Polymerized Collagen Inhibits Fibroblast Proliferation via a Mechanism Involving the Formation of a β1 Integrin-Protein Phosphatase 2A-Tuberous Sclerosis Complex 2 Complex That Suppresses S6K1 Activity

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Polymerized type I collagen suppresses fibroblast proliferation. Previous studies have implicated inhibition of fibroblast proliferation with polymerized collagen-mediated suppression of S6K1, but the molecular mechanism of the critical negative feedback loop has not yet been fully elucidated. Here, we demonstrate that polymerized collagen suppresses G1/S phase transition and fibroblast proliferation by a novel mechanism involving the formation of a β1 integrin-protein phosphatase 2A (PP2A)-tuberous sclerosis complex 2 (TSC2) complex that represses S6K1 activity. In response to fibroblast interaction with polymerized collagen, β1 integrin forms a complex with PP2A that targets TSC2 as a substrate. PP2A represses the level of TSC2 phosphorylation and maintains TSC2 in an activated state. Activated TSC2 negatively regulates the downstream kinase S6K1 and inhibits G1/S transit. Knockdown of TSC2 enables fibroblasts to overcome the anti-proliferative properties of polymerized collagen. Furthermore, we show that this reduction in TSC2 and S6K1 phosphorylation occurs largely independent of Akt. Although S6K1 activity was markedly suppressed by polymerized collagen, we found that minimal changes in Akt activity occurred. We demonstrate that up-regulation of Akt by overexpression of constitutively active phosphatidylinositol 3-kinase p110 subunit had minor effects on TSC2 and S6K1 phosphorylation. These findings demonstrate that polymerized collagen represses fibroblast proliferation by a mechanism involving the formation of a β1 integrin-PP2A-TSC2 complex that negatively regulates S6K1 and inhibits G1/S phase transition.

Control of cell proliferation is a critical event for normal development as well as physiologic repair of tissue following injury. Pathologic proliferation underlies a variety of disease states including tumorigenesis and fibroproliferative disorders involving the lung, liver, kidney, heart, vasculature, and integument (1). In addition to peptide growth factors and cell-cell interactions, the interaction of a cell with the matrix microenvironment is a key regulator of cell proliferation (2–5).

Seminal studies have demonstrated that polymerized type I collagen acts as a physiological negative regulator of fibroblast and smooth muscle cell proliferation (6–8). Acting via a negative feedback mechanism, fibroblast synthesis, deposition, and subsequent contact with polymerized collagen restrains their proliferation during normal tissue repair. This provides an effective physiologic mechanism to limit fibroproliferation after tissue injury. Currently, the molecular mechanism of the critical negative feedback loop has not yet been fully elucidated. However, investigation into the mechanism by which polymerized collagen inhibits fibroblast proliferation indicates that integrin-polymerized collagen interaction leads to up-regulation of the cdk2 inhibitors p21 and p27 and subsequent inhibition of cyclin E-cdk2 activity and suppression of the G1/S phase transition (8). Furthermore, polymerized collagen was noted to rapidly and potently decrease the level of S6K1 phosphorylation. The mechanism by which polymerized collagen suppresses S6K1 phosphorylation remains unknown. However, the serine/threonine phosphatase inhibitor calyculin A blocked the reduction in S6K1 phosphorylation, suggesting that a protein phosphatase may mediate the effect of polymerized collagen on S6K1 (8).

The integrin signal pathways regulating fibroblast responses to type I collagen have begun to be characterized. Opposite to polymerized collagen, fibroblast interaction with monomeric collagen via β1 integrin activates the PI3K/Akt2 pathway, promotes S6K1 phosphorylation, and supports fibroblast proliferation (8–11). S6K1 is a kinase downstream of PI3K/Akt that regulates ribosomal biogenesis and global translational activity. S6K1 activity is regulated by the upstream activity of Rheb-mTOR (12–14). Control of Rheb GTPase activity is regulated by TSC2, which together with TSC1 functions as a tumor suppressor complex (15, 16). TSC2 is the catalytic subunit having GTPase-activating protein activity toward Rheb. TSC2 can be phosphorylated by multiple kinases including Akt (17–19). An increase in the level of TSC2 phosphorylation by PI3K/Akt decreases TSC2 activity, whereas a reduction in the level of TSC2 phosphorylation by inhibition of PI3K/Akt increases its activity (18).

The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; siRNA, small interfering RNA; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSC2, tuberous sclerosis complex 2.
PP2A is a serine-threonine phosphatase with important roles in regulating kinase pathways and tissue development and differentiation (20–23). It consists of a catalytic C subunit, a scaffold A subunit, and one of many variable regulatory B subunits. The specific B regulatory subunit that binds the A and C subunits helps determine cellular localization and substrate specificity. Several studies indicate that PP2A can associate with β1 integrin and can be activated by integrin-type I collagen interaction (24–26). Furthermore, several studies have linked PP2A with regulation of the PI3K/Akt signal pathway (26–29). On the basis of this information, we examined the effects of fibroblast interaction with polymerized collagen on the ability of PP2A to associate with integrin and regulate S6K1 activity. We have discovered that in response to fibroblast attachment to polymerized collagen, a β1 integrin-PP2A complex forms and targets TSC2 as a substrate. This reduces the level of TSC2 phosphorylation, activating TSC2, inhibiting S6K1, and repressing G1/S phase cell cycle transit.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—For this study, the primary human lung fibroblast line HLF 210 (ATCC) was utilized. The cells were cultured in high glucose DMEM containing 10% fetal calf serum. The fibroblasts were used between passages 5 and 8 for all experiments.

**Antibodies and Chemicals**—Anti-Cyclin D1 and PP2Ac antibodies were obtained from Santa Cruz Biotechnology Company. Anti-Akt, S6K1, TSC2, HA tag, and anti-phospho-Akt, S6K1, and TSC2 antibodies were obtained from New England Biolabs. Okadaic acid was purchased from Calbiochem. Wortmannin was from Sigma.

**Collagen Matrices**—To prepare two-dimensional monomeric collagen matrices, tissue culture dishes were coated with 100 μg/ml type I collagen solution (PureCol, INAMED, Fremont, CA) in 1× phosphate-buffered saline and incubated (37 °C, 45 min). Three-dimensional polymerized collagen matrices (final concentration, 2 mg/ml) were prepared by neutralizing the collagen solution with 1/6 volume of 6 M HCl and diluting to a final volume with 1× DMEM to which fetal calf serum was added at a final concentration of 1% fetal calf serum. Gels formed following incubation of this solution at 37 °C for 1–2 h as described previously (9–11).

**Lentivirus Vector and Adenoviral Vectors**—Lentivirus control and PP2Ac siRNA (hairpin sequence: 5′-CCG GCA CAC AAG TTT ATG GTT TCT ACT CGA GTA G AA ACC ATA AAC TTG TGT GTG TTT TT-3′) vectors were ordered from OPEN Biosystems (Boston, MT). Lentivirus was generated in the 293 cell line by three co-transformations (Lentivirus vector, pCMV, and pMDG). Adenoviral vectors containing constitutively active PI3K p110 (Ad-PI3Kp110-GFP) and control (Ad-GFP) constructs were purified according to the manufacturer’s instructions (Takara Shuzo Co, Ltd., Kyoto, Japan). An adenoviral vector containing an HA-tagged PP2A catalytic (c) subunit (Ad-PP2Ac HA) was kindly provided by Dr. Alexander Verin (University of Chicago). The cells were infected with adenoviral vectors at a multiplicity of infection of 1:20.

**PP2A Catalytic (c) Subunit Activity Assay**—Briefly, the cells were lysed in lysis buffer containing 20 mM imidazole (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, and 1× protease inhibitor mixture (Calbiochem). PP2A catalytic C subunit was immunoprecipitated from lysates containing an equal number of cells by incubation with an anti-PP2A catalytic C subunit antibody overnight at 4 °C. The immunoprecipitates were washed three times with Tris-buffered saline buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10 mM KCl), followed by two additional washes with phosphate buffer (20 mM Tris, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). PP2A c activity was then analyzed using a PP2A immunoprecipitation phos-
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**RESULTS**

**Inhibition of Fibroblast Proliferation by Polymerized Collagen Is Associated with a Reduction in the Level of S6K1 Phosphorylation**—Prior studies have demonstrated that compared with monomeric collagen, polymerized collagen displays anti-proliferative properties toward fibroblasts and smooth muscle cells (6–8). Consistent with these studies, we have found that the proliferation of lung fibroblasts is suppressed by polymerized collagen compared with monomeric collagen (Fig. 1A). We have previously found that when fibroblasts interact with monomeric collagen via β1 integrin, the level of Akt and S6K1 phosphorylation is high. However, when we examined the effect of fibroblast attachment to polymerized collagen on the state of Akt/S6K1 activity, the pattern of signaling differed from that observed with monomeric collagen. The level of phosphorylated Akt increased modestly as a function of time when fibroblasts first attached to polymerized collagen, but S6K1 did not become phosphorylated (Fig. 1B). These findings indicate that compared with monomeric collagen, the amplitude of S6K1 phosphorylation on polymerized collagen is attenuated. This demonstrated a correlation between suppression of fibroblast growth by polymerized collagen and a reduction in the activity of S6K1, but not in Akt activity, and suggested that polymerized collagen may mediate its anti-proliferative effect by suppressing S6K1 activity.

The Serine/Threonine Phosphatase PP2A Mediates the Repressive Function of Polymerized Collagen toward S6K1—Because polymerized collagen suppresses the level of phosphorylation of threonine 389 of S6K1, this suggested that a serine/threonine phosphatase may be important in regulating the activity of S6K1. To examine this issue, fibroblasts were treated with the serine/threonine phosphatase inhibitor okadaic acid and plated on polymerized collagen. We found that the level of S6K1 phosphorylation increased in a dose- and time-dependent fashion, whereas the level of Akt phosphorylation remained relatively stable (Fig. 2A and B). These data are consistent with a previous study that found that the protein phosphatase inhibitor calyculin A enhanced the level of S6K1 phosphorylation and supports the concept that a serine/threonine phosphatase activity may be involved in regulating the repressive function of polymerized collagen toward S6K1 (8).

PP2A is an ubiquitous serine/threonine phosphatase whose activity is inhibited by okadaic acid. One prior study found that PP2A activity increased in response to fibroblast interaction with polymerized collagen (24). Therefore, we were interested in determining whether PP2A may be responsible for the repressive effect of polymerized collagen on S6K1. Consistent with this prior study, we found that PP2A activity increased in a time-dependent fashion when fibroblasts attached to polymerized collagen (Fig. 2C). We next knocked down PP2A by using siRNA directed against the PP2A catalytic subunit (PP2Ac). PP2A siRNA markedly knocked down PP2Ac expression (Fig. 2D). Similar to the effect of okadaic acid, knockdown of PP2A robustly increased the level of S6K1 phosphorylation in fibroblasts adhering to polymerized collagen but had minimal effect on the level of Akt phosphorylation (Fig. 2E). Control siRNA did not affect the level of S6K1 phosphorylation, and S6K1 phosphorylation was markedly suppressed by polymerized collagen. It is important to note that inhibition of PP2A by either okadaic acid or by siRNA had a minimal effect on the level of Akt phosphorylation. Therefore, these data support the concept that polymerized collagen suppresses proliferation by inactivation of S6K1 in a PP2A-dependent fashion.

Inhibition of S6K1 phosphorylation by PP2A may be by direct interaction with S6K1. However, we could not identify a direct association of PP2A with S6K1 (or vice versa) by co-immunoprecipitation experiments. This suggested that PP2A indirectly inhibits S6K1.

**Polymerized Collagen-mediated Inactivation of S6K1 Involves PP2A-mediated Augmentation of TSC2 Tumor Suppressor Activity**—The TSC1-TSC2 complex functions as a tumor suppressor (30). TSC2 negatively regulates the mTOR/S6K1 signal pathway by stimulating the conversion of Rheb-GTP to Rheb-GDP (15). This inactivates Rheb and represses mTOR and S6K1 activity. The activity of the TSC2 catalytic subunit is regulated by its phosphorylation state (17–19). P13K/
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Akt-mediated TSC2 phosphorylation reduces TSC2 activity, whereas TSC2 actively inhibits Rheb when the level of TSC2 phosphorylation is suppressed by inhibition of PI3K/Akt. We sought to determine whether the repressive effects of polymerized collagen on S6K1 activity and fibroblast proliferation were regulated via PP2A-mediated activation of TSC2 tumor suppressor activity. We first examined the effect of fibroblast interaction with polymerized collagen on the level of TSC2 phosphorylation. We found that there was a slight increase in the level of TSC2 phosphorylation (Thr1462) in response to fibroblast attachment to polymerized collagen (Fig. 3A). However, when we pretreated the fibroblasts with the PP2A inhibitor okadaic acid and permitted the fibroblasts to adhere to polymerized collagen, we found that the level of phosphorylated TSC2 increased substantially more (Fig. 3B). This increase in TSC2 phosphorylation (decrease in TSC2 activity) correlated with the increase in S6K1 phosphorylation seen in response to okadaic acid treatment (Fig. 2B). To confirm this finding, we knocked down PP2A with PP2Ac siRNA and examined the effect on TSC2 and S6K1 phosphorylation in response to fibroblast interaction with polymerized collagen. Consistent with the effect of okadaic acid, knockdown of PP2Ac markedly increased the level of TSC2 phosphorylation (Fig. 3C) and enhanced the level of S6K1 phosphorylation (Fig. 2E). There was a greater than 16-fold increase in the level of TSC2 phosphorylation at 30 min in fibroblasts treated with PP2A siRNA compared with control, indicating that PP2A has a robust suppressive effect on the level of TSC2 phosphorylation in response to fibroblast interaction with polymerized collagen. Together, these data strongly suggest that the mechanism of polymerized collagen-mediated inactivation of S6K1 involves PP2A-mediated suppression of TSC2 phosphorylation, thereby augmenting TSC2 tumor suppressor activity.

To verify the role of TSC2 in suppressing S6K1 activity in response to polymerized collagen matrices, we knocked down TSC2 expression by transfecting the fibroblasts with TSC2 siRNA. TSC2 siRNA knocked down TSC2 expression in a dose-dependent fashion (Fig. 3D). Furthermore, we found that the level of S6K1 phosphorylation (Thr389) increased as a function of time when TSC2 siRNA treated fibroblasts interacted with polymerized collagen (Fig. 3E). In contrast, in control siRNA-treated fibroblasts, the level of S6K1 phosphorylation was suppressed when these fibroblasts attached to polymerized collagen matrices. The TSC2 knockdown studies are consistent with the concept that TSC2 is exerting a suppressive effect on S6K1 in response to fibroblast interaction with polymerized collagen.

**PP2A Complexes with TSC2 in Fibroblasts Adhering to Polymerized Collagen**—Our data indicate that PP2A decreases TSC2 phosphorylation when fibroblasts interact with polymerized collagen. To determine whether PP2A reduces the level of TSC2 by directly interacting with TSC2, we performed immunoprecipitation experiments. We infected our fibroblasts with an adenoviral vector containing a PP2Ac HA-tagged construct and permitted the cells to interact with polymerized or monomeric collagen. In response to fibroblast adhesion to polymerized collagen, we identified an association of PP2A with TSC2 (Fig. 4A). However, no PP2A-TSC2 complex could be detected in fibroblasts transfected with the PP2Ac HA-tagged construct and plated on monomeric collagen.

**FIGURE 2.** The serine/threonine phosphatase PP2A mediates the repressive function of polymerized collagen toward S6K1. A, serum-starved fibroblasts were pretreated with okadaic acid (OA) (0–100 nM, 45 min) and plated on polymerized type I collagen matrices. The levels of Akt (Ser473) and S6K1 (Thr389) phosphorylation were examined by Western analysis. GAPDH is shown as a loading control. NT, no treatment. B, serum-starved fibroblasts were pretreated with okadaic acid (10 nM, 45 min) and plated on polymerized type I collagen matrices. The levels of Akt (Ser473) and S6K1 (Thr389) phosphorylation were examined by Western analysis as a function of time. C, serum-starved fibroblasts were cultured on monomeric (MC) or polymerized collagen (PC) for the indicated times. PP2A catalytic C subunit activity was analyzed by using a PP2A immunoprecipitation/phosphatase assay kit. D and E, fibroblasts were infected with PP2Ac siRNA (L-PP2Ac siRNA) or control siRNA (L-EV siRNA) in a lentiviral vector for 72 h. Total PP2Ac and actin levels were measured by Western analysis (D). The cells were then plated on polymerized collagen (PC). The levels of Akt (Ser473) and S6K1 (Thr389) phosphorylation were examined by Western analysis as a function of time. E, densitometric analysis showing the fold change in phospho-Akt (Ser473) compared with GAPDH. DMSO, dimethyl sulfoxide.
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A β1 Integrin-PP2A Complex Forms and Associates with TSC2 in Response to Fibroblast Attachment to Polymerized Collagen—Our data indicate that in response to fibroblast attachment to polymerized collagen, PP2A associates with TSC2. Fibroblast attachment to polymerized collagen is mediated through cell surface integrin matrix receptors. α2β1 is a major integrin receptor mediating adhesion to polymerized collagen (31, 32). Several studies suggest that under various conditions PP2A may complex with β1 integrin (24–26). Therefore we sought to determine whether PP2A forms a complex with β1 integrin in response to fibroblast attachment to polymerized collagen. We found that in response to fibroblast attachment to polymerized collagen, PP2A associates with β1 integrin (Fig. 4B), but no complex was detected on monomeric collagen. We next sought to determine whether PP2A together with TSC2 forms a complex with β1 integrin during fibroblast adhesion to polymerized collagen. To examine this issue, fibroblasts were transfected with a PP2Ac HA-tagged construct, PP2A was immunoprecipitated, and Western analysis for TSC2 and β1 integrin was performed. Consistent with our findings that PP2A can associate with TSC2 and β1 integrin when fibroblasts attach to polymerized collagen, we found that TSC2 can be found in a complex with β1 integrin and PP2A (Fig. 4C). Together, these data indicate that in response to fibroblast attachment to polymerized collagen, a β1 integrin-PP2A-TSC2 complex forms. These data are consistent with a scenario in which integrin-polymerized collagen interaction facilitates the association of PP2A with TSC2 in an integrin multi-protein complex. The association of PP2A with TSC2 reduces TSC2 phosphorylation, thereby enhancing TSC2 tumor suppressor activity and inactivating S6K1.

We sought to identify the specific integrin involved in complex formation and regulation of fibroblast proliferation. We first examined which integrin facilitates fibroblast adhesion to polymerized collagen using integrin blocking antibodies. Both the α2 and β1 integrin blocking antibodies inhibited the adhesion and spreading of serum-starved fibroblasts on polymerized collagen, whereas the other blocking antibodies were largely ineffective (Fig. 5A, left and right panels). We also examined which integrin fibroblasts utilize to interact with polymerized collagen during the proliferation assay in the presence of serum. We used GD25 null cells, GD25-β1 cells, and GD24-α2β1 cells for these experiments. Interestingly, only GD25 cells expressing α2β1 were capable of proliferating on polymerized collagen (Fig. 5B). Both GD25 null cells, which lack β1 integrins but express αβ3, and GD25-β1 cells, which express some β1 integrins including α5β1 but not α2β1 integrin, did not proliferate on polymerized collagen. We next examined which specific integrin is involved in PP2A complex formation during fibroblast attachment to polymerized collagen. When the PP2Aa (scaffolding) subunit was overexpressed and fibroblasts were cultured on polymerized collagen, we found an association of PP2A with the β1 integrin subunit and the α2 integrin subunit (Fig. 5C). We did not detect other α integrin subunits associated with PP2A including α11. Together, these data suggest that in response to

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**FIGURE 3.** Polymerized collagen-mediated inactivation of S6K1 involves PP2A-mediated augmentation of TSC2 tumor suppressor activity. **A**, serum-starved fibroblasts were plated on polymerized collagen. The level of phosphorylated TSC2 (Thr1462) was determined as a function of time by Western analysis. Total GAPDH expression is shown as a loading control. B, serum-starved fibroblasts were pretreated with okadaic acid (OA) (10 nM, 45 min) or DMSO (DMSO) as control and then plated on polymerized collagen for the indicated times. The level of phosphorylated TSC2 (Thr1462) was determined by Western analysis. GAPDH is shown as a loading control. C, densitometric analysis displays the fold change of phospho-TSC2 compared with GAPDH. **B**, serum-starved fibroblasts were transfected with various concentrations of TSC2 or control siRNA. Total TSC2 levels were measured by Western analysis. Actin is shown as a loading control. D, the level of phosphorylated S6K1 (Thr389) was examined by Western analysis in fibroblasts transfected by TSC2 or control siRNA and plated on polymerized collagen. GAPDH is shown as a loading control. E, densitometric analysis displaying the relative change of phospho-S6K1 (Thr389) compared with GAPDH.
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FIGURE 4. A β1 integrin-PP2A complex forms and associates with TSC2 in response to fibroblast attachment to polymerized collagen. A, serum-starved fibroblasts were infected by an adenoviral vector containing a HA-tagged PP2A catalytic C subunit construct and plated on monomeric (MC) or polymerized collagen (PC) for 30 min (0 time = cells in suspension prior to plating). HA-tagged PP2Ac was immunoprecipitated (IP) and Western analysis (WB) for total TSC2 or HA performed. Immunoprecipitation analyses indicate that PP2Ac targets TSC2 as a substrate when fibroblasts attach to polymerized collagen at 30 min but not when fibroblasts adhere to monomeric collagen. B, serum-starved fibroblasts were plated on polymerized collagen for 30 min (0 time = cells in suspension prior to plating). PP2Ac was immunoprecipitated and Western analysis for β1 integrin performed. C, serum-starved fibroblasts were infected by an adenoviral vector containing a HA-tagged PP2A catalytic C subunit construct and plated on polymerized collagen for 30 min (0 time = cells in suspension prior to plating). HA-tagged PP2Ac was immunoprecipitated, and Western analysis for total TSC2 and β1 integrin was performed. HA is shown as a control.

We have found that in response to fibroblast attachment to polymerized collagen, PP2A predominantly associates with the α2β1 integrin.

Knockdown of TSC2 Enables Fibroblasts to Elude the Antiproliferative Effects of Polymerized Collagen—We have demonstrated that polymerized collagen represses the proliferation of normal lung fibroblasts, and this is associated with suppression of TSC2 phosphorylation by PP2A, resulting in an increase in TSC2 activity (Fig. 2, A and B). To determine whether this increase in TSC2 activity is responsible for suppressing proliferation, we knocked down TSC2 using TSC2 siRNA and examined proliferation on polymerized collagen. For these experiments, serum-starved fibroblasts were transfected with TSC2 or control siRNA. The cells were then plated on monomeric or polymerized collagen matrices in DMEM + 10% FBS. To quan-

integrin occupancy by ligand, but not clustering, may be necessary.

The β1 Integrin-PP2A-TSC2 Complex but Not PI3K/Akt Activity Appears to be the Key Regulator Modulating TSC2 Function in Fibroblasts Adhering to Polymerized Collagen—The integrin-PI3K/Akt pathway regulates cell survival and proliferation (9–11, 33–40). Akt has been shown to phosphorylate TSC2 at threonine residues 989 and 1462 (17, 18). An increase in the level of TSC2 phosphorylation by Akt decreases TSC2 activity, whereas a reduction in the level of TSC2 phosphorylation by inhibition of Akt increases its activity. Because the level of TSC2 phosphorylation is suppressed during fibroblast interaction with polymerized collagen, we sought to determine whether a decrease in PI3K/Akt activity contributed to this suppression. As shown in Fig. 1B, we found that the level of phospho-Akt (Ser473) increased modestly when fibroblasts attached to polymerized collagen. Because Akt activity increased, this suggested that the suppression of TSC2 phosphorylation could not be explained by changes in Akt activity. To verify this finding, fibroblasts were infected with an adenoviral vector containing a constitutive active PI3K p110 construct and permitted to attach to polymerized collagen. As expected, up-regulation of PI3K activity maintained phospho-Akt (Ser473) at a high level (Fig. 6A). However, similar to control fibroblasts infected with an empty vector construct, the level of phospho-TSC2 (Thr1462) was minimally altered. Interestingly, the level of phospho-S6K1 only increased slightly in fibroblasts overexpressing the PI3K p110 subunit and attaching to polymerized collagen (Fig. 6A). This suggests that during fibroblast attachment to polymerized collagen, PI3K/Akt activity has minimal effects on both TSC2 and S6K1 activity. To further address this issue, we examined the effect of the PI3K/Akt inhibitor, wortmannin, on the level of Akt, TSC2, and S6K1 phosphorylation during fibroblast attachment to polymerized collagen. As shown in Fig. 6B, the level of TSC2 phosphorylation (Thr1462) was minimally altered by wortmannin pretreatment. However, the level of phosphorylated Akt was completely suppressed by wortmannin. The level of S6K1 phosphorylation was suppressed in both wortmannin-treated and control cells in response to attachment to polymerized collagen. Taken together, these results strongly suggest that the reduction in TSC2 and S6K1 phosphorylation in response to fibroblast interaction with polymerized collagen occur largely independent of Akt. Thus the predominant effect of polymerized collagen on TSC2 is to suppress TSC2 phosphorylation by a PP2A-dependent mechanism.
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A

![Graph showing cell area (μm²) for IgG, α1, a1+β1, a2, a2+β1, a2+β3, av, avβ3, β1, and β3](image)

B

**FIGURE 5.** PP2A associates with α2β1 during fibroblast interaction with polymerized collagen. A, serum-starved fibroblasts were pre-incubated with the indicated α or β integrin blocking antibody (Ab) either alone (α1, α2, α3, α5, αv, β1, or β3) or in combination (α1β1, α2β1, α3β1, α5β1, or αvβ3) or IgG control antibody (IgG). The cells were then plated on polymerized collagen (PC) and allowed to spread for 30 min. Left panel, cell areas of a random 150 cells were quantified by tracing the cell border using National Institutes of Health Image software. Right panel, representative photomicrographs displaying the effects of integrin blocking antibodies (β1, α2, and α1) on fibroblast spreading on polymerized collagen. β1 blocking antibody inhibited spreading, whereas α2 blocking antibody alone had no significant effect. IgG is shown as a control. B, GD25, GD25-β1, and GD25-α2β1 fibroblasts were cultured on polymerized collagen (right) in the presence of serum and cell numbers quantified. C, fibroblasts were infected with adenovirus containing a PP2Aa construct and plated on polymerized collagen. PP2Aa was immunoprecipitated (IP) and Western analysis (WB) for β1 and various α subunits was performed. The data are shown for the α2 integrin subunit demonstrating an association between PP2A and α2. D, serum-starved fibroblasts were allowed to adhere to monomeric collagen (MC) for 30 min. The cells were then incubated (30 min) with either TS2/16 β1-activating antibody (TS2/16), PTD2 β1 integrin antibody (PTD2), or IgG control antibody (IgG) in the presence or absence of secondary antibody. PP2A was immunoprecipitated, and Western analysis for α2 or β1 integrin or PP2Ac was performed.

Knockdown of TSC2 by TSC2 siRNA Enhances G1-S Phase Transition in Fibroblasts Cultured on Polymerized Collagen—We have shown that polymerized collagen inhibits DNA synthesis. We next examined the effect of polymerized collagen on fibroblast cell cycle transit. Fibroblasts were serum-starved for 48 h, labeled with propidium iodide, and then plated on monomeric or polymerized collagen in DMEM + 10% FBS for 24 h. Cell cycle transit was then analyzed by FACS. As shown in Fig. 8A, 24% of fibroblasts cultured on monomeric collagen entered S phase, whereas only 6% of fibroblasts cultured on polymerized collagen entered into S phase. Cyclin D facilitates the G1-S phase transition (41). Consistent with this, we found that the expression of cyclin D1 was inhibited when fibroblasts were cultured on polymerized collagen compared with monomeric collagen (Fig. 8B). The levels of cyclin A and E were minimally altered, consistent with the results from a prior study (8) (data not shown). These data confirm that polymerized collagen inhibits fibroblast G1-S phase transit. We next examined the effect of knockdown of TSC2 by TSC2 siRNA on G1-S phase transition in thymidine synchronized fibroblasts cultured on polymerized collagen. DNA analysis by FACS demonstrated that the percentage of fibroblasts treated with TSC2 siRNA and successfully transiting to S phase was 11.7% at 36 h compared with 6.7% of fibroblasts treated with control siRNA (Fig. 8C). Consistent with this, the level of cyclin D was substantially increased in TSC2 siRNA-treated fibroblasts cultured on polymerized collagen compared with control siRNA-treated fibroblasts (Fig. 8D). Knockdown of TSC2 by TSC2 siRNA also augmented fibroblast proliferation on polymerized collagen over a 48-h time period as determined by a significant increase in BrdUrd synthesis (Fig. 8E). These data are consistent with the idea that the mechanism by which polymerized collagen inhibits G1/S phase cell cycle progression involves TSC2-mediated suppression of S6K1 activity.

Figure 5 shows the effects of integrin blocking antibodies on fibroblast spreading on polymerized collagen. The panel includes representative photomicrographs displaying the effects of integrin blocking antibodies (β1, α2, and α1) on fibroblast spreading on polymerized collagen. β1 blocking antibody inhibited spreading, whereas α2 blocking antibody alone had no significant effect. IgG is shown as a control. The figure also includes graphs showing cell area (μm²) for IgG, α1, α1+β1, α2, α2+β1, α2+β3, av, avβ3, β1, and β3. The data are shown for the α2 integrin subunit demonstrating an association between PP2A and α2. The graph shows the percentage of fibroblasts successfully transiting to S phase, which was 11.7% at 36 h compared with 6.7% of fibroblasts treated with control siRNA. Consistent with this, the level of cyclin D was substantially increased in TSC2 siRNA-treated fibroblasts cultured on polymerized collagen.
attachment of fibroblasts or smooth muscle cells to polymerized type I collagen suppresses their proliferation (6–8). This provides an effective mechanism to limit fibroproliferation after tissue injury. However, the molecular mechanism of the critical negative integrin feedback loop has not been completely elucidated. Here we demonstrate that in response to fibroblast attachment to polymerized type I collagen, an integrin-PP2A complex forms that is capable of targeting TSC2 as a substrate. This suppresses TSC2 phosphorylation and increases its activity. Active TSC2 blocks S6K1 and inhibits G1-S phase transition and fibroblast proliferation.

Although it has long been recognized that polymerized type I collagen suppresses fibroblast proliferation, the mechanism by which it does so had not been explored. Recently several studies have begun to shed light on the mechanism by which polymerized collagen represses fibroblast proliferation. In 1996, Ross and co-workers (8) discovered that polymerized collagen promotes arrest in the G1 phase of the cell cycle by increasing the levels of cyclin-dependent kinase inhibitors p27 and p21. This up-regulation of p21 and p27 was found to involve integrin signaling, but the integrin signaling pathway was not defined. However, they found that polymerized collagen inactivated S6K1, and this was associated with a reduction in the level of S6K1 phosphorylation. Moreover, their preliminary experiments suggested that the serine/threonine phosphatase inhibitor calyculin A was capable of blocking the reduction in S6K1 phosphorylation brought about by cell contact with polymerized collagen (8). This implicated a serine/threonine phosphatase in mediating the repressive action of polymerized collagen on S6K1. In addition, one other study found that polymerized collagen activated the serine/threonine phosphatase PP2A in an integrin-dependent fashion (24). However, in this study PP2A was found to associate with and inhibit Akt. Inhibition of Akt was associated with dephosphorylation of glycogen synthase kinase-β. The effect on S6K1 or cell proliferation was not examined. Nevertheless, taken together, these studies suggested that the mechanism by which polymerized collagen represses fibroblast proliferation involves integrin-mediated activation of the serine/threonine phosphatase PP2A and suppression of S6K1 activity.

A major cell surface receptor that fibroblasts utilize to adhere to type I collagen is the α2β1 integrin (31, 32). When fibroblasts adhere and spread on matrices such as monomeric type I collagen, which supports cell proliferation, the PI3K/Akt pathway is activated in an integrin-dependent manner (9–11). Activation of Akt leads to phosphorylation and inactivation of TSC2 (18). This relieves the repression of TSC2 on Rheb, permitting activation of the downstream kinases mTOR and S6K1. In contrast, consistent with prior studies, we have found that when fibroblasts adhere to polymerized or fibrillar type I collagen, the level of S6K1 phosphorylation is markedly suppressed, and the activity of the serine/threonine phosphatase PP2A increased. Knockdown of PP2A by siRNA and inhibition of PP2A by okadaic acid enhanced S6K1 phosphorylation, thereby confirming a relationship between activation of PP2A and suppression of S6K1 phosphorylation. However, we could not demonstrate...
that S6K1 is a direct substrate of PP2A. This suggested that the suppressive effect of PP2A on S6K1 occurs by an indirect mechanism. Prior studies have demonstrated that PI3K/Akt-mediated phosphorylation of TSC2 inactivates TSC2, whereas a reduction in the level of TSC2 phosphorylation by inhibition of PI3K/Akt is associated with TSC2 activation and inhibition of S6K1 (18). We have found that inhibition of PP2A activity during fibroblast attachment to polymerized collagen enhances the level of TSC2 phosphorylation. Importantly, this was associated with an increase in the level of S6K1 phosphorylation. This indicates that polymerized collagen-mediated activation of PP2A represses the level of TSC2 phosphorylation. This activates TSC2 and suppresses S6K1. Furthermore, instead of directly targeting S6K1 as a substrate, we have found that when fibroblasts attach to polymerized collagen, PP2A targets TSC2 as a substrate. This ability of PP2A to associate with TSC2 is specific for polymerized collagen, because fibroblast attachment to monomeric collagen did not promote the formation of a PP2A-TSC2 complex.

Under specific conditions PP2A has been found to associate with β1 integrin (24–26). We have found that during fibroblast attachment to polymerized collagen via integrin, PP2A complexes with β1 integrin. This association is specific for fibroblast interaction with polymerized collagen, because fibroblast attachment to monomeric collagen did not promote the formation of a β1 integrin-PP2A complex. Moreover, TSC2 can be found associated with this complex. These data indicate that fibroblast adhesion to polymerized collagen via integrin promotes the formation of a β1 integrin-PP2A complex that recognizes TSC2 as a substrate.

Our studies indicate that the level of TSC2 activation appears to be critical in regulating the level of S6K1 activity, cell cycle transit, and proliferation. We have demonstrated that knockdown of TSC2 increases S6K1 activity, promotes the G1-S phase transit, and confers on fibroblasts the ability to circumvent the anti-proliferative effects of polymerized collagen. As we have demonstrated, integrin-mediated activation of PP2A and association with TSC2 suppresses the level of TSC2 phosphorylation and maintains a high level of TSC2 activity. Because Akt has been shown to modulate TSC2 phosphorylation levels, we were also interested in examining the role of the PI3K/Akt pathway in regulating TSC2 activity during fibroblast attachment to polymerized collagen. Somewhat surprisingly, we have found that fibroblast attachment to polymerized type I collagen is associated with phosphorylation of Akt, albeit in an attenuated fashion compared with monomeric collagen. Our findings of attenuated Akt phosphoryla-
Polymerized Collagen Matrix

β1 Integrin/PP2A/TSC Complex Present

PI3K/Akt → TSC1/2

mTor

S6K1

Cyclin D

Proliferation/Cell Cycle ↓

FIGURE 9. Diagrammatic representation of the molecular mechanism by which polymerized collagen suppresses fibroblast proliferation. In response to fibroblast attachment to polymerized collagen matrices via β1 integrin, a β1 integrin-PP2A complex forms that targets TSC2 as a substrate. This suppresses the level of TSC2 phosphorylation (Thr1462), thus maintaining a high TSC2 activity level. In turn, TSC2 inhibits the downstream kinase S6K1. This suppresses cyclin D, G1/S phase transit, and fibroblast proliferation.

which are characterized by the ability of fibroblasts to circumvent the negative regulatory properties of polymerized type I collagen.

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