Transforming growth factor β (TGF-β) is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures*

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Transforming growth factor β (TGF-β) modulates a number of cellular phenotypes as divergent as growth stimulation and growth inhibition. Although the Smad pathway is critical for many of these responses, recent evidence indicates that Smad-independent pathways may also have a critical role. One such protein previously known to regulate TGF-β action independent of the Smad proteins is the c-Abl nonreceptor tyrosine kinase. In the current study we determined that TGF-β receptor signaling activates c-Abl kinase activity in a subset of fibroblast but not epithelial cultures. This type cell-specific response occurs in a membrane-proximal mitochondrial independent of receptor internalization and upstream of dynamin action. Although c-Abl activation by TGF-β is independent of Smad2 or Smad3, it is prevented by inhibitors of phosphatidylinositol 3-kinase or PAK2. Thus, c-Abl represents a target downstream of phosphatidylinositol 3-kinase-activated PAK2, which differentiates TGF-β signaling in fibroblasts and epithelial cell lines and integrates serine/threonine receptor kinases with tyrosine kinase pathways.

Signaling pathways for TGF-β are routinely classified as being either Smad-dependent or Smad-independent. Smad proteins function as co-modulators of transcription and have been classified as receptor-regulated (R-Smad), co-, and inhibitory (I-Smad) Smads (1, 2, 7, 8). Binding of ligand to the type II TGF-β receptor results in the recruitment of the type I receptor into a heterotetrameric complex. The constitutively active type I receptor then transphosphorylates the type I receptor, which activates the receptor kinase and promotes R-Smad (i.e. Smad2 or Smad3) binding. Although the cellular locale where the type I receptor phosphorylates the associated R-Smad(s) may differ in various cell types (9–13), for the models employed in the present study this occurs in a compartment downstream of dynamin-mediated vesicle scission (9, 10, 14). The activated R-Smad(s) can then associate with the co-Smad (Smad4) and translocate to the nucleus to modulate gene expression (1, 2, 7).

In addition to Smad-dependent signaling, a number of responses stimulated by TGF-β occur independently of the Smad proteins (8, 15, 16). Initial work in this area implicated Ras and various mitogen-activated protein kinases including Erks, p38, and Jnk mediating TGF-β effects on cell proliferation and extracellular matrix gene expression (16–18). More recently, components of the mitogen-activated protein kinase family have been observed to control various apoptotic responses stimulated by TGF-β, and a direct link between the TGF-β receptor complex and Rho-mediated dissolution of tight junctions during epithelial-mesenchymal transitions has been reported (8, 15, 19). Of note, although these responses can be defined as Smad-independent, there is often cross-talk with the Smad pathway to obtain the full biologic response. An additional complexity to the concept of “Smad-independent” signaling is indicated by the recent work of Levy and Hill (20). These investigators documented two functional categories of TGF-β target genes dependent (or not) upon Smad4. As the canonical model for Smad signaling (discussed above) requires Smad4 to promote R-Smad nuclear translocation, this manuscript illustrates both the importance and difficulty in defining a Smad from a non-Smad response.

A fundamental question in understanding TGF-β action is how similar pathways can be activated in various cell types with completely different biological outcomes. For instance, although epithelial and mesenchymal cells are routinely growth-inhibited or growth-stimulated, respectively, by

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2 The abbreviations used are: TGF, transforming growth factor; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; GM-CSF, granulocyte/macrophage-colony-stimulating factor; BMP, bone morphogenetic protein; MDCK, Madin-Darby canine kidney cells.
TGF-β, Smad phosphorylation, translocation, and transcriptional modulation of numerous genes occur in both. Although a number of models can be proposed to account for this apparent inconsistency (7), we have investigating the hypothesis that activation of cell type-specific signaling pathways might be one means to address this paradox. In that regard, recent studies have shown that p21-activated kinase 2 (PAK2) and phosphatidylinositol 3-kinase (PI3K) are activated by TGF-β independent of Smad2 or Smad3 in a subset of mesenchymal but not epithelial cultures (14, 21). PI3K was found to function upstream of PAK2 and represent a branch point in the TGF-β response. For instance, although PI3K was required for both PAK2 and Akt activation, TGF-β morphologic transformation and monolayer proliferation resulted from signals emanating downstream of PAK2. Of note, although each pathway is necessary, functional integration with the Smad pathway is required as none are sufficient to induce these responses (14, 21, 22).

The c-Abl proto-oncogene is a member of the Src family of nonreceptor tyrosine kinases implicated in a wide variety of cellular processes (23–25). Although Abl associates with several receptors in neurons (25), cytokine regulation of c-Abl has only been shown for platelet-derived growth factor (26). As it was subsequently determined that (i) activation of PAK2 by the nonreceptor tyrosine kinases implicated in a wide variety of signaling pathways might be one means to address this paradox. In that regard, recent studies have shown that p21-activated kinase 2 (PAK2) and phosphatidylinositol 3-kinase (PI3K) are activated by TGF-β independent of Smad2 or Smad3 in a subset of mesenchymal but not epithelial cultures (14, 21). PI3K was found to function upstream of PAK2 and represent a branch point in the TGF-β response. For instance, although PI3K was required for both PAK2 and Akt activation, TGF-β morphologic transformation and monolayer proliferation resulted from signals emanating downstream of PAK2. Of note, although each pathway is necessary, functional integration with the Smad pathway is required as none are sufficient to induce these responses (14, 21, 22).

The c-Abl proto-oncogene is a member of the Src family of nonreceptor tyrosine kinases implicated in a wide variety of cellular processes (23–25). Although Abl associates with several receptors in neurons (25), cytokine regulation of c-Abl has only been shown for platelet-derived growth factor (26). As it was subsequently determined that (i) activation of PAK2 by the Rho family GTPase Cdc42 induced c-Abl activity (27) and (ii) TGF-β activated PAK2 independent of Smad2 and Smad3 (21), we extended these observations and investigated whether TGF-β might also regulate c-Abl. The data demonstrated not only that TGF-β induced c-Abl kinase activity but also that inhibition of that response with imatinib mesylate (Gleevec®) prevented in vivo lung and kidney fibrosis due to bleomycin instillation or ureter ligation, respectively (28, 29). These findings suggested that c-Abl might represent an important component of the mesenchymal cell response to TGF-β. As such, because c-Abl regulates many similar phenotypes as TGF-β (23, 25, 30, 31), we wished to determine (i) if c-Abl activation by TGF-β occurred in a cell type-specific manner and (ii) the spatial relation of c-Abl to PI3K, PAK2, and/or Akt in TGF-β signaling. In the present study we report that TGF-β activation of c-Abl was not prevented by inhibitors of clathrin- or raft-mediated internalization, displayed a distinct cell tropism, and occurred downstream of PAK2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells were obtained from ATCC (Rockville, MD) and grown in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA). Unless otherwise stated, 2.5 × 10^6 cells were seeded on 100-mm culture dishes, grown to confluence, washed with phosphate-buffered saline, and serum-starved in 0.1% FBS/DMEM or serum-free DMEM for 24 h prior to use. LY294002, PD098059, cycloheximide, and actinomycin D were purchased from Sigma, whereas Akt IV was obtained from Calbiochem.

**Western Blotting**—Total c-Abl or Smad3 protein and phosphorylated Smad3 were determined by Western blot analysis as described previously (14, 28). Briefly, cultures were treated overnight in serum-free DMEM prior to stimulation with the indicated reagents. Lysates were prepared (50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4, 1× Complete protease inhibitor; Roche Applied Science), and equivalent protein (~100 μg determined by Pierce BCA protein assay kit) was separated by SDS-PAGE. Western antibody for c-Abl was purchased from BD Biosciences (catalog no. 554148 used at 1:200 dilution) whereas total Smad3 antibody was from Zymed Laboratories (catalog no. 51-1500). A rabbit anti-phosphorylated Smad3 antibody (used at 1:3000 dilution) to the peptide COOH-GLPSIRCpSVpS was generated in our laboratory (14, 21). Secondary goat anti-mouse (1:2000 dilution) or donkey anti-rabbit (1:2000 dilution) antibody was from Santa Cruz Biotechnology (catalog no. sc-2005) and Amersham Biosciences (catalog no. NA934V), respectively.

**c-Abl Kinase Assays**—c-Abl kinase assays were performed on cell lysates essentially as described by Daniels et al. (28). Briefly, cells were lysed in 750 μl of kinase lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.1% SDS, 1% sodium deoxycholate, 0.1 trypsin inhibitory unit/ml aprotonin, 50 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mM sodium vanadate), and equivalent protein (~500–700 μg) was incubated overnight at 4 °C with an antibody to c-Abl (Santa Cruz Biotechnology, catalog no. sc-13076 used at 1:150 dilution). Immune complexes were collected with protein A-Sepharose (Sigma) and washed twice in kinase lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol) prior to incubation in 40 μl of kinase buffer supplemented with 5 μM ATP, 2 μg of glutathione S-transferase-Crk, and 0.5 μCi of [γ-32P]ATP. Following incubation at 37 °C for 5 min the reaction was terminated by the addition of 40 μl of 2× Laemmli buffer.

**Adenovirus Constructs**—Dominant negative PAK2 (PAK2K299R) or Ras (RasN17) adenovirus was generated by transfection of pAdCMV into 293Cre cells. Recombinant clones were determined by cytopathic effects, plaque-purified in 293Cre cells, and titered by limiting dilution in 293 cells. Cell lines were infected by incubation for 24 h in serum-free DMEM with virus at multiplicity of infection 200. Control GFP-expressing adenovirus was purchased from Riken GenBankTM (Japan). Ad-Empty was provided by Dr. Richard Pagano (Mayo Clinic), and adenovirus-expressing dominant negative dynamin 1 (Ad-DynK44A) was from Dr. Jeffrey Pessin (Stony Brook).

**Transfections**—Cells were seeded in six-well dishes at 2.5 × 10^5 per dish for 24 h prior to 4 h of transfection (TransIT-LT1; Mirrus Bio, Madison, WI) with 2 μg of luciferase vector plus 0.5 μg of CMV-β-galactosidase. The medium was replaced with 10% FBS/DMEM, and the cultures were allowed to recover overnight. Following incubation in serum-free DMEM for 12–18 h, the cells were left untreated or stimulated with the indicated reagents for 24 h. Normalized luciferase activity was assayed as described (10).

As described previously (14, 21), the p85 subunit of PI3K was inhibited by using antisense morpholinol oligonucleotides representing nucleotides –22 to +3 of mouse p85 (5′-CATGTGTCGGACAGTTCCCTGCTGC-3′). Similarly, PAK2 expression was prevented by transfecting antisense oligonucleotides to

\[ \text{PIK3} \]

\[ \text{PAK2} \]

\[ \text{c-Abl} \]

\[ \text{TGF-β} \]

\[ \text{Smad} \]

\[ \text{DMEM} \]

\[ \text{FBS} \]

\[ \text{Gleevec®} \]

\[ \text{IMRT} \]

\[ \text{GFP} \]

\[ \text{Ad-DynK44A} \]

\[ \text{Riken GenBankTM} \]

\[ \text{Luciferase} \]

\[ \text{TransIT-LT1} \]

\[ \text{Mirrus Bio} \]

\[ \text{Mayo Clinic} \]

\[ \text{Stony Brook} \]

\[ \text{Ad-Empty} \]

\[ \text{GSK3β} \]

\[ \text{PI3K} \]

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\[ \text{c-Abl} \]

\[ \text{TGF-β} \]

\[ \text{Smad} \]

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\[ \text{Riken GenBankTM} \]

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\[ \text{Stony Brook} \]

\[ \text{Ad-Empty} \]
nucleotides –1 to +24 of mouse PAK2 (5′-TTCTAGCTCTCCGTTATCAGACATG-3′) (Gene Tools, Philomath, OR). Scrambled (5′-GTACAGACTATTGCTCTCGATTT-3′) control oligonucleotides were used for p85 and PAK2. Cos7 cells were plated in six-well dishes at a density of 1.0 × 10⁶ well in 10% DMEM and incubated at 37 °C for 24 h. Confluent cells were transfected with the indicated oligonucleotide (6 μm) along with plasmids expressing the type I (1 μg) and II (3 μg) TGF-β receptors with Lipofectamine 2000 (Invitrogen) for 6 h. The medium was replaced with 2% FBS/DMEM for 48 h, and following a 24-h incubation in 0.1% FBS/DMEM cultures were stimulated in 2 ml of serum-free DMEM alone or containing 10 ng/ml TGF-β for the indicated times.

Soft Agar Colony Formation—Cells were suspended in 1.0 ml of 10% FBS/DMEM containing 0.4% Sea Plaque-agarose (FMC Bioproducts, Rockland, ME) in the presence or absence of TGF-β and/or imatinib mesylate. The soft agar was added to 35-mm plates containing a 0.8% agarose, 10% FBS/DMEM bottom plug, and the number of colonies greater than 25–50 μm in diameter that developed after 10–12 days was determined by microscopy using a 1.0-cm grid. Approximately 3 independent regions on each of 4 plates were counted. Imatinib was prepared as described previously (28).

RESULTS

TGF-β Activation of c-Abl Distinguishes Mesenchymal from Epithelial Cell Lines and Requires TGF-β Receptor Kinase Activity—A fundamental question in understanding TGF-β action is how one growth factor, binding to the same set of receptors in mesenchymal and epithelial cells, can lead to such diverse phenotypes as growth stimulation (i.e. mesenchymal cells) and growth inhibition (i.e. epithelial cells). Although there are numerous means by which this might be addressed, we have investigated the hypothesis that TGF-β induces cell type-specific signaling to regulate these outcomes. Specifically, our previous studies have shown that PI3K and PAK2 are two downstream targets activated by TGF-β in mesenchymal but not epithelial cultures (14, 21). Because we have also shown that TGF-β stimulation of c-Abl is a potential mediator of bleomycin-induced pulmonary fibrosis (28), we wished to extend that analysis by determining (i) whether c-Abl was an additional Smad-independent TGF-β signaling target that reflected a specific cell tropism of activation and (ii) whether c-Abl was activated in a similar and/or parallel pathway to PI3K and PAK2.

As c-Abl reflects an integration of Ser/Thr kinase activation with tyrosine kinase activation, we first determined if this took place in a cell type-specific manner. Accordingly, AKR-2B fibroblasts and Mv1Lu epithelial cells were stimulated with TGF-β and assayed for c-Abl kinase activity and Smad3 phosphorylation (Fig. 1A). Although c-Abl activity was detectable within 15 min of TGF-β treatment of AKR-2B cells, peaking at 30 min, no induction was observed in Mv1Lu cells. However, c-Abl protein was similarly expressed, and Smad3 phosphorylation occurred in both cell types. To determine whether the response observed for AKR-2B and Mv1Lu cells reflected a more generalized distinction of mesenchymal and epithelial cells, respectively, three representative fibroblast (i.e. AKR-2B, NIH-3T3, and IMR90) and epithelial (i.e. Mv1Lu, MDCK, and HeLa) cultures were stimulated with TGF-β and examined for effects on c-Abl activity. As shown in Fig. 1B, although there was no significant difference in the level of c-Abl protein expressed among the cell types, c-Abl activation by TGF-β only occurred in murine (AKR-2B and NIH-3T3) and human (IMR90) fibroblasts; no effect was observed in mink (Mv1Lu), canine (MDCK), or human (HeLa) epithelial cultures. This cell tropism is identical to what we have reported for PI3K and PAK2 activation (14, 21) and suggests that PI3K, PAK2, and c-Abl might be targets within the same TGF-β signaling pathway (see below).

TGF-β signaling is dependent upon the kinase activity of the type I and type II receptors and has been reported to be initiated in both membrane-proximal and -distal locales (10, 11, 13, 14). Because the mechanisms regulating c-Abl activation by TGF-β are unknown, we addressed two essential questions. First, what is the role of receptor kinase activity in c-Abl activation, and second, does c-Abl activation require protein or mRNA synthesis? To address the first of these questions we took advantage of the chimeric TGF-β receptor model we developed (32, 33). This system utilizes the ligand binding domain of

![FIGURE 1. TGF-β activates c-Abl in fibroblasts but not epithelial cells. A, AKR-2B (left panel) or Mv1Lu (right panel) cells were grown to confluence, placed in DMEM containing 0.1% FBS overnight, and stimulated with 10 ng/ml TGF-β for the indicated times. Top row, following c-Abl immunoprecipitation, c-Abl kinase activity was determined using GST-Crk as substrate (28). Remaining rows, Western analysis was performed with c-Abl, phospho-Smad3, or total Smad3 antibodies on an aliquot prior to immunoprecipitation. B, three fibroblast (AKR-2B, NIH-3T3, IMR90) and three epithelial (Mv1Lu, MDCK, HeLa) cell lines were grown to confluence, serum-starved overnight, and either left untreated (−) or treated (+) with 10 ng/ml TGF-β for 30 min. Top row, equivalent protein was immunoprecipitated with c-Abl antibodies, and kinase activity was determined as described (28). Bottom row, c-Abl protein was determined on an aliquot prior to immunoprecipitation. Data are representative of three separate experiments.](image-url)
the GM-CSF α and β receptors and fuses them to the transmembrane and cytoplasmic domains of the type I or type II TGF-β receptors. Because high affinity GM-CSF binding only occurs via an α/β heteromer, defined cytoplasmic interactions can be specifically induced. As the chimeric system has been previously shown to faithfully recapitulate native TGF-β receptor signaling, we examined c-Abl activation in AKR-2B clones expressing wild-type type I and II chimeric receptors (A105), wild-type type I and kinase-impaired type II chimeric receptors (A615), and wild-type type II and kinase-impaired type I chimeric TGF-β receptors (A708). Although activation of wild-type chimeric receptors with GM-CSF or native receptors with TGF-β similarly induce c-Abl kinase activity (Fig. 2A), the absence of type I or type II kinase activity prevents c-Abl activation (Fig. 2A, left panel). As expected, Smad3 phosphorylation similarly required chimeric receptor kinase activity (Fig. 2A, left panel) and occurred normally in the A615 and A708 cells when the endogenous receptors were stimulated with TGF-β (Fig. 2A, right panel). Moreover, inhibition of protein or mRNA synthesis by greater than 90% (data not shown) had no discernable effect on c-Abl activation by TGF-β (data not shown). Thus, the c-Abl kinase is a primary TGF-β signaling target downstream of the TGF-β receptor complex.

**c-Abl Activation Occurs in a Cellular Compartment Separate from Smad Phosphorylation and Defines Distinct Transcriptional Targets from the Smad Proteins**—The Smad proteins are known to regulate many aspects of TGF-β action (1, 2, 7). Although we previously reported that Smad2 or Smad3 phosphorylation and c-Abl kinase activity were

**FIGURE 2.** Activation of c-Abl requires TGF-β receptor kinase activity and is independent of transcription. A, AKR-2B lines stably expressing chimeric wild-type (A105) or kinase-impaired (A615, TβR2K277R, A708, TβR1K232R) chimeric TGF-β receptors (33) were either left untreated (−) or stimulated (+) for 30 min with 50 ng/ml GM-CSF or 10 ng/ml TGF-β. Lysates were processed for c-Abl kinase activity, total c-Abl or Smad3 protein, or phospho-Smad3 (p-Smad3) as described. B, AKR-2B fibroblasts were grown to confluence, placed in serum-free DMEM overnight, and pretreated with 100 μg/ml cycloheximide or 5 μg/ml actinomycin D for 30 min. Cultures were left untreated (−) or stimulated (+) with 10 ng/ml TGF-β for 30 min in the absence (Control) or presence of cycloheximide (Cyclo) or actinomycin D (Actino D) before examining c-Abl kinase activity (top) or total c-Abl protein (bottom). Data are representative of three separate experiments.

**FIGURE 3.** c-Abl and Smad3 activation are distinctly regulated by TGF-β receptor internalization. A, AKR-2B cells were plated on glass coverslips and following overnight incubation either left untreated (Control) or infected with adenovirus (multiplicity of infection 300) expressing empty vector (Ad-Empty) or dominant negative dynamin 1 (Ad-Dyn1[K44A]) for 24 h. Epidermal growth factor and albumin internalization (markers of clathrin- and raft-dependent endocytosis, respectively) were performed utilizing Alexa Fluor 594-labeled albumin and Texas Red-labeled EGF as described (14, 49). B, confluent AKR-2B cells were placed in serum-free DMEM in the absence or presence of Ad-Empty or Ad-Dyn(K44A) as in A. Following 24 h of incubation, TGF-β was added to a final concentration of 10 ng/ml for 30 min. The cells were lysed and assayed for the indicated active or total protein.
independently regulated (28), this does not preclude the possibility that their activity is similarly initiated. For instance, although Smad phosphorylation is dependent upon TGF-β receptor internalization, it is not known whether c-Abl activation is regulated by comparable membrane-proximal events or, like Akt phosphorylation (14), occurs in the absence of TGF-β receptor internalization. Accordingly, clathrin- and raft-dependent internalization was inhibited by infecting AKR-2B cells with an adenovirus-expressing dominant negative dynamin 1, and any effect on c-Abl, PAK2, or Smad activation was determined (Fig. 3). Although both endocytic pathways were significantly inhibited by the dominant negative dynamin 1 (Fig. 3A), no discernable effect on c-Abl or PAK2 activity was observed (Fig. 3B). As expected, both Smad2 and Smad3 phosphorylation was prevented by the dominant negative virus (Fig. 3B).

![Figure 4.](image-url)  
**FIGURE 4.** c-Abl- and Smad-dependent transcriptional activity are distinctly regulated. A, AKR-2B cells were seeded in six-well dishes containing glass coverslides as described (10). Cultures were left untreated or pretreated with 10 μg/ml imatinib or vehicle (PBS) for 30 min prior to addition (+) of TGF-β to a final concentration of 10 ng/ml. Following 30 min incubation the slides were processed for microscopy (10). B and C, AKR-2B cells or fibroblast lines with deletion of Smad2 (Smad2−/−), Smad3 (Smad3−/−), or Abl and Arg (Abl−/−Arg−/−) genes were transfected with the indicated luciferase constructs (28). The -fold induction (+) of 10 ng/ml TGF-β (relative to no TGF-β, which was assigned a value of 1.0) for each vector was determined following 24 h of stimulation. Parallel AKR-2B cells were also pretreated with 20 μg/ml imatinib for 30 min prior to TGF-β addition. B shows the transcriptional response of three Smad-dependent reporters and C the response of three Smad-independent reporters. Data for 3TP, Smad-binding element (SBE), α smooth muscle actin (αSMA), and type I collagen (Col I) represent the mean ± S.D. of three separate experiments and for fibronectin (FN) and type III collagen (Col III) the mean ± S.D. of two separate experiments, each done in triplicate.
although the initial event in Smad activation (i.e. phosphorylation) is controlled in a distinct manner from c-Abl (Fig. 3B), this does not preclude the possibility that these pathways intersect and/or become dependent further downstream. As such, to determine if c-Abl activity was required for Smad nuclear translocation, immunofluorescence microscopy for Smad2 or Smad3 was performed following addition of TGF-β (Fig. 4A). Similar to that observed for Smad phosphorylation, imatinib had no effect on nuclear Smad accumulation.

To further investigate whether Smad and/or c-Abl signals impact on similar transcriptional responses, three Smad-dependent (i.e. 3TP, SBE, and αSMA) and three Smad-independent (i.e. fibronectin, type I collagen, and type III collagen) luciferase reporters were examined. As previously reported (34, 35), activation of the Smad-dependent reporters was primarily dependent upon Smad3. Consistent with the data of Fig. 4A, no effect on these Smad transcriptional targets was observed by inhibiting the c-Abl kinase with imatinib or utilizing MEFs deleted in the c-Abl and Abl-related (Arg) genes (Fig. 4A). Similar transcriptional regulation was similarly observed for Smad-independent targets. As shown in Fig. 4C, TGF-β-stimulation of fibronectin, type I collagen, or type III collagen was independent of Smad2 or Smad3 expression but, as we previously reported (28), required c-Abl. Together, the data support the hypothesis that Smad and c-Abl activation occurs in distinct cellular locales and independently regulates the transcriptional response of a subset of TGF-β-dependent genes.

C-Abl Activity Is Necessary for TGF-β Anchorage-independent Growth and Is Downstream of PI3K and PAK2—TGF-β was initially isolated and characterized by its ability to stimulate normal anchorage-dependent fibroblasts to grow in an anchorage-independent manner (36, 37). To date, colony formation in soft agar is still one of the best in vitro correlates with tumorigenicity. The pathways and genes necessary for TGF-β-induced soft agar growth are currently unknown. Because the ABL tyrosine kinase has known tumorigenic potential (23) and TGF-β stimulates c-Abl kinase activity (Figs. 1–3), we examined whether inhibition of c-Abl would prevent TGF-β colony formation. As shown in Fig. 5, addition of imatinib resulted in a dose-dependent decrease in soft agar colonies with ~2–4 μg/ml required for half-maximal inhibition.

Recent publications have begun to characterize a Smad-independent pathway stimulated by TGF-β in mesenchymal but not epithelial cell lines (14, 21). The most upstream component of this pathway is PI3K, the activity of which is required for independent regulation of both Akt phosphorylation and PAK2 kinase activity (14). As c-Abl activation demonstrated the identical cell tropism and Smad independence as Akt and PAK2, we determined whether c-Abl might represent an additional target within this pathway. Accordingly, AKR-2B cells were stimulated with TGF-β in the absence or presence of LY294002, Akt IV, or PD098059. Whereas TGF-β stimulated significant c-Abl activity under conditions where Akt- and MEK-dependent signaling is inhibited (Akt IV and PD098059, respectively), inhibition of PI3K with LY294002 completely abrogated the response (Fig. 6A).

A recent publication by Wilkes et al. (14) has shown that TGF-β activation of Akt and PAK2 represents two distinct branches downstream of PI3K. Because inhibition of PI3K prevented c-Abl activity, and the drug Akt IV was without effect, this would indicate that c-Abl is spatially located either upstream of Akt and/or within the PAK2 branch dependent upon PI3K. To distinguish between those alternatives, AKR-2B cells were infected with adenovirus-expressing dominant negative PAK2 virus (PAK2K299R) completely prevented c-Abl activation, infection with control viruses (i.e. expressing EGFP alone or RasN17 as an unrelated dominant negative protein) had no effect. Additional specificity to the LY294002 and PAK2K299R findings on c-Abl is documented by the inability of either treatment to prevent Smad3 phosphorylation by TGF-β (Fig. 6A and B).

The previous data indicate that c-Abl is downstream of and dependent upon the activity of PI3K. Because the only evidence for the involvement of PI3K was the small chemical inhibitor LY294002, to further substantiate this conclusion we inhibited PI3K activity with antisense morpholinos to the regulatory p85 subunit of PI3K and assessed their effect on Smad-dependent and -independent TGF-β signaling. As shown in Fig. 6C, inhibition of p85 expression diminished both Akt phosphorylation and c-Abl kinase activity stimulated by TGF-β. However, Smad3 phosphorylation was unaffected. Furthermore, in support of the data presented in Fig.
antisense oligonucleotides to PAK2 similarly inhibited TGF-β stimulation of the c-Abl kinase but had no effect on either Smad3 or Akt phosphorylation.

A model depicting our current understanding of cell type-specific TGF-β signaling is shown in Fig. 6D. TGF-β receptor activation is distinctly integrated in the two cell types such that Smad phosphorylation occurs in both, whereas PI3K activity is only stimulated in fibroblast cultures (14). PI3K independently activates PAK2 and Akt such that PAK2 is an upstream regulator of the c-Abl tyrosine kinase. Although both Smads and c-Abl are believed to represent distinct pathways, they are both necessary for TGF-β morphological transformation, proliferation, and soft agar colony formation (Fig. 5, data not shown, and Refs. 14, 21, 28, and 35). Arrows do not necessarily indicate a direct interaction and may reflect multiple events.

**DISCUSSION**

The cellular response to TGF-β is extremely variable. Depending upon the in vitro or in vivo model one can find effects as divergent as growth-promoting versus growth-inhibitory, inducing apoptosis versus cell survival, and/or inhibiting differentiation versus stimulating maturation (1, 4, 7, 31). Thus, although defining phenotypes is relatively easy, identifying and characterizing the operative pathways is not only quite difficult, but one has to be cognizant of the particular cell context. For these reasons we have focused our studies in defining those
responses associated with the proliferative component of TGF-β observed on mesenchymal cells. This has resulted in our identifying three targets downstream of the TGF-β receptor that are activated in a subset of fibroblast, but not epithelial, cultures (14, 21). The most upstream component (to date) regulating this cascade is PI3K (14). Inhibition of PI3K activity with LY294002 or antisense morpholinos not only abolishes TGF-β-stimulated morphologic transformation and proliferation but also activation of PAK2. However, while preventing PAK2 activity has similar biological effects on TGF-β responses as inhibiting PI3K, there is no effect on PI3K signaling to Akt. As we had previously shown that TGF-β-mediated fibrosis was, in part, due to stimulating the nonreceptor c-Abl tyrosine kinase (28), we wished to extend this analysis by integrating c-Abl within the TGF-β signaling network.

C-Abl was originally identified as the cellular homolog of the v-Abl oncogene product and subsequently shown to be involved in human malignancy through translocation events that produce the constitutively active Bcr-Abl protein (38, 39). Abl kinases are conserved across evolution, and two Abl-related tyrosine kinases, c-Abl and Abl-related gene (Arg), are present in humans and mice, which encode two alternatively spliced proteins with distinct amino-terminal first exon sequences (23). Although Arg is primarily localized to the cytoplasm and associates with F-actin (40–42), c-Abl pools have been identified at the cell membrane, within the cytosol, associated with the actin cytoskeleton, and in the nucleus (24, 43). It is currently unclear as to how each of these pools impacts on the various functions (i.e. cell proliferation, migration) associated with c-Abl. However, a theme is emerging, which indicates that cell survival signals promote cytoplasmic accumulation of Abl whereas those signals that support apoptosis stimulate an increase in nuclear Abl (25). It is presently unknown if TGF-β distinctly modulates c-Abl accumulation or activation from defined subcellular locales and/or whether this response can be readily integrated within the developing model of c-Abl action.

A defining characteristic of Abl family members is the presence of distinct functional domains including (but not limited to) a SH2 and SH3 domain, three nuclear localization sequences, a nuclear export sequence, and binding regions for both F- and G-actin (23, 44, 45). Although Arg is primarily localized to the cytoplasm and associates with F-actin (40–42), c-Abl pools have been identified at the cell membrane, within the cytosol, associated with the actin cytoskeleton, and in the nucleus (24, 43). It is currently unclear as to how each of these pools impacts on the various functions (i.e. cell proliferation, migration) associated with c-Abl. However, a theme is emerging, which indicates that cell survival signals promote cytoplasmic accumulation of Abl whereas those signals that support apoptosis stimulate an increase in nuclear Abl (25). It is presently unknown if TGF-β distinctly modulates c-Abl accumulation or activation from defined subcellular locales and/or whether this response can be readily integrated within the developing model of c-Abl action.

A defining characteristic of Abl family members is the presence of distinct functional domains including (but not limited to) a SH2 and SH3 domain, three nuclear localization sequences, a nuclear export sequence, and binding regions for both F- and G-actin (23, 24). F-actin binding has been mapped to the extreme COOH terminus, and deletion of the final 32 amino acids enhances c-Abl kinase activity and actin microspike formation (41, 44, 45). Thus, association with the actin cytoskeleton may represent an essential role for c-Abl in modulating morphologic changes stimulated by polypeptide growth factors. In that regard, the transition from a flat cobblestone appearance to an elongated spindle shape reminiscent of transformed cells is a hallmark of TGF-β action in fibroblasts (21, 46). Although this “morphologic transformation” has been known for over 20 years, the mechanism(s) through which TGF-β induces this change is unknown and has received relatively little attention. However, our recent finding that c-Abl activity is required for TGF-β-induced morphologic transformation and mitogenicity documents a role for c-Abl in cytoskeletal reorganization and proliferation dependent upon TGF-β signaling (Fig. 5 and Ref. 28). As these data suggest a novel signaling paradigm whereby serine/threonine receptor kinases integrate with downstream tyrosine kinase-dependent signaling cascades, the present study was undertaken to further examine the role of c-Abl in mediating the profibrogenic action of TGF-β.

We previously reported that TGF-β stimulated c-Abl kinase activity independent of Smad2 and Smad3 or the platelet-derived growth factor-α and -β receptors (28). Moreover, inhibition of c-Abl with imatinib mesylate prevented in vivo tissue fibrosis associated with bleomycin-induced lung injury or unilateral ureteral obstruction (28, 29). As these findings suggest an important role for c-Abl in TGF-β-mediated proliferation, we addressed two fundamental questions. First, was the association of c-Abl with fibrosis indicating a cell type-specific effect of TGF-β on c-Abl activity and second, where within the TGF-β signaling cascade was c-Abl acting, was it upstream or downstream of known signaling targets?

To address the first of these questions a panel of three representative mesenchymal (AKR-2B, NIH-3T3, IMR90) and epithelial (Mv1Lu, MDCK, HeLa) cultures was stimulated with TGF-β and assessed for any change in c-Abl kinase activity (Fig. 1). Although each was capable of responding to TGF-β with increased Smad2/3 phosphorylation (Fig. 1A and data not shown), only the mesenchymal cell lines showed enhanced c-Abl activation. Moreover, as previous work has demonstrated that (in the cell lines utilized) Smad2 or Smad3 phosphorylation requires receptor endocytic activity and occurs downstream of dynamin action (9–11), when similar studies (Fig. 3) were done to examine c-Abl activation, there was no effect on c-Abl kinase activity when clathrin- or caveolar-dependent internalization was inhibited. Furthermore, this independent action of Smad proteins and c-Abl is also observed for TGF-β-dependent transcription (Fig. 4, B and C). Together with that reported by Daniels et al. (28), these results indicate that (i) the c-Abl tyrosine kinase is activated by TGF-β in a cell type-specific and Smad-independent manner and (ii) the initiation and propagation of the regulatory signal(s) emanating from the TGF-β receptor complex to (and from) Smad and c-Abl proteins are distinct. Whether the 2 signals eventually converge is unknown, however, as neither is sufficient to control the morphologic and proliferative responses of TGF-β (28, 35); it is likely that there is cross-talk between the pathways.

One fundamental question raised by the preceding discussion is whether c-Abl activation is mediated via a distinct motif(s) within the type I (or type II) TGF-β receptor similar to the L45 loop in the type I receptor that provides Smad specificity (47, 48). To identify this region conveying Smad specificity, a novel chimeric receptor strategy showed that by replacing the L45 loop region in the type I TGF-β receptor with the analogous region from the BMP receptor, addition of BMP ligand resulted in a TGF-β Smad response (i.e. Smad2/3 phosphorylation), and conversely, when the BMP receptor L45 loop was placed in the TGF-β receptor, addition of TGF-β generated a BMP Smad response (i.e. Smad1/5 phosphorylation). Although it is currently unknown whether a similar element(s) exists for c-Abl activation, identification of such a site would represent an initial step in our ability to develop agonist/antagonists to specific aspects of TGF-β receptor function where we might wish to inhibit the profibrogenic activity of TGF-β but retain its growth-inhibitory action.

Lastly, because c-Abl represented a new downstream target
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activated by TGF-β in a subset of fibroblast lines but not epithelial cultures (Fig. 1B), we wished to integrate this response within the cell type-specific signaling pathway we have been developing for TGF-β (14, 21). This was initially addressed by using pharmacologic or viral inhibitors to known TGF-β targets. As shown in Fig. 6, A and B, when Akt-, Ras-, or Erk-dependent signaling was prevented, no effect was observed on c-Abl activity. However, inhibition of PI3K or PAK2 abrogated the stimulatory effect of TGF-β on c-Abl (Fig. 6, A, B, and C). Of note, although PI3K activity is required for TGF-β activation of PAK2 and c-Abl (Fig. 6, A and C, and Ref. 14), it is not sufficient. Expression of “constitutively active” PI3K (BD-p110) or p85 regulatory subunit fused to the p110 catalytic subunit of PI3K does not activate PAK2 or c-Abl in the absence of TGF-β. It does, however, constitutively induce Akt phosphorylation and production of PI3K-dependent lipid second messengers (data not shown). The additional signals emanating from the TGF-β receptor complex required for PAK2 and c-Abl activation are currently unknown. Together with the data reported in Wilkes et al. (14), these findings are consistent with a model whereby PI3K and PAK2 represent upstream targets whose activity is necessary for c-Abl activation (Fig. 6D). As would be expected with the positioning of c-Abl downstream of PAK2, inhibition of c-Abl with imatinib would have no effect on PAK2 kinase activity, but PAK2 inhibition would prevent c-Abl activation (Fig. 6B, Ref. 29, and data not shown). Of course, presenting such a model generates a number of fundamental questions relating to TGF-β action. For instance, first, the issue of what defines a fibroblast from an epithelial cell to initiate this signaling cascade is of primary interest; second, what are the additional TGF-β-dependent signals that cooperate with PI3K to induce PAK2 and c-Abl activity; third, to what pathway and/or biologic response does Akt couple; and fourth, how are the signals from the Smad and PI3K pathways integrated to regulate the morphologic and proliferative response of TGF-β in mesenchymal cells. Addressing questions of this sort is fundamental if the plethora of TGF-β-dependent phenotypes are to be understood.

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