Mammalian Target of Rapamycin Positively Regulates Collagen Type I Production via a Phosphatidylinositol 3-Kinase-independent Pathway*

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The mammalian target of rapamycin (mTOR) is a multifunctional protein involved in the regulation of cell growth, proliferation, and differentiation. The goal of this study was to determine the role of mTOR in type I collagen regulation. The pharmacological inhibitor of phosphatidylinositol (PI) 3-kinase, LY294002, significantly inhibited collagen type I protein and mRNA levels. The effects of LY294002 were more pronounced on the collagen α1(I) chain, which was inhibited at the transcriptional and mRNA stability levels versus collagen α2(I) chain, which was inhibited through a decrease in mRNA stability. In contrast, addition of the PI 3-kinase inhibitor, wortmannin, did not alter type I collagen steady-state mRNA levels. This observation and further experiments using an inactive LY294002 analogue suggested that collagen mRNA levels are inhibited independent of PI 3-kinase. Additional experiments have established that mTOR positively regulates collagen type I synthesis in human fibroblasts. These conclusions are based on results demonstrating that inhibition of mTOR activity using a specific inhibitor, rapamycin, reduced collagen mRNA levels. Furthermore, decreasing mTOR expression by about 50% by using small interfering RNA resulted in a significant decrease of collagen mRNA (75% COL1A1 decrease and 28% COL1A2 decrease) and protein levels. Thus, mTOR plays an essential role in regulating basal expression of collagen type I gene in dermal fibroblasts. Together, our data suggest that the classical PI 3-kinase pathway, which places mTOR downstream of PI 3-kinase, is not involved in mTOR-dependent regulation of type I collagen synthesis in dermal fibroblasts. Because collagen overproduction is a main feature of fibrosis, identification of mTOR as a critical mediator of its regulation may provide a suitable target for drug or gene therapy.

A common characteristic of all fibrotic diseases, including scleroderma, is an abnormal accumulation of extracellular matrix proteins. Fibrotic lesions disrupt normal tissue architecture and contribute to organ failure. Type I collagen, the primary component of fibrotic lesions, is a triple helix composed of two α1 chains and one α2 chain. These chains, although coordinately expressed, are not regulated via the same mechanisms. It is well established that collagen protein degradation, mRNA stability, and transcription are tightly regulated during collagen biosynthesis. Signals from external stimuli such as cytokines (1–3), nutrients (4), and cell interactions (5, 6) modulate these processes via several pathways, including TGF-β, p38 (7, 8), PKC (9), and stress-activated protein kinase/c-Jun N-terminal kinase (10, 11). Despite these advances, the pathways controlling collagen biosynthesis are not fully characterized, prompting us to further examine potential signaling molecules involved in type I collagen regulation.

Prior studies suggested the involvement of phosphatidylinositol 3-kinase (PI 3-kinase), a ubiquitous lipid kinase, in collagen regulation. For example, Ivarsson et al. (12) described PI 3-kinase as a regulator of collagen production in attached, rounded fibroblasts. However, in the same study, the PI 3-kinase inhibitor LY294002 did not inhibit collagen production in spread dermal fibroblasts. Furthermore, in recent studies, Ricupero et al. (13) demonstrated that PI 3-kinase inhibitors (LY294002 and wortmannin) decreased collagen steady-state mRNA levels in lung fibroblasts. However, it has become clear that LY294002 and wortmannin have multiple targets, suggesting that PI 3-kinase may not be the relevant target. Other candidates include a family of proteins that share significant homology with the PI 3-kinase catalytic domain, a target of LY294002 and wortmannin. For the most part, these proteins act as checkpoints for DNA synthesis, protein translation, and mRNA quality and mediate cellular responses to stresses such as DNA damage and nutrient deprivation. For example, family members DNA-protein kinase (14), ataxia telangiectasia-mutated (ATM), ataxia telangiectasia-related genes are regulators of DNA repair, whereas the recently identified hSMG-1 (15, 16) regulates nonsense-mediated decay. Consistent with other members of this family, the mammalian target of rapamycin (mTOR) (17, 18) also appears to act as a checkpoint.

mTOR regulates yeast growth (19, 20), serves as a nutrient sensor (21), and is perhaps best known as a translational regulator. Although initially defined as a translational regulator that exerted its influence through p70 S6 kinase (p70S6K),

1 The abbreviations used are: TGF-β, transforming growth factor-β; 4EBP1, elongation initiation factor 4E-binding protein 1; COL1A2, type I collagen α2 chain; COL1A1, type I collagen α1 chain; FCS, fetal calf serum; PKB, PKB-binding protein; KD, kinase dead; mTOR, mammalian target of rapamycin; NMD, nonsense-mediated decay; p70S6K, ribosomal p70 S6 kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PP2A, type 2A protein phosphatase; siRNA, small-interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; ATM, ataxia telangiectasia-mutated; RT, reverse transcriptase; UTR, untranslated region.

2 This work was supported by National Institutes of Health Grants HL07280–25, AR43234, and AR44883 and the Scleroderma Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available online at http://www.jbc.org.
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(22) and elongation initiation factor 4E-binding protein (4E-BP1) (23), recent evidence suggests that the influence of mTOR is more extensive. For instance, mTOR has also been implicated in the control of protein kinase Cδ (PKCδ) (24), PP2A (25), interleukin-3 mRNA stability (26), and eIF4G protein stability (27). Significantly, mTOR inhibition by rapamycin (28) decreases extracellular matrix deposition in a rat model of fibrogenesis, suggesting a role for mTOR in collagen regulation in vivo (29). Although mTOR regulates numerous genes, in turn is regulated by a variety of stimuli and proteins. For example, c-Abl prevents mTOR phosphorylation in vitro and in vivo (30), and protein kinase B (or Akt) has been demonstrated to phosphorylate mTOR on Thr-2446 and Ser-2461, thereby linking mTOR to a mitogenic pathway (31, 32). mTOR may also regulate its own activity by autophosphorylation on Ser-2448, and mutation of residues Asp-2338 or Asp-2357, within the catalytic domain, abolishes the autophosphorylation reaction (22). In addition to its kinase function, the presence of HEAT repeats (huntington, elongation factor 2, A subunit of type 2A protein phosphatase (PP2A) and Tor) (reviewed in Ref. 33) and FAT/FATC domains (FRAP, ATM, and TRRAP C-terminal) and its large size (289 kDa) raise the possibility that mTOR also acts as a scaffolding protein.

mTOR has also been shown to shuttle between the cytoplasm and the nucleus, although the function of this translocation is not clear. Surprisingly, however, no conventional nuclear import signal or nuclear export signal has been found in the mTOR sequence. Nevertheless, this shuttling seems to be a necessary step in the phosphorylation of p70(S6K) and 4E-BP1 (34).

The PI 3-kinase inhibitors LY294002 and the structurally unrelated inhibitor wortmannin have helped to elucidate the role of PI 3-kinase in many cellular processes. However, the nonspecific nature of these inhibitors warrants caution. For this reason, rapamycin, which binds the FK506-binding protein 12 (FKBP12)-rapamycin binding domain (35) of mTOR, is useful in the characterization of PI 3-kinase-independent pathways. Based on previous work showing that PI 3-kinase and related molecules may be involved in collagen gene regulation, the aim of our study was to use these inhibitors and related reagents to ascertain the role of the PI 3-kinase pathway in collagen production. In this study, we show that mTOR inhibition decreases collagen mRNA stability via a PI 3-kinase-independent mechanism.

EXPERIMENTAL PROCEDURES

Materials—LY294002, wortmannin, and rapamycin were obtained from Biomol (Plymouth Meeting, PA). Chris Vlahos at Lilly kindly provided LY303511. PCR reagents were from Applied Biosystems (Foster City, CA). L-[2,3,4,5-3H]proline, [14C]proline, [35S]methionine, [32P]ATP were obtained from PerkinElmer Life Sciences. DNase I and QuantumRNA TM Classic 18 Standards were provided by guest on July 25, 2018http://www.jbc.org/Downloaded from

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RNase A and T1-Actin-KD Amplification— Fibroblasts (3 × 10^6) cells were resuspended in 75 μl of DMEM. A 200 ng aliquot of cDNA 3 or FLAG-tagged rat mTOR-Kinase dead (D2338A) (mTOR-KD) was kindly donated by Robert Abraham. L-Arginine Extender Plus was added to the suspension. Suspensions were added to 0.4 mm-gap electroporcuettes (Bio-Rad). Samples were electroporated at 0.36 kV and 0.5 microfarads (Gene Pulser® II Electroporation System with Capacitance Extender Plus, Bio-Rad). Samples were brought to a final volume of 3 ml in DMEM containing 10% FCS. Cells were allowed to recover 72 h before collection. Total RNA was isolated from electroporated cells and treated with DNase I. RNA (1 μg) was converted to cDNA by using a random hexamer primer. Rat mTOR-KD was amplified by using upper primer (5’-CCG CTA TGG CAC AGA GGA C-3’) and lower primer (5’-CGG GCA CTC TGG TCT TT 3’) which yields a 119 bp product.

Electroporation and Rat mTOR-KD Amplification— Fibroblasts (3 × 10^6) cells were resuspended in 75 μl of DMEM. A 200 ng aliquot of cDNA 3 or FLAG-tagged rat mTOR-Kinase dead (D2338A) (mTOR-KD) was kindly donated by Robert Abraham. L-Arginine Extender Plus was added to the suspension. Suspensions were added to 0.4 cm-gap electroporcuettes (Bio-Rad). Samples were electroporated at 0.36 kV and 0.5 microfarads (Gene Pulser® II Electroporation System with Capacitance Extender Plus, Bio-Rad). Samples were brought to a final volume of 3 ml in DMEM containing 10% FCS. Cells were allowed to recover 72 h before collection. Total RNA was isolated from electroporated cells and treated with DNase I. RNA (1 μg) was converted to cDNA by using a random hexamer primer. Rat mTOR-KD was amplified by using upper primer (5’-CCG CTA TGG CAC AGA GGA C-3’) and lower primer (5’-TCT CAT TGC CTT CCG TT 3’) which yields a 383 bp product.

Measurement of Collagen mRNA Message Stability— Fibroblasts were grown to confluence in 10-cm^2 dishes in DMEM supplemented with 10% FCS. Confluent fibroblasts were serum-starved in DMEM containing 0.1% BSA for 24 h, followed by incubation with inhibitors or MeSO at the indicated concentrations for 12 h. Actinomycin D (8 μg/ml) was added; and cells were scraped before actinomycin addition and at the indicated time points. Total RNA was extracted; 3.5 μg was analyzed by Northern blotting. Bands were quantitated using NIH ImageJ software.

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CTT TGCTG CGG CTG TTG-3' and lower primer to intron 1 (5'-GGCC CTT CCT CCT CCA CCA C-3') and yielded a 380-bp product. 18 S rRNA was amplified using the QuantumRNA™ Classic 18 S Standards (Ambion) to yield a 488-bp product. One microliter of cDNA for unspliced collagen and 0.5 µl of cDNA for spliced and 18 S rRNA were used for amplification. Amplification used primers at a final concentration of 0.6 µM and MgCl₂ at a final concentration of 1 mM in a total volume of 50 µl. Unspliced collagens were amplified in the presence of 6% formamide. Amplification was for 95 °C for 5 min, followed by cycles of 95 °C for 1 min, 59 °C for 30 s, and 72 °C for 45 s followed by a 5-min extension at 72 °C. Unspliced COL1A1, COL1A2, and 18 S rRNA were amplified for 34, 32, and 23 cycles, respectively. Product sizes were measured using a 100-bp DNA ladder.

mTOR COL1A2 Poly(A) Tail Length—Fibroblasts were grown to confluence in 10-cm² dishes in DMEM supplemented with 10% FCS. Confluent fibroblasts were serum-starved in DMEM containing 0.1% BSA for 24 h. LY294002 (40 µM) or Me₂SO was added for 6 h. Actinomycin D (8 µM) was then added, and cells were scrapped at the indicated time points. Poly(A) tails were amplified based on the procedure of Salles and Strickland (40). Total RNA was isolated, and 2 µg of RNA was converted to cDNA using the lower poly(DT) primer. Primers were utilized at a final concentration of 0.6 µM and MgCl₂ at a final concentration of 1.5 mM in a total volume of 50 µl. Amplification was for 5 min at 95 °C and then 26 cycles of 95 °C for 1 min, 59 °C for 30 s, and 72 °C for 30 s followed by a 5-min extension at 72 °C. The 3′ poly(A) tail was amplified by using upper primer (5′-CGG AGT CCG CTT TTT TTT-3′) and lower poly(D) primer (5′-GCC AGT CCG CTT TTT TTT TTT T3′). Product sizes were measured using a 50-bp DNA ladder.

**Procollagen Analysis on SDS-Polyacrylamide Gel**—Fibroblasts were seeded in 12-well plates and grown to confluence in DMEM supplemented with 10% FCS. Confluent fibroblasts were serum-starved in DMEM containing 0.1% BSA and 50 µg/ml ascorbic acid for 24 h. LY294002 was then added at the indicated concentrations for 48 h in the presence of ascorbic acid. 3′-[35S]-Proline (20 µCi/ml, 3.66 TBq/mmol) was added during the last 24 h of incubation. Medium was collected from each well, and cells were trypsinized and counted with a Zeta™ series Coulter® cell counter from Beckman-Coulter Inc. (Miami, FL). Medium normalization of cell numbers were corrected using a Speed-vac, denatured by boiling in SDS sample buffer containing 15 mM dithiothreitol, and loaded on a 6% SDS-polyacrylamide gel. Media were then electrophoresed, and gels were enhanced using Fluoro-Hance (Research Products International, Mount Prospect, IL) and visualized by autoradiography. The nature of the collagen bands served as changes in electrophoretic mobility. The 3′ polyproline was used to determine if the collagen transport to the extracellular space might result in the decrease in collagen biosynthesis, steady-state mRNA levels of COL1A1 and COL1A2, and 18 S rRNA, showed that 24 h after LY294002 (20 µM) addition, the amount of COL1A1 and COL1A2 mRNA decreased in a dose-dependent manner (Fig. 1C). The inhibition of COL1A1 was more pronounced than that of COL1A2, whereas TIMP-1 mRNA showed increased steady-state mRNA levels. This evidence suggests that LY294002 decreases type I collagen protein levels by decreasing its mRNA levels.

**RESULTS**

**LY294002 Selectively Decreases Collagen Protein and mRNA Levels**—We investigated the ability of PI 3-kinase or related proteins to regulate collagen production by using LY294002, a PI 3-kinase inhibitor. LY294002 (16 and 32 µM) was added to cells that were serum-starved for 24 h and labeled with [35S]proline. In Fig. 1A, LY294002 decreased types I and III collagen protein levels, suggesting that PI 3-kinase or a related protein regulates collagen production. However, the possibility that LY294002 was a general inhibitor of protein synthesis remained. Further experiments showed that total protein levels were not decreased after LY294002 (80 µM) addition (Fig. 1B). Taken together, these experiments suggest that LY294002 selectively inhibits collagen synthesis. Additional experiments were designed to investigate the affected point in type I collagen biosynthesis. PI 3-kinase has been shown to be involved in the sorting and transport of some lysosomal proteins (42, 43). Therefore, it was possible that a block in collagen transport to the extracellular space might result in the decrease in collagen production observed in our assays. Thus, to define further the role of LY294002 in collagen biosynthesis, steady-state mRNA levels were analyzed. Northern blot analysis with [32P]-labeled cDNA probes for COL1A1 and COL1A2, normalized to 18 S rRNA, showed that 24 h after LY294002 (20–80 µM) addition, COL1A1 and COL1A2 mRNA decreases in a dose-dependent manner (Fig. 1C). The inhibition of COL1A1 was more pronounced than that of COL1A2, whereas TIMP-1 mRNA showed increased steady-state mRNA levels. This evidence suggests that LY294002 decreases type I collagen protein levels by decreasing its mRNA levels.
To define further how LY294002 decreases type I collagen steady-state mRNA levels, the amounts of unspliced (newly transcribed) collagen message were measured. A reduction in steady-state mRNA levels could signify a decrease in either transcription or message stability. Hence, the influence of LY294002 on COL1A1 and COL1A2 transcription was explored. To measure COL1A1 and COL1A2 transcription, we utilized a previously described method based on the PCR (39).

Cells were treated for 24 h with or without LY294002 (40 μM). Primers to exon 46 and intron 46 were used to measure unspliced (newly transcribed, heterogeneous nuclear RNA) COL1A1 mRNA. Exon 1 and intron 1 primers were utilized to measure unspliced COL1A2 mRNA (Fig. 1D). Quantification of the bands by NIH image densitometry software and normalization to 18 S rRNA showed that COL1A1 unspliced mRNA levels were decreased after LY294002 treatment (average 91.3% decrease ± 6%), pointing to transcriptional regulation. However, COL1A2 mRNA levels were not significantly dimin-
decrease in collagen mRNA stability, whereas TGF-

amino acid deprivation (4) and a lack of cysteine (46) cause a

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lysis of COL1A2 at the transcriptional

collagen type I production

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Collagen Message Stability Is Decreased by LY294002—To explore additional post-transcriptional mechanism altered by

LY294002, we examined the effect on collagen message stability.

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and pulse labeling (45) yield similar results when measuring

message stability. Prior evidence that mRNA stability plays a role in collagen regulation also exists. For example,

amino acid deprivation (4) and a lack of cysteine (46) cause a
decline in collagen mRNA stability, whereas TGF-β has been implicated in the stabilization of collagen mRNA (44). To measure

messengers stability, cells were serum-starved for

4 h and then treated with LY294002 (40 μM) for 12 h. Fibro

blasts were collected at 0, 4, 6, 8, and 10 h after the addition of

actinomycin D (8 μM). RNA was electrophoresed, and the mem

branes were then hybridized with 32P-labeled cDNA probes for

COL1A1 and COL1A2. The results showed that LY294002

significantly decreased COL1A1 and COL1A2 message stabil

ity by 2.02- and 2.46-fold, respectively (Fig. 2, A and B). There

fore, LY294002 can decrease collagen type I production

through destabilization of the collagen messenger.

Numerous factors, including changes in protein binding, activ

ation of mRNA surveillance, and changes in poly(A) tail

length may regulate mRNA stability. Poly(A) tails serve to

stabilize mRNAs by protecting them from nuclease attack, and
deadenylation of the poly(A) tail precedes the degradation of

most mRNAs (47). To confirm further that LY294002 alters

COL1A2 stability, we measured poly(A) tail lengths. The

COL1A2 message contains five different polyadenylation sites

(48, 49), but for simplicity only the 3’-most poly(A) tail was

amplified. Primers were designed to encompass the end of the

3’UTR of COL1A2 and the poly(A) tail (Fig. 2, C). Reverse

transcription using a poly(dT) primer with a GC anchor, fol

lowed by PCR with 3’-UTR and poly(dT) primers, allowed vi

ualization of the COL1A2 poly(A) tail length (~75 adenosines

at 0 h). Specific amplification was demonstrated by the release of a 45-bp fragment after HindIII digestion (data not shown).

Cells were treated with or without LY294002 for 6 h; transcrip

tion was then halted with actinomycin D (8 μM). It was found

that the poly(A) tail of COL1A2 decreased at a faster rate in

LY294002-treated cells than in control cells consistent with

destabilization of the COL1A2 message (Fig. 2D).

Collagen Steady-state mRNA Levels Are Decreased after the

Addition of a LY294002 Analogue but Not Wortmannin—To evaluate further the role of PI 3-kinase in collagen production,

wortmannin, a structurally unrelated inhibitor of PI 3-kinase,

was used. In contrast to the expected decrease in collagen

synthesis, we observed no consistent effect on COL1A1 or

COL1A2 mRNA levels at concentrations reported to decrease

PI 3-kinase activity (10–1000 nM) (Fig. 3A).

Based on these results, we tested the effects of LY303511, an

inactive LY294002 analogue. LY303511 contains a one-atom

substitution that abolishes its ability to inhibit PI 3-kinase

activity (50). Its actions on other signaling molecules have not

been described. At concentrations of 20–80 μM, LY303511 de
creased COL1A1 and COL1A2 steady-state mRNA levels in a
dose-dependent manner (Fig. 3B). These results suggest that a
PI 3-kinase-related molecule that is not inhibited by wortman-
nin is responsible for the inhibitory action of LY294002.

It has been reported that wortmannin is unstable in media
for extended periods (51). Thus, it was possible that PI 3-kinase
activity returned to normal levels during the course of the
experiment, resulting in no effect on collagen mRNA steady-
state levels. To test whether the PI 3-kinase pathway remained
suppressed after incubation with the various inhibitors, we
examined the phosphorylation of Akt, a downstream target of
PI 3-kinase. Inhibitor addition for 24 h showed that LY294002
and wortmannin decreased Akt phosphorylation to a similar
extent (Fig. 3C). Furthermore, an in vitro PI 3-kinase assay
verified that LY294002 and wortmannin inhibit PI 3-kinase
activity, but neither rapamycin nor LY303511 inhibit its activ-
ity (Fig. 3D). Therefore, these observations confirm that although
wortmannin and LY294002 efficiently block PI 3-kinase
activity in our experiments, only LY294002 specifically
decreases type I collagen production. Thus, it is apparent that
LY294002 inhibits a molecule other than PI 3-kinase that is
refractory to wortmannin but regulates type I collagen.

Because our data suggested an alternative target for
LY294002, we examined the effect of inhibitor treatment on
4EBP1 phosphorylation, a translation regulator downstream of
mTOR (Fig. 3E). Within a cell, increases in phosphorylation of
4EBP1 dissociate it from its target eIF4E thus increasing cap-
dependent translation. Changes in 4EBP1 phosphorylation can
be observed as alterations in electrophoretic mobility. The more
highly phosphorylated form was arbitrarily assigned as
\[H_9254\] and the least phosphorylated form as
\[H_9251\]. LY294002 resulted in the greatest reduction in 4EBP1 phosphorylation to
almost exclusively the
\[H_9251\] form, whereas rapamycin and wort-
mannin diminished the
\[H_9254\] form of 4EBP1 and amplified the less
phosphorylated
\[H_9253\] form. In addition, the
\[H_9252\] form is now present
in the rapamycin- and wortmannin-treated cells, although
more substantially in the rapamycin-treated cells. Together
these results show wortmannin and rapamycin block mTOR
activity to a lesser degree than LY294002. This is consistent
with the observation that rapamycin is less efficacious toward
mTOR kinase activity as compared with LY294002 and clearly
demonstrates differences in their effects on mTOR and its
downstream targets. The strong effect of LY294002 on 4EBP1
dephosphorylation may also be a result of the activation of a
phosphatase not affected by wortmannin or rapamycin.

mTOR Regulates Collagen mRNA Levels—Because LY294002
can inhibit other PI 3-kinase-related molecules such as mTOR/
FRAP, ATM, ataxia telangiectasia-related, and DNA-protein ki-

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**Fig. 3.** A, wortmannin does not alter COL1A1 and COL1A2 mRNA steady-state levels. *Left panel,* cells were treated with inhibitor (10–1000 nM) and analyzed as above. A representative gel is shown. *Lane C,* control. *Right panel,* bars represent the average of at least three independent experiments with standard deviation. *B,* LY303511 decreases COL1A1 and COL1A2 mRNA steady-state levels. *Left panel,* cells were treated with inhibitor (20–80 μM) and analyzed as above. A representative gel is shown. *Lane C,* control. *Right panel,* bars represent the average of at least three independent experiments with standard deviation. C, Akt phosphorylation after LY294002, LY303511, rapamycin, and wortmannin addition. Cells were incubated with indicated inhibitors for 24 h. Protein (30 μg) from each condition was analyzed by Western blots using anti-phospho-Akt and anti-Akt antibodies. A representative gel is shown (inset). *Graph* represents quantitation of three independent experiments with standard deviation. *, p < 0.05. D, effect of LY294002, LY303511, rapamycin, and wortmannin on in vitro PI 3-kinase activity. Immunoprecipitated PI 3-kinase was incubated with the indicated inhibitors for 5 min before addition of cold ATP, PI, and [γ-32P]ATP. PI 3-kinase activity was assayed by measuring the conversion of PI to PI[γ-32P] by TLC and visualization by autoradiography. E, 4EBP1 phosphorylation after inhibitor treatment. Reagents were used at the following concentrations: LY294002, 40 μM; rapamycin, 10 nM; and wortmannin, 100 nM. Cells were incubated in the presence of inhibitors for 2 h followed by scraping and Western blot analysis with 4EBP1 antibody.
nase, we explored the possibility that mTOR is the relevant target of LY294002. The immunosuppressant rapamycin, in complex with FKBP12 (FK506-binding protein 12), inhibits mTOR at nanomolar concentrations. Therefore, the effects of rapamycin on collagen were examined. Type I collagen mRNA levels were significantly inhibited (50%) by rapamycin (10 nM) (Fig. 4A).

**Fig. 4.** A, rapamycin decreases COL1A1 and COL1A2 mRNA steady-state levels. *Left panel,* cells were treated with the indicated concentrations of inhibitor and analyzed as described under “Experimental Procedures.” *Lane C,* control. *Right panel,* bars represent the average of at least three independent experiments with standard deviation. B, mTOR-siRNA decreases endogenous mTOR levels. *Left panel,* cells were transfected with an mTOR-specific interfering RNA or non-silencing (NS) interfering RNA for 72 h. Endogenous mTOR levels were measured by quantitative real time RT-PCR. *Right panel,* bars represent the average of at least three independent experiments with standard deviation. C, decrease in endogenous mTOR levels reduces COL1A1 and COL1A2 mRNA levels. *Left panel,* cells were transfected with an mTOR-specific interfering RNA or non-silencing interfering RNA for 72 h. Experiments were performed in parallel with the RNA remaining from the real time RT-PCR experiments. Northern blots were hybridized to COL1A1 and COL1A2 probes, and mRNA levels were normalized to 28S and 18S rRNA. *Right panel,* bars represent the average of at least three independent experiments with standard deviation. D, collagen type I protein levels decrease with addition of mTOR-siRNA. For collagen protein levels, mTOR-siRNA was added for the indicated times. [14C]Proline and ascorbic acid were added to cells 24 h before media collection. Media normalized to cell numbers were electrophoresed and visualized by autoradiography. E, mTOR-KD decreases collagen mRNA levels. *Left panel,* mTOR-KD mRNA from electroporated samples was amplified using rat mTOR-specific primers. *Right panel,* cells were electroporated with 20 μg of mTOR-KD or pcDNA 3.0 and allowed to recover for 72 h. Type I collagen mRNA levels were analyzed by Northern blot.
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surprising, however, that rapamycin and LY294002 did not exhibit the same efficacy toward collagen. These differences are likely a reflection of their dissimilar mechanisms of action. Specifically, LY294002 competes for ATP binding to mTOR (50), whereas the rapamycin-FKBP12 complex, through steric hindrance, partially occludes the ATP-binding site of mTOR (17, 18, 22, 52, 53). However, the effect of rapamycin on mTOR function is still not precisely defined. For example, Peterson and co-workers (22, 54) reported that rapamycin does not inhibit the autophosphorylation of mTOR but instead may affect its translocation. Although rapamycin is generally thought to be specific for mTOR, decreases in other proteins have been shown after rapamycin addition, but this may be due to the key role of mTOR as a translational regulator (55). Regardless, our results point to the possibility that mTOR regulates collagen production.

We sought to confirm further the link between mTOR and collagen and to provide independent evidence for the role of mTOR in collagen gene expression. Therefore, we utilized an siRNA to specifically down-regulate mTOR levels. Decreased mTOR mRNA levels were confirmed by real time RT-PCR (Fig. 4B). In parallel, COL1A1 and COL1A2 mRNA levels were determined by Northern blot analysis from cells treated with non-silencing or mTOR-specific siRNA. Fig. 4C demonstrates that mTOR mRNA inhibition (56% (p = 0.08) results in a decrease in COL1A1 (75% (p = 0.0003) and COL1A2 (28%) (p = 0.0176) mRNA levels. These results are consistent with the differential effect of LY294002 on COL1A1 and COL1A2 mRNA levels as shown in Fig. 1. We extended these studies to determine the effect of decreased mTOR levels on collagen protein levels. We determined that mTOR-siRNA addition causes a marked reduction in types I and III collagen protein levels during a 48–96-h time period (Fig. 4D). β-Actin and TIMP-1 levels were not significantly affected (data not shown). The greater reduction in the protein levels as compared with the mRNA levels may be a reflection of the effect of mTOR at translational and post-translational levels in collagen biosynthesis. These results are consistent with the protein data obtained after LY294002 addition.

We also assessed the importance of the kinase domain of mTOR. Dermal fibroblasts were electroporated with a kinase-dead mTOR (D235SA) (28). This construct should compete with native mTOR proteins for their substrate but not initiate the phospho-transfer reaction. Electroporation of a green fluorescent protein containing plasmid performed in parallel revealed that ~30% of the cells received plasmid (data not shown). Electroporation of the mTOR-KD plasmid into cells was confirmed by specific amplification (Fig. 4E, left panel). Northern blot analysis measuring endogenous gene levels showed a significant decrease in type I collagen mRNA levels (10–20%, p = 0.0025, n = 7) after mTOR-KD electroporation (Fig. 4E, right panel). The relatively modest decrease in collagen mRNA after mTOR-KD electroporation, as compared with either rapamycin or siRNA, may reflect the low efficiency of plasmid delivery into primary cells and the possibility that endogenous mTOR function was not completely blocked. However, taking into account that only a proportion of fibroblasts received plasmid, the observed decrease in endogenous COL1A1 and COL1A2 levels suggests the importance of the kinase domain of mTOR. Taken together, these data provide evidence for the involvement of mTOR in type I collagen regulation.

DISCUSSION

This study demonstrates a positive role for mTOR in the regulation of type I collagen mRNA synthesis. We base this conclusion on our results showing that, in addition to LY294002, rapamycin, a mTOR-specific interfering RNA, and a kinase-dead mTOR decrease collagen steady-state mRNA levels, thereby identifying mTOR as a potential new regulator of the collagen type I gene (Fig. 4). In addition, even though the traditional linear PI 3-kinase pathway places mTOR downstream of PI 3-kinase, we demonstrate that mTOR regulates COL1A1 and COL1A2 mRNA synthesis in a PI 3-kinase-independent manner. Our results show that although both LY294002 and wortmannin inhibit PI 3-kinase, only LY294002 inhibits collagen production (Fig. 1 and Fig. 3, A and D). Furthermore, inhibition of type I collagen steady-state mRNA levels by LY303511, an LY294002 analogue that does not inhibit PI 3-kinase activity, argues against a PI 3-kinase-dependent pathway (Fig. 3B).

Significantly, earlier reports have found that wortmannin and LY294002 inhibit other PI 3-kinase-like proteins. For example, wortmannin inhibits PI 4-kinase (56, 57) and hSMG-1 (15), and wortmannin and LY294002 inhibit DNA-protein kinase (14) and mTOR (28). Although both agents inhibit PI 3-kinase and mTOR, higher concentrations of wortmannin are required for inhibition of mTOR, whereas LY294002 inhibits mTOR and PI 3-kinase with nearly identical potency (22, 28). Therefore, the disparity between type I collagen inhibition by LY294002 and wortmannin may exist due to their different efficacies toward mTOR.

Recent evidence further supports the existence of a PI 3-kinase-independent pathway leading to mTOR. Fang and colleagues (58) recently determined that mitogenic activation of mTOR by phosphatidic acid occurs independent of PI 3-kinase. Moreover, it was reported that CAMP inhibited mTOR activity via a PI 3-kinase-independent pathway (32). Most important, CAMP elevations correlate with reductions in collagen synthesis (59, 60). In addition, amino acid activation of mTOR occurs independent of Akt/protein kinase B and PI 3-kinase activation (31). Furthermore, prior evidence also exists for contrasting outcomes following LY294002 and wortmannin addition. For example, rapamycin and LY294002 inhibited nitric oxide production in Raw 264.7 cells, whereas wortmannin is ineffective (61). Likewise, studies in several different cell types have revealed that wortmannin and rapamycin exhibit distinct effects on the translation of individual mRNAs (62). Thus, it appears that there are multiple pathways leading to mTOR activation, which are utilized in different cellular processes.

On the other hand, previous studies (13) have also shown a role for PI 3-kinase in collagen regulation. Runyan et al. (63) determined that TGF-β-mediated collagen stimulation involves PI 3-kinase, but PI 3-kinase alone is not sufficient for collagen up-regulation in mesangial cells. Also, although our studies and those by Ricupero and et al. (13) conclude that LY294002 inhibits collagen production via a mechanism affecting message stability, we disagree on the pathway implicated. Ricupero et al. (13) proposed that a PI 3-kinase-dependent pathway regulates collagen based on the observation that both LY294002 and wortmannin inhibit collagen steady-state levels in lung fibroblasts. However, our data indicate that wortmannin does not inhibit collagen steady-state levels in dermal fibroblasts (Fig. 3A). Nonetheless, the literature provides numerous examples describing distinct pathways regulating the same cellular process in different cell types. It should also be noted that although the literature describes wortmannin as an unstable inhibitor, based on the phosphorylation of Akt after both LY294002 and wortmannin exposure for 24 h, we conclude that its capacity to inhibit PI 3-kinase in fibroblasts is comparable with LY294002 (Fig. 3C).

Published reports further substantiate our results that mTOR regulates collagen and provide additional connections between mTOR and collagen production. For example, recent studies linked mTOR to the signaling pathway of the α,β,γ...
mTOR Regulates Type I Collagen

in collagen regulation. It may also be relevant that cAMP elevation inhibits mTOR activity in vitro (32) as well as collagen gene expression in vitro and in vivo (59, 60). However, the mTOR and collagen studies were conducted in different experimental systems.

In the context of collagen regulation, it is unlikely that mTOR inhibits type I collagen via p70S6K or 4EBP1. COL1A2 experimental systems. the mTOR and collagen studies were performed in different collagen gene expression supports this theory. Also relevant are the findings that PKC and collagen production. Another likely explanation for the planification for the differential effects of LY294002 and rapamycin in activated hepatic stellate cells and contributes to elevated collagen levels in scleroderma fibroblasts (74, 75). Therefore, mTOR may be a suitable target for drug or gene therapy, useful in the treatment of fibrotic diseases.

Acknowledgments—We thank Drs. E. Carvile LeRoy, Kathryn Meier, and Steven Rosenzweig for critically reading this manuscript. We also thank Robert Abraham (Burnham Institute) for providing the kinase-dead mTOR, Chris Vilahos at Lilly for providing LY3053511, and Paul McDermott and Laura Spruill for their help with the real-time RT-PCR.

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J. Biol. Chem. 2004, 279:23166-23175. doi: 10.1074/jbc.M401238200 originally published online March 26, 2004

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