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$^{278}$, and Rb-Ser$^{285}$. 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) 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 Raf 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). 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, gene-activated protein kinase/extracellular signal-regulated kinase kinase; p70$^{S6K}$, p70 S6 kinase; Rb, retinoblastoma.
Role of p70^S6K in HSC Proliferation and Collagen Expression

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% CO2-humidified atmosphere at 37 °C. Growth medium was exchanged every other day. HSCs were incubated for another 24 h to reduce cell proliferation. One hour before the proliferative trigger cells were treated with 10 mM rapamycin (Sigma). Afterward, the cells were incubated in 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 mM rapamycin (Sigma). Afterward, the cells were incubated in medium containing 10% FBS for 24 h in Dulbecco’s modified Eagle’s medium without FBS supplementation to synchronize the cells. 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% bromophenol 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 electrochemiluminescence transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were stained with 0.5% Ponceau S to assure equal protein loading. Membranes were washed with 0.2N 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 glycerolaldehyde-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 autoradiography.

| Primary antibody | Dilution/Incubation | Secondary antibody | Dilution/Incubation |
|-----------------|---------------------|--------------------|---------------------|
| Polyclonal rabbit anti-human phospho-p70^S6K kinase (Thr421/ Ser422) (Upstate Biotechnology, Lake Placid, NY) | 1:1500/1 h | HRP-conjugated anti-rabbit IgG (Santa Cruz, CA) | 1:2500/30 min |
| Polyclonal rabbit anti-p70^S6K kinase (Upstate Biotechnology) | 1:1000/1 h | HRP-conjugated anti-rabbit IgG (Santa Cruz) | 1:1000/30 min |
| Polyclonal rabbit anti-human phospho-AKT (Ser473) (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-Iba1 (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) | 1:1000/8 h | HRP-conjugated anti-rabbit IgG (Santa Cruz) | 1:1000/1 h |
| Rabbit polyclonal anti-human phospho-Rb Ser^110 (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, CA) | 1:1000/1 h |

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

**TABLE I**

| Primary antibody | Dilution/Incubation | Secondary antibody | Dilution/Incubation |
|-----------------|---------------------|--------------------|---------------------|
| Polyclonal rabbit anti-human phospho-p70^S6K kinase (Thr421/ Ser422) (Upstate Biotechnology, Lake Placid, NY) | 1:1500/1 h | HRP-conjugated anti-rabbit IgG (Santa Cruz, CA) | 1:2500/30 min |
| Polyclonal rabbit anti-p70^S6K kinase (Upstate Biotechnology) | 1:1000/1 h | HRP-conjugated anti-rabbit IgG (Santa Cruz) | 1:1000/30 min |
| Polyclonal rabbit anti-human phospho-AKT (Ser473) (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-Iba1 (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) | 1:1000/8 h | HRP-conjugated anti-rabbit IgG (Santa Cruz) | 1:1000/1 h |
| Rabbit polyclonal anti-human phospho-Rb Ser^110 (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, CA) | 1:1000/1 h |

* All antibodies were diluted in 5% nonfat dry milk TBS-Tween solution unless otherwise stated.
phy, and signals were quantitated by PhosphorImager analysis (Amer-
sham 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 \(\mu\)M LY294002, 10 nM rapamycin, or
Me\(_{3}\)SO 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 \(\mu\)g/ml RNase for 5 min at room temperature.
Cells were incubated with 50 \(\mu\)g/ml propidium iodide at room temper-
ature 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
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 \(\mu\)M 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.

**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\(^{473}\)
in 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 ra-
amycin 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. 2A). Expression of phospho-
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\(_{3}\)SO control-treated
cells. Phosphorylated \(p70^{S6K}\) at Thr\(^{421}\)/Ser\(^{424}\) was not detected
before PDGF treatment (Fig. 2B); 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 \(p70^{S6K}\) phosphory-
lation. 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.

**TGFB Does Not Change Phosphorylation of \(p70^{S6K}\)**—TGF\(\beta\) is
one of the most important pro-fibrogenic cytokines for the HSC
(2). It has also been shown that TGF\(\beta\) inhibits HSC prolifera-
tion (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 TGF\(\beta\) inhibits
its FAK-PI3K-Akt signaling, leading to an inhibition of \(p70^{S6K}\)
activation. We previously showed that TGF\(\beta\) fails to inhibit
PDGF-induced Akt phosphorylation (17). HSCs were treated
with 5 ng/ml TGF\(\beta\) 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. TGF\(\beta\)
alone did not induce \(p70^{S6K}\) phosphorylation (Fig. 3, second

![Fig. 1. LY204002 inhibits PDGF-BB-induced phosphorylation of \(p70^{S6K}\).](image-url)

**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 un-
treated or treated with Me\(_{3}\)SO, or treated with 25 \(\mu\)M
LY294002 for 1 h before stimulation 20 ng/ml PDGF for the indicated
time periods. Cellular proteins (20 \(\mu\)g) 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 \(p70^{S6K}\)
before PDGF stimulation (Fig. 1) and anti-phospho-
\(p70^{S6K}\) (Thr\(^{421}\)/Ser\(^{424}\)) antibodies. Equal sample loading was assessed by Ponceau Red staining of the
membrane after protein transfer.

![Fig. 2. Rapamycin prevents phosphorylation of \(p70^{S6K}\) but not the protein kinase Akt.](image-url)

**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\(_{3}\)SO, or treated with 10 nM rapamycin for 1 h before stimulation with 20 ng/ml
PDGF. Cellular proteins (20 \(\mu\)g) were harvested after stimulation periods of 0,
10, 20, 30, and 60 min, separated on a 10% SDS-PAGE, and transferred on a ni-
trocellulose membrane. Western blot analysis was performed using anti-phos-
pho-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.
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**Fig. 3.** TGFβ alone or in combination with PDGF does not alter phosphorylation of p70S6K. 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, and 60 min of stimulation. The blots were probed against phosphorylated p70S6K (Thr421/Ser424). Equal sample loading was assessed by Ponceau Red staining of the membrane after protein transfer.

**Fig. 4.** TNFα does not phosphorylate p70S6K 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, or 30 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 (Thr421/Ser424). 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 MeSO or 10 nm rapamycin for 24, 48, or 72 h. Expression of α1(I) collagen mRNA was normalized to expression of β-actin for MeSO (DMSO) vehicle-treated HSCs. Expression in the presence of rapamycin is shown relative to that of MeSO-treated cells. Data represent means ± S.E.

**Fig. 6.** Rapamycin does not induce phosphorylation of p70S6K in HSCs. Western blot analysis was performed as described under “Experimental Procedures” with total RNA extracted from activated HSCs, treated once a day with 10 nm rapamycin or MeSO as a control. Western blot analysis showed that there is no significant difference in the level of α-SMA expression between HSCs treated with MeSO or rapamycin at day 3 or 7, indicating p70S6K is not required for activation of HSCs (Fig. 6D).

Inhibition of p70S6K 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 p70S6K pathway would prevent HSC proliferation, [3H]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 inducible 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%
serum alone. Together these data suggest that p70S6K signaling is involved in regulating DNA synthesis in HSCs.

Inhibition of PI3K and p70S6K Blocks HSC Proliferation at the G1-S Checkpoint—Both PI3K and p70S6K positively influence cell proliferation. We next wanted to assess where in the cell cycle LY294002 and rapamycin, inhibitors of PI3K and p70S6K, respectively, block HSC proliferation. To address this, activated HSCs were serum-starved 48 h then treated with 25 μM LY294002, 10 nM rapamycin, or Me2SO 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/G2/M phase of the cell cycle (Fig. 8A). Serum stimulation significantly increased the percentage of cells in the S/G2/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. Me2SO treatment had no appreciable effect on serum-induced cell cycle progression (Fig. 8B). However, both LY294002 and rapamycin significantly blocked cell cycle progression at the G1 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-p70S6K signaling pathway regulates cell cycle progression at the G1 to S restriction point in the cell cycle.

Rapamycin Inhibits Cyclin D1 and D3 and Cyclin E Expression, Whereas Expression of Cyclin D2, Rb-Ser780, and Rb-Ser795 Are Not Altered—Proliferation of mammalian cells is controlled at specific stages in the cell cycle. The G1 to S transition is generally controlled by the D cyclins and cyclin E (33). Therefore, to assess whether inhibition of p70S6K 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 Me2SO (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. 9A and C). However, no change in cyclin D2 protein levels were observed between Me2SO- and rapamycin-treated HSCs at 6, 12, 18, and 24 h (Fig. 9B). 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 G1-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 MeSO (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 G1 or S/G2/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.
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). 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. 9B) 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 G1 phase of the cell cycle (34). Phosphorylation of Rb late in the G1 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\(^{412}\), 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).
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 p70S6K, resulting in a proliferative response. Indeed, our findings demonstrated similar results with the PI3K inhibitor LY294002, confirming the downstream position of p70S6K in the PI3K/Akt pathway (Figs. 5 and 6). These results are in accordance with data in other cell types, where p70S6K 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-p70S6K 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 p70S6K activity does not reduce α1(I) collagen mRNA expression; however, inhibition of p70S6K 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 p70S6K activity is either by direct inhibition of p70S6K 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 p70S6K 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 p70S6K actions are independent of the stellate cell activation process.

As a mitogen-activated Ser/Thr protein kinase, p70S6K is required for G1 cell cycle progression and cell growth (20). p70S6K 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 G1 phase by inhibition of the G1/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 G1 (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 fluctuations depend 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 G1 phase (61). Cyclin-edk complexes play an important role in cell cycle transition from G1 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 G1/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 G1 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 G1 restriction point and is a major regulator of the G1/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 Ser780 and Ser795 was not affected by rapamycin in HSCs (Figs. 9, E and F). A possible explanation is that Ser780 and Ser795 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 Ser607/611, Ser492/252 Thr737, and Ser906. We investigated Rb phosphorylation at Ser795 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 Ser795 and the surrounding region is important for E2F binding, but mu...
tation of Ser\textsuperscript{780} 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\textsubscript{1} arrest, cell cycle progression was inhibited regardless of Rb phosphorylation at Ser\textsuperscript{780/785} (66).

In summary, our results demonstrate that the P13K-Akt-p70\textsuperscript{S6K} signaling pathway is important for transmitting PDGF-induced proliferative signaling in the activated HSC (Fig. 10). p70\textsuperscript{S6K} is positioned downstream of P13K and Akt. p70\textsuperscript{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\textsuperscript{780} and Ser\textsuperscript{789} of Rb are most likely not required for cell cycle arrest in HSCs and are independent of p70\textsuperscript{S6K} phosphorylation. Ultimately, p70\textsuperscript{S6K} might represent a therapeutic target to inhibit collagen deposition through inhibition of HSC proliferation during hepatic fibrogenesis.