TGF-β-induced RhoA and p160^{ROCK} activation is involved in the inhibition of Cdc25A with resultant cell-cycle arrest

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The ability of the transforming growth factor β (TGF-β) signaling pathways to inhibit proliferation of most cells while stimulating proliferation of others remains a conundrum. In this article, we report that the absence of RhoA and p160^{ROCK} activity in fibroblastic NIH 3T3 cells and its presence in epithelial NMuMG cells can at least partially explain the difference in the TGF-β growth response. Further, evidence is presented for TGF-β-stimulated p160^{ROCK} translocation to the nucleus and inhibitory phosphorylation of the cyclin-dependent kinase-activating phosphatase, Cdc25A. The resultant suppression of Cdk2 activity contributes to G_1/S inhibition in NMuMG cells. These data provide evidence that signaling through RhoA and p160^{ROCK} is important in TGF-β inhibition of cell proliferation and links signaling components for epithelial transdifferentiation with regulation of cell-cycle progression.

Transforming growth factor β1 (TGF-β1) causes growth inhibition in many nontransformed epithelial cell types (1). The mechanism by which this occurs involves the binding and activation of TGF-β type I and type II receptors for the subsequent induction of parallel downstream signaling pathways (2). The established paradigm of TGF-β type I receptor phosphorylation of Smad2 and Smad3 in conjunction with Smad4 recruitment is important in TGF-β-mediated G_1 cell-cycle arrest (3). The SMAD signaling pathway up-regulates the expression of cyclin-dependent kinase (cdk) inhibitors (4–6), thereby suppressing cyclin D- and/or cyclin E-associated hyperphosphorylation of Rb and S phase progression (reviewed in ref. 7). Additionally, SMAD signaling is associated with the transcriptional down-regulation of c-myc, required for cell-cycle progression (8–10). However, fibroblastic NIH 3T3 cells exhibit both TGF-β-mediated SMAD activation and *c-myc* down-regulation, yet proliferation of these cells is stimulated by TGF-β (11). Earlier findings suggest that growth stimulation of NIH 3T3 cells by TGF-β is caused by increased cyclin E-associated kinase activity (12). Other as-yet-unidentified TGF-β-associated signaling mechanisms involve the inhibition of CDK-activating kinase (13) and down-regulation of the Cdk2-activating phosphatase, Cdc25A (14).

We previously demonstrated TGF-β activation of RhoA in a number of cell types, but the NIH 3T3 cells were an exception (15). Thus, we hypothesized that RhoA signaling may be involved in TGF-β-mediated cell-cycle arrest. The role of RhoA in cytoskeletal organization is well established and has more recently emerged as a mediator of cell-cycle progression (16–18). The activation state of RhoA is positively regulated by guanine exchange factors and negatively regulated by GTPase-activating proteins (19). Among the downstream effector proteins of RhoA, the role of p160^{ROCK} (ROCK1) and mDia are better understood as mediators of the formation and maintenance of stress fibers, whereas PKN activation has been implicated in the delay of G_2/M progression (20).

In this article, we define the role of RhoA signaling-mediated inhibition of Cdc25A activity in the context of no detectable decline in Cdc25A protein levels. Our results suggest that TGF-β-mediated RhoA/p160^{ROCK} activation is involved not only in epithelial to mesenchymal transdifferentiation as we demonstrated previously (15) but also in the phosphorylation of Cdc25A and inhibition of Cdk2 activity.

Experimental Procedures

Cell Culture and Retroviral Transduction. The NMuMG (normal murine mammary gland epithelial) and NIH 3T3 (mouse embryonic fibroblast) cells were purchased from the American Type Culture Collection and propagated in DMEM with 10% FBS. Insulin (10 μg/ml) was added to the medium for culture of NMuMG cells. The Ostα (T. Miki, National Institutes of Health, Bethesda), RhoA, Rho binding domain of rhotekin (RBD; M. Schwartz, The Scripps Research Institute, La Jolla, CA), and p160^{ROCK} (S. Narumiya, Kyoto University, Kyoto) cDNAs were subcloned into the pBabe retroviral vector. These constructs were transfected into Phoenix cells (G. Nolan, Stanford University, Stanford, CA) and the virus-containing conditioned media used to transduce target cells (15, 21). Experiments were performed 48 h after transduction.

RhoA Activation Assay. Activation of RhoA was detected in NMuMG and NIH 3T3 cells by adsorbing cell lysates to RBD to enrich for GTP-bound RhoA as described (15, 22). Adsorbed and nonadsorbed lysates were then Western blotted for RhoA (26C4; Santa Cruz Biotechnology).

Thymidine Incorporation, Flow Cytometry, and Sequential Cell Counting. DNA synthesis was determined by [^{3}H]thymidine incorporation. Cells treated for 24 h with TGF-β were pulsed with [^{3}H]thymidine 2 h before harvest and measurement by scintillation counting (15). Cell-cycle determination was made by fluorescence-activated cell sorter (FACS) analysis of propidium iodide-labeled cells. Cells treated for 24 h with or without TGF-β were stained for 15 min before flow sorting of 50,000 cells per sample (23). Cell-cycle profiles were determined by using MODFIT software (Verity Software House, Topsham, ME). Sequential cell counts were performed for a time course of 3 days by using a Coulter counter on cells treated with or without TGF-β (5 ng/ml) or the p160^{ROCK} inhibitor Y27632 (5 μg/ml; Collaborative Research, Waltham, MA).

Knock-Down of p160^{ROCK} Expression with Small Interfering RNA (siRNA). The murine p160^{ROCK} sequence 5′-AAAGGUAAUCGGCAGAGGUGC-3′ was targeted for RNA interference. Dhar-

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Abbreviations: TGF-β, transforming growth factor β; cdk, cyclin-dependent kinase; NMuMG cell, normal murine mammary gland epithelial cell; RBD, Rho binding domain of rhotekin; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.

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macon (Lafayette, CO) generated the siRNA double-stranded oligonucleotides. Sixty percent confluent NMuMG cells were transfected by using Oligofectamine (Invitrogen) according to the provided protocol. Briefly, siRNA duplex, diluted in Opti-MEM (Invitrogen), was incubated with Oligofectamine (in ratio of 60 pmol:3 μl, respectively) for 25 min at room temperature. The mixture was added to cells in serum-free media, and subsequent experiments were performed 48 h later.

Immunoprecipitation, Western Blot Analysis, and *in Vitro* Kinase Assay. Cells subjected to indicated treatments were lysed by sonication in Hepes lysis buffer, pH 7.5, as described (24). Cell lysates of equivalent protein amounts (400 μg) were immunoprecipitated with the indicated antibodies and protein G-Sepharose beads (Sigma). The immunoprecipitants or cell lysates directly (40 μg) were separated by SDS/PAGE for subsequent immunodetection by blotting on to poly(vinylidene difluoride) membranes. Western blots were visualized by chemiluminescence (Pierce) or alkaline phosphatase development (Sigma). For kinase assays, immunoprecipitated p160^{ROCK} or Cdk2 was incubated at 30°C for 15 min with indicated substrates (Cdc25A or histone H1) and [γ-^{32}P]ATP (24). The reactions were terminated in Laemmli buffer, resolved by SDS/PAGE, and visualized by autoradiography.

P160^{ROCK} kinase assays were performed by using a human Cdc25A wild type and site-directed mutant of the Ser-123 residue to Ala (S123A) (25). PCR-mediated mutagenesis was performed on a GST-Cdc25A cDNA construct (A. Dutta, Harvard University, Boston) with primers 5′-GAGGAGCCATGCTGATTCTCTT-GAC-3′ and 5′-GTCAAGAGAATCAGCATGGCTCCTC-3′. The GST-Cdc25A constructs (wild type and mutant) expressed in HEK293 cells were purified by using glutathione-agarose (Sigma) and eluted before use in kinase reactions.

***In Vivo* Phosphorylation of Cdc25A.** Rapidly growing cells were preincubated in phosphate-free DMEM supplemented with 0.2% FBS for 1 h before [^{32}P]orthophosphate metabolic labeling (0.9 mCi/ml; 1 Ci = 37 GBq) for an additional 1 h. Then the cells were incubated further in the presence or absence of TGF-β (25 ng/ml) or Y27632 (5 μM). The cells were subsequently washed, harvested, and Cdc25A-precipitated by antibody (F-6, Santa Cruz Biotechnology) and protein G-Sepharose or glutathione-agarose as indicated. Then SDS/PAGE-separated proteins were visualized by autoradiography.

Cell Fractionation. Rapidly growing NMuMG cells incubated with TGF-β (5 ng/ml) were harvested through a 12-h time course by the Nuclear and Cytoplasmic Extraction Reagent (NE-PER; Pierce) per manufacturer's instructions. The respective nuclear and cytoplasmic fractions were analyzed by Western blot analysis. Immunodetection of proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology) and RhoA (Santa Cruz Biotechnology) were used as controls to identify nuclear and cytoplasmic cell fraction contamination, respectively.

Cdc25A Phosphatase Assay. Immunoprecipitated Cdc25A was incubated with substrate, histone H1, or Cdk2, for 15 min at 30°C in a total volume of 20 μl and separated by SDS/PAGE. Histone H1 (10 μg) was tyrosine-phosphorylated as above with [γ-^{32}P]ATP by Cdk2, and dephosphorylation was observed by autoradiography (26). Alternatively, immunoprecipitated Cdk2 (His-tagged) was incubated with Cdc25A, and subsequent specific phosphorylation state of Tyr-15 was determined by Western blotting (Cell Signaling Technology, Beverly, MA).

Results

Differential TGF-β-Mediated RhoA Response. Treatment of NMuMG cells with TGF-β results in growth inhibition; however,

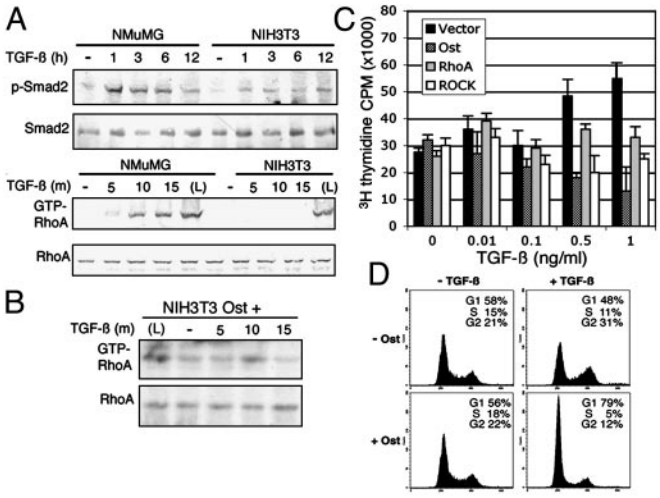

Fig. 1. Differential TGF-β-mediated RhoA responsiveness. (*A*) NMuMG and NIH 3T3 cells were incubated with TGF-β for the indicated times and lysed, and equal protein amounts of total cell lysates were analyzed by Western blotting with an antibody against the phosphorylated form and total Smad2 protein. GTP-loaded RhoA was measured by adsorbing cell lysates to GST-RBD beads and Western blotted for RhoA. As a positive control, NMuMG cells were treated for 5 min with lysophosphatidic acid (L). (*B*) Similarly, RhoA activation was examined in NIH 3T3 cells expressing Ost through a 15-min time course of TGF-β treatment. (*C*) NIH 3T3 cells expressing RhoA, Ost, p160^{ROCK}, or control cDNA constructs were treated with TGF-β and harvested for measurement of [^{3}H]thymidine incorporation. The results are representative of at least two experiments done in triplicate (mean ± SD). (*D*) Flow cytometry was performed on NIH 3T3 cells expressing Ost or empty vector grown 24 h in the presence or absence of TGF-β.

NIH 3T3 cells are growth-simulated when treated with TGF-β (12). Western blotting for the phosphorylation of Smad2 indicated Smad2 activation by 1 h of TGF-β treatment of both NMuMG and NIH 3T3 cells (Fig. 1*A*). The expression levels of Smad2 did not change in either cell line for the 12 h of TGF-β treatment. However, TGF-β stimulated GTP-RhoA accumulation in NMuMG cells by 5 min but did not induce RhoA activation in NIH 3T3 cells (Fig. 1*A*; ref. 15). The total RhoA expression levels did not change, and lysophosphatidic acid stimulated RhoA activation in both NMuMG and NIH 3T3 cells. When NIH 3T3 cells stably expressed Ost (a Rho guanine exchange factor), TGF-β activated both RhoA (Fig. 1*B*) and Cdc42 (data not shown).

To examine the disparity in TGF-β signaling and growth inhibition, the RhoA signaling pathway was artificially stimulated in NIH 3T3 cells. [^{3}H]thymidine incorporation of NIH 3T3 cells retrovirally transduced with wild-type RhoA, Ost, and p160^{ROCK} was compared with results of infecting with empty virus. In the presence of increasing concentrations of TGF-β, a 50% increase in thymidine incorporation was observed in control NIH 3T3 cells, whereas wild-type RhoA overexpressing cells were no longer growth-stimulated (Fig. 1*C*). Similarly, the expression of the downstream target, wild-type p160^{ROCK}, blocked growth stimulation of NIH 3T3 cells, but no significant growth inhibition was observed. We hypothesized that a more physiological method of RhoA/p160^{ROCK} activation might be required to mimic the transient RhoA activation by TGF-β in NMuMG cells. The transduction of Ost resulted in a 50% growth inhibition of NIH 3T3 cells in the presence of 10 ng/ml TGF-β. To further examine the role of RhoA activation in the presence and absence of TGF-β, we performed flow cytometry on rapidly growing NIH 3T3 and Ost-expressing NIH 3T3 cell. Treatment with TGF-β or expression of Ost alone did not significantly alter the cell-cycle profile compared with control cells (Fig. 1*D*). In

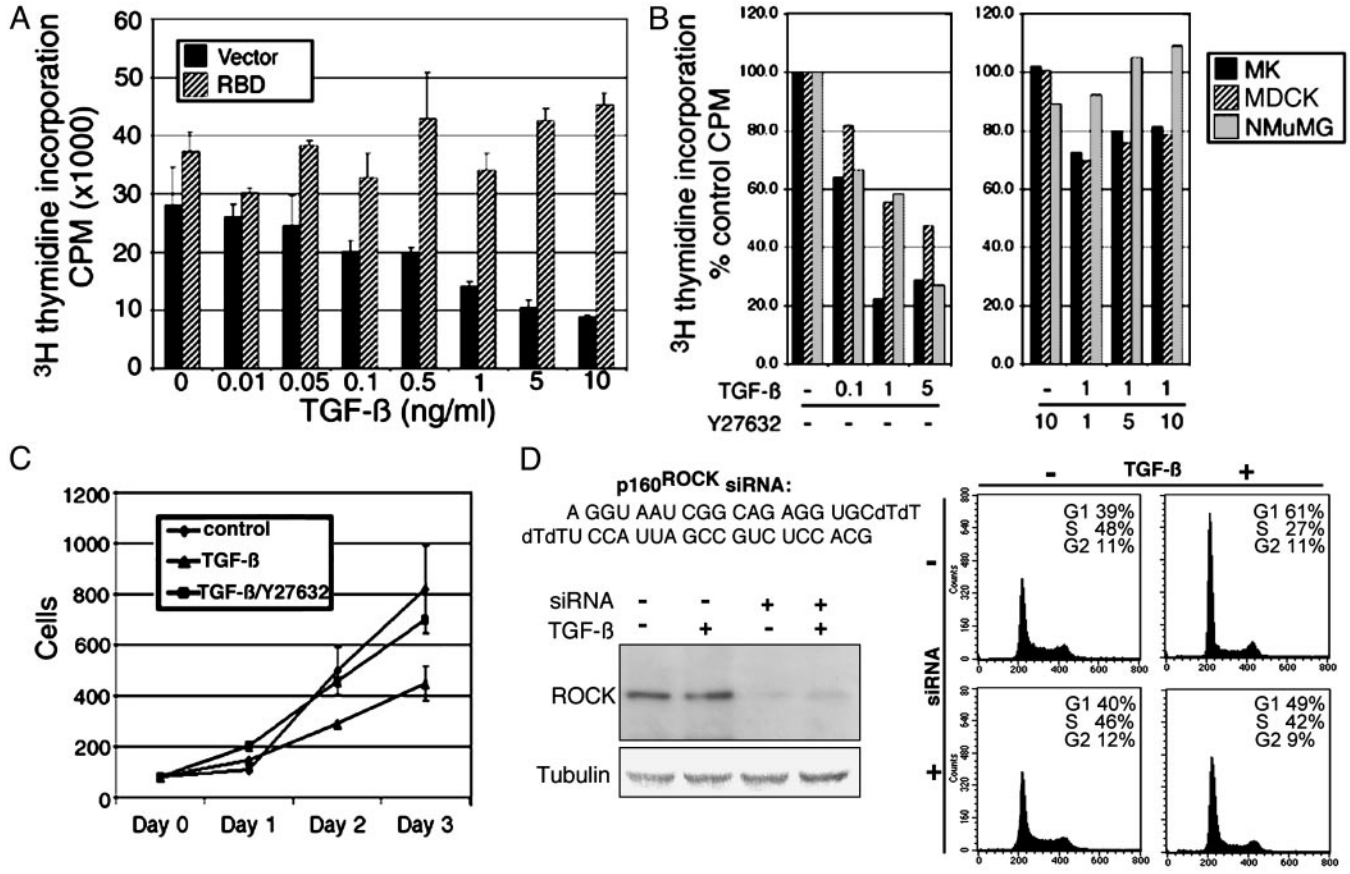

Fig. 2. Growth inhibition of NMuMG cells by TGF-β involves RhoA-p160^{ROCK} activity. (*A*) NMuMG cells expressing RBD or control cDNA constructs were treated with increasing concentrations of TGF-β and assayed for [^{3}H]thymidine incorporation. (*B*) Thymidine incorporation studies were done in MK, MDCK, and NMuMG cells in the presence of varying concentrations of TGF-β or Y23637. (*C*) Cells treated as indicated were subjected to sequential counts during a 3-day period in triplicate (mean $\pm$ SD). (*D*) At 48 h after siRNA transfection into NMuMG cells, p160^{ROCK} expression was analyzed by Western blotting. Similarly treated cells were subjected to fluorescence-activated cell sorter analysis for TGF-β-responsiveness to cell-cycle arrest.

contrast, TGF-β increased the fraction of Ost-expressing NIH 3T3 cells in the G_1 phase of the cell cycle 2-fold, indicating a G_1 arrest. The retroviral infection of GFP cDNA showed >95% infection efficiency in the NMuMG and NIH 3T3 cells (data not shown; ref. 15).

To explore the possibility of RhoA involvement in signaling in TGF-β-mediated growth inhibition, RhoA signaling was specifically inhibited by the stable introduction of RBD (22, 27) in NMuMG cells. This inhibition resulted in abrogation of TGF-β-mediated growth inhibition (Fig. 2*A*), suggesting a potential role for RhoA activation in TGF-β-mediated cell-cycle arrest.

P160^{ROCK} Contributes to TGF-β-Mediated Growth Inhibition. In agreement with reports by others (28, 29), little effect on cell growth was obtained by the treatment of epithelial cell lines, MK, MDCK, and NMuMG cells with a p160^{ROCK}-specific inhibitor, Y27632 (Fig. 2*B*). The treatment with increasing concentrations of Y27632 in the presence of 1 ng/ml TGF-β showed antagonism of growth inhibition.

To address whether Y27632-associated abrogation of TGF-β-mediated inhibition of DNA synthesis in NMuMG cells correlated with effects on cell proliferation, sequential cell-counting experiments were performed over a period of 3 days. Although TGF-β treatment resulted in reduced cell numbers, the combination treatment of Y27632 and TGF-β displayed similar rates of proliferation as those of untreated cells (Fig. 2*C*).

To specifically identify p160^{ROCK} as a mediator of TGF-β-associated G_1 arrest, p160^{ROCK} siRNA [with no crossreactivity to p150^{ROCK} (ROCK2), data not shown] was transfected into NMuMG cells. Forty-eight hours after transfection of the 21-bp double-stranded RNA oligonucleotide, the cells were treated with or without 5 ng/ml TGF-β and subsequently harvested for Western blotting or propidium iodide staining. The siRNA sequence targeted was consistently able to knock down p160^{ROCK} expression by at least 80% over an irrelevant siRNA (Fig. 2*D*). Fluorescence-activated cell sorter analysis of p160^{ROCK} knock-down cells indicated little effect on the cell cycle compared with control nontransfected cells; however, there was diminished TGF-β-induced G_1 arrest (Fig. 2*D*). In addition, expression of a dominant-negative p160^{ROCK} construct (30) in NMuMG cells resulted in reduced TGF-β-mediated growth arrest, as determined by thymidine incorporation assay (data not shown). Together these results suggest a role for p160^{ROCK} activation in TGF-β-mediated growth arrest.

TGF-β-Mediated Phosphorylation of Cdc25A Involves p160^{ROCK} Activity. Next we examined the role of p160^{ROCK} signaling on TGF-β-mediated regulation of cell-cycle regulatory proteins involved in the G_1/S transition. Rapidly growing NMuMG cells treated with 5 ng/ml TGF-β, either in the presence or absence of 5 μM Y27632, were analyzed for phosphorylation changes in Rb and Rb-related p130 by Western blotting. In the absence of TGF-β, as expected, Rb and p130 were in their hyperphosphorylated state (Fig. 3*A*). TGF-β treatment for 24 h resulted in the appearance of predominantly hypophosphorylated Rb and p130, whereas the cotreatment with Y27632 limited this process. The

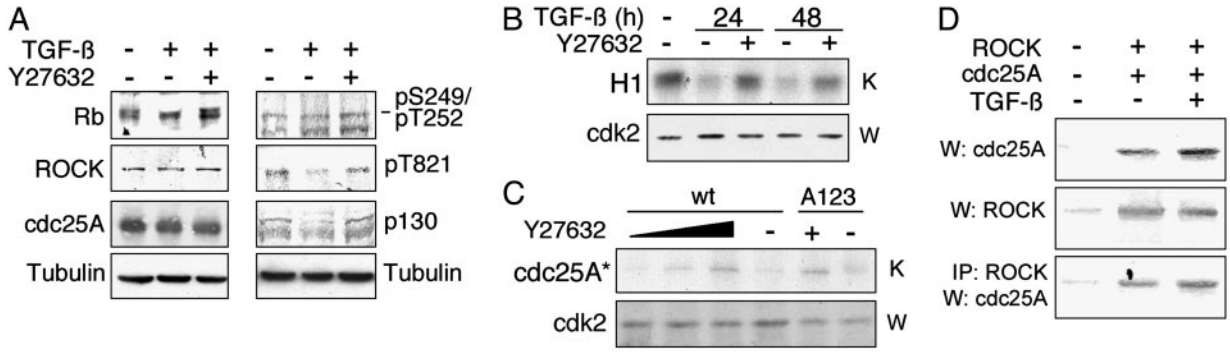

Fig. 3. TGF-β-mediated Rb hypophosphorylation involves p160^{ROCK} activity. (*A*) NMuMG cells treated with TGF-β (5 ng/ml) for 0 or 24 h in the presence or absence of Y23637 (5 μM) were harvested and analyzed by Western blotting. (*B*) *In vitro* kinase activity (K) of immunoprecipitated Cdk2 was determined by examining ^{32}P-labeling of histone H1 substrate. (*C*) Cdk2 kinase activity was examined in the presence of 5 μM, 2.5 μM, and 1 μM Y23637 in the presence of 5 ng/ml TGF-β by using Cdc25A as substrate. Western blots (W) for Cdk2 also were performed by using the same cell lysate as a control. wt, wild type.(*D*) Myc-tagged 160^{ROCK} and GST-tagged Cdc25A were overexpressed in HEK293 cells and subsequently treated with TGF-β (5 ng/ml) for 3 h. Cell lysates were Western blotted for p160^{ROCK} and Cdc25A, and anti-Myc-tag immunoprecipitants were Western blotted for Cdc25A.

use of phospho-specific Rb antibodies for Ser-249/Thr-252 (associated with Cdk4 kinase activity) and phospho-Thr-821 (associated with Cdk2 kinase activity) helped identify the pathway in which p160^{ROCK} may be involved. Because the treatment with Y27632 prevented TGF-β-associated hypophosphorylation of Rb phosphorylation on Thr-821, it suggested that TGF-β regulation of Cdk2 activity is affected by p160^{ROCK} inhibition. TGF-β or Y27632 had no effect on the cdk4-associated phosphorylation of Rb in NMuMG cells. The treatments for 24 h (Fig. 3A) and 48 h (not shown) had little effect on the expression levels of p160^{ROCK} or Cdc25A. Cdk2 kinase activity was measured directly by *in vitro* kinase assays (Fig. 3B). TGF-β-inhibition of Cdk2 kinase activity on histone H1 substrate at 24 and 48 h of treatment was antagonized by Y27632. The coincubation of TGF-β with increasing concentrations of Y27632 exhibited a concomitant elevation in Cdk2 kinase activity on its physiological substrate, Cdc25A. Similar Cdk2 kinase activity was observed when a Ser-123/Ala site-directed mutant of Cdc25A was used as a substrate (Fig. 3*C*). (Ser-123/Cdc25A is a reported residue of phosphorylation by Chk2 (25). Because Cdc25A dephosphorylation of phospho-Tyr-15/Thr-14 residues on Cdk2 (31) is a mechanism for Cdk2 inactivation and the data suggest a role for p160^{ROCK} in TGF-β-mediated Cdk2 regulation, physical interaction between Cdc25A and p160^{ROCK} were tested. Coimmunoprecipitation of endogenous Cdc25A and p160^{ROCK} suggested protein–protein interaction (Fig. 3*D*). Together, a potential role for p160^{ROCK} and Cdc25A in TGF-β-mediated Cdk2 inhibition and Rb phosphorylation is presented.

To test whether TGF-β can regulate Cdc25A activity in a p160^{ROCK}-dependent manner, TGF-β effects on the phosphorylation of Cdc25A were determined by using both *in vitro* and *in vivo* kinase assays. Although we were able to coprecipitate Cdc25A and p160^{ROCK}, the specificity of the interaction was not known. Thus, the regulation of Cdc25A phosphorylation was determined by immunoprecipitating p160^{ROCK} from NMuMG and incubating with either wild-type Cdc25A or a Ser-123/Ala mutant. Three hours of TGF-β treatment resulted in increased p160^{ROCK} kinase activity on wild-type Cdc25A, with diminishing activity by 12 h (Fig. 4*A*). In comparison, there was a reduced phosphorylation of S123A-Cdc25A by p160^{ROCK}.

To determine the kinetics of p160^{ROCK} phosphorylation of Cdc25A, *in vivo* kinase assays were performed by metabolic labeling of NMuMG and NIH 3T3 cells with [^{32}P]orthophosphate followed by immunoprecipitation of Cdc25A. This procedure showed low basal level of Cdc25A phosphorylation in rapidly growing NMuMG cells in the absence of exogenous TGF-β (Fig. 4*B*, lane 1). A successive increase in Cdc25A phosphorylation was detected in cells 30 min, 1 h, and 3 h after TGF-β addition (Fig. 4*B*, lanes 2–4). The concomitant addition of Y23637 inhibited TGF-β-stimulated Cdc25A phosphorylation (Fig. 4*B*, lanes 5 and 6).

Further evidence for a role of RhoA signaling in Cdc25A phosphorylation was determined by expressing Ost in NIH 3T3 cells and examining TGF-β stimulation of Cdc25A phosphorylation *in vivo*. [^{32}P]orthophosphate-labeled NIH 3T3 cells immunoprecipitated for Cdc25A, as predicted, exhibited little TGF-β associated phosphorylation. Ost expression in NIH 3T3 cells alone resulted in elevated Cdc25A phosphorylation, and treatment with TGF-β gave a further increased signal. However, when NIH 3T3 cells stably expressing Ost were treated with Y27632, TGF-β-associated Cdc25A phosphorylation was diminished (Fig. 4*C*). Together, these results indicate that TGF-β stimulates Cdc25A phosphorylation *in vivo* through a RhoA- and p160^{ROCK}-dependent manner.

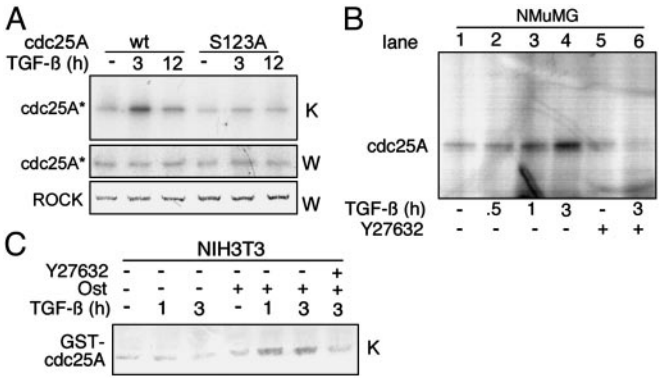

Fig. 4. p160^{ROCK} phosphorylates Cdc25A. (*A*) *In vitro* kinase activity (K) of immunoprecipitated p160^{ROCK} from TGF-β treated NMuMG cells with either wild-type Cdc25A or an S123A mutant of Cdc25A as substrate. Western blot for Cdc25A (W) also was done from the same cell lysate as a control. Cdc25A* indicates immunodetection of both S123A and wild-type (wt) isoforms. (*B*) *In vivo* kinase activity of NMuMG cells treated with TGF-β and/or Y23637 was metabolically labeled with [^{32}P]orthophosphate, and Cdc25A was immunoprecipitated from equal amounts of extracts. (*C*) *In vivo* kinase activity in NIH 3T3 cells expressing GST-tagged wild-type Cdc25A in addition to Ost or control cDNA was determined. After [^{32}P]orthophosphate labeling and TGF-β and/or Y23637 treatment, GST-Cdc25A was pulled down with glutathione beads and separated by electrophoresis. The result is representative of at least two independent experiments.

TGF-β Inhibits Cdc25A Phosphatase Activity. To better understand the mechanism of Cdc25A phosphorylation, TGF-β-mediated $p160^{ROCK}$ subcellular localization was determined by immunofluorescence and cell fractionation experiments. Immunolocalization of endogenous $p160^{ROCK}$ was primarily cytoplasmic in untreated proliferating NMuMG cells. TGF-β treatment for 12 h resulted in a distribution of nuclear and cytoplasmic localization of $p160^{ROCK}$ (Fig. 5*A*). However, dominant-negative $p160^{ROCK}$ [myc-tagged KDIA-$p160^{ROCK}$ (30), mutation resulting in no RhoA-associated activity] was localized primarily in the cytoplasm, both before and after TGF-β treatment. Subcellular fractionation of TGF-β-treated NMuMG cells resulted in elevated Smad2 in the nuclear fraction by 1 h persisting through the 12-h time course (Fig. 5*B*). Similar cellular distribution of $p160^{ROCK}$ was observed with nuclear localization apparent through the 1- to 12-h course of TGF-β treatment. Cdc25A was only detected in the nuclear fraction. The cell fractions also were examined for PCNA and RhoA expression as a control for nuclear and cytoplasmic fractionations, respectively. There was no detectable cytoplasmic expression of PCNA, and little or no RhoA was detected in the nuclear fraction.

The functional impact of TGF-β-mediated Cdc25A phosphorylation on its enzymatic activity was determined by *in vitro* phosphatase assays. NMuMG cells were harvested after treatment with TGF-β (5 ng/ml) through a 6-h time course in the presence and absence of Y23637 (5 μM). We found that the incubation of the γ-^{32}P-phosphorylated-histone H1 substrate with immunoprecipitated Cdc25A from rapidly growing cells showed efficient phosphatase activity (Fig. 5*C*). In contrast, Cdc25A from TGF-β-treated cells had diminished phosphatase activity as early as 30 min, observed by diminished dephosphorylation of the γ-^{32}P-phosphorylated-histone H1 substrate. There was progressively greater phosphatase inhibition at 6 h of TGF-β treatment. Y23637 antagonized the TGF-β inhibition of Cdc25A dephosphorylation of histone H1. Cdc25A was incubated with His-tagged Cdk2 purified from HEK293 cells to determine the consequence of Cdc25A phosphatase activity (Fig. 5*D*). Western blotting to phospho-Tyr-15/Cdk2 suggested that the knockdown of $p160^{ROCK}$ by siRNA restored Cdc25A phosphatase activity otherwise repressed by TGF-β. After immuoprecipitation for endogenous Cdc25A, we blotted for $p160^{ROCK}$ association. $P160^{ROCK}$ was specifically coprecipitated with Cdc25A in conditions where phosphatase activity was inhibited. Together these results describe a mechanism by which TGF-β may rapidly inhibit Cdc25A activity, independent of Cdc25A down-regulation, to mediate cell-cycle arrest.

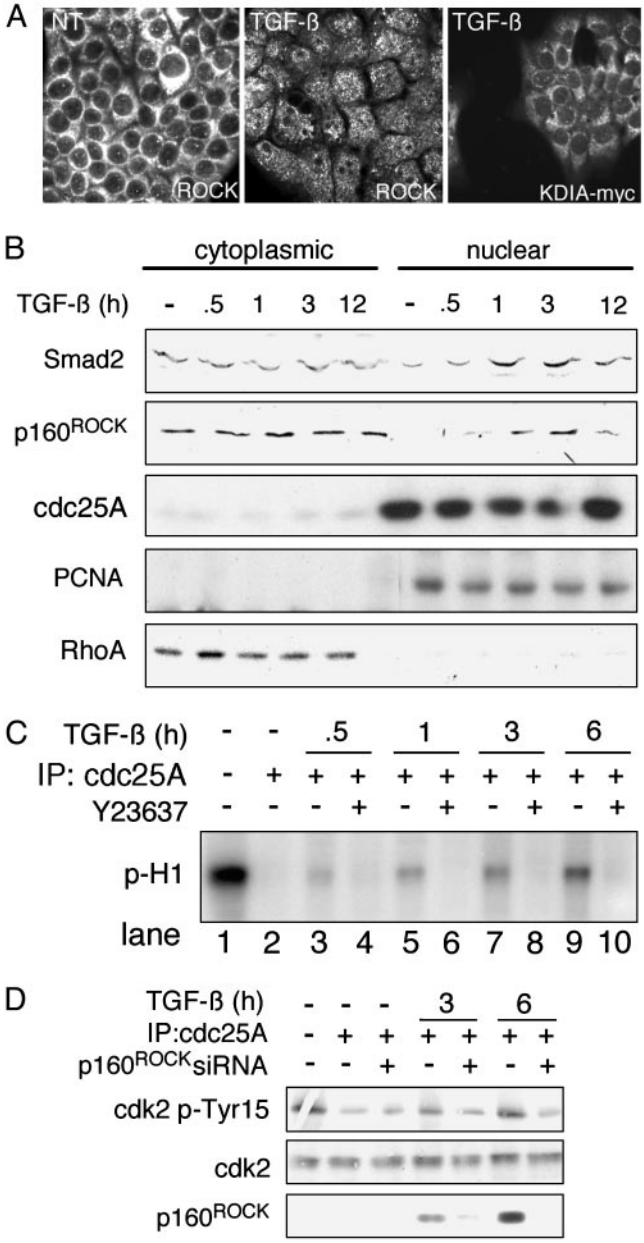

Fig. 5. $P160^{ROCK}$ mediates Cdc25A inhibition. (*A*) NMuMG cells grown on coverslips were incubated with or without TGF-β, fixed, and immunostained. Endogenous $p160^{ROCK}$ and overexpressed myc-tagged KDIA-ROCK (30) were localized by appropriate antibodies. (*B*) Nuclear and cytoplasmic fractions were isolated from TGF-β treated NMuMG cells. Equivalent amounts of protein were Western blotted for Smad2, $p160^{ROCK}$, Cdc25A, PCNA, and RhoA. (*C*) Histone H1 phosphorylated by Cdk2 with [γ-^{32}P]ATP was incubated with immunoprecipitated Cdc25A from NMuMG cells. The subsequent phosphorylation state of histone H1 was analyzed by electrophoresis and autoradiography. Note that addition of Cdc25A causes almost complete dephosphorylation of histone H1 (lanes 1 and 2), whereas treatment with TGF-β for 30 min to 6 h causes increasing inhibition of phosphatase activity (lanes 3, 5, 7, and 9). Treatment with Y23637 blocks inhibition of phosphatase activity (lanes 4, 6, 8, and 10). (*D*) Cdk2 was incubated with Cdc25A from cells either transfected with or with out $p160^{ROCK}$-siRNA or treated with or without TGF-β. Subsequently the reaction was Western blotted by using phospho-Tyr-15/Cdk2, Cdk2, and Cdc25A antibodies.

Discussion

G_1 cell-cycle progression requires the activation of the cyclin E:Cdk2 complex involving the phosphorylation of Thr-160 and dephosphorylation of phospho-Tyr-15/Thr-14 residues on Cdk2 (31). As previously reported, TGF-β signaling can inhibit Cdk2 activity both by blocking CDK-activating kinase-mediated Thr-160 phosphorylation (13) as well as down-regulating Cdc25A, involved in the dephosphorylation of Tyr-15/Thr-14 (14). However, we find that even when TGF-β does not induce Cdc25A down-regulation, Cdc25A phosphatase activity can be blocked. It was further demonstrated that inhibition of $p160^{ROCK}$ or knock down of $p160^{ROCK}$ expression in NMuMG cells attenuates the G_1 arrest induced by TGF-β. Investigations of the mechanism of RhoA/$p160^{ROCK}$-mediated growth arrest demonstrated that TGF-β rapidly stimulated $p160^{ROCK}$ translocation to the nucleus and phosphorylation of Cdc25A at a site inhibitory to phosphatase activity (25, 32, 33). This finding was associated with inhibition of Cdk2 kinase activity (23, 34). These results provide evidence that signaling through RhoA and $p160^{ROCK}$ is important in TGF-β inhibition of cell proliferation.

The TGF-βs inhibit proliferation of a variety of normal cell types, including most epithelial cells and hematopoietic cells (1). However, nontransformed dermal fibroblasts and fibroblastic

NIH 3T3 cells are growth-stimulated by TGF-β (12, 35). In this article, we report that restoration of RhoA signaling through p160^{ROCK} in NIH 3T3 cells converts the growth response to TGF-β treatment from growth stimulation to growth inhibition. The NIH 3T3 cells make an effective model system to examine TGF-β signaling because they are responsive to SMAD signaling yet refractory to TGF-β-mediated growth inhibition and RhoA activation (Fig. 1). Because we wanted to avoid epithelial differentiation of NIH 3T3 cells, as has been reported to occur under V14RhoA expression (36), a constitutively active RhoA (V14RhoA) was purposely not used. The introduction of wild-type RhoA attenuated TGF-β-mediated growth stimulation. The expression of the Rho guanine exchange factor Ost in NIH 3T3 cells resulted in TGF-β-stimulated transient increase in RhoA activity and the rescue of growth-inhibitory properties of TGF-β (Fig. 1). We also found the coincident inhibition of TGF-β-stimulated RhoA activity and growth inhibition achieved by the expression of RBD (22, 27) in NMuMG cells (Fig. 2*A*). These findings complement studies showing that blocking RhoA and p160^{ROCK} activity resulted in early cyclin D1 expression and accelerated G_1/S progression (27) and further support our hypothesis for the requirement of RhoA activity in TGF-β-mediated growth arrest. In addition, we provide evidence for RhoA signaling as an immediate mechanism of Cdc25A enzymatic inhibition through posttranslational modification that precedes the previously described TGF-β-mediated transcriptional down-regulation of Cdc25A (14).

The data suggest a two-step model for TGF-β inhibition of G_1/S progression where there is an initial inhibition of Cdc25A enzymatic activity followed by a secondary response involving SMAD-mediated transcriptional up-regulation of Cdk-inhibitory proteins (4–6) and down-regulation of c-myc (37) and Cdc25A (14). TGF-β-mediated RhoA signaling stimulates p160^{ROCK} nuclear translocation presumably through its activation of a bipartite nuclear localization signal sequence at position 1020–1037 aa. We show evidence for TGF-β-mediated *in vivo* and *in vitro* phosphorylation of Cdc25A (Fig. 4) and inhibition of Cdc25A phosphatase activity (Fig. 5 *C* and *D*). P160^{ROCK} phosphorylation of Cdc25A was blocked by the Ser-123/Ala mutation as reported for a Chk2 site of phosphorylation (25). However, detailed peptide analysis of Cdc25A needs to be performed to conclusively determine the site(s) of ROCK phosphorylation. In the event of DNA damage, the combination of Chk1/Chk2 serine phosphorylation of Cdc25A and a Chk1/Chk2-independent Cdc25A/Ser-73 phosphorylation is required to target Cdc25A for proteosome-mediated degradation (32, 38). This requirement may suggest that the preservation of genomic integrity by growth arrest is a fundamental response to both TGF-β-mediated transdifferentiation and DNA damage, analogous to a "two-wave" concept of inhibition of G_1/S progression proposed to occur during genotoxic stress, where the first wave involves inhibition of Cdc25A (39).

The context dependence of RhoA-p160^{ROCK} activation in TGF-β-mediated epithelial to mesenchymal transdifferentiation and growth arrest is particularly intriguing. RhoA and p160^{ROCK} are essential in TGF-β-mediated epithelial to mesenchymal transdifferentiation of NMuMG cells (15) in agreement with its well established role in actin cytoskeletal reorganization (19). In this article, we provide evidence for RhoA and p160^{ROCK} involvement in TGF-β-induced growth inhibition. Rho family GTPases have previously been implicated in the positive regulation of cell-cycle progression through the G_1 phase in the context of Ras-mediated transformation as well as lysophosphatidic acid stimulation of RhoA (40). This apparent discrepancy illustrates the complexity of the biological effects of RhoA and p160^{ROCK} activation.

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