PHR1 Encodes an Abundant, Pleckstrin Homology Domain-containing Integral Membrane Protein in the Photoreceptor Outer Segments*

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We cloned human and murine cDNAs of a gene (designated PHR1), expressed preferentially in retina and brain. In both species, PHR1 utilizes two promoters and alternative splicing to produce four PHR1 transcripts, encoding isoforms of 243, 224, 208, and 189 amino acids, each with a pleckstrin homology domain at their N terminus and a transmembrane domain at their C terminus. Transcript 1 originates from a 5′-photoreceptor-specific promoter with at least three Crx elements (C/T)TAATCC. Transcript 2 originates from the same promoter but lacks exon 7, which encodes 35 amino acids immediately C-terminal to the pleckstrin homology domain. Transcripts 3 and 4 originate from an internal promoter in intron 2 and either include or lack exon 7, respectively. In situ hybridization shows that PHR1 is highly expressed in photoreceptors, with lower expression in retinal ganglion cells. Immunohistochemistry localizes the PHR1 protein to photoreceptor outer segments where chemical extraction studies confirm it is an integral membrane protein. Using a series of PHR1 glutathione S-transferase fusion proteins to perform in vitro binding assays, we found PHR1 binds transducin βγ subunits but not inositol phosphates. This activity and subcellular location suggests that PHR1 may function as a previously unrecognized modulator of the phototransduction pathway.

In response to a single photon, mammalian photoreceptors produce an electrochemical signal that is integrated with many others, transmitted to higher visual centers in the brain, and ultimately perceived as vision. This elegant system depends on the coordinated expression of a large set of genes and on the interaction of their protein products. Many components of this system are expressed only in photoreceptors, and several are associated with retinal degeneration when defective. Mutations in genes encoding proteins involved in the phototransduction pathway (e.g. rhodopsin (Refs. 1 and 2), transducin (Refs. 3 and 4), or phosphodiesterase (Refs. 5–8)), photoreceptor structure (e.g. peripherin/RDS and ROM1 (Refs. 9–12)), interactions between photoreceptors and RPE (e.g. ABCR (Refs. 13–15)), or in photoreceptor-specific gene expression (e.g. CRX (Refs. 16–18)) result in inherited retinal degenerations (19, 20). Accordingly, we reasoned that genes expressed preferentially or exclusively in retina would be important for the normal retinal structure and function, and would be likely candidates for retinal degenerations. To identify genes with these expression characteristics, we implemented a differential hybridization screen (9, 21). One of the clones identified in this screen, designated PHR1 (for pleckstrin homology domain retinal protein) was of interest because it showed preferential expression in retina and brain. We subsequently cloned and sequenced human and murine full-length PHR1 cDNAs and found that they encode proteins with an N-terminal PH domain and a hydrophobic, putative transmembrane domain at the C terminus. PHR1 is the first PH domain-containing protein found to be preferentially and abundantly expressed in retina and, as far as we know, the first to be identified as an integral membrane protein. During the preparation of this report, a report describing cloning of cDNAs encoding two of the four PHR1 transcripts (designated evectin-1) appeared (see “Discussion”) (22).

PH domains were first recognized in pleckstrin, a platelet structural protein phosphorylated by PCK (23–25). Since then, more than 100 PH domain-containing proteins have been identified, including serine/threonine and tyrosine kinases (e.g. Btk, Akt, βAr), phospholipases (phospholipase C), regulators of small G proteins (e.g. Ras-GAP, SOS, Ras-GRF), adapters (3BP2), endocytic GTPases (e.g. dynamin), and some cytoskeletal molecules (e.g. spectrin, pleckstrin) (26–29). Functional

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The abbreviations used are: RPE, retinal pigment epithelium; PH, pleckstrin homology; ARNO, ADP-ribosylation factor nucleotide binding site opener; PEVR, familial exudative vitreoretinopathy; OS, outer segments; OSBP, oxysterol-binding protein; PCR, polymerase chain reaction; PIP, phosphatidylinositol phosphate; RACE, rapid amplification of cDNA ends; ROS, rod outer segments; RT, reverse transcription; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase pair(s); nt, nucleotide(s); IP, inositol phosphate; PI, phosphoinositol; PBS, phosphate-buffered saline; MBP, myelin basic protein; aa, amino acid(s); UTR, untranslated region.
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studies of PH domains show that the motif mediates binding to certain phosphotyrosinositols phosphates or inositol phosphates (30–33) and/or interacts with G protein α (34, 35) or βγ subunits (36–42) subunits. Both of these functional categories are potentially relevant to phototransduction. Transducin, an abundant photoreceptor G protein, plays a key role in the mammalian phototransduction cascade; a phosphoinositide pathway is utilized for phototransduction in Drosophila; and components of this system persist in mammalian photoreceptors (43). Here, we characterize the human and murine PHR1 genes and their protein products with emphasis on a PHR1 polypeptide that is an integral membrane protein in the photoreceptor outer segment.

**EXPERIMENTAL PROCEDURES**

Cloning, Sequencing, and 5' RACE Extension on PHR1—We performed a differential hybridization screen on a human retinal cDNA library as described (21). To isolate the murine ortholog of PHR1, we screened a murine whole eye cDNA library (kindly provided by Jeremy Nathans) using a human PHR1 cDNA as probe. To isolate the corresponding structural gene, we screened a human leukocyte genomic library (kindly provided by Se-Jin Lee) and a 128SVJ murine genomic library (stratagene) at 1:10 diluted to Et-pcR (300 ng/ml total DNA) as directed by the manufacturer’s instructions. We isolated RNA with RNAsol B (Tel-Test, Inc) according to the manufacturer’s directions. For some experiments, we purchased RNA from Invitrogen.

5'-Rapid Amplification of cDNA Ends (RACE)—To extend the 5'-ends of the human and murine PHR1, we utilized the Marathon cDNA amplification kit (CLONTECH) according to the manufacturer’s specifications. The generic primers for amplification of the murine cDNA were: m-r-7: GAACCTTTCCAGGAGTGTGAT (exon 3); m-r-6, GAACCTTTCCAGGAGTGTGAT CAG (exon 4); m-r-11, GATTCACGCCGTTCACGT (exon 5); and m-r-4, CTACAGAACAAAAAGGCCGAC (exon 9).

The primers for human PHR1 were: 38RT, CTGCTTCATCCGTCTACTAC (exon 4); 38R6, GCATCTACCCCTGTCCTCGC (exon 5); and 38R9, GGCCTTTCCCCGTCCTCGC (exon 9). We TA cloned (Invitrogen) and sequenced the products.

Mapping Human and Murine PHR1 Structural Genes—To map human PHR1, we developed a STS in the 3'-UTR in PHR1 gene and used GeneBridge4 radiation hybrid panel (Research Genetics, Inc.). The two forward primers were: 5'-GAAGCTAACATCTCAGGCAC-3' (exon 5); and m-r-4, CTCAGAACCAACAAGGCGAC (exon 9). CATTGAAGTGGATGACTAC (exon 4); m-r-11, GTCTCAGGCGTGTC-...
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**RESULTS**

**PHR1 Is Preferentially Expressed in Retina and Brain**—We identified PHR1 in a differential hybridization screen of an arrayed retinal cDNA library designed to identify cDNAs expressed preferentially or exclusively in retina (9, 21). To confirm and extend our initial result, we examined the expression of PHR1 on Northern blots of RNA from multiple human tissues (Fig. 1A). We found an ~2.0-kb PHR1 transcript(s) highly expressed in retina and brain, with much lower expression in several other tissues and no detectable expression in lymphoblasts or fibroblasts. With β-actin as normalization control, expression of PHR1 in human retina is ~4.5-fold higher than in brain and 100–170-fold higher than in testis and kidney.

In contrast to what appeared to be a single PHR1 transcript in human tissues (Fig. 1A), in mouse (Fig. 1B), there appeared to be two PHR1 transcripts: a predominant 2.1-kb mRNA, expressed only in retina; and a 1.8-kb mRNA, expressed at lower level in retina and as the only detectable transcript in brain and other tissues (e.g., liver and lung; data not shown). To determine if the retinal expression of murine Phr1 showed diurnal variation, we isolated total retinal RNA at 3 p.m. (light-adapted) and 3 a.m. (dark-adapted) in animals on a 12-h light/dark cycle transitioning at 7 a.m. and 7 p.m. Using β-actin as a normalization control, we found the ratio of the 2.1-kb transcript in dark-adapted to light-adapted retina is ~1.8:1, while that of the 1.8-kb transcript is ~1:1. Thus, the expression of the 2.1-kb transcript increased modestly (~2-fold) in the dark. We found no periodic alteration of PHR1 mRNA levels in brain.

**PHR1 Is a PH Domain-containing Protein**—We subsequently cloned and sequenced full-length human and murine PHR1 cDNAs. The human cDNA (U89715) is 2004 bp, and the murine cDNA (AF000272) is 2068 bp. Both have a 729-bp open reading frame encoding a 243-amino acid protein with 94% amino acid identity (Fig. 2A). The murine cDNA has an in-frame stop codon at −15 (where +1 is the A of the putative initiation codon), supporting our designation of the initiation methionine. The putative initiation methionine codons in human and murine PHR1 cDNAs are surrounded by acceptable Kozak sequences (ACC ATG A) (51).

The deduced PHR1 amino acid sequence has a PH domain near the N terminus (residues 21–128 in isoforms 1 and 2). The diagnostic features of this motif in PHR1 include a highly conserved Trp residue at the C terminus of the PH domain (Trp-119), two negatively charged residues located 4 and 5 amino acids upstream (Asp-114 and Asp-115), and a hydrophobic residue located 4 amino acids downstream (Leu-123) (Fig. 2B). Musacchio and colleagues divided PH domains into six conserved blocks or subdomains (26). The subdomains of the PHR1 PH domain compared with those from the four most similar PH domains identified in a BLAST search are shown in Fig. 2B. These include: Akt, a serine-threonine protein kinase (52–54); oxyester-binding protein (OSBP), a Golgi membrane-associated regulator of cholesterol metabolism (55, 56); cytohesin, a member of the ARNO (ADP-riboseylation factor nucleotide-de-binding-site opener) family involved in the budding of transport vesicles from the Golgi (57–59); and dynamin I and II, GTPases involved in the formation of endocytic coated vesicles (60–63).

An additional sequence feature of the PHR1 protein is a 27-amino acid hydrophobic α-helical segment at the C terminus (Gly-216 to Phe-243), suggestive of a transmembrane domain. Additionally, there are four consensus casein kinase II phosphorylation sites (11–14 SALE, 15–18 SPFE, 58–61 TAQD, 112–115 TKDD) upstream of or in the PH domain, and a potential PKC phosphorylation site (141–143 SRR) just C-terminal of the PH domain.

**Two Promoters and Alternative Splicing Produce Four PHR1 Transcripts**—To determine the organization of the murine and human PHR1 structural genes, we subeloned genomic fragments from both species. We sequenced ~18 kb of the murine Phr1 structural gene (AF071001) extending from 1.3 kb upstream of the transcription start site to the 3′ end of the gene. We also sequenced the exons and flanking intron junctions of the human structural gene (AF179306). The organization of both genes is summarized in Table I and the murine Phr1 gene is illustrated in Fig. 3. There are nine exons distributed over 18 kb. The exon/intron boundaries are all consistent with the splice site consensus sequences. The translation start site is in exon 2 (Fig. 3A). The PH domain is encoded by sequence extending from mid-exon 3 to exon 6.

To explain the origin of the multiple transcripts produced from the PHR1 gene, we performed RT-PCR using RNA isolated from mouse and human retina and brain as a template. We found that exon 7 was alternatively spliced in both mouse and human PHR1. This results in transcripts differing by 105 bp, which are incompletely resolved on Northern blots of murine RNA and account for the major upper band in the retinal...
Our RT-PCR results indicate that, in human retina, transcripts containing or lacking exon 7 are present in roughly equal amounts, while, in mouse retina, the transcript containing exon 7 predominates (Fig. 3B). In both human and murine brain, the transcript lacking exon 7 predominates. Exon 7 encodes a 35-amino acid segment of the PHR1 protein located immediately C-terminal to the PH domain (Ala-131 → Trp-166). Interestingly, the corresponding region of some other PH domain proteins has been shown to influence binding to Go (34) or to Gβγ (39, 42). The sequence encoded by exon 7 also contains a consensus PKC phosphorylation site (SRR 141–143) (Fig. 2A).

Alternative splicing of exon 7 does not fully explain the 200–300-nt difference in murine retinal and brain transcripts (Fig. 1B). Using RT-PCR and primer pairs corresponding to multiple overlapping segments covering the entire cDNA, we found no evidence for additional alternative splicing (data not shown). Therefore, we performed 5' RACE on total RNA from mouse retina and brain and found that the 5' ends of PHR1 transcripts in these two tissues are different. In retina, nearly
scripts produced by the murine PHR1 gene. The scripts present at low abundance in the 5'1824 nt without exon 7 (AF100613). We found identical intron 2 functions in brain and to a much lesser extent in Northern blot (Fig. 1)

retinal RNA accounting for the smaller hybridizing band on the bottom)

The size of the products and the segment of the cDNA amplified are PCR products with primers flanking the segment of the PHR1 cDNA encoded by exon 7 using RNA from human and mouse brain and retina.

The placement of the 3'-primer downstream of several introns precluded the amplification of any contaminating genomic DNA. We obtained a 571-bp product with sequence corresponding to murine exon 1, intron 1, exons 2 and 3, and part of exon 4. Thus, the first exon of the human PHR1 gene (exon 1/2) comprises sequence corresponding to murine exon 1, intron 1, and exon 2 (Fig. 3C). Examination of the human PHR1 sequence corresponding to murine intron 1 reveals a G → A transition at the +1 position (GG gtgac → GG ggtac). This transition inactivates the donor splice site and accounts for persistence of the sequence corresponding to murine intron 1 in the 5'-UTR of the human transcripts.

The use of two transcription start sites and alternative splicing of exon 7 results in four PHR1 transcripts in both humans and mice (Fig. 3A). Transcripts 1 and 2 use the same 5'-promoter and differ only in presence or absence of exon 7, respectively. Transcript 3 and 4 use an internal transcription start site in intron 2 and contain or lack exon 7, respectively. In mouse, the internal transcription start site deletes exon 1 and 2 (276 nt in total) and adds 143 nt of new 5'-UTR sequence to exon 3; the resulting transcript has a shorter open reading frame, lacking 19 5' codons and utilizing Met-20 as an initiation codon. The N-terminal truncated protein still contains the complete PH domain (Fig. 3D). All four transcripts are expressed in retina with transcripts 1 and 2 predominating. In brain, only transcripts 3 and 4 are expressed with transcript 4 predominating. The region from bp −200 to −1 in the 5'-flanking sequence of both murine and human PHR1 genes contains at least three copies of the photoreceptor-specific Crx element (consensus C/T TAATCC) (17, 18), suggesting that transcripts 1 and 2 are driven by a photoreceptor-specific promoter (Pr) (Fig. 3A), while transcripts 3 and 4 are driven by an internal promoter (Pi) with more general function.

Location of the Human and Murine PHR1 Structural Genes—We localized the human PHR1 structural gene to 11q13–14 by fluorescence in situ hybridization analysis with an 11-kb human PHR1 genomic clone as a probe (not shown) and to 11q14.1, between D11S916 and WI-6189 by radiation hybrid mapping (Fig. 4A). To localize the mouse PHR1 gene, we identified a MspI site in the 3'-UTR, present in C57BL/6J but absent in M. spreitus. Using this marker and the Jackson Laboratory Backcross mapping panels, we localized the murine Phr1 on chromosome 7, 3.72 (±1.38) cM telomeric to Tyr, and 1.06 (±0.75) cM centromeric to D7Mit17 (Fig. 4B). This region of mouse 7 has homology of synteny with human chromosome 11q13.5–14.1.

PHR1 Expression in Retina and Pineal Body—To determine the cellular localization of PHR1 expression in the retina, we first performed in situ hybridization (Fig. 5). In mouse retina,
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***Fig. 4.*** Map location of human (A) and murine (B) PHR1 structural genes.

The Phr1 antisense probe hybridized predominately to transcripts in the photoreceptor inner segment (Fig. 5A). This pattern resembles that of rhodopsin (Fig. 5B), but is quite different from that of cone blue pigment (Fig. 5C). These results indicate Phr1 (and by inference, PHR1) is expressed in rods, but do not rule out the possibility that it is also expressed in cones. To answer this question, we performed additional in situ hybridization studies using monkey retina (Fig. 5, D–F), where cones are more abundant and easier to recognize than in mouse. Our results show that PHR1 is also highly expressed in cones (Fig. 5D). In additional in situ hybridization studies, we found that PHR1 is expressed at low levels in retinal ganglion cells (data not shown). Interestingly, we found that PHR1, like several components of the phototransduction pathway, is also expressed in pineal body (Fig. 5G) (64, 65).

Using immunohistochemistry with a polyclonal antibody developed against full-length human PHR1, we found that nearly all PHR1 protein in retina is located in the OS of rods and cones (Fig. 6). We also detected some PHR1 protein in the plasma membrane of retinal ganglion cells (Fig. 6D). These results localizing PHR1 protein agree well with our in situ hybridization data and indicate that PHR1 is an abundant protein in photoreceptor OS.

**PHR1 Isoforms Have Different Tissue Distribution**—By immunoblot analysis we detected three PHR1 isoforms (1, 2, and 4) of 32, 27, and 23 kDa in extracts of murine and bovine retina (Fig. 7A). In mouse brain, we only detected isoform 4 (23 kDa). Consistent with our RT-PCR results showing a much lower expression of transcript 3, we did not detect isoform 3 on the immunoblot. In both retina and brain, the antibody also reacts with a protein slightly larger than isoform 4, which could represent phosphorylation or some other post-translational modification of isoform 4.

To localize PHR1 in the photoreceptor OS, we isolated rod OS (ROS) and ROS membranes from bovine retina. Immunoblot analysis of these subcellular fractions showed that isoform 1 and 2 are enriched in ROS and particularly in ROS membranes (Fig. 7A). By contrast, isoform 4 is less abundant in ROS and ROS membranes compared with total retina. Considering these results, together with our in situ and immunohistochemistry findings, we conclude that PHR1 isoforms 1 and 2 are photoreceptor-specific and associated with ROS membranes, while isoform 3 and 4 are expressed in retinal ganglion cells and in unspecified cells in brain.

**PHR1 Is an Integral Membrane Protein in Photoreceptor OS**—To determine the relationship of the PHR1 isoforms with ROS membranes, we extracted these samples with either 1% cholate or 100 mM Na₂CO₃. These reagents remove most membrane-associated proteins but do not extract integral membrane proteins (48, 49). We found that PHR1, like rhodopsin, is not extracted from ROS membranes by either of these reagents (Fig. 7B). By contrast, >50% of the β subunit of transducin, a membrane-associated protein, was eluted from the ROS membranes by these treatments. We conclude that PHR1 is an integral membrane protein in ROS and, by inference, predict a similar localization in cones.

**PHR1 Binding Studies**—PH domains are ancient motifs that have been shown to bind to inositol phosphates and/or G protein α or βγ subunits (26–28, 34–42). To search for PHR1 ligand(s), we made a series of PHR1-GST fusion proteins (Figs. 3D and 8B) and used them in an in vitro binding assay with radiolabeled inositol phosphates (1-IP, 1,4-IP 2, 1,4,5-IP 3, 1,3,4,5-IP 4) and transducin βγ subunits. As a positive control for these experiments, we used a GST fusion protein with the N-terminal PH domain and downstream 30 amino acids of Btk, a PH domain protein previously shown to bind 1,3,4,5-IP 4 and Gβγ (36, 38). We found no significant binding of PHR1 to any of the four inositol phosphates tested (Fig. 8A), while, as expected, the Btk fusion protein bound 1,3,4,5-IP 4 strongly and specifically (32, 33).

In contrast to our results with inositol phosphates, we found binding to transducin βγ subunits to all members of our series of PHR1-GST fusion proteins (Fig. 8D). Under the conditions of our assay, GST alone did not bind while a fusion protein containing the N-terminal 137 residues of PHR1 isoform 1 was sufficient for binding, suggesting that this activity is mediated by the PHR1 PH domain (residues 21–128). Although there appears to be a modest quantitative increase in the amount of
transducin βγ bound by PHR1 fusion proteins containing exon 7 as compared with those without (compare binding by Hsf1.L with Hsf1.S and HsPH.L+30 with HsPH.S+30), no clear qualitative difference in binding can be attributed to alternative splicing of exon 7.

**DISCUSSION**

We have identified a gene, designated *PHR1*, preferentially expressed in human and murine retina and brain. In retina, we localized nearly all PHR1 protein to the OS of both cones and rods where it is an integral membrane protein. The sequence of PHR1 suggests that it is anchored to the membrane by a C-terminal transmembrane domain. This topology would make PHR1 one of a small group of integral membrane proteins with a C-terminal anchor (66) and for all PHR1 isoforms would expose the N-terminal PH domain. We showed that this PH domain enables PHR1 to bind to transducin βγ subunits in *vitro*, suggesting a possible *in vivo* interaction between these proteins in the photoreceptor OS. Similar interactions with other G protein βγ subunits could also take place for PHR1 expressed in non-photoreceptor cells.

Transcriptional control and alternative splicing are two major mechanisms of regulation of gene expression. Both mechanisms are utilized by *PHR1*, which produces four different transcripts, each with different tissue or cell type-specificity. *PHR1* transcripts 1 and 2 are driven by a 5′-promoter, include exons 1 and 2, contain or lack exon 7, and are the predominant forms in photoreceptors. In membrane fractionation studies, we found that isoforms 1 and 2 were at least 5–10-fold more abundant in the ROS fraction compared with total retina (Fig. 7A) and were another 2–5-fold more abundant in ROS membranes compared with the ROS fraction.Taken together, these results indicate that the 5′-promoter is photoreceptor-specific.

Consistent with this hypothesis, we identified at least three Crx-like elements in bp -200 to -1 of the 5′-flanking region of both the human and murine *PHR1* genes. Crx-like elements have been identified in the promoters of several photoreceptor-specific genes and are recognized by Crx, a photoreceptor-specific transcription factor (17, 18).

*PHR1* transcripts 3 and 4

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**Fig. 5. In situ hybridization studies in retina and pineal gland:** murine retina (A–C), monkey retina sections (D–F), and rat pineal body sections (G–I). A, D, and G were hybridized with PHR1 antisense probe; B, E, and H were probed with rhodopsin antisense probe; C, F, and I were hybridized with cone blue pigment antisense probe. No signal was observed in control experiments using the corresponding sense probes (data not shown).
are driven by an internal promoter in intron 2, lack exons 1 and 2, and differ only by the presence or absence of exon 7, respectively. Northern blot (Fig. 1), RT-PCR (Fig. 3), and immunoblot (Fig. 7) analysis showed that isoform 3 is expressed at very low levels or not at all in retina and brain. In retina, isoform 4 is reduced in ROS and ROS membranes compared with total retinal extracts. Our in situ hybridization (Fig. 6) and immunostaining studies (Fig. 7) showed that PHR1 is also expressed in the retinal ganglion cells where its protein product localized to the plasma membrane. Combining all these data, we postulate that, in retina, PHR1 isoform 4 is mainly expressed in ganglion cells.

Interestingly, these alternative transcription initiation and splicing events alter the N-terminal or C-terminal context of the PHR1 PH domain, respectively, without changing the core PH motif (residues 21–128) (Fig. 3). The N-terminal 19 amino acids of the photoreceptor isoforms (isoforms 1 and 2) are absent in isoforms 3 and 4. We identified no significant homol-

![PHR1 Encodes Integral Membrane Protein in Photoreceptor OS](image)

**Fig. 6.** Immunohistochemistry. A, transverse section of murine retina stained with a polyclonal anti-human PHR1 antibody. B, transverse section of murine retina stained with pre-immune serum. C, detail of photoreceptors showing intense staining of OS. In murine retina, cone OS do not extend out as far as ROS and are located at the level of rod inner segments (90). Staining of structures consistent with cone OS is indicated by the arrowheads. D, detail of retinal ganglion cell staining. The arrowhead indicates staining of the plasma membrane.

![Immunoblot analysis of PHR1 proteins](image)

**Fig. 7.** Immunoblot analysis of PHR1 proteins. A, detection of PHR1 isoforms in bovine retina and retinal fractions and murine retina and brain. B, 1% cholate (left) and 100 mM Na₂CO₃ (right) extraction of bovine ROS membranes.

![Inositol phosphate binding to PHR1-GST fusion proteins](image)

**Fig. 8.** A, inositol phosphate binding to PHR1-GST fusion proteins. We used 3H-labeled 1-IP (IP), 1,4-IP₂ (IP₂), 1,4,5-IP₃ (IP₃), and 1,3,4,5-IP₄ (IP₄) in the binding assay. B, Coomassie staining of purified fusion proteins used in the binding assays separated by 10% SDS-PAGE. The intensity of the lower band in the lane labeled Hsfl.L increases with the age of the extract, but co-purifies with the full-length fusion protein on glutathione 4B beads. For these reasons, we conclude it is an N-terminal degradation product of the full-length fusion protein. C, Coomassie staining of 5 μg of the transducin extract used in the binding assay separated on 10% SDS-PAGE. D, binding of transducin βγ subunits by PHR1-GST fusion proteins.
ogy of this peptide with any other known functional domains. The alternatively spliced exon 7 encodes the 35 amino acids (amino acid 131–166) just two amino acids downstream of the PH domain. The corresponding region of other PH domain proteins has been shown to influence binding of the PH domain to Gβγ subunits (42). Additionally, there is a putative PKC phosphorylation site (141–143 SRR) in this 35 amino acid segment. These observations suggest that the alternative splicing event may have some functional consequence.

The PH domain is the most distinctive functional motif in PHR1, comprising about half of the entire PHR1 protein. PH domains are multifunctional motifs found in numerous proteins, many of which are involved in signal transduction pathways. Two major interactions of PH domains are with G protein βγ subunits and with lipid molecules, mainly phosphatidylinositol phosphates (PIPs) and their head groups, inositol phosphates. To begin to understand PHR1 function, we performed several in vitro assays. We did not find binding activity between PHR1-GST fusion proteins with four inositol phosphates (1-IP, 1,4-IP2, 1,4,5-IP3, and 1,3,4,5-IP4) (Fig. 8A). In parallel experiments, a Btk-GST fusion protein showed the expected specific binding to 1,3,4,5-IP4 (32, 33). Rameh et al. suggested that PH domains with clusters of basic residues in subdomains 1 and 2 (e.g., Btk has 8 basic out of 34 residues in this region) tend to have higher affinity for PI-3,4,5-P3 (whose head group is 1,3,4,5-IP4). PH domains with five or fewer basic residues in this region have lower affinity for PI-3,4,5-P3. PHR1 has only four basic residues in this region (Fig. 2).

To determine if PHR1 interacts with Gβγ, we performed binding assays with a series of PHR1-GST fusion proteins and the βγ subunit of transducin extracted from bovine retina. We found all PHR1 fusion proteins tested bound transducin βγ to an extent similar to that by the Btk-GST control. Most importantly, the PHR1 fusion protein containing the N-terminal 137 residues of isoform 1 bound transducin βγ. This result strongly implicates the PHR1 PH domain (residues 21–128) as the binding site. Although we noted modest quantitative differences in the amount of βγ bound (greater binding by fusion proteins containing the segment of PHR1 encoded by exon 7), we found no clear qualitative effect of alternative splicing on this binding property. The results of our in vitro binding assays and the location of retinal PHR1 in the photoreceptor OS suggest an in vivo interaction between PHR1 and transducin βγ and a possible role for PHR1 as a modulator of phototransduction. If so, PHR1 may modulate phototransduction. The prominent expression of PHR1 in pineal gland and the modest changes in the amount of PHR1 transcript 1 and 2 in retina during the light/dark cycle are consistent with this hypothesis (67). If PHR1 influences phototransduction, what might its effect be? Phosducin, a cytosolic phosphoprotein, has also been shown to interact with transducin βγ (68–73). Diphosphorylated phosducin forms a complex with transducin βγ, blocking its reassociation with transducin α. This interaction prevents continued activation of transducin and down-regulates the cGMP cascade in photoreceptors (73–77). If PHR1 interacts with transducin βγ in vivo, it may have an effect similar to that of phosducin on phototransduction.

Alternatively, PHR1 could interact with other members of the Gβγ family in the photoreceptor OS. Gβ5, present in brain and retina, has 53% identity with transducin β (78). Gβ5L, a Gβ5 isoform with a 42-amino acid N-terminal extension, is specifically expressed in RDS and forms a complex with RGS9 (regulator of G protein signaling protein 9) (49, 79, 80). The Gβ5L/RGS9 complex cooperates with PDE6 to enhance the GTPase activity transducin α. This interaction is essential for inactivation of the phototransduction cascade during the recovery from a photoreponse (80). If, in vivo, PHR1 prefers to bind to Gβ5L/RGS rather than transducin βγ, it could influence recovery time following photoactivation.

We found PHR1 has limited but greatest homology to four PH domain-containing proteins (Fig. 2B): 1) Akt (also known as PKB or RAC-PK), 2) ARNO, 3) dynamin, and 4) OSBP. The fact that these four proteins show affinity to different or similar PIPs might suggest that PHR1 may interact with some form of PIP in vivo. The PH domain of Akt, a serine/threonine kinase, binds PI-3,4-P2 (81, 82) in addition to Gβγ subunits PKC (83) and the head group of myosin II (84). ARNO family proteins catalyze guanine nucleotide exchange of ADP-ribosylation factor regulating the budding of transport vesicles in the Golgi apparatus. The ARNO PH domain binds to PI-3,4,5-P3 (85). Dynamin is a GTPase required for clathrin-dependent receptor-mediated endocytosis. Its PH domain binds PI-4,5-P2, which, in turn, localizes dynamin to the plasma membrane and facilitates oligomerization with other dynamin molecules (62, 86, 87). OSBP, a regulator of cholesterol homeostasis (85), translocates to Golgi membranes following binding to oxysterol in the cytosol. The OSBP PH domain binds to PI-4,5-P2 in the Golgi membrane (56). Although ARNO and dynamin both participate in vesicle trafficking, it is difficult to postulate that PHR1 could be involved in a comparable pathway since the functions of ARNO and dynamin are executed by other domains (i.e., the sec 7 domain in ARNO and GTPase domain in dynamin), neither of which are present in PHR1.

During the preparation of this manuscript, a report describing cDNAs encoding two of the four PHR1 transcripts (our PHR1 transcripts 3 and 4) was published by Krampa et al., who designated the encoded protein as evectin-1 and the gene as EVT-1 (22). By Northern blot analysis of RNA from rat tissues, they found expression of EVT-1 was limited to the peripheral and central nervous system. Using in situ hybridization in sections of rat tissues, they described expression of EVT-1 in photoreceptors, pigment epithelium, pinealocytes, oligodendrocytes, and ependymal cells. In addition, in immunoblot studies of a presumptive evectin-1 ortholog in density gradient separated fractions of frog photoreceptors, they found that about one third of the protein was bound to post-Golgi membranes, one third to membranes in the OS and the remainder to other Golgi membranes, with a small (~10%) fraction soluble. Based on these observations and on the similarity of evectin-1 to PH domain-containing proteins involved in vesicle trafficking, they speculated that evectin-1 plays a role in trafficking proteins and/or lipids into membranous organelles at the level of the post Golgi. Our sub-cellular localization data in murine and primate retinas (Figs. 5–7) do not support this speculation. Our immunohistochemistry results demonstrate that virtually all photoreceptor PHR1 is localized to the OS, a site distal to the Golgi-containing region of the photoreceptor inner segment. Furthermore, in retinal ganglion cells, the PHR1 protein is present in or contiguous to the plasma membrane (see Fig. 6).

Regardless of its function, the abundance of PHR1 in photoreceptors indicates that it is a strong candidate gene for inherited retinal degeneration phenotypes mapped to the region of the PHR1 structural gene (11q14.1). One disorder in this region is familial exudative vitreoretinopathy (FEVR) (MIM 133780), an autosomal dominant disorder characterized by abnormal development of the retinal vasculature (88). Although this phenotype is difficult to relate to the expression of PHR1 in photoreceptors, our mapping data place the FEVR gene within 1 cm of PHR1. An alternative disease consideration is Usher syndrome, type 1B (MIM 276903), an autosomal recessive disorder characterized by pigmentary retinopathy and hearing loss. Mutations in the myosin VIIA gene, which, like PHR1, maps to 11q13–14, have been
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It is shown to be responsible for most Usher 1B patients (89). As described above, the PH domain of PHR1 is most similar to that found in Atk and recent studies suggest that the Atk PH domain binds the head group of myosin II (84). By analogy, it is possible that PHR1 binds the head group of myosin VIIA in the region of the photoreceptor cilium and is involved in trafficking rhodopsin and other proteins to the OS. If PHR1 participates in this pathway, deleterious PHR1 mutations might cause an Usher-like phenotype in retina.

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