Phosphodiesterase 1A Modulates Cystogenesis in Zebrafish

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ABSTRACT

Substantial evidence indicates the importance of elevated cAMP in polycystic kidney disease (PKD). Accumulation of cAMP in cystic tissues may be, in part, caused by enhanced adenylyl cyclase activity, but inhibition of cAMP degradation by phosphodiesterases (PDE) likely has an important role, because cAMP is inactivated much faster than it is synthesized. PDE1 is the only PDE family activated by Ca2+, which is reduced in PKD cells. To assess the contribution of the PDE1A subfamily to renal cyst formation, we examined the expression and function of PDE1A in zebrafish. We identified two splice isoforms with alternative starts corresponding to human PDE1A1 and PDE1A4. Expression of the two isoforms varied in embryos and adult tissues, and both isoforms hydrolyzed cAMP with Ca2+/calmodulin dependence. Depletion of PDE1A in zebrafish embryos using splice- and translation-blocking morpholinos (MOs) caused pronephric cysts, hydrocephalus, and body curvature. Human PDE1A RNA and the PKA inhibitors, H89 and Rp-cAMPS, partially rescued phenotypes of pde1a morphants. Additionally, MO depletion of PDE1A aggravated phenotypes in pkd2 morphants, causing more severe body curvature, and human PDE1A RNA partially rescued pkd2 morphant phenotypes, pronephric cysts, hydrocephalus, and body curvature. Together, these data indicate the integral role of PDE1A and cAMP signaling in renal development and cystogenesis, imply that PDE1A activity is altered downstream of polycystin-2, and suggest that PDE1A is a viable drug target for PKD.


Autosomal dominant polycystic kidney disease (PKD) is characterized by the development and enlargement of kidney cysts, which progress and interfere with renal function. It affects from 1 in 400 to 1 in 1000 people, and it is the most common genetic cause of renal failure and the fourth leading cause of kidney failure overall, accounting for approximately 5%–10% of patients receiving dialysis or kidney transplants.1–4 Most cases have mutations in the PKD1 gene, encoding Polycystin-1 (PC1), or the PKD2 gene, encoding Polycystin-2 (PC2). PC1 and PC2 comprise a subfamily of transient receptor potential channels. They form a complex with Ca2+ channel activity located at multiple subcellular locations, including primary cilia.

Among the most consistently described changes associated with PKD is increased cAMP levels, which contribute to cystogenesis by multiple mechanisms. These findings have led to the identification of vasopressin V2 and somatostatin receptors as possible therapeutic targets because of their modulation of cAMP synthesis by adenylyl cyclase. Resulting preclinical and clinical trials have indicated the promise of this approach.5–9 A recent clinical trial found that the V2 receptor antagonist, Tolvaptan, slowed disease progression over 3 years, which was indicated by a 50% reduction in total kidney volume increase and a 30% slower decline in kidney function indicated by

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serum creatinine. Lowering cAMP levels by additional means may prove even more effective.

A powerful additional approach to lowering cAMP levels is through cAMP hydrolysis by phosphodiesterases (PDEs). As therapeutic targets, PDEs have several advantages. The capacity for hydrolysis of cyclic nucleotides by PDEs far exceeds the maximum rate of synthesis by adenyl cyclases. Additionally, the complexity of the PDE superfamily (11 families, 21 genes, and 100 isoforms) provides an opportunity for cell-specific or even subcellular compartment-specific regulation of cAMP levels and control of cystogenesis with relative safety.

Of all the PDE families, only one (PDE1) is regulated by Ca\(^{2+}\). It is highly expressed in the kidney and likely inhibited in response to decreased Ca\(^{2+}\) associated with PC1/PC2 mutations. The PDE1 family is, therefore, a logical candidate affecting renal cystogenesis. In mammals, the PDE1 family has three subfamilies: PDE1A, PDE1B, and PDE1C.

The ease of monitoring cystic phenotypes in zebrafish makes it an ideal model to dissect the function of individual PDE superfamily members. Experimental approaches are straightforward for either down- or upregulation of specific transcripts through antisense morpholinos (MOs) or exogenous RNA expression, respectively. Additionally, several zebrafish resources are available that facilitate these studies, including validated genetic models of PKD and transgenic lines with fluorescence highlighting kidney anatomy and morphology.

We have focused here on PDE1A, showing that depletion was associated with cystogenic phenotypes and that overexpression partially rescued phenotypes from PC2 depletion. These data validate PDE1A as a target for therapeutic intervention for PKD.

RESULTS

Identification of Zebrafish pde1a Isoforms, Expression Patterns, and Catalytic Activity

**Tblastn** searches of the zebrafish genome with human and mouse isoforms of PDE1A identified two isoforms with alternative starts constituting a single gene on chromosome 9, and full-length sequences of the two isoforms were amplified by RT-PCR. The transcript from the upstream start (start 1) was predicted to encode a protein with 78% similarity overall to its closest human ortholog, PDE1A2 (determined by protein–protein BLAST). The protein encoded by the downstream start of pde1a (start 2) had 79% similarity overall to its closest human ortholog, PDE1A4 (determined by protein–protein BLAST). By ClustalW alignment, the start 1 isoform had the greatest similarity to human PDE1A1, and the start 2 isoform had the greatest similarity to PDE1A4 (Figure 1A). The zebrafish and human isoforms have highly conserved catalytic domains, with 98% similarity, but they apparently differ at the C terminus. We identified an additional 73 amino acids in the zebrafish sequence after the region corresponding to the human stop codon.

Quantitative PCR (qPCR) showed that relative expression of the two isoforms varied in embryo and adult tissues. In particular, the **start 1 pde1a** isoform was expressed at a level 14 times that of the **start 2** isoform in the head of 2-day postfertilization (2-dpf) embryos (Figure 2A). Among the adult tissues tested, the **start 1** isoform was expressed at a higher level than the **start 2** isoform only in brain, where its expression was 100 times that of **start 2**.

To examine the cAMP hydrolytic activity of zebrafish PDE1A, RNA was made from either the start 1 or the start 2 isoform and injected into Xenopus oocytes, and oocyte lysates were tested for cAMP hydrolytic activity. Lysates of oocytes injected with either isoform had Ca\(^{2+}\)/calmodulin-dependent cAMP catalytic activity, whereas lysates of water-injected oocytes did not (Figure 2B).

**PDE1A Depletion Caused Pronephric Cysts and Hydrocephalus**

To determine the role of PDE1A during renal development, splice- and translation-blocking MOs were developed. Splice-blocking MOs targeted each end of exon 7, the second exon encoding the conserved phosphohydrolase domain including the critical conserved HD motif required for metal binding and hydrolytic activity (Figure 3A). The efficacy of both of these splice-blocking MOs was shown by RT-PCR and sequencing of individual embryos, showing an in-frame deletion of exon 7 (Figure 3B). Injections of either MO (10 ng) caused significant development of pronephric cysts and hydrocephalus compared with the control MO (10 ng) (Figure 3, C–H), and some embryos showed apparent tubule dilation. Sectioned splice acceptor morphants showed compression of the brain underlying hydrocephalus and compression of the epithelium lining pronephric cysts (Figure 3, F and G). The relatively milder apparent effect of the acceptor MO on splicing contrasts with its more potent effect on phenotypes, suggesting that the altered transcript may have a disproportionately strong effect on translation. Additionally, it is possible that unanticipated splice forms resulted that were not detected.

Translation-blocking MOs targeting either start 1 or start 2 caused pronephric cysts, and some embryos showed apparent tubule dilation (Figure 4). Cystogenesis resulting from the start 1 MO was dose-dependent, with a greater effect on cystogenesis at the maximum dose compared with start 2. Additionally, the start 1 MO caused hydrocephalus and curvature, whereas the start 2 MO did not. Both dose dependence and the more potent effects overall in start 1 morphants are consistent with higher expression of this isoform during embryogenesis (Figure 2). In particular, the lack of hydrocephalus in start 2 morphants is consistent with low expression of this isoform in the head at 2 dpf.

To test whether depletion of PDE1A affects cilia, we compared cilia beating in 2-dpf pde1a morphants (5 ng each start 1 and start 2 MOs) containing pronephric cysts with controls. The average beat frequency measured in undilated...
regions of the tubule in *pde1a* morphants was the same as in controls (32 ± 1 versus 30 ± 1 Hz in *pde1a* versus control MO; *P*=0.24 by two-tailed *t* test; *n*≥9 fish in three batches), and in these regions in both cases, cilia were bundled and beat in a coordinated fashion. In contrast, cilia in dilated regions of the tubule of *pde1a* morphants were not bundled, and instead, beat individually in an uncoordinated fashion (Supplemental Movie 1).

Rescue of PDE1A Depletion Phenotypes with PDE1A RNA or Protein Kinase A Inhibitors

Coinjection of human PDE1A RNA into one-cell embryos with *pde1a* start 1 and start 2 MOs significantly decreased the

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**Figure 1.** Alignment and overview of zebrafish PDE1A shows substantial similarity to human PDE1A. (A) Protein encoded by exons 1α/2α/3α of zebrafish PDE1A start 1 isoform and 1β/2β of zebrafish PDE1A start 2 isoform each aligned with the closest human ortholog. Arrowheads show the zebrafish exon boundaries. (B) Alignment of zebrafish PDE1A with human PDE1A1 starting with the protein encoded by human exon 2. Additional 73 amino acids of C-terminal zebrafish sequence is not shown. (C) Overview of genomic structure showing alternative starts and the conserved domain of the phosphohydrolases. Boxed region indicates the conserved domain of the metal-dependent phosphohydrolases. *Hs* PDE1A1 is translated from Genbank accession number NM005019.3, and *Hs* PDE1A4 is translated from Genbank accession number NM_001258313. *Hs*, *Homo sapiens*; *Dr*, *Danio rerio*.

**Figure 2.** Zebrafish *pde1a* isoform expression varies among tissues and both isoforms have cAMP catalytic activity. (A) qPCR showing relative expression levels with expression set at one for the start 2 isoform in 2-dpf embryo head. Data are shown as means±SEMs of triplicate samples using tissue pooled from 50 embryos or 3–10 adults. (B) Enzyme assays on lysates from *Xenopus* oocytes injected with water or zebrafish *pde1a* RNA. Ca²⁺/calmodulin-dependent cAMP hydrolysis is shown expressed as mean±SEM (*n*=3 tubes containing 10 oocytes each).
percent of embryos with pronephric cysts, hydrocephalus, and curvature (Figure 5, A and B). This rescue was most effective for cystogenesis (55% lower) but also decreased hydrocephalus by approximately 30% and body curvature by approximately 40%. The same PDE1A RNA injected into Xenopus oocytes increased PDE1 activity compared with water-injected oocytes (Figure 5C). The protein kinase A (PKA) inhibitors H89 and Rp-cAMPS were tested by adding them to the water when embryos reached 50% epiboly (approximately 6 hours postfertilization) (Figure 5, A and B). Data shown were obtained using 1–5 μM H89, but similar levels of rescue were seen in preliminary experiments using 1–25 μM H89. Similar to PDE1A RNA, H89 partially rescued the phenotypes, decreasing cystogenesis by approximately...
Figure 4. Two translation-blocking MOs targeting either start 1 or start 2 of pde1a caused pronephric cysts in 2-dpf embryos. (A) Representative phenotypes of 5 and 10 ng control and pde1a translation-blocking morphants. MO targeting start 1 caused pronephric cysts, hydrocephalus, and curvature, whereas MO targeting start 2 caused pronephric cysts without hydrocephalus or curvature. Arrows show cysts and tubules, and the arrowhead shows hydrocephalus. Scale bar, 100 μm. (B) Quantification shows dose responses and greater maximal penetrance of start 1 MO compared with start 2 MO. *P<0.05; **P<0.005 by chi-squared test (n=173 embryos in three or more batches of 29–62 embryos each; data shown are the total percents of embryos).

30%, hydrocephalus by 35%, and curvature by approximately 40%. Similar to H89, Rp-cAMPS decreased cystogenesis by approximately 30%, although it did not rescue hydrocephalus or curvature.

DISCUSSION

We report here the identification, expression, and requirement of two zebrafish PDE1A isoforms for preventing renal cysts and associated phenotypes. Both PDE1A isoforms have a high level of conservation with mammalian orthologs, particularly in the conserved phosphohydrolase domain. Depletion of either isoform caused renal cystogenesis, which was rescued by human PDE1A RNA. In addition, PDE1A depletion aggravated and PDE1A overexpression rescued pkd2 morphant phenotypes, indicating a functional interaction between PDE1A and PC2. To our knowledge, this work is the first demonstration of an interaction between a PDE and PC2 in zebrafish, indicative of a role for PDE1A downstream of PC2 leading to renal cystogenesis.

The specificity of the pde1a morphant phenotypes is supported by the similar effects of four distinct MOs, particularly regarding induction of pronephric cysts, and the rescue of pde1a morphant phenotypes by PDE1A RNA. In depletion studies, loss of PDE1A is expected to cause elevated cAMP levels and PKA activity. Rescue studies using the PKA inhibitors H89 and Rp-cAMPS tested the contribution of PKA signaling to PDE1A depletion phenotypes. Rescue was only partial but significant, indicating the contribution of PKA signaling to the induced cystic phenotypes. Additional cAMP effectors (i.e., exchange proteins activated by cAMP) may contribute to cystogenesis.19 Importantly, PDE1A hydrolyzes cGMP as well as cAMP, and cGMP is elevated in mouse and rat PKD models.10–13 Therefore, it is also possible that PDE1A depletion causes elevated cGMP, which contributes directly to the phenotypes reported here. As with PDE1A depletion, the rescue of PC2 depletion by PDE1A RNA was partial, suggesting that PC2 acts through cAMP as well as other mechanisms to cause renal cystogenesis, hydrocephalus, and body curvature. This finding is not surprising, because PC2 regulates intracellular...
Ca\(^{2+}\), which has many cellular effects, including activation of the Ca\(^{2+}\)/calmodulin-dependent protein kinase type II that also acts downstream of PC2 in the development of the zebrafish pronephros.\(^{20}\)

The two isoforms of \(pde1a\) identified here in zebrafish correspond to at least two of five human isoforms. Alignment of five human \(PDE1A\) isoforms listed by the National Center for Biotechnology Information (NCBI) shows that there are four distinct N termini, one of which varies by only four additional N-terminal amino acids from two others. Therefore, there are three substantially distinct N termini in humans, two of which have clear zebrafish orthologs. Human \(PDE1A\) also has two distinct C termini, neither of which is similar to the zebrafish \(pde1c\) terminus. The poorly conserved C terminus of zebrafish \(pde1a\), after the conserved phosphohydrolase domain, adds a substantial polypeptide after the stop codon in human \(PDE1A\) isoforms, but its functional significance is unclear.

The second exon in the start 1 isoform encodes 9 amino acids not present in human \(PDE1A\) but present in human \(PDE1C\). Nevertheless, the sequence reported here is most similar to human \(PDE1A\) by protein–protein BLAST. Additionally, by ClustalW, it has greater identity to \(PDE1A\) than \(PDE1C\) (using the closest isoforms \(PDE1A1\) and \(PDE1C1\)) overall (66% versus 60%) and in the hydrolase domain (89% versus 79%). Finally, we amplified a distinct partial zebrafish \(pde1c\) predicted to encode 314 N-terminal amino acids, including the start ATG (C.R. Sussman, unpublished data). The \(pde1c\) transcript was 95% similar to human \(PDE1C\) and mapped most closely to loci on chromosome 24 and an unmapped chromosome (i.e., distinct from zebrafish \(pde1a\) on chromosome 9). These data are consistent with inclusion of exon 2 in an ancestral \(pdel\), which was subsequently deleted in human \(pde1a\).

The functional significance of the two different zebrafish \(pde1a\) starts is not clear. We observed pronephric cystogenesis from depletion of either isoform but hydrocephalus and body curvature only from depletion of the start 1 isoform. Isoform differences in causing hydrocephalus and body curvature are consistent with the relative expression levels of their transcripts in the head versus body of 2-dpf embryos and the brain in the adult. Similar to their human orthologs, both isoforms had Ca\(^{2+}\)/calmodulin-dependent cAMP hydrolytic activity.\(^{10–12}\) It remains unknown whether there are additional differences in the biochemistry or regulation of the two isoforms.

By showing that depletion of \(PDE1A\) alone induces renal cysts in zebrafish, our data indicate that cAMP is a primary
Figure 7. PDE1A RNA rescued phenotypes from PC2 depletion. (A) Extent of body curvature in 3-dpf morphants was classified as in Figure 6. Coinjection of human PDE1A RNA (125 pg) with pkd2 MO (2 ng) significantly increased the percent of embryos with mild body curvature and decreased the percent of embryos with moderate body curvature compared with pkd2 MO alone. (B) Human PDE1A RNA (125 pg) co-injected with pkd2 MO (5 ng) partially rescued cysts and hydrocephalus at 2 dpf. **P<0.005 by chi-squared test (n=88 embryos in three batches of 27–51 embryos each; data shown are the total percents of embryos).

determinant of cystogenesis in an immature kidney with vigorous epithelial cell proliferation, similar to that shown in metanephric organ cultures treated with 8-Br-cAMP.21,22 Studies on metanephric epithelial cell proliferation, similar to that shown in metanephric mesenchyme in explant culture have additionally shown that PKA activation disrupts tubulogenesis and induces cystic dilations.23 Together, these studies suggest that a highly proliferative background alters the cellular response to cAMP, predisposing to cystogenesis. Additional studies are needed to test the contributions of proliferation and fluid transport to cystogenesis in these settings.

The effects of PDE1A and PC2 depletion were synergistic, implying that the proteins act in a common pathway. For example, body curvature was more severe in pde1a and pkd2 double morphants compared with pkd2 single morphants, and pde1a MO alone caused no dorsal curvature, even at high doses (10 ng). Moreover, PDE1A RNA partially rescued pronephric cysts, hydrocephalus, and body curvature resulting from PC2 depletion. Although the details of the mechanism responsible for this rescue are unknown, these data imply that increased cAMP is involved in causing these phenotypes of PC2 depletion. Zebrafish body curvature in pkd1a/b or pkd2 morphants involves changes in collagen expression, which is regulated in a complex relationship with cAMP levels.14,24

Similarly, high-speed video microscopy showed that pde1a MO did not affect cilia organization or beat frequency, except in tubule regions that were dilated, suggesting these effects were secondary effects of dilation. These observations are consistent with an effect downstream of cilia and PC2 and similar to other studies showing that PC2 depletion does not affect cilia.15,17

Together with previous studies, these data support a model for renal cystogenesis that is similar to mammalian PKD (i.e., disruption of cilia or PC2 function affects Ca2+ signaling, PDE1A activity, cAMP levels, and PKA activity).15,17,18,25 These data establish zebrafish as a model for studies of PDE and cAMP signaling in PKD and show the central role of cAMP signaling in pronephric development and cystogenesis in an intact vertebrate. Interestingly, another recent study showed that MO depletion of PDE6D, one of several genes mutated in Joubert syndrome, also causes pronephric cysts.26

The large size of the PDE family makes the rapid dissection in zebrafish an attractive way to explore the significance of family members. Although it is not clear whether PDE1A will prove to be among the more potent PDEs regulating cystogenesis, it is worth noting that depletion of PDE1A alone was sufficient to induce cysts. That is, another PDE family member did not compensate for its depletion, suggesting that PDE1A has a unique function in kidney maintenance and/or development and prevention of cystogenesis. These data support the hypothesis that different PDEs have unique effects on cystogenesis and may provide relatively specific targets for PKD therapy.

**CONCISE METHODS**

**Zebrafish**
Zebrafish were maintained and raised as described.27 All animal work adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Mayo Clinic Institutional Animal Care and Use Committee. Embryos were developed at 28.5°C with 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) added at 24 hours postfertilization to prevent pigmentation for facilitate cyst visualization. Embryos were anesthetized using 0.02% tricaine (Aquatic Habitats) before RNA extraction or observation by microscopy. Fish were obtained from Segrest Farms (Gibsonston, FL).

**Pde1a Cloning, PCR, and Catalytic Activity**
Zebrafish pde1a genomic contigs were identified in a tblastn search with human PDE1A. Putative exons were used to design primers, and zebrafish pde1a was amplified by RT-PCR and sequenced. cDNA template was synthesized using SuperScript III (Invitrogen) from total RNA isolated using TRIzol (Invitrogen). Primers used for PCR are shown in Table 1. PCR products were purified (Qiagen) and sequenced.

For qPCR, tissues from 50 zebrafish embryos or 3–10 adults were pooled, total RNA was extracted using RNeasy (Qiagen), and first-strand cDNA was synthesized using SuperScript III (Invitrogen). qPCR was done using an ABI 7900HT (Applied Biosystems) with SYBR GreenER qPCR Supermix (Invitrogen). Primers used are shown in
Table 1. PCR primers

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<sup>a</sup>Figure 1.<br>
<sup>b</sup>Figure 2.<br>
<sup>c</sup>Exons in the start 1 isoform.<br>
<sup>d</sup>Figure 3.

Pharmacologic Inhibitors

H89 or Rp-cAMPS (Sigma-Aldrich) was diluted to final concentration in embryo water, added to embryos at 50% epiboly, and replaced daily.

Analysis of the Embryonic Phenotype

Embryos were examined at 2 or 3 dpf, as indicated, for pronephric cysts, hydrocephalus, and body curvature using a Zeiss Lumar stereo fluorescence microscope and Axiovision software. Cysts were defined as being larger than otolith crystals (15 ± 1.0 μm, mean ± SEM, n=15).

Cilia beating was examined in 2-dpf embryos, anesthetized in tricaine, embedded in 3% methylcellulose, and examined on coverslips. Cilia beating was recorded using high-speed video microscopy on a Zeiss Axiowert with a Zeiss AxioCam HSR camera and Axiovision software. Images were recorded at 80 frames/s and replayed at 6 frames/s for measurement of beat frequency. At least two measurements were made on each fish to obtain an average beat frequency for each fish. Beat frequencies from 9 to 15 fish were averaged to obtain the average and SEM for each treatment.

Embryos were sectioned in JB4 (Electron Microscopy Sciences) and stained with hematoxylin and eosin as described. Sections were photographed on a Zeiss Axiowert with Axiovision software.

Statistical Analyses

Experiments were repeated in at least three unique clutches of at least 20 embryos each. Phenotypes were scored as present or absent. The total percent of embryos with phenotypes across batches is shown in graphs, and statistics were calculated using chi-squared tests. Cilia beat frequency was analyzed using a two-tailed t test.
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DISCLOSURES

None.

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