Roles of PINCH-2 in regulation of glomerular cell shape change and fibronectin matrix deposition

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Shi X, Qu H, Kretzler M, Wu C. Roles of PINCH-2 in regulation of glomerular cell shape change and fibronectin matrix deposition. Am J Physiol Renal Physiol 295: F253–F263, 2008. First published May 14, 2008; doi:10.1152/ajprenal.0070.2008.—The PINCH-1-integrin-linked kinase (ILK)-α-parvin (PIP) complex plays important roles in the regulation of glomerular cell behavior, including podocyte shape change, apoptosis, and mesangial fibronectin matrix deposition. In this study, we show that PINCH-2, a protein that is structurally related to PINCH-1 but encoded by a different gene, is coexpressed with PINCH-1 in podocytes. Treatment of podocytes with transforming growth factor (TGF)-β1 elevated the level of PINCH-2, resulting in increased association of PINCH-2 with ILK and α-parvin and concomitant displacement of PINCH-1 from the PIP complex. To gain insights into the functional consequences of elevated PINCH-2 expression, we overexpressed PINCH-2 in podocytes by infection with an adenovirus encoding PINCH-2. Overexpression of PINCH-2 resulted in displacement of PINCH-1 from the PIP complex and compromised podocyte spreading. The PINCH-2-mediated displacement of PINCH-1, however, did not prompt apoptosis. Interestingly, the effect of PINCH-2 on podocyte spreading depends on differentiation status, as overexpression of PINCH-2 in podocytes that were not fully differentiated did not alter cell spreading. Finally, we show that overexpression of PINCH-2 in mesangial cells resulted in displacement of PINCH-1 from the PIP complex but impaired neither mesangial cell spreading nor fibronectin matrix deposition. These studies suggest that PINCH-2 can substitute for PINCH-1 in at least certain processes in glomerular cells (e.g., podocyte survival signaling and mesangial fibronectin matrix deposition), albeit that an aberrantly high level of PINCH-2 may contribute to TGF-β1-induced alteration in podocyte shape modulation.

TGF-β1: ILK; parvin; podocytes; mesangial cells; cell spreading; fibronectin matrix assembly

Despite the progress, our understanding of the molecular mechanisms underlying glomerular failure remains rather primitive, which is due in part to the incomplete understanding of the basic mechanisms governing the behavior of glomerular cells.

Integrins and integrin-proximal proteins play important roles in regulation of glomerular cell behavior, including cell shape change, survival, and pericellular matrix deposition. At the molecular level, integrin-mediated cell-extracellular matrix adhesion leads to clustering of focal adhesion proteins at the matrix adhesion sites, where they physically link the extracellular matrix to the intracellular actin cytoskeleton and mediate bidirectional transmembrane signaling. PINCH-1 is a widely expressed evolutionally conserved focal adhesion protein, consisting of five LIM domains (reviewed in Refs. 15, 36, and 38). We previously demonstrated that PINCH-1 forms a ternary complex with integrin-linked kinase (ILK) and α-parvin (also known as CH-ILKBP or actopaxin) (31, 32). The formation of the PINCH-1-ILK-α-parvin (PIP) complex is mediated by two direct interactions, one mediated by the PINCH-1 NH2-terminal-most LIM1 domain and ILK NH2-terminal ankyrin repeat (ANK) domain, and the other mediated by the ILK COOH-terminal kinase domain and α-parvin CH2 domain (35). The PIP complex couples integrins to the actin cytoskeleton and signaling proteins and hence is crucial for many integrin-mediated processes (reviewed in Refs. 15, 36, and 38). Depletion of either PINCH-1 or ILK results in early embryonic lethality (16, 18, 22), underscoring the importance of both proteins in embryogenesis. In podocytes, the PIP complex is involved in regulation of cell adhesion, cytoarchitecture, and apoptosis. Overexpression of the PINCH-1-binding ANK fragment of ILK disrupted the PIP complex and compromised podocyte shape change and survival signaling (42). The importance of the PIP complex is further manifest by recent studies in podocyte-specific ILK knockout mice. Ablation of ILK from podocytes resulted in heavy albuminuria, progressive focal segmental glomerulosclerosis, and kidney failure, which ultimately led to animal death (3, 5, 11). In mesangial cells, forced disruption of the PIP complex substantially reduced fibronectin matrix deposition (7), a process that is known to require integrin-actin cytoskeleton interaction (39).

Previous studies by us and others have identified a focal adhesion protein (termed as PINCH-2) that shares a high level of sequence similarity with PINCH-1 (2, 8, 43). Despite the sequence similarity, PINCH-1 and -2 are encoded by different genes. Furthermore, PINCH-2, unlike PINCH-1, is dispensable...
for embryonic development (30). Importantly, PINCH-2 binds ILK through a site that is identical to that of PINCH-1 (43). Thus PINCH-2 could potentially play a role in glomerular cells. However, in contrast to the accumulating evidence that points to a prominent role of ILK and PINCH-1, nothing was known about PINCH-2 in glomerular cells. In this study, we show that PINCH-2 is expressed in podocytes. Furthermore, we have found that TGF-β1 elevates the level of PINCH-2 in podocytes, resulting in displacement of PINCH-1 from the PIP complex. Finally, we provide functional evidence showing that PINCH-2 can substitute for PINCH-1 in at least certain processes in glomerular cells, albeit an aberrantly high level of PINCH-2 may contribute to TGF-β1-induced alteration in podocyte shape change.

MATERIALS AND METHODS

Antibodies, cells, and other reagents. Mouse monoclonal anti-ILK antibody (clone 65.1), anti-α-parvin antibodies (clones D4 and 3B5), rabbit polyclonal anti-PINCH-1 antibody, and rabbit polyclonal anti-fibronectin antibody were previously described (7, 31, 32). Rabbit polyclonal anti-human PINCH-2 antibody was raised against a human PINCH-2 COOH-terminal peptide (N-AQPKATDLNLSA-C) conjugated to KLH. Rabbit polyclonal anti-mouse PINCH-2 antibody was raised against a mouse PINCH-2 COOH-terminal peptide (N-AQPKSSDVMNSL-C) conjugated to KLH. Mouse monoclonal anti-FLAG antibody (M5) was purchased from Sigma. Immortalized mouse glomerular podocytes and rat glomerular mesangial cells were prepared as described (6, 7, 9, 14, 19, 42). Human TGF-β1 was purchased from Chemicon. Cell culture media were purchased from Mediatech/Cellgro (Herndon, VA).

Glomerular cell culture. Rat mesangial cells were plated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% insulin-transferrin-selenium A solution supplement (Life Technologies, Grand Island, NY) as described elsewhere (6, 7). Conditionally immortalized mouse glomerular podocytes were propagated under permissive condition at 33°C in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml mouse recombinant interferon-γ (9, 14, 19, 42). To induce differentiation, the cells were cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin but lacking interferon-γ (nonpermissive condition). For TGF-β1 treatment, podocytes that were cultured under nonpermissive condition for 12 days were serum starved for 24 h and then cultured in RPMI 1640 medium containing 5 ng/ml TGF-β1 or RPMI 1640 medium lacking TGF-β1 (as a control) for 48 h. The cells were then harvested and analyzed by immunoprecipitation and Western blotting as specified in each experiment.

Adenoviral expression vectors and infection. An adenoviral-expression vector encoding the ANK fragment of ILK was described elsewhere (12, 21, 31, 33). The cells were lysed with lysis buffer (1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM Na3VO4, 2.5 mM Na2PO4, 100 mM NaF, and protease inhibitors). The cell lysates were incubated with mouse monoclonal anti-α-parvin antibody 1D4. The samples were incubated for 2 h, mixed with protein A/G-conjugated beads (Santa Cruz Biotechnology), and then incubated for one additional hour. The beads were washed four times, and the proteins bound were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with antibodies as specified in each experiment.

Caspase-3 assay. Caspase-3 activity was measured using fluorogenic caspase-3 substrate VII (Ac-DEVD-APC; Calbiochem) following the manufacturer’s protocol. Briefly, the cells were lysed with lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA). The cell lysates were mixed with fluorogenic caspase-3 substrate VII, and fluorescence (excitation at 400 nm and emission at 505 nm) was measured using a TECAN GENios PRO fluorescence microplate reader.

Cell spreading. Cell spreading was analyzed as previously described (42). Briefly, cells (as specified in each experiment) were harvested with trypsin and washed with RPMI 1640 containing 10% FBS and then twice with serum-free Opti-MEM (Life Technologies). The cells (2.25 × 104/well) were plated in fibronectin (10 μg/ml)-or Matrigel (10 μg/ml)-coated 96-well plates and incubated at 37°C under a 5% CO2-95% air atmosphere for 60 min. The cell morphology was observed under an Olympus IX70 microscope equipped with a Hoffman Modulation Contrast system and recorded with a DVC-1310C Magnafire digital camera (Optronics). Unspread cells were defined as round cells, while spread cells were defined as cells with extended processes as described elsewhere (12, 21, 31, 33). The percentages of cells adopting spread morphology were quantified by analyzing at least 300 cells from three randomly selected fields (>100 cells/field) compared with that of control cells (normalized to 1). A paired Student’s t-test was used for statistical analyses of the results. P values <0.05 were considered statistically significant.

Fibronectin matrix assembly. Rat mesangial cells infected with PINCH-2 adenovirus or the control β-galactosidase adenovirus were seeded in 60-mm tissue culture plates in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% insulin-transferrin-selenium A solution supplement (Life Technologies) for 2 days. The cell monolayers were

PacI. The positive plasmids were transformed into DH10B cells by electroporation for large-scale amplification. The plasmid DNA was digested with PacI, ethanol-precipitated, and used to transfect 293 cells with LipofectAmine PLUS. The transfected cells were harvested 10 days after transfection. The cells were lysed by three cycles of freezing in a methanol/dry ice bath and rapid thawing at 37°C, and the lysates containing the recombinant adenovirus were collected. The control adenoviral expression vector encoding β-galactosidase was kindly provided by Drs. Tong-Chuan He and Bert Vogelstein (Howard Hughes Medical Institute, The Johns Hopkins Oncology Center, Baltimore, MD).

For adenoviral infection experiments, podocytes were cultured under permissive or nonpermissive condition as specified in each experiment. The glomerular cells (podocytes or mesangial cells) were infected with various adenoviruses as specified in each experiment. The infection efficiency was monitored by the expression of green fluorescent protein encoded by the adenoviral vectors, which typically reached 80–90% within 3 days. Three days after adenoviral infection, the cells were harvested and the expression of human PINCH-2, FLAG-PINCH-1 or FLAG-PINCH-1 was analyzed by Western blotting. The formation of the PIP complex, caspase-3 activity, cell spreading, and fibronectin matrix assembly were analyzed as described below.

Analysis of PIP complex formation. The formation of the PIP complex in mesangial cells and podocytes was analyzed by immunoprecipitation and Western blotting as described elsewhere (31, 42, 44). Briefly, the cells were lysed with lysis buffer (1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM Na3VO4, 2.5 mM Na2PO4, 100 mM NaF, and protease inhibitors). The cell lysates were incubated with mouse monoclonal anti-α-parvin antibody 1D4. The samples were incubated for 2 h, mixed with protein A/G-conjugated beads (Santa Cruz Biotechnology), and then incubated for one additional hour. The beads were washed four times, and the proteins bound were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with antibodies as specified in each experiment.

Cell culture media were purchased from Mediatech/Cellgro (Hernando, VA).

Glomerular cell culture. Rat mesangial cells were plated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% insulin-transferrin-selenium A solution supplement (Life Technologies, Grand Island, NY) as described elsewhere (6, 7). Conditionally immortalized mouse glomerular podocytes and rat glomerular mesangial cells were prepared as described (6, 7, 9, 14, 19, 42). Human TGF-β1 was purchased from Chemicon. Cell culture media were purchased from Mediatech/Cellgro (Hernando, VA).

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Adenoviral expression vectors and infection. An adenoviral-expression vector encoding the ANK fragment of ILK was described elsewhere (7). Adenoviral vectors encoding untagged human PINCH-2 or FLAG-tagged human PINCH-1 and PINCH-2 were generated following a protocol that was previously described (7, 42). Briefly, cDNA encoding human PINCH-2, FLAG-tagged human PINCH-1, or PINCH-2 was cloned into the pAdTrack-CMV shuttle vector. The shuttle vector encoding the ANK fragment of ILK was described elsewhere (7, 9, 14, 19, 42). Human TGF-β1 (as a control) for 12 days were serum starved for 24 h and then cultured in RPMI 1640 medium containing 5 ng/ml TGF-β1 or RPMI 1640 medium lacking TGF-β1 (as a control) for 48 h. The cells were then harvested and analyzed by immunoprecipitation and Western blotting as specified in each experiment.
washed three times with PBS containing 1 mM 2-aminoethylbenzenesulfonyl fluoride (AEBSF) and harvested with a cell scraper. Fibronectin matrix was isolated as previously described (7, 37). Briefly, the cells were extracted sequentially with 1) 3% Triton X-100 in PBS containing 1 mM AEBSF; 2) 100 μg/ml DNase I in 50 mM Tris, pH 7.4, 10 mM MnCl2, 1 M NaCl, and 1 mM AEBSF; and 3) 2% deoxycholate in Tris, pH 8.8, and 1 mM AEBSF. Fibronectin in the Triton X-100-soluble fraction and deoxycholate insoluble extracellular matrix fraction was analyzed by Western blotting with a polyclonal rabbit anti-fibronectin antibody.

**Immunofluorescent staining.** Cells were fixed with 4% paraformaldehyde and then either permeabilized (for ILK and phallolidin staining) with 0.1% Triton X-100 in PBS-HCl (pH 7.4) containing 150 mM NaCl and 1 mg/ml BSA, or rinsed with the Tris buffer in the absence of Triton X-100 (nonpermeabilized condition for fibronectin matrix staining). The cells were stained with primary mouse monoclonal anti-ILK or rabbit anti-fibronectin antibodies and rhodamine red-conjugated anti-mouse or anti-rabbit IgG secondary antibodies. Actin filaments were stained with rhodamine-conjugated phalloidin.

**RESULTS**

**PINCH-2 is coexpressed with PINCH-1 and forms a complex with ILK and α-parvin in podocytes.** We used conditionally immortalized podocytes (19) to investigate the role of PINCH-2 in podocytes, as fully differentiated podocytes are unable to proliferate in culture. The conditionally immortalized podocytes proliferated under permissive conditions, but they were growth arrested and exhibited many characteristics of fully differentiated podocytes including expression of podocyte-specific cytoskeletal proteins and formation of cytoskeletal structures resembling foot processes after a switch to nonpermissive conditions (19). PINCH-1 (Fig. 1C, lanes 1 and 2), ILK (Fig. 1B, lanes 1 and 2), α-parvin (Fig. 1A, lanes 1 and 2) were detected in differentiated as well as undifferentiated podocytes. Furthermore, PINCH-1 (Fig. 1C, lanes 3 and 4) was communoprecipitated with ILK (Fig. 1B, lanes 3 and 4) and α-parvin (Fig. 1A, lanes 3 and 4) from both differentiated and undifferentiated podocytes, confirming the formation of the PIP complex in these cells. Probing the cell lysates with an anti-PINCH-2 antibody showed that a small amount of PINCH-2 was present in differentiated podocytes (Fig. 1D, lane 1). No PINCH-2 was detected in the lysates of undifferentiated podocytes (Fig. 1D, lane 2). To confirm this, we analyzed the α-parvin immunoprecipitates by Western blotting with the anti-PINCH-2 antibody. Again, PINCH-2 was detected in the α-parvin immunoprecipitates derived from differentiated (Fig. 1D, lane 3) but not undifferentiated (Fig. 1D, lane 4) podocytes. Thus, while undifferentiated podocytes express primarily the PIP complex, differentiated podocytes express both the PIP and PINCH-2-ILK-α-parvin complexes.

**TGF-β1 promotes PINCH-2 complex formation in podocytes.** TGF-β1 is critically involved in the pathogenesis and progression of renal failure. It elicits multiple effects on podocytes, including induction of podocyte shape change and promotion of apoptosis (1, 17, 20, 23, 24, 28, 29, 34, 41, 45). We previously found that treatment of podocytes with TGF-β1 inhibits the formation of the PIP complex (9). It was not known, however, whether TGF-β1 influences the level of the PINCH-2-ILK-α-parvin complex. To test this, we treated podocytes with TGF-β1 and found that it increased the cellular level of PINCH-2 (Fig. 2D, compare lane 2 with lane 1) but not that of PINCH-1 (Fig. 2C, compare lane 2 with lane 1). ILK (Fig. 2B, lanes 1 and 2), α-parvin (Fig. 2A, lanes 1 and 2), PINCH-1 (Fig. 1C, lanes 1 and 2), ILK (Fig. 1B, lanes 1 and 2), and α-parvin (Fig. 1A, lanes 1 and 2) were detected in differentiated as well as undifferentiated podocytes. Furthermore, PINCH-1 (Fig. 1C, lanes 3 and 4) was communoprecipitated with ILK (Fig. 1B, lanes 3 and 4) and α-parvin (Fig. 1A, lanes 3 and 4) from both differentiated and undifferentiated podocytes, confirming the formation of the PIP complex in these cells. Probing the cell lysates with an anti-PINCH-2 antibody showed that a small amount of PINCH-2 was present in differentiated podocytes (Fig. 1D, lane 1). No PINCH-2 was detected in the lysates of undifferentiated podocytes (Fig. 1D, lane 2). To confirm this, we analyzed the α-parvin immunoprecipitates by Western blotting with the anti-PINCH-2 antibody. Again, PINCH-2 was detected in the α-parvin immunoprecipitates derived from differentiated (Fig. 1D, lane 3) but not undifferentiated (Fig. 1D, lane 4) podocytes. Thus, while undifferentiated podocytes express primarily the PIP complex, differentiated podocytes express both the PIP and PINCH-2-ILK-α-parvin complexes.

![Fig. 1](http://ajprenal.physiology.org/)

**Fig.1**. PINCH-2 is coexpressed with PINCH-1 and forms a complex with integrin-linked kinase (ILK) and α-parvin in podocytes. Mouse podocytes were cultured under permissive (lanes 2 and 4) or nonpermissive (lanes 1 and 3) conditions for 12 days. The cell lysates were mixed with monoclonal anti-α-parvin (A), ILK (B), PINCH-1 (C), and mouse PINCH-2 (D), respectively. Lanes 1 and 2 were loaded with cell lysates (20 μg/lane). The asterisk in D denotes a nonspecific protein band. Note that PINCH-2 (which was slightly smaller than the nonspecific protein) was weakly detectable in the lysates of differentiated podocytes (D, lane 1). The PINCH-2 band was more prominent in the immunoprecipitates derived from the differentiated podocytes (D, lane 3). No PINCH-2 was detected in either the lysates (D, lane 2) or the immunoprecipitates (D, lane 4) derived from the undifferentiated podocytes. The sample loaded in lane 5 was prepared like those in lanes 3 and 4 except that cell lysates were omitted (to show the Ig bands derived from the antibody used in the immunoprecipitation).
1 and 2) or α-parvin (Fig. 2A, lanes 1 and 2). Noticeably, the amount of PINCH-2 (Fig. 2D, compare lane 4 with lane 3) associated with ILK (Fig. 2B, lanes 3 and 4) and α-parvin (Fig. 2A, lanes 3 and 4) was increased in response to TGF-β1. Concomitantly, the amount of PINCH-1 (Fig. 2C, compare lane 4 with lane 3) associated with ILK and α-parvin was reduced. These results suggest that TGF-β1 plays opposite roles in regulation of the PINCH-1 and PINCH-2 complexes (i.e., it promotes the formation of the PINCH-2-ILK-α-parvin complex but inhibits the formation of the PIP complex).

**Forced overexpression of PINCH-2 inhibits the PIP complex formation in podocytes.** To test whether increased expression of PINCH-2 in podocytes is sufficient to promote the formation of the PINCH-2-α-parvin complex and concomitantly inhibit the formation of the PIP complex, we sought to overexpress PINCH-2 in podocytes. Toward this end, we generated an adeno viral vector encoding human PINCH-2. Mouse podocytes were infected with the PINCH-2 adenovirus and an adenovirus encoding β-galactosidase as a control, respectively. Overexpression of human PINCH-2 in mouse podocytes infected with the PINCH-2 adenovirus (Fig. 3C, lane 2) but not those infected with the β-galactosidase adenovirus (Fig. 3C, lane 1) was confirmed by Western blotting with an antibody recognizing human PINCH-2. As expected, human PINCH-2 (Fig. 3C, lane 4) was readily coimmunoprecipitated with ILK (Fig. 3B, lane 4) and α-parvin (Fig. 3A, lane 4). Importantly, the amount of PINCH-1 associated with ILK and α-parvin was substantially reduced in podocytes overexpressing PINCH-2 (Fig. 3D, compare lane 4 with lane 3). In control experiments, similar amounts of α-parvin (Fig. 3A, lanes 3 and 4) and ILK (Fig. 3B, lanes 3 and 4) were detected in anti-α-parvin immunoprecipitates derived from the PINCH-2-overexpressing and control cells. These results suggest that the PINCH-2 level is crucial for maintaining proper levels of the PINCH-1 and PINCH-2 complexes in podocytes. An elevated level of PINCH-2 (either induced by treatment of podocytes with TGF-β1) (Fig. 2D) or by adeno viral-mediated gene transfer (Fig. 3C) shifted the balance toward increased association of PINCH-2 with ILK and α-parvin and consequently reduced association of PINCH-1 with ILK and α-parvin.

**PINCH-2 influences podocyte shape modulation.** The PIP complex plays a crucial role in the regulation of podocyte shape change and apoptosis (42). The finding that overexpression of PINCH-2 inhibits the formation of the PIP complex prompted us to test whether it functions in these processes. To test the effect on podocyte shape change, we plated PINCH-2-overexpressing podocytes and control podocytes on fibronectin and Matrigel, substrata that are known to promote podocyte spreading. Noticeably, the spreading of the PINCH-2-overexpressing podocytes was significantly reduced compared with the control podocytes (Fig. 3, E and F). These results suggest that overexpression of PINCH-2, which inhibits the formation of the PIP complex (Fig. 3D), compromises podocyte shape modulation. Although overexpression of PINCH-2 significantly reduced podocyte spreading, abundant ILK clusters (Fig. 4, B and D) and actin filaments (Fig. 4, F and H) were detected in both PINCH-2- and β-galactosidase adenovirus-infected podocytes.

To further test this, we generated adenoviruses encoding FLAG-PINCH-1 and FLAG-PINCH-2, respectively, and infected mouse podocytes with the adenoviruses. The expression of FLAG-PINCH-1 (Fig. 5A, lane 2) and FLAG-PINCH-2 (Fig. 5A, lane 3) in the corresponding infectants was confirmed by Western blotting. As expected, FLAG-PINCH-1 (Fig. 5A, lane 5) and FLAG-PINCH-2 (Fig. 5A, lane 6) were coimmunoprecipitated with α-parvin (Fig. 5C, lanes 2 and 3) and ILK (Fig. 5D, lanes 2 and 3). Probing the same samples with an anti-PINCH-1 antibody revealed that the majority of PINCH-1 in the α-parvin immunoprecipitates was displaced by FLAG-PINCH-1 (Fig. 5B, compare lane 5 with lane 4) or FLAG-PINCH-2 (Fig. 5B, compare lane 6 with lane 4) in the corresponding infectants. Consistent with the results shown in Fig. 3, E and F, overexpression of FLAG-PINCH-2 significantly reduced podocyte spreading on fibronectin (Fig. 5E) and
Matrigel (Fig. 5F), suggesting that an aberrantly high level of PINCH-2 is detrimental to proper modulation of podocyte shape. In contrast, overexpression of FLAG-PINCH-1 did not significantly alter podocyte spreading (Fig. 5, E and F).

We next analyzed the effect of PINCH-2 overexpression on caspase-3, a key executioner caspase that is activated in response to dominant negative inhibitors (e.g., ILK ANK fragment) of the PIP complex (42). To do this, we infected mouse
Fig. 5. Overexpression of FLAG-PINCH-2 but not that of FLAG-PINCH-1 reduces podocyte spreading. Mouse podocytes cultured under nonpermissive conditions were infected with adenoviral vectors encoding β-galactosidase control (lanes 1 and 4), FLAG-PINCH-1 (lanes 2 and 5), or FLAG-PINCH-2 (lanes 3 and 6). The PINCH-ILK-α-parvin complexes were immunoprecipitated with monoclonal anti-α-parvin antibody 1D4. Cell lysates (lanes 1–5) and immunoprecipitates (lanes 4–6) were analyzed by Western blotting with anti-FLAG (A) or anti-PINCH-1 (B) antibodies. Note that the majority of endogenous PINCH-1 in the PIP complex was displaced by FLAG-PINCH-1 (B, lane 5) or FLAG-PINCH-2 (B, lane 6). In control experiment, the membranes used in A and B containing the samples of immunoprecipitates were reprobed with anti-α-parvin (C) or anti-ILK (D) to show the presence of α-parvin and ILK in the PIP complex. E: β-galactosidase control, FLAG-PINCH-1-, or FLAG-PINCH-2-overexpressing podocytes were plated on fibronectin-coated surfaces for 60 min. Cell spreading was analyzed as described in Fig. 3E. Values are means ± SD from 5 (for FLAG-PINCH-2-overexpressing cells) or 4 (for FLAG-PINCH-1-overexpressing cells) independent experiments. *P < 0.05 vs. control. F: β-galactosidase control. FLAG-PINCH-1-, or FLAG-PINCH-2-overexpressing podocytes were plated on Matrigel-coated surfaces for 60 min. Cell spreading was analyzed as described in Fig. 3E. Values are means ± SD from 4 (for FLAG-PINCH-2-overexpressing cells) or 3 (for FLAG-PINCH-1-overexpressing cells) independent experiments. *P < 0.05 vs. control.

PINCH-2 regulates podocyte spreading in a differentiation status-dependent manner. Conditionally immortalized podocytes cultured under permissive condition are deficient in certain podocyte-specific cytoskeletal proteins, and they do not form extensive processes that are characteristics of differentiated podocytes (19). To test whether PINCH-2 influences cell shape modulation in podocytes cultured under permissive condition (i.e., undifferentiated podocytes), we infected these cells with PINCH-2 and β-galactosidase control adenoviruses, respectively. As expected, overexpression of PINCH-2 substantially reduced the amount of PINCH-1 (Fig. 7C, compare lane 2 with lane 1) associated with ILK (Fig. 7F) and α-parvin (Fig. 7A). In control experiments, PINCH-2 was readily detected in the α-parvin immunoprecipitates derived from the PINCH-2-overexpressing cells (Fig. 7D, lane 2). However, unlike overexpression of PINCH-2 in differentiated podocytes (Fig. 3), overexpression of PINCH-2 in undifferentiated podocytes did not alter cell spreading, either on fibronectin (Fig. 7E) or on Matrigel (Fig. 7F). These results suggest that the ability of PINCH-2 to influence podocyte spreading is dependent on differentiation status. Immunofluorescent staining analyses showed that abundant ILK clusters (Fig. 8, B and D) and actin filaments (Fig. 8, F and H) were present in both PINCH-2 and β-galactosidase adenoviral-infected undifferentiated podocytes.

PINCH-2-mediated displacement of PINCH-1 from the PIP complex does not inhibit mesangial fibronectin matrix assembly. The PIP complex is crucial for fibronectin matrix deposition in mesangial cells (7), a process that is involved in the pathogenesis and progression of diabetic nephropathy. We previously showed that disruption of the PIP complex with dominant negative inhibitors reduced mesangial fibronectin matrix deposition (7). To test whether the PINCH-2-mediated
displacement of PINCH-1 from the PIP complex influences fibronectin matrix deposition, we infected rat mesangial cells with adenoviruses encoding human PINCH-2 and β-galactosidase, respectively. The expression of human PINCH-2 in mesangial cells infected with the PINCH-2 adenovirus (Fig. 9C, lane 2) but not in those infected with the control β-galactosidase adenovirus (Fig. 9C, lane 1) was confirmed by Western blotting. As expected, human PINCH-2 was readily coimmunoprecipitated with ILK (Fig. 9B) and α-parvin (Fig. 9A) from lysates of the PINCH-2-overexpressing mesangial cells (Fig. 9C, lane 4) but not those of the control cells (Fig. 9C, lane 3). Consistent with the results in podocytes, overexpression of PINCH-2 resulted in almost complete displacement of PINCH-1 from the PIP complex (Fig. 9D, compare lane 4 with lane 3). To test whether the PINCH-2-induced displacement of PINCH-1 from the PIP complex affects fibronectin matrix
assembly, we isolated fibronectin extracellular matrix from PINCH-2-overexpressing and control mesangial cells, respectively. Western blotting analyses showed that the amount of fibronectin in the extracellular matrix fractions derived from the PINCH-2-overexpressing mesangial cells was not reduced compared with that derived from the control mesangial cells (Fig. 9E, lanes 1 and 2). To confirm this, we immunofluorescently stained the cells with an anti-fibronectin antibody. Consistent with the biochemical analyses (Fig. 9E), similar levels of fibrillar fibronectin matrix were detected in PINCH-2 and β-galactosidase adenoviral-infected mesangial cells (Fig. 10, B and D). Additionally, overexpression of PINCH-2 did not alter mesangial cell spreading (Fig. 9F). Similar levels of actin filaments (Fig. 10, F and H) and ILK clusters (Fig. 10, J and L) were detected in these cells. Thus the PINCH-2-mediated displacement of PINCH-1 from the PIP complex impairs neither fibronectin matrix deposition nor cell spreading in mesangial cells, suggesting that PINCH-2 could functionally substitute for PINCH-1 in these processes.

**DISCUSSION**

The PIP complex is emerging as a key regulator of glomerular cell behavior, including podocyte shape modulation, survival, and mesangial fibronectin matrix deposition (3, 5–7, 9, 11, 13, 14, 42). In this study, we have investigated the role of PINCH-2 in these processes. Our studies demonstrate the following. First, PINCH-2 is coexpressed with PINCH-1 and forms a complex with ILK and β-parvin in podocytes. Consequently, two distinct PINCH-ILK-β-parvin complexes are formed in podocytes, one consisting of PINCH-1 (i.e., the PIP complex) and the other consisting of PINCH-2 (i.e., the PINCH-2-ILK-β-parvin complex). Second, the level of PINCH-2 in podocytes is increased in response to TGF-β1,
which is known to alter podocyte behavior, including cell shape modulation and survival (9, 17, 23, 25, 26, 40). The TGF-β1-induced increase in the PINCH-2 level promotes the association of PINCH-2 with ILK and consequently displaces PINCH-1 from the PIP complex. Third, gene transfer-induced overexpression of PINCH-2 resulted in a similar displacement of PINCH-1 from the PIP complex and compromised podocyte spreading. Fourth, the effect of PINCH-2 overexpression on cell spreading is cell type and differentiation status dependent, as cell spreading was not compromised in PINCH-2-overexpressing mesangial cells or undifferentiated podocytes, despite displacement of PINCH-1 from the PIP complex in these cells. Finally, the PINCH-2 overexpression-induced displacement of PINCH-1 from the PIP complex does not impair podocyte survival and mesangial fibronectin matrix deposition.

It is interesting to compare the effects of PINCH-2-induced displacement of PINCH-1 from the PIP complex with those induced by overexpression of dominant negative inhibitors of PINCH-ILK interaction (e.g., ILK ANK fragment). We have previously shown that overexpression of an ILK ANK fragment, which binds PINCH-1 and PINCH-2 and therefore competitively inhibits the binding of the PINCH proteins to ILK, prompts apoptosis in podocytes (42) and inhibits fibronectin matrix deposition in mesangial cells (7). Thus the fact that displacement of PINCH-1 from the PIP complex by PINCH-2 does not impair podocyte survival and mesangial fibronectin matrix deposition strongly suggests that the PINCH-2-ILK-α-parvin complex can substitute for the PIP complex in these processes.

Although overexpression of PINCH-2 does not impair podocyte survival and mesangial fibronectin matrix deposition, it does compromise podocyte spreading. The simplest explanation is that the PINCH-2-ILK-α-parvin complex, while competent for mediating podocyte survival signaling and mesangial fibronectin matrix deposition, cannot substitute for the PIP complex during podocyte cytoskeletal remodeling. This could result from either qualitative or quantitative differences between interactions mediated by the two PINCH proteins. Consequently, forced displacement of PINCH-1 from the PIP complex by PINCH-2 could potentially alter the dynamics of the podocyte cytoskeleton and therefore compromises podocyte spreading. In this regard, it is worth noting that RSU-1, which is known to regulate cell adhesion and cytoskeleton organization (4, 10), has been reported to interact with PINCH-1 but not PINCH-2 (4). A second possible explanation is that PINCH-2, when overexpressed, influences podocyte cytoskeletal remodeling through other mechanisms. For example, PINCH-2 is capable of entering into nuclei (43) and therefore could potentially influence podocyte cytoskeletal remodeling through regulation of nuclear activities (e.g., transcription). It is worth noting that the ability of PINCH-2 to alter cell spreading is cell type and differentiation status dependent, as overexpression of PINCH-2 in mesangial cells or podocytes cultured under permissive condition, unlike those cultured under nonpermissive condition, does not impair cell spreading. Because mesangial cells and podocytes cultured under permissive conditions lack a number of podocyte-specific cytoskeletal and signaling proteins, it is attractive to propose that PINCH-1 and -2 differ in their activities toward certain podocyte-specific proteins, resulting in altered cell spreading in PINCH-2-overexpressing podocytes.

TGF-β1 is an important factor in the pathogenesis and progression of diabetic nephropathy (1, 27–29, 34, 41, 45). It has been well established that TGF-β1 can influence podocyte...
behavior through regulation of multiple signaling pathways (9, 17, 23, 25, 26, 40). In this study, we have shown that treatment of podocytes with TGF-β1 increases the level of PINCH-2. Thus PINCH-2 likely functions as one of the downstream effectors of TGF-β1 in podocytes. Because elevated PINCH-2 expression alters podocyte shape modulation (Figs. 3 and 5), the TGF-β1-induced increase in the PINCH-2 level likely contributes to the effect of TGF-β1 on podocyte cytoskeletal remodeling (23). However, because elevated PINCH-2 expression is not sufficient for inducing podocyte apoptosis (Fig. 6), TGF-β1 likely promotes podocyte survival through regulation of other signaling intermediates including other components of the PIP complexes (9, 25, 26, 40).

In summary, the studies presented herein suggest that PINCH-2, whose presence and functions in glomerular cells were previously unknown, is an important component of the cellular machinery in glomerular cells. It can substitute for PINCH-1 in several cellular processes (e.g., podocyte survival signaling and mesangial fibronectin matrix deposition). Furthermore, the level of PINCH-2 is upregulated by TGF-β1 in podocytes. Because elevated PINCH-2 expression alters podocyte spreading, an aberrantly high level of PINCH-2 may contribute to TGF-β1-induced alteration in podocyte shape modulation.

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