p21-activated Kinase 2 (PAK2) Inhibits TGF-β Signaling in Madin-Darby Canine Kidney (MDCK) Epithelial Cells by Interfering with the Receptor-Smad Interaction*

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Background: PAK2 is a mediator of TGF-β in mesenchymal cells. However, whether PAK2 could modulate TGF-β signaling remains elusive.

Results: PAK2 associates with Smad2/3 and phosphorylates Smad2 at Ser417, thus inhibiting the TGF-β-induced Smad2-TβRI association and signal transduction in MDCK cells.

Conclusion: PAK2 inhibits TGF-β signaling in MDCK epithelial cells.

Significance: This study unravels a novel regulatory mechanism of R-Smad activity.

The serine/threonine kinase p21-activated kinase 2 (PAK2) (transforming growth factor β) plays a variety of cellular functions mainly through the Smad pathway. Phosphorylation of the carboxyl SXS motif in R-Smads (Smad2 and Smad3) by the type I receptor TβRI is a key step for their activation. It has been reported that the serine/threonine kinase PAK2 (p21-activated kinase 2) can mediate TGF-β signaling in mesenchymal cells. Here, we report that PAK2 restricts TGF-β-induced Smad2/3 activation and transcriptional responsiveness in MDCK epithelial cells. Mechanistically, PAK2 associates with Smad2 and Smad3 in a kinase activity-dependent manner and blocks their activation. PAK2 phosphorylates Smad2 at Ser-417, which is adjacent to the L3 loop that contributes to the TβRI-R-Smad association. Consistently, substitution of Ser-417 with glutamic acid attenuates the interaction of Smad2 with TβRI. Together, our results indicate that PAK2 negatively modulates TGF-β signaling by attenuating the receptor-Smad interaction and thus Smad activation.

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine regulating diverse cellular processes in a cell type- and context-dependent manner (1–5). To achieve this, TGF-β elicits multiple signaling pathways, and the R-Smads (receptor-regulated Smad proteins) (Smad2 and Smad3 for TGF-β signaling)-mediated canonical pathway is involved in most of TGF-β-mediated functions (6–10). TGF-β initiates signal transduction via binding to a pair of serine/threonine kinase receptors, the type II receptor (TβRII), and the type I receptor (TβRI), which is phosphorylated by TβRII for activation. TβRI then activates Smad2 and Smad3 through phosphorylation at the farmost C-terminal SXS motif, leading to their oligomerization with Co-Smad (common Smad, Smad4), nuclear translocation and transcriptional activation.

R-Smad phosphorylation by TβRI serves as a key step for Smad activation and requires the direct association between the receptor and Smads. The L45 loop of TβRI and the L3 loop of R-Smads were shown to determine the specificity of this interaction (10–14). R-Smads have been reported to be phosphorylated at various sites by other kinases, such as MAPks (mitogen-activated protein kinases), CDK2/4 (cyclin-dependent kinase2/4), GSK-3β (glycogen synthase kinase 3β), and ROCK (Rho-associated protein kinase), which finely regulate the activities of R-Smads and integrate different signal inputs (reviewed in Refs. 15, 16).

The serine/threonine kinase p21-activated kinase 2 (PAK2) is a member of the group I PAK family (17–19). At the N terminus, it contains a Cdc42/Rac1 GTPase binding domain and an auto-inhibitory domain, which binds to the C-terminal kinase domain and keeps it at an inactive state. Binding of Cdc42/Rac1 to PAK2 changes its conformation, relieves the auto-inhibition and frees the kinase domain, resulting in its activation. In addition, apoptotic stimuli such as DNA damage lead to caspase-mediated cleavage of PAK2, generating a constitutively active kinase fragment p34 and an N-terminal regulatory fragment p27 (20, 21). Several substrates of PAK2 have been identified in various contexts and mediate its wide range of cellular events, from cytoskeletal rearrangements to survival promotion and cellular transformation (19, 22). Interestingly, PAK2 was reported to mediate TGF-β signaling in mesenchymal functions

The abbreviations used are: TGF-β, transforming growth factor-β; PAK2, p21-activated kinase 2; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor; R-Smad, receptor-regulated Smad protein; WT, wild-type; ca, constitutively active; KR, kinase-deficient; IP, immunoprecipitation; IB, immunoblotting; WCL, whole cell lysates.

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PAK2 and Smad7 were able to attenuate TGF-β-induced transcription, whereas PAK1 had little effect, suggesting a specific inhibitory effect of PAK2. These results altogether suggested that PAK2 negatively regulates TGF-β signaling in MDCK cells, and this inhibition depends on its kinase activity. Similar results were obtained with constitutively active PAK2 mutant (T402E, TE) which showed a stronger inhibition while the kinase-deficient PAK2 mutant (K278R, KR) had no effect, supporting that this inhibitory effect of PAK2 requires its kinase activity. Similar results were obtained with CAGA-luciferase (Fig. 1B). PAK2 also inhibited the constitutively active TGF-β type I receptor (ca-TβRI)-driven expression of ARE-luciferase and CAGA-luciferase (data not shown).

Next we consolidated these findings by testing the effect of Rac1, which is an upstream activator of PAK2, on TGF-β-induced expression of ARE-luciferase in MDCK cells. Like PAK2, wild-type Rac1 attenuated the reporter expression to some extent, and the constitutively active Rac1(V12) decreased it further, alone or in cooperation with PAK2 (Fig. 1C), whereas the dominant-negative Rac1(N17) enhanced the reporter expression. Furthermore, IPA-3, an allosteric PAK inhibitor (29), enhanced the reporter expression in a dose-dependent manner. Since Rac1 and IPA-3 could also act on PAK1, which is closely related to PAK2, we examined whether PAK1 could affect TGF-β-induced transcriptional response. As shown in Fig. 1D, both PAK2 and Smad7 were able to attenuate TGF-β-driven expression of CAGA-luciferase reporter, whereas PAK1 had little effect, suggesting a specific inhibitory effect of PAK2. These results altogether suggested that PAK2 negatively regulates TGF-β signaling in MDCK cells, and this inhibition depends on its kinase activity.

To examine whether PAK2 has a more general inhibitory effect on TGF-β-driven transcription, we carried out CAGA-luciferase reporter assays in other cell lines. PAK2 attenuated the reporter expression in HEK293, NMuMG, and HaCaT cells.
in varying degrees, but showed no effect in NIH3T3 fibroblasts (Fig. 1E).

**PAK2 Inhibits TGF-β-induced R-Smads Activation and Signaling**—Having showed the inhibitory effect of PAK2 on TGF-β-induced transcription in MDCK cells, we continued to investigate whether PAK2 affects TGF-β-induced Smad2 phosphorylation, using an antibody specifically recognizing the phosphorylated carboxyl S465MS467 motif of Smad2. As shown in Fig. 2A, both of wild-type and TE mutant PAK2 decreased TGF-β-induced phospho-Smad2 levels, but PAK2(KR) had little effect. Consistently, IPA-3 treatment enhanced the phospho-Smad2 level (Fig. 2B).

Next we tested whether PAK2 regulates the R-Smads/Smad4 interaction. Constructs encoding GST-fusion Smad4, Flag-tagged Smad2, HA-tagged ca-TβRI, and Myc-tagged PAK2 (WT, TE and KR) were transfected into HEK293T cells as indicated in Fig. 2C. At 40 h post-transfection, the cells were harvested for GST pulldown followed by anti-Flag immunoblotting (IB). Both WT- and TE-PAK2, but not KR-PAK2, attenuated the Smad2-Smad4 interaction. In addition, an immunoprecipitation-immunoblotting assay showed that PAK2 inhibited Smad3-Smad4 oligomerization dependently on the kinase activity (Fig. 2D). These results demonstrated that PAK2 interferes with Smad2 activation and attenuates TGF-β signaling in MDCK epithelial cells.

**PAK2 Associates with Smad2/3 in a Kinase Activity-dependent Manner**—As PAK2 attenuated TGF-β-induced Smad2 phosphorylation and subsequent Smad2-Smad4 complex formation, it is likely to function at or upstream of the Smad level. To explore the underlying mechanism, we attempted to test
whether PAK2 associates with components of the TGF-β/Smad pathway. It was showed that PAK2 does not associate with TGF-β receptors TβRI or TβRII (23), thus we examined if PAK2 interacts with Smad proteins. GST pulldown assay in HEK293T cells revealed that only Smad2 and Smad3 interacted with PAK2 (Fig. 3A). This interaction was further confirmed at the endogenous protein level in MDCK cells (Fig. 3B). Blocking of PAK2 kinase activity with IPA-3 completely abolished their interaction, while TGF-β treatment had no effect, suggesting the interaction is regulated by the PAK2 kinase but not by TGF-β.

GST pulldown assay also showed the kinase-active PAK2(TE) had a stronger interaction with Smad2 in comparison to wild-type PAK2, while the kinase-deficient mutants K278R (KR) and T402A (TA) dramatically decreased the interaction. Co-expression of constitutively active Rac1(V12) also strengthened the PAK2-Smad2 interaction, whereas dominant-negative Rac1(N17) and IPA-3 diminished their binding (Fig. 3D), indicating the kinase activity of PAK2 is essential for its binding to Smad2. A similar experiment showed the kinase activity was also required for PAK2 to associate with Smad3 (Fig. 3E).

The effect of TGF-β signaling on the PAK2-Smad2/3 interaction was then examined by co-immunoprecipitation experiments in HEK293T cells. As shown in Fig. 3F, the interaction between PAK2 and Smad2 or Smad3 were at the similar levels in the presence of the constitutively active (CA) and wild-type TβRI, which is consistent with the above observation that TGF-β had no effect on this interaction. These results together supported the notion that the PAK2-Smad2/3 interactions are induced by the kinase activity of PAK2, but not affected by TGF-β signaling.

PAK2 Phosphorylates Smad2 at Ser417 and the Phosphorylation Blocks TGF-β-induced Smad2 Activation—The notion that PAK2 associates with R-Smads in a kinase activity-dependent manner suggests a kinase-substrate relationship. To study whether PAK2 could phosphorylate Smad2 directly, His-Smad2 (Linker-MH2, 185–467aa) and GST-Smad2 (full-length, FL) were expressed and purified from Escherichia coli cells and used in an in vitro kinase assay as substrates for PAK2, in the presence of recombinant caspase 3, which is an activator for PAK2 (20). As shown in Fig. 4A, PAK2 could phosphorylate Smad2 and this phosphorylation was greatly enhanced by caspase 3. To identify the phosphorylation sites, PAK2 phosphorylated Smad2 proteins were subjected to MALDI-TOF spectrometric analysis. Identified peptides corresponded to phosphorylated CS464S465MS467 (with one or two phosphates) and MS417FVK (Fig. 4B).

It is intriguing that PAK2 might phosphorylate serines in the CS464S465MS467 motif of Smad2, of which the Ser465/467 residues are known to be phosphorylated by TβRI (30, 31). However, overexpression of WT- or TE-PAK2 could not phosphorylate these two serine residues in MDCK cells (Fig. 2A). In addition, mimicking phosphorylation of Ser464 by mutating it to glutamic acid (E) did not affect Smad2 C-terminal phosphorylation by TβRI (data not shown).
To study the function of Smad2 Ser\(^{417}\) (Smad3 Ser\(^{375}\)) phosphorylation, this serine was mutated to alanine (A) or E to make it resistant to phosphorylation or to mimic phosphorylation. As shown in Fig. 4C, ca-T\(\beta\)RI induced the carboxy phosphorylation of WT-Smad2 and S417A mutant to a similar extent, while S417E mutation completely abolished this phosphorylation. S417E mutation also decreased the ca-T\(\beta\)RI-induced Smad2-Smad4 interaction to the basal level in HEK293T cells (Fig. 4D), and eliminated Smad2-mediated transcriptional induction in MDCK cells (Fig. 4E). Finally, we tested the expression of several TGF-\(\beta\) target genes, including Smad7, c-Myc, and Bim (32–36). As shown in Fig. 4F, wild-type Smad2/3 could stimulate the expression of Smad7 and Bim and repress Myc expression in cooperation with Smad4, but Smad2(S417E) and Smad3(S375E) lost the stimulatory ability in MDCK cells.

Together, these results demonstrated Ser\(^{417}\) phosphorylation of Smad2 is critical for PAK2 to antagonize TGF-\(\beta\)/Smad signaling.

**PAK2-mediated Ser\(^{417}\) Phosphorylation of Smad2 Interferes with the T\(\beta\)RI-Smad2 Interaction**—It is notable that Ser\(^{417}\) locates just at the juncture of B10 sheet and the L3 loop in Smad2 (Fig. 5A), and the L3 loop contributes to and determines the specificity of the T\(\beta\)RI-Smad2 association (11). Thus we reasoned that phosphorylation of Ser\(^{417}\) may alter the conformation of the L3 loop, thereby affecting T\(\beta\)RI-Smad2 association. To test this hypothesis, HEK293T cell lysates containing overexpressed kinase-deficient (K232R) T\(\beta\)RI-His and Flag-Smad2 were subjected to precipitation with nicked beads followed by anti-Flag immunoblotting, and T\(\beta\)RII was co-expressed to activate T\(\beta\)RI and induce its interaction with Smad2. As shown in Fig. 5B, co-expression of WT- or TE-PAK2, but not KR-PAK2, decreased the T\(\beta\)RI-Smad2 interaction.

To study whether this effect was mediated by Smad2 Ser\(^{417}\) phosphorylation, we compared the interaction between T\(\beta\)RI and wild-type or mutant Smad2. Accordantly, S417E mutation diminished the interaction, while S417A mutation had no effect (Fig. 5C). These data indicated that PAK2 interferes with the T\(\beta\)RI-Smad2 interaction by phosphorylating Smad2 at Ser\(^{417}\).

**DISCUSSION**

TGF-\(\beta\) regulates various aspects of cellular events, and thus playing pivotal roles in different pathophysiological processes (1–3). Although the canonical Smad-mediated signaling is relatively simple, it is precisely controlled at different levels from the availability and activation of extracellular ligands, the activity and stability of membrane receptors to the activity, stability and localization of Smad proteins in the cytoplasm or in the nucleus (15, 35, 37–39).

PAK2 has been shown to be a mediator of TGF-\(\beta\) in a set of mesenchymal cells, but not in epithelial cells (23, 24). Moreover, TGF-\(\beta\)-induced activation of PI3K and fibroblast responses (such as enhanced proliferation and morphologic changes) are mediated by PAK2 (40, 41), and it was also reported that TGF-\(\beta\)-activated PAK2 is required for Ras-dependent ERK phosphorylation and Elk-1-driven transcription (42). The fibroblast-specific activation of PAK2 by TGF-\(\beta\) could be due to the absence of an epithelial cell specific protein Erbin, which traps PAK2 in the Erbin-Merlin-PAK2 complex (26). However, whether PAK2 could modulate TGF-\(\beta\) signaling stays an open question.

In this study, we provided evidence showing that PAK2 antagonizes TGF-\(\beta\)-elicited R-Smad activation, R-Smad-Smad4 oligomerization and transcriptional induction in MDCK epithelial cells. In addition, the inhibitory effect of PAK2 was also observed in HEK293T, NMuMG and HaCaT cells in varying degrees, but not in NIH3T3 fibroblasts, suggesting this effect is context-dependent. Intriguingly, the inhibitory effect of PAK2 depends on its kinase activity, as overexpression of a kinase-deficient (KR) mutant had little effect. Accordingly, blocking of endogenous PAK2 kinase activity through IPA-3 or overexpression of a dominant-negative Rac1 mutant (N17) enhanced TGF-\(\beta\) signaling.
PAK2 associates with both Smad2 and Smad3 in a kinase activity-dependent manner. In vitro kinase assay suggested that PAK2 phosphorylates Smad2 at Ser\(^{417}\) or serine residues in the C-terminal SSXS region. However, overexpression of wild-type or constitutively active PAK2 in MDCK cells could not phosphorylate the C-terminal Ser\(^{465/467}\), and mimicking phosphorylation of Ser\(^{464}\) did not affect Smad2 activation either. Instead, substitution of Ser\(^{417}\) with Glu completely abolished TGF-\(\beta\)-induced Smad2 activation and signaling. In addition, Glu substitution of the corresponding serine (Ser\(^{375}\)) in Smad3 inhibited TGF-\(\beta\)-induced Smad3 phosphorylation and downstream signaling (data not shown), and co-expression of both Smad2/3 mutants destroyed Smad2/3/4-mediated target gene transcription, together demonstrating that Ser\(^{417/375}\) in Smad2/3 could be functionally targeted by PAK2 to modulate TGF-\(\beta\) signaling.
Biochemical studies revealed that the interaction between the L3 loop of R-Smads and the L45 loop of type I receptors determines the specificity of the receptor-Smad interaction (11, 12, 43). As Ser417 is adjacent to the L3 loop in Smad2, we reasoned that its phosphorylation would affect the TβRI-Smad2 association. Indeed, mimicking phosphorylation of Ser417 with Glu substitution abolished this interaction, coinciding with the notion that PAK2 inhibition of the Smad2-TβRI interaction requires its kinase activity.

Besides TβRI and PAK2, R-Smads are also targeted by other kinases to finely control their activities and TGF-β signaling. For instance, CDK2/4 are shown to phosphorylate Smad3 at Thr8, Thr178, and Ser212 to control Smad3 activity in transcriptional induction and cell cycle regulation (44); GSK-3β-mediated phosphorylation of Smad3 modulates its stability at the basal level (45), and GRK2 (G-coupled receptor kinase 2) phosphorylates Smad2/3 to control their activities (46). Moreover, MAPKs-mediated R-Smad phosphorylation in the linker regions has been extensively studied, to promote or inhibit TGF-β signaling depending on cell types (reviewed in Refs. 15, 16). Other kinases reported include PKC, ROCK, CaMKII, and CK1y2. Phosphorylation of R-Smads by these kinases may occur independently or sequentially. Therefore, it will be interesting to study whether and how PAK2-mediated phosphorylation of R-Smads integrates with phosphorylation induced by other kinases.

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FIGURE 5. PAK2 inhibits the TβRI-Smad2 association via phosphorylating Ser417 of Smad2. A, schematic diagram of structures around Ser417 in Smad2 and the corresponding site in Smad3. B, PAK2 interferes with TβRI-Smad2 association. HEK293T cell lysates containing TβRI (K232R, KR)-His, TβRII-HA, Flag-Smad2, and WT, TE- or KR-PAK2 were subjected to precipitation with nickel beads followed by anti-Flag immunoblotting. C, S417E substitution abolishes TβRI-Smad2 association. The experiment was performed as in B.
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