Assembly of fibronectin fibrils selectively attenuates platelet-derived growth factor–induced intracellular calcium release in fibroblasts

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Cellular responses to platelet-derived growth factor (PDGF) are altered in a variety of pathological conditions, including cancers, fibroses, and vascular diseases, making PDGF-induced signaling pathways important therapeutic targets. The limited success of therapies designed to impact PDGF pathways may be overcome with a clearer understanding of how cells integrate signals from PDGF and the extracellular matrix (ECM). Here, we assessed the effects of fibronectin matrix assembly on the responsiveness of mesenchymal cells to PDGF. Our results indicate that fibroblast-mediated assembly of fibronectin fibrils attenuates intracellular calcium release in response to PDGF. The dose-dependent inhibition of PDGF-induced intracellular calcium release was specific to the ECM form of fibronectin. Further, a recombinant protein engineered to mimic ECM fibronectin similarly attenuated intracellular calcium release in response to PDGF. Of note, fibronectin attenuated the PDGF–fibronectin similarly attenuated intracellular calcium release in response to PDGF.

PDGF-induced intracellular calcium release was specific to the ECM form of fibronectin. Further, a recombinant protein engineered to mimic ECM fibronectin similarly attenuated intracellular calcium release in response to PDGF. Of note, fibronectin attenuated the PDGF–calcium signaling axis at the level of phosphoinositide 3-kinase (PI3K) activation. Interestingly, ECM fibronectin did not alter other intracellular signals activated by PDGF, including activation of PDGF receptor β, AKT Ser/Thr kinase, phospholipase Cγ1, and extracellular signal–regulated kinase 1/2 (ERK1/2).

Platelet-derived growth factors (PDGFs)3 play critical roles in mammalian development and tissue repair, yet uncontrolled activity in adult tissues is associated with a broad range of diseases, including cancers, fibroses, and vascular diseases (1). PDGF is a 30-kDa dimeric glycoprotein, composed of two disulfide-linked polypeptide chains (denoted A–D), which functions as a major mitogen and chemotactic factor for a variety of cells of mesenchymal origin. Exposure of fibroblasts and smooth muscle cells to PDGF isoform BB (PDGF-B) promotes cell growth, survival, chemotaxis, extracellular matrix (ECM) deposition, collagen matrix contraction, and calcium signaling (1). PDGF can also regulate cell metabolism (2) and actin organization (3). PDGF-B and its receptor (PDGF-Rβ) contribute to cardiovascular development and blood vessel integrity via recruitment and proliferation of vascular mural cell progenitors (1). In adult tissues, PDGF stimulates wound healing (4) and triggers aortic vasoconstriction (5). The biological effects of PDGFs are mediated by receptor tyrosine kinases (PDGF-Rα and PDGF-Rβ) that dimerize upon ligation, triggering receptor autophosphorylation and initiating a variety of signaling cascades (1).

PDGF-induced intracellular signals are frequently dysregulated in chronic diseases, making PDGF signaling pathways important therapeutic targets (1, 6). The PDGF isoform PDGF-BB is approved by the Food and Drug Administration for clinical treatment of diabetic ulcers (7). However, to date, clinical successes using PDGF to enhance wound healing have been limited, and the large doses of PDGF required for clinical efficacy may contribute to malignancy (7). Other pathologies for which PDGF signaling is an attractive target include lung and kidney fibroses, which are characterized by overexpression of PDGF (1), and pulmonary epithelial tumors, which arise from cells that have increased responsiveness to PDGF (8). The difficulties in employing PDGF signaling as a therapeutic target arise from the complexity of the cellular responses to PDGF,
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wherein broadly up- or down-regulating PDGF signaling often produces off-target effects due to the number and impact of signaling pathways affected. A greater understanding of the mechanisms by which PDGF signaling is regulated and, in particular, how cells exert selective control over which PDGF-induced signaling pathways are activated will allow for the development of more targeted therapies.

PDGF-B binds to and activates PDGF-Rβ, triggering a well-characterized intracellular signaling cascade that leads to release of calcium from intracellular stores (6). Intracellular calcium release requires concurrent binding of phospholipase Cγ1 (PLCγ1) and phosphoinositide 3-kinase (PI3K) to phosphorylated tyrosine residues on activated PDGF-Rβ (6). Upon binding to the PDGF-Rβ, PLCγ1 and PI3K become active via tyrosine phosphorylation (1). Activated PI3K catalyzes the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) within the plasma membrane (9). In turn, PIP3 serves as a docking site to recruit active PLCγ1 to the plasma membrane, where PLCγ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate, resulting in generation of inositol trisphosphate (IP3) (10, 11). Free IP3 then binds the IP3 receptor on the endoplasmic reticulum (ER), resulting in release of calcium from the ER into the cytosol (6). PDGF-induced calcium signaling can contribute to a variety of cell behaviors, including cell differentiation, proliferation, and migration (1), and stimulates the expression of ECM-associated genes, including collagen A1 and fibronectin 1 (12).

ECM-derived signals can alter cell and tissue responsiveness to PDGF (13). Fibronectin is a principal component of the ECM, where it binds to and influences the activity of various cells, other ECM components, and numerous growth factors and cytokines (13, 14). Cells secrete fibronectin in a soluble, protomeric form that is then assembled into insoluble ECM fibronectin fibrils by a tightly regulated, cell-mediated process termed fibronectin matrix assembly (14). Importantly, both the process of fibronectin fibril assembly and the resultant conformation of assembled ECM fibrils regulate tissue homeostasis. As examples, ECM fibronectin fibrils stimulate cell proliferation (15), migration (16), and differentiation (17), whereas transient changes in ECM fibronectin fibril conformation mediate local vasodilatation in response to skeletal muscle contraction (18). Although there is evidence that fibronectin fragments and protomeric fibronectin can regulate cell responsiveness to PDGF, the effects of cell-assembled ECM fibronectin on PDGF signaling are not known.

The goal of this study was to determine whether and how the active assembly of a fibronectin matrix affects intracellular signals generated in response to PDGF. Intracellular calcium release in response to PDGF was assessed quantitatively, as this pathway mediates several of the downstream effects of PDGF (12, 19). Results demonstrate that cell-assembled ECM fibronectin selectively attenuates PDGF-induced intracellular calcium release without affecting activation of PDGF-Rβ. Moreover, ECM fibronectin attenuated PDGF-induced PI3K activation specifically, without altering PDGF-induced activation of other intracellular signaling molecules. Targeted inhibition of PDGF–PI3K–calcium signaling by ECM fibronectin represents a previously unknown mechanism by which cells can tune specific responses to PDGF and thus may serve as a therapeutic target for specifically regulating one branch of the PDGF-signaling cascade.

Results

Effect of ECM fibronectin on PDGF-induced intracellular calcium release

Exposure of mesenchymal cells to PDGF triggers calcium release from intracellular stores, resulting in a single, transient increase in cytosolic calcium concentration that typically becomes evident ∼1 min post-PDGF treatment and lasts for ∼3 min (19, 20). Here, effects of ECM fibronectin on PDGF-induced intracellular calcium release were assessed using fibronectin-null mouse embryonic fibroblasts (FN-null MEFs). These cells do not produce fibronectin, but they are capable of assembling exogenously supplied fibronectin into ECM fibrils (15), allowing for the analysis of PDGF-induced intracellular signals in both the absence and presence of ECM fibronectin.

In the absence of fibronectin, the addition of PDGF to FN-null MEFs evoked a single transient increase in intracellular calcium that became apparent ∼1 min post-PDGF treatment and peaked ∼3.5 min post-PDGF treatment (Fig. 1A). This PDGF-induced intracellular calcium release was observed in the presence of the calcium chelator, EGTA, indicating release from intracellular stores (21). As expected (22), increasing concentrations of PDGF reduced the time to peak intracellular calcium concentration (Fig. 1A). Additionally, increasing concentrations of PDGF evoked a dose-dependent increase in fluorescence intensity that was statistically significant versus vehicle control at a PDGF concentration of 5 ng/ml (Fig. 1B). In agreement with previous studies of PDGF-Rβ activation (23) and PDGF-induced intracellular calcium release (19, 20), the response to PDGF saturated at 20 ng/ml PDGF (Fig. 1B). These results serve to establish that the dynamics of PDGF-induced intracellular calcium release in FN-null MEFs is similar to that observed with other mesenchymal cell types.

We next asked whether the presence of a fibronectin matrix influences intracellular calcium release following PDGF exposure. To do so, FN-null MEFs were treated with increasing concentrations of fibronectin and cultured overnight to allow for assembly of fibronectin fibrils (24). No changes in intracellular calcium were detected following treatment with vehicle alone, in either the absence or presence of fibronectin (Fig. 1C, + Vehicle). Pretreatment with fibronectin resulted in a dose-dependent attenuation of PDGF-induced intracellular calcium release (Fig. 1C) that was statistically significant at fibronectin concentrations of 12.5 nM and greater (Fig. 1D). To determine whether the matrix form of fibronectin was responsible for the attenuation of PDGF-induced intracellular calcium release, fibronectin matrix assembly was inhibited using two well-characterized approaches. First, a peptide derived from the adhesin F1 of Streptococcus pyogenes (termed FUD) was used to inhibit initial cell–fibronectin interactions and block fibronectin matrix assembly (25). Deletion of a single amino acid from the 49-amino acid sequence of FUD at position 29 (termed “del29”) renders FUD inactive (26); thus, del29 was used as a negative control. In the second approach, the anti-fibronectin mAb, 9D2, was used to block fibronectin–fibronectin self-interac-
tions and, thus, inhibit fibronectin fibril formation; 9D2 mAb does not affect initial cell–fibronectin interactions (27). FN-null MEFs were treated overnight with fibronectin in the presence of either FUD or 9D2 mAb or their respective controls. The addition of FUD peptides or 9D2 mAb blocked the ability of fibronectin to attenuate PDGF-induced intracellular calcium release (Fig. 2A). In contrast, the addition of the corresponding controls, del29 or IgG, had no effect on the attenuation of PDGF-induced intracellular calcium release by fibronectin (Fig. 2A). 

Fibronectin matrix assembly did not alter PDGF-Rβ expression or the ability of PDGF to stimulate phosphorylation of PDGF-Rβ (Fig. 2B), indicating that fibronectin does not block ligation and activation of the PDGF-Rβ. The addition of the FUD peptides or 9D2 mAb blocked the ability of fibronectin to attenuate PDGF-induced intracellular calcium release (Fig. 2A). In contrast, the addition of the corresponding controls, del29 or IgG, had no effect on the attenuation of PDGF-induced intracellular calcium release by fibronectin (Fig. 2A).

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During fibronectin matrix assembly, fibronectin undergoes a series of conformational changes that result in exposure of a matricryptic site within the first type III repeat (FNIII1) (28). To mimic the structure and signaling capacity of ECM fibronectin, a recombinant fibronectin fragment was developed previously by directly coupling an “open” FNIII1 fragment having constitutive heparin-binding activity (FNIII1H) to the cell-binding FNIII8–10 domain (FNIII1H,8–10) (28). FNIII1H,8–10 triggers a number of cell and tissue responses to a similar extent as ECM fibronectin (18, 28). Thus, studies were conducted to compare effects of ECM fibronectin and FNIII1H,8–10 on PDGF-induced intracellular calcium release. Treatment of FN-null MEFs for 4 h with FNIII1H,8–10 attenuated PDGF-induced intracellular calcium release compared with pretreatment with the control protein, glutathione S-transferase (GST; Fig. 2E). In contrast to FNIII1H,8–10, the ability of fibronectin to attenuate PDGF-induced intracellular calcium release was not evident at 4 h but, rather, was observed 20 h after fibronectin addition (Fig. 2E). These effects are consistent with the additional time required for soluble fibronectin to undergo assembly into ECM fibrils (24). Taken together, these data indicate that it is the ECM form of fibronectin that attenuates PDGF-induced intracellular calcium release.

Fibronectin does not affect PDGF-induced PDGF-Rβ, ERK1/2, or PLCγ1 activation

To determine whether fibronectin alters the time course of PDGF-Rβ phosphorylation in response to PDGF, FN-null
MEFs were incubated overnight in the absence or presence of fibronectin and then stimulated with PDGF for 2, 5, 10, or 20 min. Total PDGF-Rβ expression and PDGF-Rβ Tyr<sup>5857</sup> phosphorylation were assessed at various times after PDGF addition via immunoblot analysis. Fibronectin had no effect on either PDGF-Rβ expression or PDGF-induced PDGF-Rβ Tyr<sup>5857</sup> phosphorylation for any of the PDGF treatment durations tested (Fig. 3, A–C).

PDGF-induced PDGF-Rβ phosphorylation activates ERK1/2, a member of the mitogen-activated protein kinase family (6). In the absence of PDGF, ERK1/2 phosphorylation was not observed in either the absence or presence of fibronectin (Fig. 3A, 0 min). Two min after PDGF addition, ERK1/2 phosphorylation was detected and remained elevated for at least 20 min (Fig. 3A). Fibronectin did not affect PDGF-induced ERK1/2 phosphorylation at any of the time points tested (Fig. 3A). Together, these results indicate that fibronectin does not alter the rate or extent of PDGF-induced PDGF-Rβ autophosphorylation and, moreover, that fibronectin is not a general inhibitor of PDGF-Rβ signaling.

Intracellular calcium release in response to PDGF or ATP requires IP<sub>3</sub> binding to IP<sub>3</sub> receptors located on the ER (29). Intracellular calcium release in response to PDGF depends upon PLCγ1-generated IP<sub>3</sub> (6), whereas ATP-induced intracellular calcium release utilizes PLCβ-generated IP<sub>3</sub> (30). Thus, to determine whether fibronectin attenuates intracellular calcium release by globally blocking IP<sub>3</sub> receptors, ATP-induced intracellular calcium release was measured in FN-null MEFs cultured in the presence or absence of fibronectin. Treatment of cells with ATP caused a dose-dependent release of intracellular calcium, which was not affected by the presence of fibronectin (Fig. 4A). Thus, fibronectin does not affect the ability of free IP<sub>3</sub> to bind to IP<sub>3</sub> receptors and evoke intracellular calcium release.

To next determine whether fibronectin attenuates PDGF-induced PLCγ1 activation, PLCγ1 was immunoprecipitated from FN-null MEFs cultured in the presence or absence of fibronectin and stimulated with PDGF. Immunoblot analysis of the phosphotyrosine content of PLCγ1 immunoprecipitates indicated that fibronectin does not affect either PLCγ1 expression or PDGF-induced PLCγ1 phosphorylation (Fig. 4, B and C).

**Fibronectin attenuates PDGF-induced PI3K activation via a substrate-independent mechanism**

PLCγ1-mediated IP<sub>3</sub> generation also requires targeting of PLCγ1 to the plasma membrane (10), which is mediated by PIP<sub>2</sub>, a product of class IA PI3Ks (9, 10). To determine whether fibronectin affects PI3K activation, FN-null MEFs were incubated overnight in the absence or presence of fibronectin and then stimulated with PDGF for 2, 5, or 10 min. In the absence of fibronectin, PDGF caused a time-dependent increase in tyrosine phosphorylation of the p55 regulatory subunit of PI3K (Fig. 5, A and B). In contrast, PDGF-induced PI3K phosphorylation was significantly attenuated in cells cultured overnight in the presence of fibronectin (Fig. 5, A and B). Tyrosine phosphorylation of the alternatively spliced PI3K p85 regulatory subunit in response to PDGF was largely undetected, in either the presence or absence of fibronectin (Fig. 5A). Importantly, AKT phosphorylation was observed 2 min after PDGF treatment, in both the absence and presence of fibronectin (Fig. 5A), indicating that fibronectin selectively blocks PI3K activation.
Fibronectin attenuated PDGF-induced PI3K p55 phosphorylation in a dose-dependent manner (Fig. 6, A and B). In cells pretreated with 200 nM fibronectin, the relative band density of PDGF-induced phospho-PI3K was 21\% of that detected in cells lacking a fibronectin matrix (Fig. 6, A and B). Parallel studies showed that fibronectin did not reduce the expression of either the PI3K p55 regulatory subunit (Fig. 6 C) or the PI3K p110 catalytic subunit (Fig. 6 D). Taken together, these data indicate that fibronectin blocks activation of PI3K by PDGF.

Fibronectin fibril structure and, hence, function are influenced by the underlying adhesive substrate (24). Thus, studies were conducted to compare the ability of fibronectin to attenuate PDGF-induced calcium release in cells adherent to either collagen (an \alpha1\beta1 integrin-binding substrate) or vitronectin (an \alphav\beta3 integrin-binding substrate) (31). Collagen- or vitronectin-adherent FN-null MEFs were incubated for 20 h in the absence or presence of fibronectin, and then PDGF-induced intracellular calcium release was measured. Importantly, collagen- and vitronectin-adherent FN-null MEFs assemble fibronectin fibrils into the ECM at a similar rate (24). PDGF-induced calcium release was significantly attenuated by fibronectin in both vitronectin- and collagen-adherent cells (Fig. 7A). As with collagen-adherent cells (Figs. 5 and 6), fibronectin attenuated PDGF-induced PI3K p55 phosphorylation of vitronectin-adherent cells (Fig. 7B, 2 min). Again, fibronectin treatment did not affect PDGF-R \beta protein levels or PDGF-induced phosphorylation of PDGF-R \beta Tyr857 in vitronectin-adherent cells (Fig. 7B). Notably, and in contrast to collagen-adherent cells (Fig. 5A), phosphorylation of PI3K p55 was detected in vitronectin-adherent cells in the absence of PDGF (Fig. 7B). This basal PI3K activity of vitronectin-adherent cells was also attenuated by fibronectin (Fig. 7B), providing evidence that fibronectin-mediated attenuation of PI3K p55 phosphorylation is not specific to PDGF/PDGF-R signaling. 

Fibronectin regulates the PDGF-R signaling cascade at the level of PI3K activation

PDGF-induced PI3K activation requires phosphorylation of PDGF-R \beta on Tyr740 and Tyr751 (6). To determine whether fibronectin blocks PDGF-induced phosphorylation of PDGF-R \beta Tyr740 or Tyr751, FN-null MEFs were incubated overnight in the absence or presence of fibronectin and then stimulated with PDGF for various times (1–5 min). PDGF-R \beta expression levels and the phosphorylation state of PDGF-R \beta Tyr740 and Tyr751 were assessed by immunoblot analysis. Both the time course and extent of PDGF-induced phosphorylation of PDGF-R \beta on Tyr740 or

Figure 3. Fibronectin does not alter PDGF-R \beta expression or PDGF-induced PDGF-R \beta Tyr857 phosphorylation. Collagen-adherent FN-null MEFs (6 \times 10^4 cells/cm^2) were incubated for 20 h with 25 nM FN or an equal volume of PBS. Cells were then stimulated with vehicle control (0 min) or with 10 ng/ml PDGF for 2, 5, 10, or 20 min. Cell lysates were analyzed by immunoblotting and densitometry. A, immunoblots shown represent one of three independent experiments performed in duplicate. B, the ratio of the average net intensity of PDGF-R \beta bands to the average net intensity of vinculin bands was determined for each treatment condition. Values were normalized to the +PBS; 0 min PDGF condition, which was set to 1. Data are presented as the average ratio ± S.E. (error bars) of three experiments performed in duplicate. C, the ratio of the average net intensity of phospho-PDGF-R \beta Tyr857 bands to the average net intensity of PDGF-R \beta bands was determined for each treatment condition. Values were normalized to the +PBS; 2 min PDGF condition, which was set to 1. Data are presented as the average ratio ± S.E. of three experiments performed in duplicate. No significant differences were observed by ANOVA.
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Figure 4. Fibronectin does not attenuate ATP-induced intracellular calcium release or PDGF-induced PLCγ1 activation. A, FN-null MEFs (1.1 x 10^5 cells/cm^2) were seeded on collagen-coated substrates and allowed to adhere for 4 h. Cells were treated with either FN (25 nm) or an equal volume of PBS and incubated for 20 h. Cells were loaded with Fluo-4, stimulated with ATP or 30 ng/ml PDGF, and monitored for changes in fluorescence. Data are presented as relative change in fluorescence from baseline (ΔF/F₀) and are expressed as mean ± S.E. (error bars) of five experiments performed in triplicate. Values were normalized to the +PBS; +10 μM ATP condition, which was set to 1. *, significantly different by ANOVA (p < 0.05). B, 3 days post-seeding, cells were treated overnight with either FN (25 nm) or an equal volume of PBS and then exposed to 30 ng/ml PDGF (+) or the vehicle control (−) for 2 min. Proteins were extracted with SDS-RIPA buffer, immunoprecipitated using an anti-PLCγ1 mAb, and analyzed by immunoblotting using anti-PLCγ1 and phosphotyrosine monoclonal antibodies. Immunoblots shown represent one of three independent experiments performed in duplicate. C, the ratio of the average net intensity of phosphotyrosine bands to the average net intensity of PLCγ1 bands was determined. Values were normalized to the +PBS; +2 min PDGF condition, which was set to 1. Data are presented as the average ratio ± S.E. of three experiments performed in duplicate.

Figure 5. Fibronectin attenuates PDGF-induced PI3K p55 phosphorylation. FN-null MEFs (6 x 10^4 cells/cm^2) were seeded on collagen-coated substrates and allowed to adhere for 4 h. Cells were treated with FN (25 nm) or an equal volume of PBS and incubated for an additional 20 h. Cells were then treated with vehicle control (0 min) or with 30 ng/ml PDGF and incubated for indicated times. Cell lysates were analyzed by immunoblotting. A, immunoblots shown represent one of three independent experiments performed in duplicate. B, the ratio of the average net intensity of phospho-PI3K p55 (Tyr199) bands to the average net intensity of vinculin bands was determined. Values were normalized to the +PBS; +2 min PDGF condition, which was set to 1. Data are presented as the average ratio ± S.E. (error bars) of three experiments performed in duplicate. *, significantly different by Student’s or one-sample (2-min time point) t-test (p < 0.05).

Tyr^751 were similar in the absence and presence of fibronectin (Fig. 8, A–C). These data indicate that the attenuation of PDGF-induced PI3K activation by fibronectin is not due to loss of docking sites on PDGF-Rβ. Rather, these data provide evidence that fibronectin inhibits PI3K activation directly.

Thus, to determine whether ECM fibronectin acts as a general inhibitor of agonist-induced PI3K activation, effects of fibronectin on PDGF-, epidermal growth factor (EGF)-, and fibroblast growth factor-2 (FGF-2)-induced PI3K activation were determined. Each of these growth factors triggers intracellular calcium release via the PI3K signaling axis (29, 32). FN-null MEFs were incubated overnight in the presence or absence of fibronectin, and PI3K p55 phosphorylation in response to PDGF, EGF, and FGF-2 was examined. Fibronectin inhibited EGF-induced PI3K p55 phosphorylation (Fig. 9, A and B), providing further evidence that inhibition of PI3K activity by fibronectin is not mediated at the level of the PDGF-Rβ. Of note, FGF-2 did not activate either PI3K p55 or AKT but did stimulate ERK1/2 activation (Fig. 9A). These results are expected, as FGF-2 activates class II PI3Ks, which do not contain the PI3K p55 regulatory subunit (9).

Discussion
In this study, we report that cell-assembled, ECM fibronectin fibrils selectively attenuate intracellular calcium release in response to PDGF. The inhibitory effects of fibronectin were striking, as intracellular calcium release in response to a saturating concentration of PDGF was blocked completely by an overnight exposure to 50 μg/ml fibronectin. Remarkably, the inhibitory activity of fibronectin was confined to the PI3K–
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Calcium signaling axis, as the presence of a fibronectin matrix did not inhibit or alter the kinetics of several other PDGF-induced intracellular pathways, including ERK1/2, AKT, and PLCγ. It is important to note that the inhibitory effects of ECM FN were not due to binding and sequestration of PDGF within the ECM, as PDGF-induced phosphorylation of PDGF-Rβ Tyr857, Tyr740, and Tyr751 showed similar kinetics in the absence and presence of fibronectin. A schematic of the PDGF-intracellular calcium release cascade is shown in Fig. S2.

Several lines of evidence indicate that the inhibitory effect on calcium release is a specific property of ECM fibronectin fibrils. First, two different inhibitors of fibronectin matrix assembly (FUD peptides and 9D2 mAbs) ablated the ability of fibronectin to attenuate PDGF-induced intracellular calcium release. Similarly, intracellular calcium release in response to PDGF was enhanced in human dermal fibroblasts as well as mouse embryonic fibroblasts when fibronectin matrix assembly was inhibited. Second, incubation of FN-null MEFs with fibronectin for 20 h, but not for 4 h, attenuated PDGF-induced intracellular calcium release. During this 16-h time period, there is a 3-fold increase in the amount of fibronectin matrix deposited by FN-null MEFs (24), suggesting that fibronectin-mediated attenuation of PDGF-induced intracellular calcium release requires extensive fibronectin matrix assembly and maturation of fibronectin fibrils. Third, treatment of FN-null MEFs with the recombinant fibronectin matrix mimetic, FNIII1H,8–10, for 4 h was sufficient to attenuate PDGF-induced intracellular calc-
An important example of the specificity of ECM fibronectin in attenuating the PDGF–PI3K–calcium signaling cascade is illustrated by studies comparing the effect of fibronectin on PDGF- versus ATP-induced intracellular calcium release. The PDGF- and ATP-induced calcium release pathways are very similar but are mediated by different isoforms of PI3K and PLC\(_{6, 9, 10, 30}\). Therefore, the different isoforms of PI3K that mediate PDGF- versus ATP-induced intracellular calcium release could be important for understanding the differential regulation of the calcium response to these two agonists. ATP activates PLC\(_{\beta}\) and class IB PI3Ks\((9, 30)\), whereas PDGF activates PLC\(_{\gamma1}\) and class IA PI3K\((6, 9)\). Class IA PI3Ks have either 55- or 85-kDa regulatory subunits, whereas class IB PI3Ks utilize 101- or 85-kDa regulatory subunits\((9)\). Thus, the ability of fibronectin to attenuate PDGF- but not ATP-induced calcium release is consistent with the inhibition of the PI3K p55 regulatory subunit that was observed, as PI3K p55 is not involved in the ATP-induced calcium release pathway.

Yet another example of the specificity of ECM fibronectin in attenuating the PDGF–PI3K–calcium signaling axis is the absence of an effect of fibronectin on PDGF-induced AKT (protein kinase B) activity. Activation of AKT is classically achieved via translocation of AKT to the PI3K product, PIP\(_3\), and subsequent phosphorylation of AKT on Thr\(^{308}\) (by PDK1) and on Ser\(^{473}\) (by mTORC2)\((37)\). Interestingly, our results indicate that although fibronectin clearly attenuated PDGF-induced PI3K p55 phosphorylation, fibronectin did not attenuate PDGF-induced AKT activation. AKT is an important mediator of cell survival\((38)\), and as such, cells have developed multiple mechanisms of AKT activation, which allow PI3K and AKT to operate independently\((39)\). One such mechanism is activation of the ubiquitously expressed nonreceptor tyrosine kinase Ack1, which phosphorylates AKT on Tyr\(^{176}\), causing AKT to be activated by phosphatidic acid, rather than PIP\(_3\)\((40)\). Activation of PDGF–R\(\beta\) has been shown to activate Ack1–AKT signaling in cancer cells\((41)\), suggesting that PDGF-induced AKT activation can occur without PI3K activation\((40, 41)\). Alternatively, our data indicate that fibronectin attenuates, but never completely ablates, PDGF-induced PI3K p55 phosphorylation. Agonist-induced PIP\(_3\) production is not the rate-limiting step in AKT activation\((23)\), and relatively small amounts of PIP\(_3\) can facilitate normal levels of AKT activation\((39)\). Therefore, ECM fibronectin-mediated attenuation of PDGF-induced PI3K activation may be sufficient to inhibit PDGF-induced intracellular calcium release but insufficient to block PDGF-induced AKT activation.

The mechanism by which ECM fibronectin inhibits activation of the PI3K p55 regulatory subunit is not yet known. However, we can identify a number of mechanisms that are not responsible for attenuation of PI3K activation by fibronectin. One of these mechanisms involves Src homology region 2 domain–containing phosphatase-2 (SHP2), which dephosphorylates the phosphotyrosine residues on PDGF-R\(\beta\) that are associated with activation of PI3K (Tyr\(^{740}\) and Tyr\(^{751}\))\((42)\). PDGF-induced phosphorylation of PDGF–R\(\beta\) Tyr\(^{740}\) and Tyr\(^{751}\) was not affected by ECM fibronectin, strongly suggesting that fibronectin-mediated SHP2 activity is not the mechanism by which fibronectin attenuates PI3K activity.

**Figure 7.** Fibronectin-mediated attenuation of PDGF-induced intracellular calcium release is independent of the adhesive substrate. A, FN-null MEFs (1.1 × 10^6 cells/cm^2) were seeded on vitronectin-coated (VN) or collagen-coated (CL) wells. Four h after seeding, cells were treated with either PBS or 25 nm FN. Cells were loaded with Fluo-4, stimulated with 30 ng/ml PDGF, and monitored for changes in fluorescence. Data are presented as relative change in fluorescence from baseline (ΔF/ΔF₀) and are expressed as mean ± S.E. (error bars) of three experiments performed in quadruplicate. Values were normalized to the CL; +PBS condition, which was set to 1. *p < 0.05. B, vitronectin-adherent FN-null MEFs (6 × 10^5 cells/cm^2) were treated with 25 nm FN for 20 h (+FN) or an equal volume of PBS (+PBS) and then stimulated with vehicle (0 min PDGF) or with 30 ng/ml PDGF for 2 min. Cell lysates were analyzed by immunoblotting. Immunoblots shown represent one of three independent experiments performed in duplicate.

lyzed fibronectin fragments may serve to further amplify the effects of PDGF on cell behaviors.

PDGF-induced intracellular calcium release is initiated by PDGF-induced PDGF–R\(\beta\) trans-autophosphorylation of Tyr\(^{857}\)\((6)\). Our studies indicate that ECM fibronectin did not affect PDGF-induced PDGF–R\(\beta\) Tyr\(^{857}\) phosphorylation and that PDGF did not induce phosphorylation of PDGF–R\(\alpha\) (195 kDa) in FN-null MEFs. Furthermore, ECM fibronectin did not block PDGF-induced phosphorylation of tyrosine residues that bind and activate PI3K, such as PDGF–R\(\beta\) Tyr\(^{751}\) and Tyr\(^{740}\), and did not inhibit PDGF-induced activation of other intracellular signaling molecules, such as AKT or ERK1/2. Rather, fibronectin specifically attenuated PDGF-induced activation of PI3K, which is required for PDGF-induced intracellular calcium release\((6)\). In addition, fibronectin attenuated EGF-induced PI3K activation in collagen-adherent cells and reduced basal PI3K activity in vitronectin-adherent cells, indicating that fibronectin specifically attenuates PI3K activity, without affecting other PDGF-generated signals. In support of this inhibitory mechanism, others have shown that PI3K inhibition can attenuate growth factor–induced calcium signaling without affecting PLC\(_{\gamma1}\) activation\((36)\).

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**Diagram:**
- **Figure 7:** Fibronectin-mediated attenuation of PDGF-induced intracellular calcium release is independent of the adhesive substrate. (A) FN-null MEFs (1.1 × 10^6 cells/cm^2) were seeded on vitronectin-coated (VN) or collagen-coated (CL) wells. Four hours after seeding, cells were treated with either PBS or 25 nm FN. Cells were loaded with Fluo-4, stimulated with 30 ng/ml PDGF, and monitored for changes in fluorescence. Data are presented as relative change in fluorescence from baseline (ΔF/ΔF₀) and are expressed as mean ± S.E. (error bars) of three experiments performed in quadruplicate. Values were normalized to the CL; +PBS condition, which was set to 1. *p < 0.05. (B) Vitronectin-adherent FN-null MEFs (6 × 10^5 cells/cm^2) were treated with 25 nm FN for 20 h (+FN) or an equal volume of PBS (+PBS) and then stimulated with vehicle (0 min PDGF) or with 30 ng/ml PDGF for 2 min. Cell lysates were analyzed by immunoblotting. Immunoblots shown represent one of three independent experiments performed in duplicate.
Alternatively, downstream targets of AKT can feedback to inhibit agonist-induced PI3K activation (43). However, fibronectin did not affect basal or PDGF-induced AKT phosphorylation, indicating that an AKT-mediated negative feedback loop is unlikely to play a role in fibronectin-mediated attenuation of PDGF-induced PI3K activation. Rather, we speculate that attenuation of PI3K p55 activity by fibronectin may be due to activation of Src homology region 2 domain-containing phosphatase-1 (SHP1), which dephosphorylates and inactivates PI3K directly (44, 45). SHP1 is a 68-kDa prototyrosine phosphatase expressed predominantly by hematopoietic cells (46), but also produced at low levels by epithelial cells (47) and mesenchymal stem cells (48). Preliminary studies show that phosphorylated SHP1 was not detected in cells cultured in the absence of fibronectin (Fig. S3A). In contrast, fibronectin treatment induced a significant increase in the phosphorylation of a 47-kDa protein that was recognized by both phospho-SHP1 and nonphospho-SHP1 antibodies (Fig. S3, A and B). FN-null MEFs expressed the nonphosphorylated 47-kDa form of SHP1 in both the absence and presence of fibronectin (Fig. S3A). SHP1 is produced in multiple isoforms (49) and can be partially activated by proteolytic cleavage, and active SHP1 fragments...
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have been reported (50). Thus, future studies to characterize the 47-kDa form of SHP1 will advance our understanding of SHP1 expression and function and knowledge of how fibronectin and SHP1 may contribute to the integration of growth factor signaling in mesenchymal cells.

In summary, PDGF is a potent intercellular signaling molecule that can initiate a cascade of intracellular signals, leading to a variety of cell and tissue behaviors both in vitro and in vivo. The PDGF–PLCγ1–PI3K–calcium signaling axis exerts control over cell differentiation, proliferation, migration, and changes in gene transcription (1). The ability of ECM fibronectin to selectively regulate this arm of the PDGF signaling cascade brings to light a new mechanism by which cells utilize ECM-derived signals to tune intracellular signaling pathways. A greater understanding of the precise mechanisms by which cells integrate signals from specific ECM components to control which PDGF-induced signaling pathways are activated may allow for the development of more targeted PDGF-based therapies.

Experimental procedures

Reagents

Fibronectin was purified from outdated human plasma (American Red Cross, Rochester, NY) using gelatin-Sepharose (GE Healthcare) affinity chromatography (51). Collagen I was extracted from rat tail tendons with 0.5 M acetic acid and precipitated with sequential NaCl precipitation (52). Tissue culture plates were purchased from Corning (Lowell, MA). Unless otherwise indicated, chemicals were obtained from J. T. Baker or Sigma-Aldrich. PDGF-BB was purchased from Thermo Fisher Scientific. EGF and FGF-2 were from Peprotech (Rocky Hill, NJ). ATP was purchased from GE Healthcare. Antibodies and their sources are as follows (catalogue numbers in parenthesis): 9D2 mAb (27) was a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI); mouse IgG (I5381), anti-α-tubulin mAb (T6199), anti-vinculin mAb (V4505), and anti-fibronectin pAb (E3648) antibodies were purchased from Sigma-Aldrich; anti-phospho-PDGF receptor α (Tyr349)/PDGF receptor β (Tyr857) mAb (3170), anti-phospho-PDGF receptor β (Tyr749) mAb (3168), anti-phospho-PI3K pAb (4228), anti-PI3K P110 mAb (4249), anti-PI3K p55 mAb (11889), anti-phospho-SHP1 mAb (8849), and anti-SHP1 mAb (3759) were from Cell Signaling Technology (Danvers, MA); anti-PDGF-Rβ mAb (32570) was from Abcam (Cambridge, UK); anti-phospho-PDGF receptor β (Tyr751) mAb (9027) was from R&D Systems (Minneapolis, MN); anti-PLCγ1 mAb (610027), anti-phospho-AKT mAb (550747), and anti-phospho-Tyr mAb (610000) were from BD Biosciences; anti-phospho-ERK1/2 pAb (9101) was from New England Biolabs (Ipswich, MA); anti-vinculin mAb (MA35374) was from Millipore, and horseradish peroxidase–conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Bio-Rad. Recombinant GST-tagged FNIII1H,8–10 and vitronectin were produced in Escherichia coli and isolated by affinity chromatography, as described previously (28, 53).

Cell culture

FN-null and WT MEFs were from Dr. Jane Sottile (University of Rochester, Rochester, NY) (15). FN-null and WT MEFs were cultured under serum- and fibronectin-free conditions on collagen I–coated tissue culture flasks in a 1:1 mixture of Aim V (Invitrogen) and SF Medium (Corning). Adult NHDFs were purchased from Lonza (Walkersville, MD) (CC-2511). NHDFs were cultured in fibroblast basal medium (Lonza) supplemented with 2% fetal bovine serum, human FGF-B, and insulin (Lonza). Medium was changed every 3 days, and cells were passaged at 70–80% confluence.

Intracellular calcium release

FN-null MEFs, WT MEFs (1.1 × 10⁷ cells/cm²), or NHDFs (3.5 × 10⁵ cells/cm²) were seeded in Dulbecco’s modified Eagle’s medium onto black, clear-bottom 96-well plates precoated overnight at 4 °C with either 0.05 mg/ml type I collagen in 0.02 N acetic acid or 64 nM vitronectin in PBS (24). Four h post-seeding, fibronectin and/or inhibitors of fibronectin matrix assembly were added to the media. Following an overnight incubation (~23 h post-seeding), cells were well spread and confluent. Following removal of media, cells were loaded with 4.6 μM Fluo-4/AM (Molecular Probes, Inc., Eugene, OR) in a HEPES-based imaging buffer (127 mM NaCl, 0.56 mM MgCl₂, 4.7 mM KCl, 0.55 mM Na₂HPO₄, 1.28 mM CaCl₂, 10 mM HEPES-NaOH, 11 mM D-glucose, pH 7.4) (54). Following a 40-min incubation, the Fluo-4–containing imaging buffer was replaced with calcium-free imaging buffer containing 200 μM EGTA, and cells were incubated for 20 min to allow for intracellular de-esterification of Fluo-4. Fluorescence intensity was measured using a FlexStation multimode microplate reader (Molecular Devices). This device allows for concurrent scanning of all eight wells within a single column of the 96-well plate, taking a fluorescence reading for each of the eight wells every 1.5 s. Baseline fluorescence intensity measurements were recorded for 30 s prior to the addition of PDGF-BB or ATP. Control wells received an equal volume of the vehicle alone (PBS containing 0.1% BSA).

To quantify changes in intracellular calcium signaling in response to PDGF, raw data were filtered to reduce noise inherent in the fluorescence signal by taking a 30-s running average of the raw data (55). The PDGF-induced fluorescence increase was defined as the peak increase in fluorescence intensity (ΔF) above baseline fluorescence (F₀). Data are presented as the relative change in fluorescence (ΔF/F₀) to account for shifts in baseline fluorescence due to independent factors, including dye uptake, excitation intensity, or detector efficiency (55). ΔF/F₀ values from individual trials were normalized to a control condition (e.g. “+PBS”), which was set to 1.

Immunoprecipitation and immunoblot analysis

Cells were either solubilized directly in reducing Laemmli sample buffer (0.05 M Tris, 10% glycerin, 2% SDS, 0.1% bromphenol blue, 3.5% β-mercaptoethanol, pH 6.8) or extracted with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA containing Sigmafast™ protease inhibitor mixture (Sigma-Aldrich)). For immunoprecipitation studies, cells were washed with ice-cold PBS and extracted on ice for 15 min with 18 μl/cm² SDS-RIPA.
buffer. Protein concentrations of the soluble fractions were determined using a colorimetric bicinchoninic acid assay (Pierce® BCA protein assay, Thermo Scientific). Immunoprecipitations were performed by incubating cell lysates (750 μg) with 1 μg of anti-phospholipase Cγ1 antibodies (BD Biosciences) for 3 h at 4 °C. Protein G-Sepharose (GE Healthcare) was added, and lysates were incubated for 1 h at 4 °C. Immunoprecipitates were washed with SDS-RIPA buffer and then solubilized with reducing Laemmli sample buffer.

Cell lysates (40 μg of protein/lane) or immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (24). Immunoblots were blocked with either 5% milk (Carnation, Glendale, CA) or 3% BSA (heat-shock fraction V, pH 7.5; Affymetrix, Cleveland, OH) in TBS, pH 7.6, with 1% Tween 20 and 1 mM sodium orthovanadate. Immunoblots were incubated overnight with primary antibody at 4 °C. Blots were then washed, incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-linked secondary antibodies, and developed using SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific). After detection, blots were stripped either with 0.2 M glycine, 0.1% SDS, 1% Tween, pH 3.8, or by incubation for 1 h at 65 °C with 0.7% β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.7. Stripped blots were reprobed with secondary antibodies alone before reprobing with primary antibodies. The average net band intensity for duplicate samples was determined by densitometry using Carestream molecular imaging software (Rochester, NY). Relative band densities were averaged across multiple experiments to determine the average relative band density of each protein of interest for each treatment condition.

Statistical analyses

Unless otherwise stated, data are presented as mean ± S.E. relative to control treatment. Experiments were performed in quadruplicate (calcium signaling) or duplicate (immunoprecipitation and Western blotting) for a minimum of three independent experiments. Statistical analysis was performed using GraphPad Prism version 4 (La Jolla, CA). Unless otherwise indicated, statistical significance was determined using either an analysis of variance (ANOVA) with a Bonferroni post-test or Student’s t test. Results were considered statistically significant if p values were <0.05.

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