# Smad7 is required for TGF-β-induced activation of the small GTPase Cdc42

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## Summary

**Transforming growth factor β (TGF-β) is a potent regulator of cell growth and differentiation in many cell types. The Smad signaling pathway constitutes a main signal transduction route downstream of TGF-β receptors. The inhibitory Smads, Smad6 and Smad7, are considered to function as negative regulators of the TGF-β/Smad signaling cascade. In a previous study, we found that TGF-β induces rearrangements of the actin filament system in human prostate carcinoma cells and that this response requires the small GTPases Cdc42 and RhoA. On the basis of the current view on the function of Smad7 in TGF-β signaling, we hypothesized that Smad7 would function as a negative regulator of the TGF-β-induced activation of Cdc42 and RhoA, but instead we found that the reverse is the case; Smad7 is required for the TGF-β-induced activation of Cdc42 and the concomitant reorganization of the actin filament system. These observations propose a novel role for Smad7 in TGF-β-dependent activation of Rho GTPases.**

Key words: TGF-β, Cdc42, Rho, Actin, phosphatidylinositol-3 kinase, Smad7

## Introduction

Small GTPases have been shown to be pivotal signaling intermediates in cell migration, cell cycle progression, cell trafficking and cell survival (Ridley, 2001; Takai et al., 2001; Etienne-Manneville and Hall, 2002). The potential of small GTPases to function as signaling switches resides in their ability to cycle between active, GTP-bound states, and inactive, GDP-bound states. This cycling is tightly orchestrated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Sasaki and Takai, 1998; Schmidt and Hall, 2002; Moon and Zheng, 2003). GEFs stimulate the replacement of GDP by GTP, whereas GAPs stimulate the intrinsic GTP hydrolysis of the GTPase. GDIs act by blocking GDP-dissociation. The activation of Rho GTPases is, to a large extent, under the influence of external stimuli and factors; thus, cell adhesion, in particular via activation of the integrin complexes, has been shown to activate specific members of the Rho GTPases (Ridley, 2001; Arthur et al., 2002). In addition, several ligand:receptor systems have been shown to activate specific members of the Rho GTPase superfamily (Ridley, 2001; Schmidt and Hall, 2002). For example, activation of the receptor for transforming growth factor β (TGF-β) specifically activates Cdc42 and RhoA (Edlund et al., 2002); however, the precise mechanism underlying this TGF-β-dependent activation is currently not known.

TGF-β, a potent regulator of cell growth and differentiation in a wide variety of cell types, is a member of a large family of cytokines that, in addition to the TGF-β isoforms, includes activins, inhibins and bone morphogenetic proteins (BMPs) (Heldin et al., 1997; Massagué and Chen, 2000). These soluble ligands exert their actions by binding to transmembrane receptors with intrinsic serine/threonine protein kinase activity. Receptor binding triggers a signaling cascade involving the Smad proteins, which on receptor activation are translocated to the cell nucleus, where they are able to affect the transcription of specific genes by direct or indirect binding to their promoters (Zhang and Derynck, 1999; Attisano and Wrana, 2000; Massagué and Chen, 2000; Moustakas et al., 2001). Although the Smad signaling cascade is considered to form the principal signaling avenue downstream of the TGF-β receptor, there is an increasing awareness of the existence of alternative Smad-independent signaling pathways (Mulder, 2000; Moustakas et al., 2001). One example of a Smad-independent response was shown in our previous work on TGF-β-induced rearrangements of the actin filament system (Edlund et al., 2002). Interestingly, this response was shown to require the activity of the Rho GTPases Cdc42 and RhoA. We could divide the effect in a short-term and a long-term response, where the short-term response, seen as a formation of membrane ruffles, required the activity of Cdc42 and p38 MAPK (mitogen-activated protein kinase). By contrast, the long-term response required signaling via Cdc42/p38 MAPK, RhoA/Rho-associated coiled-coil-containing protein kinase (ROCK) and Smad proteins (Edlund et al., 2002). The long-term response was also dependent on active TGF-β-dependent gene transcription.

We wanted to study further the involvement of the Smad signaling pathway in the reorganization of the actin filament system, as well as on the activation of the Rho GTPases. The inhibitory Smads, Smad6 and Smad7, have been shown to function in a negative feedback loop, turning down the general Smad signaling cascade (Moustakas et al., 2001; Nakao et al., 2002). For this reason, we studied the potential interference by Smad7 of the activation of Rho GTPases, as well as

reorganization of the actin filament system. We found that Smad7 instead had a positive effect on the activation of Cdc42 and, to a lesser extent, of RhoA. Cells over-expressing Smad7 induced activation of Cdc42 in a Smad7-dependent manner. By contrast, in cells with reduced Smad7 expression, TGF-β was unable to activate Cdc42 and to elicit the concomitant mobilization of the actin filament system. These observations propose a novel role for Smad7 in TGF-β signaling via small GTPases.

## Materials and Methods

### Reagents, antibodies and DNA work

TGF-β1 was from R&D systems, the phosphoinositide 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) and the p38 MAPK α and β inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(pyridyl)1*H*-imidazole (SB203580) were from Calbiochem. Mouse monoclonal anti-Myc (9E10) was from Santa Cruz Biotechnology; mouse monoclonal anti-Flag antibodies (M5 and M2) and rabbit polyclonal anti-Flag antibodies were from Sigma Chemical Company. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies were from DAKO. TRITC-conjugated phalloidin was from Sigma Chemical Company. Antibodies against Akt1 and Akt2 (total Akt) were from Santa Cruz. Antibodies recognizing Akt phosphorylated on serine residue 473 (phospho-Akt) and antibodies recognizing p38 MAPK were from Cell Signaling Technology. Rabbit polyclonal antibodies recognizing Smad2 and Smad2 phosphorylated on serine residues 465 and 467 were generous gifts from A. Morén at our Institute. Goat anti-Smad7 antibodies were from Santa Cruz. Dominant negative p38 MAPKα, harboring T180A and Y182F amino acid substitutions, has been described previously (Edlund et al., 2002).

### Cell cultivation, transfection and immunohistochemistry

The following cell lines were employed: human prostate cancer cell line (PC-3U), which is a sub-line originating from PC-3 cells (Franzén et al., 1993), PC-3U cells stably expressing Flag-Smad7 under control of the $Cd^{2+}$-inducible metallothionein promoter (PC-3U/pMEP4-S7 also called clone I cells) and PC-3U cells expressing antisense Smad7 (PC-3U/AS-S7) (Landström et al., 2000). The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine and penicillin/streptomycin, in the presence of hygromycin (PC-3U/pMEP4-S7 cells) or G418 (PC-3U/AS-S7 cells) to maintain selection pressure. Cells were cultivated at 37°C in an atmosphere of 5% $CO_2$. The PC-3U/pMEP4-S7 cells were treated with 1 μM $CdCl_2$ to induce expression of Flag-Smad7, as previously described (Landström et al., 2000). PC-3U cells stimulated with 1 μM $Cd^{2+}$ were used as control. Cells were stimulated with TGF-β1 at concentrations of 5-10 ng/ml in medium containing 1% FBS. The inhibitors SB203580 and LY294002 were used at concentrations of 10 μM. The inhibitors were added to media 1 hour before stimulation of cells with TGF-β1 or $CdCl_2$. PC-3U cells were transiently transfected by LipofectAMINE (Invitrogen, Carlsbad, CA) employing the protocol provided by the manufacturer. Immunohistochemistry were performed as described before (Edlund et al., 2002). Cells were photographed by a Hamamatsu ORCA CCD digital camera employing the QED Imaging System software using a Zeiss Axioplan2 microscope. Confocal analysis was performed on a Zeiss Axiovert 200M equipped with a LSM 510 laser.

### Protein production and GST pull-down assays

The construction of GST-rhotekin (amino acid residues 1-89), GST-PAK-CRIB (amino acid residues 56-267 of human PAK1B) and GST-WASP-CRIB (amino-acid residues 201-321 of human WASP) has been described previously (Edlund et al., 2002). The GTPase activation assay to detect GTP-bound Cdc42, Rac1 and RhoA was performed as described previously (Edlund et al., 2002).

## Results

### Increased expression of Smad7 is associated with a mobilization of the actin filament system

Previous results have implicated the small GTPases Cdc42 and RhoA in the reorganization of the actin filament system after TGF-β stimulation (Edlund et al., 2002). This study formed the starting point for a further characterization of the involvement of the Smad signaling cascade in the activation of Rho GTPases and the actin filament system. The inhibitory Smads, Smad6 and Smad7, have been shown to function as negative regulators of TGF-β signaling (Moustakas et al., 2001; Nakao et al., 2002). To study the effect of Smad7 on TGF-β-induced actin reorganization and activation of Rho GTPases, we employed PC-3U cells stably transfected with Flag-Smad7 under control of the $Cd^{2+}$-inducible metallothionein promoter (PC-3U/pMEP4-S7 cells) (Landström et al., 2000).

Smad7 expression was induced by the addition of 1 μM $CdCl_2$ to serum-starved PC-3U/pMEP4-S7 cells, after which the cells were fixed at different time-points and filamentous actin was visualized with TRITC-conjugated phalloidin. Unexpectedly, increased Smad7 expression induced an accumulation of actin filaments to the cell periphery, in particular in those cells expressing high amounts of Smad7 (Fig. 1). This response was already seen after a couple of hours (data not shown) but was more pronounced after 12-24 hours when a formation of membrane ruffles was seen; these structures also contained thin filopodia protruding from the ruffles (Fig. 1, arrowheads and detail). By contrast, $CdCl_2$-treatment of parental PC-3U cells for up to 12 hours did not have any effect on the mobilization of the actin cytoskeleton (Fig. 2C). A proportion of Smad7 in the PC-3U/pMEP4-S7 cells was translocated out of the nucleus to the cytoplasm and towards the cell edge where it was localized to the membrane ruffles (Fig. 1). To confirm the Smad7 localization to membrane ruffles, we performed confocal microscopy analysis of cells treated with $CdCl_2$ for 12 hours. The Flag-Smad7 fluorescence intensity across a section of the cell was calculated and plotted in a histogram, which further emphasized the localization of Smad7 to membrane ruffles (Fig. 2A). We also counted cells with membrane ruffles and stress fibers; in cells treated with $CdCl_2$ for 12 hours, more than 80% of the cells had formed ruffles compared with roughly 20% in the control cells, indicating that Smad7 expression was important for the response (Fig. 2B). TGF-β stimulation of the PC-3U/pMEP4-S7 cells did not result in any increase in the membrane ruffle activity; however, the cell edges appeared wider and more spread-out after 12-24 hours of TGF-β stimulation (Fig. 1, Fig. 2B). Few stress fibers were seen in cells treated with $CdCl_2$ only. Instead, stress fibers emerged after prolonged stimulation with TGF-β. These data suggest that Smad7 has an active and positive role in the mobilization of the actin filament system downstream of the activated TGF-β receptor.

### Increased expression of Smad7 is associated with activation of Cdc42

TGF-β stimulation has previously been shown to mobilize the actin filament system through the Rho GTPases Cdc42 and

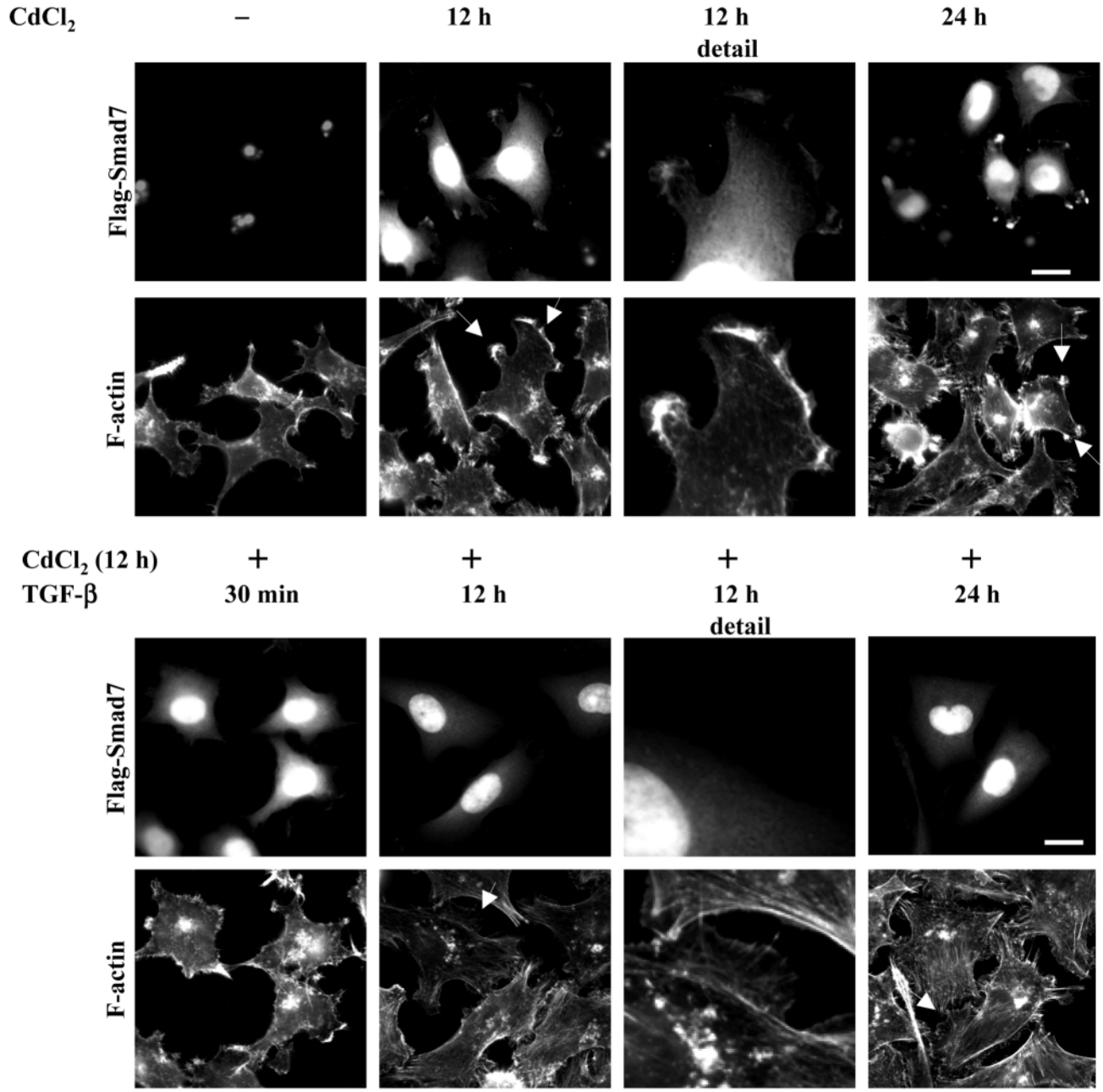



**Fig. 1.** Smad7-dependent activation of the actin filament system. PC-3U/pMEP4-S7 cells were serum-starved in 1% FBS for 12 hours and treated with 1 μM $CdCl_2$ for 12 hours or 24 hours, or with 1 μM $CdCl_2$ for 12 hours together with TGF-β for 30 minutes and 12 hours or 24 hours. The expression of Flag-tagged Smad7 was visualized by a Flag-specific mouse antibody (M5) followed by a FITC-labeled anti-mouse antibody. Filamentous actin was visualized by TRITC-labeled phalloidin. Arrowheads indicate cells with lamellipodia. Bars, 20 μm.

RhoA (Edlund et al., 2002). We therefore examined the activation of Rho GTPases in PC-3U/pMEP4-S7 cells (Fig. 3A). For these studies we used the GST pull-down activation assay to detect GTP-loaded Rho GTPases (Edlund et al., 2002). PC-3U/pMEP4-S7 cells were starved and treated with 1 μM $CdCl_2$ for 12 hours. This time period was chosen because it was shown previously to give the highest amount of expressed Smad7 (Landström et al., 2000). As a control, starved parental PC-3U cells were stimulated with 5 ng/ml of TGF-β and mock-treated with $CdCl_2$ for 12 hours, to rule out the possibility that $CdCl_2$ alone induced activation of the Rho GTPases. The amount of GTP-Rac1 was not affected by TGF-β stimulation or $CdCl_2$ treatment (Fig. 3A,B), thus the PC-3U/pMEP4-S7 did not differ from the parental PC-3U cells in this respect. In stark contrast, the amount of GTP-loaded Cdc42 and, to a lesser extent, of GTP-loaded RhoA increased in a Smad7-dependent manner. TGF-β stimulation did not lead to an increase in the activation of Cdc42 above the Smad7-dependent activation. However, an increased RhoA activation was seen after 6-24 hours, which followed the appearance of stress fibers (Fig. 1, lower panel).

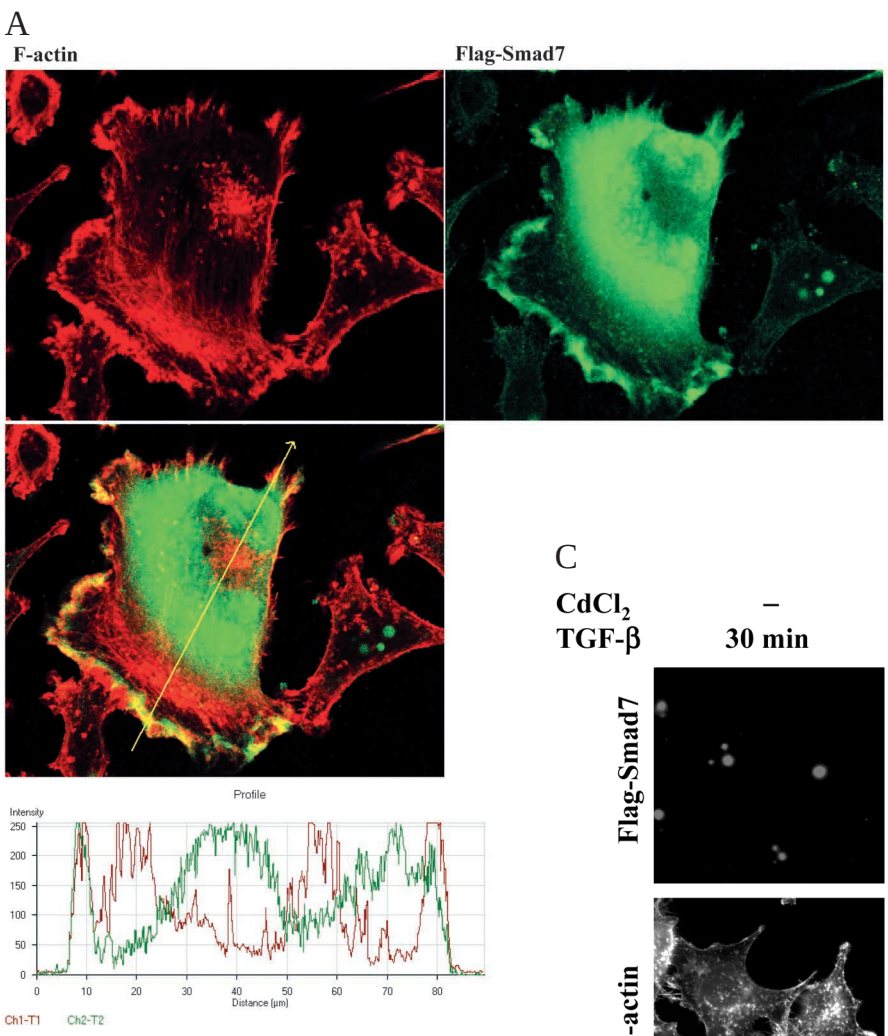



**Fig. 2.** Quantification of the Smad7-dependent activation of the actin filament system. (A) Confocal image of a representative PC-3U/pMEP4-S7 cell treated with 1 μM $CdCl_2$ for 12 hours. Flag-Smad7 and filamentous actin was detected as in Fig. 1. The fluorescence intensity changes across the yellow line in the direction of the arrowhead were plotted as the line intensities in the histogram in the lower panel. The red channel (Ch1-T1) represents filamentous actin and the green channel (Ch2-T2) represents Flag-Smad7. (B) Quantification of the effect of $CdCl_2$ and TGF-β treatment of the PC-3U/pMEP4-S7 cells in Fig. 1 on membrane ruffling and stress-fiber formation. At least 300 cells were counted under the microscope for each condition. (C) PC-3U/pMEP4-S7 cells were serum-starved in 1% FBS for 12 hours and stimulated with 5 ng/ml TGF-β for 30 minutes, 12 hours and 24 hours to show the effect on the actin filament system in the absence of $CdCl_2$-induced Smad7 expression. Parental PC-3U cells were mock-treated with 1 μM $CdCl_2$ for 12 hours or stimulated with 5 nM TGF-β for 12 hours to show that $CdCl_2$ alone did not induce any effect on the actin filament system. Bars, 20 μm.

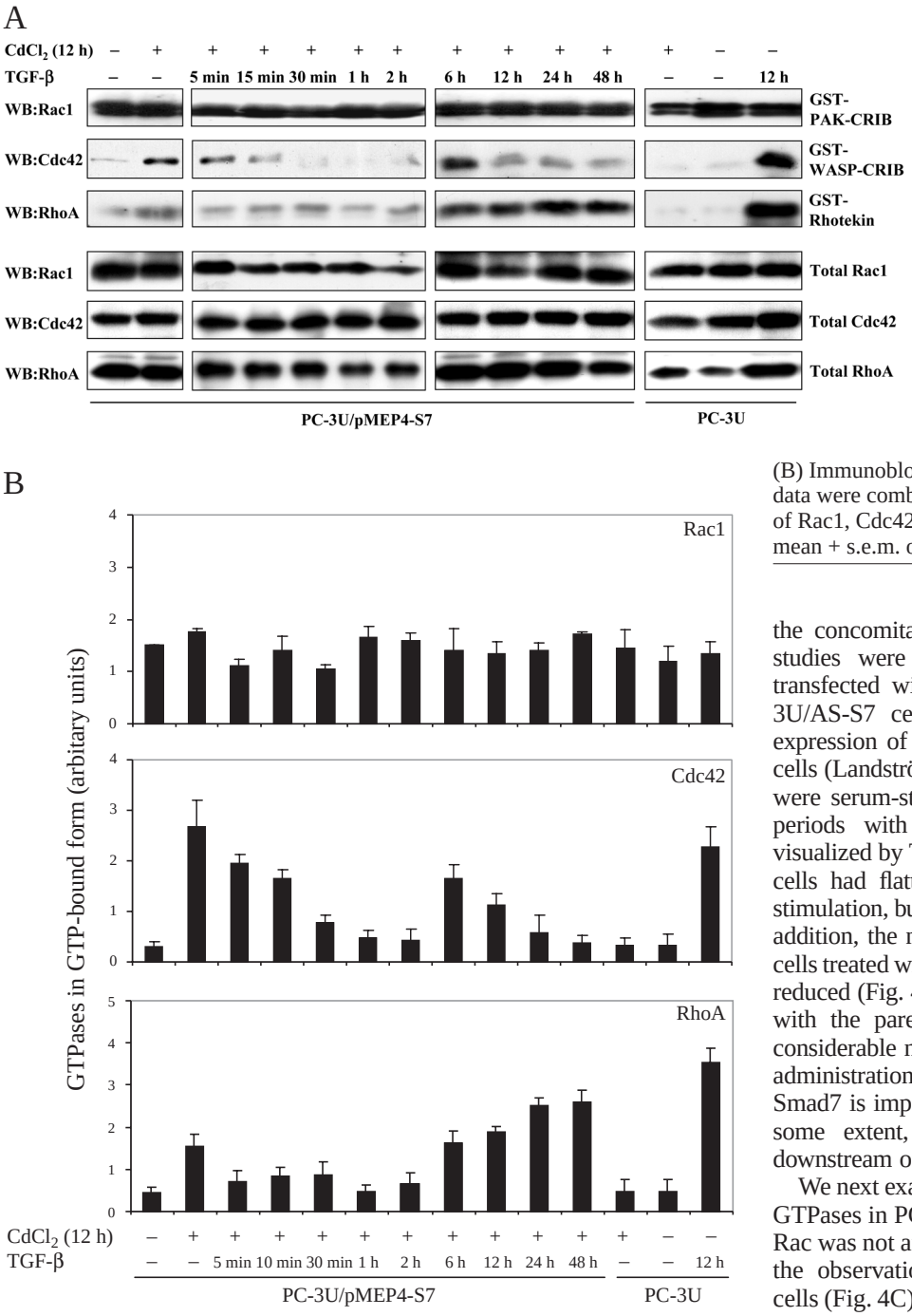



**Fig. 3.** Smad7-dependent activation of the Rho GTPases. (A) PC-3U/pMEP4-S7 cells were serum-starved in 1% FBS for 12 hours and treated with 1 μM $CdCl_2$ for 12 hours or with 1 μM $CdCl_2$ together with 5 ng/ml TGF-β for 15 minutes up to 48 hours. The control cells, PC-3U, were stimulated with 5 ng/ml of TGF-β for 12 hours as positive control, and with $CdCl_2$ for 12 hours as negative control. The amount of active, GTP-loaded Rac1, Cdc42 and RhoA was determined by GST pull-down assays with GST-PAK-CRIB, GST-WASP-CRIB or GST-Rhotekin, respectively. Rac1, Cdc42 and RhoA were detected by immunoblotting using antibodies specific for the respective GTPase. (B) Immunoblots were analyzed by densitometry and the data were combined into diagrams showing the activation of Rac1, Cdc42 and RhoA. Each column represents the mean + s.e.m. of three independent experiments.

These observations suggest that the TGF-β-induced membrane ruffles and stress fibers follow distinct pathways, where Cdc42 is involved in membrane ruffling, whereas RhoA is involved in the formation of stress fibers. In addition, these observations show that Smad7 is a signaling component with a capacity to actively induce actin reorganization primarily by influencing the activation of Cdc42 and, to lesser extent, RhoA.

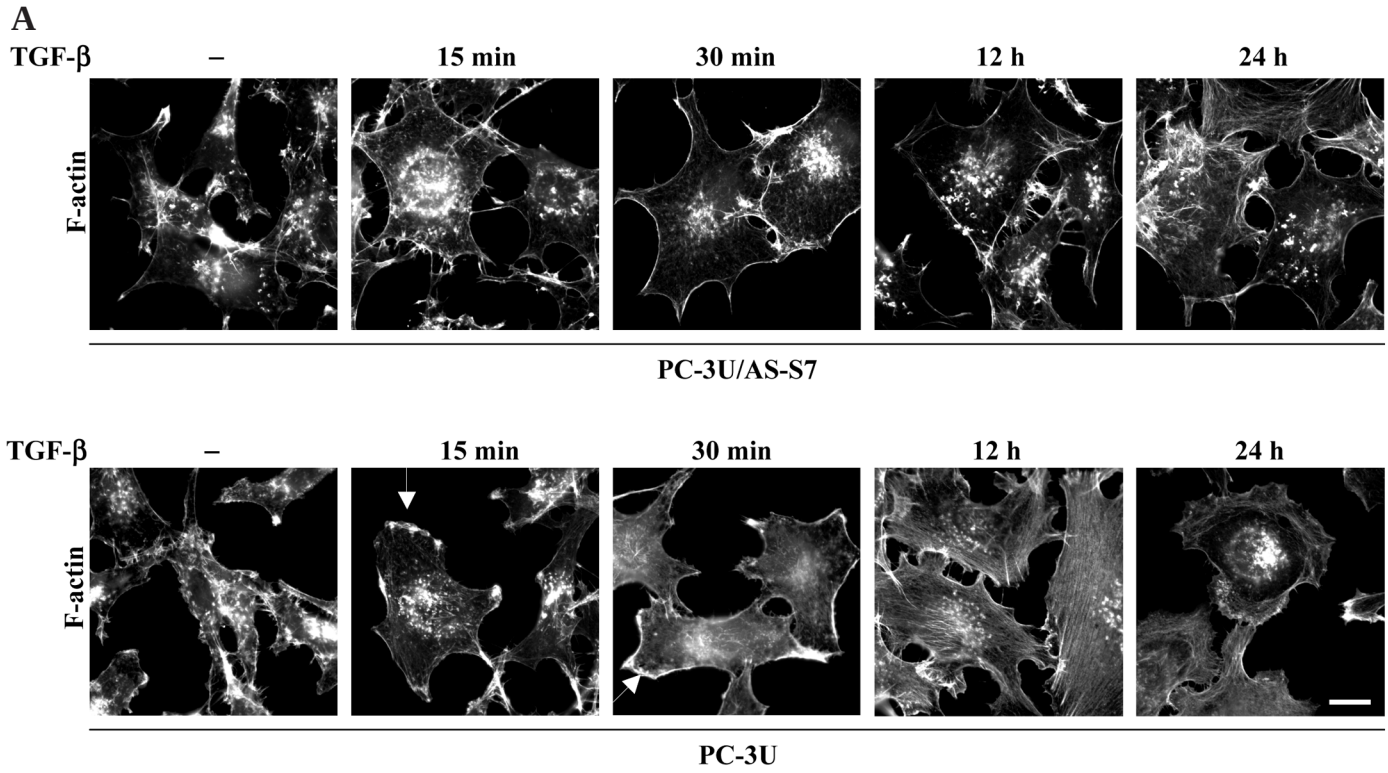
## Smad7 is necessary for TGF-β-induced activation of Cdc42 and mobilization of the actin filament system

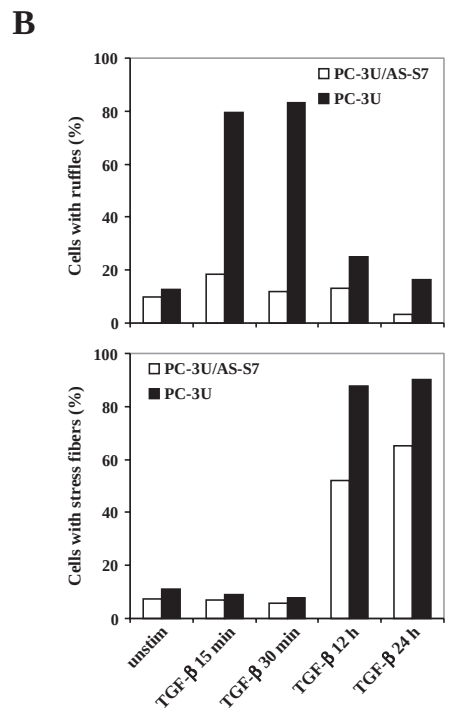
To confirm that Smad7 is important for activation of Cdc42 and the concomitant formation of membrane ruffles, the studies were continued using PC-3U cells stably transfected with an antisense Smad7 construct (PC-3U/AS-S7 cells). These cells have a much lower expression of Smad7 compared with parental PC-3U cells (Landström et al., 2000). The PC-3U/AS-S7 cells were serum-starved and stimulated for different time periods with TGF-β, and filamentous actin was visualized by TRITC-labeled phalloidin (Fig. 4A). The cells had flattened out already after 15 minutes of stimulation, but no membrane ruffles were observed. In addition, the number of stress fibers in PC-3U/AS-S7 cells treated with TGF-β for 24 hours were significantly reduced (Fig. 4A, quantification in Fig. 4B), compared with the parental PC-3U cells, which developed a considerable number of stress fibers 12-48 hours after administration of TGF-β. These data thus indicate that Smad7 is important for the membrane ruffling and, to some extent, also for the stress fiber formation downstream of the TGF-β receptor.

We next examined the GTP-bound status of the Rho GTPases in PC-3U/AS-S7 cells. The amount of GTP-Rac was not affected by TGF-β stimulation, similar to the observations in PC-3U and PC-3U/pMEP4-S7 cells (Fig. 4C). Interestingly, TGF-β stimulation of the PC-3U/AS-S7 cells did not result in any Cdc42 activation, in contrast to parental PC-3U cells where GTP-Cdc42 increased 5 minutes after the addition of TGF-β (Edlund et al., 2002) (Fig. 4C,D). The TGF-β-induced activation of RhoA was unaffected by the absence of Smad7 and followed the same biphasic manner seen in parental PC-3U cells (Fig. 4C,D). These data leave us with the conclusion that Smad7 is required for TGF-β-dependent activation of Cdc42.

## TGF-β-induced membrane ruffling and stress fiber formation requires the phosphatidylinositol-3 kinase pathway

The type I phosphatidylinositol-3 kinase has previously been



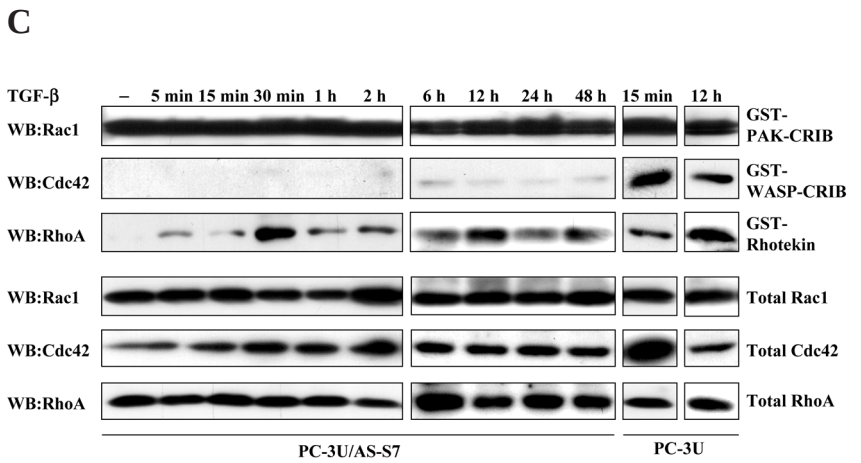
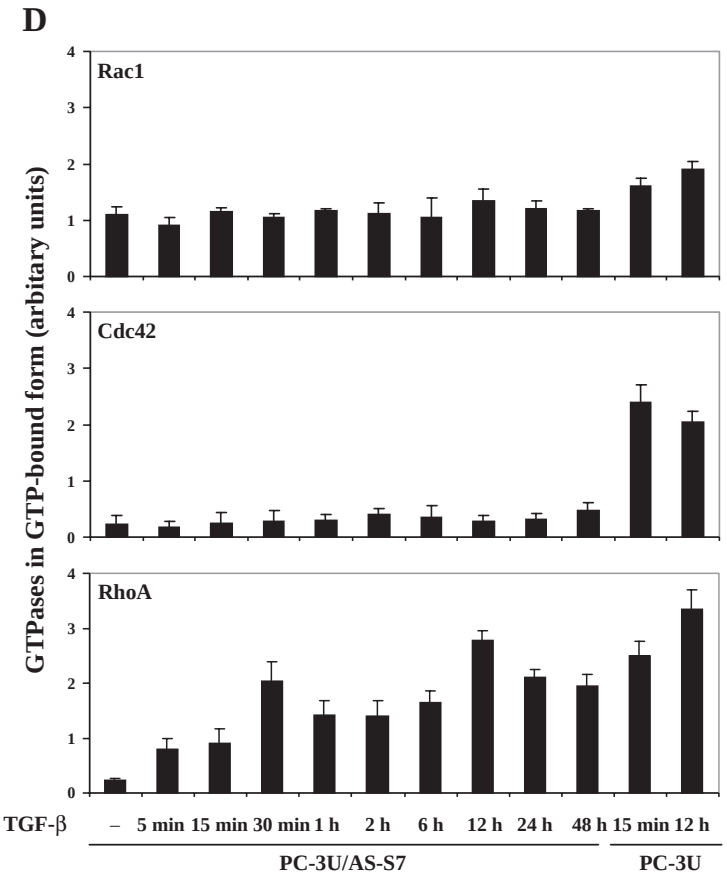




**Fig. 4.** Effects of Smad7 depletion on actin organization and activation of Rho GTPases. (A) PC-3U/AS-S7 cells (upper panel) or parental PC-3U cells (lower panel) were serum-starved in 1% FBS for 12 hours and treated with 5 ng/ml TGF-β for 15 minutes, 30 minutes, 12 hours and 24 hours. Actin filaments were visualized by TRITC-labeled phalloidin. Arrowheads in the lower panel indicate parental cells with membrane ruffles, a response absent in PC-3U/AS-S7 cells. Bar, 20 μm. (B) Quantification of the effect of TGF-β treatment of the PC-3U/AS-S7 and PC-3U cells in panel A on membrane ruffling and stress-fiber formation. At least 300 cells were counted under the microscope for each condition. (C) PC-3U/AS-S7 cells were serum-starved in 1% FBS for 12 hours and treated with 5 ng/ml TGF-β for 5 minutes to 48 hours. As a positive control, PC-3U cells were stimulated with 5 ng/ml of TGF-β for 15 minutes and 12 hours. The amount of active, GTP-loaded Rac1, Cdc42 and RhoA was determined by GST pull-down assays with GST-PAK-CRIB, GST-WASP-CRIB, or GST-Rhotekin, respectively. Rac1, Cdc42 and RhoA were detected by immunoblotting using antibodies specific for the respective GTPase. (D) Immunoblots were analyzed by densitometry and the data are combined into diagrams showing the activation of Rac1, Cdc42, and RhoA. Each column represents the mean + s.e.m. from three independent experiments.

shown to mediate the platelet-derived growth factor (PDGF)-induced activation of Rho GTPases (Hawkins et al., 1995; Tolias et al., 1995; Reif et al., 1996). For this reason, we decided to look into the possible involvement of phosphatidylinositol-3 kinase in TGF-β-mediated signaling and actin reorganization. We pretreated serum-starved PC-3U cells with 10 μM of the




phosphatidylinositol-3 kinase inhibitor LY294002, 1 hour before TGF-β stimulation (Fig. 5A). There were no membrane ruffles or stress fibers formed in cells treated with LY294002, in contrast to nontreated cells (Fig. 5A). These results indicate that an intact phosphatidylinositol-3 kinase signaling pathway is required for the TGF-β-induced mobilization of the actin filament system. Therefore, we continued the studies by looking at the activated status of the phosphatidylinositol-3 kinase effector protein Akt after TGF-β stimulation. Activation of type I phosphatidylinositol-3 kinase has been shown to result in a phosphorylation of Akt at serine residue 473, which in turn leads to activation of the Akt kinase domain (Cantley, 2002; Stephens et al., 2002). We serum-starved and stimulated PC-3U cells with TGF-β for different time periods, whereafter the cells were lysed. Phosphorylated Akt was detected by immunoblotting, employing an antibody specifically recognizing phospho-Akt, after which the filter was stripped and reblotted with an antibody against Akt-1 and Akt-2 to detect the total amount of Akt in the cell lysates (Fig. 5B). Akt was phosphorylated in a biphasic manner with the first peak after 30 minutes of TGF-β stimulation and a second peak after 12 hours. As a control experiment, we serum-starved and stimulated PC-3U cells with TGF-β for 30 minutes and 24 hours, with or without pretreatment of 10 μM of the phosphatidylinositol-3 kinase inhibitor LY294002, 1 hour before TGF-β stimulation, a treatment that totally abolished the phosphorylation of Akt (Fig. 5C).

We wanted to rule out the possibility that the observed inhibition of LY294002 treatment on TGF-β-induced membrane ruffling was caused by interference of other types of phosphatidylinositol-3 kinase-dependent signals, as LY294002 has been shown also to inhibit the type II and III phosphatidylinositol-3 kinases, which phosphorylate phosphatidylinositol to phosphatidyl-3-phosphate (Cantley, 2002; Stephens et al., 2002). The formation of this phospholipid is a crucial step in the SARA-dependent presentation of Smad2 and Smad3 for the TGF-β receptor (Tsukazaki et al., 1998); the effect seen with LY294002 pretreatment could therefore potentially be accounted for by a direct inhibition of the signaling capacity of the TGF-β receptor via the inhibition of SARA. We therefore analyzed the TGF-β-dependent phosphorylation of Smad2, which is mediated by the activated TGF-β receptor. PC-3U cells were serum-starved and stimulated with TGF-β for different time periods and the cells were lysed and subjected to immunoblotting. Phosphorylated Smad2 was detected by an antibody specifically recognizing Smad2 phosphorylated on serine residues 465 and 467. In nontreated cells, Smad2 phosphorylation was seen already after 5 minutes of TGF-β stimulation and peaked after 1 hour, but after 24 hours the amount of phospho-Smad2 was back to baseline values (Fig. 5D). In cells treated with LY294002, the initial phosphorylation of Smad2 was similar to nontreated cells; however, the phosphorylation at 1 hour and later was reduced compared with nontreated cells (Fig. 5D). Thus, the inhibition of the rapid mobilization of the actin filament system in LY294002-treated cells was due to the inhibition of type I phosphatidylinositol-3 kinase rather than by an inhibition of type II and III phosphatidylinositol-3 kinase causing a decreased phosphorylation of Smad2.

### Smad7-induced mobilization of the actin filament system requires the p38 MAPK pathway

We have previously shown that p38 MAPK is required for TGF-β-induced reorganization of the actin filament system and proposed that p38 MAPK may act downstream of Cdc42 (Edlund et al., 2002). In agreement with this observation, treatment of PC-3U cells with toxin B 10463, which inactivates the Cdc42, Rac and Rho small GTPases, abrogated the TGF-β-induced activation of p38 MAPK (data not shown) (Eichel-Streiber et al., 1995; Just et al., 1995). In addition, we have recently shown that Smad7 directly interacts with and activates p38 MAPK (Edlund et al., 2003). To test the involvement of p38 MAPK in the Smad7-induced activation of Cdc42, PC-3U/pMEP4-S7 cells were pretreated for 1 hour with 10 μM SB203580 before the addition of 1 μM $CdCl_2$ for 12 hours. We used parental PC-3U cells stimulated with either $CdCl_2$ or TGF-β as controls. Interestingly, SB203580 treatment abrogated the Smad7-induced activation of Cdc42, suggesting that p38 MAPK could, under certain conditions, also reside upstream of Cdc42 (Fig. 6A,B). In addition, SB203580 treatment resulted in an increased activation of RhoA. Pretreatment with 10 μM LY294002 did not visibly affect the Smad7-induced activation of Cdc42, thereby indicating that phosphatidylinositol-3 kinase functions either upstream of Smad7 or in a parallel pathway leading to the activation of Cdc42 (Fig. 6A,B).

We next investigated the involvement of p38 MAPK in

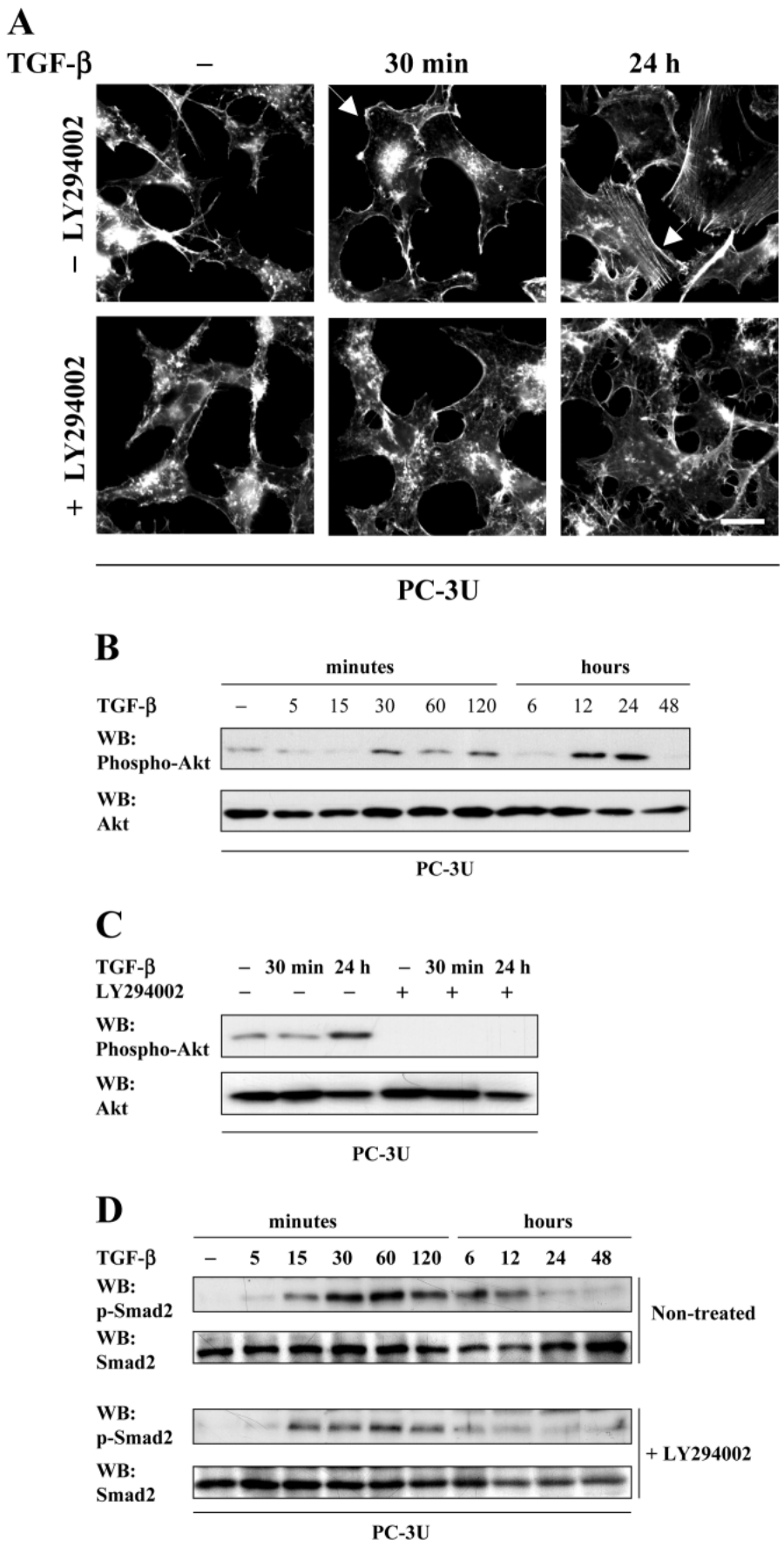



**Fig. 5.** Involvement of phosphatidylinositol-3 kinase in the TGF-β-induced activation of actin filament system. (A) PC-3U cells were serum-starved in 1% FBS for 12 hours, treated or not for 1 hour with 10 μM of the phosphatidylinositol-3 kinase inhibitor LY204009, before stimulation with 10 ng/ml of TGF-β for 30 minutes or 24 hours. Filamentous actin was visualized by TRITC-labeled phalloidin. Arrowheads indicate cells with lamellipodia (30 minutes) and stress fibers (24 hours). Bar, 20 μm. (B) PC-3U cells were starved in 1% FBS for 12 hours and stimulated with 10 ng/ml of TGF-β for different time periods as depicted in the panel. The total cell lysates were subjected to SDS-PAGE followed by immunoblotting using specific Akt antibodies. In the upper and lower panels, the phosphorylated and the nonphosphorylated forms of Akt are shown, respectively. (C) PC-3U cells were starved in 1% FBS for 12 hours, pretreated for 1 hour with or without 10 μM of the phosphatidylinositol-3 kinase inhibitor and stimulated with 10 ng/ml of TGF-β for 30 minutes and 24 hours. The total cell lysates were subjected to SDS-PAGE followed by immunoblotting with specific Akt antibodies. (D) PC-3U cells were starved in 1% FBS for 12 hours, pretreated for 1 hour with or without 10 μM of the phosphatidylinositol-3 kinase inhibitor and stimulated with 10 ng/ml TGF-β for up to 48 hours. The total cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for Smad2 phosphorylated on serine residues 465 and 467 or Smad2.

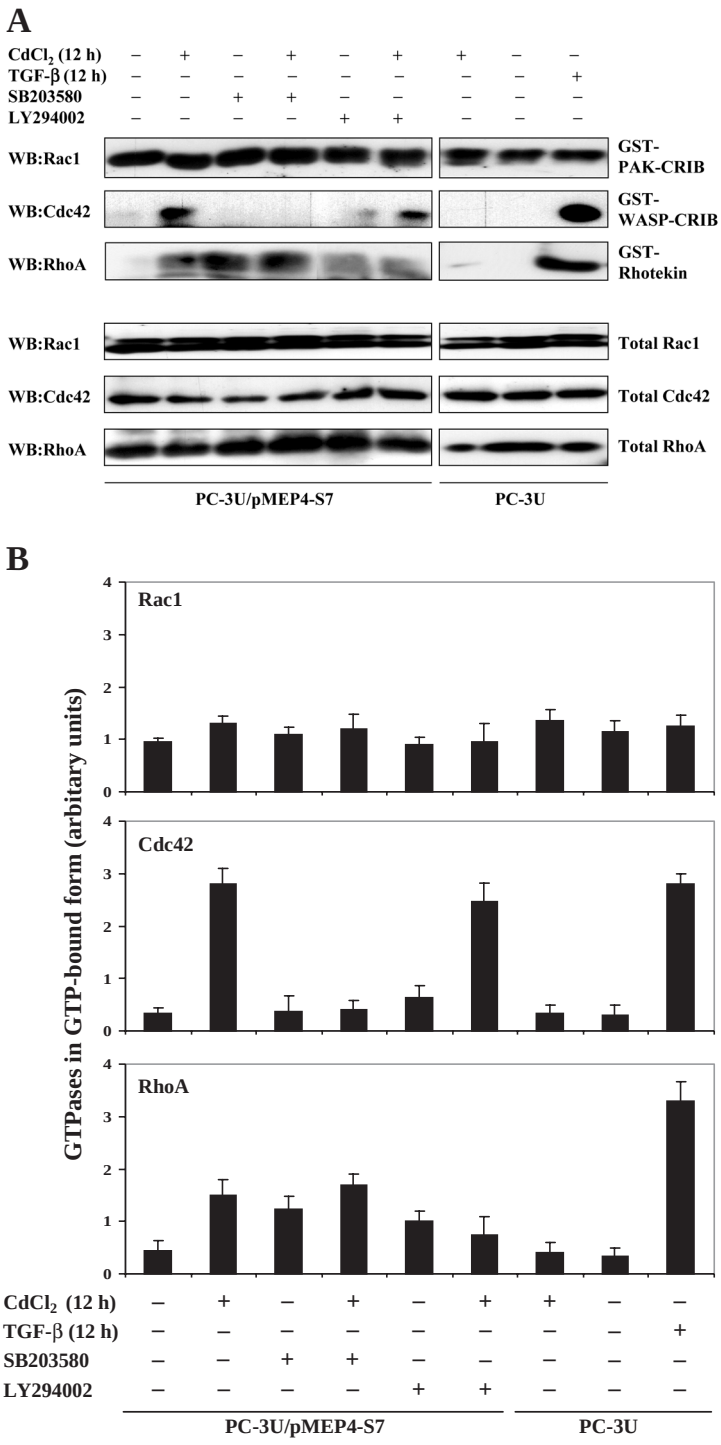



**Fig. 6.** The effect on activation of Rho GTPases of inhibitors of p38 MAPK and phosphatidylinositol-3 kinase. (A) PC-3U/pMEP4-S7 were starved in 1% FBS for 12 hours, pretreated with or without either 10 μM SB203580 or 10 μM LY294002 for 1 hour, and stimulated with 1 μM of $CdCl_2$ for 12 hours. The parental cells, PC-3U, were stimulated with 5 ng/ml of TGF-β for 12 hours as positive control and $CdCl_2$ for 12 hours as negative control. The amount of active, GTP-loaded Rac1, Cdc42 and RhoA was determined by GST pull-down assays with GST-PAK-CRIB, GST-WASP-CRIB or GST-Rhotekin, respectively. Rac1, Cdc42 and RhoA were detected by immunoblotting using antibodies specific for the respective GTPase. (B) Immunoblots were analyzed by densitometry and the data are combined into diagrams showing the activation of Rac1, Cdc42, and RhoA. Each column represents the mean + s.e.m. of three independent experiments.

Smad7-induced membrane ruffling. We used serum-starved PC-3U/pMEP4-S7 cells and pretreated the cells with 10 μM of the p38 MAPK inhibitor SB203580 for 1 hour, before adding 1 μM $CdCl_2$. The p38 MAPK inhibitor totally abrogated the Smad7-induced membrane ruffling (compare Fig. 7A and Fig. 1). Interestingly, there was a marked increase in the number of stress fibers seen 12-24 hours after the induction of Smad7 expression in the presence of the p38 MAPK inhibitor (Fig. 7A), in contrast to PC-3U/pMEP4-S7 cells not treated with SB203580 (Fig. 1). The p38 MAPK inhibitor also abrogated the TGF-β-induced membrane ruffles and stress fibers after 24 hours of stimulation (Fig. 7A). We also transiently transfected PC-3U/pMEP4-S7 cells with a dominant negative p38 MAPK. The cells were thereafter treated with 1 μM $CdCl_2$ for 12 hours. Cells expressing dominant negative p38 MAPK did not develop membrane ruffles (Fig. 7B). In conclusion, these data show that an activated p38 MAPK pathway is needed for Smad7-induced activation of Cdc42 to form membrane ruffles at the plasma membrane.

## Discussion

The studies in this paper were performed employing cell lines derived from PC-3U cells. However, TGF-β-induced activation of small GTPases such as Cdc42 can also be detected in other cell lines, such as human breast carcinoma MDA468 cells, indicating that this is a more general phenomenon (our unpublished observations). Several observations have implicated Smad7 as a negative regulator of TGF-β signaling, and on the basis of these observations Smad7 and the homologous protein Smad6 are referred to as inhibitory Smads (Moustakas et al., 2001; ten Dijke et al., 2002). The inhibitory effect is a result of the binding of inhibitory Smads to TGF-β receptors, which prevents the phosphorylation and activation of Smad2 and Smad3, and brings the associated ubiquitin ligase Smurf close to the receptor, thereby allowing ubiquitinylation and degradation of the receptors (Kavsak et al., 2000; Ebisawa et al., 2001). The results presented in this study show a different role for Smad7 as a mediator of a rapid Cdc42 and RhoA activation, a response that is concomitant with a mobilization of the actin filament system. Studies of epithelial-to-mesenchymal transdifferentiation of mouse mammary epithelial cells showed that ectopically expressed Smad7 was unable to block the morphological alterations seen in this process (Bhowmick et al., 2001). These observations are consistent with our findings and suggest that Smad7 has different and important roles in addition to its inhibitory function in TGF-β signaling.

Our previous work showed that p38 MAPK is involved in the Cdc42- and Rho-dependent activation of the actin filament system, and as Cdc42 is known to activate the p38 MAPK, we hypothesized Cdc42 to function upstream of p38 MAPK in this

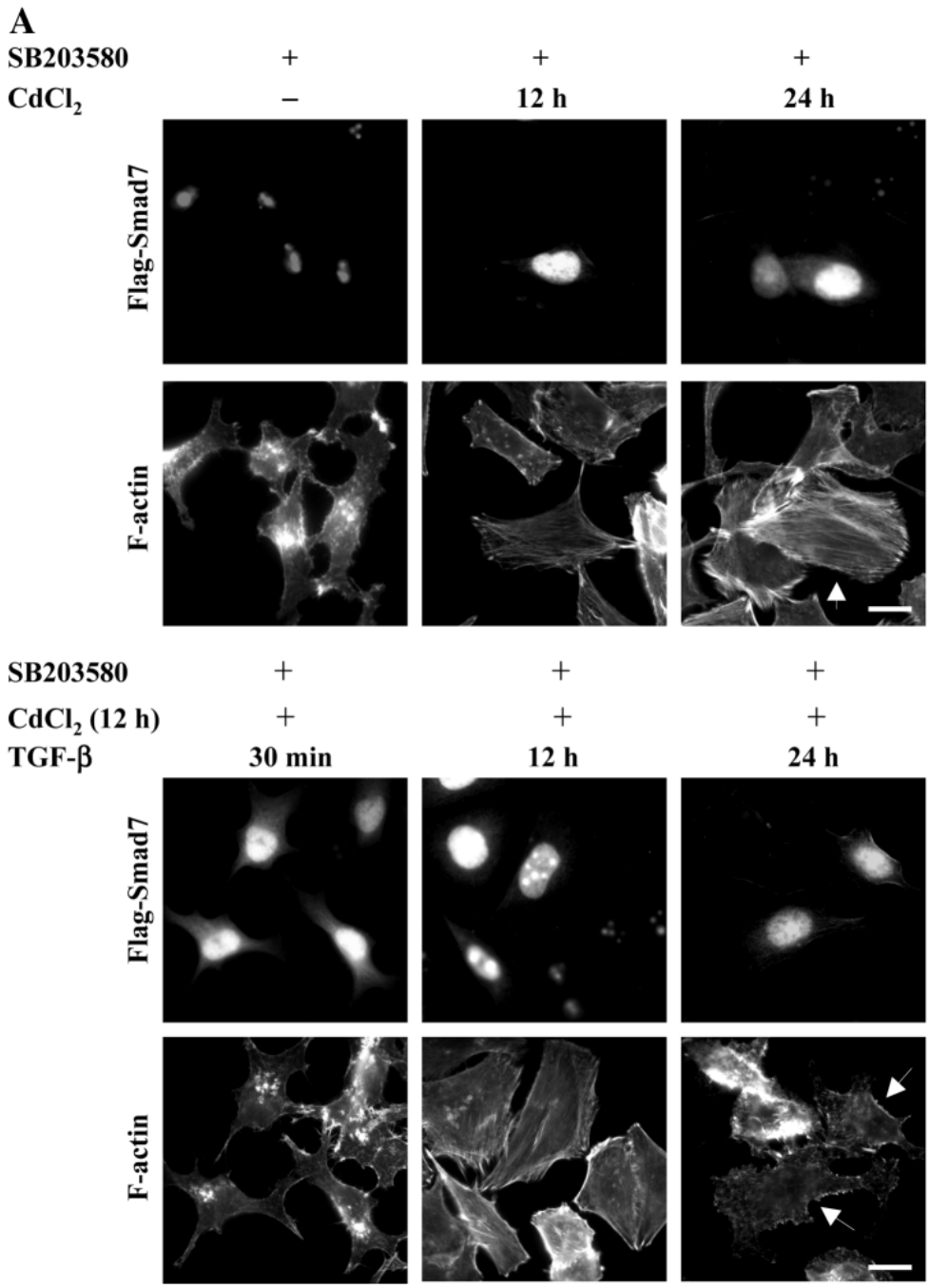
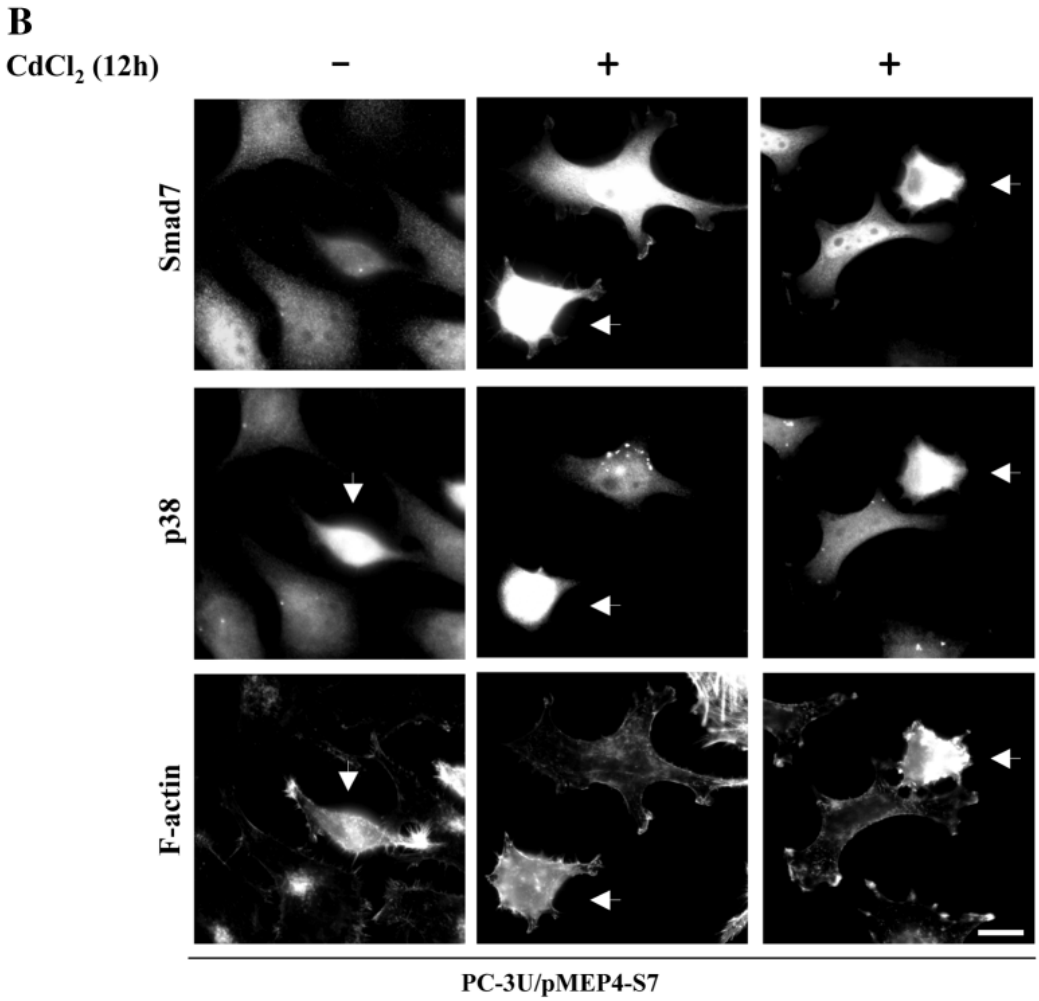



**Fig. 7.** The effect of p38 inhibitors MAPK and PI3K on Smad7-dependent activation of the actin filament system. (A) PC-3U/pMEP4-S7 cells were starved in 1% FBS for 12 hours and pretreated for 1 hour with 10 μM SB203580, before the addition of 1 μM of $CdCl_2$ for 12 hours or 24 hours, or 1 μM $CdCl_2$ for 12 hours together with 30 minutes, 12 hours or 24 hours of TGF-β. Flag-tagged Smad7 was visualized by a Flag-specific antibody followed by an FITC-labeled anti-mouse antibody. Filamentous actin was visualized by TRITC-labeled phalloidin. Arrowheads indicate cells with stress fibers (24 hours, upper panel) and cells lacking stress fibers (24 hours, lower panel). Bars, 20 μm. (B) PC-3U/pMEP4-S7 cells were transiently transfected with dominant negative mutant p38 MAPK and starved in 1% FBS for 12 hours. The cells were treated with 1 μM $CdCl_2$ for 12 hours. The expression of dominant negative p38 MAPK was visualized with a mouse monoclonal anti-p38 MAPK antibody followed by a TRITC-labeled anti-mouse antibody. Smad7 was detected by a goat anti-Smad7 antibody followed by an Alexa Fluor 488-labeled anti-goat antibody. Arrowheads denote transfected cells. Bar, 20 μm.

process (Edlund et al., 2002). Indeed, inactivation of small GTPases including Cdc42 with toxin B inhibited the TGF-β-induced activation of p38 MAPK, suggesting that Cdc42 resides upstream of p38 MAPK. In the present study, we noticed that under certain conditions p38 MAPK could reside upstream of Cdc42, as SB203580 treatment abolished the Smad7-dependent activation of Cdc42. The reason for this is not clear, but might be related to the ability of Smad7 to function as an adaptor




protein binding to several components in the p38 MAPK signaling pathway (Edlund et al., 2003). The signaling components linking the activated TGF-β receptor and Smad7-p38 MAPK to the activation of Cdc42 is currently not clear.

In addition, activation of phosphatidylinositol-3 kinase appears to be involved in Cdc42 activation. Our data show that stimulation of the TGF-β receptor triggered the activity of phosphatidylinositol-3 kinase, seen as the appearance of Akt phosphorylated on serine 473. Moreover, pretreatment with the phosphatidylinositol-3 kinase inhibitor LY294002 abolished the TGF-β-induced mobilization of the actin filament system. This latter response is analogous to signaling by tyrosine kinase receptors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), where the formation of membrane ruffles requires the conversion of phosphatidylinositol-4,5-bisphosphate to inositol-3,4,5-trisphosphate by a type I phosphatidylinositol-3 kinase (Tolias et al., 1995; Reif et al., 1996), an effect that is highly sensitive to phosphatidylinositol-3 kinase inhibitors (Stephens et al., 2002). Our data suggest that type II and type III phosphatidylinositol-3 kinase, which are important for the ability of SARA to present Smad2 and Smad3 for the TGF-β receptor (Tsukazaki et al., 1998), are not involved in the TGF-β-induced activation of Cdc42.

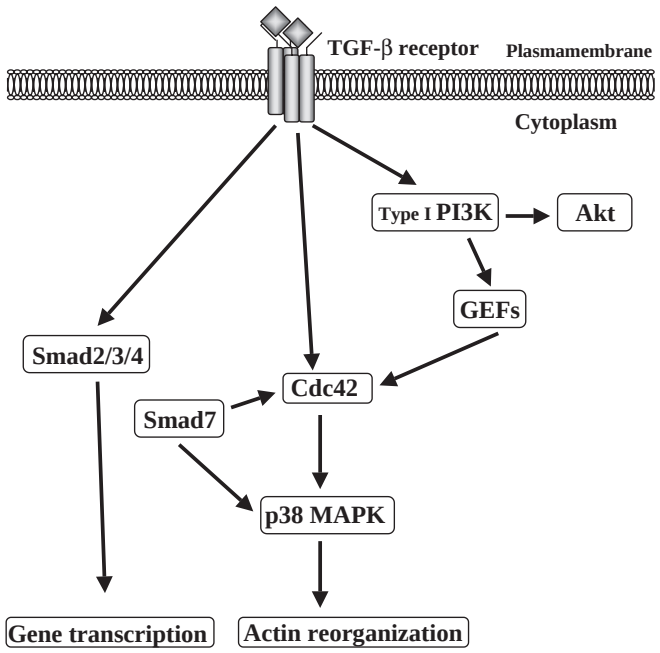



**Fig. 8.** The signaling components involved in TGF-β-induced activation of Cdc42 in PC-3U cells.

The activation of Rho GTPases is usually catalyzed by a GEF, and the activity of these proteins is dependent on phosphatidylinositol-3 kinase. There are several potential Cdc42-specific GEFs in mammalian cells. However, the currently available literature suggests the βPix/Cool GEF as a potential activator of Cdc42 (Bagrodia et al., 1998; Manser et al., 1998; Kim et al., 2001; Koh et al., 2001; Schmidt and Hall, 2002). Interestingly, βPix has been shown to enhance the p38 MAPK activation by a Cdc42/Rac/PAK/MKK3/6-mediated pathway, implicated in the regulation of membrane ruffling (Lee et al., 2001). However, it remains to be studied to what extent αPix or βPix participate in TGF-β-induced formation of membrane ruffles.

## Conclusion

We have shown that the activation of the Rho GTPase Cdc42 and the concomitant mobilization of the actin filament system require Smad7 and p38 MAPK. Moreover, we showed that TGF-β stimulation resulted in an activation of phosphatidylinositol-3 kinase, and we propose a model in which phosphatidylinositol-3 kinase activates a GEF, which in turn participates in the activation of Cdc42 (Fig. 8).

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