Retinoic acid-dependent activation of the polycystic kidney disease-1 (PKD1) promoter

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Islam MR, Puri S, Rodova M, Magenheimer BS, Maser RL, Calvet JP. Retinoic acid-dependent activation of the polycystic kidney disease-1 (PKD1) promoter. Am J Physiol Renal Physiol 295: F1845–F1854, 2008. First published October 15, 2008; doi:10.1152/ajprenal.90355.2008.—The retinoic acids all-trans retinoic acid (AT-RA) and 9-cis retinoic acid (9C-RA) and the retinoic acid receptors RAR and RXR significantly induce transcriptional activity from a 200-bp PKD1 proximal promoter in transfected mammalian cells. This PKD1 promoter region contains Ets, p53, and GC box motifs, but lacks a canonical RAR/RXR motif. Mutagenesis of the Ets sites did not affect RA induction. In contrast, GC box mutations completely blocked stimulation by AT-RA and by RXRβ or RXRB. Mithramycin A, which prevents Sp1 binding, significantly reduced basal promoter activity and suppressed upregulation by AT-RA and RXR. The 200-bp proximal promoter could not be induced by AT-RA in Drosophila SL2 cells, which lack Sp1, but could be activated in these cells transfected with exogenous Sp1. Small interfering RNA knockdown of Sp1 in mammalian cells completely blocked RXRβ upregulation of the promoter. These data indicate that induction of the PKD1 promoter by retinoic acid is mediated through Sp1 elements. RT-PCR showed that AT-RA treatment of HEK293T cells increased the levels of endogenous PKD1 RNA, and chromatin immunoprecipitation showed the presence of both RXR and Sp1 at the PKD1 proximal promoter. These results suggest that retinoids and their receptors may play a role in PKD1 gene regulation.

all-trans retinoic acid; 9-cis retinoic acid; RAR; RXR

Autosomal dominant polycystic kidney disease (ADPKD) is a human genetic disorder with a frequency of 1 in 200–1,000 individuals and accounts for ∼10% of end-stage renal disease (2, 6, 22, 29, 47, 53, 58, 65). Approximately 85% of all ADPKD cases are due to mutations in the PKD1 gene, whose gene product is a large, membrane-spanning protein, polycystin-1. The remaining cases are due to mutations in the PKD2 gene. All ADPKD patients develop bilateral cystic kidneys. They can also have a variety of other extrarenal manifestations, including liver cysts, coronary artery disease, cardiac hypertrophy, hypertension, and cerebral aneurysms (17, 18, 20, 26, 48, 61).

While a two-hit loss-of-function mechanism has been hypothesized as the genetic cause of cyst initiation (5, 51, 52), there are conflicting observations as to whether cysts always result from abnormally decreased expression of the PKD genes and whether loss of both alleles is always required. On the one hand, Pkd1(−/−) mice usually die in utero with massively cystic kidneys and cardiovascular abnormalities (4, 32, 38, 39, 44). On the other hand, there is evidence that haploinsufficiency at the Pkd1 locus can cause disease (36); there is immunocytochemical evidence for overexpression of polycystin-1 in cystic kidneys (46), and transgenic mice overexpressing a functional human PKD1 gene develop renal cysts (49, 60). Thus it is possible that ADPKD may be caused either by abnormally decreased (1- or 2-hits) or abnormally increased expression of the PKD1 gene.

Polycystin-1 appears to be developmentally regulated, with high expression during early embryogenesis in many tissues and organs, which decreases postnatally and is maintained at low levels in adults (9). Furthermore, the levels of PKD1 gene expression and polycystin-1 protein appear to govern in vitro cystogenesis (45). Thus any abnormality in the tight regulation of polycystin-1 protein levels may have important implications for normal physiological function and for cyst formation. Taken together, these observations signify the need to understand the transcriptional mechanisms regulating polycystin-1 levels. Earlier, our laboratory showed that the human PKD1 gene can be upregulated by β-catenin via a TCF/LEF consensus motif present within the PKD1 promoter (54). A number of other studies have also implicated β-catenin in the pathogenesis of PKD (28, 31, 62). We have also shown that the PKD1 proximal promoter contains two Ets binding elements, which are able to respond to the Ets factors Ets-1 and Fli-1 (50). It has also been recently demonstrated that p53 is a transcriptional repressor of PKD1, acting at the proximal promoter region (63). To further understand the mechanisms regulating PKD1 transcription, we have now looked for additional elements that could potentially regulate the expression of the PKD1 gene.

The retinoids, including all-trans retinoic acid (AT-RA) and 9-cis retinoic acid (9C-RA), are biologically active derivatives of vitamin A (13, 41). They have profound effects on the regulation of cell growth, differentiation, and apoptosis during embryonic development and within epithelial tissues in later life (8, 40, 42). The retinoids exert their effects through isoforms of RAR and RXR, the retinoic acid receptors, which are members of the nuclear hormone receptor superfamily. The RAR/RXR isoforms are ligand-dependent transcription factors that bind to DNA sequences, called RAR-elements (RAREs) or RXR-elements (RXREs), present in the promoter regions of target genes, including the RAR and RXR genes themselves.

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Upon ligand binding, the RARs/RXRαs upregulate transcription of target genes through a variety of coactivators, such as steroid receptor coactivator-1 and p300/CPB [cAMP-response element binding protein (CREB)-binding protein] (10, 21, 24, 27, 66). In genes without canonical RA response elements, the RARs/RXRαs have been shown to bind to and modulate activity of the transcription factor Sp1, resulting in an Sp1-mediated response to RA signaling (56, 59). Sp1 binds to a hexanucleotide sequence GGGCGG, called the “GC box” motif, or to variations of this sequence in which one or two nucleotides are substituted. Sp1 then activates transcription of GC box-containing genes by recruiting RNA polymerase II (19, 35). Disruption of one RXR isoform, RXRα, containing genes by recruiting RNA polymerase II (19, 35). Variations of this sequence in which one or two nucleotides are substituted. Sp1 then activates transcription of GC box-containing genes by recruiting RNA polymerase II (19, 35).

In the present study, we have found that AT-RA, 9C-RA, and the RA receptors RAR and RXR activate the PKD1 promoter through a 200-bp proximal promoter region. We determined that there is an absence of canonical RAR or RXR elements in the 200-bp PKD1 proximal promoter, but that promoter activity is mediated through the ubiquitous transcription factor Sp1 and its binding sites in the proximal PKD1 promoter.

**MATERIALS AND METHODS**

Plasmid constructs. A 3.3-kb human PKD1 promoter fragment (−3,346 to +33) subcloned in the promoterless luciferase reporter vector pGL2-Basic (Promega) was originally generated in our laboratory (54). Promoter deletion constructs (1.3 kb, 2.0 kb, and 200 bp) cloned in pGL2-Basic and/or pGL3-Basic were described previously (50, 54). The mammalian expression construct for Sp1 was from Dr. Yu-Chung Yang (Dept. of Pharmacology and Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, OH). The constructs for RAR and RXR were from Dr. Ronald M. Evans, (Howard Hughes Medical Institute, the Salk Institute). The Sp1 pPac clone, for expression in Drosophila SL2 cells, was from Dr. Yu-Chung Yang.

**Mutagenesis.** Two potential Ets binding sites (Ets-A and Ets-B) (50) and four of the six Sp1 sites (Sp1-A through Sp1-F) in the 200-bp proximal promoter region were mutated using the QuikChange site-directed mutagenesis kit (Stratagene) and the following double-stranded primers: Ets-A, 5′-GTC GGG CGG AGC Tct CGG AGG CCC CGC C-3′; Ets-B, 5′-GGA GGG TGA AGC CTC gtG ATG CCA GTC CAT CG-3′; Sp1-A, 5′-CCG GTG Gtt CGG AGC TTC CG-3′; Sp1-B, 5′-GAG GCC aaG CCC TGC TGC CG-3′; Sp1-C, 5′-G CGA AGG Gtt CGG AGC ATG CTC-3′; and Sp1-F, 5′-CC GTC taC CCC CGG CGG Cgc-3′. The lowercase nucleotides represent the mutations. The 200-bp mutant fragments were subcloned into pGL2 and/or pGL3 and verified by sequencing.

Cells, transfections, and reporter assays. HEK293T cells were cultured in DMEM with 4.5 g/l glucose, supplemented with 10% fetal bovine serum at 37°C. HCT116 cells were transfected using the Lipofectamine 2000 (Invitrogen) method according to the manufacturer’s instructions. Vector DNA or RXRβ expression plasmid (0.4 μg DNA) and the PKD1 promoter-luciferase reporter with or without siRNA duplex (80 pmol) against human Sp1 (SC-29487, Santa Cruz Biotechnology) were mixed with DMEM (total volume 150 μl/well) without serum and antibiotics (solution 1). Lipofectamine 2000 (6 μl/well) was diluted in DMEM (150 μl/well) without serum and antibiotics (solution 2). Solutions 1 and 2 were mixed and incubated for 25 min at room temperature. The mixed solution was added to and the cells were harvested overnight at 37°C. Following incubation, the medium was replaced with fresh DMEM containing 5% serum and antibiotics, and the cells were harvested at 40 h.

SDS-PAGE and western blotting. Expression of Sp1 in AT-RA-treated HEK293T cells was confirmed by SDS-PAGE and immunoblotting (1% formaldehyde in PBS at room temperature. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 0.2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 0.2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min.

Chromatin immunoprecipitation. HEK293T cells were cross-linked for 10 min in 1% formaldehyde in PBS at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. Cells were washed with ice-cold PBS, harvested by centrifugation for 2 min at 5,000 rpm, resuspended in 0.5 ml of chromatin immunoprecipitation (ChIP) lysis buffer (10 mM Tris-·HCl, pH 8.0, containing 10 mM NaCl, 0.5% NP-40, 1 mM EDTA, and protease inhibitor cocktail), and incubated on ice for 15 min. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 0.2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 0.2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min. The crude nuclear extract collected by centrifugation at 2,500 rpm for 5 min was washed once with 0.2 ml of PBS, resuspended in 500 μl of 1× IP buffer (0.5% Triton X-100, 0.5% NP-40, 0.1% SDS in PBS, and protease inhibitor cocktail), and incubated on ice for 15 min.

The fragmented chromatin was incubated with 5 μl of rabbit preimmune sera (R9133, Sigma), rabbit anti-RXR (D-20, Santa Cruz Biotechnology), or rabbit anti-Sp1 (PEP2, Santa Cruz Biotechnology). Following washing 3× with wash buffer (50 mM Tris-·HCl, pH 8.0, containing 0.15 M NaCl, 0.5% Triton X-100, 0.5% NP-40, 0.1% SDS, 1 mM EDTA), the cross-links were reversed with elution buffer (1% SDS, 0.1 M NaHCO3, 0.4 M NaCl) for 3 h at 67°C. The DNA in the supernatant was purified by using a High Pure PCR Product Purifi-
cation Kit (Boehringer Mannheim) and amplified for 35 cycles of 94°C for 45 s, 62°C for 45 s, 72°C for 45 s, with a final extension of 7 min at 72°C using the forward primer 5'-CTG CTG CCG ACC CTG TGG AG-3' and the reverse primer 5'-GCC GCC GCC GCC GGC GGA CGG-3' (135-bp product). The products were confirmed by sequencing.

**Endogenous PKD1 expression.** HEK293T cells were treated with ethanol or AT-RA in DMEM containing 2% serum for 40 h. Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer’s protocol, and the samples were treated with DNase I (Ambion). RT-PCR was carried out with 1 μg RNA in a total volume of 25 μl using random primers and Superscript reverse transcriptase (Invitrogen) as described previously (54). Primers specific for PKD1 were forward, 5'-CCG TTC ACT AGC TTC GAC-3' and reverse, 5'-AGC CTC CAG AGG GAG TCC AC-3', giving a 260-bp product; primers specific for the ribosomal protein L7 were forward, 5'-GCT TCG AAA GGC AAG GAG GAA GC-3' and reverse, 5'-GCG GCG GCG CGG GGC TGG AG-3' and reverse, 5'-GCT TCG AAA GGC AAG GAG GAA GC-3' and reverse, 5'-TCC TTC ATG CAG ATG ATG C3-3', giving a 440-bp product. Amplified PCR fragments were electrophoresed on 2% agarose gels containing ethidium bromide, and the bands were analyzed by National Institutes of Health Image software. Quantified band intensity was normalized to values for ribosomal protein L7 mRNA and plotted as relative units. The data were plotted with Microsoft Excel as the average ± SD of three independent experiments.

**RESULTS**

The PKD1 promoter is activated by retinoic acids and their receptors. The 3.3-kb PKD1 promoter and its two proximal fragments, 2.0 kb and 200 bp, were found to have high basal promoter activity in transfected HEK293T cells and to be significantly induced by AT-RA treatment (Fig. 1, A and B). In contrast, the distal 1.3-kb fragment was found to have lower basal activity and to be less responsive to AT-RA. Our previous studies showed that the 200-bp proximal fragment has high promoter activity, suggesting that it contains functionally important transcriptional elements (54). Indeed, it has been recently shown that the 200-bp proximal fragment has functionally responsive Ets and p53 sites (50, 63). The relatively strong response to AT-RA of the 200-bp proximal promoter fragment suggests that the primary retinoic acid response is mediated by elements within the first 200 bp. Deletion analysis (data not shown) of the 2.0-kb promoter confirmed that the 200-bp proximal fragment is responsible for AT-RA induction. This AT-RA induction varied from 3-fold to up to >10-fold, depending on the experiment.

The 200-bp PKD1 promoter fragment responded to both 9C-RA and AT-RA in a dose-dependent fashion (Fig. 2). This PKD1 promoter fragment also responded to AT-RA in monkey kidney COS-1 cells and human colon carcinoma HCT116 cells (Fig. 3), indicating that the response is not specific to HEK293T cells. It was also possible to stimulate the 200-bp promoter-reporter ~2.5–6-fold by transfection of retinoic acid receptors alone, with RARβ showing the highest activity (Fig. 4). The only exception was RARγ, which elicited no increase over basal promoter activity. These data suggest that sequences in the 200-bp PKD1 proximal promoter can respond to vitamin A metabolites and their nuclear receptors.

The 200-bp PKD1 proximal promoter lacks a canonical RAR/RXR response element. Sequence analysis revealed that the 200-bp proximal promoter region does not have an RAR/RXR response element (Fig. 5). We recently showed that this proximal promoter sequence does have two consensus Ets elements (EREs), Ets-A and Ets-B, and there are two consensuss p53 elements (Fig. 5) (50, 63). As there are reports that Ets factors can mediate a retinoic acid response and can modulate transcription of RA target genes (30), we thought that the EREs might be involved in RA-mediated transcriptional upregulation of the PKD1 promoter. To test this idea, site-directed mutagenesis was used to selectively disrupt the core EREs at each Ets site. Mutations were introduced either at Ets-A or Ets-B, and at both Ets-A and Ets-B (Fig. 5). The effects of these mutations were then determined in HEK293T cells following RA treatment. None of these ERE mutants, however, was able to suppress the transcriptional stimulation by AT-RA (data not shown).

Sp1 elements mediate the RA response. The 200-bp sequence was also found to contain six canonical “GC box” (GGGCGG) motifs (Sp1-A to Sp1-F). Sp1 binds the canonical GC box motif with high affinity. The PKD1 promoter lacks TATA and CAAT boxes, and thus its basal transcription, like numerous other TATA-less promoters, may depend on Sp1. In genes without canonical RA response elements, the RARs/
RXRs, have been shown to modulate the activity of Sp1, resulting in an Sp1-mediated response to RA signaling (56, 59). Thus we thought that RA might activate the PKD1 promoter through one or more of these Sp1 sites. Mutations were introduced into Sp1-A, Sp1-B, Sp1-C, and Sp1-F (Fig. 5). A double mutant at Sp1-B and Sp1-C (Sp1-BC) and a triple mutant at Sp1-B, Sp1-C, and Sp1-F (Sp1-BCF) were also generated. As shown in Fig. 6, mutations at either Sp1-A or Sp1-B alone did not cause a decrease in basal or RA-induced promoter activity. While the Sp1-C single mutation seemed to have little or no effect on basal or induced promoter activity, the Sp1-BC double mutation had significantly decreased basal activity and a reduced AT-RA responsiveness compared with wild-type (Fig. 6, bottom). The Sp1-F single mutation also had significantly decreased basal activity and a reduced AT-RA responsiveness. All of the single or double mutants could also be induced by cotransfected RXRα (data not shown). In contrast to the above results, the triple mutant (Sp1-BCF), showed an almost complete loss of both basal promoter activity and induced promoter activity in response to either AT-RA or 9C-RA (Fig. 7A) or to the nuclear receptors RXRβ or RARβ (Fig. 7B). This result suggested that the RA response is mediated primarily through a combination of Sp1 sites that includes but is not restricted to Sp1-F. As mutations at site Sp1-B alone had no effect on basal and AT-RA- or RARβ-stimulated activities (Fig. 6 and data not shown), and mutations at sites Sp1-C or Sp1-F alone caused some reductions in

Fig. 2. The 200-bp proximal PKD1 promoter is stimulated by retinoic acid in a dose-dependent manner. HEK293T cells were transfected with 0.5 μg of the 200-bp PKD1 promoter-luciferase reporter construct in pGL3. The transfected cells were incubated with either ethanol (−) or the indicated concentrations of 9-cis retinoic acid (9C-RA) or AT-RA for 6 h. Luciferase activities were normalized to β-galactosidase activities. The average RLU value obtained for the ethanol-treated (−) cells was set at 1.0. Each bar is the mean ± SD of a representative experiment done in triplicate. The 200-bp construct was induced by both 9C-RA and AT-RA (**P < 0.05, *P < 0.01, ***P < 0.001).

Fig. 3. The proximal PKD1 promoter is activated by retinoic acid in COS-1 and HCT116 cells. COS-1 cells were transfected using Lipofectamine, with 1.0 μg of the 200-bp PKD1 promoter-luciferase reporter construct in pGL3. The cells were incubated for 2 h in serum-free/antibiotic-free growth medium containing DNA-Lipofectamine and then were placed in growth medium containing 2% serum (no antibiotics), with ethanol, or with 5 μM AT-RA, and were harvested after an additional 38 h. HCT116 cells were transfected using the calcium phosphate method and were treated with ethanol or with 5 μM AT-RA. Luciferase values were normalized to β-galactosidase activities. The average RLU value obtained for the ethanol-treated cells was set at 1.0. Each bar is the mean ± SD of a representative experiment done in triplicate. The 200-bp construct was induced in both COS-1 and HCT116 cells (**P < 0.01).

Fig. 4. Retinoic acid receptors can induce the 200-bp proximal PKD1 promoter. HEK293T cells were transfected with 0.2 μg of pGL3 or the 200-bp promoter-luciferase reporter construct together with 0.2 μg of pcDNA3 and retinoic acid receptors RXRβ, RARα, RARβ, or RARγ. The transfected cells were then incubated with growth medium containing 2% serum for 40 h. Luciferase values were normalized to protein concentration. The average RLU value obtained for the control (pGL3 + pcDNA3) transfected cells was set at 1.0. Each bar is the mean ± SD of a representative experiment done in triplicate. The 200-bp construct was induced by RXRβ, RARα, and RARβ, but not RARγ relative to the 200-bp + pcDNA3 control (**P < 0.01). RARβ also seemed to induce pGL3 (*P < 0.05); however, this is likely to be nonspecific.

Fig. 5. Sequence of the 200-bp proximal promoter region of the human PKD1 gene. Two consensus Ets binding sites (Ets-A and Ets-B), two p53 motifs (p53), and six potential GC boxes (Sp1-A to Sp1-F) upstream of the transcription start site (bold arrow) are shown. The core sequence corresponding to each element is underlined (Ets and Sp1) or overlined (p53). Mutations were generated by replacing 2 base pairs at each site. The locations of the forward primer (F-primer) and the reverse primer (R-primer) for chromatin immuno-precipitation (ChIP)-PCR analysis are indicated by the long arrows.
Exogenous Sp1 activates the 200-bp PKD1 promoter. As Drosophila SL2 cells lack Sp1, but contain RAR/RXR homologs, we exploited this unique property of these cells to test the response of the 200-bp PKD1 promoter to exogenous Sp1. As shown in Fig. 10, the activity of the 200-bp promoter was significantly increased in the absence or presence of AT-RA after the cells were supplied with exogenous Sp1. In contrast, the promoter was not induced at all by AT-RA in the absence of Sp1. Taken together, these data support the idea that Sp1 can activate this promoter and thus can mediate the retinoid response.

To determine in mammalian cells whether exogenous Sp1 in combination with exogenous RXRβ or AT-RA treatment can stimulate the 200-bp PKD1 promoter, we cotransfected Sp1-transfected HEK293T cells with RXRβ, or treated Sp1-transfected cells with AT-RA. As shown in Fig. 11A, additional Sp1 led to activation of the 200-bp promoter, and this induction by exogenous Sp1 was further augmented by RXRβ, and even more so by AT-RA. As shown in the Western blot (Fig. 11B, left), HEK293T cells contain endogenous Sp1, which was not increased in AT-RA-treated cells or in RXRβ-transfected cells, thus indicating that the responses of the PKD1 promoter to AT-RA induction, it is likely that a combination of Sp1-C and Sp1-F mediate the RA response in concert. Repeated attempts to introduce mutations into Sp1-D/Sp1-E failed, making it impossible to test these sites; however, the fact that the triple mutant, Sp1-BCF, was almost completely inactive makes it unlikely that Sp1-D/Sp1-E has a role in RA responsiveness.

**MthA completely blocks RA and RXR responses.** To test whether any of the GC boxes present in the 200-bp promoter sequence are induced by endogenous Sp1, we treated promoter-transfected HEK293T cells with MthA, a GC box inhibitor that specifically blocks interaction between Sp1 and the GC motif by masking GC-rich sequences (7, 23). As shown in Fig. 8, MthA treatment reduced the basal activity of the 200-bp PKD1 promoter by ~70%, suggesting that one or more of the GC boxes is functional and that Sp1 is involved in basal transcription. As expected, MthA treatment had virtually no effect on the already very low basal activity of the Sp1-BCF mutant.

To further establish a link between RA-mediated activation and Sp1, cells transfected with the wild-type 200-bp PKD1 promoter were treated with AT-RA in the absence or presence of MthA. As shown in Fig. 9 (left), the 10-fold activation of the promoter by AT-RA was completely suppressed by MthA. Induction of promoter activity by cotransfected RXRβ was also fully abolished by MthA (Fig. 9, right). These data further support the idea that Sp1 mediates the stimulation of the 200-bp PKD1 promoter by retinoid treatment.
RXXβ and AT-RA did not involve upregulation of endogenous Sp1. In contrast, Sp1 was significantly increased in the Sp1-transfected cells (Fig. 11B, right), thus explaining the augmentation of promoter activity in Sp1-transfected cells.

To further demonstrate the involvement of Sp1 in promoter activation by the RA receptor, siRNA knockdown experiments were carried out. As shown in Fig. 12, activation of the 200-bp PKD1 promoter by RXXβ was completely abolished by Sp1-specific siRNA knockdown. Western blot analysis of cells in parallel experiments showed a significant reduction of Sp1 compared with a β-actin control.

The PKD1 gene is upregulated by AT-RA and binds RXX. To determine whether expression of the endogenous PKD1 gene responds to RA, total RNA was isolated from AT-RA-treated HEK293T cells and analyzed by RT-PCR. As shown in Fig. 13 (A–C), PKD1 mRNA levels were increased ~1.6-fold, whereas the ribosomal protein L7 mRNA was not affected by the treatment. As shown in Fig. 13D, there was a dose-response to AT-RA which increased PKD1 mRNA levels up to 2.6-fold at the maximum dose of 5 μM AT-RA.

To determine whether RXX binds the proximal promoter in vivo, ChIP-PCR assays were carried out with an anti-RXX antibody. As shown in Fig. 14 (lane 3), a PCR product similar in size to that obtained with the input DNA was seen, indicating that RXX is bound to a region within the PKD1 proximal promoter. This 135-bp promoter region includes Sp1-C and Sp1-F (the locations of the forward and reverse primers are shown in Fig. 5). Because this promoter region does not contain an RAR/RXX element, it is likely that RXX is bound indirectly through Sp1. As shown in lane 4, Sp1 was also found present on this 135-bp promoter region (Fig. 14). These results suggest that the PKD1 gene is a target of retinoid activation mediated through Sp1.

DISCUSSION

In the present study, we report that the human PKD1 promoter is a target for vitamin A metabolites. This response was localized to the proximal 200-bp region upstream of the transcription start site. A 200-bp PKD1 promoter-reporter construct was typically induced up to >10-fold by RA (AT-RA or 9C-RA) treatment in transient transfection assays using three different cell lines. Activation of the 200-bp promoter-reporter was also observed in response to cotransfected RARα, RARβ, and RXXβ (but not RARγ). These results suggested that activation of the proximal region of the PKD1 promoter involves activation of RA receptors and that the PKD1 gene may be a target of RAR/RXX. Sequence analysis of the 200-bp PKD1 proximal promoter region identified two Ets elements, two p53 elements (63), and six GC boxes (Sp1-A thru Sp1-F), but no canonical RARE. However, not all effects of RAs are directly regulated by interactions between RAR/RXX and...
canonical GC boxes and one atypical GC box within a 42-bp proximal promoter sequence. Mutation of the distal-most (canonical) site had no effect on either basal or hormone-induced activity, whereas a combination of mutations in the other three sites caused elimination of most basal and receptor-stimulated activity. Thus not all canonical GC box motifs are necessarily functional. Furthermore, a number of studies (11, 33, 34, 56) have identified the importance of 3′-flanking sequences immediately adjacent to the hexanucleotide core GC box motif in determining functionality. The presence of five or more purines or pyrimidines in the 3′-flanking region may reduce or completely abolish function, as observed in transforming growth factor (TGF)-β1 (CTCCCC and CCCCC), TGF-β RII (AGGGGG), TGF-β RII (AGAGAGG), and MDR1 (GGAGCAG), as they may interfere with binding of Sp1 to the GC box (56). In contrast, a mixture of purines and pyrimidines in the 3′-flanking region may support Sp1 binding. In the PKD1 promoter, the 3′-flanking regions of Sp1-A (AGCTTCC) and Sp1-B (GGCCTCC) contain five continuous pyrimidines, while those of Sp1-C (AGCCTGCAC) and Sp1-F (ACGGGGCGA) have mixed purine and pyrimidine sequences consistent with functional GC box motifs. We did not directly test the combined Sp1-D/Sp1-E sequence. However, the fact that the Sp1-BCF triple mutant completely abrogated promoter activity argues that the Sp1-D/Sp1-E sequence is not functional in either basal regulation or in the RA response.

MthA blocks interactions between Sp1 and GC boxes by obscuring GC-rich sequences (7, 23). MthA treatment significantly reduced (~70%) basal activity of the wild-type PKD1 promoter, suggesting that it does so by preventing the binding of Sp1 to functional GC box motifs, most likely Sp1-C and Sp1-F. However, the incomplete inhibition of basal activity suggests that MthA was not completely effective at these sites, since both basal and induced promoter activities were almost completely abolished in the Sp1-BCF triple mutant. MthA completely inhibited both AT-RA and RXRβ induced-pro

RAREs, and a number of mechanisms through which the retinoids stimulate gene transcription have been reported. These include inactivation of AP-1 via a CREB-binding protein-regulated pathway (66), inhibition of JNK (66), modulation of histone acetylation (27), and Ets-1-mediated activation (30, 56, 59).

ChIP showed that both Sp1 and RXR were bound to the PKD1 promoter in a 135-bp region that lies within 170 bp of the transcription start site, suggesting that RXR interacts with DNA-bound Sp1 at the proximal PKD1 promoter. Mutation of three of the GC boxes in the Sp1-BCF triple mutant eliminated all basal promoter activity and completely abolished the response to both retinoids (AT-RA and 9C-RA) and to both RXRβ and RARα. Since the Sp1-B mutation alone had no effect on basal or induced activity, whereas the individual Sp1-C and Sp1-F mutations decreased promoter activity and the Sp1-BCF triple mutation had no promoter activity, it is likely that both Sp1-C and Sp1-F are functional Sp1 sites that are responsive to retinoid activation.

Similar observations were described for the urokinase plasminogen activator promoter (56). This promoter contains three
moter activity, suggesting that the RA response of the PKD1 promoter is dependent on Sp1.

Further support for an involvement of Sp1 RA-induced PKD1 promoter activity was obtained using Drosophila SL2 cells, which lack Sp1 (12) but contain RAR/RXRs (37). Quite robust induction of the PKD1 promoter was obtained following transfection of these cells with Sp1, and this Sp1-dependent induction could be further augmented by AT-RA treatment. Exogenous Sp1 was also able to upregulate the PKD1 promoter in HEK293T cells. While it could be argued that the action of AT-RA and RXRβ are to upregulate Sp1, this was ruled out by showing that neither AT-RA nor RXRβ changed Sp1 protein levels (Fig. 11B). Furthermore, siRNA knockdown of Sp1 in HEK293T cells blocked RXRβ induction of the promoter (Fig. 12). Taken together, these results suggest that RA and its receptors facilitate Sp1 activation of the PKD1 promoter.

Transactivation of the PKD1 promoter by retinoids may occur in a similar manner to that seen for the urokinase transglutaminase, TGF-β, and TGF-β RI promoters, by mediation of the interaction between Sp1 and GC box motifs (11, 33, 34, 56). In those studies, it was found that RA and its receptors failed to increase Sp1 mRNA or protein but that a physical interaction between Sp1 and the RARs/RXRs receptor dimer is an intermediate to induction by RA. In this case, RA treatment first induced expression of RA receptors through

![Fig. 14. RXR and Sp1 bind within the proximal promoter of the PKD1 gene](http://ajprenal.physiology.org/)

RARE sites (8, 40, 42). The RARs then physically interacted with Sp1 together with RXR and enhanced Sp1 binding to GC box motifs, resulting in Sp1-mediated upregulation of gene transcription (56). Both types of receptors had the same ability to modulate Sp1 binding, and in vivo they appeared to act synergistically. Similar interactions between Sp1 and the estrogen receptor have also been observed (16, 55, 57, 64). In contrast, it has been shown that RA activation of thrombo-modulin gene transcription can occur by induction of both Sp1 mRNA and protein in RA-treated cells (25).

Sp1 family transcription factors play a key role in regulating transcription initiation at TATA-less promoters (3), and they are temporally and spatially regulated during vertebrate morphogenesis and are important for normal organogenesis (67). Similarly, the retinoids have profound effects on many essential biological programs, including cellular growth, somatic cell differentiation, and morphogenesis (42). In situ hybridization and RNA protection analyses have indicated distinct and often mutually exclusive expression patterns of some of the RA receptor isoforms (14, 15). We have demonstrated that expression of the endogenous PKD1 gene was elevated in response to AT-RA treatment in HEK293T cells (Fig. 13), supporting the idea that the endogenous PKD1 gene is a target of vitamin A-derived metabolites. It has been reported that targeted disruption of RXRα causes cardiac conotruncal defects identical to the cardiac phenotype found in Pkd1 null mouse embryos (44), suggesting that the Pkd1 gene may be a critical target of RA signaling in the heart. It has also been shown that a retinoid, N-(4-hydroxyphenyl) retinamide, markedly inhibited cystogenesis of ADPKD cyst-derived cells, Madin-Darby canine kidney cells, and rat epithelial cells in collagen matrix cultures (1). As such, PKD therapies may include treatment with retinoic acid derivatives and could be beneficial in cases of PKD1 haploinsufficiency.

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