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The Role of p70^{S6K} in Hepatic Stellate Cell Collagen Gene Expression and Cell Proliferation*

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During fibrosis the hepatic stellate cell (HSC) undergoes a complex activation process characterized by increased proliferation and extracellular matrix deposition. The 70-kDa ribosomal S6 kinase (p70^{S6K}) is activated by mitogens, growth factors, and hormones in a phosphatidylinositol 3-kinase-dependent manner. p70^{S6K} regulates protein synthesis, proliferation, and cell cycle control. Because these processes are involved in HSC activation, we investigated the role of p70^{S6K} in HSC proliferation, cell cycle control, and type I collagen expression. Platelet-derived growth factor (PDGF) stimulated p70^{S6K} phosphorylation, which was blocked by LY294002, an inhibitor of phosphatidylinositol 3-kinase. Rapamycin blocked phosphorylation of p70^{S6K} but had no affect on PDGF-induced Akt phosphorylation, positioning p70^{S6K} downstream of Akt. Transforming growth factor-β, which inhibits HSC proliferation, did not affect PDGF-induced p70^{S6K} phosphorylation. Rapamycin treatment did not affect α1(I) collagen mRNA but reduced type I collagen protein secretion. Expression of smooth muscle α-actin was not affected by rapamycin treatment, indicating that HSC activation was not altered. Rapamycin inhibited serum-induced DNA synthesis ~2-fold. Moreover, rapamycin decreased expression of cyclins D1, D3, and E but not cyclin D2, Rb-Ser^{780}, and Rb-Ser^{795}. Together, p70^{S6K} plays a crucial role in HSC proliferation, collagen expression, and cell cycle control, thus representing a potential therapeutic target for liver fibrosis.

Hepatic fibrosis is a pathologic response of the liver to acute and chronic insults such as ethanol, viral infection, cholestasis, and metabolic diseases (1). Hepatic stellate cells (HSCs)[1] play a crucial role in liver fibrosis, as they are responsible for excessive deposition of extracellular matrix proteins, of which type I collagen predominates (2). After a fibrogenic stimulus, HSCs transform from a quiescent vitamin A storing cell to an activated myofibroblast-like cell (2). Morphological changes associated with HSC activation include a loss of vitamin A stores and appearance of the cytoskeletal protein smooth muscle α-actin (α-SMA) (3, 4). Two major events occur after HSC activation that substantially contribute to their active role in liver fibrosis. First, they are the primary cell type responsible for increased synthesis and deposition of extracellular matrix proteins in the liver (5). Second, activated HSCs proliferate thereby effectively increasing the population of fibrogenic cells and amplifying the fibrotic response (6).

The most potent mitogenic factor for HSCs is platelet-derived growth factor (PDGF) (7). Multiple signaling pathways are implicated in HSC proliferation. Activation of Ras due to PDGF is followed by sequential activation of Raf, MEK, and extracellular signal-regulated kinase (8). Blocking extracellular signal-regulated kinase activity after PDGF stimulation inhibits HSC proliferation (9). Stress-activated kinases also regulate HSC proliferation. Inhibition of JNK in quiescent or culture-activated HSCs prevented increases in the cell population; however, inhibition of p38 increased HSC proliferation (10).

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is also activated after PDGF treatment of HSCs (11, 12). After PDGF stimulation PI3K, a heterodimeric protein composed of an 85-kDa regulatory and 110-kDa catalytic subunit associates with the PDGF receptor and becomes activated by phosphorylation. PI3K activation results in the generation of phosphorylated inositol lipids, essential second messengers for intracellular signaling (13). Phosphorylated inositol lipid bind to Akt, a downstream target in the PI3K pathway, and induce its translocation to the plasma membrane (14). There Akt becomes activated by phosphorylation at residues Thr^{308} and Ser^{473} by phosphoinositide-dependent kinase 1 (14, 15). A role of PI3K in HSC proliferation has been confirmed since inhibition of PI3K by LY294002 or wortmannin blocks HSC proliferation (12, 16, 17). We have shown that serum or PDGF activates Akt in HSCs by phosphorylating Ser^{473}. Inhibition of PI3K blocks this activity. In addition, inhibiting both PI3K and Akt blocks HSC proliferation and type I collagen synthesis (17).

Another downstream target in the PI3K pathway, via Akt, is p70 S6 kinase (p70^{S6K}), a ribosomal 70-kDa protein that is activated by mitogens, growth factors, and several hormones (18). Furthermore, it is a key regulator of mRNA translation and protein synthesis and is required for cell cycle progression,

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[1] The abbreviations used are: HSC, hepatic stellate cell; α-SMA, smooth muscle α-actin; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; TNF, tumor necrosis factor; FACS, fluorescent-activated cell sorting; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; p70^{S6K}, p70 S6 kinase; Rb, retinoblastoma.

TABLE I
HRP, horseradish peroxidase.

Primary antibody	Dilution[a]/Incubation	Secondary antibody	Dilution[a]/Incubation
Polyclonal rabbit anti-human phospho-p70^{S6} kinase (Thr^{421}/Ser^{424}) (Upstate Biotechnology, Lake Placid, NY)	1:1500/1 h	HRP-conjugated anti-rabbit IgG (Santa Cruz, Santa Cruz, CA)	1:2500/30 min
Polyclonal rabbit anti-p70^{S6} kinase (Upstate Biotechnology)	1:1000/1 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:1000/30 min
Polyclonal rabbit anti-mouse phospho-AKT (Ser^{473}) (Cell Signaling Technology, Beverly, MA)	1:1000 in 5% bovine serum albumin /12 h	HRP-conjugated anti-mouse IgG (Santa Cruz)	1:2000 in 5% bovine serum albumin/60 min
Polyclonal rabbit anti-IκBα (Santa Cruz)	1:1000/1 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:1000/1 h
Monoclonal rabbit anti-rat collagen Type I (Biodesign, Saco, ME)	1:1000/1 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:2000/1 h
Mouse anti-human smooth muscle α-actin (Dako)	1:1000/1 h	HRP-conjugated anti-mouse IgG (Santa Cruz)	1:1000/30 min
Mouse monoclonal IgG antibody against anti-human cyclin D1 (Santa Cruz)	1:1000/1 h	HRP-conjugated anti-mouse IgG (Santa Cruz)	1:1000/1 h
Rabbit polyclonal anti-human cyclin D2 (Santa Cruz)	1:400/10 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:400/1 h
Rabbit polyclonal anti-human cyclin D3 (Santa Cruz)	1:300/1 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:1000/1 h
Polyclonal rabbit anti-human cyclin E (Upstate Biotechnology)	1:1000/6 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:1000/1 h
Rabbit polyclonal anti-human phospho-Rb Ser^{780} (Cell Signaling)	1:1000/2 h	HRP-conjugated anti-rabbit IgG (Santa Cruz)	1:1000/1 h
Rabbit polyclonal anti-human phospho-Rb Ser 795 (Cell Signaling)	1:1000/2 h	HRP-conjugated anti-rabbit IgG (Santa Cruz, Santa Cruz, CA)	1:1000/1 h

[a] All antibodies were diluted in 5% nonfat dry milk TBS-Tween solution unless otherwise stated.

cell differentiation, and cell growth. Activation of p70^{S6K} occurs through a complex series of phosphorylation events on several serine or threonine residues (19). Phosphorylation of these sites is inhibited by wortmannin, LY294002, rapamycin, and by amino acid deprivation (20–22). Rapamycin, a lipophilic bacterial macrolide with immunosuppressive properties, inhibits proliferation of yeast and several mammalian cell types including B and T lymphocytes (8, 23). In HSCs, rapamycin effectively blocked insulin-like growth factor-induced DNA synthesis (24). Furthermore, rapamycin blocked extracellular matrix deposition in CCl_4-induced liver fibrosis (25). Yet, the molecular mechanism has not been clarified. Here we report that rapamycin treatment of HSCs inhibits phosphorylation of p70^{S6K}, leading to an inhibition of type I collagen synthesis, DNA synthesis, and cell cycle progression.

EXPERIMENTAL PROCEDURES

HSC Isolation and Culture—HSCs were purified from male Sprague-Dawley retired breeder rats (>400 g) as previously described (26). Isolated HSCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), standard antibiotics, and 2 mM L-glutamine in a 95% air, 5% CO_2-humidified atmosphere at 37 °C. Growth medium was exchanged every other day. HSCs were activated by culturing on plastic for 10–14 days. The purity of culture-activated HSCs was assessed by immunostaining cells using mouse anti-human smooth muscle α-actin (Dako, Carpinteria, CA). All animal procedures were performed under the guidelines set by the University of North Carolina Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Experimental Protocol—After an activation period of 10–14 days in culture, HSCs were cultured for 48 h in Dulbecco's modified Eagle's medium without FBS supplementation to synchronize the cells. After synchronization, cells were treated with 25 μM LY294002 (Calbiochem), 10 nM rapamycin (Sigma), or the vehicle Me_2SO (Sigma). After 1 h, HSCs were treated with 10% FBS or 20 ng/ml PDGF-B/B (Roche Applied Science). In some of the experiments HSCs were stimulated with 10 ng/ml TNFα (R&D Systems, Minneapolis, MN) or 5 ng/ml TGFβ (R&D Systems).

Western Blot Analysis—Cultured HSCs were washed with phosphate-buffered saline, and the cells were lysed using protein sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol). Protein concentrations were measured by the Bradford method (Bio-Rad). Protein samples were heated at 95 °C for 5 min, and 20 μg was applied to a 10% SDS-polyacrylamide gel (7.5% SDS-polyacrylamide gels were used for collagen analysis). After electrophoresis the proteins were electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were stained with 0.5% Ponceau S to assure equal protein loading. Membranes were blocked for 1 h with 5% powdered nonfat dry milk in TBS-T (25 mM Tris-HCl, pH 8.0, 144 mM NaCl, 0.1% Tween 20). Afterward, membranes were incubated with primary and secondary antibodies, each diluted in TBS-T unless specified otherwise according to Table I. After incubation with the secondary antibodies, the membranes were briefly washed twice and then 3 times for 10 min each with TBS-T. Immunodetected proteins were visualized using the enhanced chemiluminescent ECL assay kit (Amersham Biosciences) according to the manufacturer's recommended protocol.

Analysis of DNA Synthesis—DNA synthesis was assessed by [^{3}H]thymidine incorporation essentially as previously described (17). Culture-activated HSCs (7–10 days in culture) were seeded at a density of 2×10^4 cells/well in 24-well plates in growth medium containing 10% FBS. After 24 h the medium was changed to 0.2% FBS, and the cells were incubated for another 24 h to reduce cell proliferation. One hour before the proliferative trigger cells were treated with 10 nM rapamycin (Sigma). Afterward, the cells were incubated in medium containing either 0.2 or 10% FBS for 48 h with 1 μCi/ml [^{3}H]thymidine present during the last 24 h. At the end of the incubation 10% trichloroacetic acid was added, and the cells were maintained on ice for 15 min. Cells were washed twice with cold 10% trichloroacetic acid and solubilized in 0.2 N NaOH, and radioactivity was measured using a scintillation counter. Experiments were performed in quadruplicate.

RNase Protection Assay—RNase protection assays were performed as described previously (27). Radiolabeled probes were prepared for rat α1(I) collagen (27) and glyceraldehyde-3-phosphate dehydrogenase (pTRI-GAPDH-Rat, Ambion; Austin, TX) and mixed with 5 μg of total RNA. Protected fragments were analyzed using standard 6% sequencing gels. After electrophoresis, bands were visualized by autoradiogra-

phy, and signals were quantitated by PhosphorImager analysis (Amersham Biosciences).

Fluorescent-activated Cell Sorting (FACS) Analysis—Rat HSCs were cultured for 10 days then serum-starved for an additional 48 h. Cells were subsequently treated with 25 μM LY294002, 10 nM rapamycin, or Me_2SO as a vehicle control for 1 h. Afterward, 20 ng/ml PDGF-BB was added, and the cells were incubated for 24 h. For the 48-h time point, the medium was changed after 24 h, and fresh LY294002, rapamycin, and PDGF-BB were added. Cells were harvested by scraping, washed in phosphate-buffered saline, then incubated in 2 mM EDTA in phosphate-buffered saline for 20 min, washed twice with phosphate-buffered saline, and treated with 100 μg/ml RNase for 5 min at room temperature. Cells were incubated with 50 μg/ml propidium iodide at room temperature in the dark for 30 min. Cell cycle state was assessed by flow cytometry using a FACScan instrument (BD Biosciences).

Type I Collagen Protein Expression—Proteins from culture media were precipitated with the addition of ammonium sulfate to a final concentration of 40% at 4 °C. Samples were centrifuged at 10,000 $\times$ *g* at 4 °C for 30 min, and the pellet was suspended in 0.5 M acetic acid. Aliquots of the protein sample were digested with 130 units of pepsin (Sigma), neutralized with 1.5 M Tris-HCl, pH 8.8, and digested with 8 units of collagenase (Sigma) at room temperature for 30 min. Samples were applied on a 7.5% polyacrylamide gel, and Western blot analysis was performed for type I collagen as described above.

Statistical Analysis—Student's *t* test was used for determination of statistical significance as appropriate. Statistical values of $p \leq 0.05$ were considered to be significant before analysis. Data are presented as the means ± S.E.

RESULTS

LY294002 Treatment Inhibits PDGF-induced p70^{S6K} Phosphorylation—To determine whether p70^{S6K} is involved in the PI3K pathway in HSCs, activated HSCs were serum-starved for 48 h and then stimulated for 10, 20, 30, and 60 min with 20 ng/ml PDGF in the presence or absence of 25 μM LY294002, a specific PI3K inhibitor. Western blot analysis was performed to evaluate the effect of LY294002 on phosphorylation of p70^{S6K}. As shown in Fig. 1, phosphorylated p70^{S6K} is weakly detectable before stimulation with PDGF. However, stimulation of HSCs with 20 ng/ml PDGF (Fig. 1) induced phosphorylation of p70^{S6K} at Thr^{421}/Ser^{424} after 10, 20, 30, and 60 min of treatment, whereas LY294002 completely blocked phosphorylation of p70^{S6K} at all time points. These data demonstrate that PDGF (and serum, data not shown) stimulate phosphorylation of p70^{S6K} and that PDGF-induced activation of p70^{S6K} is inhibited by LY treatment. In addition, our data show that p70^{S6K} is located downstream of PI3K in PDGF-induced cell signaling in HSCs.

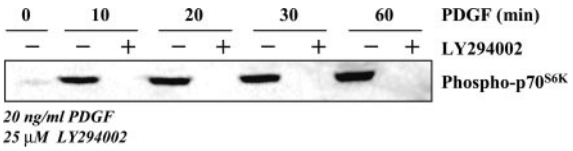

FIG. 1. **LY204002 inhibits PDGF-BB-induced phosphorylation of p70^{S6K}.** Rat HSCs were cultured for 10–14 days then serum-starved in medium containing 0% serum for 48 h. HSCs were either left untreated as a control, preincubated with Me_2SO, or treated with 25 μM LY294002 for 1 h before stimulation 20 ng/ml PDGF for the indicated time periods. Cellular proteins (20 μg) were analyzed by Western blot analysis under reducing conditions after 0, 10, 20, 30, and 60 min of stimulation. The blots were probed for phosphorylated p70^{S6K} (Thr^{421}/Ser^{424}). Equal sample loading was assessed by Ponceau Red staining of the membrane after protein transfer.

Rapamycin Blocks PDGF-induced Phosphorylation of p70^{S6K} but Not Phosphorylation of Akt—Rapamycin has been shown to inhibit activation of p70^{S6K} in several cell types (19, 21). Akt, a downstream target in the PI3K pathway, is activated by PI3K, resulting in phosphorylation of residues Thr^{308} and Ser^{473} within the activation loop of Akt (14, 15, 28). In HSCs we have previously shown that serum and PDGF both activate Akt by phosphorylation of Ser^{473}, which is inhibited by treatment with LY294002 (17). To assess the effect of rapamycin on Akt and p70^{S6K} phosphorylation in HSCs, culture-activated HSCs were serum-starved for 48 h followed by treatment with 10 nM rapamycin for 1 h. Cells were subsequently stimulated with 20 ng/ml PDGF. Western blot analysis demonstrated that 20 ng/ml PDGF resulted in the phosphorylation of Akt (Ser^{473}) throughout the 1-h time period (Fig. 2*A*). Expression of phosphorylated Akt was highest within 10 min after PDGF treatment and decreased slightly over the experimental period. No difference was found in the expression of phospho-Akt after rapamycin treatment compared with Me_2SO control-treated cells. Phosphorylated p70^{S6K} at Thr^{421}/Ser^{424} was not detected before PDGF treatment (Fig. 2*B*); however, after 10 min of PDGF treatment an induction of p70^{S6K} phosphorylation was observed that persisted up to 60 min after stimulation. At all time points, rapamycin completely blocked p70^{S6K} phosphorylation. These data show that PDGF stimulates phosphorylation of p70^{S6K} in HSCs and that p70^{S6K} is positioned downstream of Akt in PDGF-induced signaling in the HSC.

TGFβ Does Not Change Phosphorylation of p70^{S6K}—TGFβ is one of the most important pro-fibrogenic cytokines for the HSC (2). It has also been shown that TGFβ inhibits HSC proliferation (29, 30); however, the mechanism is unknown. Because we have previously shown that the focal adhesion kinase (FAK)-PI3K-Akt-signaling pathway transduces proliferative signaling in the HSC, we wanted to determine whether TGFβ inhibits FAK-PI3K-Akt signaling, leading to an inhibition of p70^{S6K} activation. We previously showed that TGFβ fails to inhibit PDGF-induced Akt phosphorylation (17). HSCs were treated with 5 ng/ml TGFβ alone 1 h before a 20 ng/ml PDGF stimulation simultaneously with a 20 ng/ml PDGF stimulation or 10 min after a 20 ng/ml PDGF stimulation, and the cells were harvested and assessed for p70^{S6K} phosphorylation. TGFβ alone did not induce p70^{S6K} phosphorylation (Fig. 3, *second*

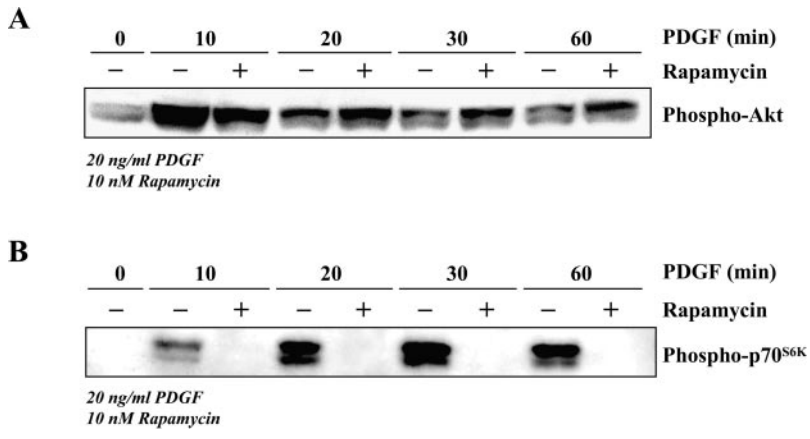

FIG. 2. **Rapamycin prevents phosphorylation of p70^{S6K} but not the protein kinase Akt.** Rat HSCs were cultured for 10–14 days and then serum-starved for 48 h. HSCs were either left untreated as a control, preincubated with Me_2SO, or treated with 10 nM rapamycin for 1 h before stimulation with 20 ng/ml PDGF. Cellular proteins (20 μg) were harvested after stimulation periods of 0, 10, 20, 30, and 60 min, separated on a 10% SDS-PAGE, and transferred on a nitrocellulose membrane. Western blot analysis was performed using anti-phospho-Akt (Ser^{473}) (*A*) and anti-phospho-p70^{S6K} (Thr^{421}/Ser^{424}) (*B*) antibodies. Equal sample loading was assessed by Ponceau Red staining of the membrane after protein transfer.

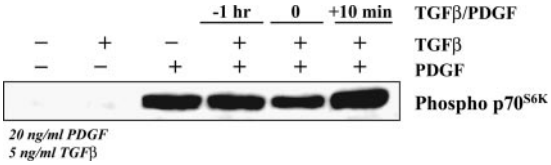

FIG. 3. **TGFβ alone or in combination with PDGF does not alter phosphorylation of p70^{S6K}.** HSCs were cultured for 10–14 days after isolation, serum-starved for 48 h, then left untreated (*first lane*), treated with 5 ng/ml TGFβ (*second lane*), or treated with 20 ng/ml PDGF (*third lane*), TGFβ 1 h before PDGF stimulation (*fourth lane*) simultaneously with TGFβ and PDGF (*fifth lane*), and TGFβ 10 min after PDGF stimulation (*sixth lane*). Cellular proteins (20 μg) were analyzed by Western blot under reducing conditions after 0, 10, 20, 30, and 60 min of stimulation. The blots were probed against phosphorylated p70^{S6K} (Thr^{421}/Ser^{424}). Equal sample loading was assessed by Ponceau Red staining of the membrane after protein transfer.

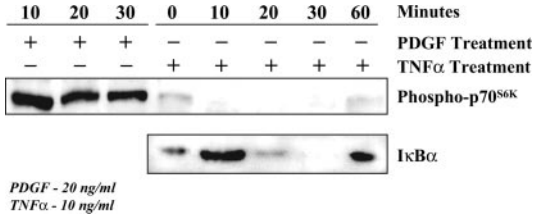

FIG. 4. **TNFα does not phosphorylate p70^{S6K} in HSCs.** HSCs were cultured and serum-starved under the same conditions as previously mentioned. As a control HSCs were treated for 10, 20, and 30 min with 20 ng/ml PDGF (*first through third lanes*). HSCs were either left untreated (*fourth lane*) or treated with 10 ng/ml TNFα for 10, 20, 30 or 60 min (*fifth through eighth lanes*). Whole cellular proteins (20 μg) were separated on a 10% SDS-PAGE, then transferred to nitrocellulose membranes. Immunoblots were probed with polyclonal anti-phospho-S6K (Thr^{421}/Ser^{424}). The same blot was stripped and re-probed with polyclonal anti-IκBα.

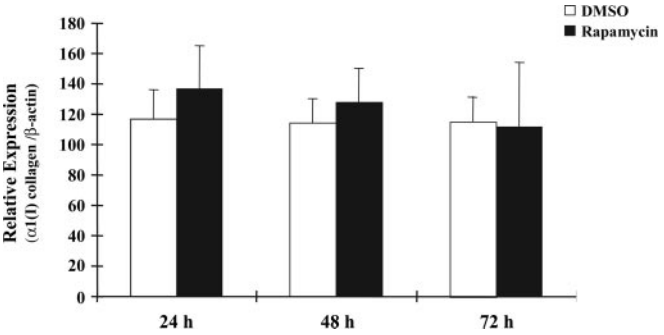

FIG. 5. **Rapamycin does not inhibit α1(I) collagen mRNA expression in activated rat HSCs.** RNase protection assays were performed as described under "Experimental Procedures" with total RNA extracted from activated HSCs, treated once a day with the Me_2SO or 10 nM rapamycin for 24, 48, or 72 h. Expression of α1(I) collagen mRNA was normalized to expression of β-actin for Me_2SO (*DMSO*) vehicle-treated HSCs. Expression in the presence of rapamycin is shown relative to that of Me_2SO-treated cells. Data represent means ± S.E.

lane from left); however, as demonstrated above, PDGF stimulation induced p70^{S6K} phosphorylation (Fig. 3, *third lane*). The addition of TGFβ 1 h before (Fig. 3, *fourth lane*), at the same time (Fig. 3, *fifth lane*), or 10 min after stimulation with PDGF (Fig. 3, *sixth lane*) did not alter p70^{S6K} phosphorylation. These data indicate that TGFβ does not inhibit HSC proliferation via the p70^{S6K} signaling pathway in HSCs.

TNFα Does Not Induce Phosphorylation of p70^{S6K} in HSCs—TNFα has been reported to activate PI3K signaling in several cell types (31). Therefore, we wanted to evaluate the effect of TNFα on p70^{S6K} activation in activated HSCs. HSCs were serum-starved in 0% serum for 48 h then treated with 10 ng/ml TNFα for 10, 20, 30, and 60 min or 20 ng/ml PDGF for 10, 20, and 30 min, which served as a control for p70^{S6K} phosphorylation. As expected, PDGF lead to phosphorylation of p70^{S6K} at all time points (Fig. 4, *first through third lanes*). In contrast, phosphorylated p70^{S6K} was not detected after TNFα stimulation (Fig. 4, *fifth through eighth lanes*). Degradation of IκBα served as a control for TNFα activity. IκBα protein was detected at 0 and 10 min after TNFα stimulation; however, after 20 and 30 min following TNFα treatment, degradation of IκBα was noted. IκBα expression was again detected after 60 min of TNFα treatment. This transient degradation of IκBα is a typical response to TNFα treatment previously shown in HSCs (32). Together, these results demonstrate that TNFα does not stimulate p70^{S6K} activity in activated HSCs.

Inhibiting p70^{S6K} Kinase Does Not Attenuate α1(I) Collagen mRNA Expression but Does Decrease Type I Collagen Protein Expression—Because HSCs are the major source of collagen during hepatic fibrogenesis, we investigated the influence of p70^{S6K} on α1(I) collagen mRNA and type I collagen protein levels. We have previously shown that inhibition of PI3K decreases α1(I) collagen mRNA and type I collagen protein secretion from culture-activated HSCs (17). HSCs were cultured on plastic, and after 10 days in culture the cells were treated with Me_2SO or 10 nM rapamycin for up to 72 h, with fresh media and treatments replaced every 24 h. Inhibiting p70^{S6K} activity with rapamycin did not significantly attenuate α1(I) collagen mRNA expression (Fig. 5).

To assess the effects of blocking PI3K or p70^{S6K} activity on type I collagen production, Western blot analysis was performed for type I collagen from cultured media. Treatment of the cells with 25 μM LY294002 significantly reduced type I collagen protein levels within 48 and 72 h compared with control Me_2SO-treated cells (Fig. 6*A*). Treatment of the cells with 10 nM rapamycin also inhibited extracellular type I collagen protein expression although not to the extent that was observed in the LY294002-treated cells (Fig. 6*A*). HSC cellular extracts were also examined for intracellular type I collagen protein levels after treatment with Me_2SO, 25 μM LY294002, or 10 nM rapamycin. LY294002 treatment significantly reduced type I collagen intracellular protein levels both at days 3 and 7 compared with control values (Fig. 6*B*). Moreover, rapamycin similarly blunted collagen I protein expression (Fig. 6*C*).

To determine whether p70^{S6K} is required for activation of HSCs in culture, HSCs were cultured and treated with 10 nM rapamycin or Me_2SO as a control. Western blot analysis showed that there is no significant difference in the level of α-SMA expression between HSCs treated with Me_2SO or rapamycin at day 3 or 7, indicating p70^{S6K} is not required for activation of HSCs (Fig. 6*D*).

Inhibition of p70^{S6K} Decreases the Relative Proliferation Rate in HSCs—We have previously shown that inhibition of PI3K or Akt attenuates HSC proliferation (17). To investigate whether inhibition of p70^{S6K} pathway would prevent HSC proliferation, [^{3}H]thymidine incorporation was assessed to measure DNA synthesis, a classical indicator of cell proliferation, 10–14 days after isolation. After a 24-h period of serum starvation, DNA synthesis of HSCs was stimulated using 10% FBS. We have shown a similar induction of cell proliferation when either serum or 20 mg/ml PDGF is used as a proliferative stimulus (data not shown). Before adding the proliferative stimulus, cells were treated with 10 nM rapamycin for 1 h. After stimulation with 10% serum, the relative proliferation rate was increased 2.5-fold compared with cells treated with 0.2% serum (Fig. 7). In contrast, HSCs stimulated with 10% serum and pretreated with 10 nM rapamycin showed a significant reduction in the proliferative rate compared with HSCs stimulated with 10% serum. Cells stimulated with 0.2% serum that were treated with 10 nM rapamycin exhibited a proliferation rate that was not significantly different from cells treated with 0.2%

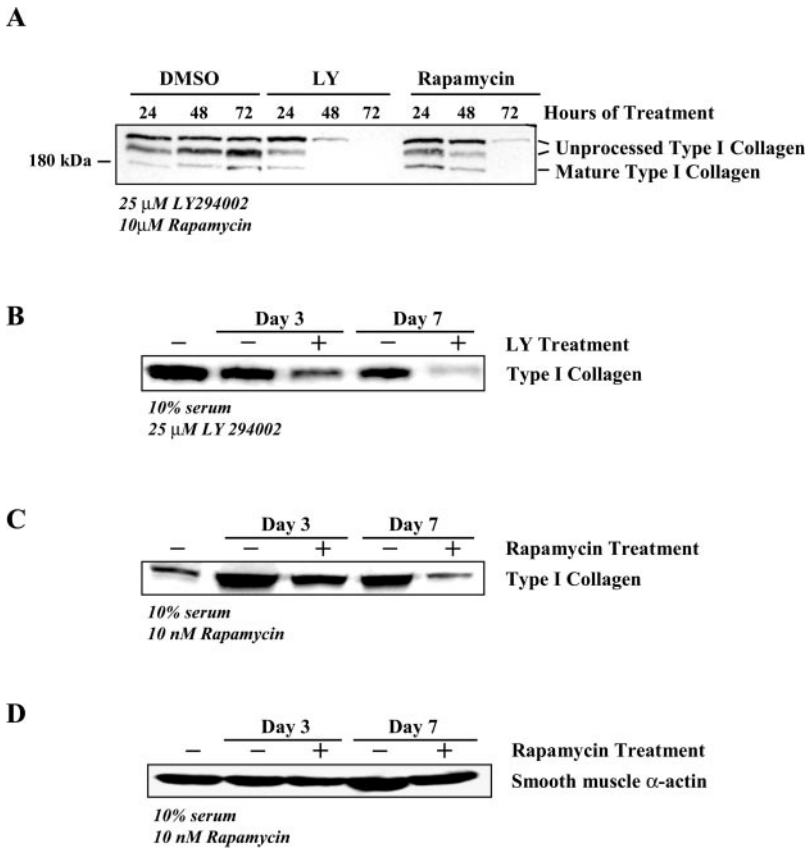

FIG. 6. **LY294002 and rapamycin decreases PDGF-induced type I collagen protein expression but does not affect α-SMA expression.** *A*, Western blot of proteins in the culture media from HSCs treated for 24, 48, and 72 h with Me_2SO, 25 μM LY294002, or 10 nM rapamycin and probed for type I collagen. Culture media was replaced daily. Western blot of cellular proteins (20 μg/lane) probed for type I collagen (*B* and *C*) or α-SMA (*D*) from HSCs treated daily with Me_2SO (*DMSO*) or 25 μM LY294002 (*B*) or 10 nM rapamycin (*C* and *D*) for 3 and 7 days. The culture media was changed daily. Equal sample loading was assessed by Ponceau Red staining of the membrane after protein transfer.

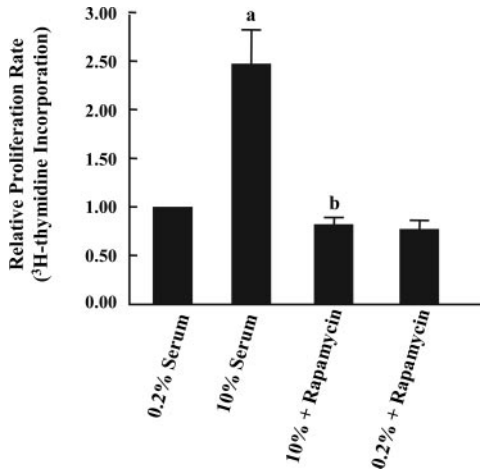

FIG. 7. **Rapamycin inhibits serum-induced DNA synthesis in HSCs.** HSCs were cultured for 10–14 days and then serum-starved for 48 h. HSCs were preincubated with Me_2SO or 10 nM rapamycin for 1 h and then stimulated with 10% FBS for 24 h in medium containing 1 μCi/ml [^{3}H]thymidine. HSCs maintained in 0.2% serum served as a control. Experiments were performed in quadruplicate. Data represent means ± S.E. *a*, $p < 0.05$ compared with HSCs treated with 0.2% serum; *b*, $p < 0.05$ compared with HSCs stimulated with 10% serum.

serum alone. Together these data suggest that p70^{S6K} signaling is involved regulating DNA synthesis in HSCs.

Inhibition of PI3K and p70^{S6K} Blocks HSC Proliferation at the G_1-S Checkpoint—Both PI3K and p70^{S6K} positively influence cell proliferation. We next wanted to assess where in the cell cycle LY294002 and rapamycin, inhibitors of PI3K and p70^{S6K}, respectively, block HSC proliferation. To address this, activated HSCs were serum-starved 48 h then treated with 25 μM LY294002, 10 nM rapamycin, or Me_2SO as a control for 1 h. Afterward, the cells were stimulated to proliferate with 10% fetal calf serum for 24 and 48 h. Cells were harvested and analyzed by FACS analysis to determine the percentage of cells in the $S/G_2/M$ phase of the cell cycle (Fig. 8*A*). Serum stimulation significantly increased the percentage of cells in the $S/G_2/M$ cell cycle phases from 17.0 ± 7.0% to 33.0 ± 4.0% after 24 h. A similar increase was observed after 48 h of serum stimulation, indicating increased cell proliferation. Me_2SO treatment had no appreciable effect on serum-induced cell cycle progression (Fig. 8*B*). However, both LY294002 and rapamycin significantly blocked cell cycle progression at the G_1 to S restriction point after both 24 and 48 h of treatment to levels similar to those observed in untreated cells. These data show that the PI3K-Akt-p70^{S6K} signaling pathway regulates cell cycle progression at the G_1 to S restriction point in the cell cycle.

Rapamycin Inhibits Cyclin D1 and D3 and Cyclin E Expression, Whereas Expression of Cyclin D2, Rb-Ser^{780}, and Rb-Ser^{795} Are Not Altered—Proliferation of mammalian cells is controlled at specific stages in the cell cycle. The G_1 to S transition is generally controlled by the D cyclins and cyclin E (33). Therefore, to assess whether inhibition of p70^{S6K} by rapamycin reduces expression of cell cycle control proteins, HSCs were cultured on plastic for 10–14 days, serum-starved for 48 h, then treated with 10 nM rapamycin for 1 h before stimulation with 20 ng/ml PDGF for 6, 12, 18, and 24 h. Western blot analysis showed that cyclins D1, D2, and D3 were present before PDGF stimulation and at all time points in cells treated with Me_2SO (Fig. 9). Treatment of the cells with PDGF did not affect protein levels of the three D cyclins analyzed (Fig. 9). In contrast, cells treated with rapamycin led to a significant reduction in the expression of cyclins D1 and D3 protein levels throughout the experiment (Fig. 9, *A* and *C*). However, no change in cyclin D2 protein levels were observed between Me_2SO- and rapamycin-treated HSCs at 6, 12, 18, and 24 h (Fig. 9*B*). Cyclin E protein levels were not detected in unstimulated cells; however, after PDGF stimulation, cyclin E protein

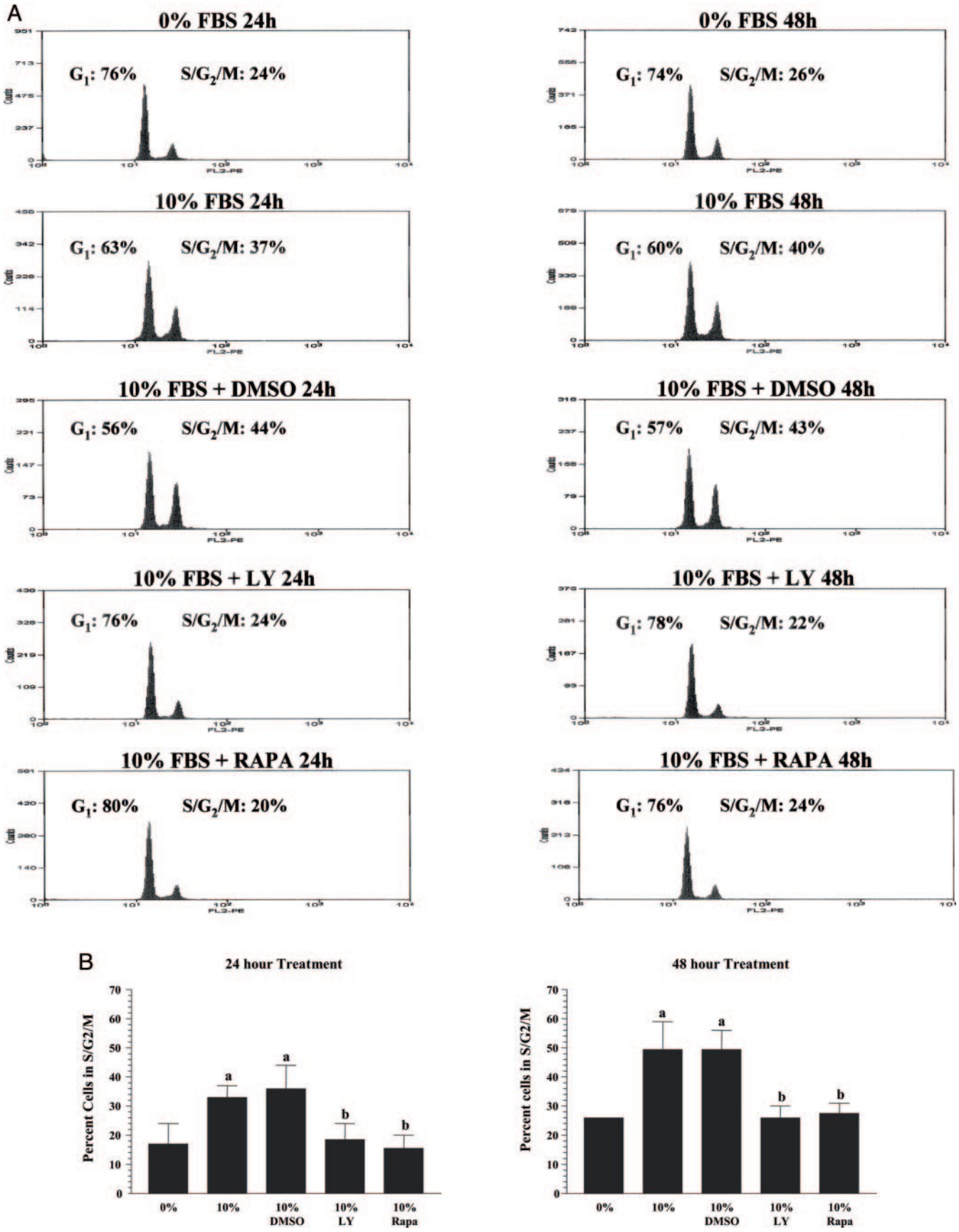

FIG. 8. **LY and rapamycin both inhibit HSC proliferation at the G_1-S cell cycle checkpoint.** Activated HSCs were serum-starved for 48 h and then subsequently treated with 25 μM LY294002 or 10 nM rapamycin (*RAPA*) or Me_2SO (*DMSO*) as a vehicle control for 1 h. Cells were stimulated to proliferate with the addition of 10% fetal calf serum (*FBS*). After 24 or 48 h, cells were harvested, and FACS analysis was performed. *A*, representative FACS scan charts showing the percent of cells in G_1 or $S/G_2/M$ cell cycle phases. *B*, a graphical representation of the FACS data from an average of three independent experiments presented. *Error bars* represent S.E. *a* represents statistically significant values compared with unstimulated cells (0%). *b* represents statistically significant values compared with cells stimulated with 10% serum.

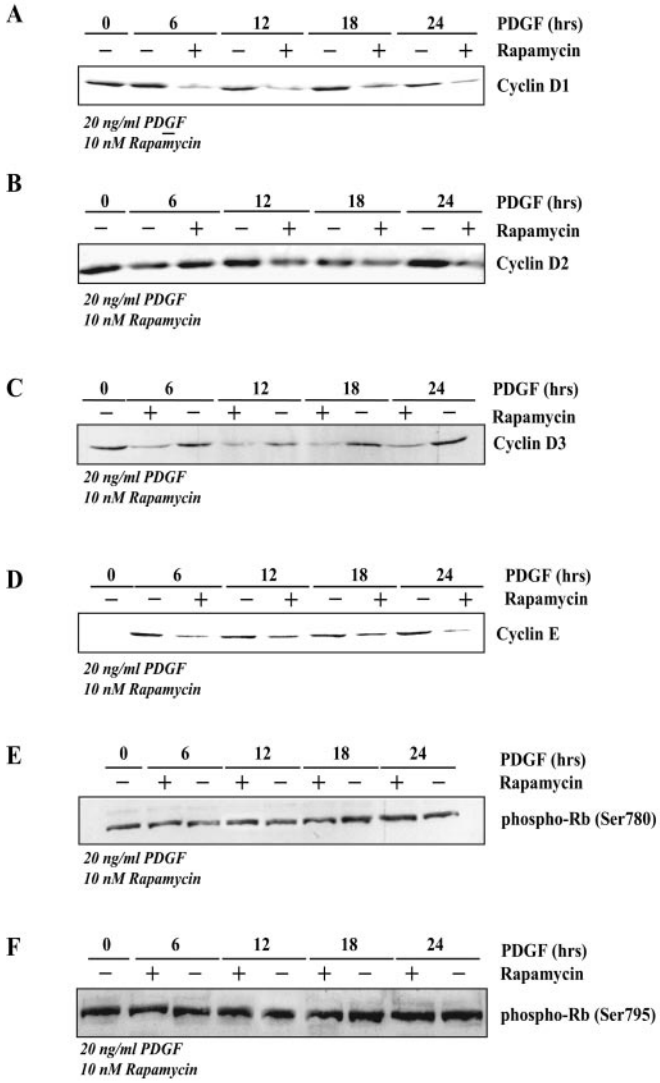

FIG. 9. **Rapamycin reduces cyclin D1, D3, E protein levels but not cyclin D2, Rb-Ser^{780}, or Rb-Ser^{795} protein levels after PDGF stimulation.** HSCs were isolated and cultured for 10–14 days and then synchronized by culturing in medium containing 0% serum for 48 h. HSCs were either left untreated as a control or preincubated with Me_2SO or 10 nM rapamycin for 1 h before stimulation with 20 ng/ml PDGF. Cellular proteins (20 μg) were analyzed by Western blot after 0, 6, 12, 18, and 24 h of stimulation. Immunoblots were probed for cyclin D1 (*A*), cyclin D2 (*B*), cyclin D3 (*C*), cyclin E (*D*), Rb^{Ser780} (*E*), Rb^{Ser795} (*F*).

expression was induced during the 24-h stimulation period. Rapamycin treatment resulted in a clear reduction of cyclin E protein levels at each time point (Fig. 9*D*). Together, we found that cyclin D1, D3, and E protein levels were reduced when HSC proliferation was blocked by rapamycin. In addition, reverse transcription-PCR analysis demonstrated that mRNA levels for cyclins D1 and E were accordingly reduced (data not shown); however, cyclin D2 protein (Fig. 9*B*) and mRNA levels (data not shown) were unaffected.

The retinoblastoma gene (Rb) product regulates cell proliferation by controlling progression through the restriction point within the G_1 phase of the cell cycle (34). Phosphorylation of Rb late in the G_1 phase induces Rb to dissociate from the transcription factor E2F, thereby permitting transcription of S-phase genes and cell cycle progression (35, 36). Therefore, we performed Western blot analysis to assess Rb phosphorylation status in HSCs after PDGF and rapamycin treatment. PDGF did not increase Rb-Ser^{780}- and Rb-Ser^{795}-phosphorylated protein levels compared with unstimulated HSCs (Fig. 9, *E* and *F*). Moreover, rapamycin did not affect Rb phosphorylation at these sites throughout the experimental period. These data indicate that inhibition of p70^{S6K} activity by rapamycin leads to a decrease in the expression of cyclins D1, D3, and E, whereas expression of cyclin D2, and phosphorylation of Rb-Ser^{780} and Rb- Ser^{795} are not changed.

DISCUSSION

After a fibrogenic stimulus the HSC changes from a quiescent non-proliferating cell to an activated and proliferating cell. The proliferative response of most cells is mediated by the presence of mitogenic growth factors such as PDGF. On the cell surface, PDGF binds to its receptor thereby activating a variety of intracellular pathways, with considerable cross-talk between the signaling pathways. From the cytoplasm these signals are transmitted to the nucleus, inducing genes required for cell cycle progression, ultimately leading to increased proliferation. Here we demonstrate that p70^{S6K} is a crucial downstream target in HSC proliferation and cell cycle control after PDGF stimulation.

The PI3K/Akt pathway is activated after PDGF stimulation of HSCs (11, 12, 17). A role of PI3K in HSC proliferation is supported by *in vivo* studies in rats where CCl_4 treatment lead to autophosphorylation of the PDGF receptor and increased PI3K activity (12). Furthermore, wortmannin and the more specific PI3K inhibitor LY294002 blocked mitogenesis in response to PDGF, supporting the involvement of this pathway in HSC proliferation (12, 16). We demonstrated that p70^{S6K} is positioned downstream of PI3K in HSCs since inhibition of PI3K by LY294002 prevented PDGF-induced p70^{S6K} phosphorylation (Fig. 1). This observation is supported by data in other cell types, where p70^{S6K} is also positioned downstream of PI3K and the target of rapamycin (FRAP/mTOR) and is a distinct signaling pathway of the Ras/mitogen-activated protein kinase cascade (20). Akt is another key downstream survival factor in several cell types. Overexpression of a constitutively active form of Akt stimulates p70^{S6K} and promotes cell proliferation and survival (37–39). Recently, we were able to show that Akt becomes activated in HSCs following PDGF stimulation after phosphorylation of Ser^{473}. Inhibition of PI3K using LY294002 blocked this activity (17). Inhibition of PI3K and Akt activation also markedly reduced HSC proliferation, α1(I) collagen mRNA expression, and secretion of type I collagen protein. Transduction of HSCs with an adenovirus expressing a constitutively active form of Akt induced HSC proliferation in low serum conditions, suggesting Akt is positioned downstream of PI3K and confirming its role in HSC proliferation (17).

To evaluate the role and the position of p70^{S6K} in the PI3K-Akt signaling pathway, we treated HSCs with rapamycin followed by PDGF stimulation. Rapamycin, a bacterial macrolide with antifungal and immunosuppressant activities, has been shown to lead to the dephosphorylation and subsequent inactivation of p70^{S6K} (23, 40). Phosphorylation of Thr^{412} is important for p70^{S6K} function *in vivo* (41). Ser^{411}, Thr^{421}, and Ser^{424} lie within a Ser-Pro-rich region located within the pseudosubstrate region (20). Rapamycin, therefore, represents a validated and adequate tool for blocking p70^{S6K} activity as it has been used on a regular basis in other cell types for inhibition of p70^{S6K} (42, 43). We show that rapamycin inhibited PDGF-induced phosphorylation of p70^{S6K} but not Akt phosphorylation, suggesting that p70^{S6K} lies downstream of Akt (Fig. 2). Inhibition of p70^{S6K} phosphorylation by rapamycin did not influence α1(I) collagen mRNA expression but did reduce both intracellular and extracellular type I collagen protein expression, unlike PI3K or Akt, which showed little effect on regulation of α1(I) collagen gene transcription. Thus, collagen gene expression appears to be more dependent on PI3K activity,

which suggests that a divergence in PI3K activities is present in HSCs, one branch leading to collagen expression and the other continuing through Akt and p70^{S6K}, resulting in a proliferative response. Indeed, our findings demonstrated similar results with the PI3K inhibitor LY294002, confirming the downstream position of p70^{S6K} in the PI3K/Akt pathway (Figs. 5 and 6). These results are in accordance with data in other cell types, where p70^{S6K} stimulation was dependent on PI3K (44–46). Similar results were obtained in HSCs stimulated by acetaldehyde, which is generated by oxidation of ethanol *in vivo* (47). Because ethanol is one of the major causes of liver fibrosis in patients, the biologic effects of this active ethanol metabolite are of great importance (48).

One major component of liver fibrosis is the increased proliferation of HSCs. This response is at least partially responsible for increased expression and deposition of extracellular matrix proteins (48). The data presented here show that rapamycin is potently able to block HSC proliferation. Inhibition of PI3K and Akt also blocked HSC proliferation (Fig. 7) (17). Together, these data suggest that the PI3K-Akt-p70^{S6K} pathway is crucial in proliferative signaling in HSCs. We previously demonstrated that inhibition of PI3K activity inhibits α1(I) collagen gene expression (17). However, in this study we showed that inhibiting p70^{S6K} activity does not reduce α1(I) collagen mRNA expression; however, inhibition of p70^{S6K} blocks intracellular as well as extracellular type I collagen protein expression. Therefore, it is possible that rapamycin alters translational efficiency in HSCs, as previously shown in other cell types (21, 49). The likely mechanisms by which rapamycin blocks p70^{S6K} activity is either by direct inhibition of p70^{S6K} phosphorylation in the PI3K pathway or by inhibition of the mTOR/FRAP-induced phosphorylation (21, 23). Rapamycin represses translation of mRNA subsets including transcripts of the translational apparatus, *i.e.* ribosomal proteins and elongation factors required for protein synthesis. Ultimately this results in an impairment of mRNA translation and reduction of protein levels (18, 21, 44, 50).

As a potent profibrogenic cytokine, TGFβ is able to stimulate collagen mRNA and protein synthesis (51, 52). Furthermore, TGFβ is anti-proliferative in HSCs (29). Surprisingly, TGFβ did not influence the phosphorylation status of p70^{S6K} induced by PDGF (Fig. 3). Therefore, the anti-proliferative actions of TGFβ must be independent of the PI3K pathway, possibly mediated through Ras, extracellular signal-regulated kinase, and MEK signaling. After a fibrogenic stimulus, HSCs become activated and start to express smooth muscle α-actin (4). Our results show that rapamycin treatment did not change α-SMA protein levels, indicating that p70^{S6K} actions are independent of the stellate cell activation process.

As a mitogen-activated Ser/Thr protein kinase, p70^{S6K} is required for G_1 cell cycle progression and cell growth (20). p70^{S6K} phosphorylates the S6 protein of the 40 S ribosomal subunit and is involved in translational control of 5′-oligopyrimidine tract mRNAs (20). It has been shown that rapamycin is effectively able to block protein synthesis and to arrest cell cycle progression in the G_1 phase by inhibition of the G_1/S transition (18, 53). This inhibition is the consequence of rapamycin effect on cyclin D1 mRNA and protein stability (54). The various forms of cyclins comprise the regulatory subunits of kinase complexes, which control cell proliferation at specific stages in the cell cycle. These kinase complexes mediate phosphorylation of Rb during G_1 (55, 56). The controlled activation of the kinase complexes at various intervals of the cell cycle is regulated by the availability of the cyclins to the catalytic subunit. Unlike the catalytic subunit, which is expressed continually, the expression and stability of the regulatory subunit

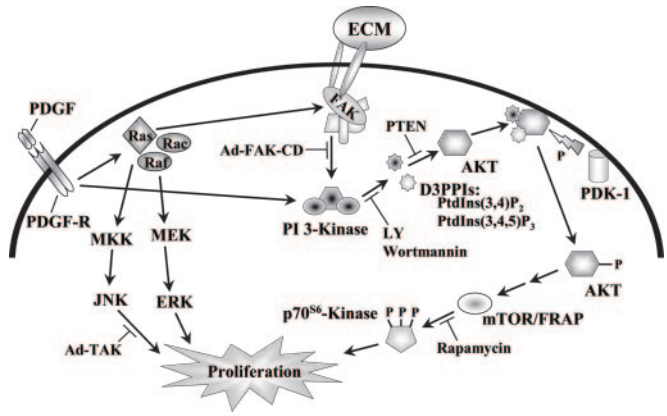

FIG. 10. **Proliferative signaling in hepatic stellate cells.** *FAK*, focal adhesion kinase; *PDK-1*, phosphoinositide-dependent kinase 1; *JNK*, c-Jun NH_2-terminal kinase; *ERK*, extracellular signal-regulated kinase; *PtdIns(3,4)P_2*, phosphatidylinositol 3,4,-phosphate; *PtdIns(3,4,5)P_3*, phosphatidylinositol 3,4,5-phosphate; *ECM*, extracellular matrix; *D3PPI*, D3-phosphorylated phosphoinositides; *PTEN*, phosphatase and tensin homolog deleted on chromosome ten; *MKK*, mitogen-activated protein kinase kinase.

fluctuates depending on the stage of the cell cycle, thereby regulating kinase activity. Accumulation evidence suggested that D-type cyclins play an important role in cell cycle progression (34, 57). Overexpression of cyclin D1 in rat fibroblasts caused a more rapid entry into the S phase (58, 59). Moreover, cyclin D expression is controlled post-transcriptionally via the PI3K/Akt-dependent pathway (60). For example, cyclin E works as a cofactor for cyclin-dependent kinase 2 (cdk2) in late G_1 phase (61). Cyclin-cdk complexes play an important role in cell cycle transition from G_1 to S since microinjection of these complexes induced DNA synthesis in human fibroblasts (62). In murine HSCs it has been shown that expression of cyclin D1, D2, and E correlated with proliferation and LY294002 inhibited expression of cyclin D1 (63). We were able to show that rapamycin treatment lead to a decrease in expression of cyclin D1, cyclin D3, cyclin E but not cyclin D2 mRNA and protein levels (Fig. 9). One possible explanation could be that expression of cyclin D2 is influenced by other kinases and that rapamycin treatment alone is not able to block cyclin D2 expression. Alternatively, cyclin D2 might not be involved in G_1/S transition in this cell type. The ability of rapamycin to inhibit expression of cyclin D1, D3, and E correlated with an arrest in the G_1 phase of the cell cycle as demonstrated by FACS analysis (Fig. 8).

As stated above, the tumor suppressor Rb is an important factor in the cell cycle. Rb controls progression through the late G_1 restriction point and is a major regulator of the G_1/S transition (34). Cell cycle progression is induced by phosphorylation of Rb at several specific sites by cyclin-dependent kinases and their regulatory subunits. Phosphorylation of Rb leads to the dissociation of transcription factors, which induce genes required for cell cycle progression (35, 36). However, Rb phosphorylation at Ser^{780} and Ser^{795} was not affected by rapamycin in HSCs (Figs. 9, *E* and *F*). A possible explanation is that Ser^{780} and Ser^{795} are not the primary phosphorylation sites involved in cell cycle arrest. Rb has numerous known phosphorylation sites and potentially yet undiscovered ones that might be unphosphorylated, thereby still preventing cell cycle progression. Such sites potentially include Ser$^{807/811}$, Ser$^{249/252}$, Thr^{373}, and Ser^{608}. We investigated Rb phosphorylation at Ser^{795} since the role of this site is important for regulating cell cycle progression in other cell types as mitogen-induced phosphorylation of Rb at this site (64). However, it has also been shown that Ser^{795} and the surrounding region is important for E2F binding, but mu-

tation of Ser^{795} alone is not sufficient to inhibit E2F binding (65). Thus, multiple phosphorylation sites seem to regulate protein binding activity of Rb toward E2F and subsequent cell proliferation. Finally, in a model of heat-induced G_1 arrest, cell cycle progression was inhibited regardless of Rb phosphorylation at Ser$^{780/795}$ (66).

In summary, our results demonstrate that the PI3K-Akt-p70^{S6K} signaling pathway is important for transmitting PDGF-induced proliferative signaling in the activated HSC (Fig. 10). p70^{S6K} is positioned downstream of PI3K and Akt. p70^{S6K} phosphorylation is required for maximal induction of cyclin D1, D3, and E, which are required for cell cycle progression in this cell type. However, phosphorylation sites Ser^{780} and Ser^{795} of Rb are most likely not required for cell cycle arrest in HSCs and are independent of p70^{S6K} phosphorylation. Ultimately, p70^{S6K} might represent a therapeutic target to inhibit collagen deposition through inhibition of HSC proliferation during hepatic fibrogenesis.

REFERENCES

1. Friedman, S. L. (1998) in *Diseases of the Liver* (Schiff, E., Sorrell, M., and Maddrey, W., eds) 8th Ed., pp. 371–386, Lippincott-Raven, Philadelphia
2. Friedman, S. L. (2000) *J. Biol. Chem.* **275,** 2247–2250
3. Mak, K. M., Leo, M. A., and Lieber, C. S. (1984) *Gastroenterology* **87,** 188–200
4. Ramadori, G., Veit, T., Schwogler, S., Dienes, H. P., Knittel, T., Rieder, H., and Meyer zum Buschenfelde, K. H. (1990) *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **59,** 349–357
5. Takahara, T., Kojima, T., Miyabayashi, C., Inoue, K., Sasaki, H., Muragaki, Y., and Ooshima, A. (1988) *Lab. Investig.* **59,** 509–521
6. Geerts, A., Lazou, J. M., De Bleser, P., and Wisse, E. (1991) *Hepatology* **13,** 1193–1202
7. Pinzani, M., Gesualdo, L., Sabbah, G. M., and Abboud, H. E. (1989) *J. Clin. Investig.* **84,** 1786–1793
8. Pinzani, M., and Marra, F. (2001) *Semin. Liver Dis.* **21,** 397–416
9. Marra, F., Arrighi, M. C., Fazi, M., Caligiuri, A., Pinzani, M., Romanelli, R. G., Efsen, E., Laffi, G., and Gentilini, P. (1999) *Hepatology* **30,** 951–958
10. Schnabl, B., Bradham, C. A., Bennett, B. L., Manning, A. M., Stefanovic, B., and Brenner, D. (2001) *Hepatology* **34,** 953–963
11. Marra, F., Pinzani, M., DeFranco, R., Laffi, G., and Gentilini, P. (1995) *FEBS Lett.* **376,** 141–145
12. Marra, F., Gentilini, A., Pinzani, M., Choudhury, G. G., Parola, M., Herbst, H., Dianzani, M. U., Laffi, G., Abboud, H. E., and Gentilini, P. (1997) *Gastroenterology* **112,** 1297–1306
13. Parker, P. J., and Waterfield, M. D. (1992) *Cell Growth Differ.* **3,** 747–752
14. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes Dev.* **13,** 2905–2927
15. Vanhaesebroeck, B., and Alessi, D. R. (2000) *Biochem. J.* **346,** 561–576
16. Gentilini, A., Marra, F., Gentilini, P., and Pinzani, M. (2000) *J. Hepatol.* **32,** 227–234
17. Reif, S., Lang, A., Lindquist, J. N., Gäbele, E., Scanga, A., Brenner, D. A., and Rippe, R. A. (2003) *J. Biol. Chem.* **278,** 8083–8090
18. Berven, L. A., and Crouch, M. F. (2000) *Immunol. Cell Biol.* **78,** 447–451
19. Dufner, A., and Thomas, G. (1999) *Exp. Cell Res.* **253,** 100–109
20. Pullen, N., and Thomas, G. (1997) *FEBS Lett.* **410,** 78–82
21. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) *EMBO J.* **16,** 3693–3704
22. Polakiewicz, R. D., Schieferl, S. M., Gingras, A. C., Sonenberg, N., and Comb, M. J. (1998) *J. Biol. Chem.* **273,** 23534–23541
23. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) *Genes Dev.* **15,** 807–826
24. Skrtic, S., Wallenius, K., Gressner, A. M., and Jansson, J. O. (1999) *Endocrinology* **140,** 5729–5735
25. Zhu, J., Wu, J., Frizell, E., Liu, S. L., Bashey, R., Rubin, R., Norton, P., and Zern, M. A. (1999) *Gastroenterology* **117,** 1198–1204
26. Rippe, R. A., Almounajed, G., and Brenner, D. A. (1995) *Hepatology* **22,** 241–251
27. Rivera, C. A., Bradford, B. U., Hunt, K. J., Adachi, Y., Schrum, L. W., Koop, D. R., Burchardt, E.-R., Rippe, R., and Thurman, R. G. (2001) *Am. J. Physiol. Gastrointest. Liver Physiol.* **281,** 200–207
28. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) *Annu. Rev. Biochem.* **68,** 965–1014
29. Bachem, M. G., Riess, U., and Gressner, A. M. (1989) *Biochem. Biophys. Res. Commun.* **162,** 708–714
30. Saile, B., Matthes, N., Knittel, T., and Ramadori, G. (1999) *Hepatology* **30,** 196–202
31. Hanna, A. N., Chan, E. Y., Xu, J., Stone, J. C., and Brindley, D. N. (1999) *J. Biol. Chem.* **274,** 12722–12729
32. Hellerbrand, C., Jobin, C., Licato, L. L., Sartor, R. B., and Brenner, D. A. (1998) *Am. J. Physiol. Gastrointest. Liver Physiol.***275,** 269–278
33. Reed, S. I. (1997) *Cancer Surv.* **29,** 7–23
34. Sherr, C. J. (1996) *Science* **274,** 1672–1677
35. Lundberg, A. S., and Weinberg, R. A. (1998) *Mol. Cell. Biol.* **18,** 753–761
36. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) *Cell* **98,** 859–869
37. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell. Biol.* **21,** 893–901
38. Kulik, G., Klippel, A., and Weber, M. J. (1997) *Mol. Cell. Biol.* **17,** 1595–1606
39. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) *Biochem. J.* **335,** 1–13
40. Chung, J., Kuo, C. J., Crabtree, G. R., and Bleins, J. (1992) *Cell* **69,** 1227–1236
41. Weng, Q. P., Kozlowski, M., Belham, C., Zhang, A., Comb, M. J., and Avruch, J. (1998) *J. Biol. Chem.* **273,** 16621–16629
42. Gerasimovskaya, E. V., Tucker, D. A., Weiser-Evans, M., Wenzlau, J. M., Klemm, D. J., Banks, M., and Stenmark, K. R. (2004) *J. Biol. Chem.* **280,** 1838–1848
43. Ganesan, L. P., Wei, G., Pengal, R. A., Moldovan, L., Moldovan, N., Ostrowski, M. C., and Tridandapani, S. (2004) *J. Biol. Chem.* **279,** 54416–54425
44. Braun-Dullaeus, R. C., Mann, M. J., Seay, U., Zhang, L., Der Leyen, H. E., Morris, R. E., and Dzau, V. J. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21,** 1152–1158
45. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14,** 4902–4911
46. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) *Nature* **370,** 71–75
47. Svegliati-Baroni, G., Ridolfi, F., Di Sario, A., Saccomanno, S., Bendia, E., Benedetti, A., and Greenwel, P. (2001) *Hepatology* **33,** 1130–1140
48. Gäbele, E., Brenner, D. A., and Rippe, R. A. (2003) *Front. Biosci.* **8,** 69–77
49. Lee, Y., Broxmeyer, H. E., Mantel, C., Kwon, H. J., Kim, J. W., Kim, J. S., Kwon, D., and Choe, I. S. (2003) *Exp. Mol. Med.* **35,** 222–226
50. Jefferies, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4441–4445
51. Armendariz-Borunda, J., Katayama, K., and Seyer, J. M. (1992) *J. Biol. Chem.* **267,** 14316–14321
52. Matsuoka, M., Pham, N. T., and Tsukamoto, H. (1989) *Liver* **9,** 71–78
53. Dennis, P. B., Fumagalli, S., and Thomas, G. (1999) *Curr. Opin. Genet. Dev.* **9,** 49–54
54. Hashemolhosseini, S., Nagamine, Y., Morley, S. J., Desrivieres, S., Mercep, L., and Ferrari, S. (1998) *J. Biol. Chem.* **273,** 14424–14429
55. Lees, J. A., Buchkovich, K. J., Marshak, D. R., and Anderson, C. W. (1991) *EMBO J.* **10,** 4279–4290
56. Lin, B. T., Gruenwald, S., Morla, A. O., Lee, W. H., and Wang, J. Y. (1991) *EMBO J.* **10,** 857–864
57. Stillman, B. (1996) *Science* **274,** 1659–1664
58. Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M., and Weinstein, I. B. (1993) *Oncogene* **8,** 3447–3457
59. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F., and Sherr, C. J. (1993) *Genes Dev.* **7,** 1559–1571
60. Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N., and Rosen, N. (1998) *J. Biol. Chem.* **273,** 29864–29872
61. Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M., and Roberts, J. M. (1991) *Cell* **66,** 1217–1228
62. Connell-Crowley, L., Elledge, S. J., and Harper, J. W. (1998) *Curr. Biol.* **8,** 65–68
63. Kawada, N., Ikeda, K., Seki, S., and Kuroki, T. (1999) *J. Hepatol.* **30,** 1057–1064
64. Garnovskaya, M. N., Mukhin, Y. V., Vlasova, T. M., Grewal, J. S., Ullian, M. E., Tholanikunnel, B. G., and Raymond, J. R. (2004) *J. Biol. Chem.* **279,** 24899–24905
65. Knudsen, E. S., and Wang, J. Y. (1997) *Mol. Cell. Biol.* **17,** 5771–5783
66. Miyakoda, M., Nakahata, K., Suzuki, K., Kodama, S., and Watanabe, M. (1999) *Biochem. Biophys. Res. Commun.* **266,** 377–381