Loss of Podocyte aPKC\(\lambda/\iota\) Causes Polarity Defects and Nephrotic Syndrome

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ABSTRACT

Atypical protein kinase C (aPKC) is a central component of the evolutionarily conserved Par3-Par6-aPKC complex, one of the fundamental regulators of cell polarity. We recently demonstrated that these proteins interact with Neph-nephrin molecules at the slit diaphragm of the glomerular filtration barrier. Here, we report that podocyte-specific deletion of aPKC\(\lambda/\iota\) in mice results in severe proteinuria, nephrotic syndrome, and death at 4 to 5 wk after birth. Podocyte foot processes of knockout mice developed structural defects, including mislocalization of the slit diaphragm. In the glomerulus, aPKC\(\lambda/\iota\) was primarily expressed in developing glomerular epithelial cells and podocyte foot processes. Interestingly, under physiologic conditions, aPKC\(\lambda/\iota\) translocated from the apical surface to the basolateral side of developing podocytes, and this translocation preceded the development of foot processes and formation of slit diaphragms. Supporting a critical role for aPKC\(\lambda/\iota\) in the maintenance of slit diaphragms and podocyte foot processes, aPKC\(\lambda/\iota\) associated with the Neph-nephrin slit diaphragm complex and localized to the tips of filopodia and leading edges of cultured podocytes. These results suggest that aPKC signaling is fundamental to glomerular maintenance and development.

Polarity is a common feature of many different cell types and a prerequisite for the development of multicellular organisms. The evolutionarily conserved Par3-Par6-aPKC complex is required for the establishment of cell polarity. The core of this complex consists of the atypical protein kinase C (aPKC) and Par6 together with the scaffold protein Par3. Both Par6 and Par3 belong to the PDZ family of adaptor proteins. Par6 seems to be necessary for activation of aPKC. The interaction with the GTP-bound form of the small GTPases Rac and Cdc42 results in a conformational change of Par6,\(^1,2\) facilitating the phosphorylation and subsequent activation of aPKC.\(^3\) Par3 is thought to act as a scaffolding protein necessary to recruit Par6/aPKC to sites where its activity is required.\(^4\) We recently demonstrated that the glomerular slit diaphragm molecules Neph1 and nephrin directly bind to Par3, suggesting that Par3 recruits the Par polarity complex to the slit diaphragm.\(^5\)

The aPKC is a key player in the generation of almost all forms of polarity.\(^6--8\) Two types of aPKC are known, aPKC\(\xi\) and aPKC\(\lambda/\iota\), which are highly related but encoded by separate genes.\(^9\) It is unclear whether both aPKCs are functionally redundant.

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and thus mediate the various aPKC functions or distinct roles are performed by each aPKC. In *Drosophila*, loss of aPKC results in polarity defects in different cell types and lethality of the embryo. In mammalian epithelia, aPKC plays a critical role in the development of the junctional structures, and in neurons the Par3-Par6-aPKC complex seems to specify axon development.

The glomerular filtration barrier is a unique structure characterized by a precise three-dimensional framework of podocytes that elaborate long, regularly spaced, interdigitating foot processes, enveloping the glomerular capillaries. All podocytes are connected through the slit diaphragm, a specialized cell junction. The slit diaphragm is the only cell–cell contact of mature podocytes, representing a signaling platform that regulates podocyte survival, endocytosis, and cytoskeletal organization. Mutations in genes encoding slit diaphragm proteins result in severe loss of protein in the urine (nephrotic syndrome) in both animal models and patients. Among these slit diaphragm proteins, Nep1 and nephrin have been shown to be indispensable for the maintenance of the architecture of podocytes—both proteins participate in intracellular signaling networks to support cytoskeletal organization, cell adhesion, and cell polarity. It is intriguing to speculate that the cellular programs used to maintain the complex podocyte architecture are similar to the programs that drive podocyte morphogenesis and repair after foot process effacement, a condition that can be rapidly reversible after some forms of glomerular injury.

Here we demonstrate that podocyte-specific deletion of aPKCζ/ζ in mice results in slit diaphragm displacement, foot process effacement, proteinuria, and accelerated renal failure. We document a unique transition of the Par polarity complex in glomerular podocytes from an apical to a basal localization. During glomerular development, this polarity conversion seems to precede podocyte foot process development. In agreement with a critical role of aPKCζ for foot process development and maintenance, aPKC associates with the Nep1-nephrin complex and localizes to the leading edge and filopodia tips of cultured podocytes. We propose that aPKC signaling is a fundamental mechanism for glomerular maintenance and development.

RESULTS

Podocyte-Specific Deletion of aPKCζ/ζ Results in Severe Glomerular Disease

Recently we identified the Par3-Par6-aPKC polarity complex as novel components of the glomerular slit diaphragm. To define further the function of aPKCs in podocytes, we first examined the expression of the two aPKC isoforms in isolated murine glomeruli and podocytes by reverse transcriptase–PCR (RT-PCR). We found that both isoforms are expressed in a glomerular-enriched fraction as well as in cultured murine podocytes (Figure 1A). Because aPKCζ-deficient mice display no renal phenotype, we examined the role of aPKCζ in more detail. The complete knockout is embryonic lethal; therefore, we crossed aPKCζ−/− mice to podocin-cre mice to induce a podocyte-specific deletion in these animals.

To confirm the podocyte-specific loss of aPKCζ protein, we used isotype-specific aPKCζ antibodies on kidney sections of 6-d-old knockout and wild-type (WT) mice, respectively. Podocin Cre-mediated aPKCζ deletion could be demonstrated as early as in the capillary loop developmental stage (Figure 1B), which is in agreement with the start of podocin expression in developing glomeruli (see also Figure 4E). Lysates of 293T cells expressing either aPKCζ or aPKCζ were used to test for the isotype specificity of aPKCζ antibodies. As demonstrated in Supplemental Figure 1, the indicated antibodies specifically detected aPKCζ but not aPKCζ. The aPKCζ conditional knockout mice were born healthy and indistinguishable from littermates up to 2 wk after birth. After this point, growth retardation became evident in these mice, and all aPKCζ conditional knockout mice died within 4 to 5 wk of birth. Six-day-old aPKCζ conditional knockout mice did not display obvious morphologic alterations on histology (Figure 1C). In contrast, at approximately 4 wk of age, aPKCζ conditional knockout mice exhibited significant growth retardation and significant proteinuria as confirmed by Coomassie gel (Figure 1, D and E). In agreement with these clinical findings, histology revealed generalized glomerulosclerosis, kidney tubule dilation, and proteinuric casts in the tubule system (Figure 1F).

Loss of aPKCζ in Podocytes Causes Disruption of Regular Foot Process Architecture

To examine the effects of aPKCζ disruption on ultrastructural glomerular morphology, we performed electron microscopic analysis on 4-wk-old proteinuric PodCre-aPKCζ/ζ mice and control littermates (Figure 2, A and B). In knockout mice, foot processes were globally effaced (Figure 2B). Intact foot processes were detectable in some areas at higher magnifications, although neighboring foot processes often displayed significant slit diaphragm displacement as well as severe junctional abnormalities (Figure 2C).

Altered Distribution of Slit Diaphragm Molecules in Podocyte-Specific aPKCζ Knockout Mice

By confocal laser microscopy, we observed the expression intensity and distribution pattern of the polarity protein Par3, the tight junction markerZO-1, and the slit diaphragm molecules nephrin and podocin. The staining of these markers revealed a linear pattern along the glomerular capillary wall in control mice. In contrast, expression of Par3, ZO-1, nephrin, and podocin was significantly impaired in 4-wk-old PodCre-aPKCζ/ζ mice with a mainly granular distribution along the glomerular basement membrane (Figure 3). In addition, nephrin expression seemed considerably reduced in PodCre-aPKCζ/ζ mice (Figure 3C).
Translocation of the Polarity Proteins aPKC ε and Par3 Precedes Slit Diaphragm Formation and Podocyte Foot Process Development

As the glomerulus matures, podocytes undergo a remarkable transformation. They lose their lateral cell contacts, except at the very basolateral aspect of immature podocytes adjacent to the basal membrane. Subsequently, when the podocyte cell bodies have become isolated from each other, they start to extend large projections. These projections divide into intermediate branches, which then divide into multiple smaller foot processes to interdigitate with the foot processes of adjacent podocytes. Although these early changes have morphologically been described in detail, little is known about the molecular pathways and mechanisms accompanying these podocyte transformations. It is conceivable to speculate that the morphogenetic switch from a simple polygonal cell to a very complex three-dimensional architecture with the octopus-like cell shape of mature podocytes relies at least in part on evolutionarily conserved polarity signaling. To examine the spatiotemporal expression of the Par polarity complex and slit diaphragm molecules during glomerular developmental stages, we stained frozen kidney sections of newborn Wistar rat (day 1) using antibodies against aPKC ε, Par3, ZO-1, nephrin, podocin, and the podocyte marker WT-1 and subjected them to confocal laser microscopy. Because newborn rats simultaneously display all stages of glomerular development, they have been extensively used as a model to study glomerular development. The polarity proteins aPKC ε and Par3 and
the tight junction protein ZO-1 were localized at the apical membrane during early stages of glomerular development (Figure 4, A through C, I, comma-shaped body). Interestingly, during the s-shaped body stage, Par3, aPKC/\(\zeta\)/H9261/H9259, and ZO-1 moved along the lateral side to the cell basis (Figure 4, A through C, II, s-shaped body). In contrast, nephrin and podocin expression started at the late s-shaped body stage and early capillary loop stage and immediately localized to the basolateral side of podocytes (Figure 4, D and E, III, early capillary loop stage). Subsequently, the polarity proteins Par3 and aPKC/\(\zeta\)/H9261/H9259 as well as ZO-1 and the slit diaphragm proteins nephrin and podocin co-localized at the developing podocyte foot processes (Figure 4, IV, capillary loop stage, and V, immature glomerulus). Supplemental Figure 2 further illustrates the timing of the “podocyte polarity conversion” with a complete translocation of typical apical epithelial markers such as the Par3 to the basal side of developing podocytes (Supplemental Figure 2A, I, comma-shaped body, II, s-shaped body, III, early capillary loop stage). Interestingly, this polarity conversion seems to precede the targeting of the slit diaphragm molecule nephrin to the basal side of podocytes (Supplemental Figure 2, II, s-shaped body).}

**DISCUSSION**

The establishment and maintenance of cell polarity is important for the development and function of many cell types. The podocyte as part of the glomerular filtration barrier is one of the most highly differentiated and polarized cell types, divided morphologically and functionally into three distinct parts: The cell body and primary and secondary foot processes. The polarity signaling pathways that establish and maintain the specialized three-dimensional podocyte foot-process network of the filtration barrier, however, are largely unknown. These mechanisms not only seem important for the understanding of glomerular development and homeostasis, but also it is likely that repair mechanisms in response to glomerular damage use these programs. Here we analyzed a conditional aPKC/\(\zeta\)/H9261/H9259 knockout mouse to define the critical role of polarity and aPKC/\(\zeta\)/H9261/H9259 signaling for glomerular maintenance in vivo.

We identified the presence of both known aPKC isoforms in podocytes. Because aPKC\(\zeta\)-deficient mice display no renal...
phenotype and constitutive knockouts of aPKC/α are embryonic lethal, we generated a podocyte-specific deletion of aPKC/α for our studies. Mice with podocytes lacking aPKC/α developed renal failure and proteinuria and lost their regular podocyte architecture at an early age. To our knowledge, this is the first genetic model to demonstrate the critical importance of known regulators of apicobasolateral polarity for glomerular maintenance. Remarkably, at birth, these mice displayed no proteinuria and relatively normal glomeruli, suggesting a maintenance rather than a developmental defect underlying this mouse model. Similar phenotypes have been described, for example, for CD2AP-deficient mice. One explanation for

**Figure 3.** Immunofluorescence studies of WT and aPKC/α knockout glomeruli. Frozen mouse kidney sections from 4-wk-old WT mice (control, top) versus knockout mice (bottom) were stained using antibodies against the Par complex protein Par3, the tight junction marker ZO-1, the slit diaphragm molecules nephrin and podocin, and nidogen, a glomerular basement membrane marker, and subjected to confocal laser microscopy. (A through D) In contrast to the linear staining of Par3, ZO-1, nephrin, and podocin in WT mice, these molecules exhibited a significantly impaired, granular distribution along the glomerular basement membrane in knockout mice. In addition, nephrin expression seemed to be reduced in knockout mice (C). Bars = 5 μm.
Figure 4. Translocation of the polarity proteins aPKCα and Par3 precedes slit diaphragm formation and podocyte foot process development. (A through E) Frozen kidney sections of newborn Wistar rat (day 1) were stained using antibodies against aPKCα, Par3, ZO-1, nephrin, podocin, and the podocyte marker WT-1 and subjected to confocal laser microscopy. Newborn rats display various stages of glomerular development because glomerular development is asynchronous. Each panel displays the expression pattern of the accordant proteins during glomerular development (from left to right): Developmental stages ranging from comma-shaped body (I), s-shaped body (II), capillary loop stage (III to IV), to a maturing glomerulus (V). (A through C) Whereas aPKCα, Par3, and ZO-1 are apically expressed during early developmental stages (I, arrows) and translocate to the basal side of glomerular podocytes at later glomerular development (II to III, arrows), nephrin (D) and podocin (E) expression begins at the late s-shaped body stage/early capillary loop stage (III, arrows) on the basolateral side of podocytes. Bars = 5 μm.
such a phenotype could be the challenge of the postnatal changes in hemodynamics and the sudden exposure to enormous mechanical stress, requiring the efficient and constant remodeling of podocyte foot processes; however, there are alternative reasons to explain the lack of a developmental phenotype in our mouse model: First, podocytes seem to express both functionally redundant aPKC isoforms, and deletion of aPKC could be compensated by aPKC during development. This functional redundancy has previously been observed and is an indicator for the evolutionary conservation of the functional aPKC domains. Second and perhaps more likely, the podocin promoter activates Cre expression relatively late during glomerular development, masking a developmental phenotype of podocyte-specific deletion of aPKC.

During development, podocytes undergo extensive morphologic changes necessary to form the glomerular filtration barrier. By studying the localization of Par proteins during these morphologic changes, we identified an unprecedented apical-to-basal translocation of the Par polarity complex during glomerular development. This polarity conversion seems to be a unique feature of glomerular podocytes; as in other epithelial cells, Par3, aPKC, and apical junction markers such as ZO-1 always mark the apical membrane of the epithelial cell. Interestingly, this polarity conversion seemed to precede the targeting of the nephrin/podocin complex to the slit diaphragm and podocyte foot process development. In agreement with these data, an apical-to-basal migration of podocyte junctions preceding the formation of slit diaphragms was reported several decades ago. On the basis of our observations in the newborn rat, we propose the following model of sequential molecular steps of podocyte transformation: (1) Simple polygonal podocytes form a monolayer connected by tight junctions with an apicobasolateral polarity maintained by the localization of the Par3-Par6-aPKC complex at the apical cell surface; (2) the Par3-Par6-aPKC complex and tight junction proteins such as ZO-1 migrate and translocate to the basal side of the premature podocyte; (3) the Par complex subsequently controls the basal targeting of slit diaphragm molecules such as nephrin; (4) together, the Par complex and the slit diaphragm molecules initiate the formation of foot processes.

Indeed, aPKC localized to the leading edge and the tip of filopodia of differentiated podocytes in culture (arrows).
Recent studies have changed our conception of the podocyte from a relatively static to a dynamic epithelial cell, whose complex three-dimensional structure depends on signaling mechanisms at the filtration barrier. The coordinated translocation of the Par3–Par6–aPKC complex during podocyte development could potentially be a prerequisite for the development of foot processes and targeting of molecules to the slit diaphragm. Our findings demonstrate that aPKCα/λ is essential for a normal podocyte homeostasis and suggest that aPKCα/λ function is critical for differentiation and remodeling of podocyte foot processes.

CONCISE METHODS

Reagents and Plasmids
Mouse Neph1 and human nephrin were described previously. To create N-terminally GST-tagged constructs of the cytoplasmic domains, Nephrin (bp 3259 to 3723 and bp 3259 to 3480) and Neph1 (bp 1657 to 2367 and bp 1657 to 2358) were cloned in pGEX-4T-3 by standard cloning procedures. Polyclonal antibody specific for a normal podocyte homeostasis and suggest that aPKCα/λ function is critical for differentiation and remodeling of podocyte foot processes.

RT-PCR
mRNA was isolated from mouse glomeruli, from adult C57BL/6 mice by using the dynabead method, or from differentiated immortalized mouse podocytes using RNA easy kit (Qiagen, Hilden, Germany). mRNA was transcribed in cDNA by reverse transcriptase. The following PCR primers were used: aPKCα/λ fp CCGCATGTGTAAGGAGGAT, aPKCα/λ rp GCGACGCTGACATGACACA, aPKCγ fp AAGTGGGTGGACAGTGAGG, aPKCζ rp TCGTGGACACTGC-CTTCTT. No reverse transcriptase was added in the negative control.

Urinary Protein Measurements
BSA standard (1, 5, 10, and 20 μg) and 1 μl of urine of aPKCα/λ WT and podocyte-specific knockout mice were separated by 10% SDS-PAGE. The gel was stained by Coomassie Blue.

Histology
Kidneys of aPKCα/λ WT and podocyte-specific knockout mice were fixed in 4% paraformaldehyde and embedded in paraffin or in Lowicryl K4M resin (Electron Microscopy Sciences; Hatfield, PA) and further processed for periodic acid-Schiff staining or electron microscopy, respectively.

Immunofluorescence Staining of Kidney Sections
Because newborn rats display various stages of glomerular development, rat kidneys of 1-d-old Wistar rats or mouse kidneys of aPKCα/λ WT and podocyte-specific knockout mice were frozen in OCT compound and sectioned at 6 μm (Leica Kryostat; Leica, Wetlazar, Germany). The sections were fixed with 4% paraformaldehyde, blocked in PBS containing 5% BSA, and incubated for 1 h with primary antibodies as indicated. After PBS rinse several times, fluorophore-conjugated secondary antibodies were applied for 30 min. Confocal images were taken using a Zeiss laser scan microscope equipped with a ×63 water immersion objective.

Pulldown Assay
Mouse kidneys were lysed in a 1% Triton X-100 lysis buffer containing 0.5 M CHAPS and glass homogenized. After centrifugation (15,000 × g, 15 min, 4°C), ultracentrifugation at 100,000 g for 1 h with primary antibodies as indicated. After PBS rinse several times, fluorophore-conjugated secondary antibodies were applied for 30 min. Confocal images were taken using a Zeiss laser scan microscope equipped with a ×63 water immersion objective.

Immunofluorescence Staining of Podocytes
Immortalized human podocytes split on Collagen A–coated cover glasses were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked in PBS containing 5% BSA, and incubated for 1 h with primary antibodies as indicated. After PBS rinse several times,
fluorophore-conjugated secondary antibodies were applied for 30 min. Confocal images were taken using a Zeiss laser scan microscope equipped with a ×63 water immersion objective.

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DISCLOSURES

None.

REFERENCES


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