TβRI Independently Activates Smad- and CD2AP-Dependent Pathways in Podocytes

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ABSTRACT

TGF-β regulates differentiation, growth, and apoptosis of podocytes and mediates podocyte depletion in glomerulosclerosis. TGF-β promotes proapoptotic signaling mediated by Smad3 but also activates prosurvival pathways such as phosphoinositide-3 kinase (PI3K)/AKT; the latter requires the CD2-associated adaptor protein (CD2AP) in podocytes. Whether the opposing activities mediated by Smad proteins and CD2AP involve molecular cross-talk is unknown. Here, we report that CD2AP-dependent early activation of the antiapoptotic PI3K/AKT pathway does not require TGF-β receptor-regulated Smad2 and Smad3. We found that the C-terminal region of CD2AP interacts directly with the cytoplasmic tail of the TGF-β receptor type I (TβRI) in a kinase-dependent manner and that the interaction between the TβRI and the p85 subunit of PI3K requires CD2AP. Consistent with the proapoptotic function of Smad signaling, Smad2/3-deficient podocytes were hyperproliferative and resistant to TGF-β-induced growth inhibition and apoptosis. In contrast, CD2AP-deficient cells were hypoproliferative and hypersensitive to TGF-β-induced apoptosis. In vivo, to determine the effects of reduced Smad3 or CD2AP gene dosage on podocyte apoptosis and proteinuria characteristic of TGF-β1 transgenic mice, we generated TGF-β1 transgenic mice deficient for Smad3 or heterozygous for CD2AP. Smad3 deficiency ameliorated podocyte apoptosis, and CD2AP heterozygosity increased both podocyte apoptosis and proteinuria. These data define distinct canonical (Smad) and noncanonical (CD2AP/PI3K/AKT) pathways that arise from direct, independent interactions with the TβRI and that mediate opposing signals for podocyte death or survival.


TGF-β is a dimeric cytokine that controls fundamental aspects of cell behavior in embryonic development and tissue homeostasis.1,2 Similar to other epithelial cell types, TGF-β stimulation induces apoptosis, growth inhibition, or differentiation in podocytes.3–5 TGF-β1 transgenic mice (TGF-β1tg) are characterized by podocyte apoptosis at the onset of proteinuria and progressive glomerulosclerosis associated with progressive podocyte depletion.3 Podocyte depletion is considered a hallmark of glomerular diseases characterized by segmental or global glomerulosclerosis.6–8 TGF-βs initiate signaling across the plasma membrane by inducing heteromeric complexes of type I and II (TβRI and TβRII) receptors,9–11 leading to recruitment and activation of major TGF-β signal transducers, the Smad proteins.9–12 Phosphorylated R-Smads form

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heteromeric complexes with a common-partner Smad4 (co-Smad) and translocate to the nucleus, where these heteromeric complexes bind to DNA to control transcription of target genes. Apoptosis is a major cell response that is induced by TGF-β in many progressive kidney diseases apart from its fibrogenic and inflammatory responses. Although these pro-apoptotic responses to TGF-β are largely mediated by Smad-dependent regulation of proapoptotic target genes, TGF-β also activates prosurvival pathways such as mitogen-activated protein kinases (MAPKs) and phosphoinositide-3 kinases (PI3Ks). Previously, we showed that Smad3 mediates apoptotic signaling of TGF-β via the p38 MAPK pathway in podocytes and reported that the adaptor molecule CD2-associated protein (CD2AP) is required for rapid, early activation of the antiapoptotic PI3K/AKT pathway in podocytes; however, it is not known whether or how Smad proteins and CD2AP interact or how PI3K/AKT is linked with TGF-βR complexes.

Here we report that TGF-βR–regulated Smad2 and Smad3 are not required for CD2AP-dependent early activation of antiapoptotic PI3K/AKT. Furthermore, we describe a direct interaction between the C-terminal region of CD2AP and the cytoplasmic tail of TGF-βR that is essential for interaction and activation of the p85 subunit of PI3K, transducing antmitogenic/proapoptotic versus antiapoptotic TGF-β responses, respectively. Balance of these two independent TGF-β signaling pathways specifies podocyte death or survival in vitro and in vivo. These findings provide a rationale for future therapeutic strategies to prevent podocyte depletion by targeting selectively proapoptotic TGF-β signaling.

RESULTS

Receptor-Regulated Smad2 and Smad3 Are not Required for CD2AP-Mediated Early Activation of AKT but Are Required for Sustained, Long-Term AKT Activation and Its Downstream Transcriptional Activity in Podocytes

R-Smads are considered central mediators of TGF-β–stimulated transcription and cellular responses in various cell types. Whereas TGF-β also stimulates PI3K/AKT activation, it is not known whether R-Smads have a role in these alternative pathways. To examine the respective requirements for Smad2 and Smad3 versus CD2AP, we established a genetically defined cell culture system including conditionally immortalized podocytes derived from wild-type (WT), Smad2/Smad3 double-knockout (DKO), and CD2AP-deficient (CKO) mice, as described previously. Early (up to 1 h) and sustained (8 to 24 h) phosphorylation patterns of Smad2 and Smad3 induced by TGF-β were comparable in WT and CKO cells. As expected, phospho-Smad2 and phospho-Smad3 were absent in DKO (Figure 1, A and B). Phospho-Smad3 antibody cross-reacts with phospho-Smad1 a mediator of bone morphogenic protein receptor signaling and is detectable as a higher molecular weight band in DKO cells (Figure 1, A and B, *). TGF-β–induced early and late phosphorylation profiles of AKT (p-AKT) were dysregulated, respectively, in DKO and CKO, compared with WT cells. The early peak of TGF-β–induced p-AKT, observed at 30 min in WT cells, was rapidly induced at 15 up to 60 min in DKO (Figure 1A), TGF-β failed to induce p-AKT up to 1 h in CKO (Figure 1A). In contrast, at 8 h, p-AKT was induced in CKO but not in DKO when compared with WT, whereas at 24 h, the peak p-AKT observed in WT cells was considerably reduced in DKO and CKO cells. The transient late peak in DKO at 8 h in p-AKT was consis-
tently observed in more than one experiment (Figure 1B). Phosphorylation of the transcription factor serum response factor (SRF) is mediated by AKT. The early TGF-β-induced activation profiles of phospho-SRF (p-SRF) and p-AKT were highly similar across WT, DKO, and CKO cells, whereby phosphorylation was increased and extended in DKO but absent in CKO cells (Figure 1A), indicating that SRF may be activated predominantly by PI3K/AKT signal transduction in response to TGF-β in podocytes. TGF-β had no effect on p-SRF at 8 and 24 h in WT, DKO, and CKO cells (data not shown).

Next, we examined the effect of the temporally defined, distinct patterns of dysregulation of PI3K/AKT activation by TGF-β, observed in DKO and CKO podocytes (see Figure 1), on transcriptional activities of Smad2-, Smad3-, or AKT-dependent reporters. We used transient luciferase reporter assays to measure transcriptional activities of Smad3/Smad4 complexes (Smad-binding element 4 [SBE4]-luciferase reporter), respectively. TGF-β significantly increased activities of the three reporters in WT but not in DKO podocytes (Figure 1C). In contrast, the TGF-β–stimulated inductions of the ARE-Luc and the SBE4-Luc reporter activities both were significantly increased, whereas induction of the PI3K/AKT-dependent SRE5-Luc reporter was significantly decreased in CKO podocytes (Figure 1A), on transcriptional activities of Smad3/Smad4 complexes (Smad-binding element 4 [SBE4]-luciferase reporter), respectively.

CD2AP Interacts Directly with TβRI through Its Proline-Rich C-Terminal Domain in a Kinase-Dependent, Ligand-Inducible Manner and Mediates Interaction of TβRI and p85 Subunit of PI3K in Podocytes

By double-immunofluorescence labeling and confocal microscopy of podocytes, we observed punctate cell surface–associated labeling for CD2AP and TβRI that was perfectly co-localized in WT podocytes and DKO cells, indicating co-localization of CD2AP and TβRI in membrane-associated vesicular structures (Figure 2, A through F). Absence of CD2AP had no effect on TβRI localization in CKO podocytes (Figure 2, G through I). We performed GST pulldown assays using GST-CD2AP fusion proteins containing full-length, C-terminal, or N-terminal regions and lysates derived from HEK293T cells transfected with hemagglutinin (HA)-tagged TβRI. TβRI bound to full-length GST-CD2AP and C-terminal GST-CD2AP but not N-terminal GST-CD2AP fusion proteins (Figure 3A). To confirm these interactions by co-immunoprecipitation, we co-transfected HEK293T cells with HA-TβRI together with myc-tagged full-length N- and C-terminal regions of CD2AP. HA-TβRI interacted with C-terminal CD2AP and full-length CD2AP but not with N-terminal region of CD2AP (Figure 3B), suggesting that the proline-rich C-terminal region but not the N-terminal SH3 domains mediate interaction of CD2AP with TβRI. WT HA-TβRI and constitutively active mutant HA-TβRI T204D interacted strongly with full-length myc-CD2AP, whereas WT HA-TβRII and the kinase-deficient mutant HA-TβRI K232A did not interact with myc-CD2AP (Figure 3C), suggesting that CD2AP does not interact with TβRII and that the interaction with TβRI is serine-threonine kinase dependent.

To determine whether CD2AP-TβRI interaction was direct, we generated recombinant 35S-labeled full-length N-terminal and C-terminal CD2AP proteins using rabbit reticulocyte lysate. Full-length and C-terminal recombinant CD2AP but not N-terminal CD2AP interacted with the cytoplasmic tail of a GST-TβRI fusion protein under cell-free...
conditions (Figure 3D). In contrast, the cytoplasmic region of GST-TβRII did not interact with recombinant CD2AP (Figure 3D). Together, these results demonstrate that the C-terminal proline-rich region of CD2AP mediates direct interactions of CD2AP with the cytoplasmic region of activated TβRI.

Interaction of endogenous TβRI and CD2AP was induced by TGF-β treatment within 1 min in WT but not in CKO cells (Figure 3E). TβRI also co-immunoprecipitated with the p85 subunit of PI3K in WT but not in CKO cells (Figure 3E). Thus, endogenous CD2AP directly binds TβRI via its C-terminal proline-rich region and the p85 subunit of PI3K through its N-terminal SH3 domains, as described previously, and is essential for the rapid activation of the PI3K/AKT pathway.

Baseline Proliferation Is Increased in DKO and Decreased in CKO Podocytes, whereas Growth Inhibition Mediated by TGF-β in WT Cells Is Abrogated in Both DKO and CKO Podocytes

Autocrine and paracrine TGF-β induces G0/G1 arrest in podocytes in a Smad3-dependent manner. To examine the relative roles of Smad2/3 and CD2AP in baseline and TGF-β–stimulated growth regulation of podocytes, we quantified growth rates of WT, DKO, and CKO podocytes using tetrazolium salt WST-1–based proliferation assays as described previously. Growth of untreated CKO cells was significantly reduced compared with WT podocytes, whereas untreated DKO podocytes proliferated at a significantly increased rate (Figure 4A). Growth of WT podocytes was significantly inhibited by TGF-β (Figure 4B). Pretreatment of cells with the caspase inhibitor zVAD-fmk had no effect on TGF-β–induced inhibition of growth, indicating that reduced proliferation but not TGF-β–induced apoptosis accounted for the growth inhibition induced by TGF-β. In contrast, TGF-β had no significant effect on growth in DKO and CKO podocytes, respectively (Figure 4, C and D). Together, these results demonstrate that Smad2/3 mediate antimitogenic signaling by TGF-β in podocytes, whereas CD2AP promotes baseline proliferation, suggesting opposing activities of both pathways.

To identify the regulators mediating opposing cell-cycle control by Smad2/3 and CD2AP, we examined mRNA and protein levels of known regulators of cell cycle. Levels of cyclin D1, a critical promoter of early cell-cycle progression in G1/S phase, were considerably increased in DKO and absent in CKO, compared with WT podocytes (Figure 4E). Protein and mRNA levels of the cyclin-dependent kinase inhibitor (cdki) p15Ink4b were induced by TGF-β in WT podocytes (Figure 4, E and F), consistent with our previous observations. In contrast, TGF-β had no effect on p15Ink4b protein and mRNA in either DKO or CKO podocytes (Figure 4, E and F). Finally, baseline p21Cip1/Waf1 mRNA and protein levels were considerably lower in DKO and CKO podocytes compared with WT podocytes (Figure 4, E and G). In contrast to the loss of TGF-β responsiveness of p15Ink4b cdki and the dramatically reduced baseline levels of p21Cip1/Waf1 in both DKO and CKO podocytes, TGF-β responsiveness of the proapoptotic target gene Gadd45β was selectively absent in DKO but increased in CKO compared with WT podocytes (Figure 4H). Thus, the increased proliferative rate observed in untreated DKO was associated with increased cyclin D1 and decreased p21Cip1/Waf1 cdki, whereas the reduced proliferative rate of untreated CKO was associated with lack of cyclin D1 expression compared with untreated WT podocytes. The lack of growth-inhibitory signaling by TGF-β observed in DKO
anti–TGF-β antibody eliminated this increased baseline apoptosis, demonstrating that CD2AP deficiency sensitizes podocytes to autocrine proapoptotic TGF-β signaling. Using the DKO and CKO culture system, we found that TGF-β had no effect on apoptosis in DKO cells, whereas CKO podocytes were hypersensitive to TGF-β–induced apoptosis (Figure 5A), consistent with previous in vitro observations using Smad3-deficient and CD2AP-deficient podocytes. Although these results and our previous work suggest Smad3 and CD2AP as opposing pro- and antiapoptotic transducers of TGF-β signaling in podocytes in vitro, respectively, the pathophysiologic relevance of these observations in experimental models and renal disease remains unclear. Kidneys of mice carrying a TGF-β1 transgene under control of the albumin promoter/enhancer, resulting in elevated circulating TGF-β1, are characterized by podocyte apoptosis at the onset of proteinuria and progressive glomerulosclerosis. To determine the effects of reduced Smad3 or CD2AP gene dosage on podocyte apoptosis and proteinuria in TGF-β1tg mice, we generated TGF-β1tg mice deficient for Smad3 (TGF-β1tg/Smad3−/−) or heterozygous for CD2AP (TGF-β1tg/CD2AP+/-), respectively. Rates of apoptotic podocytes at 2 wk of age were significantly increased in TGF-β1tg/CD2AP+/- (n = 8) and decreased in TGF-β1tg/Smad3−/− mice (n = 10), compared with TGF-β1tg/CD2AP+/-/Smad3+/- mice (Figure 5B). In contrast, rates of apoptosis of endocapillary cells, including endothelial and mesangial cells, were not significantly different across these groups (Figure 5B). Together, these results demonstrate that TGF-β–induced apoptosis is mediated through Smad3-dependent signaling, whereas CD2AP-dependent pathways mediate antiapoptotic signaling in podocytes in vivo. Furthermore, TGF-β1tg/CD2AP+/- was associated with significantly increased incidence of albuminuria at 2 wk of age (Figure 5C) and increased levels of albuminuria after weaning (Figure 5D), compared with TGF-β1tg/CD2AP+/-, respectively. Together with our previously reported in vitro observations, these in vitro and in vivo results suggest that already partial loss of CD2AP in podocytes may sensitize to TGF-β–induced apoptosis and proteinuria at the onset of glomerular injury in TGF-β1tg mice.

To verify the observed differences by molecular level analysis, we examined mRNA levels for podocyte markers and a 19–gene set expression signature previously identified in 2-wk-old TGF-β1tg and found to be predictive of subsequent progression of glomerulosclerosis in TGF-β1tg mice. Podocyte differentiation markers podocin, nephrin, and podocalyxin but not synaptopodin were consistently and significantly reduced in TGF-β1tg/CD2AP+/- compared with TGF-β1tg
CD2AP Interacts with TβRI in Tubular Epithelial Cells and Protects against TGF-β–Induced Tubular Epithelial Apoptosis In Vitro and In Vivo

TGF-βRs and CD2AP both are ubiquitously expressed in various renal tubular epithelial segments in vivo (data not shown). We wondered whether the antiapoptotic, noncanonical TβRI-CD2AP signaling module, characterized in podocytes, might also modulate apoptosis in tubular epithelial cells. We found that endogenous CD2AP and TβRI co-immunoprecipitated in TGF-β–stimulated tubular epithelial cell lines, including inner medullary collecting duct (IMCD) cells and proximal tubular epithelial (murine cortical tubular [MCT]) cells (Figure 7A). To examine the functional relevance of this interaction, we used small interfering RNA (siRNA) for effective knockdown of CD2AP in inner medullary collecting duct cell line IMCD (Figure 7B) and proximal tubular epithelial cell line MCT (data not shown). Similar to podocytes, phosphorylation of AKT was rapidly induced by TGF-β in IMCD cells transfected with nonspecific control siRNA but not in CD2AP siRNA–transfected cells (Figure 7C), indicating that CD2AP is required for activation of PI3K/AKT signaling by TGF-β in tubular epithelial cells. In contrast, TGF-β–stimulated phosphorylation of Smad2/3 was comparable between CD2AP siRNA and control siRNA-transfected IMCD cells and proximal tubular epithelial MCT cells (data not shown). Induction of the SBE4-Luc transcriptional reporter (Figure 7D) and of the ARE-Luc transcriptional reporter (data not shown) by TGF-β was significantly elevated in IMCD cells transfected with CD2AP siRNA compared with random siRNA. In contrast, the significant induction of the PI3K/AKT-dependent SRE5-Luc transcriptional reporter by TGF-β observed in random siRNA-transfected cells was abrogated in CD2AP siRNA-transfected cells (Figure 7E). Baseline and TGF-β–stimulated apoptosis rates were significantly increased in IMCD cells transfected with CD2AP siRNA compared with random siRNA (Figure 7F). Finally, kidneys of 2-wk-old CD2AP heterozygous Tβ1tg mice (TGF-β1tg/CD2AP+/−) manifested significantly increased rates of apoptosis in tubular epithelia compared with CD2AP WT Tβ1tg littermate mice (Figure 7G). Together, these results suggest that the antiapoptotic TβRI/CD2AP/PI3K/AKT signaling module is also functional in tubular epithelial cells and protects tubular epithelial cells against TGF-β–induced apoptosis in vitro and in vivo.
Activation of autocrine and paracrine TGF-β signaling cascades in glomerular and/or tubulointerstitial compartments is a hallmark of most forms of chronic renal injury.\(^8\) TGF-β initiates a variety of cell type–dependent signaling and response profiles in renal epithelial cells, including apoptosis and epithelial-to-mesenchymal transition that lead to inflammation, mesangial and interstitial cell activation, and progressive fibrogenesis in chronic renal injury.

In addition to ubiquitous Smad signaling, multiple Smad-independent TGF-β signaling pathways have been proposed.\(^9\) Reports provide insights into the molecular link of extracellular signal–regulated kinase pathway activation by the TGF-β receptor complex.\(^17\) In contrast, the physical link of PI3K/AKT activation with TGF-β receptor remains only partially understood.\(^13,20,31–33\) Although an indirect association of the regulatory p85 subunit of PI3K with TGF-βR complexes and a functional requirement of CD2AP in PI3K/AKT activation have been demonstrated,\(^20,33\) it is unknown whether CD2AP provides the physical link between p85 and the TGF-βR complex and whether activation of PI3K/AKT requires TβR1-regulated Smad2 and/or Smad3. Finally, the pathophysiologic relevance of relative changes in Smad versus CD2AP protein in renal epithelia and their effect on apoptosis in vivo remain unknown.

Our study defines a noncanonical TGF-β signaling pathway characterized by direct, ligand-inducible interaction of TβR1 with CD2AP. By binding the p85 subunit of PI3K through its SH3 domain–containing region and TβR1 through its proline-rich region, CD2AP enables the binding and activation of PI3K, resulting in AKT phosphorylation. Interestingly, sustained activation of PI3K/AKT after prolonged TGF-β stimulation beyond 60 min is CD2AP independent but Smad dependent, indicating a temporally defined switch between initial CD2AP-dependent, Smad-independent and delayed CD2AP-independent, Smad-dependent mechanisms of AKT activation. These findings define truly Smad-dependent and Smad-independent signaling and conclusively show that Smads are not required for CD2AP-dependent early PI3K/AKT activation.

The observed effects of Smad2/3 or CD2AP deficiency on baseline and TGF-β–regulated cell growth and apoptosis in kidney epithelial cells are consistent with this model. Baseline proliferation is increased in DKO cells and decreased in CKO cells, and TGF-β–induced growth inhibition is observed only in WT cells. Progressive kidney disease in TGF-β1tg or CD2AP-deficient mice is associated with dramatically increased apoptosis in epithelial cells in vivo,\(^3,20\) suggesting epithelial apoptosis as a disease-initiating defect. We demonstrate that the induction of apoptosis by TGF-β is Smad dependent and counterbalanced by simultaneous antiapoptotic PI3K/AKT signaling mediated by CD2AP/TβR1 interactions. For example, the increased baseline apoptosis observed in CD2AP-deficient podocytes was eliminated by neutralizing anti-TGF-β antibodies in vitro.\(^5,20\) Consistent with the proposed model, we demonstrate here that partial loss of CD2AP func-

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**Figure 6.** Molecular profiles of podocyte-specific markers and TGF-β1tg kidney disease progression markers in whole kidneys of TGF-β1tg/CD2AP\(^{++/+}\) and TGF-β1tg/CD2AP\(^{++/-}\) mice. (A) Histogram shows the average ± SD of mRNA levels of podocyte differentiation markers, determined by quantitative real-time PCR analysis in kidneys of 2-wk-old TGF-β1tg/CD2AP\(^{++/+}\) or TGF-β1tg/CD2AP\(^{++/-}\) mice (\(P < 0.001\)). (B) Dendrogram/heatmap shows unsupervised hierarchical cluster analysis of 11 kidney disease progression marker genes\(^50\) with significantly different mRNA levels in kidneys of 2-wk-old TGF-β1tg/CD2AP\(^{++/+}\) (WT) or TGF-β1tg/CD2AP\(^{++/-}\) (Tg-Cd2ap HT) mice. Gene expression profiles provide highly significant (100% resampling rate) clustering into sample cluster I (turquoise bar), containing 6 Tg-Cd2ap WT and 1 Tg-Cd2ap HT sample, and sample cluster II, including 7 Tg-Cd2ap HT and 1 Tg-Cd2ap WT sample, respectively.
CD2AP interacts with TβRI in tubular epithelial cells. CD2AP knockdown or loss sensitizes tubular epithelial cells to TGF-β–induced apoptosis in vitro and in vivo. (A) Western blots showing co-immunoprecipitation of CD2AP and TβRI in IMCD and MCT cells treated with TGF-β for indicated time points. (B) CD2AP expression knockdown using siRNA in IMCD cells. (C) Western blot analysis of phospho-AKT in total cell lysates of IMCD cells after time points. (B) CD2AP expression knockdown using siRNA in IMCD cells. (C) Western blot analysis of phospho-AKT in total cell lysates of IMCD cells after 24 h and 48 h. (D) Relative fold change in luciferase activity of IMCD cells transfected with reporters SBE4-Luc (D) and SRE-Luc (E) treated with TGF-β for 24 h (*P < 0.05, TGF-β-treated random siRNA versus TGF-β-treated CD2AP siRNA transfected IMCD cells). All transfections were normalized to renilla luciferase activity. Results are representative of three independent experiments. (F) Histograms show the average ± SD of TUNEL-positive cells per 20 fields in untransfected IMCD cells (■) or IMCD cells transfected with random siRNA (□) or CD2AP siRNA (▲) left untreated or treated with TGF-β for 24 h (*P < 0.05 between TGF-β–treated or untreated random siRNA-transfected versus CD2AP siRNA-transfected IMCD cells). (G) Fifty high-power fields for tubular sections of kidneys from 2-wk-old CD2AP+/− (■), CD2AP+/+ (▲), TGF-β1tg/CD2AP+/− (□), and TGF-β1tg/CD2AP−/− (checked bars) mouse kidneys. (*P < 0.05, WT versus TGF-β1tg or CD2AP+/− versus TGF-β1tg/CD2AP+/−).

Proteinuria Measurement

Proteinuria measurements were made using Strips of Albustix (Bayer Corp., Mishawaka, IN). An albumin concentration of 100 mg/dl was
considered positive, and incidence of proteinuria in these mice at 2 wk is depicted as percentage proteinuric mice. Urinary albumin and creatinine were measured using mouse albumin-specific ELISA and creatinine companion kits in 5-wk-old mice (Exocell Laboratories, Philadelphia, PA). Proteinuria was expressed as average ratios of albumin and creatinine concentrations in urine samples.

**Cell Culture**

Smad3 KO/Smad2 floxed podocyte cell line was established by a standard protocol23 from the Smad3−/− mice37 harboring a homologous exon 2 floxed allele for Smad2.14 After 5 d of primary culture, cells were trypsinized and passed through a 33-mm pore size sieve to remove remaining glomerular cores (consisting predominantly of mesangial and endothelial cells). Cells were propagated at 33°C in RPMI 1640 containing 10% FCS, 100 U/ml penicillin and streptomycin, and 30 U/ml recombinant mouse γ-IFN. Smad2 deletion was achieved through Cre-mediated recombination by infecting podocytes with Ad5 CMV Cre (Gene Transfer Vector Core Services Branch of the University of Iowa). Single clones were picked, and deficiency for Smad2 and Smad3 was verified by quantitative real-time PCR analysis and Western blotting. Immortalized podocytes derived from CD2AP−/− mice and CD2AP+/+/ littermates were cultured as described previously.23 IMCD cells (ATCC) and MCT cells from Fuad Ziyadeh (American University, Beirut, Lebanon) were grown in DMEM/F12 and DMEM containing FCS, penicillin, and streptomycin, respectively.

**Constructs**

Myc-tagged full-length CD2AP (1 through 641); Myc-tagged C-terminal–deleted CD2AP (1 through 322);26 Myc-tagged N-terminal–deleted CD2AP (323 through 641); and GST-CD2AP (1 through 641), GST-3Xflag CD2AP (1 through 324), and GST-CD2AP (324 through 641) were used. HA-tagged TβRII, kinase dead HA-TβRII(K232A), constitutively active HA-TβRII(204D),19 and HA-TβRIII were provided by Dr. Joan Massague (Sloan Kettering Institute, New York, NY). GST-TβRI cytoplasmic domain (152 through 503) was from Dr. Rik Derynck (University of California, San Francisco, San Francisco, CA). The 5XSRE-Luc reporter plasmid was from Stratagene.

**GST Pull-Down Experiments**

Protein extracts from 293T cells transfected with HA-TβRI were diluted 1:2 in Co-immunoprecipitation buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 15 mM EGTA, 0.1% Triton X-100, 1 mM dithiothreitol, and protease inhibitors) and applied to the GST columns bound with GST proteins isolated from Escherichia coli B21 (Stratagene). Protein was eluted with 4 Vol of elution buffer (10 mM reduced glutathione in 50 mM Tris [pH 8.0]) and analyzed by Western blotting.

**Co-immunoprecipitation**

293T cells were transiently transfected with myc-tagged CD2AP and HA-tagged TβRI/TβRII constructs using Superfect (Qiagen). Protein extracts were incubated with precipitating antibodies in co-immunoprecipitation buffer for 3 h at 4°C. Protein A/G-Agarose beads (Santa Cruz Biotechnology) was added to the supernatants and incubated overnight at 4°C. Bound proteins were eluted by boiling samples in sample buffer.

**In Vitro Translation Assays**

A total of 1 μg of myc-tagged CD2AP (full length or truncated) was transcribed and translated using the TNT-coupled reticulocyte lysate system (Promega) using [35S]-methionine. GST fusion proteins bound to Sepharose beads were incubated with radioactive-labeled CD2AP overnight at 4°C. Samples were washed with co-immunoprecipitation buffer. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography.

**Immunofluorescence Staining**

For co-localization studies, podocytes were plated on collagen-coated coverslips. Cells were fixed, blocked, and incubated with primary antibodies followed with Alexa Fluor–conjugated secondary antibodies (Molecular Probes). Coverslips were mounted using Fluoromount G, and images were acquired by Leica TCSSP confocal microscope.

**siRNA Transfections**

For CD2AP knockdown in IMCD cells, siRNA oligos as described previously40 were used. Transfections were performed using Nucleofector II (Amaxa Biosystems) using 1.5 or 3.0 μg of siRNA in 100 μl of basic nucleofector solution.

**Western Blotting**

Proteins were analyzed by Western blotting using the following antibodies: Polyclonal antibody anti-HA tag (Sigma) or anti-Myc-tag (Sigma), rabbit anti-p-AKT (Ser 473) (Santa Cruz), rabbit anti-total AKT (Santa Cruz Biotechnology), mouse anti-Smad2/3 (BD Transduction Laboratories), rabbit anti-phosphoSmad2 (Cell Signaling), rabbit anti-phosphoSmad3 (Cell Signaling), rabbit anti-TGF-βRI (Santa Cruz Biotechnology), rabbit anti-TGF-βRII (Santa Cruz Biotechnology), rabbit anti-CD2AP (Santa Cruz Biotechnology), rabbit anti-P13K p85 (Santa Cruz Biotechnology), mouse anti-p21cip1 (Chemicon), rabbit anti-p15INK4b (Santa Cruz Biotechnology), rabbit anti-Cyclin D1 (Santa Cruz Biotechnology), mouse anti–glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), and mouse anti–β-tubulin antibody (Sigma).

**Reporter Assays**

Podocytes or siRNA-transfected IMCD cells were transfected with reporter constructs using Effectene transfection reagent (Qiagen) and analyzed using the Dual-Luciferase Reporter Assay kit (Promega) and normalized to renilla luciferase using a FLUOstar Optima multimode microplate reader (BMG Labtech).

**Quantitative Real-Time PCR analysis**

Total RNA from cell lysates or whole mouse kidney was prepared using Qiagen RNaseasy mini-columns, and quantitative real-time PCR was performed as described previously.5,30 Statistical analysis of gene expression profiles in mice was performed by nonparametric Mann-Whitney test using P < 0.05. Expression values for genes at P < 0.05 were log2-transformed and normalized by median centering before hierarchical clustering with resampling (bootstrapping) using Pearson correlation metrics, all implemented in the TIGR Multi Experiment Viewer (TMEV) software version 4.3.41
Quantification of Apoptotic Nuclei in Vitro
Podocytes or siRNA-transfected IMCD cells were seeded on glass cover slips in 12-well dishes, stimulated with TGF-β1, and analyzed by TUNEL assay using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon Int.). TUNEL-positive cells were counted in 20 fields per sample and normalized for the total number per high-power field. Fluorescence images were acquired using a Zeiss Axioskop fluorescence microscope.

Proliferation Assays
Podocytes (1000 cells/well) were seeded in 96-well plates and treated with TGF-β in the presence or absence of 5 μM Z-DEVD-FMK. Cell proliferation was monitored with a VICTOR³ V Multilabel Counter (Perkin Elmer) at a wavelength of 450 nm using the WST-1 tetrazolium salt cleavage assay kit (Chemicon).

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DISCLOSURES
None.

REFERENCES


