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Modulation of Collagen and *MMP-1* Gene Expression in Fibroblasts by the Immunosuppressive Drug Rapamycin

*A DIRECT ROLE AS AN ANTIFIBROTIC AGENT?**

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We have examined whether rapamycin, an immunosuppressive drug, may exert part of its antifibrotic activity by directly targeting fibroblast extracellular matrix deposition. Incubation of human lung fibroblast (WI-26) cultures with rapamycin led to dose- and time-dependent reduction in the expression of types I and III collagens, both at the protein and mRNA levels. Rapamycin had no effect on collagen promoter activity but accelerated mRNA decay, indicating post-transcriptional control of collagen gene expression. In contrast, rapamycin significantly enhanced the expression of interstitial collagenase (MMP-1) at the protein and mRNA levels and transcriptionally. We determined that rapamycin efficiently activates AP-1-driven transcription by rapidly inducing c-jun/AP-1 phosphorylation with activation of the c-Jun N-terminal kinase (JNK) cascade, resulting in enhanced binding of AP-1·DNA complex formation and AP-1-dependent gene transactivation. Conversely, the JNK inhibitor SP600125 inhibited rapamycin-induced *MMP-1* gene transactivation and AP-1/DNA interactions. A c-*jun* antisense expression vector efficiently prevented rapamycin-induced *MMP-1* gene transcription. Pharmacological inhibition of either ERK or p38 MAPK pathways was without effect on rapamycin-induced *MMP-1* gene expression. It thus appears that rapamycin may exert direct antifibrotic activities independent from its immunosuppressive action.

Fibrosis is a reactive process involving different pathophysiological events such as attraction of blood-born cells (*e.g.* leukocytes, platelets, activated lymphocytes), alteration of the microvasculature, and activation of resident mesenchymal cells (fibroblasts, endothelial cells, pericytes) leading to excessive extracellular matrix (ECM)[2] deposition (1–4). Possible explanations for the excessive deposition of collagen observed during the fibrotic process include both an increased biosynthesis or reduced degradation of ECM components, particularly that of fibrillar collagens, by fibroblasts. An accumulation of collagen may originate from accelerated production of collagen resulting from enhanced collagen gene transcription and/or mRNA stabilization in response to soluble factors present in the microenvironment. Alternatively, reduced matrix metalloproteinase (MMP) expression and subsequent inhibition of collagen degradation may also contribute to the fibrotic process (1). Thus, identifying molecules that may either affect collagen production negatively or MMP expression positively is of utmost importance to define novel therapeutic means against fibrosis. In this context, rapamycin (sirolimus), a *Streptomyces* fungus macrolide antibiotic with potent immunosuppressive properties, is currently used for the prevention of graft rejection in kidney transplant recipients (5–7). Several experimental studies have shown that rapamycin is also effective in preventing liver or pulmonary fibrogenesis in animal models (8–10).

At the cell membrane, rapamycin binds to the immunophilin FK506-binding protein (FKBP12). This rapamycin·FKBP12 complex interacts with the rapamycin binding domain of mTOR, a serine-threonine kinase, and thus inactivates mTOR known to control proteins that regulate mRNA translation initiation and G_1 progression in T cells (11). mTOR is a transducer that may be initiated by insulin, growth factors, and amino acids to activate downstream targets and regulate cell growth and proliferation as well as metabolic homeostasis (12, 13). It has been shown that ribosomal protein S6 kinases 1 and 2 (S6K-1 and S6K-2) and the eukaryotic initiation factor 4E-binding protein (4E-BP1) are downstream targets of mTOR. Rapamycin induces translational arrest by preventing phosphorylation of S6K-1 and 4E-BP1 by mTOR (14).

Investigations have demonstrated some anti-inflammatory effects of immunosuppressive drugs, including rapamycin, suggesting that they may be considered as antifibrogenic (15, 16). However, the molecular mechanisms underlying the effects of rapamycin on ECM gene expression remain poorly understood. Thus, the aim of this work was to investigate the direct effects of rapamycin on fibrosis-associated genes in fibroblasts and to elucidate the molecular mechanisms associated with the modulation of ECM gene expression by rapamycin. We identify distinct mechanisms by which rapamycin modulates both the expression of fibrillar collagen genes and that of interstitial collagenase/MMP-1.

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[2] The abbreviations used are: ECM, extracellular matrix; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; CAT, chloramphenicol acetyltransferase; RT, reverse transcription; EMSA, electrophoretic mobility shift assay.

TABLE 1
Primer sequences used for quantitative RT-PCR

Name	Forward sequence	Reverse sequence
COL1A1	5′-GGGCAAGACAGTGATTGAATA-3′	5′-ACGTCGAAGCCGAATTCCT-3′
COL1A2	5′-TCTCTACTGGCGAAACCTGTA-3′	5′-TCCTAGCCAGACGTGTTTCTT-3′
COL3A1	5′-CGCTCTGCTTCATCCCACTAT-3′	5′-CGGATCCTGAGTCACAGACAC-3′
MMP-1	5′-CCCCAAAAGCGTGTGACAGTA-3′	5′-GGTAGAAGGGATTTGTGCG-3′
TIMP-1	5′-GCTGACATCCGGTTCGTCTAC-3′	5′-GTTGTGGGACCTGTGGAAGTA-3′
GAPDH	5′-GCTCCTCCTGTTCGACAGTCA-3′	5′-ACCTTCCATGGTGTCTGA-3′

EXPERIMENTAL PROCEDURES

Cell Cultures—Human lung fibroblasts (WI-26) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G and 0.25 μg/ml Fungizone™) in 5% CO_2 at 37 °C.

Cell Viability Assessment—Equal numbers of WI-26 fibroblasts were cultured in 96-well cell culture clusters and treated with various concentrations of rapamycin for 12, 24, and 48 h. Cell viability was measured using an MTS assay according to the manufacturer's protocol (Promega, Madison, WI).

Reverse Transcription-PCR Analysis—Total RNA was extracted according to the manufacturer's instructions using RNeasy mini kit (Qiagen, Hilden, Germany). DNase I treatment (25 units, 15 min) of total RNA was performed to eliminate genomic contamination of the RNA samples. One microgram of total RNA was used for first strand cDNA synthesis using a RT-for-PCR kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA) using SYBRGreen PCR core reagents (Applied Biosystems). Reaction mixtures were incubated for 2 min at 50 °C followed by 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 45 s at 60 °C, 1.5 min at 68 °C, and finally 15 s at 95 °C, 20 s at 63 °C, and 15 s at 95 °C. For each sample, gene expression was corrected against glyceraldehyde-3-phosphate dehydrogenase mRNA level. Primers used for PCR reactions are shown in Table 1.

Transient Cell Transfections and Reporter Assays—Transient cell transfections were performed with jetPEI™ according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). pRSV-β-galactosidase was cotransfected in every experiment to monitor transfection efficiency. CAT activity was measured using [^{14}C]chloramphenicol as substrate followed by thin layer chromatography and quantitation with a PhosphorImager (Amersham Biosciences). Luciferase activity was determined with a commercial assay kit (Promega). For high transfection efficiency of the pRSV-AS-c-*jun* expression vector, cells were electroporated with a Nucleofector™ (Amaxa GmbH, Köln, Germany) according to the manufacturer's protocol. Transfection efficiency was estimated to be 80% by fluorescence-activated cell sorter analysis of a cotransfected green, fluorescent protein expression vector (data not shown).

Plasmid Constructs—−3500COL1A2/CAT (gift from Francesco Ramirez, Mount Sinai School of Medicine, New York, NY), −2300COL1A1/CAT (gift from John Varga, Northwestern University Feinberg School of Medicine, Chicago, IL), −400COL3A1/CAT (gift from Benoit de Crombrugghe, University of Texas M. D. Anderson Cancer Center, Houston, Texas), −517MMP-1-lux and corresponding AP-1 mutant

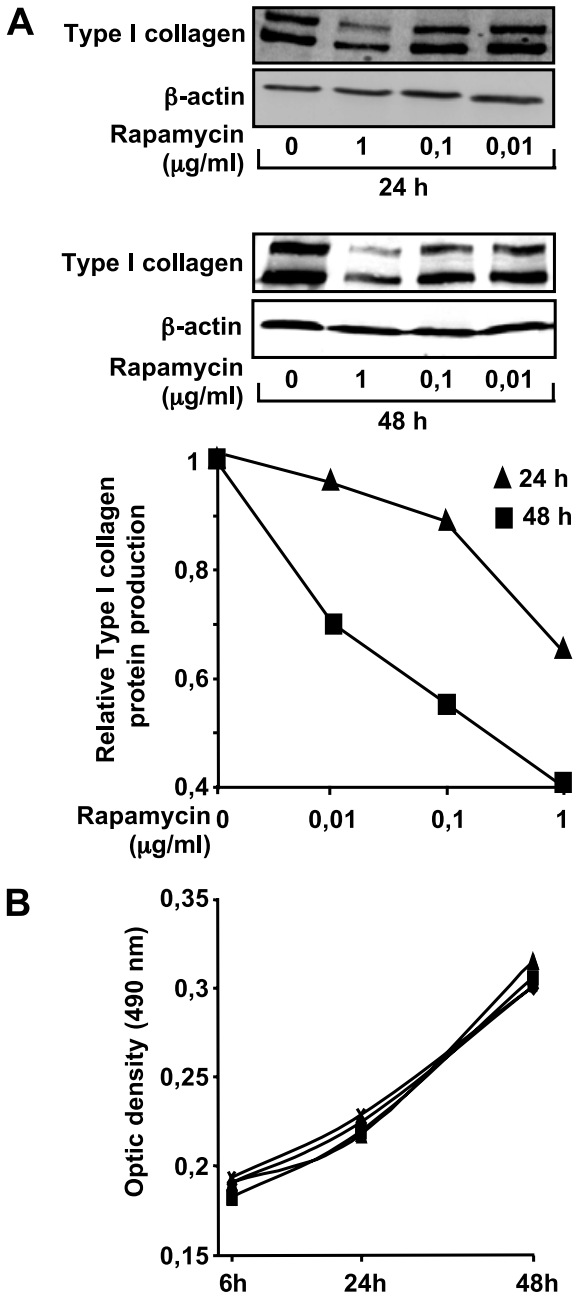

FIGURE 1. **Rapamycin decreases type I collagen protein synthesis.** *A*, serum-starved fibroblast cultures were treated with various concentrations of rapamycin (1, 0.1, or 0,01 μg/ml) for 24 (▲) and 48 h (■) as indicated. After incubations, type I collagen production was detected by Western blot analysis of whole cell lysates (*upper panel*). Specificity of the modulation was confirmed with an anti-actin antibody. The ratio of type I collagen to actin is plotted from the values of one representative experiment of four experiments (*lower panel*). *B*, fibroblast cultures were treated with various concentrations of rapamycin (10 μg/ml (■), 1 μg/ml (▲), or 0.1 μg/ml (X)) or not (◆) for 6, 24, or 48 h, after which cell viability was measured using an MTS assay. Values are the mean of three independent experiments, each performed with triplicate samples.

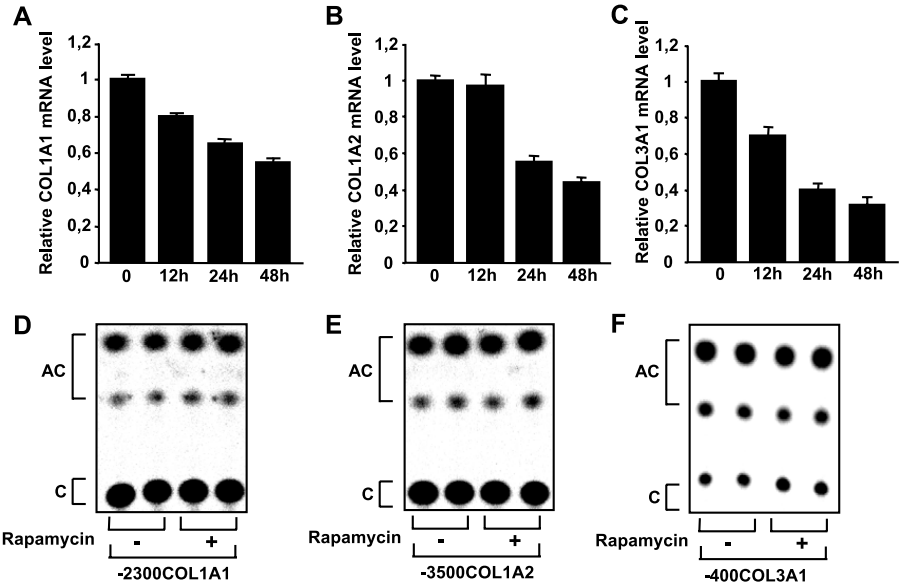

FIGURE 2. **Rapamycin decreases *COL1A1*, *COL1A2*, and *COL1A3* mRNA steady-state levels without repressing the activity of the corresponding promoters.** *A–C*, serum-starved subconfluent fibroblast cultures were treated with rapamycin (1 μg/ml) for 12, 24, or 48 h. After incubations, *COL1A1* (*A*), *COL1A2* (*B*), and *COL3A1* (*C*) mRNA steady-state levels were determined by quantitative RT-PCR. *Bars* indicate mean ± S.D. of at least three independent experiments performed, each with duplicate samples. *D–F*, serum-starved fibroblast cultures were transfected with −2300COL1A1/CAT (*D*), −3500COL1A2/CAT (*E*), and −400COL3A1/CAT (*F*) constructs. Twelve hours after transfections, rapamycin (1 μg/ml) was added and incubations continued for another 48 h. Autoradiograms shown are from a representative experiment, each for the three experiments being performed with duplicate samples.

construct (gifts from E. F. Wagner, Research Institute for Molecular Pathology, Vienna, Austria), and pRSV-AS-c-*jun* have been described previously (17–20). pAP1-TA-lux (Mercury pathway profiling vector; BD Biosciences) was used to evaluate AP-1-driven transcription.

Western Bot Analyses—Total protein cell extract (30 μg) in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) was denatured by heating at 95 °C for 3 min before resolution by SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose filters (Amersham Biosciences), immunoblotted with either anti-type I collagen (Southern Biotech, Birmingham, AL), anti-phospho-c-Jun, -c-Jun, -phospho-JNK, -JNK, -phospho-ERK, -ERK, -phospho-p38, -p38, or -actin (Sigma) antibodies, all at a dilution of 1:1000 in phosphate-buffered saline/5% nonfat milk for 1 h. Anti-phospho-c-Jun, -phospho-JNK, -phospho-ERK, -phospho-p38, -ERK, and -p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-c-Jun and -JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After incubation, filters were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000 for 1 h. Filters were then washed, developed according to chemiluminescence protocols (ECL, Amersham Biosciences), and revealed with a PhosphorImager (Amersham Biosciences).

Quantative Determination of MMP-1 Production—The MMP-1 production in cell supernates was determined using the Quantikine Human pro-MMP-1 Immunoassay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assays (EMSA)—A consensus AP-1 binding oligonucleotide (Promega) was used as a probe to detect AP-1/DNA interactions. Nuclear extracts were isolated using a small-scale preparation (21). For supershift experiments, nuclear extracts (5–7 μg) were incubated overnight at 4 °C with an anti-c-Jun antibody (Santa Cruz Biotechnology). Binding mixtures were separated electrophoretically on native 4% acrylamide gels.

Reagents—Rapamycin was purchased from Sigma. The p38 and ERK inhibitors SB203580 and PD98059 were purchased from Tocris (Ellisville, MO). The JNK inhibitor SP600125 was from Calbiochem.

RESULTS

Rapamycin Inhibits Type I Collagen Production in Human Lung Fibroblasts—We first wanted to determine whether rapamycin had a direct effect on collagen production by fibroblasts. To address this point, WI-26 human lung fibroblast cultures were incubated with various concentrations of rapamycin, and type I collagen production was measured by Western blot analysis. As shown in Fig. 1*A*, total type I collagen production was reduced by rapamycin in a time- and dose-dependent manner. Specifically, a decrease of 10 and 45% with a rapamycin concentration of 0.1 μg/ml and a decrease of 37 and 60% with a concentration of 1 μg/ml were respectively observed after 24 h (*upper panel*) and 48 h (*lower panel*) of treatment. Interestingly, even the smallest concentration of rapamycin (0.01 μg/ml) had a significant inhibitory activity after 48 h of incubation, suppressing collagen production by as much as 30%.

To ensure that the inhibition of collagen production was not because of a cytotoxic effect of rapamycin, WI-26 fibroblasts were incubated for 6, 24, and 48 h with rapamycin concentrations ranging from 0.1 to 10 μg/ml, and cell viability was determined at various time points. As shown in Fig. 1*B*, no significant cell mortality was observed at any of the concentrations tested over a 48-h period.

Rapamycin Decreases COL1A1, COL1A2, and COL3A1 mRNA Steady-state Levels by Decreasing mRNA Stability and without Affecting Gene Transcription—To determine whether the reduction of fibrillar collagen production by rapamycin occurred via modulation of the corresponding genes, we first measured *COL1A1*, *COL1A2*, and *COL3A1* mRNA steady-state levels following rapamycin treatment by quantitative RT-PCR. As shown in Fig. 2, rapamycin at a concentration of 1 μg/ml decreased *COL1A1* (Fig. 2*A*), *COL1A2* (Fig. 2*B*), and *COL3A1*

(Fig. 2*C*) mRNA levels in a time-dependent manner, the extent of inhibition reaching 48, 56, and 70%, respectively, after 48 h.

To determine whether the effect of rapamycin on *COL1A1*, *COL1A2*, and *COL3A1* expression takes place at the transcriptional level, transient cell transfections were performed with the collagen promoter/gene reporter constructs −2300COL1A1/CAT, −3500COL1A2/CAT, and −400COL3A1/CAT. As shown in Fig. 2, *COL1A1* (Fig. 2*D*), *COL1A2* (Fig. 2*E*), and *COL3A1* (Fig. 2*F*), gene promoter activities were not altered by rapamycin, suggesting that the effect of rapamycin observed at the mRNA levels (see above) does not occur via transcriptional regulation of the corresponding genes.

We thus investigated whether rapamycin may affect the stability of *COL1A1*, *COL1A2*, and *COL3A1* mRNAs. For this purpose, fibroblasts were incubated with the transcription inhibitor actinomycin D for one hour prior to addition of rapamycin. mRNA decay was then measured in the absence or presence of rapamycin. As shown in Fig. 3, treatment with rapamycin accelerated *COL1A1* (Fig. 3*A*), *COL1A2* (Fig. 3*B*), and *COL3A1* (Fig. 3*C*) mRNA decay, thus significantly reducing their half-lives. *COL1A1*, *COL1A2*, and *COL3A1* mRNA half-lives were estimated to be about 16 h in the absence of rapamycin and dropped to around or slightly below 6 h with rapamycin. Together, these results indicate that rapamycin inhibits fibroblast collagen production via post-transcriptional mechanisms that affect *COL1A1*, *COL1A2* and *COL3A1* mRNAs stability.

Rapamycin Increases MMP-1 Gene Expression and Protein Synthesis—We first wanted to determine whether rapamycin had direct effect on MMP-1 synthesis. To address this point, MMP-1 protein production was measured using MMP-1 immunoassay approach. As shown in Fig. 4*A*, rapamycin treatment of WI-26 fibroblasts led to increase in MMP-1 protein production, up to 2.2- and 4.5-fold, respectively, after 48 and 72 h at the concentration of 1 μg/ml.

We next determined whether rapamycin modulates *MMP-1* and/or *TIMP-1* gene expression. To address this point, the effects of rapamycin on *MMP-1* and *TIMP-1* mRNA steady-state levels were studied by quantitative RT-PCR. Rapamycin treatment of WI-26 fibroblasts led to a time- and dose-dependent increase in *MMP-1* mRNA levels, up to 3.5-fold after 48 h at the concentration of 1 μg/ml (Fig. 4, *B* and *C*). On the other hand, *TIMP-1* gene expression remained unaltered over the same incubation period (not shown).

c-Jun Mediates Rapamycin-driven MMP-1 Gene Transcription—Most MMP genes exhibit promoter regions that are characterized by a functional AP-1 binding site around position −70 relative to the transcription initiation site that is critical for transcriptional regulation by a variety of stimuli, including cytokines (22). The effect of rapamycin on *MMP-1* promoter transactivation was examined in parallel transient cell transfection experiments using either a wild-type *MMP-1* reporter construct −517MMP-1-lux or an AP-1 mutant not able to bind AP-1 proteins (23). Results shown in Fig. 5*A* demonstrate that the effect of rapamycin on *MMP-1* gene expression was transcriptional, as rapamycin induced a 2.5-fold transactivation of the *MMP-1* promoter. Furthermore, we determined that the integrity of *MMP-1* promoter AP-1 binding site is necessary for transactivation by rapamycin that fails to transactivate the AP-1 mutant construct.

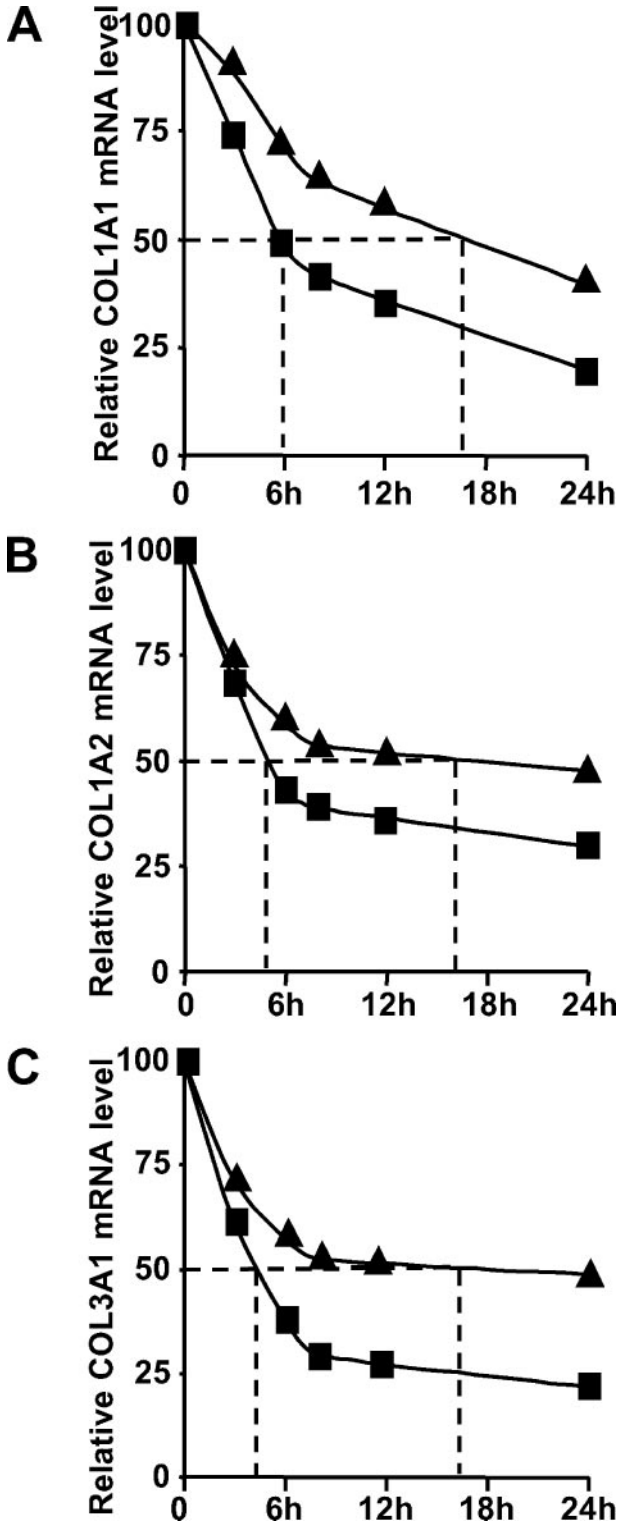

FIGURE 3. **Rapamycin decreases *COL1A1*, *COL1A2*, and *COL3A1* mRNA stability.** Serum-starved fibroblast cultures were treated with the transcription inhibitor actinomycin D (1 μg/ml). One hour later, cells were treated (■) or not (▲) with rapamycin (1 μg/ml) for 3, 8, 12, and 24 h. *COL1A2* (*A*), *COL1A2* (*B*), and *COL3A1* (*C*) mRNA decay was measured by quantitative RT-PCR. Results are shown as a percentage of mRNA remaining compared with time 0 before addition of rapamycin. Values of a representative experiment are shown (out of 3).

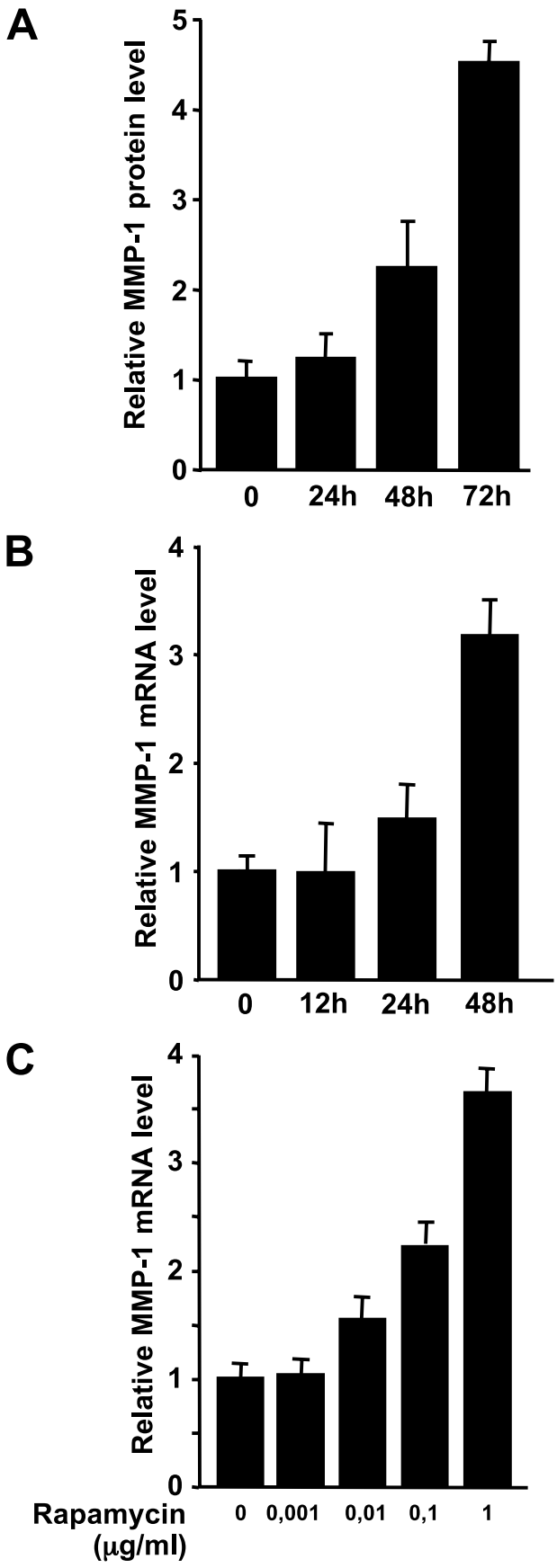

FIGURE 4. **Rapamycin increases MMP-1 both at mRNA and protein levels.** *A*, serum-starved subconfluent fibroblasts were treated with rapamycin (1 μg/ml) for various times (24, 48, and 72 h). After incubation cell culture supernates were collected, and MMP-1 protein levels were estimated by immunoassay. *Bars* indicate mean ± S.D. of two independent experiments performed, each with sixplicate samples. *B* and *C*, serum-starved subconfluent fibroblast cultures were treated with rapamycin (1 μg/ml) for various times (12, 24, and 48 h) (*B*) or with various concentrations of rapamycin (0.001, 0.01, 0.1, and 1 μg/ml) for 48 h (*C*). *MMP-1* mRNA levels were estimated by quantitative RT-PCR. *Bars* indicate mean ± S.D. of two independent experiments performed, each with duplicate samples.

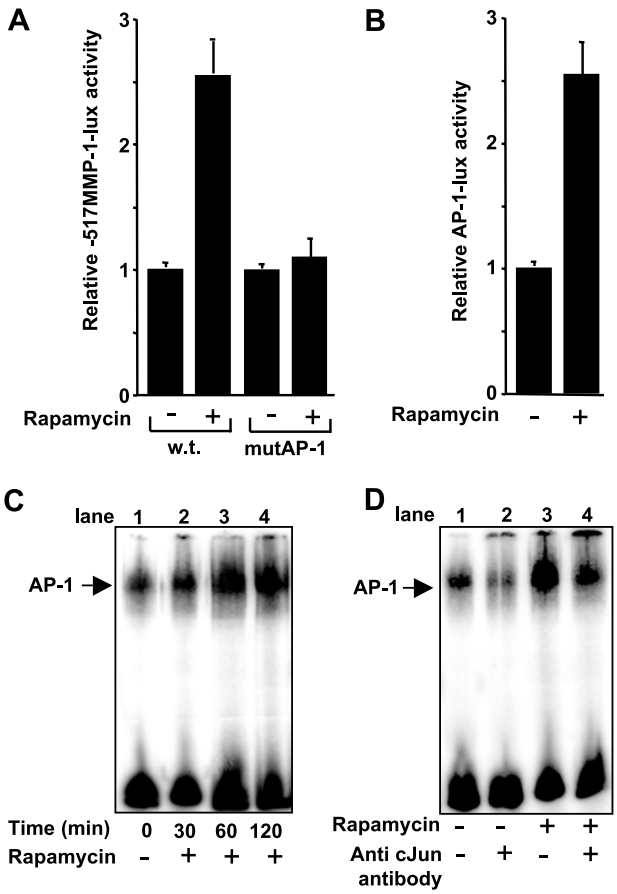

FIGURE 5. **Critical role for AP-1 in mediating rapamycin-driven transcription of MMP-1.** *A*, serum-starved fibroblast cultures were transfected with either wild-type (*w.t.*) or AP-1 mutant (*mutAP-1*) *MMP-1* promoter constructs. Six hours later, cells were treated or not with rapamycin (1 μg/ml) for 24 h. *Bars* indicate mean ± S.D. of three independent experiments performed, each with duplicate samples. *B*, serum-starved fibroblast cultures were transfected with pAP-1-lux in presence or absence of rapamycin (1 μg/ml, 24 h). *Bars* indicate mean ± S.D. of three independent experiments performed, each with duplicate samples. *C* and *D*, EMSA experiments were performed using a consensus AP-1 oligonucleotide as a probe, together with nuclear extracts from control (*C*, *lane 1*; *D*, *lanes 1* and *2*) and rapamycin-treated (1 μg/ml for 30, 60, and 120 min, respectively (*C*, *lanes 2*, *3*, and *4*) and for 30 min (*D*, *lanes 3* and *4*)) fibroblast cultures. Supershift assay was carried out with an anti-c-Jun antibody (*D*, *lanes 2* and *4*).

Next, the effect of rapamycin on AP-1-driven transcription was examined in transient cell transfection experiments with an artificial, multimerized AP-1 reporter construct, pAP1-TA-lux. As shown in Fig. 5*B*, rapamycin enhanced pAP1-TA-lux activity ~2.6-fold above control levels, attesting for the capacity of rapamycin to transactivate AP-1-dependent promoters.

To determine whether rapamycin modulates AP-1/DNA interactions, EMSA experiments were carried out with a radiolabeled consensus AP-1 oligonucleotide as a probe. As shown in Fig. 5*C*, AP-1·DNA complexes were detected with nuclear

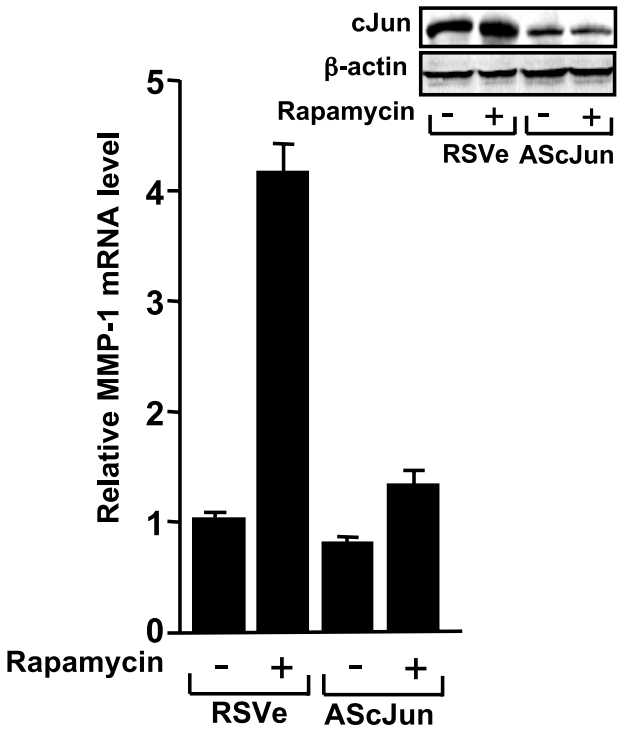

FIGURE 6. **Critical role for c-Jun in mediating rapamycin induced *MMP-1* gene expression.** Serum-starved subconfluent fibroblast cultures were electroporated with pRSV-AS-c-*jun* or RSVe. 24 h later, cells were treated with rapamycin (1 μg/ml) for 48 h. After incubations, *MMP-1* mRNA levels were detected by quantitative RT-PCR. *Bars* indicate mean ± S.D. of two independent experiments performed with duplicate samples. The expression of c-Jun was controlled by Western blot in parallel cultures (*inset*).

extracts from unstimulated control WI-26 fibroblast cultures (*lane 1*). Rapamycin induced a 1.5-, 4.2-, and 6.5-fold elevation of AP-1·DNA binding complexes after 30 min, 1 h, and 2 h of treatment, respectively (*lanes 2–4*).

To identify whether c-Jun, the main component of AP-1 complexes (24), was present in the protein·DNA complex identified with the AP-1 probe, supershift experiments were performed with an anti-c-Jun antibody. Results presented in Fig. 5*D* indicate that the retarded band obtained with nuclear extracts from control extracts (*lane 1*) and elevated in nuclear extracts from rapamycin-treated fibroblasts (*lane 3*) is significantly reduced by the anti-c-Jun antibody (*lanes 2* and *4*), attesting for the presence of c-Jun in the complexes.

To verify the implication of c-Jun in the activation of *MMP-1* gene expression by rapamycin, an antisense c-*jun* expression vector, which specifically targets c-*jun* expression and no other Jun family member (19, 25), was transfected into WI-26 fibroblasts prior to rapamycin treatment. Inhibition of c-Jun expression in antisense-transfected cells, treated or not with rapamycin, was verified by Western blotting (Fig. 6, *inset*). Quantitative RT-PCR analysis of *MMP-1* mRNA steady-state levels indicated a 95% reduction in rapamycin effect on *MMP-1* gene expression when cells received the antisense c-*jun* vector prior to incubation with rapamycin (Fig. 6). Taken together, these results demonstrate the critical role played by c-Jun in mediating rapamycin-dependent *MMP-1* gene expression at the transcriptional level.

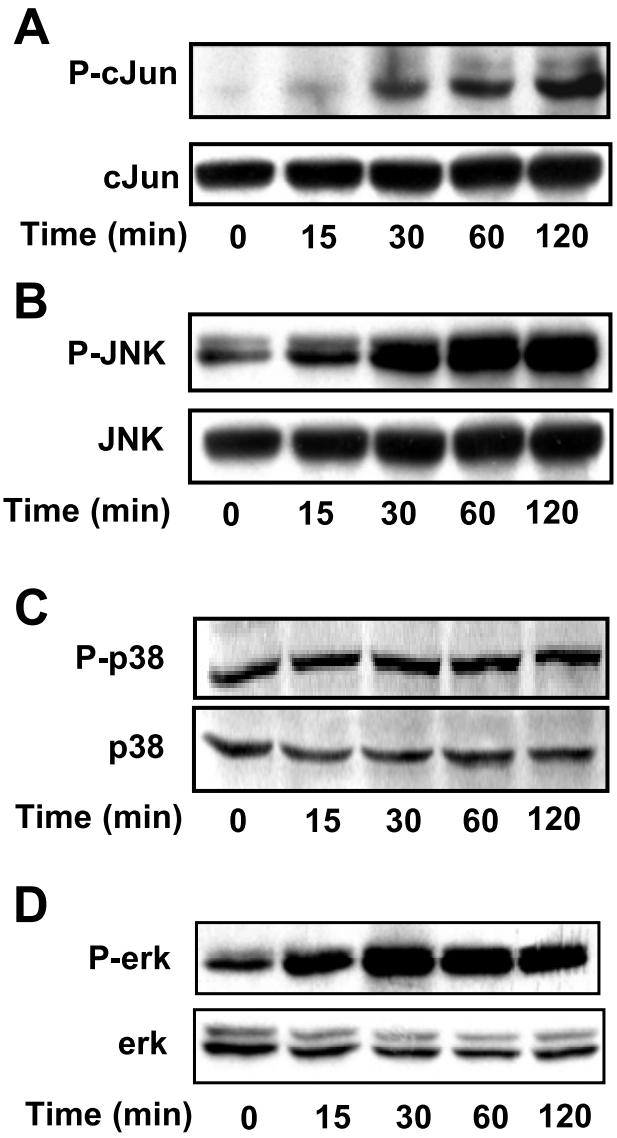

FIGURE 7. **Rapamycin stimulates c-Jun, JNK, and ERK phosphorylation.** Serum-starved fibroblast cultures were treated with rapamycin (1 μg/ml) for 15, 30, 60, or 120 min as indicated. After incubations, Phospho-c-Jun (*A*), phospho-JNK (*B*), phospho-p38 (*C*), and phospho-ERK (*D*) levels were detected by Western blot analysis of whole cell lysates (*upper panels*). Specificity of the modulation was confirmed respectively with an anti-c-Jun, anti-JNK, anti-p38, and anti-ERK antibodies (*lower panels*).

JNK Mediates Rapamycin-driven AP-1 Transcriptional Activity and Subsequent Modulation of MMP-1 Gene Expression—MAPKs act as signal transducers originating from numerous extracellular stimuli. MAPKs of the c-Jun N-terminal kinase family are critical determinants of the strength of AP-1-dependent transcription, as JNK phosphorylation of Jun protein members confers them maximal transcriptional potential (26, 27). Likewise, p38 and ERK contribute to potentiate Fos-mediated transcription (27). At present, little is known about their possible role downstream of rapamycin.

Given the effects of rapamycin on AP-1-dependent transcription and the implication of the *MMP-1* promoter AP-1 binding site in mediating rapamycin effect on *MMP-1* gene expression, we investigated the capacity of rapamycin to induce

c-Jun phosphorylation and JNK activation. As shown in Fig. 7*A*, rapamycin addition to WI-26 fibroblast cultures induced a rapid and prolonged phosphorylation of c-Jun on Ser-63, detectable as early as 15 min following stimulation and persisting at least 2 h. Likewise, rapamycin efficiently and rapidly induced JNK phosphorylation with a very similar kinetic (Fig. 7*B*). On the other hand, rapamycin had no effect on p38 MAPK activation (Fig. 7*C*), whereas ERK phosphorylation was stimulated as early as 15 min, peaked at 30 min, and slowly came down to basal level over the 2-h period following rapamycin addition (Fig. 7*D*).

To assess the role played by MAPKs in mediating rapamycin-driven induction of *MMP-1* gene expression, we investigated the ability of specific inhibitors of the JNK, p38, and ERK pathways to alter rapamycin-driven *MMP-1* gene expression. Results shown in Fig. 8*A* indicated that pretreatment of fibroblast cultures with the specific JNK inhibitor SP600125 totally inhibited the effect of rapamycin on *MMP-1* mRNA level. In contrast, the ERK and p38 inhibitors PD98059 and SB203580 failed to suppress rapamycin effect.

Next, in a similar experimental approach, the JNK inhibitor SP600125 prevented rapamycin-driven AP-1 transcriptional activity, as measured in transient cell transfection experiments with pAP-1-TA-lux (Fig. 8*B*), whereas the ERK and p38 inhibitors PD98059 and SB203580 did not. Finally, EMSA experiments with a radiolabeled consensus AP-1 oligonucleotide demonstrated that a 1-h pretreatment with SP600125 abolished rapamycin-driven elevation of AP-1·DNA complexes (Fig. 8*C*, *lane 4 versus lane 2*). Together, these experiments demonstrate the critical and specific role played by the JNK pathway in mediating rapamycin effects on both AP-1-dependent transcription and *MMP-1* gene expression in human lung fibroblasts.

DISCUSSION

In this report, we provide evidence that the immunosuppressive drug rapamycin, an inhibitor of mTOR, may exert direct antifibrotic activities both by down-regulating type I and type III collagen synthesis and by up-regulating *MMP-1* synthesis. In accordance with a previous report showing that a mTOR-specific interfering RNA and a kinase dead mTOR decrease type I collagen steady-state mRNA levels (28), we demonstrate that rapamycin inhibits *COL1A1*, *COL1A2*, and *COL3A1* gene expression by decreasing their mRNA stability.

Many reports demonstrated that collagen deposition is precisely controlled by collagen mRNA stabilization. For example, Krupsky *et al.* (29) demonstrated that *COL1A1* mRNA stability is reduced by amino acid deprivation in lung fibroblasts. Furthermore, retinoic acid and prostaglandin E_2, which decrease the uptake of amino acids transported, decrease the steady-state levels of *COL1A1* mRNA by decreasing its stability (30, 31). In the context of mTOR inhibition, Peng *et al.* (32) have shown that deprivation of amino acids induces a cellular stress response similar to that elicited by rapamycin, supporting the conjecture that mTOR acts as a nutritional sensor, and that pharmacological inhibition of mTOR by rapamycin, in some respects, mimics the signal induced by amino acid deprivation. Interestingly, it was recently demonstrated that FK506 signifi-

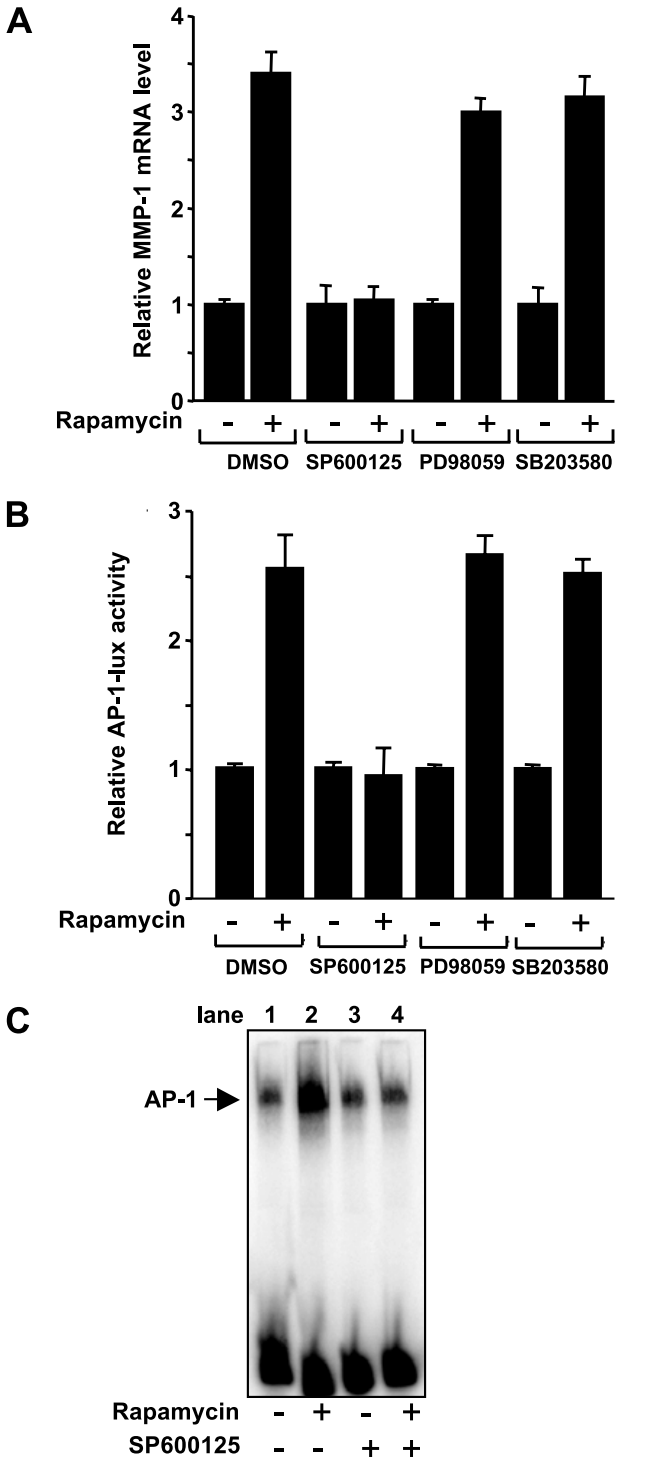

FIGURE 8. **Critical role for JNK in mediating rapamycin effects on *MMP-1* gene expression.** *A*, serum-starved subconfluent fibroblast cultures were treated with rapamycin (1 μg/ml) for 48 h in presence or absence of SP600125 (10 μM), PD98059 (10 μM), or SB203580 (10 μM) as indicated. After incubations, *MMP-1* mRNA levels were detected by quantitative RT-PCR. *Bars* indicate mean ± S.D. of three independent experiments performed with duplicate samples. *B*, serum-starved fibroblast cultures were transfected with pAP-1-lux construct. 6 h after, cells are treated with rapamycin (1 μg/ml, 24 h) in presence or absence of SP600125 (10 μM), PD98059 (10 μM), or SB203580 (10 μM) as indicated. *Bars* indicate mean ± S.D. of three independent experiments performed, each with duplicate samples. *C*, EMSA experiment was performed using the AP-1-specific oligonucleotide as a probe, together with nuclear extracts from control (*lanes 1* and *3*) and rapamycin-treated (1 μg/ml for 60 min; *lanes 2* and *4*) fibroblast cultures in presence of SP600125 (10 μM; *lanes 3* and *4*) or in absence of this inhibitor (*lanes 1* and *2*).

cantly reduces the basal expression of the human *COL1A2* mRNA levels in scleroderma fibroblasts by reducing their stability (33). Thus, our results suggest that rapamycin may be an interesting candidate drug for the treatment of fibrotic conditions because an increase in collagen mRNA stability contributes to elevated collagen levels in scleroderma fibroblasts (34).

We also demonstrated that rapamycin increases *MMP-1* gene expression. We provide definitive evidence for a direct role for the JNK/c-Jun pathway in mediating rapamycin effect at the transcriptional level through enhanced AP-1-dependent mechanisms. Consistent with our findings, it has been shown that mTOR inhibition by rapamycin induces rapid and sustained activation of JNK and consequently elevation of c-Jun phosphorylation in human rhabdomyosarcoma cell lines via 4E-BP1-dependent activation of the MKK4/7-JNK-c-Jun cascade (35).

With regard to JNK function in the context of antifibrotic properties, our findings are complementary to our recent works indicating that JNK, which is critical in conferring transcriptional activity to Jun proteins, is also instrumental in allowing these proteins to interfere with Smad-dependent gene transcription downstream of TGF-β, a major player in the development of tissue fibrosis (2, 3). We specifically demonstrated that activators of the JNK pathway, such as tumor necrosis factor-α (36–38) and 5-FU (39), antagonize TGF-β-induced collagen gene expression by preventing Smad/DNA interaction and related gene expression (2, 3). Thus, it may be speculated that JNK activation by rapamycin may have a similar inhibiting effect on TGF-β-driven collagen production. In conclusion, we have identified that rapamycin may exert direct antifibrotic activities by molecular mechanisms that affect both matrix production and degradation by fibroblasts.

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