TGF β -induced Early Activation of the Small GTPase RhoA is Smad2/3-independent and Involves Src and the Guanine Nucleotide Exchange Factor Vav2

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Key Words

TGF β • RhoA • Src • Vav2 • Smad2/3

Abstract

TGF β has been shown to induce short- and long-term actin reorganization controlled by Rho-GTPase signaling. A number of direct Smad target genes, rapidly activated by TGF β , have been previously reported to control the long-term Rho activation and actin reorganization. However, the molecular mechanisms that regulate the prompt stimulation of Rho GTPases by TGF β remain unknown. In the present study we report that TGF β rapidly stimulated RhoA and RhoB activation in JEG3 choriocarcinoma cells that lack endogenous Smad3. Inhibition of Smad2 expression via siRNA-mediated silencing or by blocking its phosphorylation using the T β RI inhibitor SB431542 did not prevent the early RhoA/B activation by TGF β indicating that this effect is Smad2/3-independent. Pre-treatment of the cells with the general tyrosine kinase inhibitor Genistein blocked the TGF β -induced early RhoA activation. In line with this finding, TGF β -stimulation resulted in a quick activation of the non-receptor tyrosine kinase Src, followed by activation of the guanine nucleotide

exchange factor (GEF) Vav2. Inhibition of Src kinase by the selective inhibitor of the Src family tyrosine kinases PP2 totally blocked the early TGF β -induced RhoA activation. Similarly, Vav2 silencing via siRNA reduced the TGF β -induced RhoA activation implying that the rapid Src/Vav2 stimulation was effective in regulating RhoA activation. Our present findings provide for the first time a clear evidence for the role of Src and Vav2-GEF in the early Smad2/3-independent Rho activation by TGF β .

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Introduction

Transforming growth factor β (TGF β) is the prototype member of a large superfamily of pleiotropic cytokines that control various physiological processes. TGF β is an important homeostatic regulator in various epithelial and endothelial cells [1-3]. TGF β acts via a “classical” pathway which involves the heterotetrameric complex of two type I (T β RI) and two type II (T β RII) Ser/Thr kinase membrane receptors. After ligand binding, the type II receptor trans-phosphorylates and acti-

vates the type I receptor, leading to phosphorylation of the downstream cytoplasmic effector proteins Smad2 and Smad3, (collectively termed as receptor-activated Smads, R-Smads), which in turn oligomerize with the common partner Smad4 and rapidly translocate to the nucleus, where they regulate gene expression [4, 5]. Apart from this well-established genomic pathway, several non-Smad signaling effectors have been described, including the mitogen-activated protein kinases ERK, JNK and p38, the phosphatidylinositol 3'-kinase (PI3-kinase) and Ras- and Rho-GTPases [6-8]. These signaling pathways operating in parallel or in direct coordination with the Smad proteins regulate various early cell responses, including rapid actin reorganization [9, 10]. An extensively investigated signaling system of TGF β -induced cytoskeleton rearrangements includes the activation of the small Rho GTPases (for a recent review see [11]).

Rapid Rho-GTPase activation was found to operate in a variety of cell models of TGF β -induced epithelial to mesenchymal transition (EMT) such as in the atrioventricular canal (AVC) of the embryonic chicken heart [12], during tubulointerstitial fibrosis [13] and in proliferative vitreoretinopathy (PVR) [14]. In addition to their role in EMT, Rho-GTPases and their downstream effectors are activated rapidly by TGF β and contribute to cytoskeletal reorganization in various cell systems. Indeed, TGF β was reported to promote the differentiation of neural crest cells into vascular smooth muscle cells via a rapid activation of RhoA and ROCK1 [15] and the differentiation of rat pulmonary arterial smooth muscle cells via a rapid activation of RhoA and its downstream kinases ROCK, PKN/PRK2 and p38 MAPK. Moreover, non-genomic signaling involving MAP kinases or the PI3 kinase as downstream effectors that cooperate with Rho-GTPases to achieve TGF β -induced cytoskeleton remodeling was reported in human prostate cancer cells [16, 17]. In previous studies we have thoroughly investigated the short-term and sustained actin cytoskeleton reorganization, the implication of early Rho-signaling and the cross-talk with Smad-pathways in TGF β treated fibroblasts [18-21]. In these cells, TGF β initiated rapid activation of the RhoA/B/ROCK/LIMK2/cofilin pathway regulating stress fiber formation [18, 19], but also a fibroblast to myofibroblast differentiation program, involving enhanced expression of α -SMA gene [20]. It was further demonstrated that the long-term actin reorganization was controlled by a Smad-dependent regulation of RhoB – but not RhoA – gene transcription in both, fibroblasts [20] and HaCaT keratinocytes [21]. While some direct Smad target genes that are rapidly activated

by TGF β , have been reported to control the long-term Rho activation and actin reorganization, the involvement of early signaling that may regulate the rapid stimulation of Rho GTPases in response to TGF β remains unknown.

In the present study we have used a cellular model that lacks endogenous Smad3 expression (JEG3 choriocarcinoma cells) [22] to identify early TGF β -activated molecular targets that may control the rapid activation of Rho-GTPases. We report that TGF β rapidly stimulated RhoA- and RhoB-GTPases. This effect was Smad2/3 independent as documented by specific silencing of Smad2, or by blocking of the T β RI by its specific inhibitor SB431542. Moreover, TGF β activates rapidly the non-receptor tyrosine kinase Src and its downstream effector, the Rho-GEF Vav2. Inhibition of Src by PP2 totally blocked the early TGF β -induced RhoA activation, while silencing of Vav2 by a specific siRNA inhibited the TGF β -induced RhoA activation. These findings imply for the first time that the rapid activation of Src/Vav2 may control the early Rho-GTPase activation upon TGF β stimulation.

Methods and Materials

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin for cell culture, Superscript II reverse transcriptase, dNTPs and Lipofectamine 2000 were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN, USA). Recombinant mature human TGF β 1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The ECL western blotting analysis system and the illustra RNAspin Mini RNA isolation kit were purchased from GE Healthcare (Uppsala, Sweden). Anti-Smad2 (sc-6200), anti-RhoA (sc-26C4), anti-RhoB (sc-180), anti-Rac1 (sc-95) and anti-Cdc42 (sc-8401) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-P-Smad2 (3101S) and anti-Vav2 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-P-Src Y418 and anti-P-Src Y529 were purchased from Biosource/Invitrogen (Carlsbad, CA, USA). The SB431542 inhibitor and Genistein were purchased from Sigma-Aldrich (St Louis, MI, USA). Anti-actin, secondary anti-mouse IgG and anti-rabbit IgG horseradish peroxidase conjugate were from Chemicon International Inc (Temecula, CA, USA). The PP2 inhibitor was purchased from Calbiochem (La Jolla, CA, USA) and the GST-RhoGTPase-RBD protein beads and the GST-PAK-PBD protein beads were purchased from Cytoskeleton Inc. (Denver, USA). Short-interfering (si) RNA for Smad2 and Vav2 were purchased from Eurofins MWG Operon (Ebersberg, Germany) and the negative control siRNA was purchased from AMBION (Austin, USA). Maxima SYBR Green/ROX qPCR Master Mix was purchased from Fermentas (Maryland, USA).

Cell culture and transient transfections

Human JEG3 choriocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, glutamine and penicillin/streptomycin at 37°C. Transient transfections in JEG3 cells with negative control siRNA or siRNAs targeting Smad2 or Vav2 were performed in two rounds with Lipofectamine 2000 according to manufacturer's instructions.

Quantitative reverse transcription PCR (qRT-PCR)

RNA extraction was performed with the illustra RNAspin Mini RNA isolation kit according to manufacturer's instructions. The first cDNA strand was synthesized by Superscript II reverse transcriptase and qRT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix. β -actin house-keeping gene was used as an internal control for mRNA normalization.

Determination of GTP-loaded RhoA, RhoB and Rac1, Cdc42 levels

For affinity precipitation with GST–RhoA/RhoB protein beads, cells were either transiently transfected in two rounds with negative control siRNA or specific siRNAs targeting Smad2 or Vav2 with Lipofectamine 2000, were serum-starved for 24 hours and then stimulated with 5 ng/ml TGF β or not for 5 minutes or alternatively pretreated with the inhibitors SB431542, PP2 and Genistein and then stimulated with 5 ng/ml TGF β or not for 5 minutes. The cells were then washed with ice-cold phosphate-buffered saline and lysed with Cell lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 0.3 M NaCl, 2% IGEPAL, supplemented with protease inhibitors). Equal volumes of cleared cell lysates were incubated with 20 μ l (60 μ g) GST–RhoA/RhoB protein beads attached to glutathione-sepharose at 4°C for 1 hour. Beads were washed 3 times with wash buffer (25 mM Tris pH 7.5, 30 mM MgCl₂, 40 mM NaCl), resuspended in sample buffer and analyzed in parallel with equal volumes of total cell lysates (TCL) on a 12% polyacrylamide gel by SDS-PAGE followed by immunoblotting with RhoA or RhoB antibody.

The same procedure was followed for the determination of the Rac1 and CDC42 activity by using instead the GST-PAK-PDB protein beads attached to glutathione-sepharose and specific antibodies for Rac1 and CDC42 in the western blots.

Quantification of the immunoblots was performed with Tinascan v.2.07d software (Raytest GmbH, Germany).

Affinity precipitation of active Vav2 GEF with GST-RhoA (G17A) sepharose beads

For affinity precipitation of active Vav2 GEF with the mutated, nucleotide-free form of RhoA, GST-RhoA (G17A) bound on sepharose beads, cells were serum-starved for 24 hours and then treated with 5 ng/ml TGF β for various time points. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer (20 mM Hepes pH: 7.4, 150 mM NaCl, 5 mM MgCl₂, 1% Triton). Cleared cell lysates were incubated with 20 μ g of freshly made GST-RhoA (G17A) sepharose beads at 4°C for 1 hour. Beads were then

Fig. 1. RhoA and RhoB are activated in Smad3 deficient JEG3 choriocarcinoma cells after short TGF β treatment. Analysis of RhoA (A) and RhoB (B) activation levels by GST–RhoA/RhoB pull-down assay in JEG3 cells treated with 5 ng/ml TGF β for various time points (5 to 30 minutes). A and B show representative figures from n=3 and n=2 distinct experiments respectively.

washed 3 times with lysis buffer, resuspended in sample buffer and analyzed in parallel with total cell lysates (TCL) on a 10% polyacrylamide gel by SDS-PAGE followed by immunoblotting with Vav2 antibody.

For preparation of the GST-RhoA (G17A) sepharose beads, competent bacteria were transformed with the pGEX-GST RhoA (G17A) expression vector (generous gift from K. Burridge) [23]. Bacteria were grown in a 200 ml culture (1:50 dilution) at 37°C for approximately 2 hours until OD_{600nm}=0.6 and the GST-RhoA (G17A) protein expression was induced with 0.5 mM IPTG at room temperature for 16 hours or at 30°C for 5 hours. Bacteria were then resuspended in 10 ml STE buffer (10 mM Tris-HCl pH: 8.0, 150 mM NaCl, 1 mM EDTA). The GST-RhoA (G17A) protein was extracted from the bacteria after sonication and addition of 1% Triton X-100 followed by incubation on ice for 20 minutes. The protein was bound to sepharose beads after incubation of the lysate with precleared glutathione-sepharose beads at 4°C for 4 hours. Finally the beads were washed 2 times with STE buffer supplemented with 1%

Fig. 2. The early activation of RhoA and RhoB by TGFβ is Smad-independent. RhoA (n=3) (A) and RhoB (n=2) (B) activation levels by GST- RhoA/RhoB pull-down assay in JEG3 cells transiently transfected with a negative control (NC) siRNA or a specific siRNA that targets Smad2 and treated or not with 5 ng/ml of TGFβ for 5 minutes. (C) Analysis of Smad2 protein levels showing the efficiency of Smad2 silencing in the RhoA and RhoB activation assays in panels A and B. Analysis of RhoA (n=3) (D) and RhoB (n=2) (E) activation levels by GST- RhoA/RhoB pull-down assay in JEG3 cells treated with 5 ng/ml TGFβ or not for 5 minutes in the presence or absence of the TGFβ type I receptor (ALK5) inhibitor SB431542 (10 μM). (F) Analysis of P-Smad2 levels showing the efficiency of SB431542 inhibitor in the RhoA and RhoB activation assays in panels D and E. β-actin serves as immunoblot loading control in panels C and F.

Triton X-100 and 1 mM DTT, 2 times with high salt buffer (10 mM Tris HCl pH: 8.0, 500 mM NaCl, 1 mM DTT) and 1 time with PBS supplemented with 1 mM DTT and analyzed with SDS-PAGE and Coomassie staining for the determination of the concentration of the GST-RhoA (G17A) protein on the sepharose beads.

Results

Early RhoA/B activation by TGFβ in the absence of Smad2 and Smad3

The rapid and sustained activation of Rho GTPases by TGFβ was reported previously in various cell models, including fibroblasts [18, 19], HaCaT keratinocytes [21], PC3 prostate cancer cells [16, 24], retinal pigment epithelial cells (RPE) [14] and rat pulmonary arterial smooth muscle cells [15]. In order to investigate the role of the Smad pathway in the early TGFβ-induced Rho

activation, we used JEG3 choriocarcinoma cells that lack the endogenous Smad3 protein. Cells were serum-starved for 24 hours and then stimulated with 5 ng/ml TGFβ for 5 to 30 minutes. The activity of RhoA was assessed by affinity precipitation using the GST- RhoA-RBD agarose beads. Fig. 1A demonstrates a clear and significant early activation of RhoA GTPase (5 to 15 minutes upon TGFβ treatment) that declined after 30 minutes. Similarly, RhoB activation was evident after 5 minutes of TGFβ addition, peaked at 10 minutes and declined after 30 minutes of TGFβ stimulation.

To investigate whether the observed rapid activation of RhoA and RhoB by TGFβ is Smad2-independent, we silenced the expression of the endogenous Smad2 gene by transfecting the Smad3-deficient JEG3 cells with a siRNA that specifically silences Smad2. As shown in Figs. 2A and 2B, the early TGFβ induced RhoA and RhoB activation remained unaffected in Smad2 knock out cells,

implying that this effect is Smad2- and Smad3-independent. The efficacy of the specific Smad2 siRNA on the expression of Smad2 is demonstrated in Fig. 2C. Furthermore, we used the specific inhibitor SB431542 that blocks the TGF β type I receptor kinase in order to provide additional experimental evidence on the role of the TGF β type I receptor. For this, cells were pretreated with the SB431542 inhibitor for 1 hour, then stimulated with TGF β for 5 minutes and the activation of both RhoA and RhoB was evaluated using the GST-RhoA pull down assay. Figs 2D and 2E document that RhoA and RhoB remained activated after stimulation with TGF β in the presence of the SB431542 inhibitor. The efficiency of the inhibitor in blocking Smad2 phosphorylation is shown in Fig. 2F. These results clearly indicate that TGF β promotes rapid RhoA and RhoB activation via a molecular mechanism that does not involve the kinase activity of the type I receptor and neither of the two specific R-Smads that this receptor phosphorylates.

TGF β induces rapid Src and Vav2 activation

Searching for molecular targets that may link TGF β to the rapid Rho activation, we first addressed the possible involvement of tyrosine kinases in this process. For this purpose, cells were initially pre-treated with the general tyrosine kinase inhibitor Genistein for 1 hour, stimulated with 5 ng/ml TGF β for 5 minutes, followed by measurement of the RhoA activity using the GST-RhoA pull down assay. As shown in Fig. 3A, increasing concentrations of Genistein significantly inhibited the early RhoA activation by TGF β implying the involvement of tyrosine kinase effectors in this process. To further explore this finding we assessed the phosphorylation levels of the non receptor tyrosine kinase Src. We focused on Src since several reports in the past have documented c-Src regulation by TGF β [25-29]. As shown in Fig. 3B, TGF β treatment induced a rapid activation of Src tyrosine kinase, as estimated by the increase in the phosphorylation of residue Y418, which is necessary for full catalytic activity. This activation peaked at 5 minutes and persisted for up to 60 minutes, while the inhibitory phosphorylation of Src in residue Y529, became evident only after 15 minutes of TGF β stimulation indicating that the activation of Src by TGF β is tightly regulated. In a control experiment we monitored the phosphorylation of Smad2 by TGF β and found that Smad2 phosphorylation could be detected after 5 minutes, became more evident after 15 minutes and persisted for at least 60 minutes (Fig. 3B).

Fig. 3. TGF β induces rapid Src and Vav2 activation. A. Analysis of RhoA activation levels by GST-RhoA pull-down assay in JEG3 cells treated with 5 ng/ml TGF β or not for 5 minutes in the presence or absence of increasing concentrations (50 and 100 μ M, triangle) of the general tyrosine kinase inhibitor Genistein (Representative figure from n=3 distinct experiments). B. Analysis of the phosphorylation levels of Src kinase in residues Y418 (auto-phosphorylation site necessary for full catalytic activity) and Y529 (negative regulatory site) in JEG3 cells treated with 5 ng/ml TGF β for various early timepoints (5 to 60 minutes) (Representative figure from n=2 distinct experiments). Immunoblotting for P-Smad2 shows the activation of the classical TGF β /Smad pathway. β -actin serves as immunoblot loading control. C. Analysis of Vav2 activation by the GST-RhoA (G17A) pull-down assay, using the mutated and nucleotide-free form of RhoA (G17A) that exhibits high affinity for activated GEFs, in JEG3 cells treated with 5 ng/ml TGF β for various early time points (5 to 60 minutes) (Representative figure from n=3 distinct experiments).

Src activation has been linked to the phosphorylation and activation of the Rho-GEF Vav2 [30-32], which in turn may be associated with the activation of the small Rho GTPases [33, 34]. Vav2 has been functionally linked to the three major Rho family members, Rac1, Cdc42 and RhoA [31, 32, 35-39]. To explore the role of Vav2 in RhoA activation by TGF β , we used an affinity precipitation assay that specifically detects activated GEFs

Fig. 4. Src and Vav2 activation is required for the TGFβ induced early RhoA stimulation. (A) Analysis of RhoA activation levels by GST- RhoA-RBD pull-down assay in JEG3 cells treated with 5 ng/ml TGFβ or not for 5 minutes in the presence or absence of the Src kinase inhibitor PP2 (10 μM) (Representative figure from n=3 distinct experiments). Immunoblotting for P-Src Y418 shows the efficiency of the PP2 inhibitor. β-actin serves as immunoblot loading control. (B) Analysis of Vav2 mRNA levels by qRT-PCR showing the efficiency of Vav2 silencing in the RhoA activation assay in panel C. (C) Analysis of RhoA activation levels by GST- RhoA-RBD pull-down assay in JEG3 cells transiently transfected with a negative control (NC) siRNA or a specific siRNA that targets Vav2 and treated or not with 5 ng/ml of TGFβ for 5 minutes (Representative figure from n=4 distinct experiments). (D) Densitometric quantification (% percentage) of RhoA activity (RhoA-GTP/RhoA total) of the immunoblot in panel C in TGFβ-stimulated versus non-stimulated cells treated with either NC siRNA or siVav2. In both parts of the graph the RhoA activity in the non-stimulated cells is set to 1.

using a mutated, nucleotide-free form of RhoA (G17A) that exhibits high affinity only for activated GEFs [23]. For this purpose, JEG3 cells were serum-starved for 24 hours, stimulated with 5 ng/ml TGFβ for 5 to 60 minutes and the activity of Vav2 GEF was estimated by pull down assay using the GST-RhoA (G17A) sepharose beads. Fig. 3C shows a rapid (5 minutes) Vav2 activation upon TGFβ treatment which peaked at 15 minutes and steadily declined afterwards. It is noted that the kinetics of the pull-down experiments (Fig. 3C) closely correlate to the kinetics of Src phosphorylation (Fig. 3B). These findings clearly show that TGFβ treatment induces rapid phosphorylation of Src and activation of Vav2 in JEG3 cells.

Src and Vav2 activation is required for the TGFβ induced early RhoA stimulation

We further explored whether the rapid activation of Src and Vav2 by TGFβ is directly involved in the early, Smad2/3-independent RhoA activation. For this, we first pretreated JEG3 cells with the specific Src kinase inhibitor PP2 for 1 hour, then stimulated with 5 ng/ml TGFβ for 5 minutes and assessed the RhoA activation with the GST-RhoA-RBD pull down assay. Interestingly, the PP2 inhibitor fully blocked the observed early RhoA activation by TGFβ (Fig. 4A), clearly indicating that the Src kinase activity is necessary for this effect. The efficacy of PP2 in inhibiting Src activity is shown in Fig. 4A, lower panel. To further explore the potential direct involvement of Vav2

Fig. 5. A model summarizing the current findings and emphasizing the short-term TGF β signaling towards RhoA regulation and actin reorganization. The long-term activation events towards RhoA/B regulation and actin reorganization involving Smad-dependent transcriptional regulation are also briefly presented.

in RhoA activation by TGF β , cells were transfected with a siRNA that targets and significantly downregulates Vav2 expression as indicated by qRT-PCR analysis (Fig. 4B). When cells were transfected with the Vav2 siRNA in the absence of TGF β , a reproducible but moderate (up to 1,8 fold) induction of RhoA activity was observed relative to the activity of RhoA after control siRNA transfection (Fig. 4C, lane 3). This finding implies that downregulation of endogenous Vav2 may increase basal RhoA activity. This observation could be attributed to the reported role of Vav2 in regulating Rac1 and explained by a competitive mechanism between Rho and Rac activity which could be triggered after Vav2 silencing [40]. Irrespective of the exact mechanism however, the measured effect of Vav2 silencing on basal RhoA activity is rather weak. Interestingly, after TGF β stimulation, silencing of Vav2 almost completely blocked the ligand-dependent activation of RhoA (Fig. 4C, lanes 3 and 4, Fig. 4D), implying that the observed Vav2 activation is directly implicated in the early activation of RhoA by TGF β .

As mentioned above, Vav2 activation by TGF β as monitored by the GST-RhoA (G17A) pull down assay could also implicate the activation of Rac1 and Cdc42, [33, 34]. To explore this possibility, we measured the activity of Rac1 and Cdc42 after short TGF β treatment in JEG3 cells by using the GST-PAK-PBD pull down assay. Interestingly, no modulation in the activity levels of either Rac1 or Cdc42 could be observed in this cell model

(data not shown), while in Swiss 3T3 fibroblasts we even observed a significant downregulation in Rac1 and Cdc42 activity (data not shown) after short treatment with TGF β . The above findings further emphasize that the TGF β induced activation of Vav2 in JEG3 cells leads to RhoA but not to Rac1 or Cdc42 stimulation.

Discussion

Rapid activation of members of the Rho-family of small GTPases by TGF β , leading to control of actin cytoskeleton rearrangements has been reported in a variety of cell types; however, the molecular mechanisms that regulate this early event remain poorly defined. In the present study we provide strong experimental evidence that TGF β rapidly activates Src/Vav2 signaling, which is directly involved in the early activation of RhoA. This conclusion is supported by a) the rapid TGF β -induced phosphorylation of Src at residue Y418, b) the pull-down experiments showing that Vav2 is activated as early as 5 minutes upon TGF β treatment and c) the inhibition of TGF β -induced RhoA activation when cells were pretreated either with the specific Src inhibitor PP2, or transfected with a small interfering RNA targeting Vav2.

We have used in this study the JEG3 choriocarcinoma cell line that lacks endogenous Smad3 thus representing a valuable model to analyze rapid TGF β effects. Treatment of these cells with TGF β generated

an early (within 5 minutes) and robust activation of RhoA/B GTPases, confirming reports in other cell models that this cytokine may influence rapidly Rho signaling. To exclude the involvement of the classical TGF β /Smad-signaling pathway, RhoA/B activation was assayed in cells pretreated either with the specific T β RI inhibitor SB431542 or in cells transfected with a siRNA targeting Smad2. Both series of control experiments revealed convincingly that the TGF β -induced early RhoA/B activation remained unaffected. Taking into account that JEG3 cells do not express Smad3, these results support the notion that the rapid activation of the RhoA/B small GTPases by TGF β is a Smad2 and Smad3-independent process. Accordingly, it can be clearly differentiated from the long-term TGF β -induced Rho activation (and actin reorganization) that has been previously reported to be controlled, at least in part, by genes that are activated by the TGF β /Smad pathway including RhoB [21], endothelin-1 [41, 42], sphingosine kinase-1 [43, 44], the Rho guanine exchange factor (GEF) Net1 [45] and the alpha-smooth muscle actin (α -SMA) [20].

Several reports have documented differential c-Src regulation by TGF β . TGF β has been shown to down-regulate Src family kinases in PC3 prostate cancer cells, playing a crucial role in the regulation of cell growth [25]. Moreover, TGF β was reported to significantly decrease v-Src kinase activity and protein abundance in Src-transformed 3Y1 fibroblasts (SR-3Y1) [26]. On the other hand, Src was activated by TGF β in mammary epithelial cells [27], in human mesangial cells [28], while TGF β -dependent Src signaling activation was also reported to control urokinase up-regulation and invasion in human ovarian cancer cells [29]. In line with the latter reports, in the present study we clearly demonstrate an early and significant increase in the autophosphorylation of Src on residue Y418 by TGF β , which is required for full catalytic activity. Moreover, this effect was shown to be directly implicated in the rapid RhoA activation by this cytokine in JEG3 choriocarcinoma cells, supporting thus the pleiotropic action of TGF β . Since RhoA activation was not inhibited by the specific T β RI inhibitor (Fig. 2A) we assume that the observed Src (and Vav2) activation is most probably triggered by the T β RII Ser/Thr kinase membrane receptor. This assumption is in line with recent reports that link Src kinase to T β RII receptor through β 3 integrins [46].

Previous studies indicated that Src activation by phosphorylation may stimulate the Rho-GEF Vav2 [30-32],

which in turn may associate with, and activate the Rho GTPases [33, 34]. In the present work we provide for the first time clear experimental evidence for a direct involvement of the Src/Vav2 signaling effectors in TGF β -induced rapid RhoA GTPase activation. It should be noted that Vav2 is not a specific GEF for RhoA, as it has been reported to be able to activate all three major small Rho GTPases (Rac1, Cdc42 and Rho) [33, 34]. Accordingly, the observed early activation of Vav2 by TGF β may also implicate the activation of Rac1 and Cdc42. However, we show that TGF β does not modulate the activation of Rac1 and Cdc42 in JEG3 cells at early time points (data not shown) or even downregulates Rac1 and Cdc42 activity after short TGF β treatment in another cell system (Swiss 3T3 fibroblasts, data not shown), which further support our conclusions that the TGF β induced activation of Vav2 is linked to RhoA activation. In other cell types it is possible that TGF β regulates Rac1 or Cdc42 activity via Vav2, which remains to be examined in future studies. Interestingly, these early effects reported in our study are clearly different from the previously reported transcriptional regulation of Vav family members by TGF β [47], or the reported TGF β -induced recruitment of the HER2/Vav2/Rac1/Pak1/actin/actinin complex to lamellipodia that may increase the metastatic properties and survival of HER2-overexpressing cells [48].

In conclusion, in the present study we provide novel mechanistic insights highlighting the early activation of the Src/Vav2 signaling that regulates the rapid and Smad2/3-independent TGF β -induced RhoA GTPase activation, providing a link between the TGF β receptor complex and the rapidly activated Rho/ROCK/LIMK2/cofilin pathway (Fig. 5). This mechanism is clearly different from the previously reported long-term transcriptional regulation of the small GTPase RhoB by TGF β involving the Smad machinery [20, 21] (Fig. 5). It will be interesting to explain how the early and late mechanisms of Rho-GTPase activation are linked with each other or whether the early and late mechanisms serve distinct functions, which are of course both based on the modulation of actin dynamics in cells that respond to TGF β .

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