Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary disorder that accounts for 8–10% of end stage renal disease. PKD1, one of two recently isolated ADPKD gene products, has been implicated in cell-cell and cell-matrix interactions. However, the signaling pathway of PKD1 remains undefined. We found that the C-terminal 226 amino acids of PKD1 transactivate an AP-1 promoter construct in human embryonic kidney cells (293T). PKD1-induced transcription is specific for AP-1; promoter constructs containing cAMP response element-binding protein, c-Fos, c-Myc, or NFkB-binding sites are unaffected by PKD1. In vitro kinase assays revealed that PKD1 triggers the activation of c-Jun N-terminal kinase (JNK), but not of mitogen-activated protein kinases p38 or p44. Dominant-negative Rac-1 and Cdc42 mutations abrogated PKD1-mediated JNK and AP-1 activation, suggesting a critical role for small GTP-binding proteins in PKD1-mediated signaling. Several protein kinase C (PKC) inhibitors decreased PKD1-mediated AP-1 activation. Conversely, expression of the C-terminal domain of PKD1 increased PKC activity in 293T cells. A dominant-negative PKC α, but not a dominant-negative PKC β or δ, abrogated PKD1-mediated AP-1 activation. These findings indicate that small GTP-binding proteins and PKC α mediate PKD1-induced JNK/AP-1 activation, together comprising a signaling cascade that may regulate renal tubulogenesis.

Insight into the function of PKD1 has been hampered by its complexity. Full-length PKD1 has not yet been expressed in vivo (14), curtailing genetic manipulation of the intact protein. Recently, we have shown heterodimeric interaction between PKD1 and PKD2 fusion proteins (15), using a heterologous integral membrane protein fused to the C-terminal cytoplasmic domain of PKD1 and PKD2. This approach has been used to delineate the effector mechanisms of various cytoplasmic domains (16–21). In the present study, we demonstrate that the C-terminal cytoplasmic domain of PKD1 triggers the activation of AP-1, a transcription factor that modulates a variety of cellular programs, including growth response and apoptosis (reviewed in Ref. 22). Furthermore, PKD1-induced AP-1 activity was mediated at least in part through activation of protein kinase C and c-Jun N-terminal kinase (JNK). Thus, we have shown that PKD1, a protein essential for normal tubulogenesis, activates a signaling cascade involved in cellular proliferation, differentiation, and apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Genistein (Calbiochem), staurosporine (Calbiochem), calphostin C (Calbiochem), BAPTA-acetoxymethyl ester (Molecular Probes), wortmannin (Calbiochem), and human epidermal growth factor (Clonetics) were used at concentrations as indicated. The C-terminal domain of PKD1 was expressed as a CD16-CD7 fusion protein. The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; JNK, c-Jun N-terminal kinase; HA, hemagglutinin; MAP, mitogen-activated protein; PKC, protein kinase C; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; AP-1, activation protein 1; NFκB, nuclear factor-κB; PAGE, polyacrylamide gel electrophoresis; BAPTA, 1,2-bis(ό-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; HNF, hepatic nuclear factor.
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protein (CD16.7.PKD1) (15). The control vector (CD16.7) encodes the extracellular domain of CD16 followed by the CD7 transmembrane domain and a short cytoplasmic tail. The AP-1 luciferase reporter construct of four AP-1-binding sites (kindly provided by D. Moore), the collagenase promoter reporter construct containing a single AP-1-binding site (kindly provided by N. H. Colburn) and the Jun2TRE construct of three 12-O-tetradecanoylphorbol-13-acetate-response elements (TRE) from the c-Jun promoter second TRE (kindly provided by S. Lewis and S. Hyman), the NFκB construct of eight NFκB-binding sites (kindly provided by B. Seed), and the c-myc construct of four c-myc-binding sites (derived from a construct kindly provided by R. N. Eisen) all contain DNA-binding sites followed by either a minimal thymidine kinase or Rouss sarcoma virus promoter directing the expression of luciferase. The c-Fos promoter construct (kindly provided by Bender GmbH) and the CAMP-response element promoter construct (kindly provided by K. Mayo) contain promoter regions of c-Fos and a-inhibin, respectively. The hemagglutinin (HA)-tagged p38 and p44 were kindly provided by J. Pouyssegur, HA-JNK1 was kindly provided by M. Karin, the dominant-negative mutants of Rac-1 (Rac-1(N17)) and Cdc42 (Cdc42(N17)) were kindly provided by J. S. Gutkind, a dominant-negative form of PKC α was kindly provided by D. Rosson, and a dominant-negative form of PKC βII m217 was kindly provided by C. E. Chalfant. The dominant-negative form of PKC δ was generated through site-directed mutagenesis replacing the lysine at 377 with arginine (23).

In Vitro Kinase Assays—293T cells were transiently transfected with a luciferase reporter construct, a FLAG-tagged PKD1, lacking both leader sequence and transmembrane domain, completely lost the ability to activate AP-1 (data not shown), suggesting that membrane localization of PKD1 is requisite for AP-1 activation. Likewise, a control protein of the extracellular domain of CD16 fused to the transmembrane domain of CD7 had no effect (Fig. 1). AP-1 activation induced by PKD1 exceeded activation by both epidermal growth factor and serum by 2-fold (data not shown). AP-1 is a transcriptional activator composed of Jun, Fos, or activating transcription factor 2 homodimers and heterodimers that bind to a common DNA sequence, the TRE. AP-1 is activated by a large variety of extracellular stimuli, including growth hormones and cytokines; its activity is controlled both at the transcriptional level and through post-translational modifications of c-Fos and c-Jun (reviewed in Ref. 22). Several kinase cascades have been demonstrated to regulate AP-1 activity (25), including the Hog1p homolog p38, the mitogen-activated protein-1 (MAPK), the Hog1p homolog p44, and members of the JNK family (reviewed in Ref. 26).

To further delineate the signaling pathway through which PKD1 may generate AP-1, we examined the activity of several kinase cascades in 293T cells expressing the C terminus of PKD1. HA-tagged p38, p44, and JNK1 were co-expressed with the C-terminal domain of PKD1 fused to CD16.7 (CD16.7.PKD1) or the control vector (CD16.7). After serum starvation for 16 h, HA-tagged kinases were immunoprecipitated, and the activity of the different kinases was determined using an in vitro kinase assay. Expression of the cytoplasmic domain of PKD1 increased JNK activity, but not that of p38 or p44 (Fig. 2). Activation of JNK was specific for PKD1 and was not detectable in 293T cells expressing full-length PKD2 (data not shown). These results together with our findings that PKD1 triggers the JunTRE, Western blot analysis with the c-Fos promoter, suggests that PKD1-induced AP-1 activation may be mediated through the formation of c-Jun and activating transcription factor 2 dimers. JNKs are typically activated by growth factors, cytokines, osmotic stress, or UV light, triggering c-Jun dimerization through phosphorylation at residues Ser-63 and Ser-73 (27, 28). Many of these conditions result in the concomitant Western Blot Analysis—293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, cells were lysed in sample buffer, fractionated on SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (NEB Life Science Products).

RESULTS AND DISCUSSION

Previous studies indicate that ADPKD epithelial cells arrest in a less than terminally differentiated state, leading to cystogenesis. To identify potential signaling pathways of PKD1, we coexpressed the cytoplasmic tail of PKD1 with luciferase reporter constructs transactivated by defined DNA-binding sites. The C-terminal 228 amino acids of PKD1 were fused to the extracellular domain of CD16 and the transmembrane domain of CD7. The combination of CD16 together with a CD7 transmembrane domain has previously been shown to target cytoplasmic domains to the plasma membrane without altering effector function (18–21) or protein-protein interaction, such as between PKD1 and PKD2 (15). The expression of CD16.7.PKD1, monitored by Western blot analysis, consistently triggered a 5–10-fold activation of the AP-1 reporter construct containing four AP-1-binding sites (Figs. 1, 3, and 5), and a more than 5-fold activation of a collagenase promoter construct containing a single AP-1 site (24) (Fig. 1). PKD1-mediated AP-1 activation was highly specific, involving only AP-1 and the Jun2TRE (Fig. 1). Cytoplastically expressed FLAG-tagged PKD1, lacking both leader sequence and transmembrane domain, completely lost the ability to activate AP-1 (data not shown), suggesting that membrane localization of PKD1 is requisite for AP-1 activation. Likewise, a control protein of the extracellular domain of CD16 fused to the transmembrane domain of CD7 had no effect (Fig. 1). AP-1 activation induced by PKD1 exceeded activation by both epidermal growth factor and serum by 2-fold (data not shown). AP-1 is a transcriptional activator composed of Jun, Fos, or activating transcription factor 2 homodimers and heterodimers that bind to a common DNA sequence, the TRE. AP-1 is activated by a large variety of extracellular stimuli, including growth hormones and cytokines; its activity is controlled both at the transcriptional level and through post-translational modifications of c-Fos and c-Jun (reviewed in Ref. 22). Several kinase cascades have been demonstrated to regulate AP-1 activity (25), including the Hog1p homolog p38, the mitogen-activated kinases p42 and p44, and members of the JNK family (reviewed in Ref. 26).

To further delineate the signaling pathway through which PKD1 may generate AP-1, we examined the activity of several kinase cascades in 293T cells expressing the C terminus of PKD1. HA-tagged p38, p44, and JNK1 were co-expressed with the C-terminal domain of PKD1 fused to CD16.7 (CD16.7.PKD1) or the control vector (CD16.7). After serum starvation for 16 h, HA-tagged kinases were immunoprecipitated, and the activity of the different kinases was determined using an in vitro kinase assay. Expression of the cytoplasmic domain of PKD1 increased JNK activity, but not that of p38 or p44 (Fig. 2). Activation of JNK was specific for PKD1 and was not detectable in 293T cells expressing full-length PKD2 (data not shown). These results together with our findings that PKD1 triggers the JunTRE, Western blot analysis with the c-Fos promoter, suggests that PKD1-induced AP-1 activation may be mediated through the formation of c-Jun and activating transcription factor 2 dimers. JNKs are typically activated by growth factors, cytokines, osmotic stress, or UV light, triggering c-Jun dimerization through phosphorylation at residues Ser-63 and Ser-73 (27, 28). Many of these conditions result in the concomitant Western Blot Analysis—293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, cells were lysed in sample buffer, fractionated on SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (NEB Life Science Products).
activation of p38 and JNK, although selective activation of JNK can occur. MEK kinases, specific mixed lineage kinases, transforming growth factor-β-activated kinase, tumor progression locus 2 (Tpl-2), and p21-activated kinases have all been demonstrated to activate the JNK pathway (reviewed in Ref. 29), whereas selective activation of JNK has been reported through the specific activation of MKK4 by germinal center kinase (30). It appears that protein-activated kinases, MEK kinases, mixed lineage kinase 3, and Raf are regulated upstream by low molecular weight GTP-binding proteins (25, 31–35). Recently, two members of this family, Rac-1 and Cdc42, have been demonstrated to activate JNK (33).

To determine the role of small GTP-binding proteins Rac-1 and Cdc42 in the PKD1-mediated activation of JNK, we co-expressed the C-terminal domain of PKD1 with the dominant-negative mutants Rac-1(N17) and Cdc42(N17) (33). Although wild-type Rac-1 and Cdc42 had no affect on the PKD1-mediated JNK activation (data not shown), both molecules nearly abrogated the PKD1-mediated JNK activation (Fig. 3A). A comparable inhibition of PKD1-mediated AP-1 activation by Rac-1(N17) and Cdc42(N17) was observed (Fig. 3B). Thus, Rho family members Rac-1 and Cdc42 appear to play a central role.
in the signaling pathway triggered by the C terminus of PKD1.

To define additional downstream components of PKD1-mediated signaling, we examined the ability of various pharmacological agents to disrupt PKD1-mediated AP-1 activation. Staurosporine, BAPTA, and calphostin C inhibited PKD1-induced AP-1 activation in a dose-dependent fashion (Fig. 4), whereas genistein and wortmannin had no effect (data not shown). Consistent with the profound effect of staurosporine and calphostin C, PKD1 caused an elevation of total protein kinase C activity; a comparable increase in PKC activity was obtained after stimulation of 293T cells with phorbol 12-myristate 13-acetate (1 μM) for 60 min (Fig. 5 A). Because PKD1-mediated PKC activation was inhibited by both staurosporine (200 nM) and BAPTA (10 μM), we speculated that PKD1-induced AP-1 activation is mediated by a calcium-dependent PKC isoenzyme. PKC is a family of proteins with at least 13 different members (36), but only the conventional PKCs, α, βI, and γ, are regulated by calcium. A dominant-negative form of the calcium-dependent PKCα (37) suppressed PKD1-mediated AP-1 activation, whereas equally well expressed dominant-negative forms of PKCβII and PKCδ had no effect (Fig. 5B). These findings suggest that PKCα may mediate PKD1 signaling. PKCα has been reported to activate MAP kinase and TRE/AP-1 in other signaling pathways (38–40), perhaps by dephosphorylating c-Jun at one or more of three critical serine/threonine residues that negatively regulate its DNA binding activity (41).

FIG. 3. Dominant-negative mutants of Cdc42 and Rac-1 block PKD1-mediated AP-1 and JNK activation in 293T cells. A, 293T cells were co-transfected with HA-tagged-JNK1, CD16.7.PKD1, or a vector control in combination with the dominant-negative mutants for Cdc42 (Cdc42(N17)) or Rac-1 (Rac-1(N17)) at equal ratios. The tagged kinase was immunoprecipitated with anti-HA antibody and incubated with GST-c-Jun (1–79) in the presence of [γ-32P]ATP. Incorporated radioactivity was visualized by 12% SDS-PAGE and autoradiography. The autoradiogram is representative of two independent experiments. B, Cdc42(N17) and Rac-1(N17) block PKD1-mediated AP-1 activation. 293T cells were co-transfected with CD16.7.PKD1, Cdc42(N17), Rac-1(N17), or a vector control at equal ratios. Transactivation of the AP-1 reporter construct was determined after 36 h of incubation and expressed as relative light units (RLU) after normalization for β-galactosidase activity. The values shown represent the means ± S.D. of six independent experiments. ***, p < 0.001; ###, significantly different from PKD1-transfected cells with p < 0.001).

FIG. 4. Staurosporine, calphostin C, and BAPTA inhibit PKD1-mediated AP-1 activation. 293T cells were transiently co-transfected with the C-terminal domain of PKD1 (CD16.7.PKD1) or a vector control (CD16.7) and exposed to increasing concentrations of staurosporine (S), BAPTA (B), and calphostin C (C) added for the last 8 h of the incubation period. Transactivation of the AP-1 reporter construct was determined after 36 h of incubation and expressed as relative light units (RLU) after normalization for β-galactosidase activity. Representative results of at least two experiments performed in triplicate (S and B) or duplicate (C) are expressed as means ± S.D. ***, p < 0.001; #, ##, and ###, significantly different from PKD1-transfected cells with p < 0.05, p < 0.01, and p < 0.001, respectively).
PKD1-mediated AP-1 Activation

PKD1, a protein kinase C (PKC) family member, has been implicated in the regulation of cellular proliferation and differentiation. PKD1 activity is regulated by various stimuli, including growth factors and cytokines. In this study, we examined the effect of PKD1 on AP-1 activation in 293T cells.

Methods:

1. 293T cells were transfected with the C-terminal domain of PKD1 (CD16.7) or a vector control (CD16.7).
2. Cells were treated with various stimuli, including phorbol 12-myristate 13-acetate (PMA), staurosporine, and BAPTA.
3. AP-1 activation was determined by measuring the transactivation of an AP-1 reporter gene.
4. PKC activity was determined in 293T cells transiently co-transfected with the C-terminal domain of PKD1 (CD16.7) and PKC isoforms.

Results:

- PKD1 activates AP-1 in a dose-dependent manner.
- PKD1-mediated AP-1 activation is inhibited by PKC inhibitors.
- PKD1 activates certain PKC isoforms, including PKCα and PKCβ.

Conclusion:

PKD1 activates AP-1 in 293T cells and this activation is mediated by PKC isoforms. The mechanism of PKD1-PKC interaction and the role of PKD1 in cellular proliferation and differentiation need further investigation.

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