted, fractionated into cytosol (cyto), membranes (mem), nuclei (nuc), and cytoskeleton (cytoSk), and 10 μg was analyzed by Western blotting, using antibodies for the proteins indicated to the left of each panel. c, cardiac myocytes were cultured in uncoated glass chambers. Cells were infected with 20 m.o.i. of adenoviruses expressing Cdk7-shRNA (lower panels), or a control Lacz-shRNA (upper panels), as indicated, in serum-free conditions. Four days later, cells were treated with 10% FBS (middle panels), or RasGAP adenovirus (right panels), for an additional 24 h. The cells were then fixed and stained with phalloidin (green, all panels). All cells were then imaged with an epifluorescence microscope at a 60× magnification. *p < 0.05 versus lacz treated, calculated by unpaired Student’s t test.
are found up-regulated in various hypertrophy models, we cannot conclude that this pathway is indispensable for all forms of growth until individually tested.

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