Role of ERK1/2 and p38 Mitogen-Activated Protein Kinases in the Regulation of Thrombospondin-1 by TGF-β1 in Rat Proximal Tubular Cells and Mouse Fibroblasts

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Thrombospondin-1 (TSP-1) inhibits angiogenesis and activates latent TGF-β1, both of which are strongly associated with progression of renal disease. Recently, it was reported that Smad2 but not Smad3 regulates TSP-1 expression in response to TGF-β1 in rat tubular epithelial cells as well as in mouse fibroblasts. This study investigated the role of ERK1/2 and p38 mitogen-activated protein kinases (MAPK). TGF-β1 activated both ERK1/2 and p38 in the rat proximal tubular cell line NRK52E. Blocking ERK1/2 and p38 inhibited TGF-β1–induced TSP-1 mRNA and protein expression. Next, the cross-talk between Smad2 and ERK1/2 or p38 was examined. Whereas blocking of ERK1/2 or p38 failed to inhibit TGF-β1–induced Smad2 activation, inhibition of Smad2 by Smad7 overexpression inhibited the phosphorylation of ERK1/2 but not p38 in response to TGF-β1. Similar results were observed using mouse fibroblasts from Smad2 knockout embryos, in that TGF-β1 was able to activate p38 but not ERK1/2 in this cell line. In conclusion, TSP-1 expression is regulated by both ERK1/2 and p38 MAPK in rat proximal tubular cells and mouse fibroblasts in response to TGF-β1. The ERK1/2 activation is dependent on Smad2 activation, whereas the p38 activation occurs independent of Smad2. Because TSP-1 is a major angiogenic molecule and an activator of TGF-β1, this provides an important insight to the mechanism by which TGF-β1 may mediate interstitial fibrosis and progressive renal disease.

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the interplay between the ERK1/2 and p38 pathway with the Smad2-dependent pathway discussed above. This is important as the interplay between Smad and MAPK (p38 and ERK1/2) pathways often define diverse patterns of cell responses to TGF-β1 (see reviews in 11,15,16).

Materials and Methods

TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Monoclonal antibodies to the following antigens were obtained: TSP-1 (TSP Ab-4, A6.1, NeoMarkers; Lab Vision Co., Fremont, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA), phosphorylated ERK (Cell Signaling, Beverly, MA), phosphorylated p38 (Cell Signaling), and Smad 2 (Pharmingen, San Diego, CA). Polyclonal antibodies to total p44/42 kinase, total p38, phosphorylated Smad2, phosphorylated MAPKAP-K2, total MAPKAP-K2 (all from Cell Signaling), and Smad 2 (Pharmingen) were used. The ERK1/2 inhibitors PD98059 (Calbiochem, San Diego, CA) and U0126 (Cell Signaling) as well as the p38 inhibitors SB203580 (Calbiochem) and NPC31169 (Scios Inc., Sunnyvale, CA) (17) were also used.

Cell Proximal Tubular epithelial cells (NRK52E) or fibroblasts derived from mouse embryo deficient in Smad2 or Smad3 or wild type were cultured in DMEM with 10% FBS, 60 µg/ml penicillin, and 100 µg/ml streptomycin in 5% CO2 at 37°C. After serum starvation, confluent cells were incubated with TGF-β1. NRK52E is a nontransformed tubular epithelial cell line that was cloned from a mixed culture of normal rat kidney cells and maintains characteristics of proximal tubular epithelial cells (18,19). All experiments were repeated at least three times.

Establishing Doxycycline-Regulated Smad7-Expression NRK52E Cell Lines

The Doxycycline (DOX)-regulated Smad7-expressing cell line was established as described previously (20). Briefly, mouse Smad7 cDNA was subcloned into a tetracycline (Tet)-inducible vector, pTRE (Clontech, Palo Alto, CA). An improved pTet-on vector, pEFpuro-Tet-on, was used, in which the gene encoding the “reverse” Tet repressor was subcloned into a pEF-BOS vector, pEF-PGKpuroAv18, thereby conferring puromycin resistance. To obtain DOX-induced (a Tet derivative) Smad7-expressing NRK52E cell lines, pTRE-Smad7 and pEFpuro-Tet-on were co-transfected into NRK52E cells by electroporation, and the stable transfected cells were selected in the presence of puromycin (4 µg/ml). As shown in Figure 2A, both 10 µM PD98059 and 5 µM Uo126 inhibited ERK1/2 activation, whereas 10 µM SB203589 blocked p38 activity, as reflected by inhibition of MAPKAP-K2 phosphorylation, a substrate of p38 (Figure 2B). At the concentrations used, none of the inhibitors had any significant effects on the basal level of ERK1/2 or p38 activation (data not shown) and none mediated cytotoxicity (LDH release). Next, we examined the role of ERK1/2 or p38 on TSP-1 expression in response to TGF-β1. As shown in Figure 2C, the inhibition of either ERK1/2 or p38 significantly inhibited TSP-1 mRNA expression by TGF-β1 at 4 h, although the effect was partial. Western blotting also confirmed that inhibition of these MAPK
partially but significantly inhibited TSP-1 protein expression in response to TGF-β1 at 24 h (Figure 2, D through F). It is interesting that the combination of inhibitors for ERK1/2 and p38 completely blocked TSP-1 protein synthesis (Figure 2G). These data document an additive role of ERK1/2 and p38 in the TSP-1 expression induced by TGF-β1 in NRK52E cells.

Cross-Talk between Smad2, ERK1/2, and p38 in NRK52E Cells

Because we have reported that Smad2 but not Smad3 regulates TGF-β1-induced TSP-1 expression in NRK52E cells (10), we examined the cross-talk between Smad2, ERK1/2, and p38. We first examined the effect of ERK1/2 and p38 inhibitors on Smad2 phosphorylation in response to TGF-β. As shown in Figure 3, Smad2 activation in response to TGF-β1 was not blocked by inhibition of ERK1/2 (10 μM PD98059 or 5 μM PD10, PD25) or 10 to 20 μM SB203589 (SB10-SB20) or 15 nM NPC31169 (NPC) was used to inhibit activation of p38. Con, control; TGF, TGF-β1 (5 ng/ml); P, phosphorylated; T, total. (A) The phosphorylation of ERK1/2 in response to TGF-β1 is inhibited by PD10 or Uo5 at 30 min. (B) p38 activation is also suppressed by SB20–10 at 120 min. (C) Two-step, real-time reverse transcriptase–PCR. PD25 and PD10 as well as SB20 and SB10 partially but significantly inhibit TSP-1 mRNA expression at 4 h in response to TGF-β1. (D through F) TSP-1 protein synthesis at 24 h. SB20 and SB10 (D), PD25 and PD10 (E), or Uo5 (F) partially inhibit TGF-β1–induced TSP-1 protein synthesis at 24 h, respectively. (G) Combination of Uo5 with either SB10 or NPC completely blocks TSP-1 protein synthesis in response to TGF-β1. Each figure is representative of a total of three separate experiments. Bar graphs (B, D, E, and F) show the densitometric analysis and are expressed related to control cells (100). aP < 0.01 versus control; bP < 0.01 ERK1/2 versus control; cP < 0.05 ERK1/2 versus control; dP < 0.05 p38 versus control.

Figure 1. Time course of Smad2, ERK1/2, and p38 activation in response to TGF-β1. (A) Western blotting for the activation of Smad2, ERK1/2, and p38. Con, control; TGF, TGF-β1 (5 ng/ml); P, phosphorylated; T, total. (B) Line graphs show the densitometric analysis with the arbitrary units from untreated control cells depicted as one-fold. Data shown are mean ± SD with three separate experiments. aP < 0.01 Smad2 versus control; bP < 0.01 ERK1/2 versus control; cP < 0.05 ERK1/2 versus control; dP < 0.05 p38 versus control.

Figure 2. Blocking ERK1/2 or p38 suppresses thrombospondin-1 (TSP-1) expression in NRK52E cells in response to TGF-β1. 10 to 25 μM PD98059 (PD10, PD25) or 5 μM U0126 (Uo5) was used to block ERK1/2, and 10 to 20 μM SB203589 (SB10-SB20) or 15 nM NPC31169 (NPC) was used to inhibit activation of p38. Con, control; TGF, TGF-β1 (5 ng/ml); P, phosphorylated; T, total. (A) The phosphorylation of ERK1/2 in response to TGF-β1 is inhibited by PD10 or Uo5 at 30 min. (B) p38 activation is also suppressed by SB20–10 at 120 min. (C) Two-step, real-time reverse transcriptase–PCR. PD25 and PD10 as well as SB20 and SB10 partially but significantly inhibit TSP-1 mRNA expression at 4 h in response to TGF-β1. (D through F) TSP-1 protein synthesis at 24 h. SB20 and SB10 (D), PD25 and PD10 (E), or Uo5 (F) partially inhibit TGF-β1–induced TSP-1 protein synthesis at 24 h, respectively. (G) Combination of Uo5 with either SB10 or NPC completely blocks TSP-1 protein synthesis in response to TGF-β1. Each figure is representative of a total of three separate experiments. Bar graphs (B, D, E, and F) show the densitometric analysis and are expressed related to control cells (100). aP < 0.01 versus TGF (TGF-β1; 5 ng/ml).
Uo126) or by inhibition of p38 (10 μM SB203580 or 15 nM NPC31169). Next, to examine the role of Smad2 in the regulation of ERK1/2 and p38, we examined the activation of ERK1/2 and p38 by blocking Smad2 activation using two different approaches. First, we suppressed Smad2 activation by overexpressing Smad7 using a DOX-inducible expression system. As shown in Figure 4, A and B, DOX-induced Smad7 inhibited Smad2 phosphorylation at the basal level as well as under TGF-β1 stimulation. It is interesting that DOX-induced Smad7 inhibited both basal and TGF-β1–stimulated phosphorylation of ERK1/2 (Figure 4C). However, p38 phosphorylation was not inhibited by DOX-induced Smad7 (Figure 4D). We also examined whether Smad2 is responsible for the activation of ERK1/2 and p38 in response to TGF-β1 with fibroblasts derived from mouse embryos deficient in Smad2 (21). As shown in Figure 5A, wild-type mouse fibroblasts showed the same response as NRK52E cells in which the activation of Smad2 by TGF-β1 was not suppressed by blocking ERK1/2 or p38. In contrast, TGF-β1 failed to induce ERK1/2 activation in Smad2-deficient fibroblasts, whereas p38 was still phosphorylated (Figure 5B). These data demonstrate that ERK1/2 phosphorylation is regulated by Smad2 but that activation of p38 in response to TGF-β1 occurs independent of Smad2.

ERK1/2 and p38 Are Distinctly Regulated in Response to TGF-β1

Finally, the relationship between ERK1/2 and p38 in response to TGF-β1 was investigated in NRK52E cells. As shown in Figure 6, ERK1/2 inhibition did not suppress p38 activation at 120 min. Similarly, inhibitors of p38 did not affect TGF-β1–induced activation of ERK1/2. These data show that neither MAPK regulates the other in response to TGF-β1.

Discussion

We previously demonstrated that TGF-β1 induces the expression of TSP-1 in a dose-dependent and time-dependent manner in tubular epithelial (NRK52E) cells (10). In the current study, we investigated the role of the MAPK pathway, especially ERK1/2 and p38, on the TGF-β1–induced TSP-1 expression in proximal tubular epithelial cells and mouse fibroblasts. Smad2 activation occurred early, which was followed by ERK1/2 activation. On the contrary, p38 was activated relatively later (120 min) in NRK52E cells. Despite different kinetics of activation, blocking either MAPK with pharmacologic inhibitors partially suppressed TSP-1 expression, and simultaneously blocking both p38 and ERK1/2 resulted in complete inhibition of TSP-1 expression. This suggests that both ERK1/2 and p38 have an additive role in mediating TGF-β1–induced TSP-1 expression in NRK52E cells. It is interesting that Smad2 activation was required for ERK1/2 activation, but p38 could be activated independent of Smad2 activation, suggesting that TSP-1 expression is regulated by two pathways, a Smad2-ERK1/2 mechanism and a p38-mediated pathway.

Because we have previously shown that Smad2 is necessary
for the TSP-1 expression in response to TGF-β1 in NRK52E cells, we investigated the cross-talk of Smad2 with ERK1/2 and p38. The role of MAPK pathway on Smad signaling is complex because the MAPK pathways may have positive or negative regulatory effects on receptor Smad (Smad2 and Smad3) depending on the cell and the type of MAPK activation (14,22–24). For example, in the kidney, TGF-β1 mediates apoptosis of proximal tubular cells via a p38-dependent but Smad-independent pathway (25). Similarly, p38 is required for TGF-β1 induction of collagen stimulation in mouse mesangial cells (26).

The reason for the different kinetics in activation of ERK 1/2 and p38 remain unclear. However, it was shown recently that TGF-β1 activation of p38 but not ERK1/2 can occur via a Smad-independent pathway involving integrin β1 (27). It is interesting that the activation of integrin-linked kinase by TGF-β1 is sustained for 24 h in proximal tubular cells (28). Therefore, the differential involvement of the integrin system might account for the different kinetics of p38 and ERK1/2 activation in response to TGF-β1.

An important finding in the study was that inhibition of either p38 or ERK 1/2 MAPK resulted in a partial inhibition of TSP-1 synthesis in response to TGF-β1 but that the inhibition of both MAPK simultaneously resulted in complete inhibition. This suggests that the two pathways are additive and can account for the full physiologic response of the cells to stimulate TSP-1 in response to TGF-β1.

With respect to ERK1/2, the current study suggests that ERK1/2 is downstream of Smad2 under TGF-β1 stimulation because we found that inhibition of Smad2 suppressed ERK1/2 activation, whereas blocking ERK1/2 failed to inhibit Smad2 activation. These studies are similar to what Schnaper and colleagues (29) showed in an epithelial cell line (NMuMG); in contrast, these authors showed that in human mesangial cells, the converse was observed, being that ERK1/2 mediated the Smad activation. ERK1/2 activation by TGF-β1 has been shown to occur early in some cell lines as a result of direct activation of Ras (11,30).

In conclusion, our studies demonstrate that TSP-1 expression in response to TGF-β1 is mediated by Smad2 activation as well as by ERK1/2 and p38. The ERK1/2-dependent pathway is directly downstream of Smad2 activation and can be viewed as a TGF-β1–Smad2–ERK1/2–dependent pathway. In contrast, p38 activation occurs independent of Smad2 activation. Finally, the complexity of the relationship is emphasized by our observation that Smad2 knockout cells make no TSP-1 in response to TGF-β1. Hence, this demonstrates that Smad2 activation is nevertheless required for TSP-1 expression in response to the activation of p38, even though the p38 activation occurs independent of Smad2 and ERK1/2. This suggests that interplay is still occurring downstream of p38 and Smad2 signaling to facilitate the expression of TSP-1.

Figure 5. Cross-talk of Smad2 with ERK1/2 or p38 in mouse fibroblasts. (A) TGF-β1–induced Smad2 activation was examined at 30 min. PD10, U05, SB10, and NPC cannot suppress Smad2 activation in response to TGF-β1 in mouse fibroblast. (B) The activation of ERK1/2 and p38 in mouse fibroblasts of wild type (WT) or Smad2 knockout (2KO) mouse fibroblasts at 30 min or at 120 min, respectively. TGF-β1–induced ERK1/2 activation is inhibited, whereas p38 phosphorylation occurs in 2KO cell. Bar graphs show the densitometric analysis and are expressed relative to control cells (100). Data shown are mean ± SD of three separate experiments. Con, control; TGF, TGF-β1 (5 ng/ml), P, phosphorylated; T, total; PD10, 10 μM PD98059; U05, 5 μM U0126; SB10, 10 μM SB203589; NPC, 15 nM NPC31169.

Figure 6. ERK1/2 and p38 are independently activated by TGF-β1 in NRK52E cells. TGF-β1–induced p38 phosphorylation was examined at 120 min and ERK1/2 at 30 min in NRK52E cells. PD10 and U05 did not block p38 activation in response to TGF-β1. ERK1/2 activation was inhibited by neither SB10 nor NPC in contrast to PD10 and U05. PD10, 10 μM PD98059; U05, 5 μM U0126; SB10, 10 μM SB203589; NPC, 15 nM NPC31169; Con, control; TGF, TGF-β1 (5 ng/ml); P, phosphorylated; T, total. *P < 0.01 versus Con.
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