Non-Smad Transforming Growth Factor-β Signaling Regulated by Focal Adhesion Kinase Binding the p85 Subunit of Phosphatidylinositol 3-Kinase*\$ 

Received for publication, February 21, 2011 Published, JBC Papers in Press, March 28, 2011, DOI 10.1074/jbc.M111.233676

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TGF-β modulates numerous diverse cellular phenotypes including growth arrest in epithelial cells and proliferation in fibroblasts. Although the Smad pathway is fundamental for the majority of these responses, recent evidence indicates that non-Smad pathways may also have a critical role. Here we report a novel mechanism whereby the nonreceptor tyrosine focal adhesion kinase (FAK) functions as an adaptor necessary for cell type-specific responses to TGF-β. We show that in contrast to Smad actions, non-Smad pathways, including c-Abl, PAK2, and Akt, display an obligate requirement for FAK. Interestingly, this occurs in Src null SYF cells and is independent of FAK tyrosine phosphorylation, kinase activity, and/or proline-rich sequences in the C-terminal FAT domain. FAK binds the phosphatidylinositol 3-kinase (PI3K) p85 regulatory subunit following TGF-β treatment in a subset of fibroblasts but not epithelial cells and has an obligate role in TGF-β-stimulated anchorage-independent growth and migration. Together, these results uncover a new scaffolding role for FAK as the most upstream component regulating the profibrogenic action of TGF-β and suggest that inhibiting this interaction may be useful in treating a number of fibrotic diseases.

As a pleiotropic cytokine, TGF-β can regulate a variety of biological processes, including tissue growth, differentiation, cell migration, angiogenesis, and immunity (1). TGF-β signaling is initiated by activating two receptor serine/threonine kinases referred to as the type I (ALK5) and type II (TβR-II) TGF-β receptors. TGF-β ligand binding to ALK5 triggers the formation of a heterotetrameric receptor complex whereby the constitutively active TβR-II phosphorylates the glycine-serine-rich region in the juxtamembrane region of ALK5 leading to kinase activation. Activated ALK5 directly phosphorylates the receptor-regulated Smad proteins (R-Smads) on a C-terminal S(M/V)S motif (2). The primary transcriptional effectors of TGF-β signaling are two paralogs, Smad 2 and Smad 3 (3, 4). Once phosphorylated, the Smad proteins form heterotrimERIC complexes with the common mediator Smad, Smad4, and translocate to the nucleus where they function as co-modulators of gene expression (3, 4).

Although many aspects of TGF-β family member signaling utilize similar Smad effectors, the majority of cell types undergo a variety of responses following TGF-β addition (5). For instance, although it has been well characterized that TGF-β mediates epithelial cell growth arrest, the same ligand stimulates cellular proliferation in subsets of fibroblast cell lines and transformation to a myofibroblast phenotype in vivo. This transition can lead to a variety of fibrotic disorders including idiopathic pulmonary fibrosis, keloids, liver cirrhosis, and systemic sclerosis among others. In addition to the aforementioned fibrotic diseases, a number of carcinomas have been shown to be associated with a fibrotic or desmoplastic reaction that promotes tumor growth, progression, and/or angiogenesis (6, 7). Of note, the histology of the cancer-associated reactive stroma is similar to that observed during normal wound healing (8). Because TGF-β is a potent inducer of desmoplasia (6, 9), the manner by which these distinct phenotypes dependent upon cell context are regulated is one of the most important, yet enigmatic, aspects of TGF-β biological action (10).

In addition to Smad-dependent TGF-β signaling, there are many signaling responses stimulated by TGF-β that occur independently of the Smad proteins. Initial work in this area suggested Ras and various mitogen-activated protein kinases including ERKs, p38 MAPK, and JNK as mediators of TGF-β-induced cell proliferation and extracellular matrix gene expression (11–13). More recently, many other targets, including (but not limited to) Rho GTPases (Cdc42 and Rac1), PI3K, PAK2, Akt, and c-Abl, have been reported to be activated by TGF-β receptors independent of the Smad proteins and in a cell type-specific manner (10, 14, 15). Although these responses are often defined as Smad-independent, a more appropriate designation might be non-Smad because there is often cross-talk between these non-Smad and Smad pathways to obtain the full biologic response (16–18).

Among all of the aforementioned non-Smad signals stimulated by TGF-β, PI3K is critical because it is the most upstream component identified necessary for the observed mesenchymal cell-specific activation of Rho family members, PAK2, c-Abl, and mTOR (17, 19–21). It should be noted, however, that TGF-β also induces PI3K signaling in epithelial cell models that undergo epithelial to mesenchymal transition (22). Regardless of the context of activation, although PI3K has a particularly significant role in regulating a number of TGF-β responses,
there is little information on the mechanism(s) by which TGF-β ligand binding transduces the signal to the regulatory and kinase subunits of PI3K. To that end, as many TGF-β generated non-Smad signals, such as phosphorylation of Akt, can be induced by growth factors that couple their responses through focal adhesion kinase (FAK)\(^2\) (23–25), we investigated whether a similar mechanism was utilized during TGF-β profibrotic signaling.

FAK and Pyk2 are the sole members of the FAK family of nonreceptor protein-tyrosine kinases (26, 27). Although FAK has been implicated in a wide variety of cellular processes (23), one of the most highly investigated is the cytoskeletal reorganization leading to cell migration and metastasis (25, 28). In such a bioprocess, integrin activation of FAK provides a platform for the recruitment of other proteins including Src and Rho family members, PI3K, as well as numerous other focal adhesion-associated proteins to the cellular leading edge (29–33). In addition to integrin-dependent FAK activation, FAK signaling is also evident for a number of growth factors, including TGF-β (24, 25, 34–36). Although in the majority of these cases PI3K/Akt represent downstream pathways whose activation is dependent upon FAK, there is relatively little information concerning whether these signals are differentially integrated in various mammalian cell types and/or if integrin-clustered and TGF-β receptors have similar or distinct FAK requirements for signal propagation.

In the current study, we have addressed both of those questions in the context of profibrotic TGF-β signaling. Data are presented documenting that (i) FAK is the most upstream component identified (so far) that couples TGF-β receptor activation to non-Smad TGF-β signaling; (ii) TGF-β induces FAK binding to the p85 regulatory subunit of PI3K in a cell type-specific manner, indicating why fibroblasts, in contrast to epithelia, can activate non-Smad pathways such as Akt, PAK2 and c-Abl; and (iii) although TGF-β stimulates FAK tyrosine phosphorylation, it is the adaptor function of FAK that is critical for non-Smad signaling because phosphorylation site mutations as well as kinase-impaired FAK constructs reconstitute TGF-β-dependent (but not integrin) FAK actions in FAK null MEFs similar to wild type. Thus, the mesenchymal cell response to TGF-β resulting in PI3K activation requires FAK acting as a signaling scaffold.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—AKR-2B, NIH3T3, Swiss 3T3, MEF cells, HeLa, Madin-Darby canine kidney, and Mv1Lu cells were grown in high glucose DMEM supplemented with 10% FBS (HyClone, Logan, UT; SV30014.03). Human TGF-β was obtained from R & D Systems (Minneapolis, MN). Unless otherwise stated, AKR-2B, NIH3T3, and Swiss 3T3 cells were plated at 2.5 \(\times\) 10^6, whereas HeLa, Madin-Darby canine kidney, and Mv1Lu cells were plated at 3.0 \(\times\) 10^6 in p100 dishes and incubated overnight at 37 °C to achieve confluence before serum starvation and stimulation. FAK null cells derived from mouse embryos homozygous for a disrupted \textit{fak} allele (37) and their wild-type counterparts were pro-

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\(^2\)The abbreviations used are: FAK, focal adhesion kinase; PRS, proline-rich sequence(s); MEF, mouse embryonic fibroblast.

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vided by Dr. Steve Hanks (Vanderbilt University). Soft agar assays were performed as described (19).

**Cell Migration**—Migration assays were performed using Transwell plates with an 8-μm-pore size membrane (Corning Costar, Cambridge, MA). Transwell membranes were coated at 37 °C with 5 μg/ml plasma fibronectin and then blocked with 1% BSA at 37 °C for 30 min. Serum-starved AKR-2B clones were treated with (or without) 10 ng/ml TGF-β supplemented DMEM for 24 h and following trypsinization suspended in 0.1% FBS/DMEM at 1×10^5 cells/ml. To assess migration, 100 μl of cell suspension was seeded into the Transwell top chamber (bottom chamber contained 0.1% FBS/DMEM) and placed at 37 °C for 4–6 h. Nonmigrated cells were wiped from the upper surface, and cells that migrated to the membrane underside were washed with PBS, fixed, stained (Hema 3 kit; Fisher) and counted in four randomly selected high power fields (100× magnification).

**Western Blotting**—Confluent cultures were treated overnight in serum-free (fibroblasts and MEFs) or 0.1% FBS/DMEM (other cell lines). Following the addition of growth factors for the indicated times, the cells were lysed in kinase lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100, 50 mM NaF, 0.1 trypsin inhibitor unit of aprotinin/milliliter, 50 μg/ml phenylmethysulfonyl fluoride, 100 μM vanadate, 1 μg/ml leupeptin) for 30 min at 4 °C and equivalent protein processed for Western analysis. Unless noted otherwise, the blots were incubated overnight with primary antibody (4 °C), washed (10 mM Tris, pH 7.4, 0.1% Tween, 140 mM NaCl), and then treated for 45 min at 25 °C with secondary antibody prior to washing and visualization with ECL reagent (Amersham Biosciences (Piscataway, NJ); RPN2209). Antibodies used were from Upstate Biotechnology (Lake Placid, NY; anti-p85 PI3K subunit 06-925; anti-Smad2, 06-829), BD Biosciences (Franklin Lakes, NJ; p130Cas, 610272), Roche Applied Science (Nutley, NJ) (anti-Myc, 11667149001;), Calbiochem (San Diego, CA; anti-phospho-Smad2, 618042), Chemicon International, Inc. (Temecula, CA; anti-GAPDH, MAB374), Zymed Laboratories Inc.-Invitrogen (Carlsbad, CA) (anti-Smad3, 51-1500), Santa Cruz Biotechnology (Santa Cruz, CA; anti-PAK2, sc1872; anti-cAbl, sc23 and sc131), BIOSOURCE-Invitrogen (Carlsbad, CA) (anti-FAK, AH01272; anti-phospho-FAK Tyr-397, 44624G; anti-phospho-FAK Tyr-577, 44614G), or Cell Signaling (Beverly, MA; anti-Akt, 9272; anti-phospho-Akt Ser-473, 9271). Secondary goat anti-mouse or donkey anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA; sc-2005) and Amersham Biosciences (Piscataway, NJ) (NA934V), respectively.

**FIGURE 2**: TGF-β-stimulated PI3K and PAK2 kinase activity requires FAK. A, quiescent FAK null and wild-type FAK+/+ MEF cells were left untreated (0) or stimulated for the indicated times with 10 ng of TGF-β/ml. PI3K kinase activity was detected using L-phosphatidylinositol (17), and Western blotting was performed with antibodies to FAK, p85 regulatory subunit of PI3K, Smad2, or phospho-Smad2 (pSmad2). B, FAK null and wild-type FAK reconstituted (WT-FAK) MEFs were left untreated (−) or stimulated (+) for 45 min with 10 ng of TGF-β/ml followed by PI3K and PAK2 kinase assays or Western blotting as described under “Experimental Procedures.”
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Co-immunoprecipitation—The cells were grown to confluence in 10% DMEM and serum-starved overnight. After the indicated treatment, the cells were lysed in modified kinase lysis buffer (pH 7.4) for 30 min at 4 °C. Normalized lysates were then incubated overnight at 4 °C in the presence of antibody before collection of the immune complex using protein A- or protein G-agarose (Upstate Biotechnology, Lake Placid, NY). After washing, the proteins were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF, and visualized by autoradiography. PI3K kinase assays were performed as previously described using L-α-phosphatidylinositol as substrate (17).

Kinase Assays—The cultures were treated as indicated and lysed for 30 min at 4 °C in 750 μl of kinase lysis buffer. Extracts were clarified, and equivalent protein (∼500–700 μg) was incubated overnight at 4 °C with antibody. Immune complexes were collected with protein G-agarose and washed twice in kinase lysis buffer and twice in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol) prior to incubation in 50 μl of kinase buffer containing 5 μM ATP, 5 μCi of [γ-³²P]ATP/μl, and 5 μg of substrate (myelin basic protein for PAK kinase assays and GST-Crk for c-Abl kinase assays). The kinase assay was allowed to proceed for 10 min at 37 °C, stopped with 50 μl of 2× Laemmlli buffer, submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. PI3K kinase assays were performed as previously described using L-α-phosphatidylinositol as substrate (17).

Luciferase Assays—MEF cells were plated in six-well plates at 2 × 10⁵/well and grown to 60–80% confluence. The cultures were subsequently transfected (PolyFect reagents; Qiagen, Valencia, CA) with 1 μg of the indicated luciferase reporters and CMV-driven β-galactosidase. Following 12 h recovery, the cells were serum-starved for 4 h in 0.1% FBS/DMEM and then treated with or without 10 ng/ml TGF-β for an additional 24 h. Normalized luciferase assays were performed on cell lysates using the luciferase assay system (Promega, Madison, WI).

RT-PCR—Total RNA was isolated using TRIzol® reagent (Invitrogen). Two micrograms of RNA were reverse transcribed with the SuperScript® III reverse transcriptase system (Invitrogen). Complementary DNAs were subjected to amplification by PCR. PCR reagents were from Denville Scientific (Metuchen, NJ). PCR products were electrophoretically resolved on 1% agarose gels and stained with ethidium bromide. Primer characteristics and PCR parameters are provided in supplemental Table S1.

RESULTS

FAK Is Required for Non-Smad TGF-β Signaling—FAK provides a key role in coupling growth factor receptors and integrins to the activation of downstream signaling targets (24, 25). Because FAK expression and/or activation has been reported to be controlled by both Smad and non-Smad pathways (35, 36, 38, 39), we investigated the requirement for FAK in regulating profibrotic TGF-β signaling. Because we had previously shown that PI3K regulates a branch point in the mesenchymal cell non-Smad response to TGF-β, with one arm leading to PAK2/c-Abl and the other to Akt/mTOR (17, 19), MEFs from wild-type (FAK+/+) and FAK null (FAK−/−) animals were obtained, and the effects on both Smad and non-Smad TGF-β targets were determined. Although a loss of FAK had no discernable effect on Smad2 activation or total Smad2 protein levels, Akt phosphorylation, and c-Abl kinase activity were completely
abrogated (Fig. 1, A and B, left panels). Most importantly, reconstitution of FAK null MEFs with wild-type FAK (WT-FAK) restored non-Smad TGF-β signaling to control levels (Fig. 1, A and B, right panels). Consistent with our previous publications showing that Akt and c-Abl activation by TGF-β are dependent upon PI3K and PAK2 (17, 20), a similar requirement was observed for induction of PI3K and PAK2 activity (Fig. 2). Identical results were obtained in AKR-2B cells expressing shRNA to murine FAK (supplemental Fig. S1).

To further assess the role of FAK in TGF-β signaling, the induction of various TGF-β-responsive genes was investigated in FAK null and wild-type FAK restored MEFs. Although FAK was not required for expression of the activin response element (Smad2-dependent) promoter, in the absence of FAK transcriptional induction of fibronectin (non-Smad regulated) (20, 40) was abrogated (Fig. 3A). Consistent with these transient reporter analyses, RT-PCR of the PAI-1 (Smad3-dependent) and fibronectin genes showed identical results (Fig. 3B). Moreover, an analogous FAK requirement is observed in FAK knockdown AKR-2B cells for TGF-β-regulated phenotypes such as soft agar colony formation and cell migration (Fig. 4). These findings indicate that although FAK is not required for a subset of Smad-dependent responses, it provides an obligatory function(s) in non-Smad TGF-β signaling and complex biological processes (Figs. 1–4).

FAK Tyrosine Phosphorylation Is Not Required for Non-Smad TGF-β Signaling—FAK is a versatile protein that can function as a tyrosine kinase to facilitate the binding of SH2-containing proteins such as Src family kinases and Grb2 or act as a scaffold through which it can associate with numerous structural and signaling molecules via various N- and C-terminal domain interactions (25, 32, 41). Although TGF-β has been shown to induce FAK tyrosine phosphorylation, and FAK is required for TGF-β-induced JNK phosphorylation and HER2 clustering (36, 39), the requirement for FAK phosphorylation, per se, on subsequent phenotypes is not well defined. To address that issue further, we first examined whether integrin- or Src family-mediated FAK phosphorylation was necessary for the aforementioned non-Smad TGF-β signaling dependent upon FAK.

SYF cells are MEFs in which the three primary Src family kinases (Src, Yes, and Fyn) have been knocked out (42). Because Src is known to have a significant role in both growth factor as well as adhesion-regulated FAK phosphorylation, we investigated the effect of Src loss on both Smad and non-Smad responses. As shown in Fig. 5A, TGF-β-stimulated FAK Tyr-397 and Tyr-577 phosphorylation was not evident in SYF cells. In contrast to FAK tyrosine phosphorylation, however, Smad and non-Smad targets including PI3K, PAK2, Akt, and Smad2 were similarly activated regardless of any effect on FAK phosphorylation.

The lack of a requirement for FAK Tyr-397 and Tyr-577 phosphorylation in TGF-β signaling was unexpected. As such, we independently extended this analysis in AKR-2B cells by using the actin polymerization inhibitor cytochalasin D. Although cytochalasin D has been shown to prevent both FAK tyrosine phosphorylation and PI3K/Akt pathway activation in integrin-dependent systems (43), whereas TGF-β-induced FAK Tyr-397 and Tyr-577 phosphorylation was similarly inhibited by cytochalasin D, there was no effect on either Smad or the indicated non-Smad targets (Fig. 5B). We were unable to examine PAK2 because the drug alone induced maximal PAK2 kinase activity, which was unaffected by TGF-β (data not shown). Because PAK family members have a major role in cytoskeletal rearrangement, this likely reflects such an interrelation.

The preceding data support the hypothesis that although TGF-β stimulates FAK tyrosine phosphorylation (Fig. 5), this does not reflect the obligate requirement for FAK in non-Smad
TGF-β signaling (Figs. 1–4). Because this conclusion would necessitate a significant change in the manner by which we perceive the inter-relation of TGF-β and FAK, to further assess the role of FAK phosphorylation and/or kinase activity in TGF-β action, we transfected FAK null cells with wild-type or site-specific FAK mutant constructs (Fig. 6A). Consistent with
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our previous data indicating a disconnect between FAK phosphorylation and a requirement for FAK protein per se, expression of phospho-site or kinase-impaired FAK mutants reconstituted TGF-β-stimulated PI3K activation and Akt phosphorylation to a similar extent as wild type (Fig. 6, B and C).

The absence of a need for FAK phosphorylation or kinase activity in non-Smad TGF-β signaling was somewhat surprising. As such, we performed analogous control studies in an integrin-dependent model where Akt activation is clearly dependent upon FAK autophosphorylation at Tyr-397 and kinase activity (32, 44, 45). FAK null and reconstituted MEFs were plated on control or fibronectin-coated wells, and Akt phosphorylation was determined. As expected, phosphorylation was significantly reduced by expression of the FAK−/− data originally followed FAK−/− but were moved for consistency with other figures. B, representative fibroblast (AKR-2B, NIH3T3, and Swiss 3T3) and epithelial (Madin-Darby canine kidney, HeLa, and Mv1Lu) cells were left untreated (−) or stimulated (+) for 45 min with 10 ng of TGF-β/ml. The cell lysates were processed in a manner identical to that described for A.

TGF-β induces the binding of FAK with PI3K through a non-canonical mechanism, and second, this association should occur in a cell type-specific manner because mesenchymal cells, but not epithelia, activate those non-Smad pathways dependent upon PI3K (17–19, 21).

To address the first issue, wild-type and FAK null MEFs were treated in the presence or absence of TGF-β, and the binding of FAK to the p85 regulatory subunit of PI3K was determined by immunoprecipitation and Western analysis. Although there was a low basal binding, TGF-β treatment increased association at 10 min with peak binding occurring by 60 min (Fig. 7A). These kinetics mirror the increase in PI3K activity that we previously reported (Fig. 2A and Ref. 17). In the absence of FAK, as expected, FAK-p85 association was not detected in the presence of TGF-β, although Smad2 phosphorylation occurred normally (Fig. 7A). Second, if the association of FAK with p85 observed in wild-type MEFs reflected a critical aspect of non-Smad TGF-β signaling, it should occur in various fibroblast lines, but not in epithelial cells. To address that point, the ligand-dependent binding of FAK and p85 was investigated in three representative mesenchymal (AKR-2B, NIH3T3, and Swiss 3T3) and epithelial (Madin-Darby canine kidney, HeLa, and Mv1Lu) cultures. As shown in Fig. 7B, although TGF-β induced the association in each of the fibroblast lines, no binding was detected in any of the epithelial cultures. Lastly, that the FAK/PI3K connection induced by TGF-β functions via an

![Figure 7. TGF-β regulates cell type-specific association of FAK with the p85 regulatory subunit of PI3K.](image-url)

A, quiescent FAK null (FAK−/−) and wild-type (FAK+/+) MEFs were left untreated (−) or stimulated (+) for the indicated times with 10 ng of TGF-β/ml. Whole cell lysates were subjected to FAK immunoprecipitation (IP, top two panels) followed by Western blotting using antibodies to the p85 subunit of PI3K or FAK. The bottom three panels show expression of total p85 or phosphorylated (pSmad2) and total Smad2 protein to document identical cellular p85 and TGF-β receptor activation, respectively. The blots with the FAK−/− data originally followed FAK−/− but were moved for consistency with other figures. B, representative fibroblast (AKR-2B, NIH3T3, and Swiss 3T3) and epithelial (Madin-Darby canine kidney, HeLa, and Mv1Lu) cells were left untreated (−) or stimulated (+) for 45 min with 10 ng of TGF-β/ml. The cell lysates were processed in a manner identical to that described for A.
alternative mechanism from that reported for integrins and receptor tyrosine kinases is further documented in Fig. 8. Additional FAK mutations (Fig. 8A) within the proline-rich sequences (PRS) previously shown to mediate various protein-protein interactions (23, 45, 46) were examined for their ability to reconstitute non-Smad TGF-β signaling and/or TGF-β-regulated FAK/p85 association. In contrast to what was observed in other receptor systems, loss of either/both PRS (in the presence or absence of wild-type Tyr-397) had no impact on FAK-dependent TGF-β responses (Fig. 8B). Western blotting with the indicated antibodies was performed as described. C, Western analysis was performed on lysates from FAK null MEFs transfected with the indicated FAK constructs as in B. D, FAK null MEFs were transfected with empty vector (first and second lanes), wild-type FAK (third and fourth lanes), or FAK mutated at Tyr-397 and both PRS domains (fifth and sixth lanes). The cultures were left untreated (−) or stimulated (+) with 10 ng/ml TGF-β for 1 h, and equivalent protein was subjected to FAK IP followed by Western blotting using antibodies to the p85 subunit of PI3K (top panel) or FAK (second panel). The bottom two panels document similar Smad activation (pSmad2).

**DISCUSSION**

One of the most fundamental questions in understanding TGF-β action is how one growth factor, binding to the same receptors, can induce such fundamentally distinct phenotypes as growth arrest (many cell types including epithelia) and cell proliferation (mesenchymal cells). The manner by which these biologies are manifested directly impacts our ability to address a number of proliferative disorders. To that end, over the past few years we have identified a variety of targets that are activated by TGF-β in mesenchymal cultures but not epithelial cultures (17, 19, 20). Although we have recently documented the presence of a regulatory mechanism in epithelia dependent upon an interaction of the epithelial enriched protein Erbin with the NF-2 tumor suppressor Merlin, which prevents expression of PAK2 kinase activity (18), this is a relatively “downstream” control that does not address how other cell type-specific activities might be regulated. Because PI3K is (i) the most upstream component (so far) identified regulating context-specific cell signaling resulting in profibrotic/desmoplastic TGF-β responses and (ii) represents a critical branch point necessary for activation of both PAK2/c-Abl and Akt/mTOR pathways following TGF-β treatment, we further investigated the manner by which its activity was modulated. Because numerous publications have shown a requirement for FAK in coupling receptor- and integrin-dependent events to PI3K (25, 39), studies were initiated to address two general questions. First, was FAK required for non-Smad TGF-β signaling? Second, might FAK help define how cell type-specific responses are obtained in fibroblasts and epithelia to TGF-β?
To address these issues, we first determined the requirement for FAK in Smad and/or non-Smad TGF-β signaling. When FAK null MEFs were treated with TGF-β, there was no discernable effect on Smad2 or Smad3 phosphorylation or induction of responsive genes. This is contrasted, however, by the inability to activate PI3K, Akt, PAK2, c-Abl, and genes dependent upon their action. Thus, although Smad pathways were unaffected, non-Smad TGF-β signaling and complex biological processes were significantly attenuated (Figs. 1–4).

FAK can function as either a tyrosine kinase affecting multiple targets or as a scaffold through its various protein-binding domains. Because various reports have documented the ability of TGF-β to induce FAK phosphorylation (25, 35, 36, 39), we investigated whether the major FAK tyrosine phosphorylation sites and/or kinase activity provided the obligate FAK requirement for non-Smad TGF-β signaling. Surprisingly, abrogation of either had no discernable effect on PI3K activation, PAK2 kinase activity, or Akt phosphorylation stimulated by TGF-β (Figs. 5 and 6, B and C). As previously reported (32, 44, 45), however, FAK autophosphorylation at Tyr-397 was necessary for integrin-dependent FAK signaling (Fig. 6D), and consistent with our interpretation that FAK is critical for the fibroproliferative actions of TGF-β, TGF-β did not induce FAK phosphorylation at Tyr-397 or Tyr-577 in epithelial cultures (supplemental Fig. S3).

The finding that non-Smad TGF-β signaling was dependent upon FAK protein (Figs. 1–6 and supplemental Fig. S1) yet was unaffected by the loss of FAK kinase activity and could occur in cells devoid of the primary Src family kinases (Figs. 5 and 6) supports the proposal that FAK is functioning as a signaling adaptor critical for integrating various non-Smad responses following TGF-β receptor activation. Because aberrant TGF-β signaling is believed to have a critical role in various fibrotic disorders as well as cancer (9, 47, 48), our findings are consistent with the hypothesis that inhibitors that target the adaptor function of FAK might be effective in TGF-β-mediated pathologies (49).

As mentioned previously, we have defined a number of non-Smad targets activated by TGF-β in a variety of mesenchymal, but not epithelial, cell lines. Because the most upstream component thus far identified required for those responses is PI3K, and our data show that the adaptor role of FAK is most critical, this raises the central question as to how FAK scaffolding activity is coupled to PI3K activation. This was directly addressed where it was shown that FAK binds the regulatory p85 subunit of PI3K in a TGF-β-dependent manner consistent with the kinetics of PI3K activation (Figs. 2A and 7A). Although we have not as yet identified the regulatory components controlling FAK/p85 binding, it is likely via a noncanonical mechanism because it is independent of FAK Tyr-397 phosphorylation and/or the proline-rich sequences in the C-terminal domain (Fig. 8). Moreover, in agreement with our finding that TGF-β does not activate PI3K in epithelia that do not undergo epithelial to mesenchymal transitions (17, 22), an analogous cell type-specific association of FAK and p85 in various mesenchymal cell lines was observed (Fig. 7B).

In summary, we present evidence that (i) FAK has an obligate role in non-Smad TGF-β signaling; (ii) in contrast to integrin signaling, activation of PI3K-dependent TGF-β targets occurs independent of FAK autophosphorylation/kinase activity and Src family kinases; and (iii) TGF-β stimulates the in vivo association of FAK and the p85 regulatory subunit of PI3K in responsive fibroblast but not epithelial cell cultures. Current studies are designed to identify and characterize the operative mechanisms mediating the FAK/p85 association.

Acknowledgments—We thank Dr. Steve Hanks (Vanderbilt University) for providing the wild-type Myc-FAK construct and FAK−/− MEFs reconstituted with wild-type FAK and Dr. Mark McNiven (Mayo Clinic) for SYF cells.

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