Transforming Growth Factor-β Activation of Phosphatidylinositol 3-Kinase Is Independent of Smad2 and Smad3 and Regulates Fibroblast Responses via p21-Activated Kinase-2

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Abstract

Transforming growth factor-β (TGF-β) stimulates cellular proliferation and transformation to a myofibroblast phenotype in vivo and in a subset of fibroblast cell lines. As the Smad pathway is activated by TGF-β in essentially all cell types, it is unlikely to be the sole mediator of cell type–specific outcomes to TGF-β stimulation. In the current study, we determined that TGF-β receptor signaling activates phosphatidylinositol 3-kinase (PI3K) in several fibroblast but not epithelial cultures independently of Smad2 and Smad3. PI3K activation occurs in the presence of dominant-negative dynamin and is required for p21-activated kinase-2 kinase activity and the increased proliferation and morphologic change induced by TGF-β in vitro. (Cancer Res 2005; 65(22): 10431–40)

Introduction

The cellular response to transforming growth factor-β (TGF-β) is dependent on the cell context (1, 2). For instance, although TGF-β stimulates apoptosis in hepatocytes and lymphocytes (3), vascular smooth muscle cells become resistant to other apoptotic cues (4). Similarly, whereas epithelial cells undergo a reversible late G1 growth arrest following TGF-β addition (5), many fibroblasts proliferate and take on a morphologically altered phenotype with characteristics similar to those of myofibroblasts (6).

With such a variety of responses, it is not surprising that TGF-β receptor (TGF-βR) activation and signaling are uniquely regulated (7, 8). There are three primary receptors for TGF-β on most cell types: types I, II, and III (β-glycan) receptor. TGF-βR activation is initiated through the binding of ligand to the type II receptor, which is a constitutively active serine/threonine kinase (9). This event facilitates the recruitment and subsequent transphosphorylation of the type I receptor in a glycine- and serine-rich region known as the GS domain. The activated type I receptor then serves as a docking site for receptor-associated Smad (R-Smad) proteins that are brought to the receptor complex associated with the FYVE domain protein Smad anchor for receptor activation (SARA; ref. 9). Following phosphorylation at a specific SSXS site in the COOH terminus by the type I receptor, the R-Smad proteins dissociate from the type I receptor and translocate to the nucleus complexed with the common mediator Smad4, where they serve as coregulators of transcription (7, 8).

Although the Smad pathway is critical for many aspects of TGF-β signaling, Smad-independent responses have also been documented (10, 11). Recently, we characterized the Smad-independent activation of the yeast STE20 homologue p21-activated kinase-2 (PAK2) by TGF-β, which occurs in mesenchymal and not epithelial cultures (1). Furthermore, the activation of PAK2 was required for the morphologic alterations and proliferative responses induced in these cells. Other Smad-independent signaling targets include TGF-β-activated kinase-1 (12), Ras (13), various Rho proteins (14, 15), c-Jun NH2-terminal kinase (10, 11), extracellular signal-regulated kinase (16), p38 (17), and the non–receptor tyrosine kinase c-Abl (18). The manner that these pathways integrate with each other and/or Smad signaling is currently not well understood.

In addition to the above targets, previous work has suggested that phosphatidylinositol 3-kinase (PI3K) might be involved in positively or negatively regulating TGF-β signaling at least in some cell types (3, 19–21). PI3Ks belong to a family of proteins that phosphorylate the 3'-hydroxyl group on the inositol ring of phosphoinositides and consist of a regulatory p85 subunit and catalytic p110 subunit (22). In addition to generating various lipid effectors, PI3K also phosphorylates several protein substrates whose activity has been linked to cell growth, differentiation, and survival (22, 23). There are multiple isoforms of PI3Ks, which originally have been subdivided into three classes (23). Class I, II, and III enzymes in the PI3K family generate specific lipid mediators that bind to FYVE and pleckstrin homology domains in a variety of proteins, affecting their localization, conformation, and activity (22). Class I PI3Ks catalyze the phosphorylation of the 3'-hydroxyl subunit of phosphoinositides. Although phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] can all act as substrates for class I PI3Ks in vitro, PtdIns(4,5)P2 is the main substrate in vivo (23). Thus, class I PI3Ks generally catalyze the conversion of PtdIns(4,5)P2 to phosphatidylinositol 3,4,5-trisphosphate. Class II PI3Ks consist of a small group of larger PI3K molecules whose cellular function is currently unclear (23). Class III PI3Ks are homologues of the yeast vesicular protein sorting protein Vps34p. These PI3Ks can only use phosphatidylinositol as a substrate in vitro and are most likely to be responsible for the generation of a large proportion of the phosphatidylinositol 3-phosphate [PtdIns(3)P] in cells (22, 24). Because the cellular levels of PtdIns(3)P remain fairly constant, this suggests that the physiologic processes in which class III PI3Ks are involved are not acutely triggered by cellular stimulation. As such, the major cellular

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function of class III PI3Ks is believed to be in intracellular trafficking, with the product of the enzyme [PtdIns(3)P] being recognized by proteins containing FYVE domain, such as EEA1, VAC1, YOTB, Hrs, and SARA (22, 24).

PI3K signaling has been implicated in the control of a wide range of cellular activities, including (but not limited to) proliferation, survival, adhesion, differentiation, and cytoskeletal organization (22, 23, 25). However, although many growth factors clearly use the PI3K pathway(s) as an integral component to their biological function, the role of PI3K in TGF-β signaling is presently unclear/unknown. PI3K has been reported to be involved (20), not involved (26), or somewhat involved (27–29) in the cytoskeletal changes associated with TGF-β-stimulated epithelial-to-mesenchymal transitions (EMT). Although the reasons for these discrepancies are presently unclear, a component of the controversy may simply reflect the fact that the majority of studies were done in epithelial cells undergoing an EMT-like change. If PI3K is primarily a response induced by TGF-β in fibroblast cultures, only a fraction of the epithelial population would be “mesenchymal” at the times tested. As such, the present study was undertaken to determine (a) whether PI3K was indeed activated in a fibroblast-specific manner to TGF-β and (b) to further clarify the requirement for PI3K signaling in the cellular response to TGF-β stimulation. The data document that (a) Smad-independent activation of class I PI3K regulates TGF-β-mediated morphologic and proliferative responses in a subset of fibroblast not epithelial cultures, (b) PI3K signaling occurs in a plasma membrane proximal compartment independent of receptor internalization, and (c) PI3K-dependent activation of PAK2 is necessary for TGF-β-stimulated morphologic transformation and cell proliferation.

Materials and Methods

Cell culture. Cells were grown in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA). DMEM and DMEM for stimulation was obtained from Dr. Anita Roberts (NIH, Bethesda, MD). Cultures were treated overnight in serum-free DMEM and stimulated for the indicated times (EMT). Although the reasons for these discrepancies are presently unclear, a component of the controversy may simply reflect the fact that the majority of studies were done in epithelial cells undergoing an EMT-like change. If PI3K is primarily a response induced by TGF-β in fibroblast cultures, only a fraction of the epithelial population would be “mesenchymal” at the times tested. As such, the present study was undertaken to determine (a) whether PI3K was indeed activated in a fibroblast-specific manner to TGF-β and (b) to further clarify the requirement for PI3K signaling in the cellular response to TGF-β stimulation. The data document that (a) Smad-independent activation of class I PI3K regulates TGF-β-mediated morphologic and proliferative responses in a subset of fibroblast not epithelial cultures, (b) PI3K signaling occurs in a plasma membrane proximal compartment independent of receptor internalization, and (c) PI3K-dependent activation of PAK2 is necessary for TGF-β-stimulated morphologic transformation and cell proliferation. 

Western blotting. Fibroblast lines with a targeted deletion of Smad3 were obtained from Dr. Anita Roberts (NIH, Bethesda, MD). Cultures were treated overnight in serum-free DMEM and stimulated for the indicated times in the presence or absence of 5 ng/mL TGF-β, 20 μg/mL Y294002, or 30 μmol/L SH-5. Cells were lysed [50 mmol/L Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na3VO4, 1× Complete protease inhibitor; Roche Applied Science, Indianpolis, IN], and equivalent protein was probed with the indicated antibody. The phosphorylated Smad2 and total Smad2 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY), respectively, whereas the total Smad3 antibody was from Cell Signaling Technology (Danvers, MA). Other antibodies were from Cell Signaling Technology (Beverly, MA) (anti-PK2, anti-Akt, and anti-phospho-Akt), Upstate Biotechnology (anti-phospho-Akt and anti-phospho-PI3K), Santa Cruz Biotechnology (Santa Cruz, CA) (anti-HA, sc-805; anti-p110, sc-8010), Stratagene (La Jolla, CA) (anti-PI3KC3 N-term), BD Transduction Laboratories (San Jose, CA) (anti-PI3K p170), Roche Applied Science (anti–green fluorescent protein (GFP)), Sigma (anti-Flag, A-1205), Amersham Pharmacia (Piscataway, NJ) (anti-His), and Clontech (Mountain View, CA) (anti-my). 

Morphologic transformation and proliferation. AKR-2B cells were plated at 2.5 × 105 per six-well dish and incubated at 37°C for 24 hours. Confluent cultures were placed in serum-free MCDB 402 (JRH Biosciences, Lenexa, KS) for 48 hours and stimulated by the addition of fresh serum-free DMEM alone or containing 5 ng/mL TGF-β and in the absence or presence of 20 μg/mL Y294002. After 48-hour incubation, images were acquired by phase-contrast microscopy (magnification, ×20). Following photography, cultures were trypsinized and the cell number was determined by hemacytometer. 

Transfections. For luciferase assays, cells were plated in six-well dishes at 2.5 × 104 per six-well dish before transfection (4 hours) with 2 μg of either 3TP-Lux, Smad-binding element (SB)-Luc, or α-smooth muscle actin (SMA)-Luc and 0.5 μg cytomeglovirus (CMV)-β-galactosidase with Fugene 6 (Roche Applied Science). Cultures were left untreated or stimulated with 5 ng/mL TGF-β in the absence or presence of 20 μg/mL Y294002 and then assayed for luciferase activity as described previously (30).

To investigate the effect of antisense p85, morpholino antisense oligonucleotides representing nucleotides –22 to +3 of mouse p85 (5′ CATGTGTTGGACAGTTTCC 3′) or a scrambled control (5′ GATACGACTATGCTCTCGATCT 3’) were synthesized with 3′ fluorescein by Gene Tools (Philomath, OR). Cos7 cells were plated in six-well dishes at a density of 1.0 × 105 per well in 10% DMEM and incubated at 37°C for 24 hours. Confluent cultures were transfected with the p85 antisense or control oligonucleotide along with plasmids expressing the type I and II TGF-βR at a final concentration of 6 μmol/L with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Following 48 hours of incubation in 2% FBS-DMEM and 24-hour incubation in 0.1% FBS-DMEM, cultures were stimulated for 60 minutes at 37°C in 2 mL serum-free DMEM alone or containing 10 ng/mL TGF-β before Western analysis (described above) or PAK2 kinase assay (described below).

Protein kinase assays. Lysates for kinase assays were prepared as described (1) and equivalent protein (500-700 μg) was incubated overnight at 4°C with the specified antibody. Immune complexes were collected with protein A-Sepharose (Sigma) and washed twice in kinase lysis buffer and twice in kinase buffer [25 mmol/L Tris (pH 7.4), 10 mmol/L MgCl2, 1 mmol/L DTT] before incubation in 50 μL kinase buffer containing 5 μmol/L ATP, 5 μCi [γ-32P]ATP per reaction, and 5 μg of either myelin basic protein (Sigma) for PAK2 assays or immunopurified glycosine synthase kinase-3 (GSK-3) to assess Akt activity. Immunopurified GSK-3 substrate was obtained using “catch-and-release” kit supplied by Upstate Biotechnology. The kinase reactions were allowed to proceed for 10 minutes at 37°C, stopped with 50 μL of 2× Laemmli buffer, and processed by SDS-PAGE and autoradiography.

In vitro lipid kinase assays. To determine the ability of P3K to phosphorylate phosphoinositides, cells were grown to 80% confluency before overnight incubation in serum-free DMEM. Cultures were treated with 5 ng/mL TGF-β for the indicated times before washing twice with cold buffer A [137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L CaCl2, 1 mmol/L MgCl2, 0.1 mmol/L sodium orthovanadate]. Cells were then lysed (buffer A containing 1% NP40 and 1% L-phenylmethylsulfonyl fluoride) with gentle agitation for 20 minutes at 4°C and centrifuged for 10 minutes at 13,000 g × g. The supernatant was immunoprecipitated with antibody to the p85 subunit of PI3K for 1 hour at 4°C, and immune complexes were collected with protein A-Sepharose and washed thrice with buffer A containing 1% NP40, thrice with 100 mmol/L Tris-HCl (pH 7.4), 5 mmol/L LiCl, and 0.1 mmol/L sodium orthovanadate, and twice with TNE [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1 mmol/L sodium orthovanadate] before incubation in 75 μL lipid kinase buffer (TNE containing 15 mmol/L MgCl2, 20 μg freshly prepared 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol, 60 μmol/L ATP, 400 μCi/mL [γ-32P]ATP). Phosphatidylinositol (Sigma) was prepared by drying the lipid under a stream of argon before the addition of 10 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EGTA and suspension by an ice bath for 10 minutes. The kinase reaction was allowed to proceed for 10 minutes at 37°C and stopped by addition of 20 μL CHCl3/ methanol (1:1; high-performance liquid chromatography grade) and brief vortexing. Organic and aqueous phases were separated by centrifugation and 50 μL of the organic phase were spotted on a 1-mm Silica gel f60 TLC plate (EM Science, Gibbstown, NJ). The plate was developed in CHCl3/methanol/H2O/NH4OH (60:47:11:3.2) and 32P-labeled phosphatidylinositol phosphate (PIP) was visualized by autoradiography and referenced to known standards. 

Adenovirus constructs. Dominant-negative PAK2-expressing and empty adenoviruses were generated by transfection of adenovirus shuttle.
vector pAdCMV into 293Cre cells plated 24 hours earlier at $9 \times 10^5$ per six-well dish. Recombinant clones were determined by induction of cytopathic effects in the monolayers, isolated, and plaque purified in 293Cre cells. Control GFP-expressing adenovirus was purchased from Riken Genbank (Japan). Dominant-negative dynamin-expressing adenovirus was generously supplied by Dr. Jeff Pessin (Stony Brook University, Stony Brook, NY).

Results

Phosphatidylinositol 3-kinase is activated by transforming growth factor-β in fibroblast cell lines. To determine whether PI3K was activated in response to TGF-β, AKR-2B fibroblasts were stimulated with TGF-β and the generation of $^{32}$P-labeled PIP was determined. Although PI3K activity was detectable within 15 to 30 minutes of TGF-β treatment, maximal incorporation occurred by 60 minutes and was maintained through the 2-hour time course (Fig. 1A). Addition of the PI3K inhibitor LY294002 prevented TGF-β-stimulated phosphoinositide phosphorylation (Fig. 1A). As expected, the appearance of phosphorylated Akt temporally followed the increase in PIP and phosphorylation was detected on both S473 and T308. Little or no effect on total Akt protein was observed (Fig. 1A) and addition of 20 μg/mL LY294002 or 50 nmol/L wortmannin prevented TGF-β-mediated Akt phosphorylation (Fig. 1A; data not shown).

Although the generation of phosphoinositides and S473 phosphorylated Akt could be detected within 15 minutes of TGF-β addition, significant activation was not observed for another 30 to 60 minutes (Fig. 1A). Although these slow kinetics are consistent with other aspects of TGF-β action, they could also indicate a requirement for de novo RNA or protein synthesis in Akt phosphorylation. To address that question, cultures were pre-treated with cycloheximide or actinomycin D before TGF-β stimulation and phosphorylated Akt determination (Fig. 1B). Despite inhibiting protein and RNA synthesis by >90% (data not shown), the treatments had no discernable effect on Akt phosphorylation.

Activation of PI3K following TGF-β treatment has not been consistently observed (20, 26, 27). As many of these studies were done on epithelial cells undergoing an EMT, we next determined whether this might reflect distinct signaling responses of the two cell types. To address this issue, three representative fibroblast (NIH3T3, Swiss3T3, and AKR-2B) and epithelial [HeLa, Mv1Lu, MDCK] cell lines were grown to confluence, placed in DMEM containing 0.5% FBS for 24 hours, and assayed for phosphorylated Akt (p-AktS473) and total Akt following addition (+) of 5 ng/mL TGF-β for 120 minutes. Following immunoprecipitation with p85 antibody, the in vitro kinase activity of PI3K was examined using l-a-phosphatidylinositol as a substrate. Both blots were stripped and reprobed for total Akt, as both showed the same result only one is displayed. Right, levels of phosphoinositide and Akt were normalized to total Akt at the indicated time points. Representative of three separate experiments. B, activation of PI3K does not require new transcription or translation. AKR-2B fibroblasts were grown to confluence, placed in serum-free medium for 24 hours, and treated with 20 μg/mL cycloheximide or 5 μg/mL actinomycin D for 60 minutes. Cultures were then stimulated with 5 ng/mL TGF-β for 120 minutes before examining PI3K activity by assaying for p-AktS473 (top) or total Akt (bottom). C, PI3K activation occurs in fibroblast but not epithelial cells in response to TGF-β. Three fibroblast (NIH3T3, Swiss3T3, and AKR-2B) and three epithelial (HeLa, Mv1Lu, and MDCK cells) cell lines were grown to confluence, placed in DMEM containing 0.5% FBS for 24 hours, and assayed for phosphorylated Akt (p-AktS473) and total Akt following addition (+) of 5 ng/mL TGF-β for 120 minutes.

Figure 1. TGF-β activates PI3K in mesenchymal but not epithelial cultures. A, TGF-β activates PI3K in AKR-2B fibroblasts. Top left, AKR-2B fibroblasts were grown to confluence, placed in serum-free medium for 24 hours, and stimulated with 5 ng/mL TGF-β for the indicated times. Parallel plates were incubated in serum-free medium in the presence of vehicle (0.3% DMSO) or 20 μg/mL LY294002 (LY) before addition of 5 ng/mL TGF-β for 120 minutes. Following immunoprecipitation with p85 antibody, the in vitro kinase activity of PI3K was examined using l-a-phosphatidylinositol as a substrate. Bottom left, before immunoprecipitation and kinase assay, protein (300 μg) was used for Western analysis against Ser473 (p-AktS473) and Thr308 (p-AktT308) phosphorylated Akt. Both blots were stripped and reprobed for total Akt, as both showed the same result only one is displayed. Right, levels of PIP (c), p-AktS473 (o), and p-AktT308 (w) were normalized to total Akt at the indicated time points. Representative of three separate experiments. B, activation of PI3K does not require new transcription or translation. AKR-2B fibroblasts were grown to confluence, placed in serum-free medium for 24 hours, and treated with 20 μg/mL cycloheximide or 5 μg/mL actinomycin D for 60 minutes. Cultures were then stimulated with 5 ng/mL TGF-β for 120 minutes before examining PI3K activity by assaying for p-AktS473 (top) or total Akt (bottom). C, PI3K activation occurs in fibroblast but not epithelial cells in response to TGF-β. Three fibroblast (NIH3T3, Swiss3T3, and AKR-2B) and three epithelial (HeLa, Mv1Lu, and MDCK cells) cell lines were grown to confluence, placed in DMEM containing 0.5% FBS for 24 hours, and assayed for phosphorylated Akt (p-AktS473) and total Akt following addition (+) of 5 ng/mL TGF-β for 120 minutes.
and Madin-Darby canine kidney (MDCK) cells] cell lines were stimulated with TGF-β and examined for effects on Akt phosphorylation. As shown in Fig. 1C, TGF-β stimulated Akt phosphorylation in each of the fibroblast cultures yet had no effect on the epithelial lines. This cell tropism is identical to what we reported previously for PAK2 activation (1) and provides evidence (albeit indirect) that PAK2 and PI3K might be integrated within the same TGF-β signaling pathway (see below).

**Phosphatidylinositol 3-kinase activation and Smad phosphorylation occur independently and in separate cellular locales.** Although Smad-independent signaling has been reported (10, 11), TGF-β signaling is primarily mediated through the Smad family of transcriptional coregulators. Because the Smad docking protein SARA contains a FYVE domain, the presence of which is essential for correct localization and activation of Smad2 in response to TGF-β (9, 31, 32), PI3K activation (Fig. 1) may be required for production of phosphoinositides to recruit SARA and Smad proteins to the activated receptor complex. To determine if PI3K activity was necessary for Smad activation, we first inhibited PI3K with LY294002 and assessed whether the kinetics of Smad2 or Smad3 phosphorylation in response to TGF-β would be modulated. As shown in Fig. 2A, detectable levels of phosphorylated Smad2 and Smad3 occur within 15 minutes of TGF-β stimulation; inhibition of PI3K by LY294002 (Fig. 2A) or wortmannin (data not shown) has no discernable effect on either the kinetics or the extent of induction. The concentration of LY294002 used was determined to be sufficient to ablate PI3K activation by TGF-β as indicated by the failure to phosphorylate phosphatidylinositol in vitro or Akt in vivo (Fig. 1A). Moreover, increasing the LY294002 concentration to levels approaching toxicity (200 μg/mL and theoretically inhibiting class I, II, and III PI3Ks) still failed to diminish Smad phosphorylation after TGF-β stimulation (data not shown).

![Figure 2](#)

**Figure 2.** Smad response is independent of PI3K activity. A, Smad phosphorylation is independent of PI3K activity. AKR-2B cells were grown to confluence and placed in serum-free medium in the absence or presence of 20 μg/mL LY294002 for 24 hours. Cultures were stimulated with 5 ng/mL TGF-β for the indicated times and assayed for phosphorylated or total Smad2 and Smad3 proteins as described in Materials and Methods. B, Smad nuclear localization is independent of PI3K activity. AKR-2B cells were treated as in (A) and Smad immunofluorescence and nuclear accumulation were determined as described (34). C, PI3K activity is not required for Smad-dependent luciferase responses. The indicated cultures were transiently transfected with either 3TP-Lux (top), SBE-Lux (middle), or α-SMA-Lux (bottom) and CMV promoter-driven β-galactosidase. Cells were grown to confluence and incubated in serum-free medium in the absence (-) or presence (+) of 20 μg/mL LY294002. After 24 hours, cells were either left untreated (-) or stimulated (+) for 24 hours with 5 ng/mL TGF-β. The mean fold induction in unstimulated (-) cells for each condition was assigned a value of 1. Columns, mean of two separate experiments done in duplicate; bars, SD.
Smad phosphorylation is only one step in the activation and subsequent signaling mediated by the Smad proteins. Once phosphorylated, Smad2 and Smad3 (R-Smads) complex with Smad4 and translocate from the cytoplasm into the nucleus (7, 8). Although the initial event(s) in Smad signaling occurred independent of PI3K (Fig. 2A), to determine if PI3K activity affected Smad nuclear translocation immunofluorescence microscopy and immunoblotting of nuclear lysates was done following addition of TGF-β and/or LY294002 (Fig. 2B). Similar to that observed for Smad phosphorylation, no discernable effect on nuclear Smad accumulation by LY294002 was detected. Lastly, as Smad proteins are known to modulate the expression of a significant number of genes, we next determined if PI3K activity had any role in Smad-mediated transcriptional activity. In agreement with data shown in Fig. 2A and B, inhibition of PI3K had no effect on the stimulation of either 3TP, SBE, or α-SMA luciferase activity in response to TGF-β (Fig. 2C). As reported previously, each of these reporter constructs required the presence of Smad3 (Fig. 2C; ref. 26).

Although Fig. 2 shows that PI3K activity is not required for Smad2 and/or Smad3 phosphorylation or action, it does not address whether Smad2 and/or Smad3 phosphorylation is required for PI3K activation. Accordingly, Smad3-null (Smad3+/−/−) mouse embryo fibroblasts (MEF) were cotransfected with a Myc-tagged dominant-negative Smad2 (Myc-Smad2 S467A) and a His-tagged wild-type Akt (Fig. 3). Following stimulation with TGF-β, cells were lysed and phosphorylation of the transfected Akt protein was determined. Although Smad2 and Smad3 activation did not occur in the knockout clone (Fig. 3, left), TGF-β-mediated Akt phosphorylation proceeded normally (Fig. 3, right). The results of

Figure 3. PI3K activation is independent of Smad proteins. Left and right, AKR-2B cells (lanes 1 and 2), AKR-2B cells transiently expressing a His epitope–tagged Akt (lanes 3 and 4), and Smad3-negative MEFs transiently expressing a His epitope–tagged Akt and a dominant-negative Smad2 (lanes 5 and 6) were grown to confluence, incubated overnight in serum-free medium, and either left untreated (−) or stimulated (+) for 120 minutes with 5 ng/mL TGF-β. Samples were lysed, normalized for equal protein, and split into nine aliquots where Western analysis was done against the indicated phosphorylated and total proteins. To detect Akt-His, the transfected protein was first immunoprecipitated with antibodies toward the His epitope before phosphorylated Akt (right, top, top) or total Akt (right, top, bottom) blotting.

Figure 4. PI3K activation occurs upstream of dynamin action. A, confluent AKR-2B fibroblasts were placed in serum-free medium in the absence (Control) or presence of adenovirus [multiplicity of infection (MOI) 300] expressing either empty vector (Ad-Empty) or dominant-negative dynamin (Ad-DynK44A). Following 24-hour incubation, TGF-β was added to a final concentration of 5 ng/mL. After 120 minutes, cells were lysed and assayed for total (Akt or Smad2) or activated (p-AktS473 or p-Smad2) Akt and Smad2 by Western blotting. B, dominant-negative dynamin prevents clathrin- and caveolar-mediated internalization. Left, AKR-2B fibroblasts were treated as in (A). Transferrin was visualized by incubation in serum-free medium with 20 μg/mL Alexa Fluor 594 (red) transferrin for 2 minutes at 37°C. Studies with BODIPY-lactosylceramide (LacCer) were done as described previously (34) using a 3-minute incubation at 37°C for internalization. Right, percent internalization of lactosylceramide (LC) or transferrin (Tfn). Columns, mean (n = 20 cells for each condition quantified five independent times); bars, SD.

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Recent data indicate that phosphorylation of Smad2 and Smad3 occurs in an endosomal compartment downstream of dynamin action (30, 32, 33). To determine whether PI3K was activated in an analogous manner, the role of internalization in PI3K activation by TGF-β was investigated. As shown in Fig. 4A, there was no effect on TGF-β-stimulated Akt phosphorylation in AKR-2B cells infected with adenovirus expressing dominant-negative dynamin. However, as would be expected, infection with the mutant dynamin virus prevented Smad2 phosphorylation as well as uptake of transferrin and lactosylceramide, readouts of clathrin- and caveolae-mediated endocytosis, respectively (Fig. 4B; ref. 34). The data (Figs. 1-4) show that TGF-β-stimulated PI3K activity (a) occurs in a subset of fibroblast but not epithelial cell cultures, (b) is independent of Smad2 and Smad3 phosphorylation, and (c) occurs in a plasma membrane compartment upstream of dynamin action.

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Phosphatidylinositol 3-kinase activation of p21-activated kinase-2 regulates transforming growth factor-β morphologic and proliferative responses in AKR-2B fibroblasts. Although TGF-β modulates several biological phenotypes, the manner in which the TGF-β signal(s) is transduced following receptor binding is poorly understood. Although both Smad-dependent and Smad-independent pathways have been shown to be critical for many aspects of TGF-β action, until recently, none of the identified pathways could readily show differences in TGF-β signaling between cell types with distinct biologies. To that end, PAK2 was shown to be activated in a Smad-independent manner in several mesenchymal but not epithelial cultures (1). Because PI3K has been reported to regulate PAK2 activation (35, 36), we determined whether TGF-β activation of PI3K and PAK2 were similarly coupled. To address if PI3K activation was required for PAK2 activation, AKR-2B cells were stimulated with TGF-β in the absence or presence of LY294002 and assayed for PAK2 kinase activity. Whereas TGF-β stimulated significant PAK2 activity peaking between 40 and 60 minutes, LY294002 completely inhibited this response (Fig. 5A). This was shown to be a specific effect on PAK2 by simultaneously assaying treated cell lysates for phosphorylated Smad3. Similar to that shown in Fig. 2A, inhibition of PI3K did not affect Smad3 phosphorylation (Fig. 5A), further supporting independent regulation of PI3K/PAK2 and Smad pathway activation by TGF-β.

A role for PI3K in PAK protein activation is well documented (35, 36), but there are a variety of reports as to which downstream signaling targets of PI3K directly (or indirectly) regulate PAK activity. For instance, it has been shown that (a) phosphorylated Akt is responsible for PAK2 activation (37, 38); (b) sphingosine activation of PAK1 is mediated through the kinase activity of phosphorylated 3-phosphoinositide-dependent kinase-1 (Pdk1), another signaling component downstream of PI3K (39); and/or (c) binding of p85 to the virally encoded Nef protein is required for the PI3K-dependent activation of PAK1 (36). To determine which, if any, of these factors were involved in TGF-β-mediated PAK2 activation, AKR-2B fibroblasts were stimulated with TGF-β and assayed for PAK2 kinase activation in the context of PI3K inhibition (LY294002), Pdk1 inhibition (transient transfection of dominant-negative Pdk1), or Akt inhibition (transient transfection of dominant-negative Akt or addition of the Akt-specific inhibitor compound SH-5). Although

Figure 6. Distinct PI3K responses regulate TGF-β-stimulated morphologic transformation and proliferation. AKR-2B fibroblasts were grown to confluence and placed in serum-free medium in the absence (Control) or presence of vehicle (0.3% DMSO), 20 μg/mL LY294002, or adenovirus (MOI 300) expressing GFP (Ad.GFP) or dominant-negative PAK2 (Ad.PAK2K278R). Following 24-hour incubation, TGF-β (+) was added to a final concentration of 5 ng/mL and representative areas were photographed (A) at ×20; phase or cell number was determined 48 hours later (B). Columns, mean of two or three separate experiments done in triplicate; bars, SD. C, TGF-β activation of PI3K in AKR-2B fibroblasts. A model depicting our current understanding of PI3K activation by TGF-β in AKR-2B fibroblasts is shown. Following ligand binding to the TGF-βR complex, both Smad and PI3K signals are activated. PI3K independently activates Akt and PAK2 such that PAK2 is required to mediate the PI3K requirement for TGF-β-stimulated morphologic transformation and proliferation. Although Smad- and PI3K-dependent pathways are believed to be distinct, both are required for the observed phenotypes (26, 27). Arrows do not necessarily indicate a direct interaction and may reflect multiple events.

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inhibition of PI3K kinase activity with LY294002 abolished PAK2 activation, inhibition of Akt or Pdk1 had little effect on TGF-β-mediated PAK2 activation (Fig. 5B). As expected, none of the PI3K pathway inhibitors had any effect on Smad2 phosphorylation (Fig. 5B).

Although Fig. 5B clearly shows that inhibiting Akt activity with SH-5 or dominant-negative AktK179A or Pdk1 does not affect PAK2 activation, it does not address the converse (i.e., whether PAK2 kinase activity is required for Akt phosphorylation). Accordingly, AKR-2B cells were infected with adenovirus expressing dominant-negative PAK2 and the level of Akt phosphorylation was determined following addition of TGF-β. Expression of dominant-negative PAK2 prevented PAK2 activation but had no effect on TGF-β-stimulated Akt phosphorylation (Fig. 5C). Lastly, although LY294002 and wortmannin are used at concentrations that are thought to selectively inhibit type I PI3Ks (Figs. 1A, 2, and 5A and B; data not shown), it is still possible that the drugs might affect other kinases. To address that issue, we transfection morpholino antisense oligonucleotides to the regulatory p85 subunit of type I PI3K and assessed their effect on TGF-β signaling. As shown in Fig. 5D, the antisense oligonucleotides, but not the scrambled control, specifically diminish p85 protein; no effect was observed on the type I PI3K catalytic p110 subunit, class II p170 protein, or class III PI3K protein PI3KC3. Most importantly, in agreement with the LY294002 and wortmannin data, loss of p85 protein resulted in an absence of TGF-β-stimulated Akt phosphorylation and PAK2 activation with no attenuation in Smad2 phosphorylation.

The previous data indicate that TGF-β-stimulated PI3K is independent of Smad protein activation and distinctly modulates both Akt and PAK2 signaling in AKR-2B cells. Of interest, however, was whether this new target could be linked to biological phenotypes regulated by TGF-β in fibroblast cultures. As shown in Fig. 6A, AKR-2B cells treated with TGF-β transform from large “cobblestone-like” geometric shapes into thin elongated cells that spread and extend over one another, reminiscent of transformed cultures. To investigate the role(s) of PI3K in this morphologic transformation, cells were treated with the PI3K inhibitor LY294002 before and during TGF-β stimulation. Whereas expression of the delivery vehicle (DMSO) was without effect, treatment with LY294002 or adenovirus expressing dominant-negative PAK2 prevented cells from morphologically transforming in the presence of TGF-β (Fig. 6A; ref. 1). As these findings indicated a direct role for PI3K and/or its downstream targets in TGF-β action, we wished to discern if the proliferative responses induced by TGF-β were similarly effected. Addition of TGF-β to confluent cultures in serum-free medium resulted in an approximate cell doubling over 48 hours, whereas treatment with LY294002 or dominant-negative PAK2 abolished TGF-β-stimulated monolayer proliferation (Fig. 6B). Analogous results were observed on fibroblasts in soft agar grown in the presence or absence of TGF-β and/or LY294002 (data not shown).

Based on our observations, we propose a model depicting Smad-independent activation of PI3K whereby PI3K defines a branch point in the TGF-β signal upstream of PAK2 and protein kinase B/Akt (Fig. 6C). Although morphologic and proliferative effects of TGF-β in these cells are dependent on signals emanating downstream of PAK2, the cellular response to Akt activation is currently unknown. In addition, although the upstream components that couple TGF-βR activation to PI3K and/or how the PI3K/PAK2- and Smad-dependent pathways interface have not been identified, it is clear that signals from both are required for TGF-β morphologic transformation and cellular proliferation.

Discussion

Downstream targets activated by the TGF-βR complex have proven difficult to define. It was not until the studies by Savage et al. (40) and Raftery et al. (41) describing the Smad and Mad proteins, respectively, that a pathway emerged (i.e., the Smad pathway) that responded to TGF-β in a consistent manner. During the ensuing years, several laboratories have described a variety of activities and mechanisms through which the Smad pathway responds to and regulates TGF-β action (7, 8). Although Smad signaling can account for much of TGF-β’s action, when one considers the plethora of activities modulated by TGF-β, it seems reasonable that additional Smad-independent pathways must exist to control the diversity of cellular responses to this cytokine. In that regard, recent work has shown that Smad-independent signaling regulates, for instance, fibronectin synthesis (10, 11) and activation of mitogen-activated protein kinase (13), PAK2 (1), and the c-Abl non–receptor tyrosine kinase (18). Evidence suggests at least some of these events are dependent on cell context in that activation of both PAK2 and c-Abl occurs in mesenchymal but not epithelial cultures (data not shown; ref. 1). Two obvious questions generated by these results are (a) what is the biological significance of such cellular responses? and (b) where are the distinct regulatory constraints manifested; are they receptor proximal or distal events? Answers to both these issues are currently unknown and under active investigation.

The present study shows that TGF-β stimulation results in the activation of class I PI3K in a subset of fibroblast cell lines. Maximal PI3K activity, as measured by the in vitro production of PIP2, occurred between 30 and 60 minutes of TGF-β treatment, with Akt phosphorylation evident by 60 minutes (Fig. 1A). Although PI3K activation was shown to be dependent on TGF-βR kinase activity (data not shown), neither the p85 nor the p110 subunit of PI3K was directly phosphorylated by either the type I or II receptor (data not shown). However, a recent article by Yi et al. (42) suggested that TGF-βS indirectly associate with p85 and previous reports have indicated a physical interaction between PI3K subunits and the TGF-βR complex under some conditions (43) but not in others (44). As such, the manner by which PI3K is activated after TGF-βR binding is unknown.

The role(s) of PI3K in TGF-β-mediated EMT is controversial (20, 26, 27). However, as TGF-β is known to induce various phenotypes dependent on the cell context, it is extremely difficult to directly compare responses between distinct cell types and transdifferentiating cultures. For that reason, the current study was designed to examine the role(s) of PI3K activation in a subset of mesenchymal cell models, which similarly respond to TGF-β by activating PI3K (Fig. 1C). Although we found no effect on PI3K signaling when TGF-β was added to epithelial cultures (Fig. 1C), it would be premature to conclude that the observed cell tropism indicates a minor role for this pathway in epithelial cell growth and/or tumor progression. There is a large body of literature supporting a paracrine role for an activated stroma in the survival and proliferation of carcinomas (45, 46) and TGF-β signaling in fibroblasts has been shown recently to modulate the oncogenic potential of specific adjacent epithelia (47, 48). The present results
support this concept and indicate a signaling activity that might regulate this response. Moreover, as many carcinomas attain a more "fibroblastic" phenotype in response to TGF-β during tumor progression (49), characterizing the targets and mediators capable of distinguishing these cell type differences is vital. PI3K may represent on such pathway.

Inhibition of PI3K with LY294002 or wortmannin had no appreciable effect on (a) the kinetics and intensity of Smad2 or Smad3 phosphorylation, (b) Smad2 or Smad3 nuclear translocation, or (c) Smad-dependent transcriptional responses (Fig. 2). Similarly, deletion/inactivation of Smad2 and/or Smad3 did not prevent Akt phosphorylation in response to TGF-β (Fig. 3; data not shown). Although these findings support the contention that PI3K activation and Smad phosphorylation are independently regulated, a recent article by Runyan et al. (50) points out a potential caveat to generating conclusions based on phosphospecific antibodies directed to a single epitope. Although those investigators observed an inhibition of Smad3 transcriptional activity by LY294002 in mesangial cells, this was reported to be independent of the COOH-terminal serine phosphorylation on Smad3 induced by TGF-β. However, as we observed no effect on either Smad2/3 phosphorylation or transcriptional activation by prior treatment with LY294002, this is consistent with distinct signals emanating from the TGF-βR complex regulating PI3K and Smad responses.

A role for the FYVE domain in correctly localizing SARA has been documented (9, 31, 32). However, the concentration of LY294002 used in the present studies would primarily be expected to disrupt class I PI3Ks as it was likely too low to inhibit class III PI3Ks that generate PtdIns(3)P in vivo, the specific phosphoinositide recognized by FYVE domain–containing proteins like SARA. To investigate this issue further, we tested the effect of a range of PI3K inhibitor concentrations (0.1-200 μg/mL) on Smad2 or Smad3 phosphorylation in response to TGF-β. Although no appreciable diminution in Smad phosphorylation was observed (data not shown), it should be noted that because class III PI3K production is constitutive the short pretreatment before ligand addition may have been insufficient to adequately reduce cellular levels of PtdIns(3)P to observe any response. Thus, although the present results do not directly address the role of phosphoinositides and SARA localization, the data clearly indicate that ligand-mediated PI3K activation by TGF-β is not required for Smad signaling in the mesenchymal cell models tested (Figs. 1-3).

The role of receptor internalization in TGF-β signaling is an area of active investigation. Whereas we and others have shown a requirement for clathrin-dependent endocytosis in Smad activation (30, 32, 33), other studies have suggested that TGF-β or activin activates Smad2 in the absence of TGF-βR internalization (51, 52). In addition, further complexity is added to this issue as a recent study reported a requirement for TGF-βR endocytosis in Smad2 nuclear translocation and transcriptional activity but not Smad2 phosphorylation (53). Although it is currently unclear why such a variety of responses has been observed, two issues that need to be considered include (a) the use of various cell types (i.e., Mv1Lu, AKR-2B, Cos1, Cos7, mesangial, and HeLa) as TGF-β's action is dependent on the cell context and (b) whether endocytic assays were done and quantitated in parallel with all signaling studies. This latter point is extremely important as the fraction of activated/endocytosed TGF-βR required to initiate Smad signaling is unknown. For instance, if internalization needs to be inhibited >80% to observe an endocytic requirement for Smad activation, unless one specifically documents that this has occurred it is difficult to directly compare the findings. In the current study, we have extended this endocytosis/signaling question to Smad-independent pathways. As shown in Fig. 4, although dominant-negative dynamin inhibited clathrin (i.e., transferrin) and caveolar (i.e., lactosylceramide) internalization by ~90% as well as Smad2 phosphorylation, no appreciable effect on Akt phosphorylation (Fig. 4A) or PAK2 activation (data not shown) was observed. This differential endocytic requirement suggests that PI3K and Smad activation occur at different cellular locales and provides the cell a unique opportunity to modulate the cellular response to TGF-β under a variety of external stimuli.

The relation of PI3K in PAK family activation is complex as both PI3K-dependent and PI3K-independent roles have been reported in various cell systems (39, 54). When fibroblast cultures were treated with 20 μg/mL LY294002 or a dominant-negative PAK2 adenovirus, TGF-β-stimulated PAK2 kinase activity was prevented, whereas the Akt inhibitor SH-5 or dominant-negative Akt or Pdk1 had no effect (Fig. 5B). Although these results are consistent with the hypothesis that PAK2 and Akt define distinct pathways downstream of PI3K, it is also possible that one or both targets reflect a nonspecific action of LY294002 on another kinase(s). To address this concern, we used morpholino antisense oligonucleotides to the regulatory p85 subunit of type I PI3K and showed that (a) p85 protein was decreased, whereas p110, class II p170, and class III PI3Kc3 protein levels were unchanged; (b) TGF-β was unable to stimulate Akt phosphorylation or PAK2 kinase activity; and (c) Smad2 phosphorylation in response to TGF-β was unchanged or slightly enhanced, potentially reflecting some negative cross-talk between Smad-dependent and Smad-independent signaling (Fig. 5D). Together, the results support the conclusion that TGF-β stimulates type I PI3K signaling in a subset of fibroblast (not epithelial) cell lines and is required for PAK2 activation.

In agreement with our previous data suggesting independent regulation of Smad and PI3K signals (Figs. 2-4), LY294002 had no effect on Smad2 or Smad3 phosphorylation (Figs. 2 and 5). Although PAK2 and PI3K activities are required for the morphologic and proliferative effects of TGF-β on AKR-2B cells, they are not sufficient. For instance, constitutively active PI3K is unable to stimulate growth or induce a morphologic change unless TGF-β is also present (data not shown). These findings agree with data from our laboratory and others (26, 27), indicating an equally important role for Smad signaling in these events.

It is currently unknown how PI3K is activated by the TGF-βR complex and/or how this results in the activation of PAK2. Although the virally encoded Nef protein has been shown to act as a scaffold capable of facilitating PAK2 activation (36), analogous proteins have not been implicated in TGF-β action. However, a model depicting our current understanding of the relation between PI3K and Smad pathways in fibroblasts is shown in Fig. 6C. Of note, whereas inhibition of PI3K abrogates TGF-β-mediated PAK2 activation (Fig. 5A, B, and D), yet dominant-negative PAK2 is unable to prevent Akt phosphorylation (Fig. 5C), these findings are consistent with a model where PI3K is spatially upstream of PAK2 and independently regulates Akt and PAK2 activation. A similar action of PI3K independent of Akt has been shown for PAK1-mediated actin phosphorylation and cytoskeleton reorganization (55). It is clear that TGF-β exerts its complex and dramatic effects on fibroblast cells through the
action of multiple signaling pathways. Understanding the synergistic and/or antagonistic manner by which these events are regulated is critical if the various cell type–dependent phenotypes induced by TGF-β are to be understood.

Acknowledgments

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