Yes-associated Protein (YAP) Promotes Cell Survival by Inhibiting Proapoptotic Dendrin Signaling*

Received for publication, January 28, 2013, and in revised form, May 8, 2013. Published, JBC Papers in Press, May 10, 2013. DOI 10.1074/jbc.C113.457390

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Background: Dendrin can relocate from the cytoplasm to the nucleus to promote podocyte apoptosis. Results: YAP is constitutively expressed in podocyte nuclei, binds to dendrin, and inhibits dendrin-mediated podocyte apoptosis.

Conclusion: YAP binding to dendrin promotes podocyte survival by inhibiting proapoptotic dendrin signaling. Significance: YAP could serve as a physiologic inhibitor of podocyte apoptosis and may open avenues for the development of podocyte protective therapies.

Kidney podocytes are highly specialized terminally differentiated cells that form the final barrier to urinary protein loss. Podocytes are a target for injury by metabolic, autoimmune, hereditary, inflammatory, and other stressors. Persistence of podocyte injury leads to podocyte death and loss, which results in progressive kidney damage and ultimately kidney failure. Dendrin is a dual compartment protein with proapoptotic signaling properties. Nuclear relocation of dendrin in response to glomerular injury promotes podocyte apoptosis. Here we show that Yes-associated protein (YAP), a downstream target of Hippo kinases and an inhibitor of apoptosis, is expressed in the nucleus of podocytes. The WW domains of YAP mediate the interaction with the PPXY motifs of dendrin. This interaction is functionally relevant because YAP binding to dendrin reduces dendrin-dependent, staurosporine-induced apoptosis in co-transfected HEK293 cells. Moreover gene silencing of YAP in podocytes increases adriamycin-induced podocyte apoptosis. It also increases staurosporine-induced caspase-3/7 activity, which is rescued by dendrin depletion in YAP knockdown cells. Our findings elucidate YAP binding to dendrin as a prosurvival mechanism. The antiapoptotic signaling properties of YAP in podocytes could hold significance in the quest for targeted therapeutics aimed at preventing podocyte loss.

YAP4 (Yes-associated protein) is a potent oncogene and is one of the two main effectors of the Hippo tumor suppressor pathway (1, 2). The YAP protein was first identified by its ability to associate with Yes and Src protein-tyrosine kinases (3). Cloning of the YAP gene and the characterization of its protein product helped to identify a modular protein domain, known as the WW domain, which mediates interaction with ligands containing PPXY motif (where X is any amino acid) (4, 5). Apart from its function in the cytoplasm, YAP is a transcriptional co-activator that interacts preferentially with the TEA domain family of transcription factors promoting expression of target genes important in cell survival, chemotaxis, differentiation, and proliferation (2, 6). YAP and its mammalian paralog Tafazzin have been shown to bind numerous other signaling regulators including 14–3–3 and Smad7 as well as transcription factors including Runx1, Runx2, a proapoptotic factor p73, and heterogenous nuclear ribonucleoprotein U (hnRPU), an RNA-binding protein implicated in apoptosis (7).

YAP phosphorylation promotes its cytoplasmic sequestration and inactivation (8, 9). Diphosphorylated YAP accumulates in the nucleus where it promotes the transcription of target genes (7). In hepatocytes, nuclear YAP increases the transcription of genes associated with proliferation such as ki67, SOX4, and H19 (10). YAP can also induce the expression of several negative regulators of apoptosis such as the IAP family members BIRC5/survivin and BIRC2/cIAP1 and the BCL2 family gene MCL1 (10). Thus, YAP can act as potent inhibitor of apoptosis in the regulation of organ size (10).

Although there are eight different isoforms of YAP, which are generated by differential splicing (7), the two major ones that differ by the presence of one or two WW domains are characterized in detail (11). Throughout this work, we used both major isoforms of YAP, which we denote as YAP1, YAP with one WW domain, and YAP2, YAP with two WW domains (12).

Podocytes of the kidney glomerulus line the outer aspect of the glomerular basement membrane (GBM) and form the final barrier to albumin, which explains why podocyte injury is typically associated with proteinuria (13). Podocytes are termi

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* This work was supported, in whole or in part, by National Institutes of Health Grants DK57683, DK062472, and DK09368201 (to P. M.), National Institutes of Health Grant DK081617 (to K. N. C.). This work was also supported by a NephCure Young Investigator grant (to K. N. C.) and by PA Breast Cancer Coalition Grants 50707 and 920093 and by a grant from the Geisinger Coalition Grants 60707 and 920093 and by a grant from the Geisinger Coalition Grants.

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nally differentiated cells that cannot undergo cell division in the adult (14). Podocytes are injured in many forms of kidney disease including membranous nephropathy, IgA nephropathy, focal and segmental glomerulosclerosis, and diabetic nephropathy.

Podocytes have a limited capacity to regenerate when they are injured, and loss of podocytes is a hallmark in the progression of proteinuric kidney disease (7, 15). Persistence of podocyte injury is manifested in the activation of cellular processes that lead to irreversible changes such as loss of adhesion to the GBM, cell hypertrophy, changes in transcription, disrupted metabolic pathways, autophagy, and cell cycle dysregulation (13).

The resulting loss of podocytes will lead to irreversible glomerulosclerosis and ultimately kidney failure. At present, the precise pathogenic mechanisms leading to loss through cell death or detachment from the GBM remain poorly understood (9–12, 16). More importantly, it is not clear whether prosurvival mechanisms exist in podocytes that could be harnessed for therapeutic benefit.

Dendrin is a PPXY motif containing dual compartment protein (17). Dendrin was originally identified in sleep-deprived rats (18), a condition that induces neuronal apoptosis (19). In normal podocytes, dendrin is a constituent of the glomerular slit diaphragm, where it binds to nephrin and CD2-associated protein (CD2AP) (17). In response to glomerular injury, dendrin relocates to the podocyte nucleus, thereby promoting apoptosis (17, 20).

As it stands, no signaling mechanisms have been identified that can antagonize dendrin-mediated cell death signaling. Here we show that YAP can bind to dendrin, thereby acting as an endogenous inhibitor of proapoptotic dendrin signaling in podocytes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—YAP1 and YAP2 plasmids have been previously described (6). The YAP mutant with deleted WW domains was generated by PCR and cloned into pFLAG5a vector. All constructs were verified by DNA sequencing.

**Cell Culture and Transient Transfection**—Podocytes were cultured as described before (21). Transient transfection of podocytes and HEK293 cells (ATCC) was done as described (22). GFP fusion proteins were analyzed by direct fluorescence microscopy in living cells or after fixation and double labeling immunocytochemistry (22).

**Yeast Two-hybrid Screening**—YAP two-hybrid screening for dendrin-interacting proteins was done as described before (22). Dendrin was cloned into bait vector pGBK7 (Clontech) to create a fusion protein with the GAL4 DNA binding domain and transformed into yeast strain AH109. Expression of dendrin-GAL4 fusion protein was verified by immunoblotting.

Transformed AH109 cells were mated with yeast strain Y187 (Clontech), which was pre-transformed with a human kidney MATCHMAKER cDNA library fused to the GAL4 activation domain. The screen was done according to the manufacturer’s protocol (MATCHMAKER two-hybrid system 3, Clontech). Prey plasmids were isolated, sequenced, and retransformed into AH109 cells in combination with the dendrin bait construct, control plasmid (pGBK7-Lamin, Clontech), or the empty bait vector pGBK7 to exclude false positives.

**Confocal Microscopy**—Immunofluorescence microscopy of mouse kidney frozen sections and cultured podocytes was done as described before (18). Double labeling of cultured podocytes, HEK293 cells, and mouse kidney sections was performed with rabbit polyclonal anti-dendrin (13), guinea pig anti-dendrin, mouse anti-WT1 (Santa Cruz Biotechnology), and rabbit polyclonal anti-YAP (Novus) antibodies. Confocal images were acquired on a Leica SP5 DM Microscope.

**Western Blotting and Co-immunoprecipitation**—Western blotting and co-immunoprecipitation (Co-IP) from transfected HEK293 cells were performed as described previously (22, 23). Briefly, GFP and FLAG-tagged cDNA constructs were transfected into HEK293 cells using FuGENE 6 (Roche Applied Science). After 48 h, cells were lysed with 1% Triton buffer and supplemented with protease (Roche Applied Science) and phosphatase inhibitors (Sigma). Lysates were then incubated for 4 h with anti-FLAG beads (Sigma). Beads were then washed five times with 1% Triton buffer to remove unbound proteins. Bound proteins were eluted with 4X Laemmlli buffer/2-mercaptoethanol. Eluates along with whole cell lysates (servicing as input) were analyzed by Western blotting. Blots were probed with anti-GFP (Clontech; 1:500) and anti-FLAG antibodies (Sigma; 1:10,000).

For HEK cells treated with staurosporine, HEK cells were first transfected with GFP- and FLAG-tagged cDNAs. After 48 h, cells were treated for 24 h with 1 μM staurosporine. DMSO was used as the vehicle control. Cells were then lysed with radioimmunoprecipitation assay (RIPA) buffer. For Western blotting, affinity-purified rabbit primary antibody against YAP (Novus) was used at 1:200, rabbit anti-dendrin (Millipore) was used at 1:500, rabbit anti-survivin (Cell Signaling) was used at 1:1000, mouse anti-GAPDH (Calbiochem) was used at 1:3000, and anti-goat human cIAP-1 (R&D Systems) was used at 1 μg/ml.

**Gene Silencing of YAP**—pLKO.1 lentiviral shRNA plasmids were purchased from Addgene for scramble control and from Sigma-Aldrich for YAP and dendrin. We used sequence 5’-CCGGGCGTTGAAAACACAGGAAATCTCGAGTATTTCTGTTTATCTTTGTTTTC-3’ to target YAP. We used sequence 5’-CCGGGATTAATGGAGACTATTCTTCAGGAAAATAGTCTTTACATTTTTGTTTTC-3’ to target dendrin. The pLKO.1 plasmids (YAP shRNA or control shRNA) along with the helper plasmids psPAX2 and pMD2.G were transfected into HEK293T cells at 70% confluence using FuGENE 6 (Promega). Medium was replaced 16–18 h after transfection.

36 and 60 h after transfection, virus-containing supernatants were harvested and centrifuged at 3,000 rpm for 5 min. Viral particles were then passed through a 0.45-μm filter (Corning). The supernatants were subsequently used for the infection of target cells in the presence of 4 μg/ml Polybrene (Sigma). Wild-type undifferentiated podocytes were infected for 24 h and then selected. Noninfected podocytes were removed by selection in 2 μg/ml puromycin (Sigma). Podocytes were selected for −1 week, after which 1 μg/ml puromycin was used as the maintenance dose.
Annexin V/Propidium Iodide Assays—24 h after co-transfection of HEK293 cells with various dendrin and YAP constructs, apoptosis was induced in by staurosporine (Sigma-Aldrich) (1 μM) treatment for 24 h (17). A commercial annexin V/propidium iodide assay (Molecular Probes) and FACS were used to measure apoptosis as described previously (17). Apoptotic cells were defined as those with high annexin and low propidium iodide binding. Podocyte assays were carried out in a similar way except that cells were treated with staurosporine for 1 h. Each experiment was repeated at least three times.

**Caspase 3/7 Activity Assay**—Stable YAP knockdown or control shRNA-expressing podocytes were differentiated on collagen-coated 10-cm dishes for 8–10 days. Cells were then trypsinized and counted. 10,000 cells in 100 μl of medium were plated into each well of the collagen-coated opaque bottom 96-well plate (BD BioCoat). Cells were allowed to attach for 2 days. Cells were then treated with either 0.5 μg/ml or 1 μg/ml staurosporine (Sigma) for 3 h in 100 μl of complete RPMI medium. At the end of incubation, 100 μl of Caspase-Glo 3/7 reagent (Promega) was added to each well, according to the manufacturer’s protocol. After a 30-min incubation at room temperature, the luminescence of each sample was measured in a plate-reading luminometer.

**Adriamycin-induced Podocyte Apoptosis**—We induced podocyte injury in YAP knockdown and control shRNA-expressing podocytes by incubating with adriamycin (0.25 μg/ml) for 48 h (20). We measured apoptosis by annexin V/propidium iodide labeling followed by flow cytometry quantification as described before (17).

**RESULTS**

**YAP Interacts with Dendrin through WW-PPXY Domain Binding**—We previously reported that in podocytes, dendrin directly binds to nephrin and CD2AP (17). To identify additional dendrin-interacting protein, we screened a human cDNA library using the yeast two-hybrid system where dendrin was fused to the DNA binding domain of the GAL4 protein according to our published protocols (22). Among others we found two cDNA clones encoding for YAP. To confirm the two-hybrid results with an independent biochemical approach, we performed Co-IP experiments in HEK293 cells co-transfected with GFP-dendrin and FLAG-YAP1 or FLAG-YAP2, respectively. We found that FLAG-YAP2, and to a lesser extent FLAG-YAP1, specifically interacted with GFP-dendrin (Fig. 1A). No interaction was found with FLAG-Raver serving as negative control (Fig. 1A).

To determine whether the WW domains of YAP mediate the interaction with dendrin, a YAP construct lacking both WW domains (FLAG-YAP2ΔWW) was generated. In contrast to FLAG-YAP2, FLAG-YAP2ΔWW did not interact with GFP-dendrin (Fig. 1A).

To map the domains in dendrin responsible for the interaction with YAP2, we created three dendrin deletion constructs lacking PPXY motifs 1 (dendrinAPPXY1), 2 and 3 (dendrinAPPXY2+3), or all three (dendrinAPPXY1+2+3) and performed Co-IP experiments in HEK293 cells (Fig. 1B). We found that GFP-dendrin and dendrinAPPX1 co-precipitated with FLAG-YAP2. In contrast, GFP-dendrinAPPXY2+3 and GFP-dendrinAPPXY1+2+3 did not interact with FLAG-YAP2 (Fig. 1B). No interaction was found between FLAG-YAP2 and GFP-Sui serving as a negative control (Fig. 1B).

**YAP Is Expressed in Podocytes**—We tested by Western blotting and confocal microscopy whether YAP is expressed in podocyte, both in vivo and in vitro. Using a YAP-specific antibody (Novus), a single protein band with an apparent molecular mass of 65 kDa was detected in isolated mouse glomeruli as well as differentiated and undifferentiated mouse podocyte cultures. D, confocal microscopy shows co-localization of YAP with the transcription factor WT-1 in nuclei of podocytes in mouse kidney (low and high power).

**FIGURE 1. YAP is expressed in podocytes and interacts with dendrin.** A, GFP-dendrin co-precipitates with FLAG-YAP1 and FLAG-YAP2, but not with FLAG-YAP2ΔWW. No interaction is found with FLAG-Raver (control) serving as negative control. B, GFP-dendrin and GFP-dendrinΔPPXY1 lacking PPXY motif#1 co-precipitate with FLAG-YAP2 from co-transfected HEK293 cells. In contrast GFP-dendrinΔPPXY2+3 lacking PPXY motifs 2 and 3 or GFP-dendrinΔPPXY1+2+3 lacking all three PPXY motifs do not interact with FLAG-YAP2. C, Western blot analysis shows YAP protein abundance in isolated mouse glomeruli as well as differentiated and undifferentiated mouse podocyte cultures. D, confocal microscopy shows co-localization of YAP with the transcription factor WT-1 in nuclei of podocytes in mouse kidney (low and high power).

Double labeling confocal microscopy with the nuclear podocyte marker WT1 (24) showed nuclear and cytoplasmic expression of YAP in mouse kidney glomeruli (Fig. 1D). In podocytes, YAP co-localized with WT1, suggesting significant base-line nuclear YAP expression (Fig. 1D, high power view). We next examined the localization of YAP and dendrin in cultured podocytes. In keeping with published results (17), dendrin (green) was found in the cytoplasm and the nucleus, whereas YAP (magenta) demonstrated predominant nuclear distribution (Fig. 2A). Similarly, dendrin was found in the podocyte foot processes in adult mouse kidney, whereas YAP was detected in podocyte nuclei (Fig. 2 B and C).
YAP Binding to Dendrin Reduces Stauroporine-induced Apoptosis in Co-transfected HEK293 Cells—We previously reported that nuclear dendrin enhances staurosporine-induced apoptosis in HEK cells (17). YAP signaling is associated with improved cell survival (10). Therefore we tested whether YAP can antagonize staurosporine-induced proapoptotic dendrin signaling in HEK293 cells co-transfected with GFP-dendrin and FLAG-YAP2, FLAG-YAP2ΔWW that cannot bind to dendrin (Fig. 1A), or FLAG-Raver serving as negative control. Co-expression of GFP-dendrin and the FLAG-Raver control enhanced staurosporine-induced apoptosis by 4.61 ± 0.12-fold when compared with DMSO-treated control cells (Fig. 3A). Co-transfection of GFP-dendrin with FLAG-YAP2 significantly reduced staurosporine-induced apoptosis to 3.82 ± 0.04-fold (p < 0.05, Fig. 3A). In contrast, FLAG-YAP2ΔWW did not significantly suppress dendrin-dependent apoptosis (4.28 ± 0.11-fold, N.S.), thereby showing that the interaction of YAP with dendrin is required for YAP to dampen proapoptotic dendrin signaling. The staurosporine-induced reduction in protein abundance of the YAP transcriptional targets survivin and cIAP1 (10) was comparable between the three groups (Fig. 3B), suggesting that YAP binding to dendrin does not affect cIAP1 or survivin expression.

YAP Enhances Podocyte Survival by Inhibiting Apoptosis—To directly test whether endogenous YAP could confer antiapoptotic properties in podocytes, we used a gene silencing approach to reduce the expression of YAP in differentiated podocytes. Gene silencing of YAP caused a near complete down-regulation of YAP protein abundance (Fig. 3C). Functionally, gene silencing of YAP significantly increased adriamycin-induced podocyte apoptosis when compared with nonsilencing control shRNA-expressing cells (control, 4.75 ± 0.49-fold increase; YAP knockdown, 7.16 ± 0.87-fold increase, p < 0.05, Fig. 3D).

To validate these findings with an independent apoptosis assay, we examined the effect of YAP on staurosporine-induced effector caspase activity (25, 26). YAP depletion significantly increased staurosporine-induced caspase-3/7 activity when compared with control-treated cells, both at 0.5 μM (control, 4.55 ± 1.02-fold increase; YAP knockdown, 13.89 ± 2.27-fold increase; p < 0.05) and at 1 μM (control, 7.02 ± 0.88-fold increase; YAP knockdown, 20.66 ± 3.53-fold increase; p < 0.05) staurosporine (Fig. 3E).

Dendrin Gene Silencing Protects YAP-depleted Podocytes from Apoptotic Stimuli—To test directly whether the interaction of YAP with dendrin mediates the antiapoptotic functions of YAP, we generated podocytes that were gene-silenced for YAP, dendrin, and both YAP and dendrin together (Fig. 4A) and exposed them to staurosporine (0.5 μM). Interestingly, YAP gene silencing was associated with a reduction in dendrin protein abundance. Likewise, dendrin knockdown podocytes showed a reduction in YAP protein abundance. Double knockdown podocytes had significantly less expression of each protein than respective single knockdown cell lysates (Fig. 4A).

Consistent with the data in Fig. 3E, YAP knockdown significantly increased caspase 3/7 activity (11.66 ± 1.86-fold), which was reduced to control levels (6.9 ± 0.64) in dendrin knockdown (6.2 ± 0.62) or YAP and dendrin double knockdown podocytes (4.79 ± 0.48-fold) (Fig. 4B). The differences among the means were statistically significant (analysis of variance,
KIBRA can inactivate Yorkie, the mediator of prosurvival signaling in podocytes. Under physiological conditions, YAP is highly expressed in podocyte nuclei, where it guards against apoptotic cell death, thereby identifying YAP as a mediator of prosurvival signaling in podocytes. These results are in keeping with findings in hepatocytes where YAP promotes the expression of antiapoptotic genes (10). In contrast to hepatocytes and many other cells, podocytes cannot replicate in the adult (14). Thus, the protection against podocyte death and ensuing podocyte loss is paramount for the prevention of progressive kidney injury (13, 16). Our results suggest that nuclear YAP contributes to prosurvival signaling in podocytes through its inhibitory effect on pro-injury dendrin signaling. This conclusion is highlighted by the fact the YAP gene silencing markedly increases the susceptibility to apoptotic cell death, thereby identifying YAP as a mediator of prosurvival signaling in podocytes.

YAP is a downstream effector of the Hippo pathway (27, 28), where Hippo kinases Mst and Lats phosphorylate YAP, leading to its cytoplasmic sequestration and inhibition of its function as a transcriptional co-activator promoting cell survival and differentiation (7, 29, 30). The functional characterization of Hippo signaling in podocytes could yield important information on the pathogenesis and progression of glomerular disease.

KIBRA is another component of the Hippo pathway (27, 28). KIBRA can inactivate Yorkie, the Drosophila YAP ortholog (31). The loss of KIBRA leads to decreased YAP phosphorylation, resulting in its activation and subsequently reduced apoptosis and improved survival in MCF10A cells (31–33). Similar to YAP, KIBRA can also interact with dendrin via its WW domains (34). KIBRA signaling in podocytes has not been extensively studied beyond the modulation of podocyte motility and polarity (35). Based on our findings, it is possible that KIBRA may potentiate proapoptotic dendrin signaling by phosphorylating YAP, thereby promoting its cytoplasmic sequestration and inactivation. Clearly, future studies will be needed to confirm or refute this signaling scenario.

A further interesting outcome of this study is the identification of the WW domains of YAP as the domain responsible for dendrin binding. In contrast to many other interactions, where YAP binds to the SH3 domain of the binding partner, including Yes, Nck, Crk, Src, Abl, and GTPase-activating protein (11), the binding to dendrin is mediated by PPXY motifs 2 and 3 of dendrin.

The downstream signaling cascade responsible for YAP inhibition of podocyte apoptosis likely involves transcriptional co-activation of target genes associated with survival, growth, and differentiation. The observation that in healthy podocytes YAP is found primarily in the nucleus raises the interesting possibility that YAP serves as a physiologic inhibitor of podocyte apoptosis, a potential role highlighted by the low levels of podocyte apoptosis seen in various cell- and animal-based glomerular injury models (20, 36–38).

The potential existence of a regulatory feedback loop between YAP and dendrin is suggested by our finding that silencing of YAP reduces the protein abundance of dendrin and vice versa. Although future studies are warranted to explore whether these changes occur at the transcriptional, translational, or post-translational level, these results underscore the importance of a YAP-dendrin interaction in the regulation of cell death signaling.

In summary, this work provides molecular insight into prosurvival signaling of YAP in podocytes. Under physiological conditions, YAP is highly expressed in podocyte nuclei, where it can antagonize proapoptotic dendrin signaling (17). YAP gene silencing renders podocytes more susceptible to apoptotic stimuli. Further characterization of the YAP signaling axis in podocytes is warranted and should pave the road for the identification of novel drug targets that increase the survival of podocytes in proteinuric kidney disease.

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