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Oncoprotein DJ-1 interacts with mTOR complexes to effect transcription factor Hif1α-dependent expression of collagen I (α2) during renal fibrosis

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Proximal tubular epithelial cells respond to transforming growth factor β (TGFβ) to synthesize collagen I (α2) during renal fibrosis. The oncoprotein DJ-1 has previously been shown to promote tumorigenesis and prevent apoptosis of dopaminergic neurons; however, its role in fibrosis signaling is unclear. Here, we show TGFβ-stimulation increased expression of DJ-1, which promoted noncanonical mTORC1 and mTORC2 activities. We show DJ-1 augmented the phosphorylation/activation of PKCβII, a direct substrate of mTORC2. In addition, coimmunoprecipitation experiments revealed association of DJ-1 with Raptor and Rictor, exclusive subunits of mTORC1 and mTORC2, respectively, as well as with mTOR kinase. Interestingly, siRNAs against DJ-1 blocked TGFβ-stimulated expression of collagen I (α2), while expression of DJ-1 increased expression of this protein. In addition, expression of dominant negative PKCβII and siRNAs against PKCβII significantly inhibited TGFβ-induced collagen I (α2) expression. In fact, constitutively active PKCβII abrogated the effect of siRNAs against DJ-1, suggesting a role of PKCβII downstream of this oncoprotein. Moreover, we demonstrate expression of collagen I (α2) stimulated by DJ-1 and its target PKCβII is dependent on the transcription factor hypoxia-inducible factor 1α (Hif1α). Finally, we show in the renal cortex of diabetic rats that increased TGFβ was associated with enhanced expression of DJ-1 and activation of mTOR and PKCβII, concomitant with increased Hif1α and collagen I (α2). Overall, we identified that DJ-1 affects TGFβ-induced expression of collagen I (α2) via an mTOR-, PKCβII-, and Hif1α-dependent mechanism to regulate renal fibrosis.

Chronic kidney disease (CKD) is a state of progressive and irreversible decline of renal excretory function due to renal tissue injury, reduced glomerular filtration rate, and nephron loss. CKD is associated with significant morbidity and mortality. In 2017, a prevalence of 9.1% was recorded globally (1). More than 37 million Americans have CKD, which contributes to increased risk of cardiovascular disease and loss of renal function resulting in end stage kidney disease (2, 3) (CDC; https://www.cdc.gov/kidneydisease/publications-resources/CKD-national-facts.html). It is also a risk multiplier in patients with diabetes and hypertension (4). In fact, diabetes is a significant contributor of CKD (4). Thus, understanding the mechanism of progression of CKD is important to develop new therapies.

Damage to kidney tubules causes histological and functional changes leading to progressive fibrosis. In fact, renal tubulointerstitial fibrosis represents the best predictor of end stage renal disease (5). The inflammatory cells recruited during the initial phases of fibrosis and the intrinsic renal cells secrete profibrogenic growth factor and cytokines including transforming growth factor-β (TGFβ). TGFβ acts in an autocrine or paracrine fashion upon proximal tubular epithelial cells among many cell types to induce renal hypertrophy. This leads to hyperfiltration and microalbuminuria and accumulation of matrix proteins due to increased production and reduced degradation of extracellular matrix components. Unabated, this process contributes to further fibrosis and greater degree of proteinuria in a vicious cycle (6, 7). In fact, hyperglycemia and angiotensin II employ TGFβ as a mediator of kidney injury in diabetic kidney disease (6, 8). Furthermore, TGFβ directly or indirectly stimulates the production of profibrotic connective tissue growth factor and inflammatory cytokines such as interleukins and TNFα (9). Action of TGFβ on proximal tubular cells induces epithelial to mesenchymal transdifferentiation to produce myofibroblasts, which generate collagen to promote fibrosis in various renal diseases (10–12). Liver specific overexpression of TGFβ with increased circulating level of the cytokine developed tubulointerstitial fibrosis with enhanced expression of matrix proteins (13, 14).

Two TGFβ receptors (type I and type II) exist with structural characteristics of dual specificity kinases although both of them functionally act as serine/threonine kinases (15). Dimeric TGFβ binds to the TGFβ receptor II due to higher affinity. Subsequently, TGFβRI is recruited to form a heterotetrameric receptor complex in a symmetric 2:2:2 ligand–receptor complex (16). Upon oligomerization, the type II receptor

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phosphorylates the type I receptor at Thr-186 and Ser-187/189/191 in the juxtamembrane glycine- and serine-rich domain upstream of the kinase domain. This phosphorylation of multiple residues causes a conformational change to release the immunophilin FK506-binding protein FKBP12 from the glycine- and serine-rich domain, relieving its inhibitory function to activate the TGFβRI kinase activity (17, 18). In turn, the activated type I receptor phosphorylates receptor-specific Smads 2 and 3 at their C-terminus to induce heterodimerization with Smad 4 for translocation to nucleus where they cooperate with other transcriptional activators or repressors to regulate gene expression (19).

In addition to the canonical serine/threonine kinase signaling, we and others have shown that TGFβ induces tyrosine phosphorylation of multiple proteins including TGFβRI (20, 21). The non-Smad signaling pathway induced by TGFβRI initiates Erk1/2, JNK, and p38 MAP kinases (19). Also, we showed that TGFβ activates PI 3 kinase–Akt–mTOR signaling in renal cells (21–24).We have established the role of mTOR in renal cell hypertrophy and matrix protein expansion, two pathological features associated with renal fibrosis (22, 24–27).

PKCs play significant role in multiple pathological states including renal fibrosis (28, 29). These groups of enzymes fall in the AGC superfamily of protein kinases and are classified into three subfamilies, classical (α, β1, βII, and γ), novel (δ, ε, η, and θ), and atypical (ζ and λ/ι). The role of different PKC isoforms has been extensively studied in renal fibrosis in the context of diabetic kidney disease (28). Induction of type 1 diabetes in the PKCβ null mice showed amelioration of renal fibrosis (30). Although various isoforms have been shown to be activated in renal cells by the fibrotic stimuli such as hyperglycemia, PKCβII plays an important role (31–33).

DJ-1 was identified as a ras-cooperating oncogene (34). Later, homozygous deletion and missense mutations were found in DJ-1 gene, which cause aggregation of the protein resulting in early onset of autosomal recessive Parkinson's disease (35–37). DJ-1 is a ubiquitously expressed homodimeric protein, which shows significant structural similarity with the bacterial protease Pfp1/PH1704. However, due to an occluded and distorted catalytic site, DJ-1 does not possess any protease activity (38). In contrast, DJ-1 has weak glyoxalase II activity to detoxify reactive carbonyl species (39). More recently, it has been shown to have deglycase activity to repair glycation damage in proteins and nucleic acids (40, 41). High sensitivity of its Cys-106 residue for oxidation protects the neurons from oxidative stress. Thus, it serves as an anti-apoptotic protein in the neurons of Parkinson's disease patients although excessive oxidation of Cys-106 render this protein inactive (42, 43). Apart from its function in neuronal cells, its role in spermatogenesis and fertilization, where it cooperates with androgen receptor, have been reported (44). As DJ-1 was originally discovered as an oncogene and since oncogene-mediated biological activities include activated serine/threonine kinases that lead to proliferation of cells during tumorigenesis, it is possible that DJ-1 may interact with the oncogenic kinases. Also, mTOR acts as an oncogenic kinase to drive tumorigenesis (45, 46). Apart from its role in cancer development, mTOR plays important role in the progression of CKD including renal fibrosis (47). In fact, we and others have shown previously that mTOR contributes to the pathogenic function of TGFβ in renal cells (22, 24, 25, 48). The direct role of DJ-1 in activation of this kinase to affect downstream signaling for renal fibrosis has not been investigated. In the present study, we examined how TGFβ activates mTOR via DJ-1. Also, we determined the role of DJ-1 in activation of PKCβII downstream of mTORC2 in mediating the expression of fibrotic protein collagen I (α2).

Results

TGFβ increases the expression of DJ-1

Renal proximal tubular epithelial cells respond to TGFβ to drive fibrosis in the kidney. Although DJ-1 is a ubiquitous protein, its expression in brain and cancer tissues have been mainly studied in association with early-onset Parkinson's disease and malignancy (35, 49). To initiate a systematic study on the role of DJ-1 in renal fibrosis, human proximal tubular epithelial cells were exposed to TGFβ. Immunoblot analysis of the lysates revealed a time-dependent increase in the expression of DJ-1 protein (Fig. 1*A* and Fig. S1*A*). Similarly, expression of DJ-1 mRNA was increased in response to TGFβ, suggesting DJ-1 expression may be regulated at the transcriptional level (Fig. 1*B*). Further immunoblot analysis revealed that TGFβ significantly stimulated the expression of DJ-1 protein in a sustained manner till 96 h (Fig. S1, *B* and *C*). Since TGFβ transmits signal via its type I receptor–mediated phosphorylation of Smad 3, we tested the effect of pharmacologic blockade of TGFβ receptor I by SB 431542 (SB). SB blocked TGFβ-stimulated DJ-1 expression along with inhibition of Smad 3 phosphorylation (Fig. 1*C*, Fig. S1*D* and Fig. 1*D*, Fig. S1*E*). To test whether Smad 3 regulates DJ-1 expression, proximal tubular epithelial cells were transfected with siRNAs against Smad 3 prior to exposure to TGFβ. Downregulation of Smad 3 abrogated TGFβ-induced DJ-1 expression (Fig. 1*E* and Fig. S1*F*). To confirm these observations in human proximal tubular epithelial cells, we performed experiments in mouse proximal tubular epithelial cells. TGFβ significantly increased DJ-1 in mouse proximal tubular epithelial cells in a sustained manner till 96 h (Fig. S2, *A*–*D*). Similar to the results in human proximal tubular epithelial cells, SB, which inhibits Smad 3 phosphorylation, and siSmad 3 blocked TGFβ-induced DJ-1 expression (Fig. S2, *E*–*J*). These results demonstrate involvement of the canonical TGFβ receptor I signaling for increased DJ-1 expression.

DJ-1 regulates TGFβ-stimulated mTORC1 and mTORC2 activities

We and others have shown a role of mTOR in renal fibrosis (24, 25, 50–52). mTOR exists in two complexes with different substrate specificities (53). Activation of mTORC1 phosphorylates its downstream substrate S6 kinase at Thr-389 and serves as a measure of mTORC1 activation (45). TGFβ increased rapid and sustained phosphorylation of S6 kinase

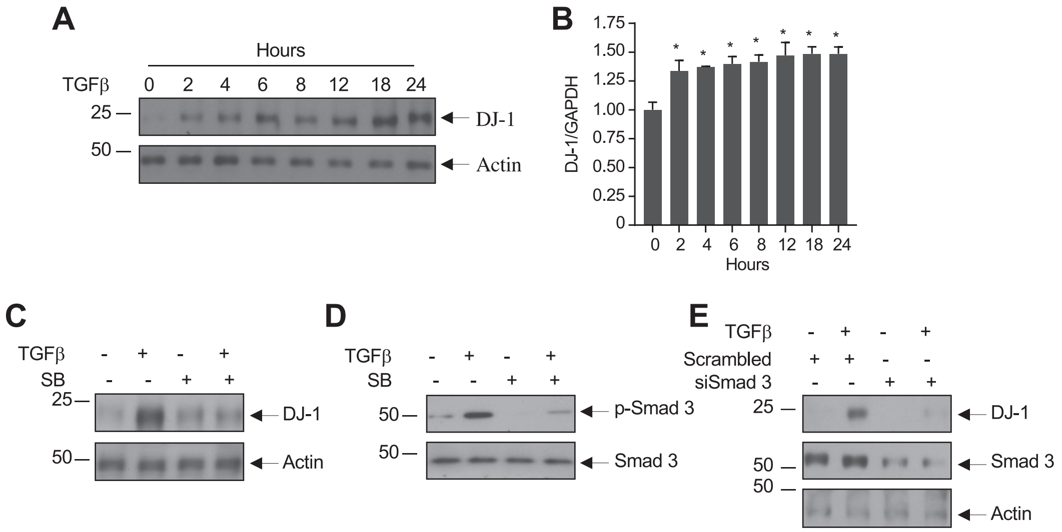

Figure 1. TGFβ increased DJ-1 expression in Smad 3–dependent manner. *A* and *B*, serum-starved human proximal tubular epithelial cells were incubated with 2 ng/ml TGFβ for the indicated periods. *C* and *D*, cells were treated with 5 μM SB for 1 h prior to incubation with 2 ng/ml TGFβ for 24 h. *E*, cells were transfected with siRNAs against Smad 3 or scrambled RNA prior to incubation with 2 ng/ml TGFβ for 24 h. In panels (*A*) and (*C–E*), cell lysates were immunoblotted with indicated antibodies to determine the expression of each protein. Molecular weight markers are shown on the *left* margins. Representative blots from three independent experiments are shown. Quantification and significance of these data are shown in Fig. S1, *A* and *D–F*. In panel (*B*), expression of DJ-1 mRNA was determined. Total RNAs were prepared and used for real tie RT-PCR to detect DJ-1 and GAPDH mRNAs as described in the Experimental procedures. Mean ± SD of triplicate measurements is shown. *$p < 0.0002$ *versus* 0 h. TGFβ, transforming growth factor β.

initiating at 15 min of stimulation, indicating activation of mTORC1 (Fig. 2*A*, Fig. S3*A* and Fig. 2*B*, Fig. S3*B*). To test whether DJ-1 regulates mTORC1 activation, we used siRNAs against this protein. Downregulation of DJ-1 blocked TGFβ-stimulated phosphorylation of S6 kinase (Fig. 2*C*, Fig. S3*C*). mTORC1-mediated phosphorylation of S6 kinase increases its activity toward rps6 (45, 53). TGFβ increased the phosphorylation of rps6, which was inhibited by siRNAs against DJ-1 (Fig. 2*D*, Fig. S3*D*). Conversely, when we transfected a vector to express FLAG-tagged DJ-1, it increased the phosphorylation of S6 kinase and its substrate rps6 similar to TGFβ (Fig. 2*E*, Fig. S3*E* and Fig. 2*F*, Fig. S3*F*).

mTORC2 phosphorylates Akt and PKCβII at the hydrophobic motif sites Ser-473 and Ser-660, respectively, resulting in their activation (54). To determine activation of mTORC2, we examined phosphorylation of these two proteins at these

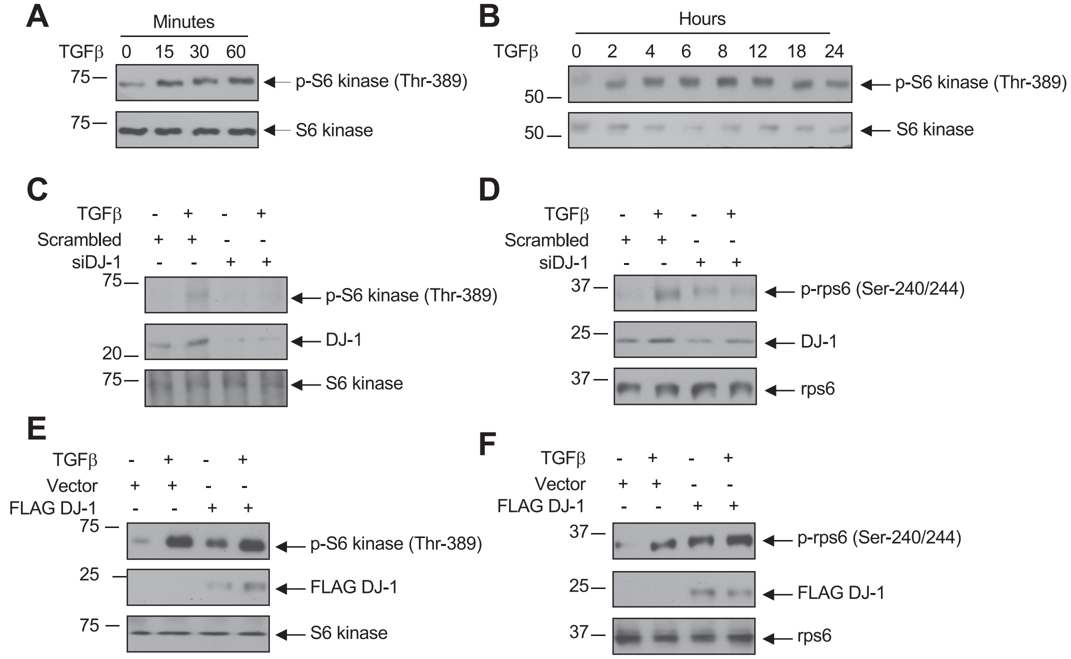

Figure 2. DJ-1 regulates TGFβ-stimulated mTORC1 activity. *A* and *B*, serum-starved human proximal tubular epithelial cells were incubated with 2 ng/ml TGFβ for indicated periods. *C* and *D*, cells were transfected with siRNAs against DJ-1 or scrambled RNA prior to incubation with 2 ng/ml TGFβ for 24 h. *E* and *F*, cells were transfected with a vector encoding FLAG-tagged DJ-1 or control vector prior to incubation with 2 ng/ml TGFβ for 24 h. Cell lysates were immunoblotted with the indicated antibodies to determine the expression of each protein. Representative of three independent experiments is shown. Quantification and significance of these data are shown in Fig. S3, *A–F*. TGFβ, transforming growth factor β.

sites. TGFβ rapidly increased the phosphorylation of these two kinases at their mTORC2 phosphorylation sites (Akt Ser-473 and PKCβII Ser-660) starting at 15 min of stimulation (Fig. 3*A*, Fig. S4*A* and Fig. 3*B*, Fig. S4*B*). Also, TGFβ enhanced the phosphorylation of Akt at Thr-308 (Fig. 3*A*, Fig. S4*A*, right panel). Prolonged incubation with TGFβ increased the phosphorylation of Akt and PKCβII in a sustained manner (Fig. 3*C*, Fig. S4*C* and Fig. 3*D*, Fig. S4*D*). In determining the role of DJ-1 in this process, as shown in Fig. 4, *A* and *B*, siRNA-mediated downregulation of DJ-1 inhibited the phosphorylation of Akt and PKCβII, respectively (Fig. S5, *A* and *B*). Phosphorylation of Akt and PKCβII at these sites increases their kinase activities (55, 56). To test their kinase activities, we examined the phosphorylation of their two respective endogenous substrates GSK3β at Ser-9 and myristoylated alanine rich PKC substrate (MARCKS) at Ser-152/156, which elicit many effects of Akt and PKCβII, respectively (57–59). TGFβ significantly increased the phosphorylation of GSK3β and MARCKS (Fig. 4*C*, Figs. S5*C* and Fig. 4*D* and Fig. S5*D*). Blockade of DJ-1 expression by siRNAs inhibited the TGFβ-induced phosphorylation of GSK3β and MARCKS (Fig. 4*C*, Fig. S5*C* and Fig. 4*D* and Fig. S5*D*). To confirm these observations, DJ-1 was overexpressed in the proximal tubular epithelial cells. Increased expression of DJ-1 significantly enhanced the phosphorylation of Akt and PKCβII at their hydrophobic motif sites, resulting in phosphorylation of their substrates GSK3β and MARCKS similar to TGFβ (Fig. 4, *E–H* and Fig. S5, *E–H*). Together, these results demonstrate that DJ-1 mediates TGFβ-stimulated mTORC1 and mTORC2 activation in proximal tubular epithelial cells.

DJ-1 forms complex with mTORC1 and mTORC2

DJ-1 contains multiple domains that can interact with other proteins to modulate their functions (60). Thus, one possible mechanism by which DJ-1 promotes the activities of mTORC1 and mTORC2 is by association with these complexes. To address this hypothesis, proximal tubular epithelial cells were transfected with vectors expressing FLAG DJ-1 and Myc Raptor to examine mTORC1. Immunoprecipitation of the cell lysates with the FLAG antibody followed by immunoblotting with anti-Myc showed association of DJ-1 with raptor (Fig. 5*A*, Fig. S6*A*). Reciprocal immunoprecipitation and immunoblotting confirmed complex formation between raptor and DJ-1 (Fig. 5*B*, Fig. S6*B*). Similarly, when lysates of cells transfected with FLAG DJ-1 and Myc Rictor were used for FLAG immunoprecipitation followed by anti-Myc immunoblotting, we detected association of rictor with DJ-1 (Fig. 5*C*, Fig. S6*C*). Reciprocal immunoprecipitation and immunoblotting confirmed this observation (Fig. 5*D*, Fig. S6*D*). These results suggest that association of DJ-1 with both mTOR complexes may regulate their activities.

TGFβ regulates association of DJ-1 with mTORC1 and mTORC2

Our results demonstrate requirement of DJ-1 in TGFβ-stimulated activation of mTORC1 and mTORC2 (Figs. 2 and 3). Given that DJ-1 interacts with both mTORC1 and mTORC2 (Fig. 5), we next investigated the responsiveness to TGFβ. Proximal tubular epithelial cells were exposed to TGFβ. DJ-1 was immunoprecipitated from the cell lysates and immunoblotted with antibody against mLST8, which is a common subunit for both mTORC1 and mTORC2. Fig. 6*A* shows increased association of DJ-1 with mLST8 (Fig. S7*A*). Reciprocal mLST8 immunoprecipitation followed by DJ-1 immunoblotting confirmed increased association of these two proteins (Fig. 6*B*, Fig. S7*B*). These results demonstrate that DJ-1 may be incorporated in both mTOR complexes. Next, we confirmed specific association of DJ-1 with mTORC1 by coimmunoprecipitating raptor with DJ-1. The results showed incorporation of DJ-1 into mTORC1 (Fig. 6*C*, Fig. S7*C* and Fig. 6*D*, Fig. S7*D*). To examine the association of DJ-1 with mTORC2 complex, we used two specific subunits of it, rictor and mSin1. Reciprocal immunoprecipitation of DJ-1 and rictor, and mSin1 showed incorporation of DJ-1 into mTORC2 (Fig. 6, *E–H* and Fig. S7, *E–H*).

DJ-1 regulates mTORC1 and mTORC2 kinase activities

The observations above indicate complex formation between DJ-1 and mTORC1/mTORC2 in which mTOR is the common kinase subunit. Therefore, we examined association

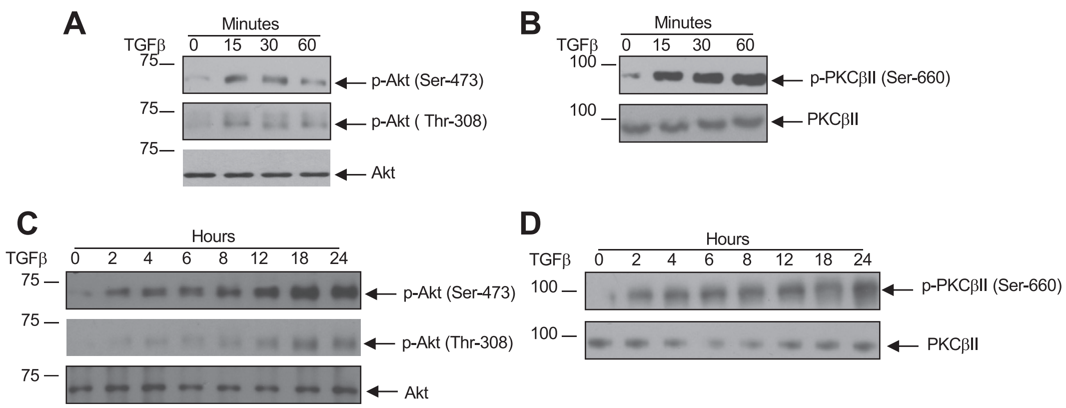

Figure 3. TGFβ stimulates mTORC2 activity in human proximal tubular epithelial cells. Serum-starved cells were incubated with 2 ng/ml TGFβ for the indicated periods of time in panels (*A–D*). The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three independent experiments is shown. Quantification and significance of these data are shown in Fig. S4, *A–D*. TGFβ, transforming growth factor β.

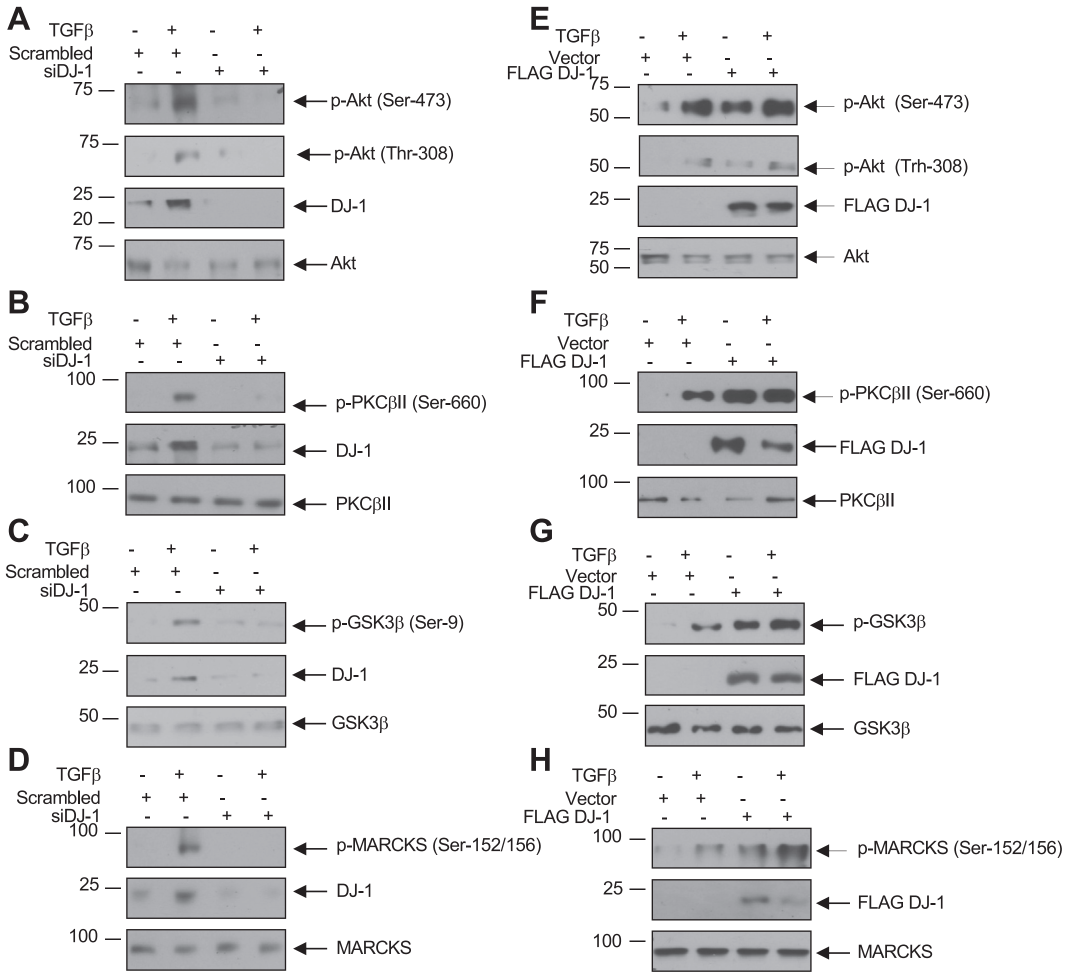

Figure 4. DJ-1 mediates TGFβ-stimulated mTORC2 activity. *A–D,* human proximal tubular epithelial cells were transfected with siRNAs against DJ-1 or scrambled RNA prior to incubation with 2 ng/ml TGFβ for 24 h *E–H,* the cells were transfected with vector-encoding FLAG-tagged DJ-1 or control vector prior to incubation with 2 ng/ml TGFβ for 24 h. The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of 3 to 5 independent experiments is shown. Quantification and significance of these data are shown in Fig. 5*A* and Fig. S5*H*. TGFβ, transforming growth factor β.

of DJ-1 with mTOR. DJ-1 immunoprecipitates were used for immunoblotting with mTOR antibody. TGFβ significantly increased association of mTOR with DJ-1 (Fig. 7*A*, Fig. S8*A*). Complementary experiment using mTOR immunoprecipitates for DJ-1 immunoblotting validated these results (Fig. 7*B*, Fig. S8*B*). Next, we determined the role of DJ-1 in both mTORC1 and mTORC2 kinase activities using immunecomplex kinase assays of mTOR immunoprecipitate *in vitro*. Proximal tubular epithelial cells were transfected with siRNAs against DJ-1 prior to incubation with TGFβ. To assay mTORC1 activity, mTOR immunoprecipitate was used in immunecomplex kinase assay with recombinant S6 kinase as substrate in the presence of ATP. Fig. 7*C* shows significantly increased S6 kinase phosphorylation at the Thr-389 site by TGFβ (Fig. S8*C*). Interestingly, DJ-1 siRNAs significantly inhibited the TGFβ-stimulated mTORC1 activity in the mTOR immunoprecipitate (Fig. 7*C*, Fig. S8*C*). Similarly, when we used recombinant Akt in mTOR immunecomplex kinase assay, siDJ-1 inhibited the TGFβ-induced phosphorylation of Akt at its mTORC2 hydrophobic motif site Ser-473 (Fig. 7*D*, Fig. S8*D*). These results demonstrate that DJ-1 is required for TGFβ-stimulated activities of both mTORC1 and mTORC2.

DJ-1 regulates TGFβ-stimulated collagen I (α2) expression via PKCβII

Using rapamycin, the role of mTOR especially mTORC1 in the expression of collagen I (α2) and in fibrotic renal diseases is established (22, 25, 50, 61–65). However, it is known that prolonged rapamycin treatment can block mTORC2 (66, 67), suggesting that a role for mTORC2 in renal disease especially in fibrosis cannot be ruled out. Our results above show that DJ-1 controls the activities of both mTORC1 and mTORC2 (Figs. 2 and 4). Therefore, we examined whether DJ-1 regulates the fibrotic protein collagen I (α2) expression in proximal tubular epithelial cells. DJ-1 siRNAs were transfected prior to exposure of cells with TGFβ. Immunoblot analysis of the cell lysates showed that inhibition of DJ-1 expression blocked TGFβ-stimulated expression of collagen I (α2) (Fig. 8*A*, Fig. S9*A*). In contrast to these results, overexpression of DJ-1 was sufficient to increase collagen I (α2) expression similar to TGFβ (Fig. 8*B*, Fig. S9*B*). Next, we studied the role of PKCβII, which is phosphorylated and activated by TGFβ via mTORC2, to augment collagen I (α2) expression. Expression of a vector containing kinase-dead mutant of PKCβII (HA-tagged PKCβII K371R) in proximal tubular epithelial cells

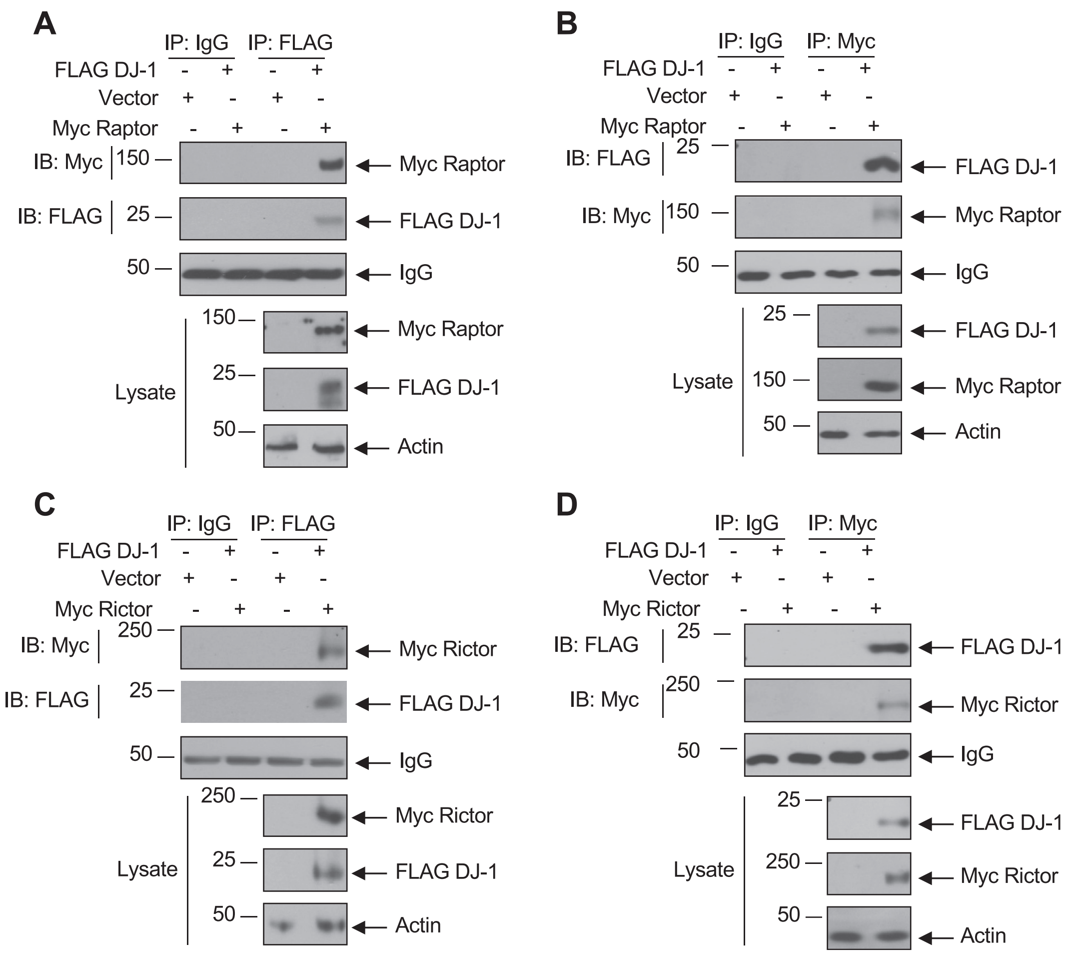

Figure 5. DJ-1 association with mTORC1 and mTORC2. *A* and *B*, FLAG-tagged DJ-1 and Myc-tagged raptor were cotransfected into human proximal tubular epithelial cells. The cell lysates were immunoprecipitated with antibodies against FLAG (panel *A*) and Myc (panel *B*) or nonimmune IgG. The immunoprecipitates were immunoblotted with anti-Myc and anti-FLAG antibodies, respectively. *C* and *D*, FLAG-tagged DJ-1 and Myc-tagged rictor were cotransfected into human proximal tubular epithelial cells. The cell lysates were immunoprecipitated with antibodies against FLAG (panel *C*) and Myc (panel *D*) or nonimmune IgG. The immunoprecipitates were immunoblotted with anti-Myc and anti-FLAG antibodies, respectively. *Bottom* panels show immunoblot analysis of each indicated proteins in the cell lysates. Representative of 3 to 4 independent experiments is shown. Quantification and significance of these data are shown in Fig. S6, *A–D*.

significantly inhibited the expression of collagen I (α2) in response to TGFβ (Fig. 8*C*, Fig. S9*C*). To complement this observation, we used siRNAs against PKCβII. Transfection of these siRNAs blocked the expression of PKCβII in proximal tubular epithelial cells resulting in the inhibition of expression of collagen I (α2) (Fig. 8*D*, Fig. S9*D*). However, TGFβ uses Smad 3 to regulate expression of genes (19). siRNAs against Smad 3 inhibited TGFβ-stimulated collagen I (α2) expression (Fig. 9*A*, Fig. S10*A*). To examine the existence of a cross talk between Smad 3 and PKCβII, we cotransfected Smad 3 and kinase-dead PKCβII. Expression of kinase-dead PKCβII significantly inhibited Smad 3-induced collagen I (α2) expression (Fig. 9*B*, Fig. S10*B*). Similarly, siRNAs against PKCβII blocked Smad-3-stimulated collagen I (α2) (Fig. 9*C*, Fig. S10*C*). These results demonstrate that both Smad 3 pathway and PKCβII contribute to the expression of DJ-1 by TGFβ.

We have shown above the regulation of PKCβII by DJ-1 (Fig. 4, *B*, *D*, *F* and *H*). To examine whether TGFβ-stimulated DJ-1 integrates PKCβII with the expression of collagen I (α2), siRNAs against DJ-1 and a mutant of PKCβII (HA-tagged PKCβII CAT) conferring constitutive catalytic activity were cotransfected into proximal tubular epithelial cells. As shown in Fig. 10*A*, siDJ-1–induced inhibition of TGFβ-stimulated collagen I (α2) expression was reversed by the expression of catalytically active PKCβII (Fig. S11*A*). In fact, when a dominant negative PKCβII was expressed, collagen I (α2) expression was inhibited in response to both FLAG DJ-1 alone and FLAG DJ-1 plus TGFβ (Fig. 10*B*, Fig. S11*B* and Fig. 10*C*, Fig. S11*C*). Similarly, siRNAs against PKCβII blocked FLAG DJ-1– as well as both FLAG DJ-1– and TGFβ-mediated expression of collagen I (α2) (Fig. 10*D*, Fig. S11*D* and Fig. 10*E*, Fig. S11*E*). Collectively, our results demonstrate a role for PKCβII downstream of DJ-1 in the expression of collagen I (α2) induced by TGFβ.

DJ-1 regulates Hif1α via PKCβII to upregulate collagen I (α2) expression

A role for Hif1α to drive renal fibrosis is established in CKD in which TGFβ is a major participant (68, 69). TGFβ increases expression of Hif1α to induce many fibrotic genes including

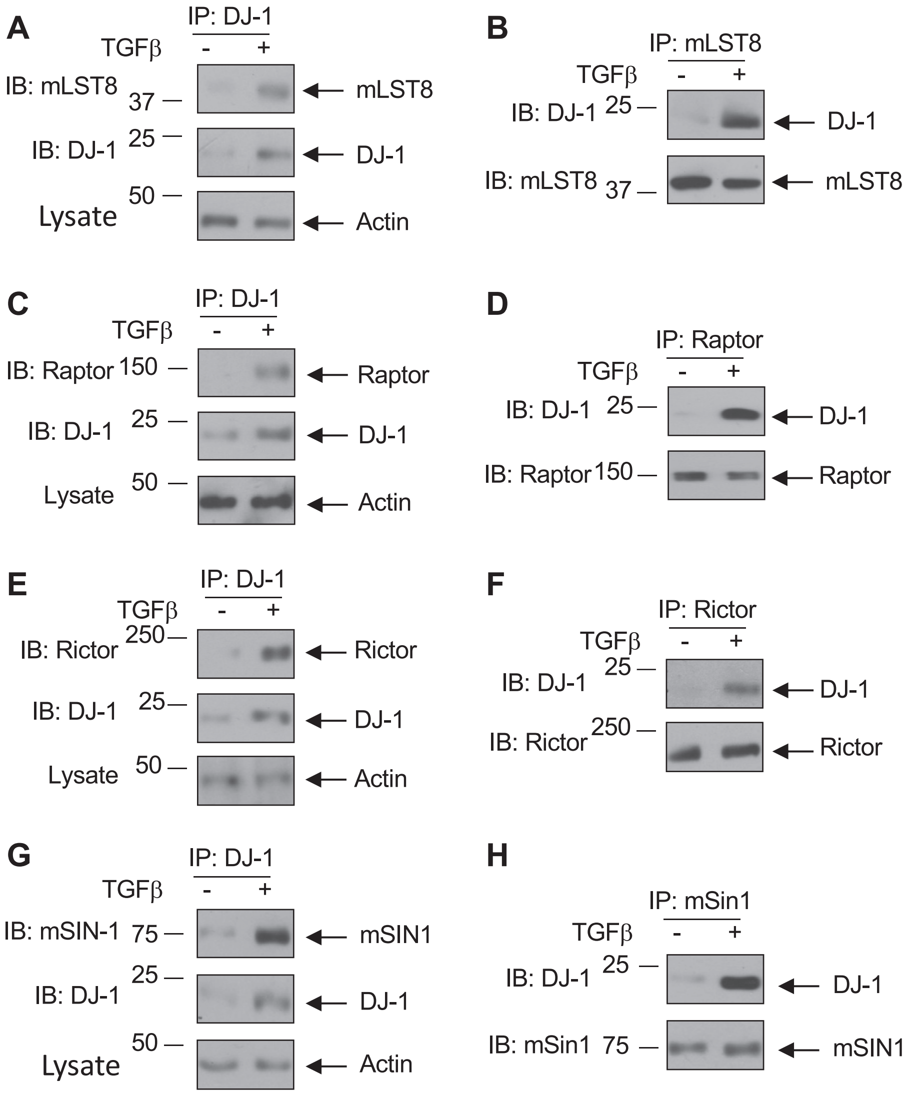

Figure 6. TGFβ induces association of DJ-1 with mTORC1 and mTORC2. Human proximal tubular epithelial cells were incubated with 2 ng/ml TGFβ for 24 h. *A, C, E,* and *G,* the cell lysates were immunoprecipitated with DJ-1 antibody followed by immunoblotting with antibodies against mLST8 (*panel A*), raptor (*panel C*), rictor (*panel E*), mSin1 (panel *G*), and DJ-1 antibodies to detect the corresponding proteins. The *bottom* panels show immunoblot analysis of the indicated proteins in the cell lysates. *B, D, F,* and *H,* the cell lysates were immunoprecipitated with antibodies against mLST8 (panel *B*), raptor (panel *D*), rictor (panel *F*), and mSin1 (panel *H*) followed by immunoblotting with DJ-1 antibody and antibodies against mLST8, raptor, rictor, and mSin1 as indicated. Representative of 3 to 5 independent experiments is shown. Quantification and significance of these data are shown in Fig. S7, *A–H*. TGFβ, transforming growth factor β.

collagen I (α2) (68, 70). Although TGFβ-stimulated canonical Smad 3 regulates expression of collagen I (α2), recently, a role of Hif1α has been shown in normoxic renal cells (71–73). Furthermore, rapamycin inhibited TGFβ-stimulated Hif1α-mediated increase in collagen I (α2), suggesting involvement of mTORC1 (65). We examined the role of mTORC2 in this process. Downregulation of rictor, which regulates mTORC2 activity (74), by two independent shRNAs, significantly inhibited TGFβ-stimulated expression of Hif1α (Fig. 11*A* and *B*, Fig. S12, *A* and *B*). We have shown above that DJ-1 regulates mTORC2. Therefore, we examined whether DJ-1 regulates Hif1α expression in proximal tubular epithelial cells. siRNAs against DJ-1 significantly impaired TGFβ-induced expression of Hif1α (Fig. 11*C*, Fig. S12*C*). In addition, overexpression of DJ-1 enhanced the level of Hif1α similar to that induced by TGFβ (Fig. 11*D*, Fig. S12*D*). Since we have demonstrated activation of PKCβII downstream of mTORC2, we determined its role in Hif1α expression. Dominant negative PKCβII inhibited Hif1α expression in response to TGFβ (Fig. 11*E*, Fig. S12*E*). Similarly, siRNAs against PKCβII blocked the expression of Hif1α (Fig. 11*F*, Fig. S12*F*). Further, expression of constitutively active PKCβII increased Hif1α similar to TGFβ (Fig. 11*G*, Fig. S12*G*). These results demonstrate independent involvement of DJ-1 and PKCβII in the expression of Hif1α.

Since mTORC2 regulates PKCβII and expression of Hif1α, we examined the involvement of mTORC2 in the expression of collagen I (α2). Expression of two independent shRNAs against rictor blocked TGFβ-stimulated collagen I (α2) expression (Fig. 12, *A* and *B*, Fig. S13, *A* and *B*). Next, to probe further whether Hif1α has any connection with DJ-1 in

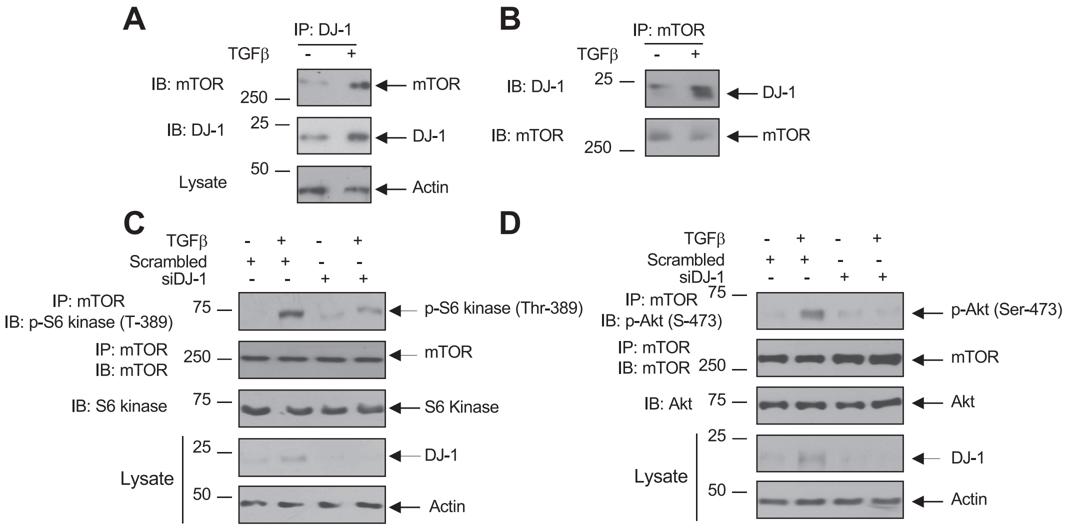

Figure 7. TGFβ induces association of DJ-1 with mTOR to increase mTORC1 and mTORC2 activities. *A* and *B*, human proximal tubular epithelial cells were incubated with 2 ng/ml TGFβ for 24 h. *A*, the cell lysates were immunoprecipitated with DJ-1 antibody followed by immunoblotting with antibodies against mTOR and DJ-1. The *bottom* panels show actin immunoblot. *B*, the cell lysates were immunoprecipitated with mTOR antibody followed by immunoblotting with DJ-1 and mTOR antibodies, respectively. *C* and *D*, immunecomplex kinase assays for mTORC1 and mTORC2. Human proximal tubular epithelial cells were transfected with siRNAs against DJ-1 or scrambled RNA prior to incubation with 2 ng/ml TGFβ for 24 h. The cell lysates were immunoprecipitated with mTOR antibody. In panel (*C*), the immunoprecipitate was assayed for mTORC1 activity using 100 ng recombinant S6 kinase substrate in the presence of ATP in an immunecomplex kinase assay as described in the Experimental procedures. In panel (*D*), the mTOR immunoprecipitate was used to assay mTORC2 activity using 100 ng recombinant Akt as substrate. For S6 kinase and Akt blots in these panels, 20 ng recombinant proteins were run in parallel. *Bottom* panels show immunoblots of the indicated proteins in the cell lysates. Representative of four to five experiments is shown. Quantification and significance of these data are shown in Fig. S8, *A–D*. TGFβ, transforming growth factor β.

expression of collagen I (α2), siRNAs against DJ-1 and a vector-expressing Hif1α were cotransfected into proximal tubular epithelial cells. Results showed overexpression of Hif1α reversed the siDJ-1–mediated inhibition of TGFβ-stimulated collagen I (α2) expression (Fig. 12*C*, Fig. S13*C*). To specifically examine the role of DJ-1, FLAG DJ-1 and siRNAs against Hif1α were cotransfected. DJ-1 was expressed alone and, in the presence of TGFβ, siHif1α inhibited the expression of collagen I (α2) (Fig. 12, *D* and *E*, Fig. S13, *D* and *E*). These results are consistent with the involvement of Hif1α in the expression of collagen I (α2) downstream of DJ-1. Given the role of PKCβII in the expression of Hif1α, and that these molecules are activated in renal fibrosis, it is important to investigate whether there is any link between them in the expression of collagen I (α2). For these studies, overexpression of Hif1α was utilized along with expression of dominant negative PKCβII. As seen above, kinase-dead PKCβII inhibited TGFβ-stimulated collagen I (α2) expression (Fig. 12*F*,

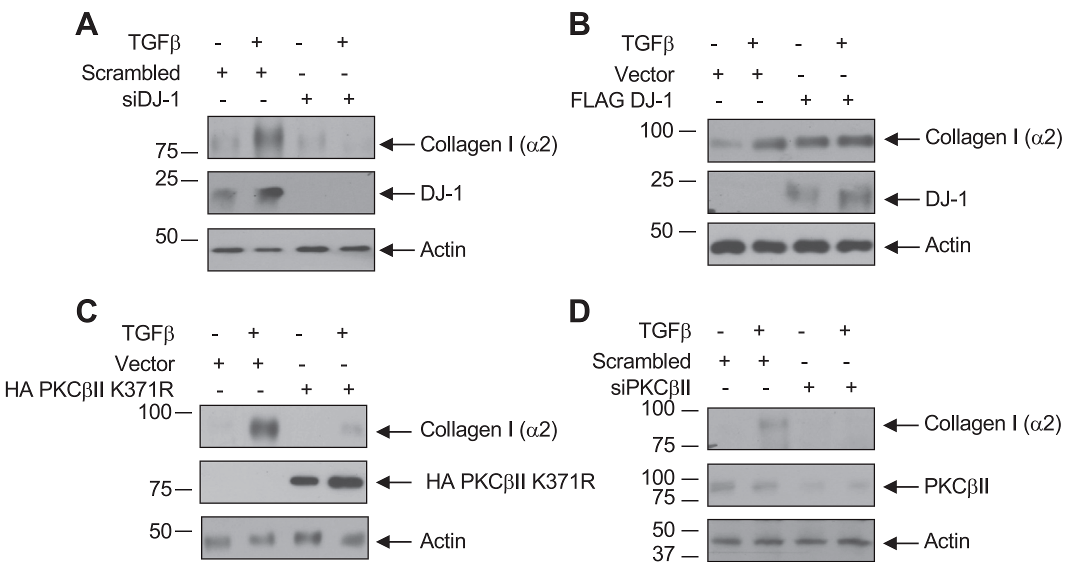

Figure 8. TGFβ-stimulated expression of collagen I (α2) is regulated by DJ-1 and PKCβII. Human proximal tubular epithelial cells were transfected with siRNAs against DJ-1 (panel *A*) and PKCβII (panel *D*) and with FLAG-tagged DJ-1 (panel *B*) and HA-tagged PKCβII K371R (panel *C*) prior to incubation with 2 ng/ml TGFβ for 24 h. The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three to four independent experiments is shown. Quantification and significance of these data are shown in Fig. S9, *A–D*. TGFβ, transforming growth factor β.

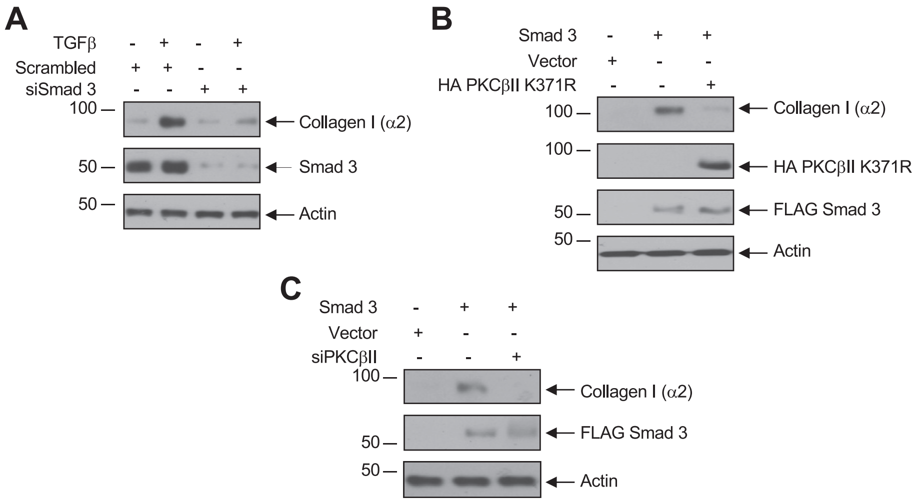

Figure 9. PKCβII cooperates with Smad 3 signaling for TGFβ-stimulated collagen I (α2) expression. *A*, human proximal tubular epithelial cells were transfected with siRNAs against Smad 3 prior to incubation with 2 ng/ml TGFβ for 24 h. *B* and *C*, cells were cotransfected with Smad 3 and PKCβII K371R (panel *B*) or siRNAs against PKCβII (panel *C*) prior to incubation with 2 ng/ml TGFβ for 24 h. The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three independent experiments is shown. Quantification and significance of these data are shown in Fig. S10, *A–C*. TGFβ, transforming growth factor β.

Fig. S13*F*). However, coexpression of Hif1α prevented this inhibition (Fig. 12*F*, Fig. S13*F*). Similarly, expression of Hif1α reversed siPCKβII-mediated inhibition of TGFβ-stimulated collagen I (α2) expression (Fig. 12*G*, Fig. S13*G*). To complement these results, siRNAs against Hif1α were cotransfected with vector containing catalytically active PKCβII into proximal tubular epithelial cells. siHif1α inhibited the expression of collagen I (α2) in cells expressing constitutively active PKCβII alone as well as in cells expressing constitutively active PKCβII treated with TGFβ (Fig. 12, *H* and *I*, Fig. S13, *H* and *I*). Taken together, the above results strongly suggest that Hif1α downstream of TGFβ-DJ-1–mTORC2–PKCβII axis contributes to the expression of collagen I (α2).

We and others have shown that TGFβ-induced expression of collagen I (α2) is regulated by transcriptional mechanism via Hif1α in renal cells (26, 68, 70, 73). To determine the role of TGFβ-stimulated DJ-1 in Hif1α-mediated transcription of collagen I (α2), we used luciferase reporter construct in proximal tubular epithelial cells. The cells were cotransfected with the reporter plasmid along with siDJ-1 and Hif1α. As shown in Fig. 13*A*, expression of Hif1α reversed the siDJ-1–mediated inhibition of TGFβ-stimulated transcription of collagen I (α2). Furthermore, siHif1α inhibited the collagen I (α2) promoter activity by FLAG-DJ-1 alone and in the presence of TGFβ (Fig. 13, *B* and *C*). Similar to the collagen I (α2) protein, expression of dominant negative PKCβII as well as siPKCβII inhibited the transcription of collagen I (α2) (Fig. 13, *D* and *E*). Coexpression of Hif1α prohibited this inhibition (Fig. 13, *D* and *E*). To confirm this observation, we used constitutively active PKCβII, which alone and with TGFβ, increased collagen I (α2) transcription (Fig. 13, *F* and *G*). Coexpression of siHif1α blocked this increase (Fig. 13, *F* and *G*). These results indicate that Hif1α downstream of DJ-1/PKCβII regulates collagen I (α2) expression in response to TGFβ in proximal tubular epithelial cells.

Increased expression of DJ-1 in the renal cortex of diabetic rats

Progressive diabetic kidney disease is characterized by renal fibrosis as a pathology (75). Hyperglycemia induces renal production of TGFβ that contributes to the pathogenesis of diabetic nephropathy (6, 75–77). Rodent models of diabetes are a useful tool to study renal fibrosis in diabetic nephropathy (78–81). Previously, we and others reported that mTOR is activated in the kidneys of streptozotocin-induced diabetes in rat and in mice models of diabetes and, in human kidney (24, 61–64, 82–85). Administration of rapamycin ameliorates the pathologies of diabetic nephropathy including renal fibrosis, suggesting a significant role of mTOR in this process (50, 62–64, 85). Our results above show a conclusive role of DJ-1 in the activation of mTOR and in the expression of proximal tubular cell collagen I (α2) expression. To investigate the *in vivo* relevance of our observations, we used streptozotocin-induced type 1 diabetic rats, which showed early changes of diabetic nephropathy including expression of fibrotic markers (86). Renal cortical lysates were used to determine the expression of TGFβ. Results showed significant increase in the expression of this fibrotic cytokine in the diabetic rats (Fig. 14, *A* and *B*). This increase in TGFβ correlated with enhanced expression of DJ-1 (Fig. 14, *C* and *D*). In proximal tubular epithelial cells, increased expression of DJ-1 by TGFβ was associated with the activation of mTORC1 and mTORC2. Consistently, the phosphorylation of S6 kinase at Thr-389 and Akt at Ser-473 as measures of mTORC1 and mTORC2 activation, respectively, was significantly elevated in the diabetic cortex (Fig. 14, *E–H*). In fact, we detected increased phosphorylation of rps6 and GSK3β, two substrates of S6 kinase and Akt, respectively, indicating activation of these kinases (Fig. 14, *I–L*). We have shown that DJ-1 promoted the hydrophobic motif site phosphorylation and activation of PKCβII in proximal tubular epithelial cells. We

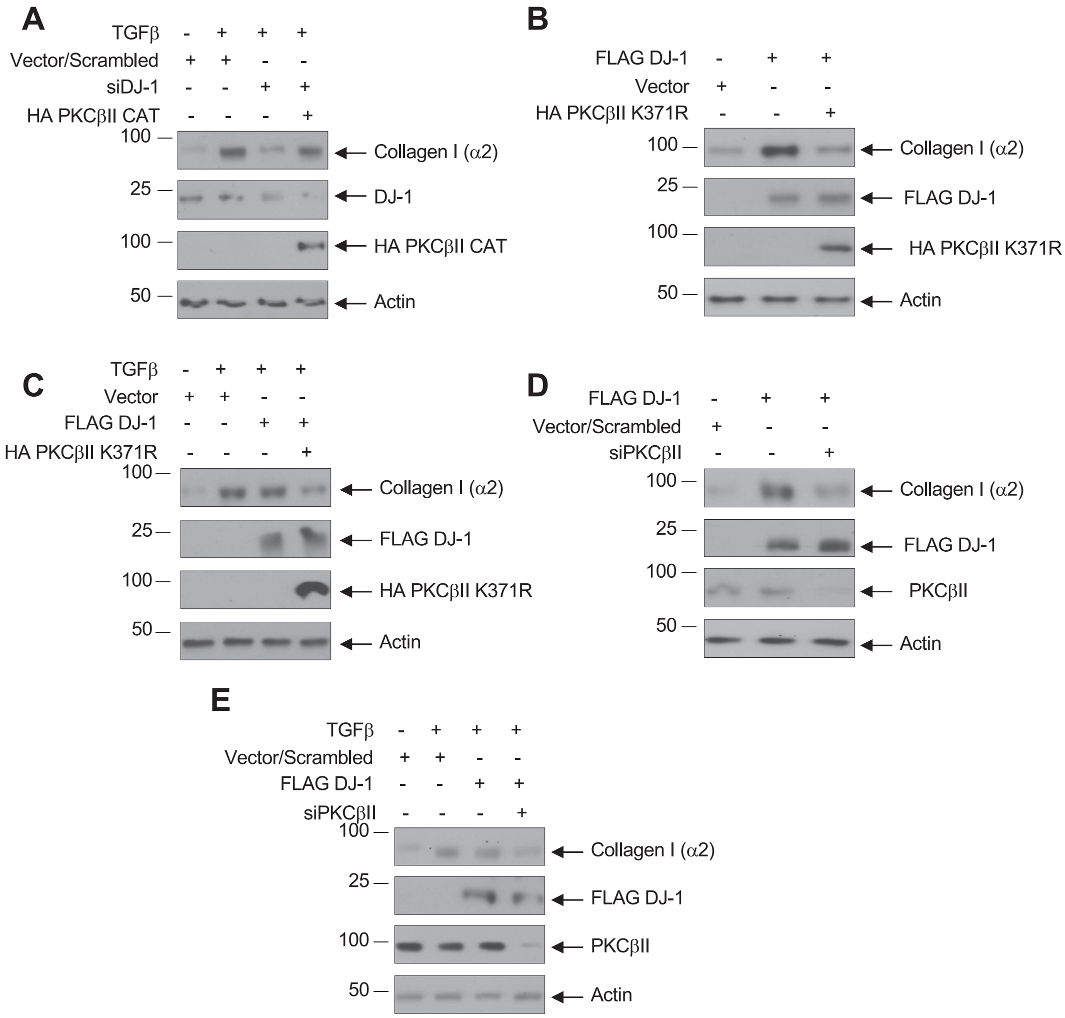

Figure 10. TGFβ-stimulated expression of collagen I (α2) is regulated by PKCβII downstream of DJ-1. Human proximal tubular epithelial cells were cotransfected with siDJ-1 plus HA-tagged PKCβII CAT (panel *A*), FLAG DJ-1 plus HA PKCβII K371R (panels *B* and *C*), and FLAG DJ-1 plus siPKCβII (panels *D* and *E*). The transfected cells were incubated with 2 ng/ml TGFβ for 24 h (panels *A, C,* and *E*). The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three independent experiments is shown. Quantification and significance of these data are shown in Fig. S11, *A–E*. TGFβ, transforming growth factor β.

examined these phenomena. The results shown in Fig. 14, *M–P* demonstrate elevated phosphorylation of PKCβII at its hydrophobic motif site, resulting in phosphorylation of its substrate MARCKS. Our results above show that DJ-1–regulated PKCβII controls the expression of Hif1α, which increases the expression of collagen I (α2) in response to TGFβ. We determined the expression of Hif1α. A significant increase in the expression of Hif1α was observed in the renal cortex of diabetic animals (Fig. 14, *Q* and *R*). This increase was associated with elevated levels of collagen I (α2) (Fig. 14, *S* and *T*). These results indicate a possible role of DJ-1 in regulation of mTOR and PKCβII for Hif1α-dependent collagen I (α2) expression in the pathology of diabetic kidney injury.

Discussion

TGFβ plays significant role in CKD (76, 87). Increased expression of TGFβ in a rat model of glomerulonephritis promoted glomerulosclerosis (88). Importantly, a TGFβ antibody ameliorated the fibrosis in this model (89). Also, significant ameliorative effect of a TGFβ monoclonal antibody was observed in the models of adriamycin- and podocyte ablation–induced nephropathy in mice (90). Renal fibrosis elicited by diabetic nephropathy shows hyperexpression of TGFβ, which contributes to the pathology (6, 91). In fact, renal hypertrophy and matrix protein expression, two features of diabetic nephropathy in mice models of type 1 and type 2 diabetes, were prevented by TGFβ neutralizing antibody (77, 92). Generation of whole body TGFβ hypomorphic and overexpression mice models with type 1 diabetes showed decreased and increased albuminuria, respectively (93). In fact, increased expression of TGFβ in type 1 diabetic mice showed significantly reduced proximal tubular expression of megalin, which endocytose albumin (93). When TGFβ expression was induced specifically in the proximal tubules of the same diabetic mouse model, albuminuria and fibrosis were affected significantly (93). Furthermore, in a more recent study where a soluble TGFβRII was administered to a model of renal fibrosis via a viral vector-mediated gene therapy, the kidney pathology was significantly ameliorated (94). These results conclusively

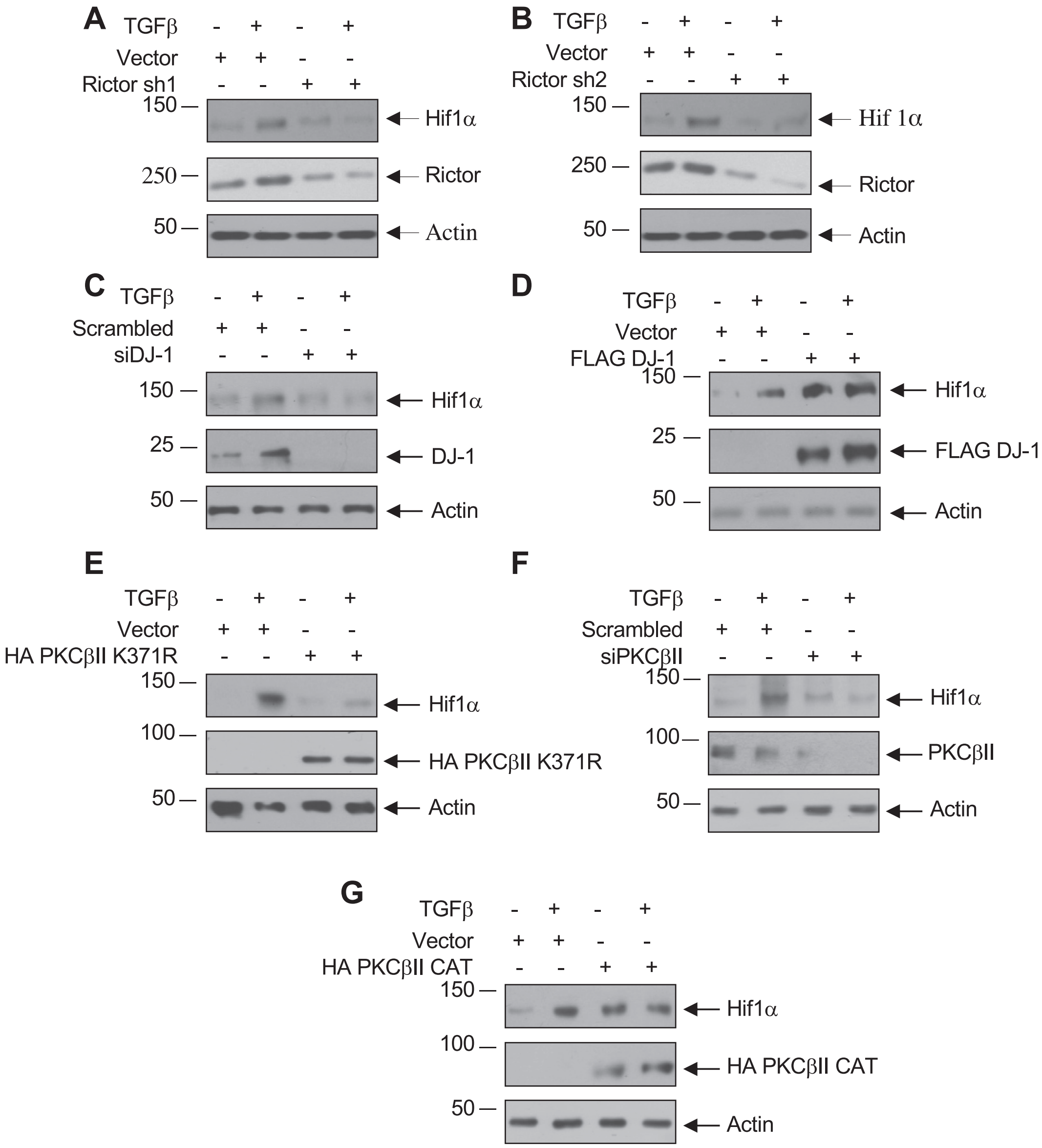

Figure 11. Rictor as well as DJ-1 and PKCβII regulate TGFβ-stimulated Hif1α expression. Human proximal tubular epithelial cells were transfected with two independent shRNAs against rictor (panels *A* and *B*), siDJ-1 (panel *C*), FLAG DJ-1 (panel *D*), HA-tagged PKCβII K371R (panel *E*), siPKCβII (panel *F*), and HA-tagged PKCβII CAT (panel *G*). The transfected cells were incubated with 2 ng/ml TGFβ for 24 h. The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three independent experiments is shown. Quantification and significance of these data are shown in Fig. S12, *A–G*. TGFβ, transforming growth factor β; Hif1α, hypoxia-inducible factor 1α.

demonstrate a role of TGFβ in renal fibrosis. Thus, blocking the action of TGFβ may be beneficial for renal fibrosis. However, because TGFβ plays an important role in many other biological responses including immune homeostasis, potential adverse effects present a major challenge for employing anti-TGFβ therapy (91). Thus, it is important to delineate intricacies of TGFβ signaling to identify alternative therapeutic strategies for inhibiting renal fibrosis.

In the present article, we identified the familial early-onset Parkinson's disease protein DJ-1 as a target of TGFβ-induced canonical Smad 3 signaling leading to its increased expression in the renal proximal tubular epithelial cells. Similar to the role of oxidative stress in Parkinson's disease, a role of reactive oxygen species in the pathogenesis of renal fibrosis has been established (6, 50, 95, 96). In fact, TGFβ has been shown to increase reactive oxygen species due to increased expression of NADPH oxidases especially Nox4 that contribute to the expression of fibrotic markers (24, 27, 97, 98). Along with quenching reactive oxygen species in neuronal cells in Parkinson's disease, DJ-1 has been shown to regulate proliferation of cancer cells including breast, lung, thyroid, pancreas, and prostate among many (60, 99). However, it was suggested that DJ-1 function is reciprocally regulated in neurodegenerative disorder and cancer (100). Molecular analysis showed that the PI 3 kinase–Akt signaling is downregulated in the Parkinson's disease, causing apoptosis of neurons while these enzymes act as the drivers of cancer cell proliferation and metastasis including glioblastoma (55, 101, 102). Previously, in a model of eye development in *Drosophila*, DJ-1 was placed upstream of Akt kinase (49). Also, DJ-1 was shown to regulate Akt

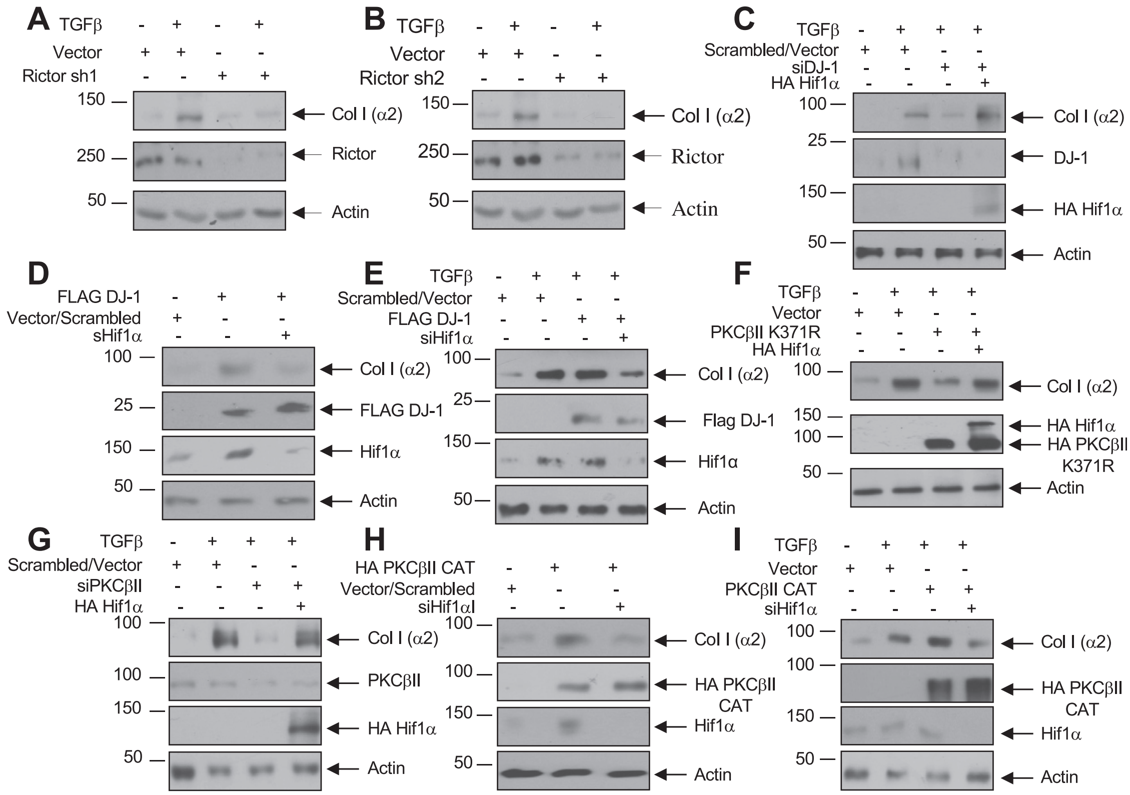

Figure 12. Rictor as well as Hif1α downstream of DJ-1–PKCβII axis regulates TGFβ-stimulated collagen I (α2) expression. Human proximal tubular epithelial cells were transfected with two independent shRNAs against rictor (panels *A* and *B*), with siDJ-1 plus Hif1α (panel *C*), FLAG DJ-1 plus siHif1α (panel *D* and *E*), PKCβII K371 R plus Hif1α (panel *F*), siPKCβII plus Hif1α (panel G), PKCβII CAT plus siHif1α (panel *H*), and PKCβII CAT plus siHif1α (panel *I*). The cell lysates were immunoblotted with antibodies to detect the indicated proteins. Representative of three to four independent experiments is shown. Quantification and significance of these data are shown in Fig. S13, *A*–*I*. TGFβ, transforming growth factor β; Hif1α, hypoxia-inducible factor 1α.

activation in cancer cells (49, 99). Recently, we and others have established a role of TGFβ-stimulated PI 3 kinase–Akt noncanonical signaling in increased synthesis of matrix protein in renal cells (21, 71). In the present study, we demonstrate a mechanism of TGFβ-stimulated Akt phosphorylation and its activation via increased DJ-1 in proximal tubular epithelial cells. In fact, we show that DJ-1 regulates the mTORC2 activity, which phosphorylates the hydrophobic motif site of Akt.

mTOR exists in three different complexes of which complexes 1 and 2 have sizes of 1.0 and 1.3 mDa respectively and contain common and distinct multiproteins which are not present in the mTORC3 (45, 47, 103). While both mTORC1 and mTORC2 manifest rapamycin sensitivity, mTORC3 is resistant (104). mTORC1 and mTORC2 phosphorylate distinct proteins and enzymes while mTORC3 uses substrates which can be phosphorylated by both mTORC1 and C2 (45, 103). Importantly, Akt is phosphorylated by the mTORC2 for its full activation (54). Our observation that DJ-1 regulates Akt hydrophobic motif site phosphorylation demonstrates DJ-1 regulation of mTORC2, which is known to regulate mTORC1 via Akt (45). In fact, we found that DJ-1 contributes to the activation of mTORC1. Interestingly, we demonstrated that TGFβ increased phosphorylation of S6 kinase at Thr-389 (measure of mTORC1) and Akt at Ser-473 (measure of mTORC2) along with Thr-308, similar to that increased by activation of a receptor tyrosine kinase platelet-derived growth factor (PDGF) receptor by its ligand (Fig. S14, *A*–*D*). Similarly, the both ligands significantly stimulated phosphorylation of PKCβII at Ser-660 (Fig. S15, *A* and *B*).

Beneficial and significant deleterious effects of different PKC isoforms have been reported in renal cells (28).Although PKCβ contributes to significant pathologies during the progression of renal fibrosis, other isoforms such as PKCε has been shown to be protective in other organ fibrosis (105). Deletion of PKCε induced renal fibrosis in mice. Furthermore, induction of diabetes in this model aggravated the pathology (106). TGFβ has been shown to activate PKCε in proximal tubular epithelial cells possibly as a protective mechanism, where hyperactivation of this kinase inhibited TGFβ-induced Smad 3 phosphorylation and increased Smad 7 expression to block fibrotic marker expression (107). In contrast, in the renal cortex, which is predominantly constituted by proximal tubular epithelial cells, increased expression and apical translocation of PKCα is observed in diabetic mice. Although in this model, increased TGFβ expression is obvious, its expression did not correlate with PKCα activation (108). Similarly, TGFβ expression was not affected in the kidneys of diabetic PKCα KO mice (109). In the proximal tubular epithelial cells, urinary protein–induced epithelial-mesenchymal transdifferentiation and fibronectin expression were mediated by PKCα and PKCβI and not by PKCβII (110). In contrast to these results,

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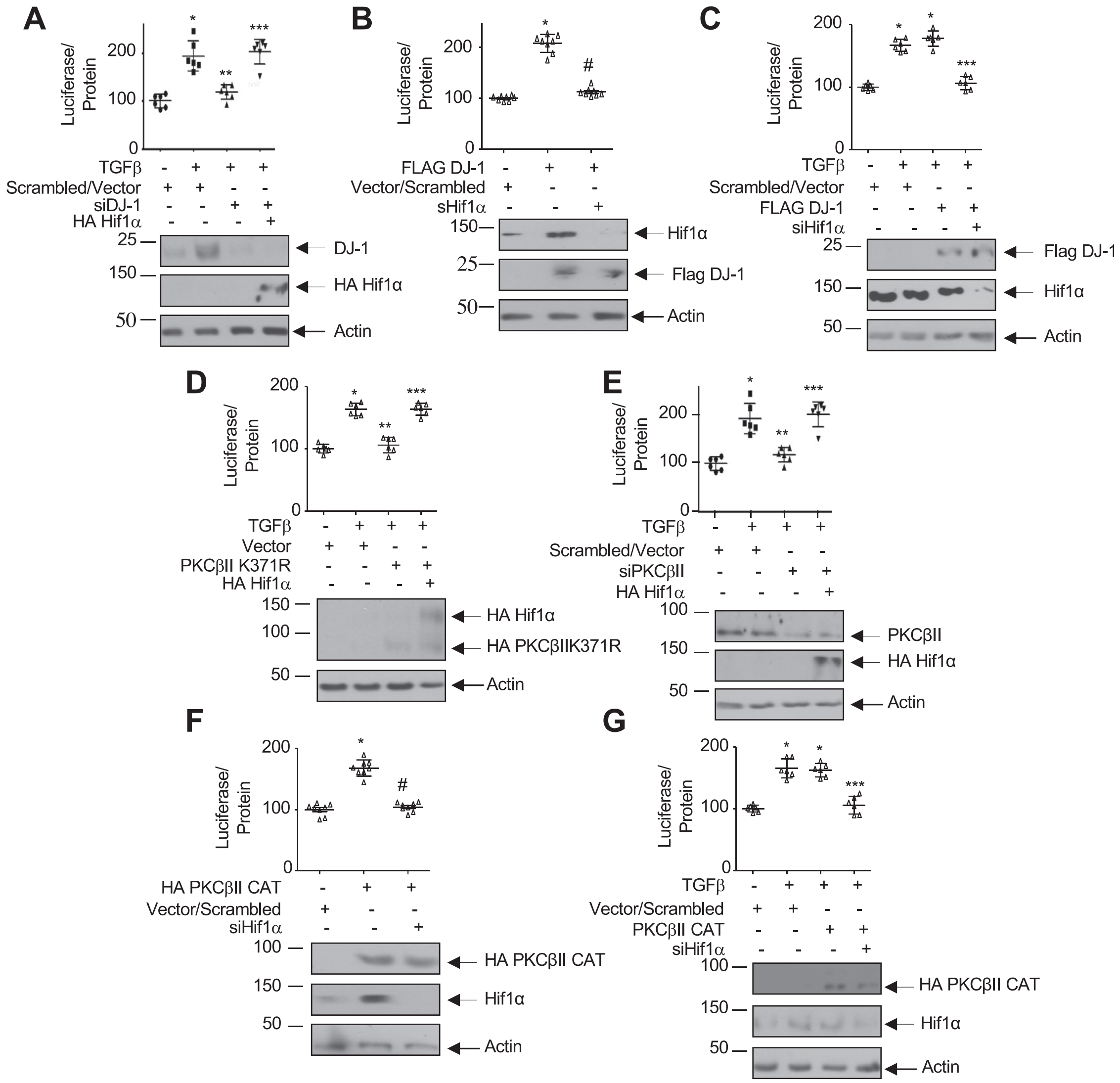

Figure 13. Rictor and Hif1α following DJ-1/PKCβII regulates TGFβ-induced transcription of collagen I (α2). Proximal tubular epithelial cells were cotransfected with collagen I (α2) promoter-driven luciferase reporter plasmid along with siDJ-1 plus Hif1α (panel *A*), FLAG DJ-1 plus siHif1α (panels *B* and *C*), PKCβII K371 R plus Hif1α (panel *D*), siPKCβII plus Hif1α (panel *E*), and HA PKCβII CAT plus siHif1α (panels *F* and *G*). The transfected cells were incubated with 2 ng/ml TGFβ for 24 h. The cell lysates were assayed for luciferase activity as described in the Experimental procedures. Mean ± SD of six to eight measurements is shown. *$p < 0.001$ *versus* control (panels *A–G*); **$p < 0.001$ *versus* TGFβ (panels *A, D, E*); #$p < 0.001$ *versus* FLAG DJ-1 or PKCβII CAT (panels *B* and *F*); ***$p < 0.001$ *versus* TGFβ plus siDJ-1 (panel *A*), *versus* TGFβ plus FLAG DJ-1 (panel *C*), *versus* TGFβ plus PKCβII K371R (panel *D*), *versus* TGFβ plus siPKCβII (panel *E*), and TGFβ plus PKCβII CAT (panel *G*). The *bottom* panels show expression of indicated proteins. TGFβ, transforming growth factor β; Hif1α, hypoxia-inducible factor 1α.

overexpression of PKCβII in proximal tubular epithelial cells increased TGFβ and fibronectin expression (111). Furthermore, in a rat model of ureteric obstruction, which predominantly involves TGFβ, inhibition of PKCβ ameliorated the pathology (112). It should be noted that phosphorylation-dependent activation of PKCβII has not been investigated in these studies related to kidney diseases. However, the major pathologic effects of PKCβ are mediated through TGFβ, suggesting that this cytokine is a downstream target of the kinase (30, 106, 111, 113–116). In contrast to these observations, we demonstrate that PKCβII is part of the TGFβ noncanonical signaling. We find that TGFβ increases the hydrophobic motif site phosphorylation of PKCβII, resulting in its activation and phosphorylation of its substrate. In fact, TGFβ increased the PKCβII hydrophobic motif phosphorylation to the same extent as by activation of PDGF receptor in response to PDGF (Fig. 15, *A* and *B*).

Importantly, we show that TGFβ-stimulated DJ-1 regulates these phenomena. Thus, with our findings that TGFβ-stimulated DJ-1 regulates phosphorylation at the hydrophobic motif sites of Akt and PKCβII and phosphorylation of S6 kinase, we conclude that DJ-1 controls the mTORC2 and mTORC1 activities in proximal tubular epithelial cells. The mechanism by which DJ-1 activates the mTOR complexes is not known. DJ-1 is a versatile protein and is able to bind or form complex with multiple signaling proteins such as ErbB3, androgen receptor, Raf, SIRT1, and more to either activate or inhibit their functions (60, 117–120). Interestingly, we identified that TGFβ stimulated complex formation between DJ-1 and raptor and mLST8 as well as with rictor and mSin1. Furthermore, we

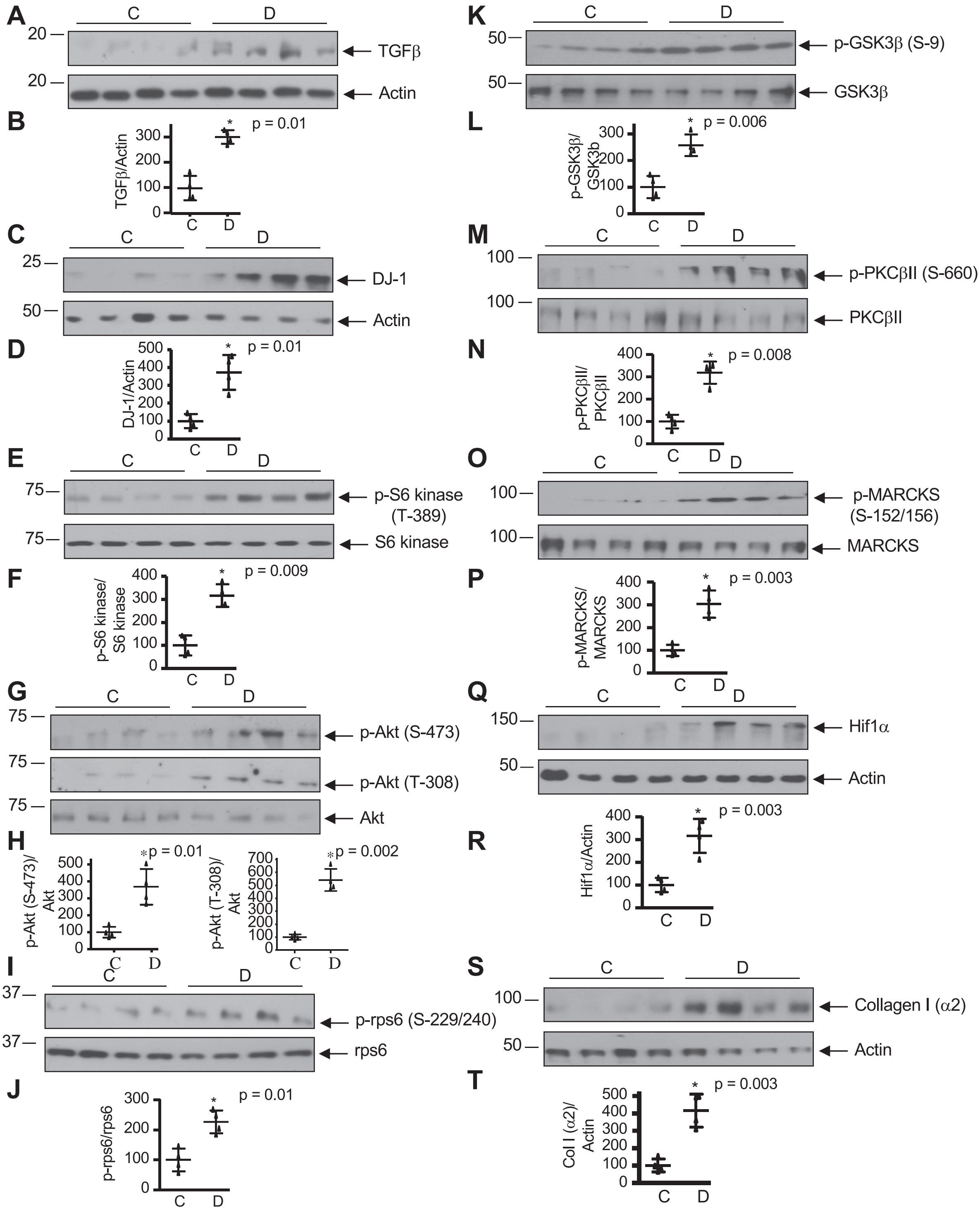

Figure 14. Increased expression of TGFβ is associated with enhanced expression of DJ-1, Hif1α, and collagen I (α2) along with phosphorylation/activation of mTORC1 (as judged by phosphorylation of S6 kinase/rps6) and mTORC2 (as judged by phosphorylation of Akt, GSK3β, PKCβII and MARCKS) in diabetic rats. Renal cortical lysates were immunoblotted with antibodies to detect the indicated proteins. C, control animal; D, diabetic animal. Scatter graphs show quantification of top immunoblot. Mean ± SD of four animals per group is shown. *p* values are indicated. TGFβ, transforming growth factor β; Hif1α, hypoxia-inducible factor 1α.

demonstrate association of DJ-1 with mTOR. Importantly, we show that DJ-1 regulates the kinase activities of both mTORC1 and mTORC2. These data provide a mechanism on how DJ-1 may regulate both mTORC1 and mTORC2 activities.

A role of TGFβ and PKCβ has been established in renal fibrosis (30, 77, 112, 121). TGFβ significantly contributes to the expression of the matrix proteins including collagen I (α2) during the progression of renal fibrosis (121, 122). Previously, a cross talk between TGFβ receptor-specific Smad 3 and mTOR has been implicated in the expression of collagen I (α2) in response to TGFβ (71, 123). In a mouse model using unilateral ureteral obstruction (UUO) of kidney, the interstitial fibrosis is mainly mediated by significant expression of TGFβ due to activation of mTOR in the macrophages and myofibroblasts but not in the proximal tubular cells (124, 125). A role of DJ-1 in preventing renal damage in UUO model has been shown using a DJ-1 KO mouse. Interestingly, the DJ-1 KO mice showed decreased TGFβ levels compared to WT UUO model. However, there was no difference in the increase in expression of collagen I (α1) (125). In contrast to these results, our results show that DJ-1 is downstream of TGFβ in which its upregulated expression contributes to the expression of collagen I (α2) in the proximal tubular epithelial cells, suggesting a possible role in tubular fibrosis. These results are opposite to that observed in cardiac fibrosis induced by ischemia reperfusion injury where DJ-1 protects the organ from fibrosis (126). However, our results are in line with the pathologic role of DJ-1 in liver fibrosis (127). Interestingly, we not only show a role of DJ-1 in regulation of collagen I (α2) expression but it regulates PKCβII to increase the expression of the matrix protein. These results for the first time provide a mechanism for expression of this matrix protein involving the upstream and downstream targets of mTOR in response to TGFβ.

TGFβ uses the canonical Smad 3 signal transduction for expression of fibrogenic genes (128). We demonstrate the presence of a cross talk between Smad 3 and PKCβII in the regulation of collagen I (α2). Hif1α, which is mainly stabilized by hypoxia, has been shown to be upregulated along with TGFβ and promotes epithelial to mesenchymal transdifferentiation during the progression of tubulointerstitial fibrosis (129). However, a beneficial role of myeloid-derived Hif1α has been reported in the UUO and remnant kidney models of renal fibrosis (130, 131). Furthermore, stable expression of Hif1α in a model of subtotal nephrectomy increased renal fibrosis (69). However, expression of Hif1α is significantly increased in the tubules of patients with diabetic nephropathy (132). In conjunction with these studies, inhibition of Hif1α in a model of diabetic nephropathy ameliorated tubular injury (133). Similarly, inhibition of Hif1α by YC1 ameliorated progression of renal fibrosis in the UUO model (69). Also, proximal tubular ablation of Hif1α blocked renal fibrosis including inhibition of expression of matrix modification enzyme PAI-1 and collagen deposition (132). Interestingly, we and others have shown hypoxia-independent activation of Hif1α in renal cells including proximal tubular epithelial cells (26, 73). In fact, an interaction between TGFβ-stimulated Smad 3 and Hif1α has been reported to regulate the expression of collagen I (α2) (65, 70, 73). Normoxic Hif1α level is regulated by PI 3 kinase/Akt and mTOR (65, 71, 134). Also, these enzymes downstream of TGFβ controls collagen I (α2) expression in renal cells (48, 71). However, the intricacies of this signal transduction that regulates Hif1α-mediated collagen I (α2) expression has not been clarified. Upstream of mTORC2, we identified DJ-1 to regulate mTOR activity. In fact, our data for the first time show that DJ-1 controls the TGFβ-stimulated Hif1α, which increases the expression of collagen I (α2). Furthermore, expression of collagen I (α2) protein was dependent upon PKCβII downstream of DJ-1. Similarly, we observed that DJ-1–regulated PKCβII increased the transcription of collagen I (α2) by Hif1α in response to TGFβ. Together, our data provide the first evidence for a role of DJ-1 to regulate mTORC2-dependent activation of PKCβII to induce Hif1α that increases proximal tubular collagen I (α2) expression. In fact, in a rat model of diabetes which exhibits features of diabetic nephropathy, we demonstrate expression of DJ-1 concomitant with the activation of mTOR–PKCβII axis and increased Hif1α in association with increase in collagen I (α2).

Due to sequence homology and structural similarities among the catalytic domains of various PKC isozymes, it has been difficult to develop selective inhibitors for specific isotypes. However, ruboxistaurin, a PKCβ-specific inhibitor was shown to ameliorate renal pathologies in preclinical models of diabetic nephropathy (31, 135). This compound was evaluated in an underpowered human study with 123 patients to test its efficacy to delay diabetic nephropathy. The albumin-creatinine ratio decreased in the ruboxistaurin group without any effect on glomerular filtration rate; however, no statistical significance was observed when the data were analyzed for between-group differences (136, 137). In a large study with patients with diabetic retinopathy, ruboxistaurin did not show any difference in kidney outcomes (138). Targeting mTOR for intervention, we and others have shown beneficial effects of rapamycin in rodent models of diabetic nephropathy (50, 61, 63, 64, 85). However, inhibition of mTOR by chronic treatment with rapamycin shows significant adverse effects including insulin resistance and glucose intolerance (139–141). Similar adverse effects may be observed in humans (141, 142). In this report, we identify a linear signaling pathway in which DJ-1 regulates mTOR, PKCβII, and Hif1α in TGFβ-stimulated fibrotic collagen I (α2) expression. We also demonstrate expression of DJ-1 in the kidneys of mice with diabetic nephropathy concomitant with the activation of PKCβII and expression of Hif1α. Thus, our results show importance of DJ-1 to be considered as an alternative therapeutic target to block the pathologic effects of mTOR and PKCβII in states of TGFβ-mediated renal fibrosis including diabetic nephropathy.

Experimental procedures

Reagents

Materials for cell culture including OPTIMEM medium for transfection, RNA spin mini isolation kit, cDNA synthesizing SuperScript VILO master mix, and PowerUp SYBR Green master mix were purchased from Thermo Fisher. TGFβ and PDGF BB were obtained from R & D Systems. The transfection reagent FuGENE HD and luciferase assay kit were acquired from Promega. NP-40, protease inhibitor cocktail, PMSF, Na_3VO_4, FLAG antibody, and GAPDH primers were obtained from Sigma. TGFβ1 was purchased from R & D Systems. SB 431542 was obtained from CalBiochem. DJ-1, Myc, collagen I (α2), and actin antibodies were acquired from Santa Cruz. Antibodies against phospho-Smad 3 (Ser-423/425), Smad 3, phospho-Akt (Ser-473), p-Akt (Thr-308), Akt, phospho-GSK3β (Ser-9), GSK3β, phospho-S6 kinase (Thr-389), S6 kinase, phospho-rps6 (Ser-240/244), rps6, phospho-PKCβII (Ser-660), PKCβII, phospho-MARCKS (Ser-152/156), raptor, rictor, mTOR, mLST8, and mSin1 were purchased from Cell Signaling. MARCKS antibody was obtained from ProteinTech. HA antibody was obtained from Covance. Pool of three siRNAs against DJ-1 and PKCβII were obtained from Santa Cruz. Recombinant S6 kinase and Akt were obtained from Novus Biological. DJ-1 primers to detect its mRNA were purchased from Qiagen. The FLAG-tagged DJ-1 expression vector was a kind gift from Dr H. Ariga (Hokkaido University). HA-tagged Hif1α plasmid was provided by Dr A. Kung (Dana-Farber Cancer Institute). HA-tagged constitutively active PKCβII CAT and dominant negative PKCβII K371R expression plasmids were purchased from Addgene. The collagen I (α2) promoter–luciferase reporter plasmid has been described previously (84).

Cell culture

The HK-2 proximal tubular epithelial cells were purchased from ATCC. These cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% bovine serum albumin (26, 143). The murine proximal tubular epithelial cells, originally obtained from Dr Eric Neilson at Northwestern University, were grown in DMEM with 5 mM glucose and 7% fetal bovine serum as described previously (144). For experiments, the cells were starved in serum-free medium for 24 h prior to incubation with 2 ng/ml TGFβ for indicated periods of time. For TGFβ receptor I inhibitor, the serum-starved cells were treated with 5 μM SB for 1 hour prior to TGFβ addition.

Animals

Sprague-Dawley rats (200–250 gm) were used for the study. Streptozotocin in sodium citrate buffer (pH 4.5) at a dose of 55 mg/kg body weight was injected through tail vein of the animal. At 24 h postinjection, the blood glucose levels were monitored (82). The rats were housed in the animal facility at UT Health San Antonio. They had free access to food and water. The rats were euthanized after 5 days of streptozotocin injection. The kidneys were removed and renal cortical sections were isolated (82). The cortical preparation was stored in an ultralow freezer at -70 °C. The animal protocol was approved by the UT Health San Antonio Animal Care and Use Committee.

Cell lysis and preparation of renal cortical lysates

At the end of TGFβ incubation, the cell monolayer was washed twice with PBS. RIPA buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4, and 0.1% protease inhibitor cocktail) was added to the cells and incubated at 4 °C for 30 min to permit cell lysis. Lysed cell debris was scraped off and collected in centrifuge tubes. Similarly, the frozen renal cortex was thawed on ice and lysed in RIPA buffer. The lysed cell debris and cortical extracts were spun at 10,000 x g for 30 min at 4 °C. The cleared supernatant was collected in a fresh tube. The protein concentration was determined in this supernatant.

Immunoblotting

Equal amounts of cell or renal cortical lysates were mixed with SDS-PAGE sample buffer, boiled for 5 min, and separated by electrophoresis. The separated proteins were transferred to PVDF membrane using an electroblotting apparatus. To perform immunoblotting, the membrane containing the separated proteins was incubated with the indicated primary antibody at 4 °C. The dilution of antibody used was 1:1000. After the incubation, the membrane was washed and further incubated with horseradish peroxidase–conjugated secondary antibody (1:10,000). The membrane was treated with enhanced chemiluminescence reagent. Subsequently, the membrane was exposed to X-ray film in a dark room to visualize the specific protein band recognized by the primary antibody (145).

Immunoprecipitation

After incubation with TGFβ, the immunoprecipitation (IP) buffer (40 mM Hepes, pH 7.5, 0.3% CHAPS, 1 mM EDTA, 120 mM NaCl, 10 mM pyrophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM glycerophosphate, and 0.1% EDTA-free protease cocktail) was added to the PBS-washed cell monolayer at 4 °C for 30 min. The cell extracts were collected and centrifuged as described above. The cleared supernatant was transferred to a fresh tube and protein concentration was determined. Equal amounts of proteins were incubated with indicated antibody at 4 °C for 30 min (84). This protein-antibody mixture was then incubated overnight with protein G-agarose on rotating device at 4 °C. The mixture was centrifuged briefly to collect the immunebeads. The beads were then washed three times with IP buffer. Finally, the beads were suspended in the SDS polyacrylamide gel sample buffer. The boiled protein sample was separated by SDS-PAGE. Subsequently, the separated proteins were transferred to PVDF membrane and immunoblotted as described above.

RNA preparation and real-time RT-PCR

Total RNAs were isolated from proximal tubular epithelial cells using RNA spin mini isolation kit as described by the vendor's protocol. Five hundred nanogram of RNA was used to prepare first strand cDNAs using Superscript VILO master mix. The cDNA was amplified in a 96-well plate using primers for DJ-1 and GAPDH in a 7500 real time PCR machine (Applied Biosystems). The conditions for PCR were 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, respectively. The relative mRNA levels were normalized to the reference GAPDH in the samples. Data analysis was carried out by the comparative $\Delta\Delta C_t$ method (84).

Immunecomplex kinase assays for mTORC1 and mTORC2

The cells were extracted in IP buffer and centrifuged as described above. The cleared cell lysates were immunoprecipitated with mTOR antibody. After washing the immunebeads with IP buffer, the beads were washed twice with immunecomplex kinase assay buffer (25 mM Hepes, pH 7.4, 100 mM potassium acetate, and 1 mM $MgCl_2$). The immunecomplexes were resuspended in 20 μl immunecomplex kinase assay buffer, which contains 100 ng of recombinant S6 kinase (for mTORC1 substrate) or recombinant Akt (for mTORC2 substrate). The reaction was started with 500 μM ATP at 37 °C and incubated for 30 min. The kinase assay was terminated by adding 4X SDS sample buffer. The reaction mixture was then separated by PAGE and immunoblotted with p-S6 kinase (Thr-389) and p-Akt (Ser-473) antibodies to detect mTORC1 and mTORC2 activities, respectively. For controls, one fifth of the recombinant S6 kinase and Akt were separated by PAGE and immunoblotted with S6 kinase and Akt antibodies, respectively.

Transfection

The cell monolayer was washed with PBS once inside the cell culture hood. OPTIMEM was added to the monolayer. The expression plasmids, vector, siRNAs, or scramble RNA were mixed with OPTIMEM and FuGENE HD in a tube and incubated at room temperature for 5 min. Subsequently, the mixture was added to the cells. The cells were then incubated at 37 °C in a humidified cell culture incubator for 6 h. Complete medium was added after this incubation period. Twenty-four hours postincubation, the cells were serum-starved for 24 h before addition of TGFβ as described above (24, 84).

Luciferase activity

Proximal tubular epithelial cells were cotransfected with collagen I (α2) promoter–luciferase reporter plasmid, siRNAs against DJ-1, Hif1α, FLAG DJ-1, siHif1α, PKCβII K371R, PKCβII CAT, vector, or scrambled RNA as described in the figure legends. The transfected cells were starved for 24 h prior to incubation with 2 ng/ml TGFβ for 24 h. The cell lysates were assayed for luciferase activity using a kit as described previously (146).

Statistics

The data were expressed as mean ± SD. The significance of the data was determined by using GraphPad Prism using analysis of variance or paired t test. A p-value of < 0.05 was considered significant. The significance of all the immunoblotting experiments has been included in the Supplementary Figures.

Data availability

All data are contained within the article.

Supporting information—This article contains supporting information.

Author contributions—F. D. and S. M. data curation; F. D. and G. G. C. formal analysis; G. G. C. conceptualization; G. G. C. supervision; G. G. C. funding acquisition; G. G. C. writing–original draft; G. G. C. project administration; N. G.-C. and G. G. C. writing–review and editing; B. S. K. formal analysis.

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Abbreviations—The abbreviations used are: CKD, chronic kidney disease; Hif1α, hypoxia-inducible factor 1α; IP, immunoprecipitation; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor β; UUO, unilateral ureteral obstruction.

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