Eukaryotic Translation Initiation Factor 4E Binding Protein 1 (4EBP-1) Function Is Suppressed by Src and Protein Phosphatase 2A (PP2A) on Extracellular Matrix*5

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Human lung fibroblasts utilize integrins to attach and proliferate on type I collagen. β1 integrin is the major integrin subunit for this attachment. Integrins coordinate cellular responses to cell-cell and cell-extracellular matrix interactions that regulate a variety of biological processes. Although β1 integrin-mediated signaling pathways in lung fibroblasts have been studied, a detailed molecular mechanism regulating translational control of gene expression by 4EBP-1 is not understood. 4EBP-1 inhibits cap-dependent translation by binding to the eIF4E translation initiation factor. We found that when lung fibroblasts attach to type I collagen, PI3K/Akt activity suppresses 4EBP-1 expression via PP2A, and the decrease of 4EBP-1 is due to protein degradation. The inhibition of Src activity dramatically increases PP2A and 4EBP-1 expression. Furthermore, ectopic expression of 4EBP-1, or PP2A silencing using PP2A siRNA confirmed that 4EBP-1 is regulated by PP2A. In addition, we found that 4EBP-1 inhibition by fibroblast attachment to collagen increases cap-dependent translation. Our study showed that when lung fibroblasts are attached to collagen matrix, the β1 integrin/Src/PP2A-mediated 4EBP-1 regulatory pathway is activated. We suggest that β1 integrin-mediated signaling pathway may be a crucial event in regulating fibroblast translational control machinery on collagen matrix.

Integrins coordinate cellular responses to cell-cell and cell-extracellular matrix interactions that regulate a variety of biological processes, including proliferation and viability (1–4). The α and β chains cooperate in signaling such that ligand binding specificity resides predominantly within the α chain and intracellular signaling molecules largely associate with the intracellular domain of the β chain (5). Among integrins, β1 integrin is the major integrin subunit that mediates fibroblast attachment to type I collagen (6). Seminal studies showed that once fibroblasts attach to type I collagen, several crucial signaling pathways are activated, conferring fibroblast fates such as proliferation, migration and apoptosis. Among them, eIF4E-binding protein-1 (4EBP-1)2 is a PI3K/Akt downstream target protein and negatively regulates the translational function of eIF4E, the mRNA cap-binding protein, and prevents binding of eIF4E to eIF4G, a large scaffold protein (7, 8). Studies showed that nonphosphorylated 4EBP-1 binds tightly to eIF4E, thereby inhibiting a key step in translation initiation (9–12).

When fibroblasts attach to type I collagen, PI3K/Akt activity increases via reduced PTEN function (13, 14). High Akt activity allows cells to progress G1 to G2 by inhibiting expression of the cell cycle inhibitor protein p27, promoting fibroblast proliferation (14, 15). Furthermore, serine/threonine phosphatase PP2A activity is also suppressed by fibroblast attachment to type I collagen (14). One of the upstream proteins that is known to regulate PP2A function is Src kinase (16–18). Src phosphorylates tyrosine 307 residue of PP2A and inactivates its activity (19, 20). These studies suggested that β1 integrin-collagen interaction may inhibit PP2A by high Src activity. Furthermore, a prior study suggested that 4EBP-1 can be a potential substrate of PP2A (21), and the inhibition of 4EBP-1 increases cell proliferation (22, 23). Thus, these findings provide a possibility that type I collagen-integrin interaction inhibits 4EBP-1 via a Src/PP2A-dependent pathway. Therefore, we hypothesized that when fibroblasts attach to collagen, 4EBP-1 function is inhibited through the suppression of PP2A as a result of high Src activity, thereby increasing the activity of cap-dependent translational control machinery.

To examine our hypothesis, we measured 4EBP-1 expression in response to fibroblast attachment to collagen as a function of time. Fibroblast interaction with type I collagen via a β1 integrin promotes a decrease in 4EBP-1 protein expression. The decrease of 4EBP-1 is due to protein degradation. We also found that β1 integrin is a crucial integrin subunit that is responsible for the suppression of PP2A expression. Furthermore, PP2A function is inhibited by high Src kinase activity when fibroblasts attached to collagen, which subsequently inhibits 4EBP-1 expression. In contrast, ectopic expression of PP2A enhanced 4EBP-1 levels in response to fibroblast attachment to collagen, and the inhibition of Src kinase increased 4EBP-1 via high PP2A activity. Our study suggests that in response to the type I collagen matrix, β1 integrin modulates translational control machinery through a Src/PP2A/4EBP-1-dependent mechanism.

* This work was supported by the Scientist Development grant and the Pulmonary Fibrosis Research grant from the American Lung Association and the American Heart Association (to R. S. N.).

1 The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

2 The abbreviations used are: 4EBP-1, eIF4E-binding protein-1; DMSO, dimethyl sulfoxide; PP2A, protein phosphatase 2A.
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MATERIALS AND METHODS

Cell Culture and Type I Collagen Matrices—For this study, the normal human lung fibroblast line HLF-210 was used (purchased from ATCC). HLF-210 fibroblasts were cultured in high glucose DMEM containing 10% FCS. The fibroblasts were used between passages 5–8 for all experiments. Type I collagen matrix was purchased from Advanced Biomatrix and prepared between passages 5–8 for all experiments. Type I collagen was coated on Petri dishes as described previously (24).

Antibodies and Chemicals—Anti-4EBP-1, Thr-35/46 4EBP-1, Thr-70 4EBP-1, 4EBP-2, Src, nonphosphorylated Src-Tyr-527 and phosphorylated Src-Tyr-416 antibodies were obtained from Cell Signaling Technologies. The eIF4E antibody was purchased from BD Transduction Laboratories. Rabbit anti-eIF4G antibody was a gift from Nahum Sonenberg. PP2A antibody was obtained from Millipore. GAPDH and actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The β integrin-activating monoclonal antibody TS2/16 was produced from hybridoma culture (ATCC, HB-243). β1 integrin-blocking antibody P5D2 was also produced from hybridoma culture. α1 to α5 and αv, β3 integrin blocking antibodies were purchased from Chemicon. Src inhibitor 1, PP2A inhibitor (okadaic acid), lactacytin, and proteasome inhibitor were purchased from Calbiochem.

Adenovirus Constructs, siRNA, and Quantitative RT-PCR—The PP2A adenovirus expressing HA-tagged PP2A catalytic subunit was kindly provided by Dr. Alexander Verin (University of Chicago). The cells were infected with adenoviral vectors at a multiplicity of infection of 20. Adenovirus expressing HA-tagged Src or wild type or transactivation domain deleted FoxO3a was purchased from Vector Biolabs. For 4EBP-1 expression, the pShuttle-CMV-4EBP-1 construct and empty vector were kindly provided from Dr. Karen Smith (University of Minnesota). For quantitative PCR assay, human lung cells were serum-starved for 2 days followed by plating on a collagen-coated plate for the indicated time as described. Cells were then collected, and total RNA was isolated using TRIzol. cDNA was prepared using random hexamer primers with purified total RNA (1 μg) from each sample, and quantitative PCR was carried out with 4EBP-1 sense primer, 5’-AAATCCCTGATGAGTGTCG-3’; 4EBP-1 antisense primer, 5’-CATCCTCAAACGAGTGCCTTGCACC-3’; and GAPDH sense primer, 5’-CTCGACTTCTTTGATG-3’; and GAPDH antisense primer, 5’-GAGTGTCG-3’ of primer-labeled radioactive proteins. The radioactive proteins were then chased from ATCC. HLF-210 fibroblasts were cultured in high collagen. We have shown previously that cell attachment to collagen, PI3K/Akt activity increases, whereas PP2A expression and activity was suppressed, promoting fibroblast proliferation (13). Because 4EBP-1 is an inhibitor of eIF4E and a potential downstream target of PP2A, we hypothesized that the integrin-collagen interaction suppressed 4EBP-1, thereby increasing cap-dependent protein translation. To test this hypothesis, serum-starved lung fibroblasts were first attached to type I collagen-coated plates, and 4EBP-1 protein expression levels were measured as a function of time. 4EBP-1 expression progressively decreased on collagen (Fig. 1A, left and right), whereas 4EBP-1 protein levels remained relatively unaltered on a non-coated plate. The attachment to type I collagen measured by a light microscope further demonstrated that cell attachment progressively increased on the collagen-coated plate, whereas cells were not able to efficiently attach to non-coated plate (Fig. 1B). These data show that fibroblast attachment to type I collagen decreases 4EBP-1 expression as a function of time. We next examined whether cell attachment to extracellular matrices were lysed in lysis buffer containing 2 mM imidazole (pH 7.0), 2 mM EDTA, 2 mM EGTA, 2% Nonidet P-40, and 1× protease inhibitor mixture (Calbiochem, La Jolla, CA). Total protein levels were measured from the resulting lysates. The PP2A catalytic subunit protein was then immunoprecipitated from lysates containing equal amounts of protein by incubation with 2 μg of anti-PP2A antibody overnight at 4 °C. The immunoprecipitates were washed twice with TBS buffer followed by two additional washes with phosphatase reaction buffer (50 mM Tris, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1 mM DTT). PP2A activity was assayed using a Malachite green phosphatase kit (Echelon) according to the manufacturer’s instructions. Src activity based on tyrosine-phosphorylated 416 in fibroblasts on collagen was performed using STAR Src activity assay kit according to the manufacturer’s protocol (Millipore). Briefly, fibroblasts attached on collagen were collected as a function of time followed by lysis using cold 1× radioimmune precipitation assay buffer containing protease inhibitors. 100 μl of standard or samples were then added to 96-well plates coated with an Src capture antibody and incubated for 2 h at room temperature. 100 μl of the detection antibody was added to each well and further incubated for 1 h. After washing, 1:100 diluted anti-rabbit IgG HRP conjugate was added to each well and further incubated for 45 min. After TMB solution was added to each well and incubated briefly for 15 min, the reaction was stopped and read at 450 nm using a 96-well reader. A cap affinity assay was done as before with minor changes (25); 300 μl (1 μg/μl) of cell lysate were incubated while mixing for 2 h at 4 °C, with 50 μl of suspended (50% mixture) 7-methyl GTP-Sepharose 4B (GE Healthcare), to capture eIF4E and its binding partners eIF4G and 4EBP-1. Following incubation, beads were thoroughly washed free of unbound materials, and bound proteins were eluted. The captured proteins were eluted with 30 μl of elution buffer (25 mmol/liter Tris-HCl (pH 7.5), 150 mmol/liter KCl) containing 100 μmol/liter 7-methylguanosine 5’-triphosphate (Sigma-Aldrich) and prepared for immunoblotting.
such as fibronectin or laminin also inhibits 4EBP-1 expression levels. Similar to type I collagen, 4EBP-1 levels decreased rapidly when fibroblasts attached to fibronectin-coated plates (Fig. 1C, upper left and right). In contrast, 4EBP-1 levels moderately decreased on laminin coated plates (lower panel). These data suggest that the degree of 4EBP-1 protein decrease was dependent upon ECM. Fibroblasts utilized integrins to attach type I collagen (2). Because α2β1 integrin is a major receptor for type I collagen, we next examined the role of α2β1 integrin on collagen matrix. Serum-starved lung fibroblasts were preincubated with α2 and/or β1 integrin blocking antibody and allowed to attach to collagen. 4EBP-1 expression level was higher when fibroblasts were preincubated with a β1 integrin-blocking antibody (Fig. 2A, lane 3, upper and lower panels). 4EBP-1 levels marginally increased in the presence of α2 integrin-blocking antibody when compared with an isotype control antibody (Fig. 2A, lane 2). The combination of α2 and β1 integrin blocking antibodies had a synergistic effect in preventing the decrease in 4EBP-1 protein expression (Fig. 2A, lane 4). In contrast, 4EBP-1 expression levels were low in fibroblasts preincubated with an isotype control antibody (Fig. 2A, lane 5). These data demonstrated that when fibroblasts are attached to

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**FIGURE 1. 4EBP-1 protein expression decreases on extracellular matrix.** A, left, serum-starved human lung fibroblasts were attached to collagen-coated plates (100 μg/ml) as a function of time. 4EBP-1 protein levels were then measured. GAPDH was used as a loading control. P, noncoated plastic plate was used as a control. Right, 4EBP-1/GAPDH expression ratio as a function of time on collagen. Assay was repeat at least three times. B, shown are the phase-contrast microscopic cell morphologies as a function of time after plating lung fibroblasts on type I collagen-coated plates. C, left, human lung fibroblasts were attached to either fibronectin (100 μg/ml) or laminin-coated plates (100 μg/ml) as a function of time, and Western analysis was carried out to measure 4EBP-1 protein expression. Right, 4EBP-1 expression (4EBP-1/GAPDH expression) was measured. The assay was repeated three times.
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A. **Collagen**

|          | S/S | α2 | β1 | α2+β1 | IgG |
|----------|-----|----|----|-------|-----|
| 4EBP-1   |     |    |    |       |     |
| Actin    |     |    |    |       |     |

![Graph showing 4EBP-1 expression](image)

**FIGURE 2. β1 integrin-collagen interaction suppresses 4EBP-1 expression.** A, upper panel, lung fibroblasts were serum-starved followed by preincubation with α2 or β1 integrin or both blocking antibodies (1 μg/ml, respectively) for 45 min. Cells were then attached to type I collagen for 30 min, and 4EBP-1 levels were measured. Actin was used as a loading control. S/S, Serum-starved lung fibroblasts. α2, α2 integrin blocking antibody; β1, β1 integrin blocking antibody. α2+β1, α2 and β1 blocking antibodies. IgG, isotype control antibody (1 μg/ml). Lower panel, 4EBP-1/Actin expression was measured. The assay was repeated three times. B, Western analysis was carried out to measure phospho-4EBP-1 (Thr-37/46), phospho-4EBP-1 (Thr-70), and 4EBP-2 expression levels on type I collagen in the presence of α2 or β1 integrin or both blocking antibodies (1 μg/ml, respectively). GAPDH was used as a loading control. The assay was repeated three times.

B. **Collagen**

|          | α2 | β1 | α2+β1 | IgG |
|----------|----|----|-------|-----|
| p-4EBP-1 (thr 37/46) |     |    |       |     |
| p-4EBP-1 (thr 70)     |     |    |       |     |
| 4EBP-2             |     |    |       |     |

![Graph showing 4EBP-2 expression](image)

**PP2A Decreases When Fibroblasts Are Attached to Collagen via β1 Integrin—Prior studies suggested that PP2A has been implicated with the regulation of 4EBP-1 (30, 31). Therefore, to test integrin function on PP2A expression, we first measured PP2A catalytic expression on collagen coated plates as a function of time. PP2A progressively decreased when fibroblasts attached to collagen-coated plates, whereas PP2A levels remained relatively unaltered on a non coated plate (Fig. 3A, upper and lower panels). We next examined whether PP2A decrease is also due to cellular attachment to collagen via various α or β integrin subunits. To test this, serum-starved lung fibroblasts were attached type I collagen in the presence or absence of α or β integrin subunit-specific blocking antibodies, and PP2A expression was measured by Western analysis. Similar to 4EBP-1 expression levels, PP2A protein expression was preserved when fibroblasts were attached to collagen in the presence of β1 integrin blocking antibody (Fig. 3B, lane 7, upper and lower panel). In contrast, when fibroblasts attached to collagen in the presence of α1, α2, and α5 integrin-blocking antibodies, PP2A proteins were moderately increased (Fig. 3B, lower panel). We further tested other collagen-binding α integrin subunits such as α10 and α11 on PP2A protein regulation. When α10 protein was silenced, PP2A protein was highly elevated (supplemental figure, A and B, upper panel). In contrast, the inhibition of α11 subunit protein had very minimum effect on PP2A expression. These findings strongly suggest that although the β1 integrin-collagen interaction plays a crucial role in the suppression of PP2A expression, the PP2A regulatory signaling pathway is also dependent upon a variety of α subunits such as α1, α2, α5, and α10, and the variable combinations of α and β1 integrin subunits differentially modulate PP2A protein expression.

**High Src Activity Suppresses PP2A Function—**Previous studies showed that Src kinase suppresses PP2A, and the expression of nonphosphorylated Src Tyr-527 and/or phosphorylation of Tyr-416 in the activation loop of the kinase domain increases its activity (32, 33). Therefore, we hypothesized that high Src activity in fibroblasts on collagen inhibits PP2A via β1 integrin. To test this, we first measured nonphosphorylated Src Tyr-527 expression in response to fibroblast attachment to collagen as a function of time. Nonphosphorylated Src level progressively increased on collagen (Fig. 4A, upper and middle panels), showing that Src activity increases when fibroblasts attach to collagen. To verify Src activity by the phosphorylation of Tyr-416 on collagen, we utilized a Src activity assay kit. Interestingly, Tyr-416 phosphorylation levels were high at the 15-min time point
**FIGURE 3.** \( \beta 1 \) integrin regulates PP2A expression on collagen. **A**, upper panel, serum-starved human lung fibroblasts were attached to collagen-coated plates as a function of time, and PP2A expression was measured. GAPDH was used as a loading control. **B**, upper panel, serum-starved human lung fibroblasts were preincubated with \( \alpha 1 \) to \( \alpha 5 \) and \( \alpha v \) integrin-blocking antibodies or \( \beta 1 \) and \( \beta 3 \) integrin-blocking antibodies (1 \( \mu g/\text{ml} \), respectively) for 45 min followed by the attachment to type I collagen for 30 min. PP2A levels were then measured, and GAPDH was used as a loading control. IgG, isotype control antibody. **Lower panel**, PP2A/GAPDH expression was measured in the presence of \( \alpha \) or \( \beta \) integrin-blocking antibodies on collagen as described above. The assay was repeated three times. *, \( p = 0.03 \) versus IgG isotype control.
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and gradually decreased at later time points (Fig. 4A, lower panel). These data suggest that when lung fibroblasts attach to type I collagen, Src becomes active at the early time point by predominantly phosphorylating Tyr-416, whereas Src activity by dephosphorylating Src Tyr-527 increased at later time points. Collectively these results show that Src activity increased when fibroblasts attach to collagen by utilizing different Tyr phosphorylation residues of the Src protein. We next examined whether Src functions to regulate PP2A expression on collagen by using a Src inhibitor. PP2A expression remained relatively unaltered when cells preincubated with the Src inhibitor were attached to collagen (Fig. 4B). Furthermore, PP2A expression progressively increased in a dose-dependent fashion (Fig. 4C, upper and lower panels). These data suggest that Src inhibits PP2A expression on collagen. To confirm these findings, we performed a PP2A activity assay in the presence of various doses of Src inhibitor. PP2A activity also progressively increased when Src inhibitor concentration was increased on collagen (Fig. 4D). These data demonstrated that Src has a function in inhibiting PP2A expression and activity on collagen.

Src Suppresses PP2A and 4EBP-1 Expression—Our results suggest the possibility that high Src activity inhibits 4EBP-1 via PP2A when fibroblasts attach to type I collagen. To explore this, we first examined whether PP2A can regulate 4EBP-1 expression. We ectopically expressed PP2A catalytic subunit and measured whether PP2A protein induction can increase 4EBP-1 expression on collagen. 4EBP-1 expression was high when PP2A was overexpressed (Fig. 5A). This finding showed that when PP2A protein is reconstituted in cells cultured on collagen, 4EBP-1 expression remains high. Our data showed that when fibroblasts were serum-starved, PP2A and 4EBP-1 expression levels were high. To further elucidate the role of PP2A in regulating 4EBP-1, we next inhibited PP2A when serum-starved condition and examined 4EBP-1 level. 4EBP-1 expression was low when PP2A was silenced by PP2A siRNA (Fig. 5B, left panel). Likewise, we found that 4EBP-1 expression was also reduced when PP2A was silenced in the presence of serum (Fig. 5B, right). These data demonstrated that PP2A regulates 4EBP-1 expression. To elucidate whether Src regulates 4EBP-1 via PP2A, we next examined 4EBP-1 expression in the presence of various doses of Src inhibitor on the collagen matrix. 4EBP-1 expression progressively increased as Src inhibitor concentration increased (Fig. 5C, left and right). To confirm this finding, we utilized adenovirus expressing Src protein and measured PP2A and 4EBP-1. When Src was overexpressed, PP2A protein level was low (Fig. 5D). Likewise, 4EBP-1 expression level was also suppressed. We further examined PP2A and 4EBP-1 levels on collagen-coated plates using Src siRNA. Unlike PP2A and 4EBP-1 expression levels on collagen-coated plates, PP2A expression increased when Src protein was silenced (Fig. 5E). 4EBP-1 protein level was also high in the presence of Src siRNA. Collectively, our results showed that when fibroblasts attach to collagen, high Src activity suppresses PP2A, and this low PP2A activity can be responsible for 4EBP-1 inhibition.

β1 Integrin Regulates 4EBP-1 through Src/PP2A-dependent Pathway—Our data suggest that β1 integrin inhibits 4EBP-1 via PP2A. To confirm this, we first examined the levels of 4EBP-1 using PP2A siRNA in the presence or absence of β1 integrin-blocking antibody on collagen. When control siRNA-transfected cells were attached to collagen in the presence of β1 integrin-blocking antibody, the 4EBP-1 level was high (Fig. 6A, lane 2, and right panel). However, when cells transfected with PP2A siRNA were attached on collagen, the 4EBP-1 protein level remained low in the presence of the β1 integrin-blocking antibody (Fig. 6A, lane 3). Similarly, we found that the 4EBP-1 level was low when the PP2A protein was silenced by PP2A siRNA in the presence of isotype control antibody (Fig. 6A, left panel, lanes 4 and 5). We further examined this finding using β1 integrin-blocking antibody and β1 integrin siRNA. Cells preincubated with β1 integrin-blocking antibody were cultured on a collagen-coated plate, and the 4EBP-1 level was examined. The 4EBP-1 level was high when β1 integrin-blocking antibody was used (Fig. 6B, upper panel). To confirm this, β1 integrin was silenced by β1 integrin siRNA, and 4EBP-1 expression was measured on collagen-coated plates. Like the case of β1 integrin-blocking antibody, the 4EBP-1 level was also high when β1 integrin siRNA was used (Fig. 6B, lower panel) (The 4EBP-1 level in the presence of α10 and α11 siRNA is also shown in the supplemental data.) Taken together, these data demonstrated that β1 integrin inhibits 4EBP-1.

We next examined whether the β1 integrin suppresses PP2A and 4EBP-1 expression via Src. Lung fibroblasts were ligated with a β1 integrin-activating antibody TS216 in the presence of various concentrations of Src inhibitor, and PP2A expression was measured. The PP2A level was decreased when cells were ligated with only TS216 (Fig. 6C, lane 2, upper and lower panels). However, PP2A protein levels progressively increased when fibroblasts attached to collagen in the presence of increasing doses of Src inhibitor (Fig. 6C, lanes 3 and 4). We next measured 4EBP-1 levels when cells were ligated with TS216 in the presence of the Src inhibitor. 4EBP-1 expression was significantly decreased at the early time point and progressively increased during the later time point, *p = 0.002; **p = 0.0002; ***p = 0.01 versus control. P, noncoated plastic plate. B, lung fibroblasts were preincubated with 100 nM of Src inhibitor (Si) for 45 min followed by the attachment to 100 μg/ml of collagen-coated plates for 30 min. PP2A and GAPDH levels were then measured. DMOS, dimethyl sulfoxide control. C, upper panel, serum-starved human lung fibroblasts were preincubated with 10 or 100 nM of Src inhibitor (Si) for 45 min. Cells were then attached to collagen-coated plates for 30 min, and Western analysis was carried out to measure PP2A expression levels. GAPDH was used as a loading control. S/S, serum-starved cells. Lower panel, PP2A expression was measured in the presence of various doses of Src inhibitor on collagen. The assay were repeated three times. *p = 0.03 versus DMSO control. D, lung fibroblasts were preincubated with various doses of Src inhibitor (Si, 0.1 to 10 nM) followed by attachment to collagen-coated plates for 30 min. Cells were then collected, and PP2A activity assay was carried out as described in under "Materials and Methods." *p = 0.04; **p = 0.03 versus DMSO control.
decreased when cells were treated with a DMSO control (Fig. 6D, lane 4, upper and lower panels). However, the 4EBP-1 level remained high when cells were pretreated with the Src inhibitor (Fig. 6D, lane 2). Collectively, our results demonstrated that cell attachment to collagen via β1 integrin suppresses PP2A function via high Src activity, which results in the inhibition of 4EBP-1 protein expression.

Our results showed that when fibroblasts attach to type I collagen, 4EBP-1 expression decreased as a function of time. We next sought to examine the underlying mechanism of the decreases in 4EBP-1 protein expression on collagen-coated plates. We first examined whether low 4EBP-1 mRNA is responsible for 4EBP-1 protein levels on collagen. Unlike the 4EBP-1 protein level on collagen, quantitative PCR analysis demonstrated that 4EBP-1 mRNA levels did not decrease as a function of time (Fig. 6E). These data showed that when fibroblasts attach to collagen, low 4EBP-1 protein expression is not due to transcriptional suppression. A prior study showed that 4EBP-1 is degraded by the proteasome (38). Therefore, we next examined whether 4EBP-1 becomes degraded in response to fibroblast attachment to collagen via proteasome. 4EBP-1 levels were not altered when cells were
preincubated with proteasome inhibitor (Fig. 6F, lane 3, upper and lower panels). 4EBP-1 expression was also moderately high in the presence of lactacystin. Furthermore, 4EBP-1 levels progressively increased when a various doses of proteasome inhibitor was used (Fig. 6G, upper and lower panels). Collectively, our data showed that when fibroblasts attach to collagen, 4EBP-1 protein becomes degraded.

Collagen-β1 Integrin Interaction Increases cap-dependent Translation via Low 4EBP-1 Function on Collagen—Our results showed that when fibroblasts attach to collagen, 4EBP-1 expression is suppressed. To examine whether the β1 integrin-collagen interaction increases eIF4G activity via low 4EBP-1 function, thereby promoting cap-dependent translation, we performed the cap-binding assay to elucidate the functional role of 4EBP-1 in the presence or absence of α2 or β1 integrin-blocking antibody on the collagen matrix. eIF4E protein levels were unaltered in the presence of both α2 and β1 integrin-blocking antibodies when cells were placed on collagen (Fig. 7A, upper panel). In contrast, when cells were treated with β1 integrin-blocking antibody, the 4EBP-1 level was high, and 4EBP-1 expression is suppressed.
expression was substantially higher when cells were preincubated with both α2 and β1 integrin-blocking antibodies (Fig. 7A, lane 4, upper panel). Similar to 4EBP-1 expression levels on collagen in the presence of integrin blocking antibodies, cap-binding assay demonstrated that 4EBP-1 activity was high when cells were preincubated with β1 or α2 and β1 integrin-blocking antibodies together (Fig. 7A, lower panel, lanes 3 and 4, respectively). The ratio of eIF4E/4EBP-1 protein levels demonstrated that when β1 integrin function was inhibited, 4EBP-1 activity was high, and eIF4G activity was suppressed (Fig. 7B). Furthermore, the combination of α2 and β1 integrin-blocking antibodies synergistically suppressed eIF4G function by high 4EBP-1 expression. In contrast, when cells were attached to collagen in the presence of iso-type control antibody, eIF4G activity was high due to low 4EBP-1 activity. Taken together, these data demonstrated that when fibroblasts attach to collagen, eIF4G activity increases as a result of low 4EBP-1 activity, thereby promoting cap-dependent translational machinery.

Src, PP2A, and 4EBP-1 Regulate Fibroblast Proliferation—We have previously shown that fibroblast proliferation increases on type I collagen (14, 24). In this study, we further elucidate that when fibroblasts attach to collagen, high Src activity suppresses PP2A function, thereby inhibiting 4EBP-1. Because the suppression of 4EBP-1 promotes cell proliferation (22, 23), we next examined whether the inhibition of 4EBP-1 as a result of high Src and low PP2A activities regulates fibroblast proliferation. To examine this, PP2A protein was silenced in fibroblasts, and cell proliferation was measured using MTS assay. Fibroblast proliferation was increased 40% in the presence of PP2A siRNA (Fig. 8A). In contrast, when PP2A was overexpressed, fibroblast proliferation was suppressed (Fig. 8B). Likewise, when cells were infected with adenovirus-expressing Src protein, ~20% of fibroblast proliferation increased (Fig. 8C, right panel). However, the proliferation was low when Src protein was silenced using siRNA (Fig. 8C, left panel). Furthermore, like the case of PP2A protein, when wild type 4EBP-1 was overexpressed, fibroblast proliferation was suppressed (Fig. 8D). Taken together, these data demonstrated that when fibroblasts attach to collagen, Src, PP2A, and 4EBP-1 functions are important to regulate fibroblast proliferation.
Cell attachment to extracellular matrix is a crucial event in matrix biology. Under normal physiological conditions, when β1 integrin interacts with type I collagen, it activates Akt and inhibits PP2A, thereby promoting fibroblast proliferation. Our study showed that these events suppress eIF4E inhibitor protein, 4EBP-1, initiating cap-dependent translation by increasing eIF4G activity. We showed that when fibroblasts interact with type I collagen via β1 integrin, activated Src suppresses PP2A, which results in the inhibition of 4EBP-1. 4EBP-1 is an
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important regulator for protein translation. 4EBP-1 is an inhibitor of eIF4E, and precise control of 4EBP-1 and eIF4E function is required for cells to regulate protein synthesis. Ribosome recruitment to mRNA is mediated by the eIF4 group of initiation factors. eIF4E recognizes the cap structure of mRNAs, initiating the translation process. 4EBP-1 can bind to eIF4E and prevents its association with eIF4G and incorporation into the eIF4F complex, which leads to inhibition of cap-dependent translation. Our prior study showed that when fibroblasts attach to type I collagen, high PI3K/Akt and low PP2A synergistically promotes fibroblast proliferation (13, 24). When Akt is activated, eIF4E function is up-regulated, thereby promoting protein synthesis. 4EBP-1 takes part in as an antagonist in this event and a negative regulator of cell growth (34). 4EBP-1 is also known to be a potential PP2A downstream protein. These findings also indicate that collagen integrin interaction may suppress 4EBP-1 function via low PP2A activity. Therefore, we hypothesized that β1 integrin plays a key role in suppressing 4EBP-1 via PP2A, thereby increasing cap-dependent translation. To test our hypothesis, we first measured 4EBP-1 expression levels in lung fibroblasts on type I collagen. When cells were attached to type I collagen, the 4EBP-1 level progressively decreased. In contrast, 4EBP-1 levels were high when PP2A was overexpressed. β1 integrin-activating and -blocking antibodies demonstrated that PP2A and 4EBP-1 function is dependent upon on β1 integrin. These results further support our previous findings that β1 integrin plays an important role in conferring cell fate.

Studies have shown that the down-regulation of 4EBP-1 activity increases cell proliferation by activating eIF4E. In particular, the role of 4EBP-1 has been studied in cancer models, and 4EBP-1 protein levels are frequently altered in a variety of tumors (35–37). This observation suggested us that fibroblast attachment to collagen matrix may alter 4EBP-1 function, thereby promoting cap-dependent translation. Because Src activity increases when fibroblasts attach to collagen, and PP2A and 4EBP-1 expression are inhibited via the β1 integrin, we further hypothesized that high Src activity inhibit 4EBP-1 function via PP2A when cells attach to collagen via β1 integrin. Our data showed that when fibroblasts attach to collagen via β1 integrin, 4EBP-1 function is inhibited by a Src/PP2A-dependent pathway, which increases cap-dependent translation and cell proliferation (Fig. 9). The cap-binding assay further demonstrated that low 4EBP-1 activity increases eIF4E activity when fibroblasts are interacted with collagen matrix and that this event is a β1 integrin-dependent. Although PP2A function has been implicated with the regulation of cell growth by modulating 4EBP-1, to our knowledge, this is the first report that 4EBP-1 function is inhibited via β1 integrin on type I collagen. Our data support the notion that when fibroblasts are cultured on collagen, the precise orchestration of a series of kinase(s) and phosphatase(s) facilitates cap-dependent translational machinery via inhibiting 4EBP-1. In summary, we demonstrated that the β1 integrin-collagen interaction is an important event in promoting cap-dependent translation by utilizing Src, PP2A, and 4EBP-1. Based on our study, future study of β1 integrin function can reveal the significant molecular mechanisms of how cells respond to extracellular matrix and how the alteration of this integrin function can contribute to certain types of diseases.

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