Nuclear relocation of the nephrin and CD2AP-binding protein dendrin promotes apoptosis of podocytes

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Kidney podocytes and their slit diaphragms (SDs) form the final barrier to urinary protein loss. There is mounting evidence that SD proteins also participate in intracellular signaling pathways. The SD protein nephrin serves as a component of a signaling complex that directly links podocyte junctional integrity to actin cytoskeletal dynamics. Another SD protein, CD2-associated protein (CD2AP), is an adaptor molecule involved in podocyte homeostasis that can repress proapoptotic TGF-β signaling in podocytes. Here we show that dendrin, a protein originally identified in telencephalic dendrites, is a constituent of the SD complex, where it directly binds to nephrin and CD2AP. In experimental glomerulonephritis, dendrin relocates from the SD to the nucleus of injured podocytes. High-dose, proapoptotic TGF-β1 directly promotes the nuclear import of dendrin, and nuclear dendrin enhances both staurosporine- and TGF-β1-mediated apoptosis. In summary, our results identify dendrin as an SD protein with proapoptotic signaling properties that accumulates in the podocyte nucleus in response to glomerular injury and provides a molecular target to tackle proteinuric kidney diseases. Nuclear relocation of dendrin may provide a mechanism whereby changes in SD integrity could translate into alterations of podocyte survival under pathological conditions.

glomerular injury | proapoptotic signaling | TGF-β signaling | slit diaphragm

Glomerular podocytes serve as the final barrier to urinary protein loss by the formation and maintenance of podocyte foot processes (FP) and the interposed slit diaphragm (SD) (1). All forms of nephrotic syndrome are characterized by abnormalities in podocytes, including retraction (effacement) of podocyte FP and/or molecular reorganization of the SD (1). The discovery of several podocyte proteins and their mutation analysis, including nephrin (2), CD2-associated protein (CD2AP) (3), α-actinin-4 (4), podocin (5), TRPC6 (6, 7), and neph1 (8), have shed light on the pathogenesis of proteinuria and emphasized the critical role of the podocyte and the SD in maintaining the function of the glomerular filtration barrier. At the cytoplasmic insertion site of the SD, ZO-1 (9), α-, β-, γ-catenins (10), podocin (11), and CD2AP (3) are present. In nephrotic syndrome, the normal podocyte substructure is lost, with effacement of podocyte FP, proteinuria (1), as well as altered phosphorylation of ZO-1 (12) and nephrin (13, 14).

Dendrin is a proline-rich protein of unknown function that was originally identified in telencephalic dendrites of sleep-deprived rats (15). In the brain, two protein variants (81 kDa, 89 kDa) were identified in cytosolic and membranous protein fractions (15). More recently, KIBRA, a WW domain-containing protein, was identified as a dendrin-interacting protein, but the functional relevance of this interaction remains to be established (16). Here we show that dendrin is a component of the SD complex. We further show that dendrin relocates to the nucleus of injured podocytes in a murine model of crescentic glomerulonephritis. Finally, we show that TGF-β promotes the nuclear translocation of dendrin and that nuclear dendrin amplifies staurosporine- and TGF-β1-induced podocyte apoptosis.

Results

Dendrin Is a Component of the SD Complex. Dendrin contains two putative nuclear localization signals (NLSs) and three PPXY motifs that are preserved among human, rat, and mouse (Fig. 1*a*). To explore the renal expression of dendrin, we generated and affinity-purified a peptide antibody against the C terminus of mouse dendrin. This antibody detected the previously described 89-kDa and 81-kDa isoforms of dendrin in the brain but only the 81-kDa isoform in isolated glomeruli (Fig. 1*b*). By immunofluorescence microscopy of adult mouse kidney, dendrin was detected exclusively in glomeruli (Fig. 1*c*), where it colocalized with the podocyte marker synaptopodin (17). During kidney development, dendrin was detected in the capillary loop stage of glomerular development (Fig. 1*d*), during which podocytes start developing their characteristic FP and the mature SD (18). Of note, we did not detect any nuclear localization of dendrin during glomerular development. The precise subcellular localization of dendrin was determined by postembedding immunoelectron microscopy of adult mouse kidney, and gold particles were found at the cytoplasmic insertion of the SD into FP (Fig. 1*e*).

The C Terminus of Dendrin Interacts with Nephrin. The immunogold labeling studies suggested an association of dendrin with the SD complex. Therefore, we performed GST pulldown and coimmunoprecipitation studies to test whether dendrin interacts with the key SD proteins nephrin, podocin, or CD2AP. When glomerular extracts were passed over GST columns, GST-dendrin, but not GST alone, specifically interacted with nephrin, CD2AP, and podocin (Fig. 2*a*). On the other hand, GST-dendrin did not interact with ZO-1, another component of the SD complex (9) (data not shown). To confirm further the interaction between dendrin and nephrin, endogenous proteins were immunoprecipitated from glomerular extracts of adult mice by using anti-dendrin and anti-nephrin antibodies as described before (11). Anti-dendrin precipitated dendrin and coprecipitated nephrin (Fig. 2*b Left*). Conversely, anti-nephrin precipitated nephrin and coprecipitated dendrin (Fig. 2*b Right*). No interaction was found with an anti-GFP antibody serving as negative control (Fig. 2*b*). These results show that endogenous dendrin–nephrin complexes are present in podocytes in the kidney. Next, FLAG-nephrin was coexpressed with GFP-tagged N- or C-terminal fragments of

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Abbreviations: CD2AP, CD2-associated protein; FP, foot processes; NLS, nuclear localization sequence; SD, slit diaphragm.

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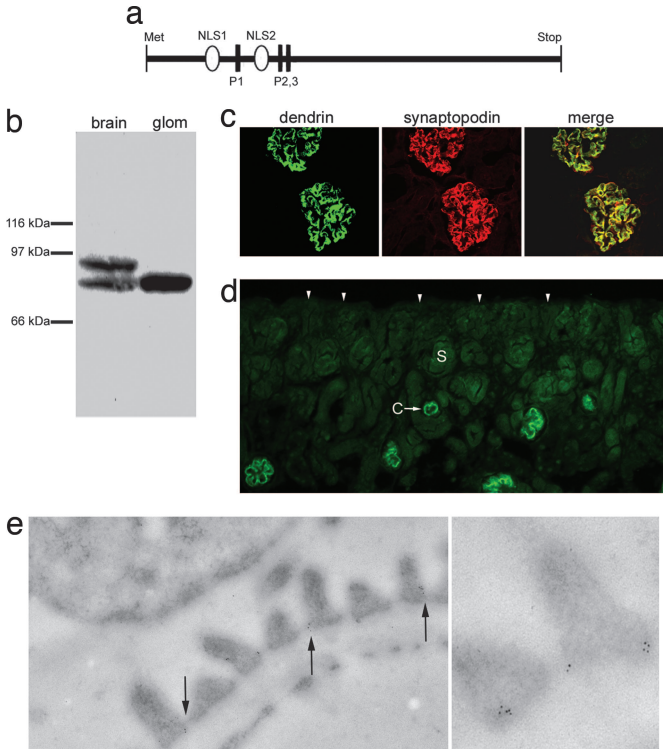

Fig. 1. Dendrin is a component of the SD complex. (*a*) Dendrin contains two putative NLSs (NLS1 and NLS2), and three PPXY motifs (P1, P2, and P3). (*b*) Western blot analysis. In contrast to the brain, which contains both dendrin isoforms, only the 81-kDa isoform is found in isolated glomeruli. (*c*) Double labeling immunofluorescence microscopy of adult mouse kidney with synaptopodin confirms the expression of dendrin in podocytes. (*d*) During kidney development, dendrin is found in differentiating podocytes of the capillary loop stage (C) but not in undifferentiated podocytes of the S-shaped body stage (S). Arrowheads mark the kidney capsule. (*e*) Immunogold labeling of ultrathin frozen sections from adult mouse kidney shows the localization of dendrin in podocyte FP at the cytoplasmic insertion site of the SD (arrows).

dendrin in HEK293 cells. As shown in Fig. 2*c*, the N-terminal fragment of dendrin did not coprecipitate with FLAG-nephrin. In contrast, the C-terminal fragment of dendrin was coprecipitated by anti-FLAG (Fig. 2*c*).

The C Terminus of Dendrin Interacts with CD2AP. The CD2AP homolog CIN85 interacts with dendrin (19). In podocytes, CD2AP interacts directly with nephrin (20) and podocin (11). To test whether the observed interaction between CD2AP and dendrin (Fig. 2*a*) is direct or indirect through nephrin or podocin, FLAG-dendrin and GFP-CD2AP were coexpressed in HEK293 cells, and GFP-CD2AP was coprecipitated with FLAG-dendrin (data not shown). Next, FLAG-tagged CD2AP was coexpressed with the GFP-tagged N- or C-terminal fragments of dendrin. Similar to nephrin, the interaction between CD2AP and dendrin occurred through the C terminus of dendrin (Fig. 2*d*). In contrast, GFP-podocin did not coprecipitate with FLAG-dendrin in coimmunoprecipitation studies (data not shown).

Dendrin Binds Directly to Nephrin and CD2AP. To explore the direct binding of dendrin to nephrin and CD2AP, we conducted *in vitro* reconstitution studies with purified GST-dendrin, FLAG-nephrin, FLAG-CD2AP, and FLAG-podocin according to our previously published protocols (21, 22). Purified GST-dendrin was immobilized on glutathione–agarose beads and incubated with purified FLAG-CD2AP, FLAG-nephrin, or FLAG-

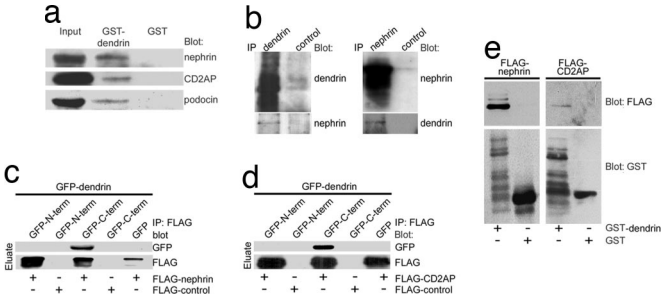

Fig. 2. The C terminus of dendrin interacts directly with nephrin and CD2AP. (*a*) Nephrin, CD2AP, and podocin from glomerular extracts (input) specifically interact with GST-dendrin but not with GST alone. (*b*) Coimmunoprecipitation experiments showing that endogenous dendrin interacts with nephrin in podocytes from isolated glomeruli. (*Left*) Immunoprecipitation (IP) with anti-dendrin. (*Right*) IP with anti-nephrin. No interaction is found with anti-GFP serving as negative control. (*c*) The GFP-tagged C-terminal (GFP-C-term) but not N-terminal (GFP-N-term) fragment of dendrin coprecipitates with FLAG-nephrin but not the FLAG control. No interaction is found between FLAG-nephrin and GFP alone. (*d*) GFP-C-term but not GFP-N-term also interacts with FLAG-CD2AP. No interaction was found with the GFP or FLAG controls. (*e*) GST-dendrin but not the GST control binds directly to FLAG-nephrin and FLAG-CD2AP, respectively.

podocin. Nephrin and CD2AP bound directly to dendrin but not the GST control (Fig. 2*e*). In contrast, we did not observe any binding of dendrin to podocin (data not shown). Taken together, the C terminus of dendrin can interact directly with nephrin and CD2AP.

Nuclear Relocation of Dendrin in Experimental Glomerulonephritis. We found structural (Fig. 1) and biochemical (Fig. 2) evidence for the association of dendrin with the SD complex under normal conditions. The presence of two putative NLSs (Fig. 1*a*) prompted us to test whether dendrin relocates to the podocyte nucleus under pathological conditions. We analyzed the expression and localization of dendrin in a murine model of glomerulonephritis that displays crescent formation, sclerosis, and proteinuria (23). In crescentic glomerulonephritis, injured podocytes form bridges between the glomerular tuft and Bowman's capsule, and these podocyte bridges are thought to represent an early event in the pathogenesis of crescent formation (24). By double labeling deconvolution microscopy with the nuclear podocyte marker WT-1 (25), we found an accumulation of dendrin in the nucleus of such injured bridging podocytes at day 7 after disease induction (Fig. 3 *Middle*). Interestingly, some of these bridging podocytes showed a dual subcellular distribution of dendrin both at the cell membrane and in the nucleus (Fig. 3 *Middle*, arrow). In PBS-injected control mice, both bridging podocytes and nuclear expression of dendrin were absent (Fig. 3 *Top*). In kidney sections from mice harvested on day 14 after disease induction, nuclear expression of dendrin was also noted in WT-1-positive nuclei of podocytes located within the crescents (Fig. 3 *Bottom*).

NLS-Mediated Nuclear Import of Dendrin. Having established an association of nuclear dendrin with inflammatory glomerulonephritis, we next wanted to explore the function of dendrin in the nucleus. To this end, we first determined the intracellular localization of dendrin in cultured differentiated mouse podocytes. Nuclear localization of dendrin in differentiated cultured podocytes was detected by double labeling confocal microscopy with DAPI (Fig. 4*a Upper*). We also conducted double labeling deconvolution microscopy with α-tubulin but found no colocalization with microtubules (data not shown). Instead, we found the association of cytoplasmic dendrin with intermediate fila-

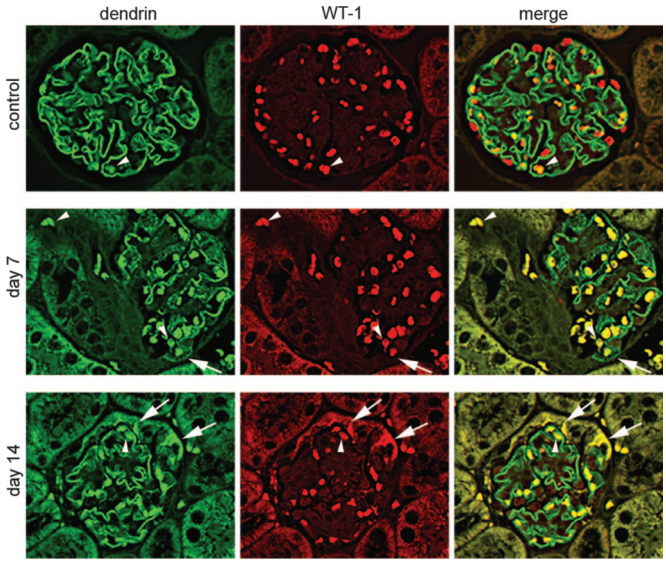

Fig. 3. Nuclear translocation of dendrin in experimental glomerulonephritis. (*Top*) In PBS-injected control mice lacking bridging podocytes, dendrin is absent from WT-1-positive podocyte nuclei. Arrowheads indicate autofluorescence of red blood cells. (*Center*) On day 7 after disease induction, dendrin colocalizes with WT-1 in the nucleus of a bridging podocyte that adheres to Bowman's capsule. Of note, there is also simultaneous labeling of the podocyte cell membrane (arrow). (*Bottom*) On day 14, in mice with active disease, dendrin can be found in the nucleus of WT-1-positive podocyte within crescents (arrows).

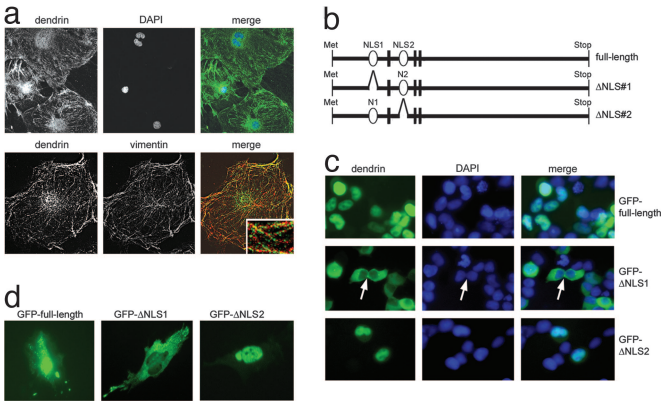

Fig. 4. NLS1-mediated nuclear import of dendrin. (*a*) (*Upper*) Confocal microscopy with DAPI shows that in differentiated cultured podocytes endogenous dendrin is found in the nucleus. (*Lower*) Cytoplasmic dendrin is associated with intermediate filaments as shown by double labeling deconvolution microscopy with vimentin. (*b*) Schematic of GFP-dendrin, GFP-dendrinΔNLS1, and GFP-dendrinΔNLS2. (*c*) Double labeling with DAPI reveals nuclear localization of GFP-dendrin and GFP-dendrinΔNLS2. In contrast, GFP-dendrinΔNLS1 is excluded from the nucleus (arrows). (*d*) Similar to HEK cells, in transfected podocytes, GFP-dendrin and GFP-dendrinΔNLS2 are imported into the nucleus. In contrast, GFP-dendrinΔNLS1 is excluded from the nucleus and accumulates in the cytoplasm.

ments as shown by double labeling deconvolution microscopy with vimentin (Fig. 4*a Lower*). Dendrin contains two putative nuclear localization sequences NLS1 and NLS2. To test the functional activity of these NLSs, we transfected HEK cells with GFP-tagged full-length dendrin and its deleted forms (ΔNLS1, ΔNLS2; Fig. 4*b*) and analyzed the subcellular distribution of dendrin by double labeling with DAPI (Fig. 4*c*). Full-length dendrin was found in the nucleus of all transfected cells (Fig. 4*c Top*). In contrast, the deletion of NLS1 (ΔNLS1) completely abrogated the nuclear import of dendrin (Fig. 4*c Middle*). The deletion of NLS2 did not impair the nuclear import of dendrin because all ΔNLS2-transfected cells showed nuclear localization of GFP-dendrin (Fig. 4*c Bottom*). Next, the relevance of the results in HEK cells for podocyte biology was assessed by transfection of podocytes with the GFP-tagged dendrin constructs (Fig. 4*d*). Similar to the endogenous protein (Fig. 4*a*), GFP-tagged full-length dendrin was found in the cytoplasm and nucleus of transfected podocytes (Fig. 4*d Left*). As in HEK cells, ΔNLS1 (Fig. 4*d Center*) but not ΔNLS2 (Fig. 4*d Center*) was excluded from the podocyte nucleus. Hence, NLS1 is necessary and sufficient for the nuclear import of dendrin in podocytes. This finding is consistent with results by others also identifying an essential NLS in dendrin (19).

Proapoptotic TGF-β Signaling Promotes Nuclear Import of Dendrin in Podocytes. Differentiated cultured podocytes that are maintained in medium containing 10% FBS (26) show a dual cytoplasmic and nuclear localization of dendrin (Fig. 4*a*), raising the possibility that a signaling factor in the serum induces the nuclear import of dendrin. Consistent with this hypothesis, we found that after serum starvation using medium containing 0.2% FBS for 24 h, dendrin was excluded from the podocyte nucleus as shown by double labeling confocal microscopy with DAPI (Fig. 5*a*, control). TGF-β1, which is present in significant amounts in serum, is a multifunctional cytokine that regulates growth, differentiation, and apoptosis in most cells, including podocytes (27, 28). We therefore tested whether TGF-β1 could rescue the nuclear import of dendrin, and we found that the addition of proapoptotic (5 ng/ml) TGF-β1 (27) to the culture medium for 24 h restored the nuclear localization of dendrin (data not shown). To test whether TGF-β can directly promote the nuclear import of dendrin, we analyzed the nuclear import at several time points up to 1 h after 5 ng/ml TGF-β1 treatment by using untreated cells grown in 0.2% FBS as a control (Fig. 5*a*). We first detected nuclear localization of dendrin after 15 min. After 30 min, the nuclear import of dendrin was completed, and no further effect was observed after 60 min (Fig. 5*a*). Of note, lower concentrations of TGF-β ($\leq$1 ng/ml) did not promote nuclear translocation of dendrin (data not shown). The quantitative analysis of these results ($n = 3$) showed that 18.71 $\pm$ 0.75% of control cells displayed nuclear dendrin versus 51.17 $\pm$ 3.83% after 15 min ($P = 0.001$; t test), versus 72.67 $\pm$ 1.42% after 30 min ($P = 0.000002$; t test), versus 80.65 $\pm$ 3.97% after 60 min ($P = 0.0001$; Fig. 5*b*). There was no significant difference between 30 and 60 min (P = not significant; t test).

Nuclear Dendrin Enhances Staurosporine and TGF-β-Mediated Apoptosis. TGF-β is often up-regulated in injured glomeruli (28), and TGF-β mediated podocyte apoptosis plays a prominent role in the progression of glomerular disease (29). Now we find that dendrin accumulates in the nucleus of injured podocytes *in vivo* (Fig. 3). Together with the observation that high-dose, proapoptotic TGF-β induces the nuclear import of dendrin (Fig. 5 *a* and *b*), these data raise the intriguing possibility that dendrin is a modulator of TGF-β-mediated apoptosis. To test this hypothesis, we transfected HEK293 cells that lack endogenous dendrin (data not shown) with GFP-dendrin, or GFP-dendrinΔNLS-1, which cannot enter the nucleus. GFP alone served as negative control. The transfected cells were subsequently treated with staurosporine (1 μM, 12 h) or TGF-β (5 ng/ml, 24 h) with transfected untreated cells serving as controls. Apoptotic cells were detected by flow cytometry after immunolabeling with an annexin V conjugate. Transfection of GFP-tagged fusion proteins allowed the analysis of GFP-expressing transfected cells only. Full-length dendrin, capable of undergoing nuclear import, caused a 5.27 $\pm$ 0.5-fold increase in staurosporine-induced

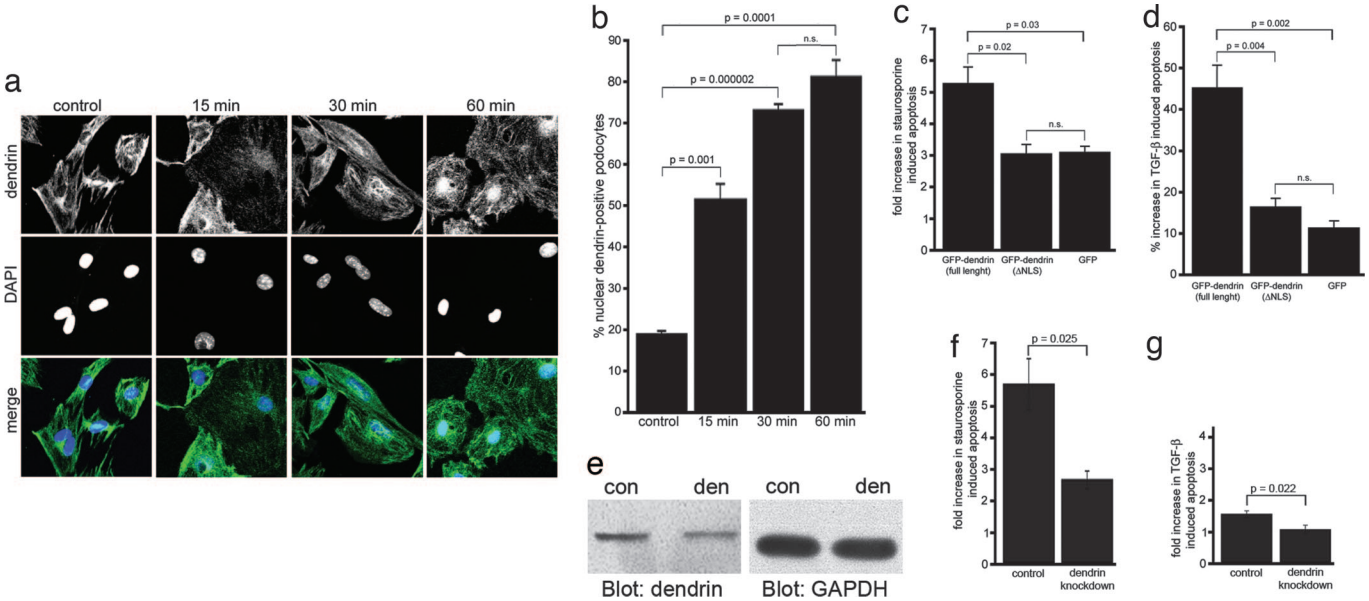

Fig. 5. Nuclear dendrin promotes staurosporine- and TGF-β-mediated apoptosis. (*a*) Time course of TGF-β-induced nuclear import of dendrin after serum starvation for 24 h. Nuclear translocation of dendrin can be observed after 15 min and is completed by 30 min. No further increase is seen at 60 min. (*b*) Quantitative analysis of TGF-β-induced nuclear import of dendrin. For details, see *Results*. (*c* and *d*) Nuclear (full-length) but not cytoplasmic (ΔNLS1) dendrin enhances staurosporine- (*c*) and TGF-β- (*d*) induced apoptosis in HEK293 cells. For details, see *Results*. (*e*) Western blot analysis reveals reduction of dendrin protein expression after lentiviral knockdown of dendrin (den) mRNA expression. GAPDH shows equal protein loading. (*f* and *g*) Knockdown of dendrin expression significantly reduces staurosporine- (*f*) and TGF-β- (*g*) induced apoptosis in podocytes. For details, see *Results*.

apoptosis, which was significantly greater compared with the effect of GFP-dendrinΔNLS1 (3.05 ± 0.37-fold increase; P = 0.02; t test) or GFP alone (3.10 ± 0.26-fold increase; P = 0.03; t test) (Fig. 5*c*). In contrast, there was no difference between GFP-dendrinΔNLS1 and GFP-transfected control cells (P = not significant; t test). The treatment of transfected HEK293 cells with proapoptotic 5 ng/ml TGF-β yielded qualitatively similar results, albeit as expected from previous studies (29), TGF-β1 was a less potent inducer of apoptosis than staurosporine. GFP-dendrin caused 45.22 ± 5.46% increase in apoptotic cells, which was significantly greater than the increase induced by GFP-dendrinΔNLS1 (16.36 ± 2.16%, P = 0.004) or GFP alone (11.30 ± 1.78%; P = 0.002). Again, there was no significant difference between GFP-dendrinΔNLS1 and GFP-transfected control cells (P = not significant; t test). To strengthen these results further, we generated dendrin knockdown podocytes by lentiviral infection that showed a significant reduction of dendrin protein expression (Fig. 5*e*). We repeated the apoptosis assays and found that both staurosporine- (Fig. 5*f*) and TGF-β-(Fig. 5*g*) induced podocyte apoptosis were significantly reduced in dendrin knockdown podocytes. For staurosporine, we found a 5.34 ± 0.77-fold increase for the control cells versus 2.49 ± 0.27 for dendrin knockdown cells (n = 3; P = 0.025, t test; Fig. 5*f*). After TGF-β, treatment, control podocytes showed a 1.54 ± 0.11-fold increase in apoptosis versus 1.05 ± 0.14 for dendrin knockdown podocytes (n = 5; P = 0.022, t test; Fig. 5*g*). Taken together, nuclear dendrin enhances both staurosporine- and TGF-β-mediated apoptosis in podocytes.

Discussion

Originally, dendrin was identified as a dendritic protein whose expression is differentially modulated by prolonged behavioral activity (15). However, so far the molecular function of dendrin remained elusive. Here we identified dendrin as an enhancer of apoptosis. The observation that dendrin, like synaptopodin (17), is found both in podocyte FP and dendritic spines adds further to the notion that podocytes and neurons share important structural and functional features. Both cell types develop prominent cell processes equipped with well organized microtubular cytoskeleton, intermediate, and actin filaments (30), and key mechanism of process formation are shared by both cell types (31). Similar to neurons, podocytes express the synaptic vesicle molecule Rab3A and its specific effector rabphilin-3a (32). They contain structures resembling synaptic vesicles, which contain glutamate, and synaptotagmin 1, and undergo spontaneous and stimulated exocytosis and recycling, with glutamate release (33). The idea that the SD is similar to a neurological synapse is further supported by the observation that densin, originally identified as brain-specific synaptic protein of the *O*-sialoglycoprotein family (34), interacts in the brain with α-actinin-4 (35), the target gene of a familial form of FSGS (4) and is also found at the SD, where it interacts with nephrin (36).

In the current work we did not find any nuclear localization of dendrin during renal development, suggesting that pathways of glomerular injury are different from developmental pathways. The developmental expression of dendrin commences later than that of nephrin or CD2AP, thereby meshing well with the hypothesis that dendrin is dispensable for SD formation but rather acts as surveillance factor similar to the WT-1-interacting protein WTIP (37) that can shuttle between adhesion junctions and the nucleus (38). WTIP translocates from cell–cell junctions to the nucleus in PAN-treated podocytes, where it suppresses WT-1-mediated gene expression. These results led to the hypothesis that WTIP monitors SD function and shuttles into the nucleus after podocyte injury (39), thereby translating changes in SD structure into altered gene expression.

The SD represents a signaling platform that contributes to the regulation of podocyte function in health and disease (40, 41). Based on recent data, it appears that at least two major SD signaling pathways can be discerned: (*i*) acute, reversible phosphotyrosine-based ZO-1 (12) and nephrin (13, 42–44) signaling leading to P-nephrin–Nck-mediated actin reorganization (14, 45). (*ii*) Chronic calcium (6, 7) and phosphoserine/threonine signaling-mediated regulation of cytoskeleton dynam-

ics and podocyte survival (29, 46, 47). Both CD2AP and nephrin interact with the p85 regulatory subunit of phosphatidylinositol 3-kinase *in vivo*, stimulating antiapoptotic Akt signaling (47). Podocytes lacking CD2AP are more susceptible to apoptosis, and mice lacking CD2AP show increased podocyte apoptosis in the early stages of progressive glomerulosclerosis (46). Hence, CD2AP appears to have an antiapoptotic role, with apoptosis considered a critically important mechanism in podocyte depletion. In fact, elegant studies from several groups have clearly established that podocyte depletion occurs in most progressive glomerular diseases and results from podocyte loss while the remaining podocytes are unable to proliferate (48–51). In the context of the current results that dendrin amplifies TGF-β-induced apoptosis, it is of particular interest that podocytes undergo apoptosis at early stages in the course of progressive glomerulosclerosis in TGF-β1-transgenic mice (29). The critical role of TGF-β1 in podocytes has been extended by the observation that dose-dependent TGF-β signaling in podocytes can be good or bad (27). At a concentration exceeding 1 ng/ml, TGF-β signaling switches from a G_0/G_1 growth arrest and differentiation promoter to a pathophysiologic inducer of G_2/M growth arrest and apoptosis in murine podocytes (27). Now, we identify nuclear, but not cytoplasmic, dendrin as a modulator of TGF-β-induced apoptosis because only high-dose TGF-β1 promotes import of dendrin, and only nuclear dendrin increases staurosporine- or TGF-β1-induced apoptosis. In summary, we propose that dendrin serves a surveillance function at the SD and relocates to the podocyte nucleus in response to glomerular injury, thereby providing a signaling mechanism whereby changes in SD function modulate podocyte survival. The identification of nuclear dendrin as an enhancer of staurosporine- or TGF-β-induced apoptosis defines an antisurvival signaling pathway.

Methods

Animal Studies. Experimental glomerulonephritis was induced in adult male 129 mice aged 14–20 weeks (body weight, 28–38 g) by two consecutive i.p. injections of a sheep anti-rabbit glomerular antiserum (0.5 ml per 20 g of body weight per day for 2 consecutive days) as described before (23). Mice ($n = 3$ per group) were killed on days 7 and 14 after the second injection of anti-glomerular antibody, and kidneys were harvested. PBS-injected age-matched 129 mice ($n = 3$) served as controls.

Plasmid Constructs. A full-length cDNA clone of rat dendrin (52) was cloned in frame into a modified pGEX vector, pEGFP-C1 (BD Bioscience Clontech, San Jose, CA), or pFLAG-CMV-5c (Sigma–Aldrich, St. Louis, MO). The N-terminal (amino acids 1–351) and C-terminal (amino acids 352–653) fragments of dendrin were generated by PCR and cloned into pEGFP-C1. Dendrin deletion constructs lacking NLS1 (amino acids 59–77) or NLS2 (amino acids 163–169) were generated by PCR and cloned into pEGFP-C1 and pFLAG-CMV-5a vectors. Mouse nephrin cDNA (53) was cloned into pFLAG-CMV-5a. Mouse CD2AP cDNA (3) was cloned into pFLAG-CMV-5a vector and pGFP-C1. All constructs were verified by DNA sequencing.

Cell Culture and Transient Transfection. Podocytes were cultured as described before (26). Transient transfection of podocytes and HEK293 cells (American Type Culture Collection, Manassas, VA) was done as described previously (54). GFP fusion proteins were analyzed by direct fluorescence microscopy in living cells or after fixation and double labeling immunocytochemistry (54).

Generation of Polyclonal Antibodies Against Mouse Dendrin. Rabbits were immunized with a keyhole limpet hemocyanin-conjugated peptide (single letter code, CEEGFIREDTRKTPQGNRERE) corresponding to the C terminus of mouse dendrin. The antiserum was affinity-purified with the corresponding peptide linked to Ultralink (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Immunofluorescence and Immunogold Electron Microscopy. Immunofluorescence microscopy of mouse kidney frozen sections and cultured podocytes as well as immunogold labeling were done as described before (11). Double labeling of cultured podocytes and mouse kidney sections was performed with mouse monoclonal anti-vimentin and mouse monoclonal WT-1 antibodies, respectively. For the quantitative analysis of TGF-β-mediated nuclear import of dendrin, pictures of 50 cells were captured by confocal microscopy for each time point. The data represent the mean ± SEM of three independent experiments.

Western Blotting, Immunoprecipitation, and GST-Binding Assays. SDS/PAGE, Western blotting, coimmunoprecipitation from transfected HEK cells, and endogenous coimmunoprecipitation from isolated glomerular extracts were done as described previously (21, 54). GST pulldown studies with GST-dendrin and glomerular protein extracts were done as described previously (21, 22). For Western blotting, the affinity-purified primary antibody against dendrin was used at 1:200. The other primary antibodies used in this work were as follows: rabbit anti-nephrin (53), rabbit anti-CD2AP (3), and rabbit anti-podocin (11). To study the direct binding of nephrin and CD2AP to dendrin, GST pulldown studies with purified recombinant proteins were done as described before (21). One microgram of GST-dendrin was immobilized on GSH-agarose beads. Beads were washed five times in 1% Triton X-100 in PBS, and 1 μg of purified FLAG-nephrin, FLAG-CD2AP, or FLAG-podocin in 500 μl of buffer (1% Triton X-100/50 mM Tris·HCl/150 mM NaCl) was added to the beads. Reactions were incubated under rotation for 2 h at 4°C, and beads were washed five times in radioimmunoprecipitation assay buffer (1% Triton X-100/50 mM Tris·HCl/200 mM NaCl/1 mM EDTA/1 mM EGTA/0.25% deoxycholate). Proteins were eluted in 100 μl of sample buffer and analyzed by SDS/PAGE and immunoblotting (21).

Apoptosis Assays in HEK293 Cells. Twenty-four hours after transfection with GFP-dendrin, GFP-dendrinΔNLS1 and GFP control, apoptosis was induced in HEK cells by human TGF-β1 (Roche, Mannheim, Germany) or staurosporine (Sigma–Aldrich). Cells were treated with 5 ng/ml TGF-β for 24 h or 1 μM staurosporine for 12 h. Transfected cells not treated with apoptotic stimuli served as controls. A commercial annexin V/propidium iodide assay (Molecular Probes, Eugene, OR) was used to quantify cells undergoing apoptosis. Transfected HEK293 cells were harvested by rinsing with complete medium (DMEM/10% FBS/penicillin/streptomycin) and washed in cold PBS. Cells were diluted to a concentration of 1×10^6 cells/ml in annexin-binding buffer (10 mM Hepes/140 mM NaCl/2.5 mM $CaCl_2$, pH 7.4) and incubated for 15 min with Alexa Fluor 647 annexin V conjugate (Molecular Probes). Necrotic cells were identified with propidium iodide staining. Cells were analyzed on a FACSCalibur flow cytometer (BD Bioscience, Mountain View, CA) with data analysis of 30,000–50,000 events done on CellQuest Pro (BD Bioscience).

Lentivirus Production. Two dendrin-specific oligonucleotides (GAGGCTCACCTGCTGTTGAGA, corresponding to nt 448–468 of the mouse dendrin-ORF, and GGAAGTGAGCAGATATGGCTT, corresponding to 961–981) were cloned into the lentiviral vector FUGW, which coexpresses GFP under the ubiquitin promoter. A control FUGW vector was constructed by using a 21-nt scrambled sequence (GACCGCGACTCGCCGTCTGCG), which has no significant homology to any mam-

malian gene sequence. Lentivirus was produced as described previously by Dittgen *et al*. (55).

Apoptosis Assays in Podocytes. Lentivirus-containing medium was added to cultured murine podocytes grown under permissive conditions at 33°C after pretreatment with 10 μg/ml Polybrene for 5 min. GFP expression indicating lentiviral infection of podocytes was noted uniformly after 48 h of transduction. Apoptotic stimuli were provided by 1 μM staurosporine for 1 h or 5 ng/ml TGF-β for 24 h. Before TGF-β treatment, podocytes were serum-starved in 0.2% FBS-containing medium for 24 h. For the quantification of apoptotic cells, only GFP-expressing infected podocytes were considered, where the infection efficiency was 70–80%.

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