RGD-containing Peptides Activate S6K1 through β3 Integrin in Adult Cardiac Muscle Cells*

The enzyme p70S6 kinase (S6K1) is critical for cell growth, and we have reported its activation during cardiac hypertrophy. Because cardiac hypertrophy also involves integrin activation, we analyzed whether integrins could contribute to S6K1 activation. Using adult feline cardiomyocytes, here we report that integrin-interacting Arg-Gly-Asp (RGD) peptides activate S6K1 as observed by band shifting, kinase activity and phosphorylation at Thr-389 and Thr-421/Ser-424 of S6K1, and S6 protein phosphorylation. Perturbation of specific integrin function with blocking antibodies and by overexpressing the β1A cytoplasmic tail revealed that β3 but not β1 integrin mediates the RGD-induced S6K1 activation. This activation is focal adhesion complex-independent and is accompanied by the activation of extracellular signal-regulated kinases 1/2 (ERK) and mammalian target of rapamycin (mTOR). Studies using specific inhibitors and dominant negative c-Raf expression in cardiomyocytes indicate that the S6K1 activation involves mTOR, MEK/ERK, and phosphatidylinositol 3-kinase pathways and is independent of protein kinase C and c-Raf. Finally, addition of fluorescent-labeled RGD peptide to cardiomyocytes exhibits its internalization and localization to the endocytic vesicles, and pretreatment of cardiomyocytes with endocytic inhibitors reduced the S6K1 activation. These data suggest that RGD interaction with β3 integrin and its subsequent endocytosis triggers specific signaling pathway(s) for S6K1 activation in cardiomyocytes and that this process may contribute to hypertrophic growth and remodeling of myocardium.

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Cell growth involves orchestrated events leading to accumulation of cell mass that is accompanied by a proportional increase in cell size and is modulated significantly by the extracellular matrix (ECM)† environment. An increase in total protein content, which is greatly influenced by translational control mechanisms, is a hallmark of cell growth (1, 2). In the case of proliferative cells, translational activation is important for the transition from G1 to S phase. However, adult cardiac muscle cells (cardiomyocytes), which are terminally differentiated, undergo predominantly hypertrophic growth in response to an applied stress (3, 4). Such hypertrophic growth of cardiomyocytes, as observed in an adult feline right ventricular pressure overload model, is associated with an overall increase in the size and mass of cardiomyocytes via translational activation (4–6). Multiple pathways including the activation of phosphatidylinositol 3-0H-kinase (PI3K), protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) contribute to cardiac hypertrophy (7, 8).

One major converging point for these pathways is S6K1, whose activation has been shown to result in increased biogenesis of translational components. By phosphorylating the 40 S ribosomal S6 protein (S6 protein), S6K1 is reported to enhance the translation of mRNA possessing unique 5′-terminal oligopyrimidine sequences in the 5′-untranslated region. 5′-Terminal oligopyrimidine mRNAs generally encode ribosomal proteins, thereby increasing the overall translational capacity of cells (9). Genetic knockout of S6K1 resulted in small organism phenotypes in mouse (10) and Drosophila (11); such findings emphasize the important role of S6K1 in determining cell growth and size. Considering the prominent role played by S6K1 in cell growth, this kinase can be anticipated to play a significant role during cardiac hypertrophy as well.

There are two isoforms of S6K1, both expressed by the same gene because of alternative start sites: a 70-kDa isoform, which is predominantly cytoplasmic, and an 85-kDa isoform, which has 23 additional N-terminal residues that direct it to the nucleus. S6K1 activation requires modular phosphorylation at multiple sites dispersed throughout the molecule, and its regulation is controlled spatiotemporally by several different kinases and phosphatases (12). Of these phosphorylation sites, Ser-411, Thr-421, and Ser-424 in the catalytic domain, Thr-229 and Ser-371 in the pseudosubstrate domain, and Thr-389 and Ser-404 in the linker region, and Thr-229 and Ser-371 in the catalytic domain have been well characterized. For example, the phosphorylation of the pseudosubstrate domain is mediated via proline-directed protein kinases, including the extracellular signal-regulated kinases 1/2 (ERK) (13); Thr-389 phosphorylation is under the control of the mammalian target of rapamycin (mTOR) (14); and Thr-229 phosphorylation is primarily mediated by the constitutively active phosphatidylinositol-dependent kinase (PDK1) (15).

Our previous studies (16) in hypertrophying myocardium of rapamycin; PDK, phosphatidylinositol-dependent kinase; BIM, bisindolylmaleimide; TBST, Tris-buffered saline plus Tween 20.
revealed that S6K1 undergoes activation as early as 30 min after the onset of pressure overload (PO) and persists up to 48 h. Although our subsequent studies (17) show S6K1 activation is initiated by a PKCε-Raf/MEK/ERK pathway, the sustained activation of this kinase could be mediated by additional signaling events. In this context, we also demonstrated integrin activation and focal adhesion complex formation 4–48 h after PO (18, 19), indicating the possibility that this process contributes at least in part to the sustained activation of S6K1. Ubiquitously expressed on the cell surface as αβ heterodimers, integrins mediate both outside-in and inside-out signaling. Integrins, apart from being critical for cell adhesion, regulate a vast array of signaling events including mechanical sensing, translational activation, cell growth, and tissue remodeling. In addition to differences arising from the extracellular milieu and cell type, the diversity in integrin signaling is attributed in part to the pairing of α and β subunits, because 8 different α subunits and 12 different β subunits constitute at least 24 integrin subtypes (20, 21). Binding of integrins to ECM ligands results in occupancy of integrin-ligand binding sites and triggers an increase in lateral integrin clustering. These events not only initiate adhesion and cytoskeletal organization via direct physical association of integrins with structural proteins, but also activate many intracellular signaling pathways that regulate numerous cellular processes (21). A majority of integrin-ligand interactions known to date are mediated through the Arg-Gly-Asp motifs of the respective ECM ligands. These motifs are either conformationally exposed or buried inside the three-dimensional structure of the intact ligands, and often proteolytic cleavage of the ECM proteins results in the exposure of many of the cryptic sites for interaction with integrins (22). Molecular entities bearing RGD motifs not only interact with integrins at the cell surface and generate signaling cascades (19, 23–25), but also undergo subsequent endocytosis to mediate various cellular events (26–28).

Integrins are expressed in a cell- and/or tissue type-specific manner, and the expression pattern is developmentally regulated. In the myocardium, expression of specific integrins is down-regulated during postnatal growth and re-expression occurs upon hypertrophic stimulation. In models of cardiac hypertrophy, dilated cardiomyopathy, and myocardial infarction, dramatic changes occur in the arrangement of ECM components and integrins (reviewed in Ref. 29). Of the various integrin subtypes expressed in cardiac muscle cells, stimulation of αβ3 (30) and αβ2 (19) integrins has been linked to a hypertrophic phenotype. In mice, heart-specific ablation of integrins results in severe cardiac abnormalities including myocardial fibrosis, depressed contractility and relaxation, intolerance of transverse aortic constriction, and development of cardiomyopathy (31). This model demonstrates the importance of the β3 integrin subtype in hypertrophic growth. However, the nature of the role played by β3 integrin in hypertrophying cardiomyocytes is yet to be understood, although our studies in pressure-overloaded myocardium demonstrate β3 integrin activation and complex formation with signaling proteins at the focal adhesion sites (18, 19).

Because we initially observe S6K1 activation in pressure overload myocardium, which is subsequently accompanied by integrin activation and focal complex formation, we hypothesized that the latter event might contribute to the sustained activation of the former. To test this hypothesis, we performed experiments to address the following questions: (i) can integrin ligation result in S6K1 activation in adult cardiomyocytes, (ii) if so, which integrin subtype is responsible for the activation, (iii) is focal adhesion complex formation necessary in this process, and (iv) what is the mechanism underlying S6K1 activation? Our study indicates that RGD stimulation of β3 integrin can lead to S6K1 activation and that integrin-mediated endocytosis of RGD might contribute significantly to this activation process.

**MATERIALS AND METHODS**

**Chemicals**—The peptides Gly-Arg-Gly-Asp-Ser (RGD) and Arg-Gly-Glu-Ser (RGE), insulin, monodansylcadaverine, protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane, and phosphatase inhibitor mixture containing cantarindin, bromotetramisole, microcystin, sodium orthovanadate, sodium molybdate, sodium taurate, and insulin were obtained from Sigma. Bisindolylmaleimide (BIM), U0126, Wortmannin, monensin, and 12-O-tetradecanoylphorbol-13-aceta (TPA) were from Calbiochem (La Jolla, CA). Alexa Fluor 594 was obtained from Molecular Probes (Eugene, OR). Thin-layer chromatographic (TLC) plates coated with silica gel LK6D and PLK18F were from Whatman Inc. (Clifton, NJ).

**Antibodies**—The following antibodies were obtained from the indicated vendors: S6K1 and LAMP-1 (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-S6K1 (Thr-389), phospho-S6K1 (Thr-421/Ser-424), phospho-S6 (Ser-235/Ser-236), phospho-mTOR (Ser-2448), phospho-ERK (Thr-202/Tyr-204), phospho-c-Src (Tyr-416), phospho-p38 MAPK (Thr-180/Tyr-182), phospho-Jun N-terminal kinase (Thr-183/Tyr-185), AKT and phospho-AKT (Ser-473) (Cell Signaling, Beverly, MA); PY-c-Jun, FAK, and β3 integrin (F11) (BD Biosciences, San Diego, CA); phospho-FAK (Thr-397) (Biosource International, Camarillo, CA); β3 integrin (clones P5D2 and V2E9) and non-immune IgG (Chemicon International Inc., Temecula, CA); CD 25 (Labvision, Fremont, CA); anti-mouse secondary antibody labeled with fluorescein isothiocyanate (Jackson Laboratories, Inc.); gallocycerase-3-phosphate dehydrogenase (Research Diagnostics Inc., Flanders, NJ); c-Src and p85 subunit-specific P3K (Upstate Biotechnology Inc., Lake Placid, NY); and horseradish peroxidase-labeled secondary antibodies (Vector Laboratories, Burlingame, CA).

**Adult Cardiomyocyte Primary Culture—**Ventricular cardiomyocytes from normal adult felines of random sex were isolated as described previously (4, 32). Unless otherwise specified, 105 cells were plated on 35-mm tissue culture dishes (Falcon, Franklin Lakes, NJ) coated with laminin (20 μg/ml, BD Biosciences) in Medium 199 (M199) (Invitrogen, Carlsbad, CA). In all experiments, the cells were maintained at 37°C in humidified air with 5% CO2. To compare the cytoskeletal assembly and activation of c-Src and FAK, freshly isolated adult cardiomyocytes were embedded three-dimensionally in type I collagen in the presence or absence of 9 mM RGD peptide for 1 h, as we described previously (19, 33).

**Adenoviral Infection of Cardiomyocytes—**The following adenoviruses were constructed and used before in our laboratories: β-galactosidase (β-gal) and dominant negative c-Raf (CB4) (17), a fusion protein containing the N-terminal regulatory domain of c-Raf and C-terminal antigenic region of B-Raf to abolish the interactions of c-Raf through cysteine finger domain located at the N-terminal regulatory region (34). In addition, an adenovirus encoding a TAC-Δβ2 chimera, which is a product of the cytoplasmic domain of human β2 integrin fused with the transmembrane and extracellular domains (TAC subunit) of the human interleukin-2 receptor (a generous gift from Dr. Robert Ross, UCCLA, Los Angeles, CA) (30), was propagated in our laboratories. In experiments involving adenoviral infection, cardiomyocytes plated on laminin-coated dishes for 4 h were infected with the indicated adenovirus in Fibro’s medium (M199 containing 2% bovine serum albumin, 5 mM creatine, 2 mM t-carnitine, 5 mM taurine, 0.25 mM t-ascorbate, 10 μM NaAsc, 40 mM insulin (all obtained from Sigma), and 200 units/ml penicillin and 200 μg/ml streptomycin (Invitrogen)). The multiplicities of infection (m.o.i.) used in the present study were 250 (CB4) and 30 (TAC-Δβ2). Cells infected with an equal m.o.i. of β-gal adenovirus served as controls. After 12–16 h in culture, the adenovirus medium was replaced with M199, pH adjusted to 7.4, and centrifuged to remove any undissolved particles at indicated concentrations for 1 h unless specified otherwise. In experiments using pharmacological inhibitors, cells were pretreated with the specified inhibitors as indicated in figure.
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legends and treated with peptides in the presence or absence of inhibitors. In the case of integrin function blocking, cardiomyocytes were preincubated with specified function-blocking or control antibodies at a concentration of 50 μg/ml for 30 min and then treated with RGD or RGE peptides in the presence or absence of function-blocking antibodies for 1 h.

Preparation of Cell Lysate—After treatment, the cells were washed with ice-cold PBS and the soluble and cytoskeletal (CSK) fractions were prepared as described previously (19). Briefly, the cells were scraped in cold Triton X-100 extraction buffer (30 mM Tris-HCl, pH 7.4, 2% Triton X-100 containing protease and phosphatase inhibitor mixtures) and centrifuged at 16,000 × g to obtain the supernatant representing the detergent-soluble fraction to which an equal volume of 2× SDS sample buffer was added and boiled. The insoluble pellet was washed with the extraction buffer and boiled in SDS sample buffer to obtain the CSK fraction. In the case of three-dimensionally cultured cardiomyocytes, cells were recovered from collagen gels by collagenase treatment, and soluble and insoluble samples were prepared as described previously (19, 33).

Western Blotting—Soluble and/or CSK fractions of cell lysates were resolved by SDS-PAGE on either conventional gels (10%) or on NuPAGE (Invitrogen) gels. Resolved proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) and blocked with a solution of 5% nonfat dry milk and 0.1% bovine serum albumin prepared in Tris-buffered saline containing protease and phosphatase inhibitor mixtures and 0.1% Tween 20 (TBST) (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The desired primary antibody was diluted in TBST, added to the blocked membrane, and shaken for 2 h at room temperature or overnight at 4°C. After washing the membranes three times with TBST for 5 min each, appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBST was added to the membranes and shaken for 1 h at room temperature. The secondary antibody was removed by five 5-min washes in TBST, and the protein bands were detected using Renaissance enhanced chemiluminescence kit (PerkinElmer Life Sciences). In all Western blotting analyses, the glyceraldehyde-3-phosphate dehydrogenase level was used to show protein loading.

Immune Complex Kinase Assay—For S6K1 activity, cytosolic samples containing RGD-, RGE-, or control peptide (Garcia-Toledano, J., and others. 2003. Immune complex kinase assay were prepared as described previously (16) and S6K1 was immunoprecipitated using polyclonal anti-S6K1 antibody (C-18, Santa Cruz Biotechnology, CA). Immune complex-bound kinase activity was then measured using a synthetic substrate peptide, RRRRLSSLRA (35), as we described previously (16). The immunoprecipitation and assays were performed in duplicate, and the results are shown as mean ± S.D.

Measurement of PI3K Activity—To measure the PI3K activity in cardiomyocytes, two approaches were taken: (i) the active pool of PI3K was immunoprecipitated using PY-biotin (biotin-labeled phosphotyrosine) antibody and then immunoblotted for the presence of p85 isoform of PI3K (Upstate Biotechnology, Inc.), and (ii) the lipid kinase activity was assayed in immunoprecipitates obtained using a polyclonal antibody against p85 isoform of PI3K (Upstate Biotechnology, Inc.) as reported earlier with minor modifications (16). Briefly, to measure the kinase activity, cardiomyocytes (4 × 106), after stimulation with RGD, RGE, or insulin, were extracted using a buffer containing 20 mM Tris-HCl, pH 7.4, 157 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM dithiothreitol, and protease and phosphatase inhibitors. The extract was centrifuged at 16,000 × g for 10 min. The cleared supernatant was rotated overnight at 4°C with 1 μg of an antibody against the p85 subunit of PI3K. Immune complex was bound to protein A-Sepharose beads (Pierce) by gently rotating at 4°C for 1 h and washed as follows: three times with the extraction buffer; three times with 0.1% Triton X-100 in a 0.1% sodium orthovanadate solution; three times with 0.1% Triton X-100, 1 mM sodium orthovanadate, and 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1 mM sodium orthovanadate. After washing, the beads were assayed for PI3K enzyme activity using phosphatidylinositol (Sigma) and [γ-32P]ATP (Easy Tides, PerkinElmer Life Sciences) as substrates. The organic phase containing lipid products was separated by TLC on silica gel L/DH50F254 with a solvent system of chloroform/methanol/ammonia (60:47:11:2 v/v/v). The TLC plate was then autoradiographed.

Fluorescent Labeling of Peptides—RGD and RGE peptides were labeled with Alexa Fluor 594 as described by Perlot et al. (36) using the Alexa Fluor 594 protein labeling kit (Molecular Probes). Briefly the peptide was dissolved in 100 mM sodium bicarbonate, added to succinimidyl ester derivative of Alexa Fluor 594 dissolved in 100 mM sodium bicarbonate (in a peptide:dye molar ratio of 40:1), and stirred at room temperature for 1 h. The resulting peptide-dye conjugate was separated from free dye by TLC on silica gel PLK138F using acetonitrile:water (85:15, v/v). The peptide-dye conjugate exhibited a slower migration than free dye when monitored under UV light. The area corresponding to the conjugated peptide was scraped and eluted from silica gel using acetonitrile:water (85:15, v/v). The solution was then lyophilized and reconstituted in M199.

Internalization of Alexa Fluor 594-labeled RGD—Cardiomyocytes plated on laminin-coated chamber slides were incubated overnight at 37°C as above, and treated with either RGD- or RGE-Alexa Fluor 594 conjugate for various periods as indicated in the figure legends. After the incubation period, the cells were washed three times with PBS and fixed in 4% formaldehyde. Fixed cells were then mounted (Pro-Long, Molecular Probes) and subjected to laser scanning confocal microscopy (LSM 510; Olympic Optical Co Ltd., Tokyo, Japan). Images were obtained at various Z sections, and the ones corresponding to the central plane of the cells were recorded and processed in Adobe Photoshop.

For colocalization studies, cells plated on laminin-coated chamber slides were treated with RGD-Alexa for 1 h, fixed in 4% formaldehyde for 15 min, and then washed three times with PBS. The fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed three times with PBS, and blocked with 20% donkey serum in PBS for 1 h at room temperature. The cells were then incubated with anti-LAMP-1 antibody overnight at 4°C and washed three times with PBS. Fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody was then added and incubated for 1 h at room temperature. After washing the cells three times with PBS, the cells were mounted and processed for confocal microscopy as described above.

RESULTS

RGD Peptides but Not Control RGE Peptide Activates S6K1—The purpose of this study was to determine whether integrin engagement can lead to S6K1 activation that might contribute to the sustained activation of this kinase in pressure-overloaded myocardium. To explore this possibility, adult feline primary cardiomyocytes were cultured on laminin-coated plates and stimulated using an integrin-binding Gly-Arg-Gly-Asp-Ser (RGD) peptide. A peptide containing an RGE motif served as control in addition to a no-peptide control. Treatment of cardiomyocytes for 1 h with RGD peptide caused a dose-dependent increase in band shifting (retarded electrophoretic mobility) of both the p70 and p85 isoforms of S6K1, as evidenced by Western blot analysis using anti-S6K1 antibody (Fig. 1A, left panel). The S6K1 band shifting was observed at concentrations of RGD peptide as low as 3 mM, and a greater effect was observed at RGD concentrations of 6–9 mM. However, the control peptide (RGE) did not cause any noticeable change in S6K1 migration even at 9 mM.

To further analyze the activation of S6K1, we measured the phosphorylation status of S6K1 at some of its kinase-regulatory sites, namely Thr-389 in the hydrophobic region, Thr-421/Ser-424 in the pseudosubstrate domain and Thr-229 in the activation loop. RGD treatment (9 mM) showed Thr-389 phosphorylation for the p70 isoform of S6K1, although such phosphorylation was not present at detectable levels for the p85 isoform. In the case of Thr-421/Ser-424 sites, phosphorylation was markedly increased in a dose-dependent manner for both the S6K1 isoforms. However, neither of these sites underwent phosphorylation by RGE treatment, even at the highest concentration (9 mM) used in this study. Because mTOR, by mediating the phosphorylation of the Thr-389 site, serves as a key upstream regulator of S6K1 (14), we analyzed its activation by assessing its phosphorylation state at Ser-2448. Phosphorylation of mTOR was evident in cardiomyocytes treated with 6–9 mM RGD but not RGE peptide. Furthermore, RGD-induced activation of S6K1 was also confirmed by checking the phosphorylation status of one of its known substrates, S6 protein of the 40 S ribosomal subunit. Phosphorylation of this protein at the Ser-235/Ser-236 site was observed in a dose-dependent manner in RGD- but not RGE-treated cells. Similar profile of
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For all the parameters described for dose-response experiment (Fig. 1A, left panel). RGD-induced S6K1 activation, as assessed by band shifting and phosphorylation of S6K1, mTOR, and S6 protein, can be observed as early as 30 min with a maximal activation occurring between 60 and 90 min. Therefore, all subsequent RGD treatments were performed at 6–9 mM peptide concentration for 60 min, unless otherwise specified. Overall, the data shown in Fig. 1A demonstrate that stimulation of adult feline cardiomyocytes with RGD peptide, known to interact with specific integrins, can result in phosphorylation of S6K1 at specific sites in a dose- and time-dependent manner.

To measure the phosphorylation status of Thr-229 in the activation loop of S6K1, phospho-antibodies from two different commercial sources were employed (Biosource and R&D Systems). Both of these antibodies were developed against the human S6K1 sequence, and one of them (R&D Systems) is described by the vendor to react at low levels with mouse and rat samples. Whereas these antibodies were shown to react with EGFr-treated HEK cell lysates, they did not react with the feline samples, prepared after treatment of cardiomyocytes with various stimulants, including RGD, TPA, insulin, and forskolin (data not shown). Because it is possible that these antibodies may not react well with feline samples and because phosphorylation of Thr-229 in the activation loop is reported to be critical for its kinase activity (14), we directly measured S6K1 activity after immunoprecipitating it from the cytosolic samples of cardiomyocytes, treated with RGD, RGE, or insulin. These studies (Fig. 1B) demonstrate that RGD stimulation of cardiomyocytes resulted in a robust activation of S6K1 (13-fold) similar to insulin stimulation, indicating the presence of Thr-229 phosphorylation and kinase activation under these conditions.

β3 Integrin Plays a Prominent Role in Mediating RGD-induced S6K1 Activation—Among the various integrin subtypes that are known to interact with RGD peptide, both αvβ3 and αvβ5 are expressed in adult heart (for review, see Ref. 29). Therefore, we sought to characterize these two integrin subtypes for S6K1 activation in adult cardiomyocytes. For this, we pretreated cardiomyocytes with blocking antibodies specific for β3 and β1 integrins and then stimulated with RGD peptide (Fig. 2A, left panel). Whereas non-immune IgG did not block S6K1 activation, an antibody raised against the β3 integrin subunit (F11) (37) significantly blocked RGD-induced S6K1 activation as evidenced by reduced levels of band shifting and Thr-389 phosphorylation. A moderate blocking effect on the RGD-induced Thr-421/Ser-424 phosphorylation was also observed, and this phosphorylation pattern, when compared with cells treated with RGD alone, exhibited a downward band shifting. Such a shift might have been caused by the loss of phosphorylation at other sites, including the Thr-389 site, upon β3 integrin antibody treatment. These data indicate that the Thr-421/Ser-424 phosphorylation is not exclusively mediated via β3 integrin and that other integrin subtype(s) may also contribute to this phosphorylation. Although β3 integrin antibody blocked S6K1 phosphorylation/activation significantly, this antibody exhibited only a low level inhibition of S6 protein phosphorylation. Similar studies using anti-β1 integrin blocking antibody caused only a partial effect on the RGD-stimulated band shifting and Thr-389 phosphorylation of S6K1 and S6 protein phosphorylation and had no effect on the RGD-stimulated Thr-421/Ser-424 phosphorylation.

Because β3 integrin antibody blocking exerted only a moderate inhibition of S6K1 activation, we further characterized the role of this integrin subtype using adenoviral gene delivery. In skeletal and cardiac muscle cells, two isoforms of β3 integrin, namely β3α1 and β3α2, have been reported, and β3α2 is believed to

Fig. 1. S6K1 activation by RGD peptide in adult cardiomyocytes. A, primary cultures of adult feline cardiomyocytes plated on laminin were treated with various concentrations of RGD or RGE peptide for 1 h (left panel) or 9 mM RGD peptide for indicated duration (right panel). Control lanes (Cont) represent cells treated with no peptide. Triton X-100-soluble cell extracts were analyzed by Western blotting using indicated primary antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, adult cardiomyocytes plated on laminin were treated with 9 mM RGD or RGE peptide for 1 h or 100 nM insulin for 20 min. S6K1 was immunoprecipitated from cytosolic fraction and assayed for S6K1 activity using an S6 peptide substrate as described under “Materials and Methods”. Activity is shown as 32P-radioactive counts/min (cpm) incorporated into substrate peptide.

Integrin-mediated S6K1 activation was observed in cardiomyocytes stimulated using another tripeptide with a sequence Arg-Gly-Asp (data not shown).

Fig. 1A (right panel) summarizes the results from a time-course experiment in which cardiomyocytes were treated with RGD peptide (9 mM) for 30, 60, and 90 min and then analyzed
be the predominant isoform in these cell types (reviewed in Ref. 29). To investigate the role of \( \beta_1 \) integrin in the RGD-stimulated S6K1 activation, we expressed, via adenoviral gene delivery, the cytoplasmic domain of \( \beta_1 \lambda \) integrin fused with extracellular and transmembrane domains of interleukin-2 receptor (TAC-\( \beta_1 \lambda \)). Previous studies demonstrate that the expression of this chimeric integrin affects cell adhesion and morphology (30). In our studies, expression of either TAC-\( \beta_1 \lambda \) or \( \beta_1 \)-gal did not affect the RGD-stimulated band shifting and Thr-389 phosphorylation of S6K1 (Fig. 2A, right panel), although substantial TAC-\( \beta_1 \lambda \) expression, as evidenced by the CD25 antibody Western blot that detects the interleukin-2 receptor extracellular domain, was observed (Fig. 2B). Indeed, RGD-stimulated S6K1 phosphorylation/activation was slightly augmented in TAC-\( \beta_1 \lambda \)-expressing cells. In the case of Thr-421/Ser-424, despite a decrease in basal phosphorylation in TAC-\( \beta_1 \lambda \), when compared with \( \beta_1 \)-gal, there is a similar augmentation of phosphorylation at these sites upon RGD treatment. Furthermore, TAC-\( \beta_1 \lambda \) did not affect the RGD-stimulated S6 protein phosphorylation. Together these data indicate that \( \beta_1 \lambda \) integrin is unlikely to contribute to S6K1 activation; however, \( \beta_1 \)-gal, as indicated by the moderate blocking effect with \( \beta_1 \) integrin antibody (Fig. 2A, left panel), may contribute at a lower level to S6K1 activation.

**RGD-induced S6K1 Activation Is Independent of Focal Adhesion Complex Formation and Accompanied by ERK and p38 MAPK Activation**—Integrin activation triggers formation of focal adhesion complexes consisting of both signaling and structural proteins. We have established the formation of such complexes during 4–48 h of PO by identifying FAK, c-Src, and p130\( \alpha\) in the Triton X-100-insoluble actin-rich cytoskeletal fraction (18, 19). In addition, we established a cell culture model in which adult cardiomyocytes are embedded three-dimensionally in type I collagen and stimulated with RGD peptide. Together these data indicate that focal adhesion complex is necessary to mediate S6K1 activation during RGD stimulation. We analyzed the Triton X-100-soluble and insoluble/cytoskeletal fractions from cardiomyocytes cultured two-dimensionally on laminin or three-dimensionally in type I collagen. Analysis of the soluble and insoluble fractions revealed that RGD treatment at various concentrations did not cause cytoskeletal recruitment of c-Src and FAK in two-dimensional culture (Fig. 3A, left panel). Similarly, RGD treatment did not result in autophosphorylation (activation) of c-Src at Tyr-416 and FAK at Tyr-397 in either fraction. Because both c-Src and FAK do not exhibit assembly to the CSK in the two-dimensional model at all concentrations of RGD, the protein bands detected in this fraction with phosphorylation state-specific antibodies represent nonspecific background. On the other hand, RGD stimulation of cardiomyocytes in the three-dimensional model resulted in cytoskeletal assembly and activation of c-Src and FAK (Fig. 3A, right panel). Furthermore, RGD treatment caused cytoskeletal assembly and activation of S6K1 in the three-dimensional model (data not shown). Overall, these results demonstrate that RGD-mediated S6K1 activation, observed in the two-dimensionally cultured cardiomyocytes, proceeds independent of both cytoskeletal recruitment and activation of c-Src and FAK.

Next, we analyzed whether the RGD-stimulated S6K1 activation is accompanied by the activation of MAPK family members, namely ERK, p38 MAPK, and c-Jun N-terminal kinase. Our previous studies (17) showed that the activation of ERK is critical for TPA-mediated S6K1 activation in adult cardiomyocytes. In the present study, RGD induced the activation of both the 44- and 42-kDa isoforms of ERK (ERK 1 and 2, respectively) in a dose-dependent manner (Fig. 3B). RGD induced the activation of p38 MAPK, as evidenced by the phosphorylation of this kinase. However, c-Jun N-terminal kinase was not activated (as evidenced by the absence of phosphorylation), even at the highest RGD concentration. These data suggest that RGD-induced S6K1 activation is accompanied by the activation of at least two MAPK family members, namely ERK and p38 MAPK.

We then analyzed whether the activation of MAPK family members (ERK and p38 MAPK) by RGD is also mediated by \( \beta_1 \) integrin. Anti-\( \beta_1 \)-integrin blocking antibody showed no effect on the RGD-stimulated activation of these two kinases (Fig. 3C), although, as shown in the previous experiment (Fig. 2A, left panel), this antibody significantly blocked the RGD-stimulated S6K1 activation (band shifting). The \( \beta_1 \) integrin blocking anti-

**Fig. 2. Role of \( \beta_1 \) integrin in RGD-induced S6K1 activation.** Western blotting was performed with indicated primary antibodies in Triton X-100-soluble cell extracts obtained from the following experiments. **A**, left panel, adult feline cardiomyocytes plated on laminin were preincubated for 30 min with no or 50 \( \mu \)g/ml amounts of the indicated blocking antibodies or non-immune IgG and then stimulated with 8 mM RGD or 8 mM RGE peptide as indicated. Right panel, adult feline cardiomyocytes plated on laminin were infected with either \( \beta_1 \)-gal or TAC-\( \beta_1 \lambda \) adenoviruses, both with an m.o.i. of 30. After 36 h of incubation, cells were treated with 9 mM RGD or 9 mM RGE peptide as indicated. Cont, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B**, cells infected with \( \beta_1 \)-gal or TAC-\( \beta_1 \lambda \) adenoviruses were analyzed for TAC-\( \beta_1 \lambda \) expression using CD25 antibody by Western blotting.
subsequently stimulated with 8 mM RGD peptide. Triton X-100-soluble
activation. Presence or absence of 9 mM RGD peptide for 1 h. Triton X-100-soluble
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Fractions were prepared as indicated under
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and insoluble (CSK) fractions were prepared as indicated under “Materials and Methods” and analyzed for both total and phosphorylated FAK and c-Src. Cont, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, Triton X-100-soluble fractions were analyzed for the activation of MAPK family members using indicated antibodies. pJNK, phosphorylated c-Jun N-terminal kinase. C, cardiomyocytes were preincubated with 50 μg/ml indicated antibodies for 30 min and subsequently stimulated with 8 mM RGD peptide. Triton X-100-soluble fractions were analyzed by Western blotting using specified antibodies.

body (P5D2) did not inhibit the activation of S6K1, ERK, or p38 MAPK. In these experiments, as an additional control, we used a non-function-blocking β3 integrin antibody (V2E9), whose treatment did not alter the RGD-stimulated activation of these kinases. Overall, these studies with blocking antibodies demonstrate that neither β2 nor β1 integrin contributes to the activation of RGD-mediated ERK and p38 MAPK, although β3 integrin plays a significant role in S6K1 activation.

S6K1 Activation by RGD Peptide Is Not Accompanied by the Activation of PKC and c-Raf but Requires MEK/ERK Pathway—In a recent report (17), we demonstrated that the PKC/c-Raf/MEK/ERK pathway controls the activation of S6K1 observed in TPA-stimulated cardiomyocytes and in 1-h PO myocardium. Therefore, we asked whether the RGD-stimulated S6K1 activation also requires the activation of this pathway. For this, we employed pharmacologic agents BIM and U0126 to block the activity of PKC and MEK, respectively (Fig. 4, left panel), and dominant negative c-Raf (C4B) to block c-Raf activation (Fig. 4, right panel). BIM treatment did not block RGD activation of S6K1 significantly, as evidenced by the retained band shifting of S6K1, Thr-389, and Thr-421/Ser-424 phosphorylation as well as S6 protein phosphorylation (Fig. 4, left panel). However, U0126 showed a moderate blocking effect on the RGD-stimulated Thr-389 and Thr-421/Ser-424 phosphorylation of S6K1, S6 protein phosphorylation, and ERK activation. Both these drugs significantly blocked the TPA-stimulated S6K1 phosphorylation at Thr-389 and Thr-421/Ser-424, S6 protein phosphorylation, and ERK activation. The partial loss of RGD-stimulated ERK activation by U0126 but not by BIM suggests that the MEK-mediated activation of ERK under these conditions occurs independent of PKC.

To explore the role of c-Raf, which mediates the PKC-dependent S6K1 activation, we used adenovirus-expressing dominant negative c-Raf (C4B), employed in our previous studies (17). Whereas C4B expression but not β-gal expression in cardiomyocytes abolished TPA-induced S6K1 activation, the RGD-stimulated S6K1 activation and S6 protein and ERK phosphorylation were not affected by the C4B expression (Fig. 4, right panel). Taken together, these studies demonstrate that the RGD-stimulated S6K1 activation proceeds independent of PKC and c-Raf and that MEK/ERK activation or their basal activity is important for the S6K1 phosphorylation at Thr-389/Ser-424 and Thr-389 sites.

RGD-induced S6K1 Activation Is Blocked by PI3K and mTOR Inhibitors—To determine whether one or more of the PI3K isoform is involved in the S6K1 activation process, we used wortmannin, a specific inhibitor of PI3K, and insulin treatment as a positive control. In these experiments, RGD treatment was carried out only for 15 min to study the effect of wortmannin, a light-sensitive drug. Both the RGD- and insulin-stimulated S6K1 activation, as evidenced by electrophoretic mobility changes and phosphorylation at Thr-389 and Thr-421/Ser-424 sites and S6 protein phosphorylation, were significantly blocked by wortmannin treatment (Fig. 5A). Similar studies with LY-294002, another inhibitor of PI3K, also exhibited a blocking effect both on the RGD- and insulin-stimulated S6K1 activation (data not shown). These data indicate the possible involvement of PI3K during RGD-stimulated S6K1 activation.

Because the p85 isoform has been shown to mediate a pre-
dominant pathway for S6K1 activation (38), we analyzed whether the activation of this isoform is critical for RGD-induced S6K1 activation. For this, we measured the tyrosine phosphorylation status of this isoform in control, RGD-, and insulin-stimulated cardiomyocytes after immunoprecipitating the kinase with biotin-conjugated phosphotyrosine antibody and then detecting either this kinase isoform by Western blot with p85 PI3K antibody or measuring the immune complex for PI3K activity. Western blot analysis (Fig. 5B) exhibits the presence of tyrosine-phosphorylated p85 isoform of PI3K only in the case of insulin treated cells, but not in the case of RGD- or control peptide RGE-treated cardiomyocytes. Measuring the PI3K activity in these immune complexes revealed a significant activation only in insulin-treated, but not in RGD- or RGE-treated, cardiomyocytes (data not shown), consistent with the Western blot data. Furthermore, we also analyzed the PI3K activity after immunoprecipitating with the p85 isoform-specific PI3K antibody (Fig. 5C). Whereas insulin treatment for 15 and 30 min caused a significant activation of PI3K, RGD- and RGE-treated cells showed only a basal level PI3K activity. Finally, in these samples, using a phosphorylation state-specific antibody, we also analyzed the activation of protein kinase B (AKT), a major downstream component of PI3K. Whereas insulin treatment caused a dramatic increase in AKT phosphorylation, RGD but not RGE treatment of cardiomyocytes showed a low level phosphorylation of this kinase, and these results correlate well with the data from PI3K assay mentioned above. Together with the wortmannin data, these studies measuring PI3K activity indicate that either the basal activity of p85 isoform of PI3K or the activation of other isoforms of this kinase is involved in the RGD-stimulated S6K1 activation.

Next, to analyze whether the mTOR, which has been shown to control the Thr-389 phosphorylation of S6K1 in several cell types, including cardiomyocytes (17), controls the RGD-stimulated Thr-389 phosphorylation of S6K1, we used rapamycin to block the activity of mTOR. Pretreatment of cardiomyocytes with 2 nM rapamycin almost completely blocked the RGD-stimulated activation (band shifting) and Thr-389 phosphorylation of S6K1 (Fig. 5D). However, rapamycin did not affect the RGD-stimulated Thr-421/Ser-424 phosphorylation, and this observation is similar to what we described for the TPA-stimulated cardiocytes (17). These studies demonstrate that mTOR activity is required for the RGD-stimulated Thr-389 phosphorylation and kinase activation of S6K1.

**RGD Undergoes Endocytosis and Inhibitors of Endocytosis Block RGD-stimulated S6K1 Activation**—Previous studies demonstrate that when peptides bearing an RGD motif are added to cells, they undergo internalization (39) and that the
internalized peptides with RGD sequence are capable of triggering cellular responses (26–28). Therefore, we studied the possibility of endocytosis of the RGD peptide in adult cardiomyocytes. To follow the internalization of RGD, we labeled both RGD and control RGE peptides with the fluorescent dye, Alexa Fluor 594 (see “Materials and Methods”). The labeled peptides (corresponding to 1 mM peptides) were added to adult cardiomyocyte cultures, and cells were then fixed after various time intervals. Confocal microscopic analysis indicated the presence of labeled RGD inside the cell as early as 5 min, increasing in a time-dependent manner, with significant intracellular staining at 60 min (Fig. 6A). However, RGE, which was labeled and added in a similar manner, did not show any appreciable intracellular staining even at the 60-min time point. These studies suggest that RGD peptide is indeed internalized following its addition to cardiomyocytes. To further analyze the fate of the internalized RGD, we undertook colocalization studies to demonstrate their intracellular localization within the endocytic vesicles (Fig. 6B). For this, we labeled cardiomyocytes treated with RGD-Alexa for 60 min with an endocytic marker LAMP-1. Although LAMP-1 was found to be uniformly distributed throughout the cell, the colocalization of RGD and LAMP-1 was predominantly observed in the perinuclear region. These results suggest the possibility that RGD is selectively localized to endocytic vesicles in the perinuclear region for subsequent processing.

Finally, we analyzed whether the endocytosis observed with RGD contributes to the S6K1 activation. For this, we used monodansylcadaverine and monensin to specifically block the endocytosis. The RGD-stimulated S6K1 activation, including the band shifting and Thr-389 phosphorylation, was significantly blocked by both types of drug treatments (Fig. 6C). However, Thr-424/Ser-424 phosphorylation was significantly blocked only by monensin and not by monodansylcadaverine. These data strongly indicate the possibility that RGD internalization is important for the S6K1 activation.

**DISCUSSION**

The purpose of the present study is to explore whether integrin-mediated signaling events that occur subsequent to S6K1 activation could contribute to the sustained activation of S6K1 in pressure-overloaded myocardium. For this, we used adult feline cardiomyocytes cultured on laminin-coated plates and stimulated with integrin-interacting RGD peptides. Although RGD peptides are conventionally used as cell adhesion blockers, our recent studies show that they can cause integrin clustering and focal adhesion complex formation in cardiomyocytes that are embedded three-dimensionally in a type I collagen matrix but not two-dimensionally on a laminin surface (19). We used both of these models (two-dimensional and three-dimensional) in our initial studies to explore whether integrin engagement with RGD (the two-dimensional model) or with subsequent focal adhesion complex formation (the three-dimensional model) is necessary for S6K1 activation.

Our studies performed in two-dimensionally cultured cardiomyocytes clearly demonstrate that integrin engagement with soluble monomeric RGD, but not a control peptide (RGE), in itself is sufficient for S6K1 and mTOR activation as well as S6 protein phosphorylation. Whereas activation of these components have been demonstrated in fibroblasts adhering to integrin ligands (40) and in other cell types stimulated with various agents including serum, amino acid, and growth factors (41), we show for the first time that a peptide with an RGD motif can trigger S6K1 activation in adult cardiomyocytes. Furthermore, although we observe activation and cytoskeletal recruitment of FAK and c-Src (Fig. 3A) and S6K1 (data not shown) in RGD-stimulated three-dimensional collagen-embedded cardiomyocytes, our data obtained from two-dimensionally cultured cardiomyocytes indicate that this activation can occur in the absence of focal adhesion complex formation and c-Src and FAK activation. This supports the notion that integrins can mediate signals independent of FAK, as reported for MAPK activation in fibroblasts (42).

Multiple integrin subtypes have been reported in the heart (for review, see Ref. 29). Of these subtypes, β1 and β3 integrin-mediated signaling have been implicated in hypertrophic growth (19, 30). In the heart, β1 and β3 integrins normally pair with αv and αv integrin subunits, respectively, and are known to interact with more than one ECM protein through the RGD motif of the ligand. Because the RGD motif interacts with multiple integrins (20) and because β1 and β3 are implicated in cardiac hypertrophy, we investigated whether one or both of these integrin subtypes mediate the S6K1 activation.

Our studies with β1 integrin blocking antibody clearly demonstrate that this integrin subtype predominantly mediates the RGD-stimulated S6K1 activation, as evidenced by the S6K1 band shifting and Thr-389 phosphorylation. However, the Thr-421/Ser-424 phosphorylation of S6K1 and the activation of ERK under these conditions were not significantly affected by the β1 integrin blocking antibody, indicating the possible involvement of other RGD-binding integrin subtypes in this process. This also confirms the notion that ERK activation is responsible for the pseudosubstrate phosphorylation at Thr-421/Ser-424 (17). Similar studies using a monoclonal antibody to β1 integrin blocked neither the S6K1 activation nor other associated signals. Furthermore, overexpression of the cytoplasmic domain of β1 integrin, which was shown to block phenylephrine-stimulated FAK activation and morphology of neonatal cardiomyocytes (30), did not block but rather augmented the RGD-stimulated S6K1 activation, indicating that β1 integrin activation is unlikely to play a primary role in S6K1 activation. Our observations are in line with earlier studies implicating β1 integrin in cell growth: promotion of growth in cancer cells by collagen fragments through αvβ3 (43), αvβ3β5-dependent translational activation in platelets for Bcl-3 synthesis (44), and redistribution of eIF4E to mRNA-rich cytoskeleton in platelets (45). Therefore, although multiple integrins may be triggered during RGD stimulation of cardiomyocytes, the pathway critical for S6K1 activation is predominantly mediated by β1 integrin.

At least three major pathways have been shown to activate S6K1 that involve PI3K, PKC, and PKA (7, 8). Our studies in 1–4-h pressure-overloaded myocardium indicate that the PKC/c-Raf/MEK/ERK pathway contributes significantly to this initial activation of S6K1 (17). Because we observed that the sustained S6K1 activation (up to 48 h of PO) is accompanied by integrin activation in pressure-overloaded myocardium (16), it is possible that this latter event contributes to S6K1 activation via one or more of the three major pathways mentioned above. Blocking PKC or its downstream component, c-Raf, affected only the TPA-stimulated but not the RGD-stimulated S6K1 and ERK activation and S6 protein phosphorylation. Whereas U0126 completely blocked TPA-induced S6K1 and S6 protein phosphorylation, this compound moderately blocked the RGD-stimulated Thr-389, Thr-421/Ser-424 phosphorylation of S6K1 and ERK phosphorylation. This indicates that the RGD-stimulated S6K1 activation occurs largely independent of PKC and c-Raf, although the MEK/ERK activity may be important for S6K1 activation via phosphorylation at Thr-421/Ser-424 and Thr-389 sites, as suggested by our previous studies (17) and by Herbert et al. (46).

Various approaches were taken in the present study to demonstrate the importance of PI3K in RGD-stimulated S6K1 ac-
tivation, because this enzyme plays a predominant role during insulin-stimulated S6K1 activation in adult cardiomyocytes (17). Additionally, recent studies implicate the class I PI3Ks in cell growth and cardiac hypertrophy: activation of class IB isoform of PI3K consisting of p110γ subunit has been observed in a mouse transverse aortic constriction model (47), and class IA PI3K consisting of p110α subunit has been shown to play a role in determining cell size (48). The p85 subunit of PI3K

Fig. 6. Internalization of RGD peptide in adult cardiomyocytes. A, adult feline cardiomyocytes were incubated for indicated duration with Alexa Fluor 594-labeled RGD or RGE peptide and analyzed by confocal microscopy as described under “Materials and Methods.” Left panel shows the phase contrast image, whereas the right panel represents the fluorescent image (decorated in red). Bar = 20 μm. B, adult feline cardiomyocytes plated on laminin and treated with RGD-Alexa Fluor 594 for 1 h were fixed and stained for LAMP-1 as indicated under “Materials and Methods.” In the figure, RGD-Alexa Fluor 594 is decorated in red, and LAMP-1 is decorated in green. The yellow/orange signal in right panel is the result of colocalization of red and green signals. Bar = 20 μm. C, cardiomyocytes were pretreated with 100 μM monodansylcadaverine (MDC) or 25 μM monensin (MON) for 4 h and subsequently stimulated with 9 mM RGD peptide for 1 h as indicated. Triton X-100-soluble cell extract was analyzed by Western blotting using the specified primary antibodies. Cont, control.
partners with p110α, -β, and -δ and constitutes the class IA PI3Ks. In our studies, although neither the p85 isoform of PI3K nor the other PI3K isoforms immunoprecipitated with phosphotyrosine antibody are activated, we conclude, based on the wortmannin and LY-294002 sensitivity and AKT phosphorylation, that active isoform(s) other than the p85 isoform is involved in RGD-induced S6K1 activation. Our notion is further supported by a recent study (49), which demonstrates that PI3K-mediated activation of PKCζ and PDK1 cooperatively controls a sustained Thr-389 phosphorylation of S6K1 in HEK 293 cells and for this, the phosphorylation of Thr-229 in the activation loop is critical. In our studies, Thr-229, as suggested by the S6K1 activity, is phosphorylated upon RGD stimulation and the RGD-stimulated Thr-389 phosphorylation remained higher up to 90 min, indicating the possibility for such a PI3K-regulated cooperative mechanism by PDK1 and PKCζ.

In the case of PKA, this enzyme is activated upon β, δ integrin clustering in HT-1080 cells (50), and it mediates S6K1 activation upon forskolin treatment in cardiomyocytes (17). In the present study, neither of the PKA inhibitors, H-89 and adenosine 3′-5′ cyclic monophosphorothioate (Rp-isomer), inhibited S6K1 activation by RGD, nor was the RI subunit of PKA phosphorylated at Ser-96 upon RGD stimulation (data not shown), thus ruling out the involvement of PKA in RGD-mediated S6K1 activation in cardiomyocytes.

Considering the fact that mitogenic signaling and mTOR activation is invoked here and that RGD peptides can undergo endocytosis, we hypothesized that RGD-containing peptides generated upon ECM degradation during tissue remodeling might undergo endocytosis and evoke an anabolic response for cell growth. Although endocytosis is a phenomenon of almost all mammalian cells (reviewed in Ref. 51), there have been only a few reports in cardiomyocytes: fluid phase endocytosis of solutes (52), phagocytosis of yeast cells (53) and Trypanosoma cruzi (54) and clathrin-mediated endocytosis of the fluorescent marker lucifer yellow (55). In addition, there has been no report on integrin-mediated endocytosis in adult cardiomyocytes despite a few reports in other cell types: endocytosis of synthetic peptide with RGD motifs by M21 melanoma cells (39) and uptake of ECM protein-bound microbeads by retinal pigment epithelial cells that implicates integrin α5β1 in ECM remodeling (28). In this context, our studies demonstrate an intracellular accumulation of Alexa Fluor 594-labeled RGD peptide in a time-dependent manner in cardiomyocytes. Such internalized RGD is localized with endocytic vesicles in the perinuclear region as evidenced by LAMP-1 staining, and this observation is in line with other studies (39). Importantly, treatment of cardiomyocytes with specific inhibitors of endocytosis, monodansylcadaverine (56) and monensin (57), results in the loss of S6K1 activation, indicating that the internalization of RGD peptide is critical for this process. Once internalized, the RGD peptide can either be degraded in the lysosomes and/or proteasome (58) or escape the endosomal compartment (27, 59). In the latter case, the intact RGD motif might be recognized by putative elements upstream of mTOR that possess Asp-Asp-X motifs (60) and could result in mTOR and S6K1 activation. On the other hand, if RGD follows lysosomal and/or proteosomal degradation, an increase in intracellular amino acid concentration may activate putative intracellular amino acid sensor(s) (61), resulting in mTOR and S6K1 activation. Recent studies have implicated a role for mTOR in integrating mitogenic and nutritional signals for cell growth (62). Furthermore, integrin activation either alone or in synergism with vascular endothelial growth factor could lead to the activation of translational process via mTOR (63). In this line, our study showing a rapamycin-sensitive pathway initiated by integrin binding RGD peptides assumes significance.

This model of endocytosis of RGD peptides is further favored by our observation that suspended cardiomyocytes when allowed to adhere on plates coated with laminin, fibronectin, or vitronectin did not result in S6K1 activation (data not shown). This suggests that integrin occupancy alone without subsequent internalization is not sufficient for S6K1 activation. In this context, ventricular remodeling as observed because of changes in hemodynamic overload, under conditions such as myocardial infarction, hypertension, and valvular defects, are known to activate multiple matrix metalloproteinases (64, 65) that could generate peptide fragments containing RGD peptides. Internalization of these peptides with RGD sequence in cardiomyocytes thus may allow the cells to undergo hypertrophic growth via S6K1 activation, while favoring the clearance of ECM surrounding these cells. Future studies will determine how the integrin-mediated internalization of RGD peptides activates S6K1 for hypertrophic growth of cardiomyocytes via ribosomal biogenesis as well as favors myocellular remodeling.

In summary, the present investigation reveals that β, δ integrin can mediate S6K1 activation in adult cardiomyocytes, which may contribute to the sustained S6K1 activation observed in pressure-overloaded myocardium and that integrins are capable of mediating the internalization of peptide fragments possessing RGD motif to potentially regulate growth and remodeling of the myocardium.

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RGD-containing Peptides Activate S6K1 through β3 Integrin in Adult Cardiac Muscle Cells

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