Phosphatidylinositol 3-Kinase Function Is Required for Transforming Growth Factor β-mediated Epithelial to Mesenchymal Transition and Cell Migration*†‡‡

Andrei V. Bakin‡§, Anne K. Tomlinson‡§, Neil A. Bhowmick‡§, Harold L. Moses‡§, and Carlos L. Arteaga‡§¶**†***‡‡

From the Departments of ‡Medicine, §Cell Biology, and ¶Pathology, Vanderbilt University School of Medicine,
**Department of Veteran Affairs Medical Center, and ¶¶Vanderbilt-Ingram Cancer Center, Nashville Tennessee 37232

We have studied the role of phosphatidylinositol 3-OH kinase (PI3K)-Akt signaling in transforming growth factor β (TGFβ)-mediated epithelial to mesenchymal transition (EMT). In NMuMG mammary epithelial cells, exogenous TGFβ induced phosphorylation of Akt at Ser-473 and Akt in vitro kinase activity against GSK-3β within 30 min. These responses were temporally correlated with delocalization of E-cadherin, ZO-1, and integrin β1 from cell junctions and the acquisition of spindled cell morphology. LY294002, an inhibitor of the p110 catalytic subunit of PI3K, and a dominant-negative mutant cell morphology. LY294002, an inhibitor of the p110 catalytic subunit of PI3K, and a dominant-negative mutant of Akt blocked the delocalization of ZO-1 induced by TGFβ1, whereas transfection of constitutively active p110 induced loss of ZO-1 from tight junctions. In addition, LY294002 blocked TGFβ-mediated C-terminal phosphorylation of Smad2. Consistent with these data, TGFβ-induced p3TP-Lux and p(CAGA)12-Lux reporter activities were inhibited by LY294002 and transiently expressed dominant-negative p85 and Akt mutants in NMuMG and 4T1 cells. Dominant-negative RhoA inhibited TGFβ-induced phosphorylation of Akt at Ser-473, whereas constitutively active RhoA increased the basal phosphorylation of Akt, suggesting that RhoA is involved in TGFβ-induced EMT. Finally, LY294002 and neutralizing TGFβ1 antibodies inhibited ligand-independent constitutively active Akt as well as basal and TGFβ-stimulated migration in 4T1 and EMT6 breast tumor cells. Taken together, these data suggest that PI3K-Akt signaling is required for TGFβ-induced transcriptional responses, EMT, and cell migration.

The transforming growth factor β (TGFβ)† family of secreted factors is involved in the control of different biological processes including cell proliferation, differentiation, and apoptosis (1). TGFβ signals through the activation of heteromeric complexes of TGFβ type I (TβRI) and type II (TβRII) receptors (1, 2). Activated TβRI phosphorylates receptor-associated Smads (Smad2 and Smad3), which then bind Smad4 and translocate to the nucleus where they regulate transcription of target genes (3, 4). TGFβ exhibits a tumor suppressor activity, and components of its signaling pathway are frequently mutated or silenced in colon and pancreatic cancers (1, 5). However, accumulating data indicate that TGFβ can positively affect tumorigenesis and contribute to the progression and invasiveness of tumors (5–8). Moreover, it was recently reported that inhibition of autocrine TGFβ signaling in carcinoma cells reduces cell invasiveness and tumor metastases (9, 10). These effects of TGFβ are associated with its ability to induce an epithelial to mesenchymal transition (EMT) and stimulate cell migration.

The EMT induced by TGFβ results in the disruption of the polarized morphology of epithelial cells, formation of actin stress fibers, and enhancement of cell migration (8, 9). Two species of TβRI, Alk2 and Alk5, have been implicated in the induction of EMT by TGFβ in mammary epithelial cells (11, 12). It has also been reported that high levels of ectopic Smad2 and Smad3 can induce some features of EMT in mammary epithelial cells in the context of expression of an activated type I receptor (12). However, considering the complexity of TGFβ signaling (3, 13–16), it is conceivable that other molecules can also contribute to EMT. For example, members of the AP-1 family of transcription factors have been shown to induce EMT and promote tumor invasiveness (17, 18). AP-1 complexes can be activated in response to TGFβ (19–21), physically interact with Smads (13, 14), and cooperate with Smads in the control of gene expression (19–21). In addition, several other downstream signaling pathways can also be activated by TGFβ, including p38Mapk (21), c-jun N-terminal kinase (22, 23), and phosphatidylinositol 3-OH kinase (PI3K) (24, 25). These signaling pathways can potentially contribute to TGFβ1-mediated EMT, but their significance for EMT and cell migration mediated by TGFβ remains unclear.

In this study, we used the NMuMG mammary epithelial cell line as a model for TGFβ1-induced EMT (11). Two metastatic breast tumor cell lines, 4T1 and EMT6, that express high levels of TGFβ ligands and TGFβ receptors were used in transcription and migration studies. We report that TGFβ-induced EMT

saline; MT, microtubule; ca, constitutively active; dn, dominant-negative; PKB, protein kinase B; SARA, Smad activator for receptor activation; FYVE domain, domain found in Fab1p, YOTB, Vac1p, and EEA1 proteins.
and cell migration depend on the PI3K-Akt pathway. We also show that the phosphorylation of Smad2 and transcriptional responses induced by TGFβ are inhibited by pharmacological and molecular antagonists of the PI3K-Akt pathway. TGFβ1 can induce phosphorylation and activation of Akt/PKB in a PI3K-dependent manner, and this activation requires the Rho GTPase function. Taken together, our data suggest that PI3K-Akt signaling is required for the morphogenic, transcriptional, and migratory activities of TGFβ.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Reagents**—TGFβ1 was from R&D Systems (Minneapolis, MN) and EGF from CLONTECH (Palo Alto, CA). Antibodies to E-cadherin and integrin β1 were from Transduction Laboratories (Lexington, KY), to p85 from Upstate Biotechnology (Lake Placid, NY), and to β-actin from Chemicon (Temecula, CA). Phalloidin-fluorescein isothiocyanate (actin) was from Molecular Probes (Eugene, OR). The TGFβ1-neutralizing 2G7 monoclonal IgG2 was a gift from B. Fendly (Genentech, Inc.) and has been described previously (26). Antibodies to phospho-Ser-473 Akt and total Akt were from New England Biolabs (Beverly, MA), to Smad2 (N19) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and to C-terminal phospho-Smad2 from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to phospho-ERK1/2 and total ERK1/2 were from Promega (Madison, WI) and New England Biolabs, respectively. Mouse monoclonal antibodies 12CA5 and M2 to HA and Flag epitopes were from Roche Molecular Biochemicals and Sigma, respectively. Anti-Myc mouse monoclonal 9E10 antibody was a gift from J. F. Primus (Vanderbilt University). LY294002, ML7, okadaic acid, PD98059, rapamycin, U0126, and U73122 were purchased from Calbiochem (San Diego, CA). Curcumin was from Sigma. The Rac1 inhibitor SCH511434 was a kind gift from C. Kumar (Schering Research Institute, Kenilworth, NJ) (27). Adenovirus vectors encoding a dominant-negative mutant of Akt (AxAktK179D), a mutant regulatory subunit of p85 (AxAap85), and a constitutively active myristoylated mutant of p110 (AxAxx-p110) were kindly provided by W. Ogawa (Kobe University), J. F. Primus (Vanderbilt University), and P. N. Tsichlis (National Institutes of Health, Bethesda, MD). Plasmid vectors encoding GST-GSK-3β, a wild-type or a mutant was a gift from P. N. Tsichlis (Thomas Jefferson University, Philadelphia, PA). Plasmid vectors encoding Q61L/RhoA and N19RhoA were obtained from Dr. Lynn Cross (National Institutes of Health, Bethesda, MD). A plasmid vector encoding a GST-GSK3β peptide fusion protein was a gift from C. L. Van Den Berg (University of Colorado, Denver).

**Cell Culture and Adenoviral Infection**—NMuMG cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FBS and 10 μg/ml insulin. 4T1 tumor cells were provided by F. Miller (Karmanos Cancer Center, Detroit, MI) and EMT6 tumor cells by B. Teicher (Lilly Research Laboratories, Indianapolis, IN); both were cultured in DMEM plus 10% FBS. For adenoviral infection of NMuMG and 4T1 cells, cells were plated in 6-well plates and transduced with adenovirus vectors at 10–100 plaque-forming units/cell as described by Sakaue et al. (29). More than 90% of the NMuMG cells infected at a similar multiplicity of infection with an adenovirus expressing β-galactosidase (Adβ-Gal) exhibited blue staining. Infected cells were subjected to further treatment 24–48 h later.

**Cell Lysis and Immunoblot Analysis**—Cells were lysed in EBC buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin), and protein concentrations in cell lysates were determined by the Bradford method. Protein extracts (50 μg/lane) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (100 mA, 2.5 h). Membranes were blocked with 5% milk in TBST buffer (containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20/v/v) for 1 h at room temperature and then incubated with primary antibodies in TBST plus 1% milk for 1 h at room temperature and then incubated with primary antibodies diluted in 1% milk/TBS (1/300 for ZO-1, 1/500 for integrin β1, and 1/2000 for E-cadherin), and then incubated with secondary antibodies (1/500) for 1 h at room temperature. Coverslips were mounted onto 25 x 75-mm microslides (WVR Scientific, West Chester, PA) using AquaPolyMount (Polysciences, War- rington, PA). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axioiplan upright microscope.

**Migration Assays**—4T1 and EMT6 tumor cells (4 x 10⁴/well) were plated in DMEM, 10%FBS in the upper chamber of 9-μm pore (24-well)-size transwell inserts and cultured in DMEM, 5% FBS on glass coverslips. The inserts cooled CCD digital camera from a Zeiss Axioiplan upright microscope.

**RESULTS**

The PI3K-Akt Pathway Is Involved in EMT Induced by TGFβ1—TGFβ1 induced a mesenchymal transition in NMuMG cells within 24 h. Cells treated with 2 ng/ml TGFβ1 changed their shape from a cuboidal to a more elongated form (Fig. 1A, D, C, D). Concomitantly, TGFβ1 induced the delocalization of E-cadherin from adherens junctions, ZO-1 from tight junctions, and the delocalization of integrin β1 from the cell surface (Fig. 1). There were no detectable differences in the intracellular staining of E-cadherin, ZO-1, and integrin β1 between treated and untreated cells. In addition, no detectable changes in E-cadherin were found by immunoblot analysis of whole cell extract (Fig. 1B).

To determine the signaling pathways that contribute to TGFβ1-induced EMT, we examined the ability of different pharmacological agents to block the changes in cell morphology and in localization of epithelial markers at cell junctions. We found that LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K (30), blocked the morphological transition, the delocalization of ZO-1 from cell junctions, and the reorganization of actin filaments (Fig. 2A). Inhibitors of MEK1/2 (PD98059) (Fig. 2A) and U0126, a specific inhibitor of MEK (Fig. 2A), the n-terminal kinase (c-Jun), AKT (oncogenic target of rapamycin), phospholipase C (PLC) (UTS22,23), Rac (SCH511434), MLCK (myosin light chain kinase; ML7), and PP2A (okadaic acid) did not affect TGFβ1-mediated transition (data not shown), suggesting that signaling pathways associated with these molecules may not contribute to EMT mediated by TGFβ1. Inhibition of EMT by LY294002 suggested that PI3K is involved in EMT induced by
TGFβ1. To further test this hypothesis, NMuMG cells were infected with adenovirus encoding a constitutively active mutant of p110 (ca-p110), the catalytic subunit of PI3K. Cells expressing Myc-tagged ca-p110 showed a higher level phosphorylation of Akt at Ser-473, confirming its functional activity (Fig. 2B). Similar to exogenous TGFβ1, infection with the ca-p110 virus resulted in the delocalization of ZO-1 from tight junctions. However, the cells retained their epithelial morphology, whereas infection with a β-galactosidase adenovirus (Δβ-gal) did not alter cell morphology nor ZO-1 staining at adherens junctions (Fig. 2B). Finally, we examined whether Akt/PKB, a downstream effector of PI3K, would affect EMT. Transduction of NMuMG cells using a dominant-negative mutant Akt (AktK179D) adenovirus inhibited TGFβ1-induced delocalization of ZO-1 from tight junctions as well as changes in cell morphology (Fig. 2C). These data suggest that the PI3K-Akt pathway is required for some of the phenotypic hallmarks associated with TGFβ1-mediated EMT.

**Activation of the PI3K-Akt Pathway in Response to TGFβ1**—To further test that the PI3K pathway is activated by TGFβ1, we examined the phosphorylation status and kinase activity of Akt. Immunoblot analyses with antibodies specific to the phosphorylated form of Akt showed that TGFβ1 induced phosphorylation of Akt at Ser-473 within 30 min, achieving a detectable maximum at 2 h (Fig. 3A). Phosphorylation of Ser-473 Akt was inhibited by 20 μM LY294002 (Fig. 3A, last lane), indicating that Akt activation requires PI3K function. The activity of Akt/PKB was measured using an *in vitro* kinase assay with GST-GSK3β fusion protein containing GSK-3β peptide in frame with GST and immobilized on agarose beads as a substrate. Treatment of NMuMG cells with TGFβ1 for 2 h stimulated a 4-fold induction in the incorporation of 32P into GST-GSK3β (Fig. 3B). Next, we tested the TGFβ1 dose dependence of phosphorylation of Akt and Smad2. Treatment with 0.5 ng/ml (20 pm) TGFβ1 was sufficient to induce a maximal phosphorylation for both Ser-473 Akt and Smad2 (Fig. 3C). TGFβ1 and EGF, a known agonist of PI3K, induced similar levels of Ser-473 Akt phosphorylation. EGF induced activating phosphorylation of ERK1/2, whereas TGFβ1 did not stimulate ERK activation at any concentration tested (Fig. 3C).

**Rho-like GTPases Mediate Activation of the PI3K-Akt Pathway in Response to TGFβ1**—Recent studies have suggested that RhoA is involved in TGFβ1-mediated transcription (22, 23) and that TGFβ1 can activate RhoA in NMuMG cells. Therefore, we tested whether RhoA GTPase affected the activation of PI3K-Akt mediated by TGFβ1. NMuMG cells transiently transfected with a dominant-negative RhoA mutant (N19RhoA) showed a significantly reduced level of Akt phosphorylation compared with a control (Fig. 3D). Transfection of the constitutively active form of RhoA (Q61LRhoA) resulted in an increase in basal phosphorylation of Akt (Fig. 3E). These results suggest that RhoA may be involved in TGFβ1-mediated activation of the PI3K-Akt pathway.

**Transcriptional Responses to TGFβ1 Involve the PI3K-Akt Pathway**—TGFβ1 transcriptional responses can be controlled through the subcellular localization of Smads. It has been shown that SARA, a recently identified mediator of TGFβ signaling, controls recruitment of Smad2 to TGFβ receptors (31). The function of SARA depends on its FYVE homology domain, which binds phosphatidylinositolos phosphatidylated by PI3K (31). In addition, recent data have suggested that microtubules (MTs) may control Smad-dependent TGFβ1 transcriptional responses (32). It has been shown that PI3K associates tightly with α- and β-tubulins (33), and it is involved in the function of MTs (34). Therefore, we next examined whether PI3K is involved in the regulation of TGFβ1-mediated transcription. Two TGFβ-responsive reporter constructs were used in transcriptional assays: p3TP-Lux, containing the firefly luciferase reporter gene under the control of three 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements and a fragment of the PAI-1 promoter (1), and p(CAGA)12-Lux, a reporter gene containing 12 repeats of Smad binding sequences from the PAI-1 promoter (35). In NMuMG cells transiently transfected with p3TP-Lux, TGFβ1-mediated induction of luciferase was inhibited by LY294002 in a dose-dependent manner at 4 and 16 h (Fig. 4A). Similar results were obtained with 4T1 and EMT6 mammary tumor cell lines (data not shown). LY294002 also inhibited TGFβ-stimulated reporter activity in both NMuMG and 4T1 cells transfected with p(CAGA)12-Lux (Fig. 4B). We next examined whether an adenovirus vector encoding a dominant-negative mutant of p85 (dn-p85), the regulatory subunit of PI3K, would emulate the effects of LY294002. Expression of dn-p85 significantly reduced a basal phosphoryla-
The PI3K-Akt pathway is involved in TGFβ-mediated EMT. A. NMuMG cells were treated or not with 2 μg/ml TGFβ1 for 24 h in the presence 20 μM PD098059 or 20 μM LY294002 where indicated. Cells were stained with antibodies to ZO-1 (1:300) or phalloidin-fluorescein isothiocyanate (1:100) to visualize actin filaments. B. Localization of ZO-1 in NMuMG cells infected with adenovirus vectors encoding β-galactosidase or the constitutively active mutant of p110 (ca-p110) for 48 h at a multiplicity of infection of 100 plaque-forming units/cell. By phase contrast (DIC, differential interference contrast), cells infected with the ca-p110 virus retained their epithelial morphology. The immunoblot analysis shows expression of Myc-tagged ca-p110 (lane 2) in cells infected with ca-p110 compared with control virus (lane 1). The lower panel shows the level of phospho-Ser-473 Akt in control cells (lane 1), cells infected with dn-p85 (lane 2), or cells infected with ca-p110 (lane 3). C. NMuMG cells were infected with AxAkt-K179D (dn-Akt) or AxδG-Gal (control) adenoviruses at a multiplicity of infection of 40; 48 h later, cells were treated with 2 ng/ml TGFβ1 for an additional 24 h followed by immunostaining for ZO-1 (1:300) as indicated under “Experimental Procedures.” The immunoblot shows expression of Flag-tagged dn-Akt in cells infected with AxAkt-K179D (lane 2) compared with the control virus (lane 1). Scale bars represent 15 μm.

The transcriptional functional activity of Ser-473 Akt (Fig. 2B), confirming its functional activity. TGFβ-induced p3TP-Lux reporter activity was reduced by 75% in both NMuMG and 4T1 cells infected with the dn-p85 adenovirus vector but not with a control adenovirus encoding β-galactosidase (Fig. 4C). Finally, transient transfection of a dominant-negative mutant of Akt (AktK179M) markedly inhibited TGFβ-induced p3TP-Lux transcription (Fig. 4D). These data suggest that the PI3K-Akt pathway is involved in TGFβ transcriptional responses.

TGFβ-mediated Phosphorylation of Smad2 Requires PI3K—The transcriptional data using the p(CAGA)₆Lux reporter (Fig. 4B) suggested that PI3K is involved in the control of Smad-dependent transcription. Therefore, we examined the effect of PI3K blockade on TGFβ-induced phosphorylation of Smad2. Immunoblot analysis with antibodies specific to Smad2 phosphorylated at the C terminus showed that C terminus phosphorylation of Smad2 was induced by TGFβ1 within 15 min, reaching a maximum by 1 h. However, co-incubation with 20 μM LY294002 markedly reduced ligand-mediated Smad2 phosphorylation without detectable changes in total Smad2 protein levels (Fig. 5A). At the same time, phosphorylation of Ser-473 Akt was completely blocked by LY294002 (Fig. 5B). The induction of the C-terminal phosphorylation of Smad2 and phosphorylation of Ser-473 Akt in response to TGFβ1 appears to occur with similar kinetics and TGFβ1 dose dependence (Figs. 3 and 5). To test whether the PI3K-Akt pathway is directly involved in the C-terminal phosphorylation of Smad2, NMuMG cells were transfected with dn-Akt followed by TGFβ1 treatment and immunoblot analysis of C terminus phosphorylation of Smad2. The level of Smad2 phosphorylation was similar in control cells and cells transfected with dn-Akt, suggesting that Akt is not involved in C-terminal phosphorylation of Smad2 (Fig. 5C). Infection of cells with ca-p110 also did not induce ligand-independent phosphorylation of Smad2 (Fig. 5D).

TGFβ1-induced Cell Migration Requires PI3K Activity—TGFβ1 can stimulate the migration of tumor and nontumor cells (7, 36, 37). PI3K has been implicated in the regulation of cell migration and chemotaxis of human neutrophils (38–40).
Therefore, we examined whether PI3K is involved in TGFβ-induced cell migration. We used 4T1 and EMT6 mouse tumor cells, which exhibit high levels of TGFβ receptors that mediate transcriptional responses (Fig. 4) but are not growth inhibited by exogenous TGFβ1. TGFβ1 enhanced migration of both cell lines in a dose-dependent manner with an EC50 of approximately 0.1 ng/ml (4 μM). LY294002 blocked both basal and TGFβ-stimulated cell migration (Fig. 6A) without an effect on tumor cell proliferation (data not shown). The TGFβ1-neutralizing 2G7 monoclonal antibody also reduced basal cell migration, suggesting that this phenotypic response was partially dependent on autocrine TGFβ signaling (Fig. 6B). Furthermore, both LY294002 and 2G7 reduced the basal level of phosphorylation at Ser-473 Akt in 4T1 and EMT6 cells (Fig. 6C), suggesting a causal association between autocrine TGFβ signaling with basal PI3K-Akt signaling and the subsequent migration of tumor cells.

**DISCUSSION**

The tumor-promoting activity of TGFβ1 associated with the induction of EMT has been documented for different tumor types (5–9). Several reports have shown that TGFβ can induce a reversible mesenchymal transition in mammary epithelial NMuMG cells (11, 12). In this study, we present data to support the role of the PI3K-Akt pathway in TGFβ-mediated EMT. We found that either the blockade of PI3K activity by a synthetic inhibitor, LY294002, or by expression of dn-Akt significantly inhibited EMT (Fig. 2). These observations led us to hypothesize that the PI3K-Akt pathway is directly involved in this transition. Similar to TGFβ1, forced expression of constitutively active PI3K (ca-p110) was sufficient to promote the disruption of cellular junctions but did not induce per se the

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3 C. L. Arteaga, unpublished data.
changes in cell morphology associated with EMT (Fig. 2B). The dissolution of tight junctions and the disruption of adherent junctions induced by TGFβ1 are relatively early processes, occurring within 4–8 h after the addition of TGFβ1, whereas changes in the cell shape occur later. This result suggests that PI3K function is required for the early changes during TGFβ-mediated EMT but that other events associated with the reorganization of cytoskeleton leading to changes in cell morphology may not depend on the PI3K-Akt pathway. The observed delocalization of E-cadherin, integrin β1, and ZO-1 from cellular junctions occurred without detectable changes in their cellular content, suggesting that these TGFβ-mediated effects may involve PI3K-dependent endocytosis. These observations are consistent with the studies implicating PI3K in endocytosis and vesicular trafficking (41–43). Similar to TGFβ, hepatocyte growth factor can also disrupt epithelial cell-cell junctions and induce the delocalization of E-cadherin from cell junctions (44).

In this process, hepatocyte growth factor induces the delocalization of both E-cadherin and the hepatocyte growth factor receptor, c-Met, via PI3K-mediated co-endocytosis (44). This co-endocytosis can be blocked by dominant-negative mutants of RhoA and Rab5, a component of early endosomes (44). In addition, Rab5-mediated endocytosis is also regulated by Akt/PKB (45). Thus, TGFβ-mediated delocalization of epithelial markers from cell junctions may involve the function of PI3K-Akt and Rho-like GTPases.

The activation of PI3K in response to TGFβ has been reported in two other cell systems (24, 25). In NMuMG cells, TGFβ1 induced phosphorylation and activation of Akt/PKB with kinetics similar to the C-terminal phosphorylation of Smad2 (Figs. 3 and 5). Activation of Akt depends on PI3K, since it can be blocked by a synthetic inhibitor of PI3K (Figs. 3 and 5) and by expression of dn-p85 (Fig. 2B, inset). These results suggest that the PI3K-Akt pathway is activated directly by TGFβ1. This conclusion is further supported by recent reports showing co-precipitation of p85, the regulatory subunit of PI3K-Akt and TGFβ-mediated EMT.
PI3K with TGFβ receptors and stimulation of PI3K activity by TGFβ in other cell types (24, 25). We also confirmed a direct association p85 with both type I and type II TGFβ receptors in NMuMG cells.4

Because of the reported role of Rho family GTPases in TGFβ signaling and their interaction with the PI3K pathway (46), we tested the role of the RhoA GTPase in TGFβ-mediated activation of Akt. Expression of dominant-negative N19RhoA mutant disrupted ligand-induced phosphorylation of Akt at Ser-473. On the other hand, expression of a constitutively active mutant, Q63LRhoA, resulted in an increase of the basal phosphorylation of Akt. These findings suggest that RhoA GTPase is involved in TGFβ1-mediated activation of Akt, which is consistent with recent reports that Rho-like GTPases can synergize with TGFβ signaling (22, 23). Therefore, RhoA may function as an upstream effector of Akt activation in response to TGFβ1.

Using two reporter constructs, p3TP-Lux and p(CAGA)12-Lux, we found that TGFβ1 transcriptional responses in NMuMG and two tumor cell lines are inhibited by both pharmacological and molecular antagonists of the PI3K-Akt pathway, including dominant-negative p85 and Akt mutants (Fig. 4, A–D). The fact that a blockade of the PI3K-Akt pathway affected Smad-dependent transcriptional responses suggested the involvement of PI3K and Akt in TGFβ1 intracellular signal transduction. Consistent with this idea, we found that LY294002 significantly reduced TGFβ1-mediated C-terminal phosphorylation of Smad2 in NMuMG cells (Fig. 5). However, neither PI3K nor Akt is involved in C-terminal phosphorylation of Smad2, since introduction of ca-p110 or dn-Akt did not affect it. These results, coupled with the inhibitory effect of LY294002 on Smad2 phosphorylation (Fig. 5), suggest that PI3K is involved indirectly in TGFβ1-mediated C-terminal phosphorylation of Smad2.

PI3K activity may also be required for the function of intracellular mediators of TGFβ signaling. Recently, two factors regulating C-terminal phosphorylation of Smad2 were described (31, 32). First, the intracellular localization of Smad2 is controlled by SARA, a recently cloned Smad2-binding protein (31). SARA co-localizes with EEA1, an early endosome marker,3 and this co-localization depends on the FYVE domain of SARA, which binds phosphatidylinositol 3-phosphates (47,48). It has been shown that deletion of the FYVE domain results in the mislocalization of Smad2 and inhibition of TGFβ1 transcriptional responses (31). We found that Smad2 co-localizes with EEA1 in the absence of TGFβ1 in NMuMG cells.3 Thus, it is conceivable that the blockade of PI3K activity in NMuMG cells with LY294002, similar to wortmannin (49), will reduce the levels of phosphatidylinositol 3-phosphate, resulting in the mislocalization of Smad2. This is a potential explanation of the inhibitory effect of LY294002 on TGFβ1-induced phosphorylation of Smad2 (Fig. 5A), whereas neither ca-p110 nor dn-Akt can directly modulate Smad2 phosphorylation (Fig. 5C, D). In addition, a recent report provides evidence that endogenous Smad2, Smad3, and Smad4 are stored in the MT network (32). It has been suggested that upon TGFβ treatment, Smad2 and Smad3 dissociate from MT, become phosphorylated by TβRII, and translocate to the nucleus where they regulate the transcription of TGFβ target genes. Moreover, destabilization of MTs with nocodazole can facilitate Smad-mediated TGFβ transcriptional responses per se in the absence of exogenous TGFβ1 (32). On the other hand, TGFβ has been reported to stabilize MTs (50), potentially limiting Smad signaling. PI3K has also been shown to control the dynamics of the MT network, which is important for intracellular trafficking, cell motility, and other cell functions (51). Therefore, PI3K antagonists may affect the MT network and interfere with TGFβ signaling. To formally demonstrate that PI3K blockade inhibits TGFβ signaling via its effects on MTs will require further investigation.

Both TGFβ and PI3K have been implicated in chemotaxis and cell migration (7, 36–40). Here, we show that putative concentrations of TGFβ enhanced the basal migration of tumor cells, whereas blockade of PI3K with LY294002 reduced both basal and TGFβ-stimulated cell migration (Fig. 6, A and B). These data are in agreement with a critical role of PI3K in cell motility and migration via the modulation of cytoskeletal organization (47, 51). These results were generated with tumor cells that exhibit high levels of TGFβ expression and TGFβ receptors as well as constitutive activation of Akt in the absence of added TGFβ ligand. Similar to LY294002, TGFβ1-neutralizing monoclonal antibodies reduced basal cell migration and Ser-473 phosphorylation of Akt, suggesting an association between autocrine TGFβ signaling with both constitutively activated Akt/PKB and cell invasiveness. Neither exogenous TGFβ, anti-TGFβ antibodies, nor LY294002 had any effect on 4T1 or EMT6 cell proliferation. These data coupled with the transcription data using TGFβ reporters in 4T1 and EMT6 cells imply that EMT can be dissociated from the anti-mitogenic effects of TGFβ. In summary, the results presented provide evidence that the PI3K-Akt pathway is causally involved in the morphogenic, transcriptional, and migratory activities of TGFβ.

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PI3K-Akt and TGFβ-mediated EMT

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