Role of Rho/ROCK and p38 MAP Kinase Pathways in Transforming Growth Factor-β-mediated Smad-dependent Growth Inhibition of Human Breast Carcinoma Cells in Vivo*

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TGF-β is a multifunctional cytokine known to exert its biological effects through a variety of signaling pathways of which Smad signaling is considered to be the main mediator. At present, the Smad-independent pathways, their interactions with each other, and their roles in TGF-β-mediated growth inhibitory effects are not well understood. To address these questions, we have utilized a human breast cancer cell line MCF10CA1h and demonstrate that p38 MAP kinase and Rho/ROCK pathways together with Smad2 and Smad3 are necessary for TGF-β-mediated growth inhibition of this cell line. We show that Smad2/3 are indispensable for TGF-β-mediated growth inhibition, and that both p38 and Rho/ROCK pathways affect the linker region phosphorylation of Smad2/3. Further, by using Smad3 mutant at the putative phosphorylation sites in the linker region, we demonstrate that phosphorylation at Ser207 and Ser205 residues is required for the full transcriptional potential of Smad3, and that these residues are targets of the p38 and Rho/ROCK pathways. We demonstrate that activation of the p38 MAP kinase pathway is necessary for the full transcriptional activation potential of Smad2/Smad3 by TGF-β, whereas activity of Rho/ROCK is necessary for both down-regulation of c-Myc protein and up-regulation of p21<sup>waf1</sup> protein, directly interfering with p21<sup>waf1</sup> transcription. Our results not only implicate Rho/ROCK and p38 MAPK pathways as necessary for TGF-β-mediated growth inhibition, but also demonstrate their individual contributions and the basis for their cooperation with each other.

Transforming growth factor-β (TGF-β)<sup>1</sup> is a pleiotropic cytokine, which modulates cell proliferation, differentiation, apoptosis, adhesion, and migration of various cell types and favors the formation of extracellular matrix (1–7). It also regulates the function and maturation of immature hematopoietic cells, activated B and T cells, macrophages, neutrophils, and dendritic cells. Since TGF-β is pleiotropic in nature, its biological activity is tightly regulated. It belongs to a family consisting of structurally related polypeptides, including TGF-βs, activins, and bone morphogenetic proteins (BMPs), all of which regulate pivotal biological functions (8, 9). The most well studied TGF-β response in normal epithelial cells is growth inhibition. TGF-β causes G<sub>1</sub> cell-cycle arrest by inhibiting cyclin-dependent kinase (CDK) activity via induction of p15<sup>INK4a</sup> and p21<sup>waf1</sup> (2, 10). Another key event in the TGF-β program of growth arrest is repression of c-Myc expression, and this response has been shown to be selectively lost in certain tumor cell lines, which have escaped from inhibition of growth by TGF-β (11, 12). TGF-β has a biphasic role in tumorigenesis (13). In early phases, when cells are still sensitive to the growth inhibitory effects of TGF-β, it typically acts as a tumor suppressor. However, in late stages where it acts as a pro-metastatic agent, cells typically have escaped selectively from the growth inhibitory effects of TGF-β (14), even though certain pathways of TGF-β signaling remain functional in these cells (13, 15, 16).

TGF-β exerts its biological effects through specific intracellular effector molecules called Smads (17–20). Binding of ligand to its receptor serine/threonine kinase leads to the formation of a heteromeric transmembrane receptor complex, which then phosphorylates Smad protein substrates. Upon activation, Smads hetero-oligomerize with Smad4, translocate to the nucleus, and either activate or repress their target genes. The genes encoding the TGF-β receptors (TβRs) and Smads have been found to be genetically altered in a small percentage of human cancers. In particular, TβR-II is frequently mutated in colon and gastric cancers with a microsatellite instability phenotype (21). Mutations and/or functional loss of Smad4, Smad2, and Smad3 all have been documented (22–26). Whether these changes lead directly to the loss of sensitivity to inhibition of growth by TGF-β is not known, although it has been shown that in pancreatic carcinoma cell lines resistant to inhibition of growth by TGF-β, Smad complexes have a shorter nuclear residence time, which results in an inability to maintain expression of the CDK inhibitor, p21<sup>waf1</sup> (14).

Although the Smad pathway is the main mediator of TGF-β signaling, recent studies have implicated other pathways such as extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein (MAP) kinases, phosphatidylinositol 3-kinase (PI 3-kinase), and p70S6 kinase either as mediators or modulators of TGF-β-dependent biological effects, the molecular details of which are still being studied (16). TGF-β can both activate MAP kinase pathways directly, and TGF-β-dependent Smad signaling can cooperate with these pathways when they are activated by other means leading to a pro-oncogenic response (27). Recent data suggest that aberrant activation of MAP kinase pathways may play an important role in diverting
the TGF-β response toward a pro-oncogenic outcome. Thus, for example, TGF-β may cooperate with activated Ras to promote invasive, metastatic disease (28, 29). In keratinocytes, stimulation of invasion by TGF-β is dependent on Ras/MAPK/MEK signaling, and secretion of several metalloproteinases is dependent on p38 MAP kinase (30). Moreover, induction of epithelial-mesenchymal transition (EMT), implicated in acquisition of an aggressive phenotype in certain cancers, can require cooperation between MAP kinase and Smad pathways (31–33). In other cases, EMT has been shown to be Smad-independent utilizing the RhoA and PI 3-kinase pathways (34, 35).

Among the other non-Smad pathways that might be implicated in a role in growth control by TGF-β, p38 MAP kinase and PI 3-kinase signaling have been shown to be involved in the pro-survival activity in mesenchymal cells/fibroblasts (36). p38 MAP kinase pathway is required for the sumoylation, and thereby transcriptional activity of Smad4 by protein inhibitors of activated Stat (PIAS) proteins (37). At the level of cell cycle progression, it was shown that p38 MAP kinase and SAP/JNK activation by TGF-β leads to the stabilization of p21\textsuperscript{waf1} protein in a Smad-independent manner and leads to growth arrest in a human colon carcinoma cell line HD3 (38). p38 MAPK is also necessary for TGF-β-mediated cell adhesion (39), collagenase-3 expression (40), thrombospondin-1 expression, and apoptosis (41, 42).

To identify the role of Smad-independent pathways that are activated by TGF-β and to study their role in Smad-dependent biological effects on growth, we have utilized the MCF10CA1h cell line, which is derived from the MCF10At1k Ras-transformed human breast cancer cell line (43–45). Our results show that in addition to Smad signaling, TGF-β activates Rho and p38 pathways and that these pathways contribute in both TGF-β-dependent and TGF-β-independent modes to inhibition of growth by TGF-β. Further investigation revealed that these
pathways modulate Smad function in a positive manner by affecting their linker region phosphorylation, as well as their transcriptional activation potential. Contrary to earlier reports, p21<sup>WAF1/CIP1</sup> was found not to be a direct target for TGF-β-mediated activation of the p38 MAP kinase pathway (38). Instead, in MCF10CA1h cells, we found the Rho/ROCK pathway to be necessary for the up-regulation of p21<sup>WAF1/CIP1</sup> protein. Interestingly, our results also showed that activation of Rho/ROCK is necessary for the down-regulation of c-Myc protein. Overall, our results demonstrate a complex interplay between Smad, p38 MAP kinase, and Rho pathways in TGF-β-mediated growth inhibition.

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**MCF10CA1h cells were obtained from Dr. Fred Miller (Barbara Ann Kermanos Cancer Institute, Detroit, MI). Cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA), 5% horse serum (Invitrogen) at 37 °C, 5% CO2. The amphotropic retroviral packaging cell line Phoenix-A was obtained from Dr. Rick Derynck. LPCX vector has puromycin resistance marker for selecting cells, which have stable retroviral integration. From Dr. Fred Miller (Barbara Ann Kermanos Cancer Institute, Detroit, MI). LPCX vector contains the SSVS-motif of Smad3) Smad3 sequence (Smad3 coding for either the full-length Smad3 or a C-terminally truncated (lack-...-mediated activation of the p38 MAP kinase pathway (38). Instead, in MCF10CA1h cells, we found the Rho/ROCK pathway to be necessary for the up-regulation of p21<sup>WAF1/CIP1</sup> protein. Interestingly, our results also showed that activation of Rho/ROCK is necessary for the down-regulation of c-Myc protein. Overall, our results demonstrate a complex interplay between Smad, p38 MAP kinase, and Rho pathways in TGF-β-mediated growth inhibition.

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**Stable Expression of Wild-type or Dominant-negative Smad3—**For retroviral packaging, Phoenix-A cells were plated 3 × 10<sup>5</sup> cells/9-cm tissue culture dish 24 h before transfection. Transfection was done by calcium phosphate method, using 10 µg of DNA per plate. Cell culture medium containing the recombinant viruses was collected 36 h after transfection and filtered through 0.45-µm filters to remove cell debris. For retroviral infection, MCF10CA1h cells were seeded at a density of 5 × 10<sup>3</sup> cells in 9-cm plates and 4 ml/plate of retroviral supernatants together with 4 µg/ml (final) of polybrene (Sigma) was added. Cells were placed in fresh growth medium after 5 h of infection and allowed to grow. Selection with 2 µg/ml of puromycin was started 48 h of infection for 5 days. Thereafter, cells were maintained in 200 µg/ml of puromycin.

**Thymidine Incorporation and Cell Growth Assays—**Cell survival was estimated by [3H]thymidine ([1 mCi/ml stock; PerkinElmer Life Sciences] incorporation for 2 h as previously described (46). In brief, MCF10 CA1h were seeded 3000 cells/well in a 96-well plate in complete growth medium and allowed to adhere properly for 5 h. Then they were shifted to low serum medium containing 0.5% horse serum and incubated overnight at 37 °C, 5% CO2. Synthetic inhibitors were added to the cells at various concentrations 1 h prior to the addition of TGF-β.

**Preparation of Nuclear Extracts—**Cells growing under subconfluence conditions in complete growth medium were starved (0.5% horse serum) for 12 h and treated with 2 ng/ml of TGF-β alone or together with inhibitors as desired. Nuclear fractions were prepared by extracting the cells (after extracting cytoplasmic fractions) with 2.5 volumes per volume pellet of hypertonic nuclear buffer (10mM Hepes, pH 7.9, 400 mM NaCl, 5% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, and was completed with 1 mM dithiothreitol, 10 mM sodium molybdate, 0.1 mM sodium orthovanadate, and a mix of protease inhibitors from Roche Applied Science). Protein quantification was done with the BCA protein assay kit (Pierce).

**Protein Extraction and Western Blotting—**For preparing total cell extracts, cells were seeded at subconfluence in complete growth medium and allowed to adhere properly. Then they were shifted to starvation medium with 0.5% horse serum for 12–18 h and then treated with 2 ng/ml of TGF-β (R&D Systems, Inc., Annapolis, MD) as desired. Total cell lysates were prepared in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, and a protease inhibitor mixture). Proteins extracts were quantified by the BCA protein assay kit separated on SDS-PAGE gels under reducing conditions and were transferred onto Immobilon<sup>™</sup> polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Blots were hybridized with specific primary antibodies, and antigen-specific signal was detected using horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Pierce). Anti-Smad2 and -Smad3 antibodies were from BD Transduction Laboratories Inc; anti-phospho-Smad2 and phospho-Smad3 antibodies were generous gifts from Dr. Michael Reiss, Cancer Institute of New Jersey; anti-total and phospho-p38, ERK, ATK-2, antibodies, and anti-total p21<sup>human</sup> antibodies are from BD Transduction Laboratories,
Antibodies against linker region phosphorylated Smad2/3 (pT178, pT8, pS212, pS207, and pS203) were generously provided by Dr. Fang Liu (Rutgers University).

In Vitro Kinase Assays—In vitro kinase assays for p38 and Rho were performed according to the manufacturer's instructions. The p38 MAP kinase assay kit is from Cell Signaling Technology, Beverly, MA; the Rho activation assay kit is from Upstate Cell Signaling Solutions, Lake Placid, NY.

Expression Vectors, Cell Transfection, and Reporter Assays—MCF10CA1h cells were seeded 2 × 10⁵ cells/well in 6-well plates 18 h prior to transfection in complete growth medium. Cells were transfected with a total of 2 μg/well of either CAGA12-Luc, full-length p21wafl promoter (kindly provided by Dr. Jane E. Trepel, National Institutes of Health), TIE-Luc (kindly provided by Dr. Xiao-Fan Wang, Duke University), or ARE-Luc and FAST-1 together with pRL-TK (Renilla, for normalization) plasmid DNA using Nucleofector™ Kit V (AMAXA Biosystems) according to the manufacturer's instructions. Linker-mutated Smad2 and Smad3 (EPSM), (47) were from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center) and linker-mutated Smad3 (C2S3), (48) was kindly provided by Dr. Fang Liu (Rutgers University). Dominant-negative p38 MAP kinase and dominant-negative RhoA constructs were kindly provided by Dr. Seong-Jin Kim, NCI, National Institutes of Health. pcDNA3 was used either as a control or as filler.

48 h after transfection, the medium was replaced with medium containing 0.5% horse serum (starvation), and cells were either treated with 2 ng/ml of TGF-β or left untreated for 16–18 h and then were lysed in passive lysis buffer (Promega). Luciferase and Renilla activities were determined by VICTOR² (PerkinElmer Life Sciences).

RESULTS

Effect of TGF-β on MCF10CA1h Cell Growth—Treatment of MCF10CA1h cells with TGF-β led to a profound inhibition of overall proliferation rate as detected by using a [³H]thymidine incorporation assay. Thymidine incorporation was suppressed in a dose-dependent manner and was reduced by about 80% in the presence of 10 ng/ml of TGF-β or left untreated for 16–18 h and then were lysed in passive lysis buffer (Promega). Luciferase and Renilla activities were determined by VICTOR² (PerkinElmer Life Sciences).
c-Myc oncprotein were down-regulated as early as 1 h after treatment with TGF-β (Fig. 1E). Another growth regulatory protein, Retinoblastoma (pRb), which is a downstream target for the p21(wt), mediates growth suppressive effects in its hypophosphorylated form. Hypophosphorylation of pRb was detected after 12 h of treatment with TGF-β in MCF10CA1h cells (Fig. 1D). These results indicate that the growth inhibition of these cells by TGF-β is accompanied by the up-regulation of p21(wt), hypophosphorylation of pRb and down-regulation of c-Myc protein, events typically associated with inhibition of cell growth by TGF-β (49).

Signaling Pathways Activated by TGF-β—In MCF10CA1h cells, both Smad2 and Smad3 were C-terminally phosphorylated after treatment with TGF-β for 5 min, and the phosphorylation persisted for about 3 h (Fig. 2A). TGF-β has also been shown to activate other pathways including the MAP kinase ERK1/2, JNK, p98, and Rho. These pathways often cooperate with Smad signaling to control gene expression and cell phenotype. Western analysis of total cell extracts from MCF10CA1h cells treated with TGF-β showed an activation of both ERK and p38 MAP kinase pathways 5 min after treatment (Fig. 2, B and C).

Involvement of Smad Signaling in TGF-β-mediated Growth Inhibition of MCF10CA1h Cells—To investigate the role of Smad proteins in TGF-β-mediated growth inhibition, we have utilized two retrovirus infected pools of MCF10CA1h cells, one overexpressing a full-length Smad3 protein and the other expressing a C-terminally truncated version of Smad3 (Smad3C), which was shown earlier to inhibit the activation (phosphorylation) of both endogenous Smad2 and Smad3 proteins (46). This was achieved by infecting MCF10CA1h cells with the retroviral vector LPCX, coding for the respective protein. Expression of the truncated version of Smad3 inhibited TGF-β-mediated phosphorylation of both Smad2 and Smad3, in agreement with our previous results (46) (Fig. 3A). In cells defective in Smad activation, both the down-regulation of c-Myc as well as the hypophosphorylation of pRb were not seen with TGF-β treatment (Fig. 3, B and C), consistent with the demonstration that these cells are refractory to the growth inhibitory effect of TGF-β (Fig. 5D) (46). In cells overexpressing Smad3, phosphorylation of Smad2 was comparable to that of control cells after TGF-β treatment while Smad3 phosphorylation was elevated strongly (Fig. 4A). But in cells overexpressing Smad3, c-Myc was rapidly down-regulated as early as 15 min after addition of TGF-β. The level of hypophosphorylated pRb was comparable to that of the control (Fig. 4, B and C). These results suggest that signaling through Smad2 and Smad3 is necessary for TGF-β-mediated growth inhibition in MCF10CA1h cells.

Smad-independent Signaling Pathways and Their Role in TGF-β-mediated Growth Inhibition of MCF10CA1h Cells—Since activation of the Smad signaling pathway might in turn activate MAP kinase pathways, we checked this possibility in MCF10CA1h cells by using cells stably expressing Smad3 ΔC′, in which activation of Smad2 and Smad3 by TGF-β treatment is suppressed (Fig. 3A). Both p38 and ERK MAP kinase pathways are activated in this cell line (Fig. 3, D and E) with similar kinetics to that seen in the parental cells (Fig. 2, B and C), suggesting that the activation of these MAP kinase pathways are independent of Smad activation. In order to investigate the role of Smad-independent pathways in the growth inhibition of MCF10CA1h cells, we employed specific inhibitors for each of these pathways together with TGF-β. Results with [3H]thymidine incorporation showed striking interference with the TGF-β growth inhibitory effect with either 5 μM p38 inhibitor SB203580 or 10 μM ROCK inhibitor Y27632. While cells treated with 10 ng/ml TGF-β showed about 80% growth suppression, treatment with either of these two inhibitors together with TGF-β reduced the extent of inhibition to about 20% (Fig. 5A). When the number of live cells was counted, an ~2-fold increase was observed when cells were treated for 48 h with TGF-β together with either SB203580 or Y27632 compared with cells treated with TGF-β alone after 48 h (data not shown). In contrast, treatment with the MEK inhibitor PD98059 had no effect on growth inhibition by TGF-β, suggesting that ERK MAP kinase may not play a role in this pathway.
Treatment of cells with 5\(^{-5}\)M Alk5 inhibitor SB431542 (22) (Fig. 5A) completely blocked the effects of TGF-\(\beta\) on growth, suggesting that activation of T\(\beta\)RI kinase is required for all these effects.

Activation of p38 MAP Kinase and Rho Pathways in MCF10CA1h Cells by TGF-\(\beta\) Treatment—Because the experiments with the inhibitors suggested a role for Rho/ROCK and p38 pathways in growth suppression, in vitro kinase assays were performed. Using GST-ATF-2 fusion protein as a substrate, we found that the kinase activity of p38 is induced by TGF-\(\beta\) treatment and reaches its maximal level after 30 min of treatment (Fig. 5B). In contrast, MCF10CA1h cells showed a slight basal level of Rho kinase activity, which was elevated strongly only late in treatment (Fig. 5C). These results suggest that the kinase activity of both Rho and p38 MAP is induced by TGF-\(\beta\) treatment and that the kinetics of their induction are different from each other.

Hierarchy of Smad, p38 MAP Kinase, and Rho Pathways in Effects of TGF-\(\beta\) on Growth Inhibition—Because we demonstrated that both p38 MAPK and Rho/ROCK signaling together with Smad activation are necessary for optimal inhibition of growth of MCF10CA1h cells by TGF-\(\beta\), we decided to investigate the hierarchy of these pathways. Results show that in cells expressing the dominant-negative Smad (Smad3\(\Delta C\)), both p38

**Fig. 5.** A, synthetic inhibitors against p38 MAP kinase, Rho/ROCK, and T\(\beta\)RI (Alk5) antagonize the anti-proliferative effect of TGF-\(\beta\) on MCF10CA1h cells. MCF10CA1h cells treated either with TGF-\(\beta\) alone or together with synthetic inhibitors against p38 MAP kinase (SB203580, 5 \(\mu\)M), MEK (PD98059, 25 \(\mu\)M), Alk5 (SB431542, 5 \(\mu\)M), or ROCK (Y27632, 10 \(\mu\)M), and cell proliferation was assessed by using \(^{3}\text{H}\) [thymidine incorporation assay. Data are the average of triplicates and are expressed as percentage of growth (thymidine incorporation relative to control experiment). B and C, TGF-\(\beta\) treatment leads to p38 MAP kinase and Rho kinase activation in MCF10CA1h cells. Cell lysates from MCF10CA1h cells either non-treated or treated for various periods of time with TGF-\(\beta\) were prepared according to the manufacturer’s instructions. B, p38 MAP kinase was immunoprecipitated from total cell lysates using phospho-specific antibodies (Thr\(^{180}\)Tyr\(^{182}\)) to p38 MAP kinase. Kinase activity was determined by using ATF-2 fusion protein as substrate. C, from MCF10CA1h cell lysates, GTP-Rho was pulled down using GST-tagged mouse Rhotekin Rho binding domain. Anti-Rho antibodies were used to detect the Rho protein, which represents the GTP-bound kinase active Rho. D and E, activation of Smad2 and Smad3 is required for TGF-\(\beta\)-mediated MCF10CA1h cell growth inhibition. Retroviral-infected MCF10CA1h cell pools expressing either C-terminally truncated Smad3 (Smad3\(\Delta C\)) (C) or ectopically expressing full-length Smad3 protein (CAS3) (D) were treated with TGF-\(\beta\) alone or together with synthetic inhibitors against p38 MAP kinase (SB203580, 5 \(\mu\)M), or ROCK (Y27632, 10 \(\mu\)M). Cell growth was assessed by using \(^{3}\text{H}\) thymidine incorporation assay. Data are the average of triplicates and are expressed as percentage of growth (thymidine incorporation relative to control experiment).
and ROCK inhibitors failed to exert any significant effect on thymidine incorporation (Fig. 5D). But in cells overexpressing Smad3, a clear and marked decrease in TGF-β-mediated inhibition of thymidine uptake was observed with the p38 inhibitor, while the ROCK inhibitor was without effect, suggesting that the Rho/ROCK pathway might be more sensitive to the levels of Smad3 in these cells (Fig. 5E). These results, together with previous experiments, suggest that Smad, p38 MAPK, and Rho pathways are activated independently of each other. But, in the context of growth regulation, the data suggest that p38 MAPK and Rho pathways are upstream to Smads and that all these pathways are necessary for optimal inhibition of growth of MCF10CA1h cells by TGF-β.

**Effect of p38 MAPK and Rho Pathways on Phosphorylation and Transcriptional Activity of Smads**—Since results from our earlier experiments suggested that Smads are downstream to p38 MAPK and Rho pathways, we investigated whether inhibition of these pathways might affect the transcriptional activation potential of Smad2 and Smad3. We addressed this by using reporter constructs specific for either Smad2 (ARE-Luc) (50) or Smad3 (CAGA12-Luc) (51). We evaluated the reporter activity in MCF10CA1h cells after treatment with TGF-β alone or together with either the p38 inhibitor or ROCK inhibitor. Results showed a 50% reduction in both ARE and CAGA12 reporter activity with the p38 inhibitor, suggesting a partial loss of transcriptional activation potential of Smad2 and Smad3 (Fig. 6, A and B) when this pathway is inhibited. The ROCK inhibitor showed no effect on TGF-β-mediated reporter activity at 10 μM concentration. The inhibitors by themselves showed no effect on either ARE or CAGA12-Luc reporter activity (Fig. 6C). To confirm the effect of these pathway-specific inhibitors, the experiments were repeated in cells transfected with either a dominant-negative p38 or a dominant-negative RhoA expression construct. Comparable results were obtained to those obtained by using synthetic inhibitors (Fig. 1 and Supplementary Data).
To understand these effects more fully, we also investigated whether the phosphorylation status of Smad2 and Smad3 was modified by the p38 MAPK and Rho pathways. Phospho-specific Smad2 and Smad3 (Ser/Thr) antibodies showed that C-terminal phosphorylation of Smad2 and Smad3 was not altered by SB203580 (p38 inhibitor) and Y27632 (ROCK inhibitor) (Fig. 6, D and E), suggesting that inhibition of these pathways does not affect the upstream interaction between these Smads and the TGF-βRI kinase. But this does not exclude the possibility that these pathways might affect phosphorylation of these Smads on other sites such as their linker regions and MH1 domain. We addressed this question by using specific antibodies to assess whether phosphorylation of Smad2 and Smad3 at sites previously identified as being sites for phosphorylation by MAPK (47) or Cdk2/4 (48) might be affected by the p38 and ROCK pathways. Using inhibitors of these pathways, we showed that of all of these sites examined, including Thr8 and Thr178 and Ser203, Ser207, and Ser212, those most likely to be involved in modulating Smad function in the presence of TGF-β were Ser203 and Ser207 (Fig. 7, A and C). Smad3 was

![Synthetic inhibitors of p38 MAP kinase and ROCK alter the phosphorylation of linker region sites in Smad2/3. Total cell lysates from MCF10CA1h cells treated with TGF-β (2 ng/ml), SB203580 (5 µM), or Y27632 (10 µM) either alone or in the combinations indicated were subjected to Western analysis with phosphospecific Smad3 antibodies against either Ser203 and Ser207 (A) or against Thr8 and Thr178 and Ser212 (B). C, tabulation of the Western blot data in A and B, in terms of the basal state of phosphorylation of the designated residues in MCF10CA1h cells, the direction of change in phosphorylation at each site brought about by the designated treatments, and the dominant effect when TGF-β is added together with either the p38 or ROCK inhibitors.]

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phosphorylated at both Ser<sup>203</sup> and Ser<sup>207</sup> in untreated MCF10CA1h cells and the p38 and ROCK inhibitors each down-regulated phosphorylation at these sites, both in the presence and absence of TGF-β/H9252. While TGF-β/H9252 increased basal phosphorylation at Ser207 in the presence or absence of the inhibitors, there was a strong reduction in the level of Smad3 phosphorylated at either Ser203 or Ser207 in the presence of TGF-β/H9252 and the inhibitors, compared with that in the presence of TGF-β/H9252 alone. As Smad proteins are not catalytic, but rather affect transcription in proportion to their amount in a cell, these reductions in the level of Smad3 phosphorylated at these positions could contribute to altered Smad activity (52). In contrast, while TGF-β reduced the phosphorylation of Smad3 at Ser<sup>212</sup> and Thr<sup>8</sup>, and had little effect on the phosphorylation level of Thr<sup>178</sup>, the total amount of Smad3 phosphorylated at these sites in the presence of TGF-β was unchanged by addition of either the p38 or ROCK inhibitor (Fig. 7, B and C). Unexpectedly, treatment of MCF10CA1h cells with the ROCK inhibitor Y27632 alone led to a strong phosphorylation of Smad3 on Ser<sup>212</sup> and Thr<sup>8</sup>, suggesting that this pathway negatively regulates the basal of phosphorylation of these residues (Fig. 7B). However, since suppressive effects of TGF-β on phosphorylation of these residues persist, even in the presence of the ROCK inhibitor (Fig. 7, B and C), these sites are likely not to contribute to effects of the inhibitors on Smad transcriptional activity or, more generally, on TGF-β-dependent inhibition of growth of the cells.

To test whether a reduction in the level of Smad3 phosphorylated on Ser<sup>203</sup> or Ser<sup>207</sup> could affect its transcriptional activity, we ectopically expressed Smad3 protein mutated either at all four of the putative middle-linker region phosphorylation sites (EPSM) (47), to mimic the dominant effects of the p38 and RhoA inhibitors on suppression of phosphorylation of Ser<sup>203</sup> and Ser<sup>207</sup> (Figs. 7C and 8A), or mutated only at Thr<sup>8</sup> and Thr<sup>178</sup> and Ser<sup>212</sup>, such that Ser<sup>203</sup> and Ser<sup>207</sup> remain phosphorylatable (C2-S3) (Fig. 8A) (48). Importantly, EPSM-Smad3 failed to induce the Smad3-dependent CAGA12-Luc reporter activity in MCF10CA1h cells while C2-S3 activated it to a level higher than that of wild-type Smad3 (Fig. 8B). Remarkably, when we used a TIE (TGF-β inhibitory element) reporter construct derived from the c-myc gene, which is suppressed by Smad3 (53), the EPSM-Smad3 mutant not only failed to down-regulate, but actually enhanced the reporter activity, possibly by interfering with the activity of endogenous Smad3 (Fig. 8C). The C2-S3 mutant showed the opposite effect by suppressing the reporter activity similar to that of wild-type Smad3 (Fig. 8C). Together these data suggest that phosphorylation at Ser<sup>203</sup> and Ser<sup>207</sup> may be important in regulating the transcriptional activity of Smad3, and that suppression of phosphorylation at these sites, as by the p38 and ROCK inhibi-
induction of p21 and total ERK was used for normalization. Values were normalized against pRL-TK plasmid using AMAXA transfection kit. 48 h after transfection, cells were treated for 16 h, and reporter activity was estimated. These inhibitors against either p38 MAP kinase (5 μM) or ROCK (10 μM) were probed with (A) anti-Myc antibodies and actin was used for normalization (B) anti-p21waf antibodies and total ERK was used for normalization. C, nuclear extracts from MCF10CA1h cells treated with TGF-β alone or together with synthetic inhibitors against either p38 MAP kinase or ROCK were probed with (C) anti-p21waf antibodies. Protein loading was normalized with proliferating cell nuclear antigen. D, MCF10CA1h cells were transfected with full-length p21waf promoter-luciferase reporter together with pRL-TK plasmid using AMAXA transfection kit. 48 h after transfection, cells were treated for 16 h, and reporter activity was estimated. These values were normalized against Renilla values and were presented as normalized average values from triplicates.

Potential Growth-related Target Genes for Rho and p38 Pathways in MCF10CA1h Cells—As we showed above, treatment of MCF10CA1h cells with TGF-β led to an increase in p21waf and down-regulation of c-Myc levels and that cells expressing dominant-negative Smad3 (Smad3ΔC) failed to do so (Figs. 1 and 3). Because both p38 and ROCK inhibitors partially reversed the effects of TGF-β on growth (Fig. 5C) we checked the direct effect of these inhibitors on transcription levels of Smad proteins are indispensable for TGF-β-mediated growth inhibition. In order to address this question, we have utilized a human breast cancer cell line MCF10CA1h, with defined oncogenic potential and functionally intact Smad signaling pathways in the anti-proliferative effects of TGF-β. Our investigations into the signaling and molecular mechanisms behind TGF-β-mediated growth regulation of this cell line have shown a role for the Rho pathway in MCF10CA1h cells and suggest that effects of this pathway on levels of Smad3 phosphorylated on Ser203 and Ser207 might play a role (Fig. 7, A and C).

**DISCUSSION**

TGF-β is a multifunctional cytokine with a plethora of biological effects, many of which cannot be attributed to Smad signaling alone. Recent studies have shown that TGF-β activates a number of signaling pathways in addition to the Smad pathway, which may affect the biological outcome either in a positive or negative manner in a cell- and context-dependent manner. But so far the interplay between these pathways has been poorly characterized, especially in terms of their contribution to more complex endpoints such as TGF-β-mediated growth inhibition. In order to address this question, we have utilized a human breast cancer cell line MCF10CA1h, with defined oncogenic potential and functionally intact Smad signaling, and which is responsive to the growth inhibitory effect of TGF-β. Our investigations into the signaling and molecular mechanisms behind TGF-β-mediated growth regulation of this cell line have shown a role for the Rho/ROCK pathway in MCF10CA1h cells and suggest that effects of this pathway on levels of Smad3 phosphorylated on Ser203 and Ser207 might play a role (Fig. 7, A and C).
MCF10CA1h cells were derived from Ras-transformed MCF10A1k cells and are highly tumorigenic in vivo (43–45). The well described effects of TGF-β on inhibition of growth of these cells allowed us to undertake a molecular analysis of the pathways and gene targets contributing to this effect (46, 54). Most commonly, activation of Smad2/3 signaling in cells by TGF-β represses c-Myc expression, induces p21\(^{\text{waf1}}\) and p15\(^{\text{INK4b}}\) and leads to the formation of repressive complexes of transcription factor E2F4 and pRb that ensure cell growth arrest at the G1 phase of cell cycle (49). In MCF10CA1h cells, TGF-β treatment led to the phosphorylation of both Smad2 and Smad3 proteins in a time-dependent manner and to the down-regulation of c-Myc, a pro-proliferation/survival gene, up-regulation of CDK inhibitor p21\(^{\text{waf1}}\) protein, and hypophosphorylation of tumor suppressor pRb. Consistent with the central role of the Smad pathway in the growth regulatory function of TGF-β in various cell lines, including tumor cells and cells of epithelial origin (21, 55–57), we showed that an inhibition of Smad2/3 signaling, with Smad3\(^{\text{C}}\), resulted in complete loss of TGF-β-mediated inhibition of cell growth, coupled with the loss of c-Myc down-regulation and hypophosphorylation of pRb protein. These results highlight the necessary and pivotal role of Smad2 and Smad3 proteins in TGF-β-mediated growth inhibitory responses in MCF10CA1h cells.

TGF-β is known to activate various non-Smad signaling pathways, including the ERK and p38 MAP kinase pathways, SAPK, and JNK pathways. In MCF10CA1h cells, TGF-β treatment leads to Smad-independent phosphorylation of both ERK1/2 and p38 proteins, because their activation was affected neither by activation of Smad pathway with ectopic Smad3 (CAS3) or by interference with the pathway by expressing dominant-negative Smad (Smad3\(^{\Delta C}\)). Moreover, since C-terminal phosphorylation of Smad2 and Smad3 is not affected by inhibitors of either p38 MAP kinase or Rho/ROCK pathways, we conclude that the MAP kinase and Smad pathways are activated in parallel and independent of each other in these cells. Recent advances in the field of TGF-β signaling have confirmed the role of Smad-independent pathways and their cooperation with Smad proteins in mediating various biological effects (3, 28–31, 34, 35, 37). In MCF10CA1h cells, use of inhibitors specific for various non-Smad signaling pathways showed that only the p38 MAP kinase and Rho/ROCK pathways and not ERK-MAP kinase pathway contribute to the Smad-dependent effects of TGF-β on inhibition of growth. TGF-β treatment led not only to the activation of RhoA and p38 MAP kinases, but also activated their kinase activities with distinctly different kinetics. These and other data suggest that even though both of these pathways target TGF-β-dependent regulation of growth, they might, in fact, be affecting different target genes, with p38 MAP kinase likely affecting early and Rho late response genes, respectively. Moreover, since inhibitors of the p38 and Rho/ROCK pathways did not completely counter the effects of TGF-β on growth, it can be assumed that they act cooperatively, presumably together with Smad2 and Smad3.

To understand the basis of the cooperative effects of Smad signaling and signaling through p38 MAP kinase and Rho/ROCK more fully, we examined effects of either overexpression or inhibition of the Smad pathway on the contributions of the p38 MAP kinase and Rho/ROCK to growth inhibition. In cells ectopically overexpressing Smad3, inhibition of p38 MAP kinase still interfered with the effects of TGF-β on growth,

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**Fig. 10.** Schematic representation of cooperative TGF-β-dependent and -independent mechanisms affecting the TGF-β-mediated inhibition of growth of MCF10CA1h cells. Note that the upstream activators of p38 and Rho/ROCK signaling responsible for their effects on basal phosphorylation of alternate sites on Smad2/3 are unknown.
whereas inhibition of Rho/ROCK had no effect in this context. The inability of the Rho inhibitor to revert the growth inhibition when Smad signaling is enhanced may be due to a higher threshold for inhibition, or may suggest that the Rho/ROCK pathway is more sensitive than the p38 MAP kinase to signaling flux through the Smad pathway. Importantly, when signaling through the Smad pathway was blocked, as in cells expressing the truncated version of Smad3 (Smad3ΔC), neither p38 MAP kinase nor ROCK inhibitors had any further effect indicating that Smad signaling is indispensable for TGF-β-mediated growth inhibition of MCF10CA1h cells. These results also indicate that hierarchically p38 and Rho pathways are likely upstream to Smad signaling and they somehow modulate Smad function (Fig. 10).

Smad proteins undergo a constant, highly regulated, nucleocytoplasmic shuttling, which is governed by the levels of activated receptors (22). p38 MAP kinase was shown to regulate cell adhesion by phosphorylating Smad3 and enhancing its nuclear translocation (39). Co-expression of Smad3 and constitutively active MKK3b and MKK6b, the upstream activators of p38, was shown to result in Smad3 nuclear translocation and induction of MMP-13 in the absence of TGF-β stimulation (58). Interestingly, Yu et al. (59), have shown that in NMuMG cells, TGF-β-induced activation of p38 MAP kinase is required for TGF-β-induced apoptosis and epithelial-to-mesenchymal transition (EMT), but not for growth arrest. In MCF10CA1h cells, effects of not only the p38 pathway but also the ROCK pathway on phosphorylation of Smad2/3 at two serine residues (Ser203 and Ser207) in the proline-rich middle linker region, appear to contribute to their effects on TGF-β-mediated gene regulation. These sites and two additional sites in the middle linker region were previously shown to be putative MAP kinase phosphorylation sites (47). In other cell lines, mutation of all four of these middle linker region MAP kinase phosphorylation sites in Smad2/3 (EPSM, Ref. 47; Fig. 8A) leads either to activation (47) or inhibition (60) of their transcriptional activity, dependent on the cell type. Recently, Matsuura et al. (48) have shown that CDK2 and 4 phosphate Smad3 at Thr4 in the MH1 domain and Thr178, Ser211 at sites in the middle linker region and that this, in turn, inhibits its transcriptional activity and antiproliferative function. By using Smad2 and Smad3 proteins with specific mutations in these putative phosphorylation sites (EPSM and C2-S3: Fig. 7A) and antibodies specific for these phosphorylation sites (48), we have demonstrated that in MCF10CA1h cells, phosphorylation of Smad3 at Ser203 and Ser207 is uniquely dependent on the Rho/ROCK and p38 pathways even in the presence of TGF-β (Fig. 7C). These TGF-β-independent effects of these pathways likely cooperate at the transcriptional level with TGF-β-dependent events such as C-terminal phosphorylation. Reporter assays utilizing the Smad3-dependent CAGA12-Luc and the p21<sup>wa/wa</sup> promoter show dependence on the p38 and Rho/ROCK pathways, respectively, suggesting that interaction between Smad signaling and these pathways may involve not only phosphorylation of specific residues in the middle linker region, but may also depend on other pathway-specific effects.

At the level of cell cycle progression, the p38 MAP kinase pathway has recently been implicated in stabilization of the p21<sup>wa/wa</sup> protein in a Smad-independent manner, leading to growth arrest in human colon cancer cell line (38), and Rho has been shown to play a role in the down-regulation of c-Myc protein (61). In our cell system, the ROCK inhibitor interfered with both TGF-β-dependent down-regulation of c-Myc and up-regulation of p21<sup>wa/wa</sup> while inhibition of the p38 pathway had little effect. Specific downstream targets for the p38 MAP kinase pathway in these cells still need to be identified.

To date, TGF-β-mediated activation of the Rho/ROCK pathway has mainly been implicated in the process of EMT and cytoskeletal rearrangements. However, two recent reports suggest that this pathway is also necessary for cell growth regulation (62, 63). Bhowmick et al., (63) have shown that TGF-β-mediated activation of RhoA and p16<sup>ROCK</sup> is involved in the inhibition of Cdc25A with resultant cell cycle arrest. They also suggest a two-step model for TGF-β inhibition of G, S progression with an initial inhibition of Cdc25A enzymatic activity followed by a secondary response involving Smad-mediated transcriptional up-regulation of Cdki inhibitory proteins and down-regulation of c-Myc and Cdc25A. We now show that in MCF10CA1h cells, down-regulation of the c-Myc oncogene and activation of the p21<sup>wa/wa</sup> promoter by TGF-β are also dependent on the Rho/ROCK pathway. Most importantly, we show that even though the activation of p38 and Rho/ROCK pathways are independent of the Smad pathway, activation of Smad2 and Smad3 are crucial for inhibition of growth of MCF10CA1h cells by TGF-β. Together with previously published data, our work now adds a new complexity to the cooperation between Smad signaling and the p38 MAP kinase and Rho/ROCK pathways in mediating the growth inhibitory effects of TGF-β (Fig. 10).

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