The Polycystic Kidney Disease-1 Promoter Is a Target of the β-Catenin/T-cell Factor Pathway*

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Autosomal dominant polycystic kidney disease (ADPKD) is a very common inherited disease worldwide, having a gene frequency of 1 in 200–1,000 individuals and causing 6–9% of all end-stage renal disease (1, 2). Mutations in the PKD1 gene are responsible for the vast majority (85–90%) of ADPKD cases (3, 4). PKD is characterized by the neoplastic growth of tubular epithelial cells, which gives rise to the formation of kidneys containing numerous fluid-filled cysts and ultimately to renal failure.

In situ and Northern hybridization and immunocytochemical analyses have shown that the PKD1 gene is widely expressed in a number of embryonic and adult tissues and organs, including the kidney (5–10). Targeted deletion of the PKD1 gene has shown that it is important for renal, pancreatic, cardiovascular, and skeletal development (9, 11–14). However, there are conflicting observations as to whether PKD results from abnormally decreased or increased expression of the PKD1 gene (15). On the one hand, there is evidence that loss-of-function germ line mutations can lead to haploinsufficiency at the PKD1 locus (16, 17) and additional evidence for a two-hit gene inactivation mechanism as a cause of PKD (18–20). On the other hand, there is immunocytochemical evidence for overexpression of the protein product of the PKD1 gene, polycystin-1, in cyst-lining epithelial cells of polycystic kidneys (15). Furthermore, transgenic overexpression of a functional PKD1 gene can cause PKD (21). At the present time, there is no information concerning the mechanisms regulating transcription of the PKD1 gene.

β-catenin is a major component of adherens junctions, linking the actin cytoskeleton to members of the cadherin family of transmembrane cell-cell adhesion receptors (22–24). Together with α- and γ-catenin, β-catenin binds to the cytosolic domain of E-cadherin to form a complex that is necessary for adhesion to occur. In addition, soluble β-catenin can translocate to the nucleus, where it associates with members of the TCF/LEF family of high mobility group (HMG)-box factors to alter the expression of certain target genes (25). By playing a dual role, a structural role at cell-cell junctions and a regulatory role in the nucleus, β-catenin can transduce changes in cell adhesion and junction formation to control transmembrane signaling and gene expression. The level of soluble β-catenin in the cell is regulated by its association with the tumor suppressor molecule adenomatous polyposis coli (APC), axin, and glycogen synthase kinase-3β (GSK-3β). Phosphorylation of β-catenin by the APC-axin-GSK-3β complex leads to its degradation by the ubiquitin-proteasome system. The failure of this degradation in cells expressing mutated β-catenin leads to the accumulation of soluble β-catenin that can result in activation of oncogenic β-catenin-responsive genes (23–25).

A number of observations have implicated β-catenin in the pathogenesis of PKD. Transgenic overexpression of either β-catenin or c-Myc, which is a downstream target of β-catenin, has been shown to give rise to PKD (26, 27). Also, high levels of expression of c-Myc mRNA and protein are found in the cysts of
both human ADPKD (28) and the cpk mouse model of PKD (29–31), and the half-life of soluble β-catenin is increased in cell lines from cpk mice (32). Transient overexpression of the C-terminal tail of polyclin-1 has been shown to stabilize β-catenin and to activate transcription of the β-catenin target gene, siamois (33). Finally, polyclin-1 has been shown to be in a complex with E-cadherin and α- and β-catenin in normal cells (34), and E-cadherin has been shown to be mis-targeted in ADPKD cells (35), possibly because of abnormal polyclin-1 function. In this study, we have investigated the transcriptional regulation of the PKD1 promoter and have determined that the PKD1 gene is a target for the β-catenin/Tcf complex.

MATERIALS AND METHODS

DNA Clones—The cosmid clone 2H2 (LANL) (GenBank accession no. AC005346) was used to amplify a 3,414-bp sequence (29,490–32,904 of AC005346 or 77–3,490 of L39891) from −3,965 to +51, relative to the established transcription start site of the PKD1 gene (36). The DNA product was cloned in pGEM-T (Promega). A KpnI site was introduced near the 5′-end of the 3.3-kb fragment during the initial PCR reaction, and a HindIII site was introduced near the 3′-end. The fragment resulting from KpnI/HindIII digestion (-3,346 to +33; Fig. 1, 1.3 kb) was subcloned into pGEM-T. The promoterless luciferase vector (BCA-AS promoter) was a gift from O. Tetsu and F. McCormick. A Myc-tagged dominant-negative (DN) TCF-4E pcDNA3 lacking the O. Tetsu and F. McCormick. A Myc-tagged dominant-negative (DN) TCF-4E pcDNA3 lacking the β-catenin-binding domain (amino acids 2–53) also was from O. Tetsu and F. McCormick. pGST-TCF4 (DNA-binding domain) was a gift from K. Kinzler and B. Vogelstein.

Cell Lines, Transfection, Immunofluorescence, and Reporter Assays—Human embryonic kidney 293T (HEK293T), human colon carcinoma HCT-116, and human fibroblasts from newborn TNI080 cells were obtained from the American Type Culture Collection. COS-1 and HeLa cells were obtained from R. Pestell, Albert Einstein College of Medicine. Human fibroblasts (HEK293T) and a long 5′-flanking region; however, a GC-rich region was identified in the 2.0-kb construct containing the QuikChange site-directed mutagenesis kit (Stratagene). All constructs, mutations, and deletions were checked by sequencing. A Myc epitope-tagged wild-type human β-catenin pcDNA3 expression vector was a gift from P. Polakis. A Myc-tagged mutant (lacking amino acids 29–48), constitutively active (CA) β-catenin pcDNA3, and a cyclin D1 reporter construct (originally from R. Pestell, Albert Einstein College of Medicine) were gifts from O. Tetsu and F. McCormick. A Myc-tagged dominant-negative (DN) TCF-4E pcDNA3 lacking the β-catenin-binding domain (amino acids 2–53) also was from O. Tetsu and F. McCormick. pGST-TCF4 (DNA-binding domain) was a gift from K. Kinzler and B. Vogelstein.

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RESULTS

The PKD1 gene lies in a complex region of DNA on chromosome 16 (36). More than two-thirds of the 5′-end of the gene is duplicated several times in an area proximal to the PKD1 gene on chromosome 16. This PKD1 homologous region appears to give rise to multiple transcripts (39). A 38,849-bp cosmid clone (LANL) 2H2 has in its 3′-end, the 5′-portion of the PKD1 gene and a long 5′-upstream region. Based on homology with available PKD1 sequence and differences with PKD1 homologous regions, we can conclude that this cosmid clone contains the genuine PKD1 gene. This cosmid DNA was used to amplify a 3,414-bp sequence from −3,363 to +51, which was analyzed for putative cis-acting elements utilizing the OMIGA sequence analysis software (Oxford Molecular) and TESS (www.eblen. upenn.edu/teess/index.html).

Typical TATA- and CAAT-boxes are absent from this proximal 5′-flanking region; however, a GC-rich region was identified within close proximity to the transcription start site. Numerous transcription factor consensus binding motifs were found in the PKD1 promoter, including AP-1, AP-2, Sp1, Ets, and four TCF binding elements (CTTTGGA/CACT). To test for promoter activity, a number of deletion constructs were made, and their activities were determined following transfection into human (HEK293T) and monkey (COS-1) kidney cell lines (Fig. 1). The constructs appeared to have similar promoter activities in the two cell lines. The 2.0-kb construct containing the −2,018 to +33 region supported the highest level of transcription in both cell lines, suggesting that there may be a negative element in the distal 1.3-kb region.

To determine whether β-catenin is potentially capable of regulating the transcription of the PKD1 gene, we transfected PKD1 promoter-luciferase reporters into HEK293T cells and measured their responses to cotransfected β-catenin expression vectors encoding wild-type or mutant CA β-catenin. HEK293T
cells had been shown previously to express TCF-4 and to support β-catenin-dependent activation of the cyclin D1 promoter (40). β-catenin induction of the 3.3-kb construct (data not shown) was less significant when compared with that of the 2.0-kb fragment. Therefore, we concentrated our studies on the 2.0-kb fragment, which contains the most proximal TCF site (TBE1). The transcriptional activity of the 2.0-kb fragment showed a dose-dependent response to increasing amounts of cotransfected wild-type β-catenin (Fig. 2A). The response of the 2.0-kb fragment to a CA β-catenin (Fig. 2B, left panel) was similar to that of the β-catenin target, cyclin D1 (right panel) under the same transfection and assay conditions. To further confirm the role of β-catenin/TCF-4 in the activation of the PKD1 promoter, we attempted to inhibit its activity in HEK293T cells with a dominant-negative TCF-4 mutant (DN TCF). As shown in Fig. 2C, cotransfected DN TCF suppressed the activity of the 2.0-kb promoter fragment in the presence or absence of exogenous β-catenin.

To determine whether the response of the 2.0-kb construct to induction by β-catenin depends specifically on the TBE1 site, we tested the activities of several deletions in the TBE1 region. An extended deletion of 109-bp at the 5′-end of the 2.0-kb fragment (gatctctggc acattttatt tgctctgtct caccacatgg attttgtttt ttgtttCTT TGTTtttt ga gatggagtct cactcttgtt gcccaggctg gagtgccat) completely abrogated β-catenin induction (Fig. 3A). Deletion of 15 bp (underlined) containing the TCF consensus motif (capital letters) did not abolish induction (data not shown), possibly because there are other AT-rich sequences in this region that could substitute as TCF binding sites. A 28-bp deletion (bold face) removing this AT-rich DNA and the TCF motif strongly reduced the effect of transfected β-catenin (Fig. 3A). To further examine the 109-bp sequence, its functional response was tested when cloned upstream of a minimal promoter. As shown, the 109-bp sequence was capable of being induced by β-catenin (Fig. 3B). These results, taken together, suggest that there might be interactions between β-catenin and/or TCF with other transcription factors within this region that are involved in conferring the observed β-catenin responsiveness.

To determine whether the TBE1 sequence is capable of binding TCF, we carried out electrophoretic mobility shift assays (EMSAs) using a synthetic double-stranded DNA, gtttCTTT-CTTGTTtttt, containing the consensus TCF binding motif (capital letters). The oligo was able to form a DNA-protein complex with a GST-fusion protein containing the DNA-binding domain of human TCF-4 (Fig. 4A, GST-TCF). As a control, a DNA-protein complex was formed with GST-TCF and an optimal TCF-4 site.
TOP (T), but failed to form with the mutated binding site, FOP (F). The TBE1 complex with GST-TCF could be competed with an excess of unlabeled TBE1. The 15-bp TBE1 oligo also formed DNA-protein complexes with nuclear extract from HEK293T cells (Fig. 4B, arrows), suggesting that the PKD1 TBE1 site can bind native TCF and β-catenin. The TBE1 complexes were completely eliminated by competition with excess unlabeled TBE1 and partially eliminated by competition with excess unlabeled TOP (the asterisk indicates a remaining DNA-protein complex). EMSA using the TOP oligo also resulted in the formation of two DNA-protein complexes, which were completely eliminated by competition with excess unlabeled TOP and from HEK293T cells transfected with mutated constitutively active β-catenin (293T). Mutation of this 15-bp oligo (gcgcCTTTGTTgggg), leaving only 5 bp of the core of the TCF motif (capital letters), abrogated the binding both with GST-TCF and with the HCT and 293T nuclear extracts (Fig. 4C).
Although we were unable to supershift the wild-type 15-bp oligo using an anti-β-catenin antibody, possibly because of other protein factors preventing an interaction with the antibody, we did supershift a probe containing the TBE1 core site (cgggCTTTGTTgggg) and nuclear extract from HCT cells (Fig. 4C, right panel). As expected, no such complex was formed using an anti-c-Myc antibody. The functional response of this 15-bp TBE1 TCF site was also tested in isolation. A single copy of the wild-type 15-bp TBE1 sequence in pGL3-Basic was able to confer β-catenin responsiveness in a dose-dependent manner, which was strongly inhibited by cotransfection with DN TCF. B, mutation of the core TCF motif in the 15-bp TBE1 sequence (gg) strongly reduced the responsiveness to CA β-catenin.

To determine whether expression of the endogenous PKD1 gene responds to β-catenin, human fibrosarcoma HT1080 cells were treated with 20 mM LiCl, a known inhibitor of GSK-3β activity that results in the stabilization of cytosolic β-catenin and elevation of nuclear β-catenin. PKD1 gene expression was assayed by RT-PCR and compared with the expression of a control that should not respond to β-catenin, the ribosomal protein L7 gene. As shown in Fig. 6A, PKD1 mRNA levels increased 3.3-fold in response to LiCl, whereas L7 mRNA did not increase. We also stably transfected HeLa cells with CA β-catenin and determined the effect of overexpression of this mutant form of β-catenin, which cannot be targeted for degradation. β-catenin levels were shown to be increased in these transfected cells by RT-PCR (Fig. 6B) and by immunofluorescence (Fig. 6C). Cells transfected with β-catenin showed ~3-fold increase in PKD1 mRNA levels, whereas L7 mRNA levels were unaffected (Fig. 6B). These results are consistent with the endogenous PKD1 gene being a target of the β-catenin pathway.

**DISCUSSION**

The 5′-flanking 3.3-kb region of the human PKD1 gene was isolated and analyzed for promoter activity. Functional analysis of deletion constructs fused to a luciferase reporter in...
HEK293T and COS-1 cells revealed strong transcriptional activity within this 3.3-kb region, with the highest activity localized to the 2.0-kb proximal fragment. Analysis of the 5′-flanking region by TESS predicted a cluster of Sp1 binding sites just upstream of the transcription start site. Sp1 plays a key role in regulating the transcription initiation of TATA-less promoters (42) and is temporally and spatially regulated during nephrogenesis in a pattern that suggests that it may be important in kidney development (43).

Sequence analysis of the PKD1 promoter also revealed four core TCF/LEF binding element (TBE1–4) consensus sequences. We focused on the most proximal element, TBE1, which is the only TCF site contained within the 2.0-kb proximal fragment. The 2.0-kb fragment showed a dose-responsive induction by β-catenin, which was inhibited by cotransfection with a dominant-negative TCF construct. Deletion of a 109-bp sequence containing the TBE1 site completely abolished the β-catenin responsiveness of the 2.0-kb fragment. In addition, both this 109-bp fragment and a 15-bp fragment within it containing the TBE1 site were able to confer β-catenin responsiveness when placed upstream of a minimal promoter. The response of the 15-bp TBE1 site was reduced or eliminated by mutation of the core consensus TCF motif or by dominant-negative TCF. Furthermore, the wild-type 15-bp sequence, but not the mutated 15-bp sequence, was able to form DNA-protein complexes using nuclear extracts from cells known to contain β-catenin/TCF and with a GST-TCF fusion protein. We conclude from these experiments that the TBE1 site in the PKD1 promoter is a β-catenin/TCF response element. Although we did not focus on the three upstream TBE elements (TBE2–4), it is quite possible that they are also β-catenin/TCF response elements because we have shown that a 1.3-kb fragment containing all three (but lacking TBE1) is also induced by β-catenin (data not shown). Further analysis will be required to determine which (one or whether all three) has the potential to act as a TCF response element. Finally, to determine whether expression of the endogenous PKD1 gene is able to respond to increases in β-catenin, we treated human fibrosarcoma HT1080 cells with LiCl, which is a known inhibitor of GSK-3β kinase. The β-catenin levels were regulated by other mechanisms such as the Wnt/β-catenin pathway (42) and is temporally and spatially regulated during nephrogenesis and may be relevant to PKD. β-catenin has been shown to regulate the reorganization of renal epithelial cell aggregates into long tubules (48) and therefore may be important in normal tubulogenesis. Increased stability of β-catenin was found in immortalized cpk cells (a mouse model of recessive PKD) (32) and in primary ADPKD cells. Transgenic mice overexpressing either β-catenin (26) or c-Myc (a target of β-catenin) (27) have PKD, and high levels of expression of c-Myc mRNA and protein are found in the cysts of both human ADPKD (28) and of the cpk mouse model of PKD (29–31). Furthermore, Wnt-4 expression was recently reported to be induced in poly cystic kidneys (50). Together, these results suggest that increased levels of soluble β-catenin may cause overexpression of the normal (and/or mutated) PKD1 gene, which in turn may contribute to cyst formation in PKD.

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