Extracellular ATP-induced Proliferation of Adventitial Fibroblasts Requires Phosphoinositide 3-Kinase, Akt, Mammalian Target of Rapamycin, and p70 S6 Kinase Signaling Pathways*

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Extracellular nucleotides are increasingly recognized as important regulators of growth in a variety of cell types. Recent studies have demonstrated that extracellular ATP is a potent inducer of fibroblast growth acting, at least in part, through an ERK1/2-dependent signaling pathway. However, the contributions of additional signaling pathways to extracellular ATP-mediated cell proliferation have not been defined. By using both pharmacologic and genetic approaches, we found that in addition to ERK1/2, phosphatidylinositol 3-kinase (PI3K), Akt, mammalian target of rapamycin (mTOR), and p70 S6K-dependent signaling pathways are required for ATP-induced proliferation of adventitial fibroblasts. We found that extracellular ATP acting in part through G1 proteins increased PI3K activity in a time-dependent manner and transient phosphorylation of Akt. This PI3K pathway is not involved in ATP-induced activation of ERK1/2, implying activation of independent parallel signaling pathways by ATP. Extracellular ATP induced dramatic increases in mTOR and p70 S6K phosphorylation. This activation of the mTOR/p70 S6 kinase (p70 S6K) pathway in response to ATP is because of independent contributions of PI3K/Akt and ERK1/2 pathways, which converge on the level of p70 S6K. ATP-dependent activation of mTOR and p70 S6K also requires additional signaling inputs perhaps from pathways operating through Gα or Gβγ subunits. Collectively, our data demonstrate that ATP-induced adventitial fibroblast proliferation requires activation and interaction of multiple signaling pathways such as PI3K, Akt, mTOR, p70 S6K, and ERK1/2 and provide evidence for purinergic regulation of the protein translational pathways related to cell proliferation.

Release of ATP from vascular wall cells has been demonstrated in response to a number of environmental stimuli including shear stress, stretch, osmotic load, and hypoxia (1). This release of ATP is speculated to play an important autocrine/paracrine role in modulating various functions of vascular wall cells, including growth. Specifically, extracellular ATP has been demonstrated to stimulate DNA synthesis in endothelial cells, smooth muscle cells, and adventitial fibroblasts, as well as in glial cells, lymphocytes, and renal mesangial cells (2–6). We recently demonstrated that hypoxia increases the release of ATP from pulmonary arterial adventitial fibroblasts and that this ATP plays a critical role in modulating hypoxia-induced fibroblast proliferation, a response previously shown to be critical in the vascular remodeling observed in response to hypoxia (3, 7). We also found that extracellular ATP acts synergistically with cytokines and peptide mitogens known to be released by vascular cells under hypoxic conditions (e.g. platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor) to stimulate growth, emphasizing the important role of ATP in hypoxia-induced vascular remodeling (3). At present it is known that extracellular ATP and hypoxia act to stimulate proliferation through activation of G proteins, extracellular signal-regulated kinase (ERK1/2),7 and the early growth response-1 transcription factor transcription factor in fibroblasts (3). However, whether ATP stimulates PI3K and/or mTOR signaling in vascular fibroblasts and whether these pathways play a role in ATP-induced proliferation are not known.

The importance of PI3K, mTOR, and p70 S6K as key regulators of cell growth in some, but not all, cell systems has been established. There is solid evidence that PI3K is involved in the mitogenic response of vascular, airway, and intestinal smooth muscle cells to a variety of growth-promoting agents (8–11). To date, several classes of structurally different PI3K isoforms have been cloned (12, 13) and shown to be differentially utilized in promoting cell growth depending on the initiating stimuli. At least two isoforms of class I PI3K (PI3Kα and PI3Kγ) can be directly activated by G protein βγ subunits (for review see Refs. 12–14). Several studies have demonstrated that G protein-coupled receptor-mediated proliferative responses occur via activation of PI3K (9, 15–18). However, only one report (19), in

1 The abbreviations used are: ERK, extracellular signal-regulated kinase; ADP/βS, adenosine 5′-O-(2-thiodiphosphate); ATPγS, adenosine 5′-O-(3-thiotriphosphate); 2MeS-ATP, 2-methylthioadenosine triphosphate; ω-ATP (AMP-CTP), adenosine 5′-(ω-methylene)triphosphate; β,γme-ATP (AMP-PCP), adenosine 5′-β,γmethylene triphosphate; Ap5A, P1,P4-di(adenosine-5′)-tetraphosphate; Bz-ATP, benzoylbenzoxy-ATP, 2′-3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate; FBS, fetal bovine serum; MEK1, ERK-activating kinase; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PI3K, phosphoinositide 3-kinase; p70 S6K, ribosomal p70 S6 kinase; S6, 40 S ribosomal S6 protein; PTX, pertussis toxin; MOPS, 4-morpholinepropanesulfonic acid; dn, dominant-negative; GFP, green fluorescent protein.
which pharmacologic inhibitors were exclusively utilized, has implicated a role for PI3K activation in ATP-mediated proliferative responses. Furthermore, there is no information on signaling events downstream from PI3K that may be involved in regulating ATP-induced cell growth. Therefore, since G protein-regulated PI3K isoforms are expressed in lung tissue and vascular cells (12, 15), we sought to test the possibility that extracellular ATP, acting through G protein-coupled pathways (P2Y receptors), may stimulate fibroblast proliferation through activation of PI3K.

Cellular proliferation requires protein translation that involves mTOR and p70 S6K pathways. Several studies have clearly demonstrated the critical importance of mTOR in cell cycle regulation, metabolic responses, transformation, tumor growth and patterning during embryonic development (20–23). The role of mTOR in cell growth is based on findings that the immunosuppressant rapamycin blocks mTOR activity through specific interaction with FK-binding protein (24). mTOR is known to act on downstream targets such as p70 S6K and 4E-BP1 (for review see Ref. 23). p70 S6K phosphorylates the 6S ribosomal protein of the 40 S ribosomal subunit and is involved in translational control of the 5′-oligopyrimidine tract mRNAs (23, 25). p70 S6K activity is regulated by phosphorylation at multiple serine/threonine residues in response to stimulation by growth factors, insulin, and G protein-coupled receptor agonists (25–29). Although the regulation of mTOR and p70 S6K by growth factors operating through receptor tyrosine kinase has been extensively studied, the regulation by G protein-coupled receptor pathways has received relatively little attention. Specific activation of mTOR and/or p70 S6K signaling by extracellular ATP or other purinergic agonists has not been reported to our knowledge.

Both PI3K and mTOR belong to the family of phosphatidylinositol kinase-like kinases. These proteins share a common structure of C-terminal lipid kinase domain and, as has been shown in in vitro kinase assays, are sensitive to wortmannin and LY294002 (20, 30). Hence, when these inhibitors are used as a pharmacological tool to define the contribution of the PI3K pathway, they often cannot distinguish the individual contribution of PI3K versus mTOR pathways to cellular responses. It has been demonstrated that PI3K and mTOR may act either via parallel independent pathways or via an interaction between PI3K and mTOR that is mediated by Akt (27, 28, 31, 32). Akt is recognized as a downstream PI3K target, which plays a central role in a number of key biological functions (for review see Ref. 33). In addition to its well known role in cell survival and metabolic responses, an important role for Akt in cell cycle regulation has also been reported (34, 35). However, the contribution of Akt to cell proliferation, and its role in mediating PI3K-dependent mTOR regulation, has been suggested to be cell type-specific and stimulus-dependent. Its role in ATP-initiated DNA synthesis and mTOR activation is unknown at the present time.

Thus, the goal of this study was to evaluate specifically the contribution of PI3K, mTOR, and p70 S6K pathways on ATP-induced adventitial fibroblast proliferation and to examine a possible role of Akt in mediating cross-talk between PI3K and mTOR pathways. By using pharmacological approaches, in vitro kinase assays, and genetic alterations in Akt expression, we show that ATP-induced adventitial fibroblast proliferation requires PI3K, Akt, ERK1/2, mTOR, and p70 S6K-dependent pathways. Furthermore, we provide evidence that ATP-induced activations of PI3K and ERK1/2 are independent events and that PI3K, Akt, and ERK1/2 play a positive regulatory role in supporting mTOR and p70 S6K activity. To our knowledge, the present findings demonstrate for the first time a role for extracellular ATP on the activation of mTOR, p70 S6K, and S6 ribosomal protein, which are well known to be key regulators of the protein translation pathway. These observations are important for understanding cell growth in the systems where extracellular nucleotide concentrations may be actively regulated by environmental stimuli such as hypoxia.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Pulmonary artery adventitial fibroblasts were isolated from tissue explants of 120–180-day gestational bovine fetuses as described previously (3). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20 mM l-glutamine (Cellgro), nonessential amino acids (1:100, v/v), 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma), and 10% fetal bovine serum (PBS) (Gemini Bio-Products, Woodland, CA). Cell cultures were maintained in a humidified atmosphere with 5% CO2 at 37 °C, and medium was changed every 3 days. For expansion, fibroblasts were cultured to confluence, harvested with trypsin/EDTA solution (0.2–0.5 g/liter), and replaced at 1:4 ratio. All studies were performed on cells between passages 2 and 6. For the assays, the cells were plated at a density of 5 × 10⁴ cells/cm², cultured to 80% confluent cells/well in DMEM, supplemented with 10% FBS. The next day the cells were rinsed with phosphate-buffered saline (PBS) and incubated in serum-deprived DMEM for 72 h. Cells were preincubated with or without LY294002 (20 µM, 60 min), wortmannin (100 nM, 2 h), rapamycin (5 nM, 60 min), UO126 (10 µM, 60 min) (Cell Signaling Technology, Beverly, MA), or pertussis toxin (100 ng/ml, 18–20 h) (List Biological Laboratories, San Jose, CA) and then stimulated with ATP (100 µM) in the presence of 1.0 µCi of [methy1-³H]thymidine incorporation. Cells were plated in 24-well plates at a density of 12 × 10⁵ cells/well in DMEM, supplemented with 10% FBS.

**DNA Synthesis Analysis—**DNA synthesis was determined by [methy1-³H]thymidine incorporation. Cells were plated in 24-well plates at a density of 12 × 10⁵ cells/well in DMEM, supplemented with 10% FBS.

**Cell Experiments and Western Blot Analysis—**Adventitial fibroblasts were cultured to near-confluence and serum-starved in DMEM for 96 h. For the experiments with PI3K, mTOR, and Akt inhibitors, cells were grown with LY294002, wortmannin, rapamycin (Cell Signaling Technology), SH-5, SH-6 (36) (Biogenio, Carlsbad, CA), or NL-71–101 (37) (Calbiochem), as described in the figure legends. For the experiments aimed at investigating the involvement of G proteins in ATP-mediated responses, cells were treated with pertussis toxin (PTX, 100 ng/ml) for 18–20 h. Cells were stimulated with ATP (100 µM) in serum-free conditions for an arbitrary period of time. After washing, cells were washed twice with ice-cold PBS and lysed with Tris-HCl buffer (40 mM, pH 7.5, 4 °C), containing 0.5 M NaCl, 0.1 M EDTA, 50 µg/ml leupeptin, 1 mM PMSF, and 0.1% Nonidet P-40. For the experiments with PI3K, mTOR, and Akt inhibitors, cells were preincubated with or without LY294002, wortmannin, PH-795 (Calbiochem), or pertussis toxin (100 ng/ml) for 18–20 h. Cells were stimulated with ATP (100 µM) in serum-free conditions for an arbitrary period of time. After washing, cells were washed twice with ice-cold PBS and lysed with Tris-Cl buffer (40 mM, pH 7.5, 4 °C), containing 0.1% Triton X-100, 0.25 M sucrose, 3 mM EGTA, 3 mM EDTA, 50 µM β-mercaptoethanol, 1 mM PMSF, and complete protease inhibitor mixture (Calbiochem). Cell lysates were centrifuged at 7500 × g for 10 min at +4 °C, and supernatant fractions were collected and stored at −80 °C. Equivalent amounts of total cell protein (10–25 µg) were subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and probed with rabbit polyclonal antibodies against phospho-ERK1/2 (Tyr-202/Thr-204), phospho-p70 S6K (Thr-421/Ser-424 and Thr-389), phospho-Akt (Thr-308 and Ser-473), phospho-mTOR (Ser-2448 and Ser-2481), and phospho-p65 (Ser-235/236) under conditions recommended by the manufacturer (Cell Signaling Technology, Beverly, MA). After washing with TBS-Tween buffer, membranes were incubated with mouse anti-rabbit peroxidase-conjugated IgG, 1:10,000 dilution (Amersham Biosciences), for 1 h at room temperature. For detection of the nonphosphorylated form of these proteins, membranes were stripped with buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 µM β-mercaptoethanol, blocked with 5% nonfat dry milk in TBS-Tween, and reprobed with antibodies against total Akt, p70 S6K, ERK1/2, mTOR, and S6 kinases. The membranes were washed (1:10,000 dilution, Cell Signaling Technology). Immunoreactive bands were detected by ECL kit (Renaissance, PerkinElmer Life Sciences) followed by exposure to Hyperfilm and then quantitatively analyzed with a densitometry system.

**Assay of in Vitro PI3K Activity—**Adventitial fibroblasts were grown to 80% confluency in 100-mm² dishes, growth-arrested in DMEM without serum for 4 days, and then stimulated with ATP (100 µM). After

**Assay of in Vitro mTOR Activity—**Adventitial fibroblasts were grown to 80% confluency in 100-mm² dishes, growth-arrested in DMEM without serum for 4 days, and then stimulated with ATP (100 µM). After
stimulation, cells were washed two times with ice-cold PBS containing 0.2 mM activated orthovanadate and incubated in 750 μl of lysis buffer containing 157 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, complete protease inhibitor mixture (Calbiochem) and incubated for 20 min at +4 °C. Cell lysates were transferred to 1.5-ml tubes and centrifuged at 10,000 × g for 10 min to sediment insoluble material. Supernatants were normalized for protein content (Bio-Rad assay kit) and incubated with anti-p85 antibody linked to protein A-agarose (Upstate Biotechnology, Lake Placid, NY). One microgram of antibody was used per 0.5 mg of total cell protein. After rocking at +4 °C for 1–2 h, agarose beads containing antibody-enzyme complexes were centrifuged at 10,000 × g for 20 s and washed three times with buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 0.1 mM sodium orthovanadate), three times with buffer B (0.1 mM Tris-HCl, pH 7.5, 5 mM LiCl, 0.1 mM sodium orthovanadate), and three times with buffer C (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate). Immuno-precipitates were resuspended in 50 μl of buffer C and combined with 10 μl (20 μg) of sonicated phosphatidylinositol and 10 μl of 100 mM MgCl₂. The phosphorylation reaction (final volume 80 μl) was started by addition of 10 μl of mixture, containing 15 μCi of [γ-32P]ATP (10 mM, 3000 Ci/mmol, PerkinElmer Life Sciences) and 0.88 mM ATP. The samples were incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 20 μl of 6 N HCl. Radiolabeled lipids were extracted with 160 μl of chloroform/methanol mixture (1:1, v/v). Organic phase was separated by centrifugation for 10 min at 12,000 × g at 4 °C, and lipids were separated on oxaolate-coated silica TLC plates (Silica Gel 60, Sigma) by chromatography in CH₂₃O/CH₃OH/0.1 M NH₄OH (60:40:11.3:2 by volume) solvent system in parallel with nonradioactive standard. Radioactive spots were revealed by autoradiography and quantitatively analyzed with a densitometry system.

Assay of in Vitro p70 S6K Activity—The p70 S6K immunocomplex kinase assay was performed according to the manufacturer’s protocol with small modifications (Upstate Biotechnology, Inc., Charlottesville, VA). Quiescent fibroblasts in 100-mm dishes were stimulated with ATP (100 μM) for the indicated times, washed twice with ice-cold PBS, and solubilized in 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM phosphatidylinositol, and 100 μM MgCl₂. The phosphorylation reaction (final volume 80 μl) was started at +4 °C for 2 h and then with 40 μl of protein A-Sepharose beads at +4 °C for 1–2 h. Beads containing antibody-enzyme complexes were centrifuged at 10,000 × g for 20 s and washed twice with lysis buffer containing 0.5 mM NaCl, twice with lysis buffer alone, and twice with Assay Dilution Buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) and 50 μl of sonicated phosphatidylinositol and 10 μl of 100 mM MgCl₂. The phosphorylation reaction (final volume 80 μl) was started by addition of 10 μl of mixture, containing 15 μCi of [γ-32P]ATP (10 mM, 3000 Ci/mmol, PerkinElmer Life Sciences) and 0.88 mM ATP. The samples were incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 20 μl of 6 N HCl. Radiolabeled lipids were extracted with 160 μl of chloroform/methanol mixture (1:1, v/v). Organic phase was separated by centrifugation for 10 min at 12,000 × g at 4 °C, and lipids were separated on oxaolate-coated silica TLC plates (Silica Gel 60, Sigma) by chromatography in CH₂₃O/CH₃OH/0.1 M NH₄OH (60:40:11.3:2 by volume) solvent system in parallel with nonradioactive standard. Radioactive spots were revealed by autoradiography and quanitatively analyzed with a densitometry system.

RESULTS

Extracellular ATP-induced Proliferative Responses in Adventitial Fibroblasts Involves PI3K and mTOR—Although ATP has been demonstrated to activate PI3K signaling in renal mesangial cells, this pathway is not thought to be directly involved in ATP-induced proliferation in these cells (38). However, in vascular endothelial cells and smooth muscle cells, PI3K signaling is thought to be important in cell proliferation (10, 19, 39, 40). Therefore, we initiated our studies using pharmacological inhibitors to determine whether PI3K and/or mTOR are critical signaling components in ATP-induced proliferation of adventitial fibroblasts. DNA synthesis in response to extracellular ATP was measured in the absence or presence of the indicated compounds. In agreement with our previous observations, we found that ATP (100 μM) significantly increased [methyl-3H]thymidine incorporation in growth-arrested fibroblasts (Fig. 1). Pretreatment with LY294002 (20 μM), wortmannin (100 nM), or rapamycin (5 nM) completely attenuated the response, suggesting that PI3K and mTOR are necessary for ATP-induced DNA synthesis in adventitial fibroblasts. The efficacy of these compounds in inhibiting ATP-induced proliferation was equivalent to U0126, a known MEK1/2-specific inhibitor. We also found that PTx significantly inhibited the effect of ATP, indicating that in adventitial fibroblasts extracellular ATP acts, at least in part through Gαi-coupled P2Y receptors.

ATP Induces PI3K Activity in Adventitial Fibroblasts—To confirm that PI3K activation is an important upstream event in ATP-induced cell proliferation, we measured PI3K activity following ATP stimulation by using an in vitro kinase assay. We demonstrated previously that ATP-induced fibroblast proliferation occurs largely through Gαi-mediated signaling (3). It is known that two isoforms of PI3K containing p110β and p110γ catalytic subunits can be regulated by G protein βγ subunits (13). By using immunoprecipitation with p85 antibodies, subsequent Western blotting analysis, and an in vitro kinase assay, we found that PI3K isoform containing p110α and p110β catalytic subunits are expressed in adventitial fibro-
blasts. No PI3K activity was found when anti-p110ψ, anti-p110γ, or anti-p101 adaptor protein antibodies were used for immunoprecipitation. Therefore, to examine the effect of ATP on PI3K activity, we stimulated quiescent fibroblasts with ATP, and we immunoprecipitated total cell lysates with anti-p85i antibodies for assessment of PtdIns-3-P production. As shown in Fig. 2, top and bottom panels, stimulation of adventitial fibroblasts with 100 µM ATP resulted in increased PtdIns-3-P accumulation. Increases in PtdIns-3-P were observed after 5 min of stimulation, with the maximal increase (3.8-fold) observed at 30 min. Wortmannin blocked basal and ATP-stimulated lipid kinase activity (Fig. 2, right panel). In similar studies using phospho-Tyr antibodies for immunoprecipitation, negligible PI3K activity was detected. These data demonstrate that adventitial fibroblasts express both p110α and p110β catalytic subunits and that p110β likely mediates the effect of ATP on PI3K activation.

PI3K Does Not Mediate ERK1/2 Activation in Adventitial Fibroblasts—We demonstrated previously that ERK1/2 phosphorylation is necessary for hypoxia- and ATP-induced proliferation of fibroblasts (2, 5) (Fig. 1). It has been shown in many, but not all, cell types that PI3K is an upstream regulator of ERK1/2 (8, 17, 19, 41, 42). However, whether PI3K activation is required for ERK1/2 phosphorylation in fibroblasts is unknown. To determine whether PI3K regulates ERK1/2 in adventitial fibroblasts, we examined the effects of wortmannin (100 nM) and LY294002 (20 µM) on the level of ATP-induced ERK1/2 phosphorylation. As shown in Fig. 3, neither wortmannin nor LY294002, used at concentrations that effectively blocked ATP-induced increases in fibroblast proliferation and in vivo PI3K activity, significantly affected ATP-induced ERK1/2 phosphorylation. In contrast, PD98059, a MEK1 inhibitor, suppressed ATP-induced ERK1/2 phosphorylation and DNA synthesis (Fig. 1). These data indicate that in adventitial fibroblasts, ATP-induced PI3K and ERK1/2 activation are likely parallel signaling events, both necessary for ATP-induced proliferation.

ATP Induces Early Akt Activation in Adventitial Fibroblasts—To begin to examine targets downstream of PI3K in the ATP-induced proliferative response, we sought to determine whether Akt was activated. Incubation of adventitial fibroblasts with 100 µM ATP for 0–120 min resulted in modest and transient Akt Thr-308 and Ser-473 phosphorylation. ATP-induced phosphorylation occurred between 5 and 10 min with the peak at 7.5 min. Phospho-Akt increased by ~300–380% compared with the basal levels for Thr-308 and Ser-473, respectively (Fig. 4A). Extended exposure of adventitial fibroblasts to ATP up for to 2 h did not induce an additional increase in Akt phosphorylation. The effect of ATP was completely abolished in the presence of the PI3K inhibitor LY294002, suggesting ATP-induced Akt phosphorylation is mediated by PI3K (Fig. 4B). It has been shown that G protein βγ subunits can interact with and activate effector proteins through interaction with their pleckstrin homology domains. As Akt has an N-terminal pleckstrin homology domain, we sought to test the role of G proteins in ATP-induced Akt phosphorylation. We found treatment of adventitial fibroblasts with PTx, which selectively ADP-ribosylates Goα and prevents release of Gβγ subunits, decreased ATP-induced Akt phosphorylation at Ser-473 (Fig. 4B) and Thr-308 (data not shown). These data suggest that in addition to 3’-phosphorylated phosphoinositides, βγ subunits derived from Goα proteins participate in ATP-induced Akt activation. These data also suggest that in adventitial fibroblasts extracellular ATP acts at least in part through Goα-coupled P2Y receptors.

Extracellular ATP Stimulates mTOR Phosphorylation—The ability of rapamycin to inhibit ATP-induced DNA synthesis indicates that the mTOR pathway is involved in the proliferative response of adventitial fibroblasts. To evaluate further the role of mTOR and to determine its relationship to PI3K and Akt activation, we performed Western blot analyses with phosphospecific mTOR antibodies that recognize the activated state of the protein. Phosphorylation of mTOR Ser-2448 implies an

FIG. 2. ATP stimulates PI3K in adventitial fibroblasts. Growth-arrested fibroblasts were exposed to 100 µM ATP (left panel) for the indicated times. Equivalent amounts of total cell protein (350–500 µg) were immunoprecipitated with a rabbit polyclonal antibody against the p85i subunit of PI3K. Lipid kinase activity was determined as described under “Experimental Procedures” with 32P-phosphatidylinositol as a substrate. 32P-phosphatidylinositol 3-phosphate (PtdIns-3-P) indicates the product of PI3K activity. To show that 1-α-phosphatidylinositol-3-phosphate formation is because of PI3K activity, cells were exposed to 100 µM ATP for 30 min, and PI3K activity was measured in the presence or absence of 100 nM wortmannin (right panel). Spots corresponding to 32P-phospholipids were verified by using unlabeled lipid standards. Data on the bottom panel show accumulated 1-α-phosphatidylinositol-3-phosphate at the indicated times (mean ± S.E.) from four independent experiments performed in three separate cell populations. WT, wild type.

FIG. 3. ATP-induced ERK1/2 phosphorylation is not mediated by PI3K. Growth-arrested fibroblasts were pretreated either with LY294002 (20 µM, 60 min), wortmannin (100 nM, 2 h), or PD98059 (10 µM, 60 min) and then stimulated with ATP (100 µM, 10 min). Following stimulation, 10% of total cell protein was subjected to 10% SDS-PAGE and Western blot analysis with anti-phospho-ERK1/2 Tyr-202/Thr-204. Phospho-ERK1/2 was normalized to total ERK1/2, and quantitative data are expressed as % of basal level. Data on the upper panel represent two typical experiments performed on separate cell populations. Similar results were reproduced in at least five different experiments (lower panel). Cont, control; WT, wortmannin.
involvement of the PI3K/Akt pathway in mTOR activation, whereas phosphorylation of mTOR Ser-2448 may be the result of autokinase activity (32, 43, 44). Stimulation of quiescent adventitial fibroblasts with ATP increased both mTOR Ser-2448 and Ser-2481 phosphorylation in a time-dependent manner with significant increases observed within 5–30 min (Fig. 5A). mTOR phosphorylation was sensitive to LY294002, consistent with the idea that PI3K and Akt are involved in mTOR activation (Fig. 5B).

**Extracellular ATP Activates p70 S6K—p70 S6K is a downstream target of mTOR in growth and metabolic signaling pathways. Activation of p70 S6K is accompanied by a complex phosphorylation of multiple Ser and Thr residues located within the catalytic linker and pseudosubstrate domain (25, 26). We sought to determine whether p70 S6K is activated in response to ATP stimulation. Growth-arrested adventitial fibroblasts were treated with 100 μM of ATP, and p70 S6K activation was assessed over time by Western blot analyses using phospho-Thr-389- and phospho-Thr-421/Ser-424-specific antibodies. As shown in Fig. 6A, stimulation of adventitial fibroblasts with ATP induced a time-dependent increase in p70 S6K phosphorylation with a maximal response (420–450% compared with the basal level) at 30 min. ATP-induced activation of p70 S6K was confirmed in an in vitro kinase assay with S6 ribosomal protein peptide as a substrate (Fig. 6B). The time course observed for S6 phosphorylation is slightly different from the time course observed for p70 S6K phosphorylation itself (compare 10 min), probably indicating that phosphorylation of p70 S6K at multiple sites is required for its full activation. The maximal increase in p70 S6K phosphorylation was observed at 30 min, which is consistent with the maximal ATP-induced p70 S6K phosphorylation determined by Western blot analyses (Fig. 6A).

**PI3K, Akt, and mTOR and ERK1/2 Mediate ATP-induced p70 S6K Activation and S6 Ribosomal Protein Phosphorylation—**To determine whether PI3K and mTOR regulate ATP-induced p70 S6K activity, we examined the effects of wortmannin and LY294002 on p70 S6K phosphorylation. LY294002 (20 μM) completely blocked ATP-induced p70 S6K Thr-389 phosphorylation and decreased Thr-421/Ser-424 phosphorylation at least by 65% (Fig. 7, A–C). Similar inhibitory effects of LY294002 were observed when 10% fetal bovine serum was used to induce a maximal phosphorylation response (Fig. 7, A and D). In contrast, wortmannin (100 nM) was not as effective as LY294002, as it decreased ATP- and serum-induced p70 S6K Thr-389 phosphorylation by ~52 and ~47%, respectively, and had no significant effect on Thr-421/Ser-424 phosphorylation. Similarly, wortmannin had only a slight inhibitory effect on serum-induced Thr-421/Ser-424 phosphorylation. The increase in p70 S6K phosphorylation was effectively blocked by the specific mTOR inhibitor, rapamycin, indicating that mTOR is an upstream regulator of p70 S6K (Fig. 7, A–D). We also found that pre-incubation of adventitial fibroblasts with the specific MEK1/2 inhibitor UO126 attenuated ATP- and FBS-induced Thr-421/Ser-424 phosphorylation by ~70 and ~65%, respectively (Fig. 7, A–D), implying a role for ERK1/2 signaling in ATP-induced mTOR activation. In addition to an effect on p70 S6K, ATP stimulated phosphorylation of S6 ribosomal protein, a known downstream target of p70 S6K (Fig. 8). The response was dramatically sensitive to inhibition by both rapamycin and LY294002, indicating PI3K and mTOR are required for S6 phosphorylation.

To determine the role of Akt in mTOR and p70 S6K activation, we examined the effects of the putative pharmacologic Akt inhibitors. We found that SH-5 (10 μM) and SH-6 (20 μM) decreased ATP-induced mTOR Ser-2448 phosphorylation by 65–72% (Fig. 9A). In addition, adventitial fibroblasts treated with another putative Akt inhibitor, NL-71-101 (20 μM), exhibited up to an 80% decrease in mTOR phosphorylation and a 65–70% decrease in p70 S6K Thr-389 and Thr-421/Ser-424 phosphorylation in response to ATP (Fig. 9B).

To evaluate further the role of Akt in ATP-induced activation of mTOR and p70 S6K, adventitial fibroblasts were transduced with adenoviruses encoding dominant-negative mutant Akt (dn-Akt) or a constitutively activated myristoylated Akt. Transduction with a GFP-expressing adenovirus as a control, had no significant effect on the ability of cells to respond to ATP, whereas transient expression of dn-Akt dramatically decreased ATP-induced mTOR Ser-2448, p70 S6K Thr-389, and Thr-421/Ser-424 phosphorylation (Fig. 9C). A nonsignificant decrease in the basal phospho-Thr-389 level was also observed in fibroblasts expressing dn-Akt. Transient expression of con-
stitutively active myristoylated Akt resulted in a small but significant increase in the basal level of phospho-mTOR and phospho-p70 S6K. mTOR and p70 S6K phosphorylation was further increased after cells were stimulated with ATP (Fig. 9D). Collectively, these data demonstrate that Akt, at least in part, mediates PI3K-dependent activation of mTOR and p70 S6K in response to extracellular ATP.

Extracellular ATP Induces p70 S6K Phosphorylation through P2Y and P2X Purinergic Receptors—It is known that vascular cells express multiple subtypes of purinergic receptors that belong to two classes, metabotropic P2Y and ionotropic P2X receptors (45). To determine P2 receptor subtypes involved in ATP-induced proliferative responses in adventitial fibroblasts, we compared the ability of adenine nucleotides and their stable analogs, as well as UTP and UDP, to induce phosphorylation of Akt, ERK1/2, and p70 S6K. We chose to examine these three kinases because our results showed that PI3K/Akt, ERK1/2, and mTOR/p70 S6K-dependent pathways are all crit-
ical in adventitial fibroblast proliferation in response to puri-
ergic stimulation. We found that ATP, UTP, UDP, ADP, ATP/H9253, 2MeS-ATP, Ap4A, and Bz-ATP had significant effects on phosphorylation of Akt, ERK1/2, and p70 S6K, although with variable potency (Table I). For instance, Ap4A was more effective in stimulating phosphorylation of Akt and ERK1/2 than p70 S6K. The ATP analogs/H9251/H9252 meATP and/H9252/H9253 meATP had small but significant effects on ERK1/2 phosphorylation, whereas they had no significant effect or lacked an effect on Akt and p70 S6K, respectively. The observed efficacy of the agonists suggests that at least P2Y1, P2Y2/4, P2Y6, and P2X7 receptor subtypes are involved in the activation of PI3K/Akt, ERK1/2, and mTOR/p70 S6K pathways. The rank order of potency of these compounds to activate each individual kinase may also suggest that P2Y1 receptors are preferably coupled to the p70 S6K pathway, whereas P2Y2/4 and P2Y6 receptors are preferably coupled to the Akt and ERK1/2 pathways. P2X7 receptors have relatively more contribution to the activation of PI3K/Akt pathway than to the activation of ERK1/2 and p70 S6K pathways. The involvement of other P2X receptor subtypes in the activation of ERK1/2 pathway cannot be excluded.

**DISCUSSION**

Extracellular nucleotides are released in response to me-
chanical forces by cells of the vasculature, airways and gut, and

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**FIG. 7. PI3K and mTOR are involved in ATP-induced p70 S6K phosphorylation.** Growth-arrested adventitial fibroblasts were preincubated either with LY294002 (20 μM, 60 min), wortmannin (100 nM, 2 h), rapamycin (5 nM, 60 min), UO126 (10 μM, 60 min), or a corresponding vehicle and then stimulated with either ATP (100 μM, 30 min) or fetal bovine serum (FBS) (100%, 30 min). A, Western blot analysis of phospho-p70 S6K (Thr-389 and Thr-421/Ser-424) in total cell lysates prepared from unstimulated cells (cont) and cells exposed to ATP or 10% FBS (ATP, FBS). Asterisks indicate p5856K, which is recognized by anti-phospho-p70 S6K (Thr-386) antibodies. Results of representative Western blots from one of at least three experiments are shown. B–D, quantitative data of Western blot analyses from experiments performed as described above. Phospho-p70 S6K was normalized to total p70 S6K, and values are expressed as % of basal level. Data represent means ± S.E. (S.E.) from three to six independent experiments. B shows effect of inhibitors on unstimulated growth-arrested cells (Cont). C shows the effect of inhibitors on ATP-induced phospho-p70 S6K in cells pretreated with inhibitors of PI3K, mTOR, and MEK1/2. D shows the effect of the same inhibitors on the FBS-induced phospho-p70 S6K. WT, wortmannin; Rapa, rapamycin.

**FIG. 8. PI3K and mTOR are involved in ATP-induced phosphorylation of S6 ribosomal protein.** Growth-arrested adventitial fibroblasts were preincubated either with LY294002 (20 μM, 60 min), rapamycin (5 nM, 60 min), or the corresponding vehicle and then stimulated either with 100 μM ATP or 10% fetal bovine serum for 30 min. 20 μg of total cell lysates were analyzed by Western blot analysis with anti-phospho-S6 Ser-235/Ser-236 antibodies (upper panel). Phospho-S6 was normalized to total S6, and quantitative data of three independent are expressed as % of basal level (lower panel). Data represent means ± S.E. of three independent experiments. Cont, control.
FIG. 9. Effect of Akt inhibitors and altered Akt expression on mTOR and p70 S6K phosphorylation. A and B, growth-arrested adventitial fibroblasts were treated with SH-5, SH-6 (20 μM, 3 h), NI-71-101 (10 μM, 6 h), or Me2SO (1:1000, 3 or 6 h), then stimulated with ATP (100 μM, 10 min), and harvested, and total cell lysates were subjected to Western blot analyses with phospho-mTOR (Ser-2448) or phospho-p70 S6K (Thr-389 and Thr-421/Ser-424) antibodies. Representative Western blots from one experiment are shown on the left. Phospho-mTOR and phospho-p70 S6K were normalized to total mTOR and total p70 S6K, respectively, and quantitative data are expressed as % of basal level. Quantitative data shown on the right represent means ± S.E. from three to five independent experiments.

C and D, adventitial fibroblasts were grown to 50–60% confluence, growth-arrested for 72 h in DMEM without serum, and transduced for 16 h with adeno-GFP, adeno-dnAkt, or adeno-myr-Akt (multiplicity of infection 125). Nontransduced cells served as a negative control. Media were replaced with fresh DMEM, and after 2–4 h cells were stimulated with ATP (100 μM, 30 min). Western blot analyses were performed with 25–40 μg of total cell protein by using anti-phospho-mTOR (Ser-2448), anti-phospho-p70 S6K (Thr-389 and Thr-421/Ser-424), anti-hemagglutinin (HA), or anti-Akt antibodies. Bands marked by asterisks correspond to p85S6K. Representative Western blots from one experiment are shown on the left. Quantitative data, expressed as % of increase of phospho-mTOR or phospho-p70 S6K of basal level, are shown on the graphs on the right. Data represent means ± S.E. (S.E.) from three to five independent experiments.
Role of PI3K and mTOR in ATP-induced Fibroblast Proliferation

The effect of various nucleotides and nucleotide analogs on phosphorylation of Akt, ERK1/2, and p70 S6K was evaluated by Western blot analysis as described under "Experimental Procedures." Adventitial fibroblasts were stimulated for either 7.5, 10, or 30 min for the detection of phospho-Akt, phospho-ERK1/2, and phospho-p70S6K, respectively. Data represent average ± S.E. from three to six independent experiments.

| Agonist (100 μM) | pAkt (Ser-473) | pERK1/2 (Thr-202/Tyr-204) | p70 S6K (Thr-421/Ser-424) |
|------------------|----------------|--------------------------|------------------------|
| Basal            | 100            | 100                      | 100                    |
| ATP              | 312.86 ± 67.59a| 1062.62 ± 119.89a        | 699.25 ± 83.64a        |
| UTP              | 277 ± 10.5a    | 778.67 ± 131.56a         | 932.25 ± 103.78a       |
| UDP              | 394.00 ± 81.63a| 1046.40 ± 218.93a        | 611.12 ± 74.62a        |
| ADPβS            | 293.25 ± 53.51b| 1264 ± 244.50a           | 977.00 ± 79.75a        |
| ADP              | 330 ± 37.27a   | 1314.50 ± 106.36a        | 324.55 ± 38.84a        |
| ATP,γ            | 352.40 ± 43.58a| 1334.80 ± 107.96a        | 335.12 ± 26.97a        |
| αβme-ATP         | 159.14 ± 37.35a| 347.43 ± 74.34a          | 90.75 ± 7.44           |
| βγme-ATP         | 184.50 ± 47.01 | 269.83 ± 63.23a          | 97.65 ± 11.81          |
| 2MeS-ATP         | 204.75 ± 40.97b| 695.83 ± 96.01a          | 219.00 ± 19.15a        |
| Bz-ATP           | 276.00 ± 75.65a| 591.29 ± 192.04a         | 425.34 ± 41.98a        |
| Ap4A             | 191.00 ± 29.21a| 349.00 ± 96.59a          | 89.00 ± 3.32           |

Suggested receptor subtypes:
- P2Y6,2/4,1P2X7
- P2Y2/4,6,1,2P2X7
- P2Y1,2,6,P2X7

<sup>a</sup> p < 0.01 compared with nonstimulated control (basal). Based on phosphorylation profiles, the receptor subtypes mediating the responses for each kinase is indicated in the bottom row (as suggested receptor subtypes).

<sup>b</sup> p < 0.05.

<sup>c</sup> P2Y receptor subtypes are listed in relative order of contribution in the activation of each kinase.

<sup>d</sup> Potential involvement of other P2X receptor subtypes

A role for the ATP receptor subtype P2Y1 in ATP-induced proliferation was examined (19). The ATP receptor was used as a positive control, and the data were compared with those from PI3K and Akt, ERK1/2, and p70 S6K. Our results demonstrate that extracellular ATP acting through P2 purinergic receptors initiates PI3K- and mTOR-dependent signaling pathways that ultimately converge at the level of p70 S6K. We show that ATP activates PI3K, which includes a role for the βγ subunits of heterotrimeric G proteins in this response. This signaling cascade activates Akt, which lies downstream of PI3K and G1 and participates in the proliferative response. In addition, mTOR and p70 S6K are activated in response to ATP and play a key role in ATP-induced proliferation. However, inputs other than those from PI3K can participate in the activation of mTOR and p70 S6K (Fig. 10). We believe that elucidation of these critical signaling pathways sheds some light on the mechanisms of ATP-induced proliferation in adventitial fibroblasts. The findings may be relevant to those pathologic conditions where ATP-induced proliferation is observed, and that ATP-induced proliferation is consistent with the previous observations of Wilden et al. (9) who demonstrated an inhibitory effect of LY294002 on ATP-stimulated DNA synthesis in rat coronary artery smooth muscle cells. It differs slightly from the report of Huiwiler et al. (38) who showed that PI3K and Akt are activated in response to ATP in renal mesangial cells but do not participate in cell proliferation. However, until now there has not been any direct evidence presented to show that ATP stimulates PI3K activity. It is known that distinct classes of PI3Ks can be selectively involved in the response to receptor tyrosine kinases and G protein-coupled receptor activation by PI3K and Akt, ERK1/2, and p70 S6K.
critical in mediating mitogen-induced growth responses. In many cell systems and mitogenic signaling pathways, ERK1/2 lies downstream of PI3K (17, 41, 42, 50). In other cell systems ERK1/2 and PI3K act independently in parallel to promote cell growth (8, 9, 19). The latter situation appears to be the case for ATP-stimulated adventitial fibroblast proliferation. We found the PI3K inhibitors wortmannin and LY294002 had no significant effect on ATP-stimulated ERK1/2 phosphorylation, yet nearly completely attenuated ATP-induced DNA synthesis. Thus, extracellular ATP appears to induce parallel activation of two pathways, both of which are necessary for ATP-induced proliferation. It seems likely that PI3K activation will have other important effects on cell function that will need to be elucidated.

Akt has been identified as one downstream target of PI3K in growth-related and survival signaling cascades (33). We therefore tested the possibility that Akt acts as a downstream target of PI3K, which is important in mediating ATP-stimulated proliferation of adventitial fibroblasts. Our data suggest that ATP rapidly, but transiently, phosphorylates Akt at both Ser-473 and Thr-308 residues, sites both known to be critical for maximal kinase activation (Fig. 4). This early response (with the peak at 7.5 min) is similar to the ATP- and UTP-induced increases in Akt phosphorylation observed in renal mesangial cells (38), chemotaxant-stimulated neutrophils (51), and µ-opioid receptor-stimulated HEK293 cells (52). The early time course of ATP-induced Akt phosphorylation and inhibition with pertussis toxin is consistent with the idea of the direct regulatory role of G proteins in this process. Although the precise roles of individual Go and Gβγ subunits are not completely understood, the possible mechanisms include a direct interaction of Gβγ with the Akt pleckstrin homology domain and with β-arrestin (53, 54). For example, Goel and Baldassare (55) demonstrated the regulatory role of β-arrestin in thombin-mediated Akt activation. By using pharmacologic and genetic approaches, we show Akt is activated by ATP through G protein- and PI3K-dependent mechanisms and is directly involved in proliferative responses.

The dramatic effect of rapamycin on ATP-stimulated DNA synthesis points out the importance of the mTOR pathway in adventitial fibroblast proliferation. The involvement of the protein translation pathway is not surprising because cell growth and cycle progression require an increase in protein synthesis. The association of mTOR and p70 S6K activation with the proliferative response has been reported in many but not all cell types. Many studies have demonstrated mTOR and p70 S6K activation in growth factor-initiated tyrosine kinase-dependent signaling pathways, whereas only a few reports have shown an activation of this pathway through G protein-coupled receptors such as carbachol (56), thrombin (28), and lysophosphatidic acid (29). Our data demonstrating the ability of extracellular ATP to activate mTOR and its downstream targets, p70 S6K and S6 ribosomal protein, provide the first evidence that protein translation can be under extracellular purinergic control. Furthermore, we demonstrate that both mTOR and PI3K are involved in the regulation of p70 S6K and ribosomal S6 protein. Most importantly, ATP-induced increases in p70 S6K and S6 phosphorylation ranged between 5- and 8-fold (Figs. 5–8) which is comparable in magnitude to the effects of 10% fetal bovine serum, used in our experiments as a positive control to evaluate the maximal stimulatory effect. Intriguing questions remain regarding the sensitivity of mTOR and p70 S6K in response to ATP stimulation, as well as questions regarding how PI3K and ERK1/2 are involved in regulating mTOR and p70 S6K activity (Fig. 10).

There is evidence in some cell systems to support a role for Akt in the regulation of the mTOR pathway. An important finding of our study is that ATP induces mTOR phosphorylation at Ser-2448, which is known as a site for Akt-dependent phosphorylation (32, 44), suggesting a signaling cross-talk between the Akt and mTOR pathways. In support of this idea, we demonstrated that transduction of adventitial fibroblasts with activated Akt resulted in an increase in mTOR and p70 S6K phosphorylation, whereas transduction with a dominant-negative mutant Akt or treatment with the putative Akt inhibitors SH-5, SH-6, and NL-71–101 had the opposite effect (Fig. 9). These data clearly demonstrate that in adventitial fibroblasts PI3K and Akt, at least in part, mediate ATP-induced activation of mTOR and p70 S6K. However, the potential involvement of cAMP-dependent protein kinase cannot be ruled out as the IC50 of NL-71-101 for Akt and cAMP-dependent protein kinase are in the same concentration range (37). Consistent with our observations, wortmannin-sensitive platelet-derived growth factor- and lysophosphatidic acid-induced activation of p70 S6K has been shown in Rat-1 fibroblasts (29) as well as in other cell systems (32, 44, 57). In contrast, there are also data to indicate that PI3K/ Akt and mTOR/p70 S6K can act as independent signaling pathways. For example, Harai et al. (58) showed that overexpression of the dominant-negative p85 subunit of PI3K in Chinese hamster ovary cells had no effect on insulin-induced activation of p70 S6K. Similarly, PI3K-independent p70 S6K activation was observed in platelet-derived growth factor-stimulated pulmonary artery fibroblasts, glucose-induced mitogenesis in pancreatic β-cells, and phenylephrine-stimulated p70 S6K in Rat-1 fibroblasts (28, 59, 60). Of note in our study is that ATP-induced p70 S6K phosphorylation at Thr-421/Ser-424 and Thr-389 residues revealed different sensitivities to the PI3K inhibitors LY294002 and wortmannin. The failure of wortmannin to block p70 S6K phosphorylation at Thr-421/Ser-424 may indicate that ATP-induced regulation of p70 S6K occurs in part through an as yet unidentified PI3K-independent signaling pathway. Most interestingly, a PI3K-independent pathway of mTOR regulation has been demonstrated for amino acids and some mitogens (61).

We observed that in adventitial fibroblasts extracellular ATP exerts more potent effects on p70 S6K activation than it does on PI3K and Akt (compare percent over the basal level in Figs. 2, 4, and 7). This observation led us to believe that the sensitivity of the mTOR and p70 S6K pathways to purinergic regulation exceeds the sensitivity of the PI3K/Akt pathway. This may imply the contribution of an additional regulatory pathway(s) that provides complementary signaling inputs into p70 S6K. Again, this possibility is supported by the observations that products of phospholipase D activity, such as phosphatidic acid and choline phosphate, are involved in up-regulation of the mTOR/p70 S6K pathway (62, 63). In addition, some studies have demonstrated the involvement of protein kinase Cζ and phosphoinositide-dependent kinase in the regulation of p70 S6K (56, 64–66). Unexpectedly, we found that the MEK1/2 inhibitor UO126 attenuated ATP-induced phosphorylation of p70 S6K at Thr-421/Ser-424 by up to 70%, implying an involvement of MEK1/2/ERK1/2 pathways in p70 S6K regulation (Fig. 7). These observations suggest that regulation of the mTOR/p70 S6K signaling pathways may include the combination of both PI3K/Akt-dependent and PI3K/Akt-independent signaling pathways (27) (Fig. 10). It is likely that some additional pathways contribute to p70 S6K regulation in a cell type and stimulus-dependent manner.

Inoki et al. (46) have recently suggested that AMP-activated protein kinase is a physiological cellular energy sensor, which senses small changes in ratios of ATP, ADP, and AMP concentrations. In response to energy starvation, AMP-activated pro-
Role of PI3K and mTOR in ATP-induced Fibroblast Proliferation

In this study, we investigate the role of PI3K and mTOR in ATP-induced fibroblast proliferation. Our results indicate that PI3K and mTOR are crucial for ATP-induced proliferation, as evidenced by the inhibition of proliferation upon inhibition of these pathways.

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Extracellular ATP-induced Proliferation of Adventitial Fibroblasts Requires Phosphoinositide 3-Kinase, Akt, Mammalian Target of Rapamycin, and p70 S6 Kinase Signaling Pathways

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