We have examined the interaction of transforming growth factor β (TGFβ) receptors with phosphatidylinositol 3-(P3) kinase in epithelial cells. In COS7 cells, treatment with TGFβ1 increased P3 kinase activity as measured by the ability of p85-associated immune complexes to phosphorilate inositol lipids, signal transducers that are essential for the regulation of cell cycle progression, glucose metabolism, cell motility, epithelial-to-mesenchymal transition, and apoptosis among others (6, 7). In Swiss 3T3 cells, TGFβ stimulates PI3 kinase activity, as measured by the ability of immune complexes precipitated with an antibody against p85, the regulatory subunit of PI3 kinase, to induce the formation of phosphatidylinositol-3-monophosphate (PI3P) in vitro (8). In mammary epithelial cells, TGFβ induces epithelial-to-mesenchymal transition and cell motility as well as phosphorylation and activation of the PI3 kinase-dependent Akt serine-threonine kinase. These responses are blocked by LY294002, a small molecule inhibitor of the p110 catalytic subunit of PI3 kinase (9). In mesenchymal and epithelial cells, TGFβ-mediated protection from apoptosis is blocked by LY294002 and/or expression of dominant-negative, kinase-dead Akt (10–12). Inhibition of PI3 kinase reverses the fibroblastoid phenotype of TGFβ-treated Ras-transformed hepatocytes to an epithelial phenotype (13). Furthermore, PI3 kinase inhibition abrogates both basal and TGFβ-induced motility in mammary cancer cells (9). Taken together, these data suggest that PI3 kinase is a major effector pathway of the transforming effects of TGFβ in epithelial cells. Therefore, in this study, we have examined the initial mechanisms of TGFβ-induced activation of PI3 kinase in epithelial cells.

MATERIALS AND METHODS

Cell Lines and Antibodies—COS7 and NMuMG epithelial cells were purchased from American Type Culture Collection (ATCC). R1B cells were provided by Harold Moses (Vanderbilt University, Nashville, TN). COS7 and R1B cells were maintained in Dulbecco’s Modified Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). NMuMG cells were maintained in 10% fetal bovine serum–Dulbecco’s modified Eagle’s medium with 10 µg/ml insulin. We utilized the following antibodies: p85 (Upstate Biotechnology), Smad2/3 (Transduction Laboratories), hemagglutinin (HA) rabbit polyclonal and TβRII (Santa Cruz Biotechnology), FLAG epitope (Sigma), Ser-473 P-Akt, total Akt, P-MAPK, total MAPK (Erk42/44), and P-Smad2 (Cell Signaling). Human recombinant TGFβ1 was obtained from R&D Systems. The PI3 kinase inhibitor wortmannin was from Calbiochem. The TβRII transactivates TβRII (1). Activated TβRI phosphorylates the receptor-specific Smad2 and Smad3, which then associate with Smad4 and, as a heteromeric complex, translocate to the nucleus where they regulate the transcription of TGFβ target genes (1). The Smad signaling pathway mediates the antiproliferative effect of TGFβ in epithelial cells (2, 3) and is the best characterized. Several non-Smad pathways have also been implicated in mediating the cellular effects of TGFβ. These include the extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 (P3) kinase, and the family of Rho GTPases (4, 5). Activated PI3 kinase increases the formation of intracellular 3'-phosphorylated inositol lipids, signal transducers that are essential for the regulation of cell cycle progression, glucose metabolism, cell motility, epithelial-to-mesenchymal transition, and apoptosis among others (6, 7). In Swiss 3T3 cells, TGFβ stimulates PI3 kinase activity, as measured by the ability of immune complexes precipitated with an antibody against p85, the regulatory subunit of PI3 kinase, to induce the formation of phosphatidylinositol-3-monophosphate (PI3P) in vitro (8). In mammary epithelial cells, TGFβ induces epithelial-to-mesenchymal transition and cell motility as well as phosphorylation and activation of the PI3 kinase-dependent Akt serine-threonine kinase. These responses are blocked by LY294002, a small molecule inhibitor of the p110 catalytic subunit of PI3 kinase (9). In mesenchymal and epithelial cells, TGFβ-mediated protection from apoptosis is blocked by LY294002 and/or expression of dominant-negative, kinase-dead Akt (10–12). Inhibition of PI3 kinase reverses the fibroblastoid phenotype of TGFβ-treated Ras-transformed hepatocytes to an epithelial phenotype (13). Furthermore, PI3 kinase inhibition abrogates both basal and TGFβ-induced motility in mammary cancer cells (9). Taken together, these data suggest that PI3 kinase is a major effector pathway of the transforming effects of TGFβ in epithelial cells. Therefore, in this study, we have examined the initial mechanisms of TGFβ-induced activation of PI3 kinase in epithelial cells.

Transforming growth factor β (TGFβ)1 binds to a hetero-meric complex of transmembrane serine-threonine kinases, the type I and type II TGFβ receptors (TβRI and TβRII). Following ligand binding to TβRII, the type I receptor is recruited to the ligand-receptor complex where the constitutively active TβRII transactivates TβRI (1). Activated TβRI phosphorylates the receptor-specific Smad2 and Smad3, which then associate with Smad4 and, as a heteromeric complex, translocate to the nucleus where they regulate the transcription of TGFβ target genes (1). The Smad signaling pathway mediates the antiproliferative effect of TGFβ in epithelial cells (2, 3) and is the best characterized. Several non-Smad pathways have also been implicated in mediating the cellular effects of TGFβ. These include the extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 (P3) kinase, and the family of Rho GTPases (4, 5).

Received for publication, November 23, 2004, and in revised form, January 13, 2005. Published, JBC Papers in Press, January 18, 2005, DOI 10.1074/jbc.M413223200
Type I TGFβ Receptor Binds to and Activates PI3 Kinase

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Plasmids and Viral Vectors—The pCMV6-p85 construct tagged with c-myc in its C terminus was provided by Lewis Cantley (Harvard Medical School, Boston, MA). The pRK5-TRII-Flag plasmid was provided by Rik Derynck, UCSF. The wild-type TRII-HA construct, from Kohei Miyazono (Japanese Foundation for Cancer Research, Tokyo), was subcloned into the retroviral expression vector pBMN-IRES-EGFP (provided by Garry Nolan, Stanford University, Palo Alto, CA) (151). Cells were transfected with plasmids using FuGENE 6 reagent (Roche Applied Sciences) according to the manufacturer's instructions. Retroviruses were prepared by transfection of 293T cells with 15 µg of DNA/100-mm dish of three plasmids encoding gag/pol, VSV-G, and the target construct (in 4:3:8 ratios, respectively). Supernatants from cells were collected for 2 days and combined, filtered through 0.22-µm filters, and stored in aliquots at −80 °C. Rb cells were infected with supernatant containing retroviruses in the presence of 8 µg/ml Polybrene (Sigma) as described previously (16). Three days after transfection of Rb cells with pBMN-IRES-EGFP, EGFP-positive cells were selected by flow cytometry. Under these conditions, >95% of selected cells expressed GFP at the time of any of our experiments. The HA-Alk5(17) and Flag-Smad7 adenoviral constructs were also from Dr. Kohei Miyazono. Stocks of recombinant adenoviruses were generated in 293T cells and titered utilizing the Takara assay (Takara, Tokyo, Japan). Cells were then infected with these or with a control β-galactosidase adenovirus at an equivalent multiplicity of infection as described previously (17).

Immunoprecipitation and Immunoblot Analysis—Cells were transfected with pCMV6-p85. After 16 h, cells were washed twice with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (1% Nonidet P-40, 120 mM NaCl, and 50 mM Tris-HCl, pH 7.4), protease inhibitor mixture (Roche Applied Sciences), 10 mM NaF, 1 mM Na3VO4 on ice for 20 min. Cell lysates were centrifuged at 14,000 rpm at 4 °C for 20 min, and protein concentrations were then determined by the BCA method (Pierce). Equal amounts of cell lysates were incubated overnight at 4 °C with primary antibodies. Immune complexes were collected with protein G-Sepharose 4B or protein A-Sepharose 4B (both from Sigma) at 4 °C for 3 h. The precipitates were washed three times with centrifugation and washed three times with lysis buffer, once with high salt buffer (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl) and once with low salt buffer (10 mM Tris-HCl, pH 7.4). The final pellets were boiled in Laemmli buffer containing 10% β-mercaptoethanol and separated by SDS-PAGE.

For immunoblot analysis, 20–30 µg of total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween 20) at room temperature for 1 h. The blots were incubated with primary antibody in 4 °C for 16 h, washed three times with TBST for 1 h, incubated for 2 h with secondary anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences), and then washed three times with TBST for an additional 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Membranes were blocked with 5% nonfat dry milk in TBST and incubated overnight at 4 °C followed by centrifugation at 12,000 rpm for 5 min. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences), and then washed three times with TBST. The blots were incubated with primary antibody in 4 °C for 16 h, washed three times with TBST for 1 h, incubated for 2 h with secondary anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences), and then washed three times with TBST for an additional 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) as described (17).

GST Pull-down Assays—The expression vectors for glutathione S-transferase (GST) fusion proteins, pGEX4T and pGEX4T-TXR2 (gift from Pran Datta, Vanderbilt University) and pGEX2T-TRII (provided by Rik Derynck, UCSF) were transformed into Escherichia coli BL21 strain. The bacterial cultures were induced by 0.1 mM isopropyl-D-thiogalactoside (Roche Applied Sciences) according to the manufacturer's instructions. Bacterial lysates were next sonicated. Bacterial lysates were brought up in 4% Triton, 10 µg of DNAse, and 8 mM MgCl2 and incubated for 2 h at 4 °C followed by centrifugation at 12,000 × g for 5 min. GST fusion proteins were purified by binding to glutathione (GSH)-Sepharose 4B beads (Amersham Biosciences) at room temperature for 1 h before washing three times with phosphate-buffered saline containing protease inhibitors. The concentration of proteins immobilized was estimated by SDS-PAGE and Western blots against bovine serum albumin standards (Sigma) after staining the SDS gel with Coomassie Blue. Binding assays were performed between GST fusion proteins and in vitro translated p85 protein. p85 was generated with a T7T-coupled reticulocyte lysate system (Promega) also containing pCMV6-p85-myc, T7 polymerase, and [35S]labeled methionine (Amersham Biosciences). 10 µl of [35S]-labeled protein and equal amounts of immobilized GST or GST fusion proteins were incubated for 2 h at 4 °C with gentle rotation. The precipitates were pelleted by centrifugation and washed three times with 1% Nonidet P-40 buffer, once with high salt buffer and once with low salt buffer. The beads were resuspended in Laemmli buffer, boiled, separated by SDS-PAGE, and analyzed by autoradiography.

PI3 Kinase Assay—PI3 kinase catalytic activity was measured in vitro as described previously (18). In brief, cells were lysed with 1% Nonidet P-40 in buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM Na3VO4) and a protease inhibitor mixture. Lysates were precipitated with a p85 antibody and protein A-Sepharose 4B (both from Sigma) at 4 °C for 3 h. The precipitates were washed twice with centrifugation and washed three times with lysis buffer (200 µl Hepes, 150 mM NaCl, 0.1 mM EDTA), the precipitates were resuspended in 80 µl of assay buffer containing 10 µl of crude brain phosphoinositides (PI, 2 mg/ml; Sigma) and 10 µl of substrate (500 µM cold ATP with 10 µCi/µl of [γ-32P]ATP (specific activity 6000 Ci/mmol; Amersham Biosciences) in 200 µl Hepes, pH 7.5, and 50 mM MgCl2). Following gentle agitation for 10 min at room temperature, the reaction was terminated by the addition of 100 µl of HCl and 200 µl of chloroform:methanol (1:1). The radiolabeled lipids were extracted, concentrated, and separated by TLC using silica gel plates pretreated with 1% v/v K oxalate in a solvent system of n-propanol:2 M acetic acid (65:35 v/v). The incorporation of [32P] into PI was detected by autoradiography.

RESULTS

p85, the Regulatory Subunit of PI3 Kinase, Can Bind Both TRII and TRII—In serum-starved COS7 cells, addition of TGFβ stimulated PI3 kinase activity in a time-dependent fashion as measured by the ability of immune complexes precipitated with p85 antibodies to stimulate formation of 3'-phosphorylated inositol lipids in vitro. This induction was maximal at 6 h and temporally correlated with phosphorylation of the serine-threonine kinase Akt in Ser-473 (Fig. 1). The kinetics of ligand-induced Smad2 phosphorylation were faster with maximal induction seen after treatment with TGFβ for 1 h.

To determine whether p85 interacted directly with TGFβ receptors, we co-transfected COS7 cells with vectors encoding p85 and TRII or TRII followed by immunoprecipitation with
receptor antibodies. p85 was detectable in TβRI and TβRII pull downs from cells co-transfected with the respective combination of vectors (Fig. 2A). To determine whether this binding is modulated by TGFβ, COS7 cells were stimulated with TGFβ for variable times. The binding between p85 and TβRI was maximal 1 h after TGFβ treatment, and this association was correlated with maximal induction of Smad2 and Akt phosphorylation (Fig. 2B, left). However, binding between p85 and TβRII was not affected by exogenous TGFβ even though in these co-transfected cells a more modest ligand-induced phosphorylation of Smad2 and Akt was still observed (Fig. 2B, right). To determine whether this p85-TGFβ receptor association was direct, we co-incubated rabbit reticulocyte 35S-labeled p85 with GST-TβRI and with GST-TβRII fusion proteins (Fig. 2C). Under these conditions, we were unable to recover labeled p85 from the immobilized GST-TGFβ receptor fusions, implying that the p85-TβRI and p85-TβRII associations, as supported by the prior co-precipitation experiments in transfected cells (Fig. 2A and B), were not direct.

TβRII and TβRI Are Required for Ligand-mediated Activation of PI3 Kinase—To investigate whether TβRII function is required for ligand-induced activation of PI3 kinase, we co-transfected COS7 cells with p85 and TβRI with or without a vector encoding a kinase-inactive TβRII mutant in which the lysine at position 277 has been mutated to arginine (14). The expression of the TβRII(K277R) construct prevented ligand-induced association of p85 with TβRI (Fig. 3A). In cells co-transfected with p85 and TβRI, we were unable to detect an increase in TGFβ-induced PI3 kinase activity associated with p85 precipitates (data not shown). We speculate that under these conditions, the majority of the overexpressed p85 protein is not associated with TβRI, and thus, the p85-associated catalytic activity does not reflect ligand-induced, TGFβ receptor-associated PI3 kinase activity. Therefore, we transfected TβRII without p85 but with or without TβRII(K277R). The expression of the kinase-dead type II receptor abrogated a ligand-mediated increase in PI3 kinase activity in vitro (Fig. 3B). This is different from the slower kinetics of ligand-induced activation observed in untransfected COS7 cells (Fig. 1). In COS7 cells transfected with TβRI, but not with TβRII, exogenous TGFβ maximally increased PI3 kinase activity at 1 h. We did not detect any PI3 kinase activity in TβRII precipitates.

To examine whether TβRI was required for ligand-induced activation of PI3 kinase, we used R1B cells, a subline of Mv1Lu cells, which lacks type I receptors (19). R1B cells were transfected with pBMN-IRES-EGFP or pBMN-TβRII (Alk5)-IRES-EGFP retroviral vectors; EGFP-positive cells were sorted by flow cytometry. In all our experiments, >95% of cells used expressed GFP. In TβRII-reconstituted cells but not in cells transduced with the control retrovirus, treatment with TGFβ induced PI3 kinase activity as well as Smad2 and Akt phosphorylation (Fig. 4).

Active TβRI (Alk5T204D) Can Induce PI3 Kinase Activity—Because p85 can bind TβRII (Fig. 2), we next determined whether overexpression of TβRII was permissive for ligand-induced PI3 kinase and P-Akt and compared these with the kinetics of activation observed in untransfected COS7 cells (Fig. 1). In COS7 cells overexpressing transfected TβRII, TGFβ maximally induced PI3 kinase activity and Ser-473 P-Akt at 6 h (Fig. 5) suggesting that an excess of type II receptor did not

**Fig. 2. p85 binds to TβRI and TβRII.** COS7 cells were transiently transfected with vectors encoding p85 and TβRII-HA or p85 and TβRII-Flag. A, cells without TGFβ treatment were lysed by a buffer containing 1% Nonidet P-40 and precipitated (IP) with TβRII or TβRIII antibodies, respectively. Immunoprecipitates were subjected to a p85 immunoblot procedure (IB, top panel). Expression of the transfected proteins in whole cell lysates was checked by p85, HA, and FLAG immunoblot (3 bottom panels). The p85 band in the control (Ctl) lane represents endogenous protein. B, Twenty-four hours after transfection with vectors encoding p85 and TβRII-HA or p85 and TβRII-Flag, the cells were serum-starved for another 24 h and then treated or not treated with TGFβ (2 ng/ml) for the indicated times. Cell lysates were precipitated with TβRI or TβRII antibodies, and the precipitates were immunoblotted with a p85 antibody (top panel only). Expression of the transfected p85, TβRII, and TβRIII was detected by p85, HA, and FLAG immunoblot, respectively. Total Smad2, P-Smad2, total Akt, and P-Akt were also determined by immunoblot analyses as indicated to the right of each panel. C, 35S-labeled p85 generated by in vitro transcription/translation in the presence of T7 polymerase was incubated with GST alone, GST-TβRI, or GST-TβRIII as indicated under “Materials and Methods.” Labeled p85 was separated from GSH beads by 8% SDS-PAGE and visualized by autoradiography. Sp6 polymerase was used as a negative control. Bottom panel shows the input of GST and GST-fusion proteins into the SDS gels.
accelerate PI3 kinase activation compared with untransfected cells. We were unable to detect any PI3 kinase activity in immune complexes precipitated with TβRII antibodies (not shown).

The studies above had shown that in cells overexpressing transfected p85 and TβRI, the association of p85 and type I receptors was maximal after 1 h of treatment with TGFβ (Fig. 2B). Moreover, in COS7 and R1B cells overexpressing transfected TβRI, maximal ligand-induced activation of PI3 kinase was evident after 1 h of treatment (Figs. 3B and 4B), suggesting that TβRI can accelerate the activation of PI3 kinase. Therefore, to test whether TβRI can activate PI3 kinase, we transduced NMuMG and R1B cells with an HA-tagged adenovirus encoding a mutant of TβRI (Alk5T204D). A substitution of threonine 204 with aspartic acid leads to constitutive activation of the type I receptor serine-threonine kinase thus allowing it to signal in the absence of added ligand (20). In both cells, transduction with a mutant Alk5T204D but not with a β-galactosidase control adenovirus resulted in a marked increase in ligand-independent, p85-associated PI3 kinase activity as well as Smad2 phosphorylation (Fig. 6).

To determine whether active Alk5 was causally associated with the enhanced PI3 kinase activity observed, we utilized small molecule TβRII serine-threonine kinase inhibitor LY580276 (21). LY580276 is a parafluorophenyl substituted dihydropyrrrolpyrazole that is highly selective for TβRI (IC_{50} 0.18 μM, K_{i} 37 nM) relative to TβRII (IC_{50} >20 μM), p38α (IC_{50} >20 μM) and a panel of 39 additional kinases with an IC_{50} >10 μM. It inhibits TGFβ-stimulated p3TP-Lux reporter activity in Mink lung cells (IC_{50} 96 nM) and NIH3T3 proliferation (IC_{50} 39 nM). The crystal structure of LY580276 in Alk5T204D has been solved, which confirms binding at the ATP site (22). Treatment with LY580276 for 24 h blocked Alk5T204D-mediated PI3 kinase activation and Smad2 phosphorylation (Fig. 6) supporting a causal association between active Alk5 and the induction of PI3 kinase catalytic activity.

Smad7 Blocks Alk5-mediated Activation of PI3 Kinase—To determine whether the activation of PI3 kinase by TβRI requires Smad function, we blocked Smad signaling with the inhibitory Smad7 (23, 24). Smad7 stably interacts with activated TβRI thereby blocking the association with phosphorylation and activation of Smad2/3. Overexpressed adenoviral Smad7 blocked Alk5T204D-induced PI3 kinase activity as well as Smad2 phosphorylation (Fig. 7).

DISCUSSION

We have examined whether TGFβ receptors interact with and/or activate PI3 kinase in epithelial cells. Exogenous TGFβ increased PI3 kinase activity as measured by the ability of p85-associated immune complexes to phosphorylate inositides in vitro. The kinetics of ligand-induced PI3 kinase activation were much slower than the phosphorylation of Smad2 with maximal detectable induction 6 h after the addition of TGFβ. Both type I and type II receptors associated with p85, but the association of TβRII appeared constitutive, as it was not enhanced by exogenous ligand. On the other hand, the interaction of TβRI with p85 was markedly enhanced by treatment with TGFβ for 1 h. This receptor association with PI3 kinase was not direct. 35S-Labeled rabbit reticulocyte p85 did not associate with fusion proteins containing type I and type II receptors. In addition, we were unable to detect any PI3 kinase activity in TβRII and TβRII precipitates from TGFβ-stimulated cells. This is consistent with a previous report using TGFβ-stimulated fibroblasts in which PI3 kinase activity was not recovered with a TβRII antibody (8). These results and the slow kinetics of induction of PI3 kinase catalytic activity by TGFβ suggest the presence of signaling intermediates between ligand-bound TGFβ receptors and PI3 kinase. Although TβRII overexpres-
**FIG. 4.** TβRI is required for TGFβ-induced PI3 kinase activity. R1B cells were stably transduced with retroviruses encoding TβRI or vector alone. Transduced R1B cells were incubated with serum-free medium for 24 h and then treated with or without TGFβ (2 ng/ml) for the indicated times. Cell lysates from vector control (A) and TβRI-reconstituted cells (B) were collected and tested in a PI3 kinase in vitro kinase assay (left panels). TβRI, total Smad2, P-Smad2, total Akt, and P-Akt were measured in whole cell lysates by immunoblot (right panels). Wn, wortmannin.

**FIG. 5.** Overexpression of TβRII does not accelerate PI3 kinase activation. COS7 cells were transiently transfected with TβRII-Flag. Twenty-four hours after transfection, the cells were serum-starved for 24 h and then treated with or without TGFβ (2 ng/ml) for the indicated times. Cell lysates were collected, precipitated with a p85 antibody, and subjected to a PI3 kinase in vitro assay (left panel). Transfected TβRII expression level was detected by FLAG immunoblot. Phosphorylation and content of Smad2, Akt, and MAPK were detected by immunoblot analysis of whole cell lysates (right panel). Wn, wortmannin.
sion did not increase TGFβ-induced or uninduced PI3 kinase activity, a kinase-dead, dominant-negative mutant of TβRII blocked ligand-induced p85-TβRII association and PI3 kinase activity suggesting that a functional type II receptor is required for TGFβ-mediated activation of PI3 kinase. These data also imply that in tumors with inactivating mutations in the TGFBR2 gene TGFβ may not be able to engage PI3 kinase and contribute to tumor progression by this effector pathway.

In TβRII-null R1B cells, TGFβ did not stimulate PI3 kinase activity. However, this stimulation was restored upon reconstitution of TβRII by transfection. Moreover, the overexpression of TβRI accelerated ligand-induced catalytic activity with maximal induction observed 1 h after the addition of TGFβ. To confirm that the serine-threonine kinase activity of TβRI can activate PI3 kinase, we transduced R1B and NMuMG epithelial cells with a dominant active mutant form of TβRII. In both cell lines, the expression of TβRII204D markedly enhanced ligand-independent PI3 kinase activity, which was blocked by the addition of the TβRI kinase inhibitor LY580276, strongly suggesting a causal link between TβRI function and PI3 kinase. Finally, overexpressed Smad7, which binds phosphorylated TβRI, prevented ligand-induced PI3 kinase activity as well as Smad2 phosphorylation, implying further that TβRI can associate with and activate PI3 kinase.

Despite this causal evidence between TβRI function and TGFβ-mediated activation of PI3 kinase, it is not clear how TβRI stimulates this activity. The level of induction with added ligand is comparable with that reported in platelet-derived growth factor-stimulated Swiss 3T3 (8) and NIH3T3 cells (25) where the p85-associated PI3 kinase activity is only increased ~20% over untreated controls. Only 3–10% of cellular PI3 kinase is activated by insulin, colony-stimulating factor-1, or erbB2 receptors (26–28). This level of activation probably reflects the large pool of PI3 kinase that is modestly affected by a single growth factor. In human airway smooth muscle cells, for example, TGFβ can induce a detectable increase in PI3 kinase only in the presence of epidermal growth factor (29). Further consistent with this notion, overexpression of p85 in COS7 cells eliminated the detectable increase in p85-associated PI3 kinase activation in response to an added ligand (data not shown). Nonetheless, the marked induction of p85-associated PI3 kinase observed in cells transduced with the constitutively active TβRIIT204D mutant, which was blocked by a pharmacological inhibitor of the type I receptor kinase, pro-

![FIG. 6. Constitutively active TβRII mutant induces PI3 kinase activity. NMuMG (A) and R1B (B) were transduced with (control) β-galactosidase (βGal) or Alk5T204D-HA adenoviruses (3 multiplicity of infection each). Twenty-four hours after transduction, the small molecule inhibitor of the TβRI kinase, LY580276 (10 μM), was added. After a 24-h incubation, cell lysates were collected, precipitated with a p85 antibody, and tested in a PI3 kinase in vitro reaction (left panels). Alk5T204D-HA, Smad2, and P-Smad2 levels were measured by immunoblot procedures using the indicated antibodies (right panels). Wn, wortmannin.](image)

![FIG. 7. Smad7 blocks Alk5T204D-induced stimulation of PI3 kinase activity. NMuMG cells were transduced with the indicated adenoviruses. After 48 h, cell lysates were collected and tested in an PI3 kinase in vitro assay as in previous figures (left panel). Expression levels of Alk5T204D-HA and Smad7-Flag were confirmed by immunoblot analysis using HA and FLAG antibodies, respectively. Smad2 and P-Smad2 were measured by immunoblot of whole cell lysates (right panel). βGal, β-galactosidase; Wn, wortmannin.](image)
vides causal evidence linking TβRI and PI3 kinase activities. Similar results (17) have been reported in MDA-231 human breast cancer cells expressing kinase-dead TβRIK277R. Transduction with an adenovirus encoding activated TβRI induced P-Akt and restored PI3 kinase-dependent cell motility (17).

PI3 kinase is usually induced by activated transmembrane tyrosine kinases such as insulin (30), nerve growth factor (31), and platelet-derived growth factor receptors (25). Activated epidermal growth factor receptors (erbB1) or erbB2 can also link to PI3 kinase activity after partnering with erbB3, a receptor with six p85-binding sites (32–34). Upon receptor activation, p85 associates via its SH2 domain with phosphorylated tyrosines within XXXM motifs in the C terminus of the receptor. Because of this, phosphotyrosine antibody precipitates from platelet-derived growth factor-stimulated cells have been shown to contain 50-fold more PI3 kinase activity than unstimulated controls (35). XXXM motifs are not present in TβRI or TβRII, which is further in line with the lack of evidence for a direct association between p85 and TGFβ receptors (Fig. 2C). In some cases, the interaction between p85 and receptor tyrosine kinases is also indirect and occurs through intermediary phosphoproteins such as that reported for the insulin receptor substrates IRS1 and IRS2 (36, 37). We were unable to recover any detectable PI3 kinase activity in phosphotyrosine receptor immunoprecipitates from TGFβ-stimulated cells (35). Yee, J. K., Miyanoohara, A., LaPorte, P., Bouic, K., Burns, J. C., and Friedmann, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9564–9568

It remains to be determined whether TGFβ-induced PI3 kinase activity is universal among epithelial cells. If in fact, as our data would suggest, PI3 kinase activation requires signal intermediates, the differential expression of those putative intermediates may specify whether TGFβ can or cannot activate this signaling pathway as well as the magnitude and time course of this activation. Indeed, we have not observed ligand-induced activation of Akt in MCF-10A and MCF-7 human mammary epithelial cells. Because Akt activity is PI3 kinase-dependent, this result would suggest that activation of PI3 kinase by TGFβ is cell type-dependent. The answer to these questions will require further investigation.

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