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Cloning and Characterization of the cDNA Encoding the α-Subunit of cGMP-Phosphodiesterase in Canine Retinal Rod Photoreceptor Cells

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Rod photoreceptor cyclic GMP-phosphodiesterase (cGMP-PDE, EC 3.1.4.17) is a key enzyme in the phototransduction cascade of the vertebrate retina. The enzyme is composed of α- and beta-catalytic subunits, and two identical inhibitory gamma-subunits. Once the phototransduction cascade is initiated by the absorption of light by rhodopsin, activated cGMP-PDE rapidly hydrolyses cGMP, depletion of which shuts cGMP-gated cation channels in the plasma membrane. The consequent hyperpolarization of the photoreceptor outer segment represents a large signal amplification and generates the visual neural impulse. Aberrant function of cGMP-PDE is causally associated with retinal degenerative diseases in man and animals. Mutations in the genes for the α- and beta-subunits of cGMP-PDE (PDEα and PDEβ, respectively) cause retinitis pigmentosa (OMIM entry) in some human families [6,8,9]. Defects in PDEβ also cause retinal degeneration in the rd mouse [3,11] and the rcd1 dog [5,13,14]. Rod-cone dysplasia 2 (rcd2), which affects the collie dog, also represents a defect of retinal cyclic GMP metabolism since retinal cyclic GMP levels are significantly elevated and cGMP-PDE activity is deficient [16]; however, rcd2 is not caused by a defect in PDEβ [1]. Thus it is likely that the rcd2 locus codes for either another cGMP-PDE structural subunit [PDEα, or PDEγ (the gene for the gamma-subunit of cGMP-PDE)], or for one of the other proteins in the phototransduction cascade that activates cGMP-PDE. Since the canine chromosomal locations of the rcd2 locus and the genes involved in phototransduction are unknown, none of these candidate genes can be ruled out based on their map location relative to the rcd2 locus.

In order to identify the mutation responsible for the rcd2 disorder, we have begun to examine the different candidate genes that code for the phototransduction cascade proteins in the dog. The cDNAs for the α-subunit of cGMP-PDE (PDEα) have been cloned and characterized from man [12], mouse [2], and cow [10,12]. However, no information is available in the literature on the canine PDEα cDNA.

In this study we characterize the canine PDEα cDNA from normal dog, present evidence for usage of alternate polyadenylation sites to generate the two different transcripts described in multiple species [2,7,12], and compare the deduced amino acid sequences for conservation through evolution.

RESULTS

Initially two different segments of canine PDEα cDNA were obtained by reverse transcription (RT) and polymerase chain reaction (PCR) using total retinal RNA and consensus primer pairs (PDEα-1/PDEα-2 and PDEα-3/PDEα-4; Table 1) based on the coding regions of known PDEα cDNA sequences from other species. These RT-PCR amplified fragments were cloned (1-PDEα and 2-PDEα) in pCRII vector (Invitrogen; San Diego, CA) and sequenced to confirm authenticity of the retinasepecific PDE cDNA sequence. From the confirmed canine cDNA sequence obtained by screening a canine retinal cDNA library using a PCR primer pair (PDEα-3/PDEα-4; Table 1) based on the coding regions of known PDEα cDNA sequence from other species, these RT-PCR amplified fragments were cloned (1-PDEα and 2-PDEα) in pCRII vector (Invitrogen; San Diego, CA) and sequenced to confirm authenticity of the retinasepecific PDE cDNA sequence.

Figure 1. Strategy for cloning the canine PDEα cDNA. The cDNA containing the coding sequence (shaded box), the untranslated regions at the 5'- and 3'-end (open boxes) and two sites where poly (A) are added, is shown as an insert in the vector pBK-CMV (Stratagene, La Jolla, CA) used for the construction of the library. Clones containing different overlapping regions of the cDNA (1-PDEα, 2-PDEα, 5'-PDEα, 3'-PDEα, PDEα-S and PDEα-L), the sizes of the cDNA fragments in the clones, and the primers (see Table 1 for sequence) used for generating those fragments are identified. The clones 1-PDEα and 2-PDEα were obtained by reverse transcription and polymerase chain reaction (RT-PCR) from canine retinal total RNA using the RT-PCR kit (Perkin-Elmer; Foster City, CA) as recommended by the manufacturer. Amplification of cDNA to obtain 1-PDEα and 2-PDEα was done for 30 cycles at 94 °C (1 min), 54 °C (2 min) and 72 °C (2 min). Other clones (5'-PDEα, 3'-PDEα, PDEα-S and PDEα-L) were obtained by screening a canine retinal cDNA library using a PCR based method. The PCR for screening the canine retinal cDNA library was done in 50 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM dNTPs, 2.0 mM MgCl2, 10% DMSO, and 1.25 units Taq polymerase (Life Technologies; Grand Island, NY). The conditions for PCR amplification of each fragment were as follows: 5'-PDEα and 3'-PDEα were obtained by 30 cycles at 94 °C (1 min), 60 °C (1.5 min), 72 °C (2 min); PDEα-S and PDEα-L were obtained by 30 cycles at 94 °C (1 min), 58 °C (2 min), 72 °C (3 min). All reactions were concluded with a single step extension reaction at 72 °C for 10 min.
PDEα cDNA sequence, new canine-specific primers were designed to amplify both the 5'- and 3'-ends of canine PDEα cDNA from a canine retinal cDNA library by PCR (Figure 1).

To amplify the 5'-end of the cDNA, forward vector-specific (pBK-II) and PDEA-specific reverse (PDEA-6) primers were used. From the PCR products, the largest fragment showing evidence for PDEα specificity, based on PCR using internal primers, was cloned (5'-PDEA). To amplify the 3'-end of the cDNA, gene specific forward (PDEA-5) and vector specific reverse (T7) primers were used, and the amplified DNA fragment was cloned (3'-PDEA). The clone 3'-PDEA, however, lacked the poly (A) tail. We therefore designed a canine-specific primer (PDEA-9) from the 5'-end of the cDNA and used it in combination with the vector-specific reverse primer (pBK-V) for amplification of the entire open reading frame (ORF) and 3'-untranslated region (UTR) of PDEα cDNA from the cDNA library. The PCR resulted in amplification of two DNA fragments (3.0 and 3.2 kb), both of which hybridized to canine PDEα cDNA in Southern blots (data not shown). These two putative PDEα cDNA fragments (PDEA-L and PDEA-S) were cloned as described above.

Sequence of the PDEα cDNA was obtained from clones 1-PDEA, 2-PDEA, 5'-PDEA, 3'-PDEA, and PDEA-S, which contain overlapping fragments, from both directions. The identity of the larger clone (PDEA-L) as PDEα was confirmed by (a) partial sequencing (800 bp) of the 3'-end and two other upstream regions of the insert; (b) amplification of multiple overlapping fragments identical in size to those obtained from PDEA-S by PCR using the same set of primers; and (c) identical and predicted restriction enzyme digestion pattern of PCR amplified DNA fragments from both the clones (Figure 2). Sequences of all the primers used and their location in the canine PDEα cDNA or vector DNA are listed in Table 1.

### Table 1. Sequence and Location of Primers Used for PCR

<table>
<thead>
<tr>
<th>Primer sequence [5’ to 3’]</th>
<th>Source of Primer</th>
<th>Name of primer</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>gTCTGGCAGAAGTCACTAACC</td>
<td>pBKCMV</td>
<td>PDEA1</td>
<td>192–215</td>
</tr>
<tr>
<td>AGTTGCGGAATCATGACGACT</td>
<td>pBKCMV</td>
<td>PDEA2</td>
<td>815–836</td>
</tr>
<tr>
<td>GCCTGCGGTACCATTTC</td>
<td>pBKCMV</td>
<td>PDEA3</td>
<td>1759–1778</td>
</tr>
<tr>
<td>ATGGATTCTTCCTGACGAC</td>
<td>pBKCMV</td>
<td>PDEA4</td>
<td>2393–2412</td>
</tr>
<tr>
<td>TCTAAGTGCGGCACT</td>
<td>pBKCMV</td>
<td>PDEA5</td>
<td>1832–1851</td>
</tr>
<tr>
<td>CAGCTACCTGCGGCACT</td>
<td>pBKCMV</td>
<td>PDEA6</td>
<td>681–706</td>
</tr>
<tr>
<td>CTCAATTGCGGCACT</td>
<td>pBKCMV</td>
<td>PDEA7</td>
<td>380–404</td>
</tr>
<tr>
<td>CTTACTAGTACGACA</td>
<td>pBKCMV</td>
<td>PDEA8</td>
<td>137–160</td>
</tr>
<tr>
<td>CGACATTGCGGCACT</td>
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<td>PDEA9</td>
<td>2032–2054</td>
</tr>
<tr>
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<td>pBKCMV</td>
<td>PDEA10</td>
<td>1036–1055</td>
</tr>
<tr>
<td>ACTCCTCGCGGCACT</td>
<td>pBKCMV</td>
<td>PDEA11</td>
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<td>pBKCMV</td>
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<td>CGGCTCTAAGTACGACA</td>
<td>pBKCMV</td>
<td>PDEA15</td>
<td>980–1000</td>
</tr>
</tbody>
</table>

Primers (PDEA-1 to PDEA-4) used for RT-PCR correspond to the human (h) PDEα cDNA sequence, selected from the consensus region in different species. All other PDEA primers correspond to canine (c) PDEα cDNA sequence. Location of all the PDEA primers are shown with respect to cPDEα cDNA sequence. Nucleotides (shown in lower case) in some primers have mismatches with the canine sequence because those primers were selected either from the human sequence, or from preliminary canine sequence. Phagemid vector (pBK-CMV) specific primers were selected either from the multiple cloning site or from the flanking region. The bold-italicized region of primer pBK-V represents the linker used to make the canine cDNA library.

Table 1. The two full length canine rod PDEα cDNAs are 2988 and 3233 nucleotides long, including the poly (A) tail (Figure 3). We have previously reported the presence of two distinct PDEα mRNAs, 3.3 and 3.0 kb, expressed in equivalent amounts in canine retina by northern blot [7]. Thus the sizes of the canine PDEα cDNAs described here (GenBank accession number U52868) are in complete agreement with our observation from northern analysis [7]. We have not formally determined the transcription start site; however, the clone containing the most upstream 5'-noncoding region contains 139 nucleotides in the 5'-UTR followed by the ATG codon for initiation of translation. The stop codon (TAA) corresponds to positions 2723 through 2725 of the cDNA sequence. The 3'-UTRs of the 3.0 kb and 3.3 kb transcripts, represented by clones PDEA-S and PDEA-L, are 263 and 508 nucleotides respectively. A non-consensus putative polyadenylation signal (A(TTTAAA) is present in the corresponding locations of the smaller (PDEA-S) and larger (PDEA-L) clones. In PDEA-S this first polyadenylation signal (Figure 3, nucleotides 2948 through 2953) is 15 nucleotides upstream of the poly (A) tail.

![Figure 2](http://www.molvis.org/molvis/v2/p3)
In the larger clone (PDEA-L) there is a second polyadenylation site (Figure 3, nucleotides 3187 through 3192) 23 nucleotides upstream of the poly (A) tail. The ORF of the canine sequence predicts a protein of 99.7 kDa containing 861 amino acids.

**DISCUSSION**

Comparison of the ORF nucleotides of the canine PDEα cDNA with that of other species shows that it shares similar nucleotide identity with the bovine (91.0%) and human (90.4%) sequences, and slightly lower identity with the mouse (86.5%) sequence. At the amino acid level, however, the similarity between the canine sequence and that of other species is higher (94%) than at the nucleotide level. Two transcripts have been reported to be present for human (4.9 and 5.3 kb), bovine (4.0 and 4.6 kb) and canine (3.0 and 3.3 kb) PDEα [2,7,12]. The reported human and bovine clones, however, contain only 2.9-3.2 kb of the PDEα cDNA sequence [10,12]. The size of the transcript in mouse has not been reported. Since only one ORF with high homology between different species has been identified for PDEα cDNAs, it is reasonable that the interspecies difference in the sizes of the transcripts could be due to either different transcription start sites or use of alternative poly (A) addition sites. The sequence presented here clearly demonstrates that two differently sized canine PDEα transcripts are generated by use of different polyadenylation signals.

Figure 3. The nucleotide sequence of PDEα cDNA from canine retina (GenBank accession number U52868), and the deduced amino acid sequence. The position of the nucleotides and the deduced amino acids are indicated on the right. The two extra amino acids that are inserted upstream of the poly (A) tail. The ORF of the canine sequence predicts a protein of 99.7 kDa containing 861 amino acids.
sites. The same mechanism could well account for the observed different transcript sizes within other species. The canine sequence does not contain the canonical polyadenylation signal (AATAAA); instead a similar sequence motif (ATTAAA) was identified in both appropriate locations (Figure 3). This alternative motif has been demonstrated to serve as a surrogate polyadenylation signal with -80% efficiency [15]. A non-consensus polyadenylation signal (AATACA) has also been reported to be present in human PDEα cDNA [12].

It is noteworthy that while PDEαt cDNA clones from 3 other species contain an ORF capable of coding for a polypeptide of 859 amino acids, the canine PDEαt polypeptide is predicted to contain 861 amino acids. With respect to the human and mouse sequences, the two extra amino acids are located as the 849th (Ala) and 850th (Gly) residues (Figure 3). Similar to the comparison made between human and bovine PDEα sequences [12], we noted that the differences in amino acid sequence among the four species (human, mouse, bovine, and canine) are clustered in the 225 N-terminal and 45 C-terminal residues (data not shown). The conserved region includes the domain present in several eukaryotic cyclic nucleotide phosphodiesterases [4]. Alignment of amino acid sequences of canine PDEαt and PDEβt shows a 72% overall identity, and the domains that are most dissimilar are at the N-terminus (first 50 residues) and C-terminus (last 30 residues).

The data presented here have been used to identify the possible mechanism for the presence of two transcripts for PDEαt in different species, and to compare nucleotide/amino acid identity among rod-specific PDEα sequences of these species. Characterization of the wild type canine PDEα cDNA sequence will allow us to detect PDEα mutations in dogs affected with rd2 or other inherited retinal degenerations. Because collie dogs affected with rd2 have elevated retinal cGMP levels secondary to low PDE activity, we are currently investigating the possibility of a PDEα mutation in this disease.

ACKNOWLEDGEMENTS


REFERENCES