PhLPs and PhLOPs in the Phosducin Family of G$\beta\gamma$ Binding Proteins†

Cheryl M. Craft,*,‡ Jun Xu,§,|| Vladlen Z. Slepak,§,⊥ Xinyi Zhan-Poe,‡ Xuemei Zhu,‡ Bruce Brown,‡ and Richard N. Lolley‡

Mary D. Allen Laboratory for Vision Research, Doheny Eye Institute, Department of Cell & Neurobiology, University of Southern California School of Medicine, Los Angeles, California 90033, and Division of Biology, 147-75, California Institute of Technology, Pasadena, California 91125

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ABSTRACT: In this study, we identify new isoforms of the retinal phosducin and investigate the expression of the phosducin family, showing that an isoform, PhLP1, has sequence homology with Phd and G$\beta\gamma$ binding capability, whereas two isoforms (phosducin-like orphan proteins, PhLOPs) share sequence homology with Phd but fail to bind G$\beta\gamma$. Original identification of PhLP1 and the PhLOPs was from a human retina cDNA library, using a PCR product for library hybridization screening that contained a predicted functional epitope domain. The screen identified Phd and three related, but distinct, recombinants (PhLP1, PhLOP1, and PhLOP2). By RT-PCR, all isoforms are expressed in either retina or forskolin-stimulated Y79 retinoblastoma cells; however, the new isoforms are below the level of detection on Northern blot analysis. The predicted amino acid translation of each homologue revealed major differences, arising from either splice variants or gene duplication of Phd. To test the functional interaction of all phosducin isoforms with G$\beta\gamma$ in vitro, a glutathione *S*-transferase (GST) fusion protein was developed for each member. Biochemical interaction with purified retinal transducin G$\beta\gamma$ was verified for GST−Phd and demonstrated for GST−PhLP1; however, neither GST−PhLOP1 nor GST−PhLOP2 bound G$\beta\gamma$. Comparable results were observed when the GST−phosducin fusion proteins selectively sequestered G$\beta\gamma$s from retinal extracts or when functional G$\beta\gamma$ interactions were assessed using surface plasmon resonance technology. Phosducin and its isoforms are widely distributed in body tissues where they may participate in signal transduction pathways. Phd and PhLP1 possess an 11-amino acid conserved epitope domain (TGPKGVINDWR) that controls the high-affinity binding of G$\beta\gamma$; these isoforms are implicated in the G-protein signaling pathway. The phosducin-like orphan proteins (PhLOPs) fail to bind G$\beta\gamma$, suggesting that the PhLOP isoforms may participate in still unidentified signaling pathways.

Phosducin (Phd)[1] binds selectively the $\beta\gamma$ complex of large G-proteins (G$\beta\gamma$), diminishing signal amplification through competition with the α-subunit and disrupting G-protein complex reassembly. Identification of long and short homologues of phosducin (phosducin-like proteins, $PhLP_{L/S}$) (*1*) suggests that phosducin is a member of a family of proteins in which the binding of G$\beta\gamma$ may be the predominant function. Phosducin was identified initially in the retina where phosducin resides within the cytoplasm of rod and cone photoreceptors (*2*−*5*), but now it has been found in all tissues examined, including the pineal gland, brain, liver, and olfactory epithelium (*6*−*12*). Phosducin is phosphorylated during darkness by protein kinase A (PKA) and dephosphorylated by type 1 or 2A phosphatases upon exposure to light, in vivo (*2*, *13*−*15*). Competition between phosducin and the α-subunit of retinal transducin (Gα) for available G$\beta\gamma$ results in downregulation of the phototransduction cascade of vision, in vitro, through interference with reassembly of the G-protein complex that is essential for G-protein recycling with activated rhodopsin (*16*; reviewed in ref *17*). Phosducin has been implicated also in the light adaptation of retinal photoreceptors (*18*, *19*).

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* To whom correspondence and reprint requests should be addressed: Cheryl M. Craft, Ph.D., Mary D. Allen Professor for Vision Research, Doheny Eye Institute, Professor and Chair, Department of Cell & Neurobiology, BMT 401, 1333 San Pablo St., Los Angeles, CA 90033. Telephone: (323) 442-1794. Fax: (323) 442-2709. E-mail: ccraft@hsc.usc.edu.

‡ University of Southern California School of Medicine.

§ California Institute of Technology.

|| Present address: Cadus Pharmaceutical Corp., 777 Old Saw Mill River Rd., Tarrytown, NY 10591. Telephone: (914) 345-3344, ext 253. Fax: (914) 345-3565. E-mail 103176.2520@compuserve.com.

⊥ Present address: Department of Molecular and Cellular Pharmacology, P.O. Box 016189, University of Miami School of Medicine, Miami, FL 31101. Telephone: (305) 243-6643. Fax: (305) 243-4555. E-mail: vslepak@newssun.med.miami.edu.

[1] Abbreviations: Phd, phosducin; $PhLP_{L/S}$, phosducin-like protein long and short; PhLOP, phosducin-like orphan protein; G$\alpha\beta\gamma$, heterotrimeric guanine nucleotide regulatory protein, with α-, β-, and γ-subunits; hr, human retina; RT-PCR, reverse transcriptase polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; EST, expressed sequence tag; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; nt, nucleotide; PMSF, phenylmethanesulfonyl fluoride; ROS, rod outer segment; NaAlF, sodium aluminum fluoride; RU, arbitrary units; PKA, protein kinase A.

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The binding of G$\beta\gamma$ by phosducin provides a source of available G$\beta\gamma$ and a cytoplasmic shuttle for transporting G$\beta\gamma$ within the cell. The crystal structure of the phosducin−transducin $\beta\gamma$ complex has recently been reported (*20*). The structural data verify the proposal that phosducin and Gα compete for overlapping binding sites on the G$\beta\gamma$ complex. No additional function or predicted enzyme activity was suggested from the structural analysis. Phosducin is comprised of two structural domains: the N-terminal domain, which contains an 11-amino acid domain that is essential for high-affinity G$\beta\gamma$ binding, and the C-terminal domain, which has the same structural folds as thioredoxin but which lacks the appropriate cysteine residues for thioredoxin activity. The C-terminal domain of phosducin or PhLP may be able to bind G$\beta\gamma$ also, but the mechanism for the interaction remains unclear (*21*, *22*).

The G$\beta\gamma$ family of proteins have gained in stature since they have been shown to participate directly in signal transduction (*23*). Likewise, the binding of G$\beta\gamma$ by Phd or a Phd isoform takes on a broader perspective in which competitive binding interactions between G$\beta\gamma$ receptor and Phd isoforms might modulate a diversity of cellular functions (*17*). Perturbation of cellular function by alcohol offers an insight into this potential interplay since alcohol induces cultured cells to express a homologue of phosducin ($PhLP_{S/L}$) (*1*). Moreover, this study was the first to demonstrate that phosducin homologues exist.

In this study, an epitope domain anchor strategy developed previously (*24*) was utilized for the cloning and identification of phosducin and other homologous proteins. Four cDNAs representing phosducin isoforms were identified, each sharing nucleotide and amino acid sequence identity (*10*). Three previously unknown isoforms were expressed in either retinas or stimulated Y79 cells. The binding of G$\beta\gamma$ with GST-recombinant, phosducin isoforms was evaluated, showing that one homologue (PhLP1) bound G$\beta\gamma$, whereas two (PhLOP1 and PhLOP2) failed to bind G$\beta\gamma$.

EXPERIMENTAL PROCEDURES

Library Screen. A total of 1×10^6 recombinants from a human retina cDNA λMAX library (5′-Stretch, catalog no. HL 1153n, lot no. 18240, Clontech Laboratories, Palo Alto, CA) was screened with a random primed, [α-^{32}P]dCTP radioisotopically labeled PCR fragment from cerebral cortex that had been identified in a tissue screen for mRNAs encoding a proposed domain for G$\beta\gamma$ binding (see the legend of Figure 1). After the tertiary round of plaque screening under low-stringency hybridization conditions, single plaque-forming units were isolated as previously described (*6*, *25*), 11 recombinants were excised, double-stranded plasmid cDNAs were isolated from bacteria, and each cDNA was sequenced on both strands by standard techniques as previously described (*24*, *25*).

Cell Culture. Y79 retinoblastoma cells (American Type Tissue Culture, Rockville, MD) were maintained in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere (*26*). For each experiment, cells were plated at 1×10^6/mL into 60 mm dishes precoated with 0.1 mg/mL poly-D-lysine 24 h prior to treatment with 10 μM forskolin (Sigma Chemical Co., St. Louis, MO) or the drug vehicle, ethanol. Following a 3 h drug treatment, cells were harvested by freezing in liquid nitrogen and processed for total RNA isolation.

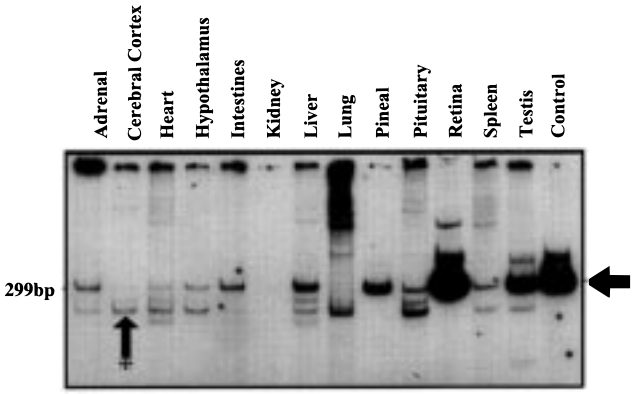


FIGURE 1: Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of phosducin homologues from selected rat tissues. Poly-A^+ mRNA from 13 selected rat tissues, identified across the top, were transcribed with RT, and the cDNA was PCR amplified with [α-^{32}P]dCTP in the mix with sense (+Phd[29−49]-21D) and antisense (−Phd[328−305]-24U) specific primers and electrophoretic separation of products on a 5% acrylamide gel. The fragments were visualized on X-ray film after a 24 h exposure. The arrow identifies the expected size (299 nt) of the control cDNA encoding rat pineal phosducin, rpPDC17 (*6*). Selected fragments from each tissue were reamplified, subcloned, and sequenced for verification. A cerebral cortex PhLP PCR fragment was selected for DNA−DNA hybridization screening of a human retina cDNA library (arrow with an asterisk).

Reverse Transcriptase Polymerase Chain Reaction Analysis (RT-PCR). Total cellular RNA was isolated using the RNAzol reagents (TEL-TEST, Inc.) as described by the manufacturer. The first-strand cDNA was synthesized from 1 μg of total RNA (Y79 retinoblastoma) or 0.5 μg of mRNA (human retina, Clontech) with the cDNA Cycle Kit (Invitrogen) using oligo-dT primer in a reaction volume of 20 μL. The RNA integrity and cDNA synthesis in every sample were confirmed by PCR analysis using human actin primers as a control in each experiment. The phosducin transcripts were PCR amplified from 1 μL of cDNA using a unique, 5′-human retina (hr) Phd, PhLP1, PhLOP1, or PhLOP2 sense primer (+) paired with a shared, 3′-Phd antisense primer (−) (identical nucleotide sequences for all four mRNAs).

Primers used for PCR are as follows: +hrPhd (93−118), 5′-CAC ATA CAG GAC CCA AAG GAG TAA TAA A-3′; +hrPhLP1 (69−98), 5′-ATG CCC GAG AGC CTG GAC AGC CCA ACC TCT-3′; +hrPhLOP1 (74−96), 5′-CAA CAA AAA GAC CAT TGA AGA AA-3′; +hrPhLOP2 (6−35), 5′-ATG TCC GAC AAG CCT GGC AGA AGG CAG ACA-3′; and −hrPhd/PhLP/PhLOP (712−690), 5′-CCA TAT TCA TTT AGG AAA GAC TC-3′.

Control primers for amplification of actin are as follows: +hβ-actin (206−223), 5′-TGA CGA GGC CCA GAG CAA-3′; and −hβ-actin (1169−1152), 5′-CTA GAA GCA TTT GCG GTG-3′.

In initial experiments, 4 μCi of [α-^{32}P]dCTP (Amersham) was added to each PCR mixture (final volume of 25 μL) to label the PCR products. Mixtures in subsequent experiments in which identical amplification conditions were used with unlabeled PCR products were resolved on a 1% agarose gel, transferred to a nylon membrane, and processed as previously described (*6*, *25*).

Electrophoresis and Immunoblot Analysis. A Bradford protein determination established the concentration of each sample, and for each sample, 50 μg of protein was utilized for electrophoretic analysis. Replicate 10% sodium dodecyl sulfate−polyacrylamide gel elecrophoresis (SDS−PAGE) resolved proteins between 5 and 100 kDa. One acrylamide gel was Coomassie Blue stained for proteins. Immunoblots were performed in replicate gels onto Immobilon-P Transfer Membrane using standard published protocols (Millipore, Bedford, MA). The immobilized specific antigens were detected using enhanced chemiluminescence (ECL) (Amersham). An appropriate dilution (1:500 to 1:5000) of the primary affinity-purified antibodies and a 1:5000 dilution of the second antibody (goat anti-rabbit IgG horseradish peroxidase conjugate, Bio-Rad) was used, following the manufacturer's recommendations.

Preparation of the Expressed GST Fusion Proteins and in Vitro Binding of G$\beta\gamma$ to GST−Phosducin and Phosducin-like Orphan Fusion Proteins. PCR fragments of each recombinant were inserted into the pGEX-3X vector (Pharmacia) using the *Bam*HI and *Eco*RI sites so that the cDNA inserts were in-frame downstream of the glutathione *S*-transferase (GST) cDNA and resequenced. The first two amino acid residues of recombinant proteins were converted to Pro and Gly in the fusion proteins. Expression of the GST fusion proteins was achieved as previously described (*27*). The soluble fractions of the GST fusion proteins were incubated with the glutathione−Sepharose 4B beads (Pharmacia) at 4 °C for 2 h, followed by washing three times with phosphate-buffered saline (PBS). In some experiments, the GST fusion proteins were used in a soluble form unbound to the Sepharose 4B beads. The cDNAs used for this study were the complete predicted amino acid translated coding regions of human Phd, PhLP1, PhLOP1, and PhLOP2, as well as a GST−bovine phosducin (GST−brPhd1−245) (*7*, *10*, *27*). A GST control without an insert was also prepared.

Preparation of the Purified Transducin G$\beta\gamma$. For affinity chromatography, the subunits (G$\beta\gamma$) of transducin were prepared according to the established methodology (*28*). The sucrose media used to suspend bovine rod outer segments (*29*) contained soluble proteins from both the outer and inner segments of bovine photoreceptors and were used as a source of the phosducin−G$\beta\gamma$ complex. This medium was subjected to DE52 (Whatman) chromatography, acid precipitation, ammonium sulfate precipitation, hydroxyapatite (Bio-Rad) chromatography, and cold Q Sepharose (Pharmacia) chromatography so the phosducin−G$\beta\gamma$ complex could be purified. A room-temperature Q Sepharose column was used to separate the phosducin from the G$\beta\gamma$. This purified G$\beta\gamma$ was used in the binding and chromatography studies. For plasmon resonance technology, purified G$\beta\gamma$ was prepared as described by Bigay and Chabre (*30*) from the bovine retina and diluted in PBS containing 0.01% Lubrol PX, and then added and used as previously described (*27*).

Purified G$\beta\gamma$ Binding to GST Fusion Proteins on Glutathione−Sepharose 4B. Ten micrograms of purified G$\beta\gamma$ in 100 μL of 10 mM HEPES (pH 7.3), 120 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 0.01% Tween 20 was combined with a 25 μL pellet of each GST fusion protein bound to glutathione−Sepharose 4B beads. The six GST fusion proteins tested were Phd, PhLP1, PhLOP1, PhLOP2, brPhd1−245, and a GST control. The samples were rotated at 4 °C for 2 h and centrifuged at 5000 rpm for 5 min, and the supernatants were saved for analysis. The pellets were washed four times with 200 μL of the HEPES buffer per wash. The supernatants, washes, and pellets were finally taken up in SDS−PAGE sample buffer, and the proteins retained on the Sepharose beads were separated on 10% SDS−polyacrylamide gels. Duplicate gels were prepared and treated as described above. The G$\beta\gamma$ was immunodetected using several sources of Gβ polyclonal antibodies made in rabbits [bovine retinal transducin Gβ (8IB), generated from purified transducin Gβ1 protein, which recognizes Gβs in retina and brain (*31*); Gβ (M-14), Santa Cruz #sc261, which identifies a conserved domain (amino acids 1−14) of the amino terminus of Gβ1 and is broadly reactive with Gβ1-4; Gβ5L, CT215, which identifies a retina-specific gene product (*32*), kindly provided by J. Chen, University of Southern California, Los Angeles]. The relative abundance of the various GST fusion proteins was determined by densitometric scanning of the appropriate protein-stained band. The autoradiographs were also densitometrically scanned to determine the relative amount of G$\beta\gamma$ bound to each GST fusion protein.

Binding of Endogenous G$\beta\gamma$ from a Bovine Retina Supernatant. The same GST fusion proteins used in the purified G$\beta\gamma$ binding study were tested for their ability to sequester G$\beta\gamma$ from a bovine retinal supernatant**.** Three bovine retina were homogenized in 3 mL of 50 mM PBS (pH 6.8). The 100000*g* soluble fraction was preadsorbed overnight with glutathione−Sepharose 4B to remove any proteins that might nonspecifically bind to the Sepharose. One-half of a milliliter of the preadsorbed supernatant was combined with a 25 μL pellet of each of the GST fusion proteins and a GST control. The mixtures were rotated overnight at 4 °C and centrifuged at 5000 rpm for 5 min, and the supernatants were saved for analysis. The supernatants, washes, and pellets were washed four times with 400 μL of the PBS buffer per wash. The pellets were resuspended in SDS−PAGE sample buffer and analyzed as described above.

Binding of Purified G$\beta\gamma$ to GST−brPhd1−245, PhLP1, and PhLOP2 on Sucrose Gradients. Ten micrograms of purified G$\beta\gamma$ was added to 50 μL of the concentrated soluble GST fusion proteins (PhLP1, PhLOP2, and control, not bound to glutathione−Sepharose 4B beads). The samples were allowed to incubate at 4 °C for 1 h before being applied to the top of 5 to 20% sucrose gradients made in 5 mM MES (pH 6.9), 100 mM KCl, 0.2 mM EDTA, 15 mM 2-β-mercaptoethanol, and 0.1 mM PMSF. The gradients were centrifuged at 51.5 K for 16 h in a SW 60 Ti rotor (Beckman). The tubes were fractionated from the bottom to the top in 27 separate, 150 μL fractions. The proteins in the fractions were electrophoretically separated on 10% SDS−PAGE and subsequently identified by Coomassie Blue and silver stain. The gels were densitometrically scanned on the Eagle Eye (Stratagene, Inc.), and the data were analyzed with the statistical package Prism 2.0 (GraphPad Software, Inc., San Diego, CA).

Kinetic Analysis of Interactions of GST−Phd, −PhLP1, and −PhLOP1 with G$\beta\gamma$ on BIAcore. G$\beta\gamma$ complexes from bovine rod outer segments (ROS) were biotinylated with NHS-LC-biotin (Pierce) and then coupled to the streptavidin-precoated sensor chip (SA5). The biotinylation protocol of

G$\beta\gamma$ was critical for protecting the functional activity since G$\beta\gamma$ complexes retained their functional activity after labeling only if they were protected from excessive modification by Gαo. Briefly, washed bovine ROS were treated with NHS-LC-biotin, and then the G$\beta\gamma$ subunits were specifically extracted with a hypotonic buffer, concentrated, and incubated with a histidine-tagged Gα_o protein immobilized on Ni−NTA beads. The pure biotinylated G$\beta\gamma$ complexes were eluted with a buffer containing NaAlF and used for immobilization on the SA5 sensor chip. The biotinylated G$\beta\gamma$ complexes were capable of interacting with G$\beta\gamma$ subunits, purified bovine phosducin, the PH domain of the β-adrenergic receptor kinase, the C-terminal fragment of the effector K-channel, and GIRK1 (data not shown). Less than 0.5 μg of the biotinylated G$\beta\gamma$ was sufficient for the immobilization during an 8 min injection at a flow rate of 5 mL/min. Immobilization densities of 500−2000 RU of G$\beta\gamma$ were used in kinetic experiments.

The running buffer for phosducin binding contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM methanol, and 0.01% Lubrol PX. GST fusion proteins were eluted from the glutathione−Sepharose 4B beads in 50 mM Tris-HCl (pH 8.0) with 10 mM reduced glutathione. In a standard experiment, a GST fusion protein preparation was diluted in the running buffer to achieve a desired concentration of the protein and injected over the surface for 8 min at a flow rate of 5 mL/min. The sensor surface regenerated after a lengthy (>1 h) wash with the running buffer, allowing complete wash-out of the bound GST−Phd or GST−PhLP1. In the kinetic experiments using the injections with different concentrations of Phd, an equal amount of G$\beta\gamma$ was immobilized on separate channels of the sensor chip and various concentrations of GST−Phd or GST−PhLP1 were perfused through the channels. Usually, G$\beta\gamma$ retained about 50% of the activity after 24 h on the chip and up to 10 injections.

The specificity of binding was verified by analyzing the blank (bovine serum albumin or GST control) injections over the surface with the immobilized G$\beta\gamma$ and by injecting the analyte over the surface without active G$\beta\gamma$. Although we did not observe significant nonspecific binding, the background was routinely subtracted from the sensograms prior to kinetic analysis which was performed using the BIAevaluation 2.1 software (Biosensor).

RESULTS

Molecular Identification and Characterization of Phosducin Isoforms. First-strand cDNA was synthesized by reverse transcriptase (RT) from poly-A$^+$ mRNA isolated from rat tissue and amplified by PCR using Phd primers designed from the nucleotide regions surrounding the encoded amino acid domain that has been implicated in G$\beta\gamma$ binding. Using rat retina phosducin cDNA as a reference, the predicted RT-PCR product should consist of 299 nucleotides with the following primers (Figure 1): +Phd[29−49]-21D, 5′-GAG GAA GAT TTT GAA GGA CAG-3′ (amino acids 10−16, EEDFEGQ); and −Phd[328−305]-24U, 5′-AGG CCC AAA ACT CAG CTT CTG GTG-3′ (amino acids 102−109, HQKLSFGP) (*6*, *7*). The predicted PCR amplification product for Phd was observed in all samples; however, multiple PCR products were also amplified from the cDNAs (Figure 1), including one from kidney that required an exposure for detection longer than that needed for the other transcripts. Selected PCR fragments were chosen for analysis and reamplified, subcloned into appropriate vectors, and sequenced for verification (data not shown). A phosducin-like protein (PhLP) cDNA, representing an mRNA from rat cerebral cortex (Figure 1, asterisk), was chosen as a molecular probe for library screening.

Eleven cDNAs, ranging in size from 1200 to 1600 nucleotides (nt), were isolated in the DNA−DNA hybridization screen of a human retina (hr) library. Six isolates amplified with the original antisense, −Phd[328−305] primer were coupled with a sense primer that targeted the vector. Upon sequence analysis in both orientations of the respective isolates, three were dissimilar to Phd, and they constitute the focus of this report. Upon characterization of these isolates and analysis of their capacity to bind G$\beta\gamma$, they were renamed: hrPhd 11-7 = Phd, hrPhLP 10-5 = PhLP1, hrPhLP 12-11 = PhLOP1, and hrPhLP 1-1 = PhLOP2 [accession numbers AF076463 (PhLP1), AF076464 (PhLOP1), and AF076465 (PhLOP2), data not shown] (*10*). The cDNAs for each isolate, including 5′- and 3′-noncoding regions, are aligned in Figure 2A−D: (A) PhLP1, 1341 nt; (B) PhLOP1, 1222 nt; (C) PhLOP2, 1440 nt; and (D) Phd, 1199 nt. The amino acid sequences are translated into their respective predicted single-amino acid sequences and aligned in Figure 3.

The longest open reading frame is depicted in uppercase letters, and the first, in-frame, ATG encoding a methionine (M) was chosen for each predicted amino acid translation and is bold and underlined. For each cDNA, the corresponding predicted ATG start codon is displayed as is the termination codon (***). Each cDNA contained a 5′- and 3′-noncoding sequence depicted in lowercase letters, a polyadenylation signal (aataaa), upstream from a poly-A$^+$ tail. Published exon−intron splicing sites for the Phd gene (*33*−*35*) are identified and labeled with a box and an arrow designating the published exons, E1−E4 (Figure 2A−D).

The nucleotide sequence for Phd (Figure 2D) that was obtained in this study is almost identical to that of the published mRNA encoding human phosducin (*33*−*36*). The mRNA was obtained from pooled human retinas, and eight nucleotide variants or polymorphisms were observed: one (nt 30) in exon 2 and seven (nt 353, 488, 511, 976, 977, 1040, and 1173) in exon 4 (Figure 2D, bold and underlined). Only one amino acid was altered by the nucleotide variants observed (nt 511, T to C; amino acid 158, L to P), and this occurred in a highly conserved amino acid. From published work, the Phd gene is organized into four exons; exons 2−4 are displayed in Figure 2D, but exon 1 is absent from this figure since it is situated in the 5′-flanking region, which was not included in our Phd cDNA isolate.

PhLP1 is also organized into at least four predicted exons. From published information about the 5′-flanking region of the human Phd gene, exon 1 begins at nt 116 (double arrows in Figure 2A,D). Thus, exon 1 of PhLP1 contains nucleotides (nt 116−155) that are present in the 5′-flanking region of the human Phd gene (*34*). Additional exons upstream of the published exon 1 must contain nucleotides 1−115, which are novel for this Phd isoform. This sequence was used to generate a unique probe for identification of the PhLP1 isoform [Figure 2A, in the far right box, the sense +Phd

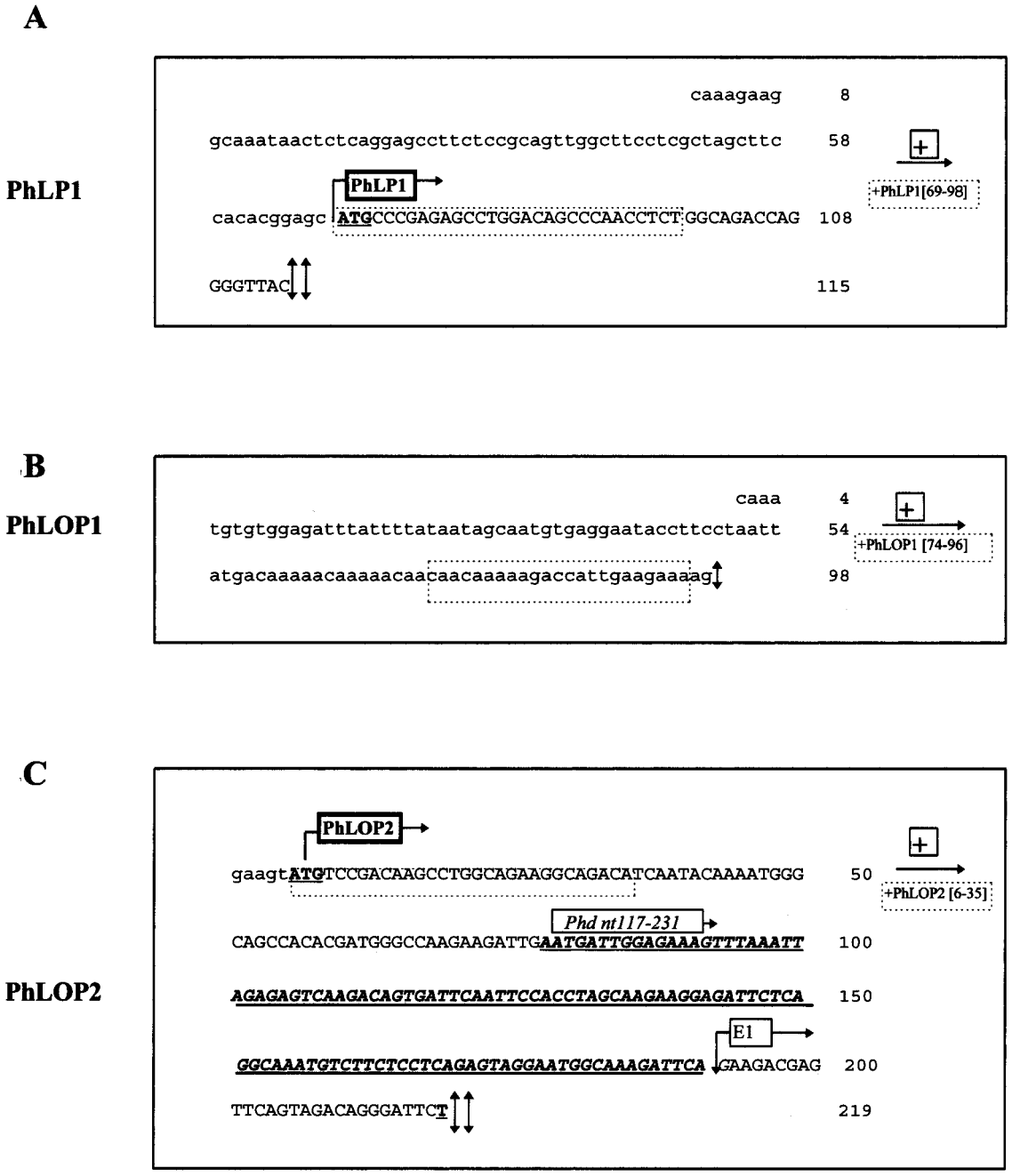
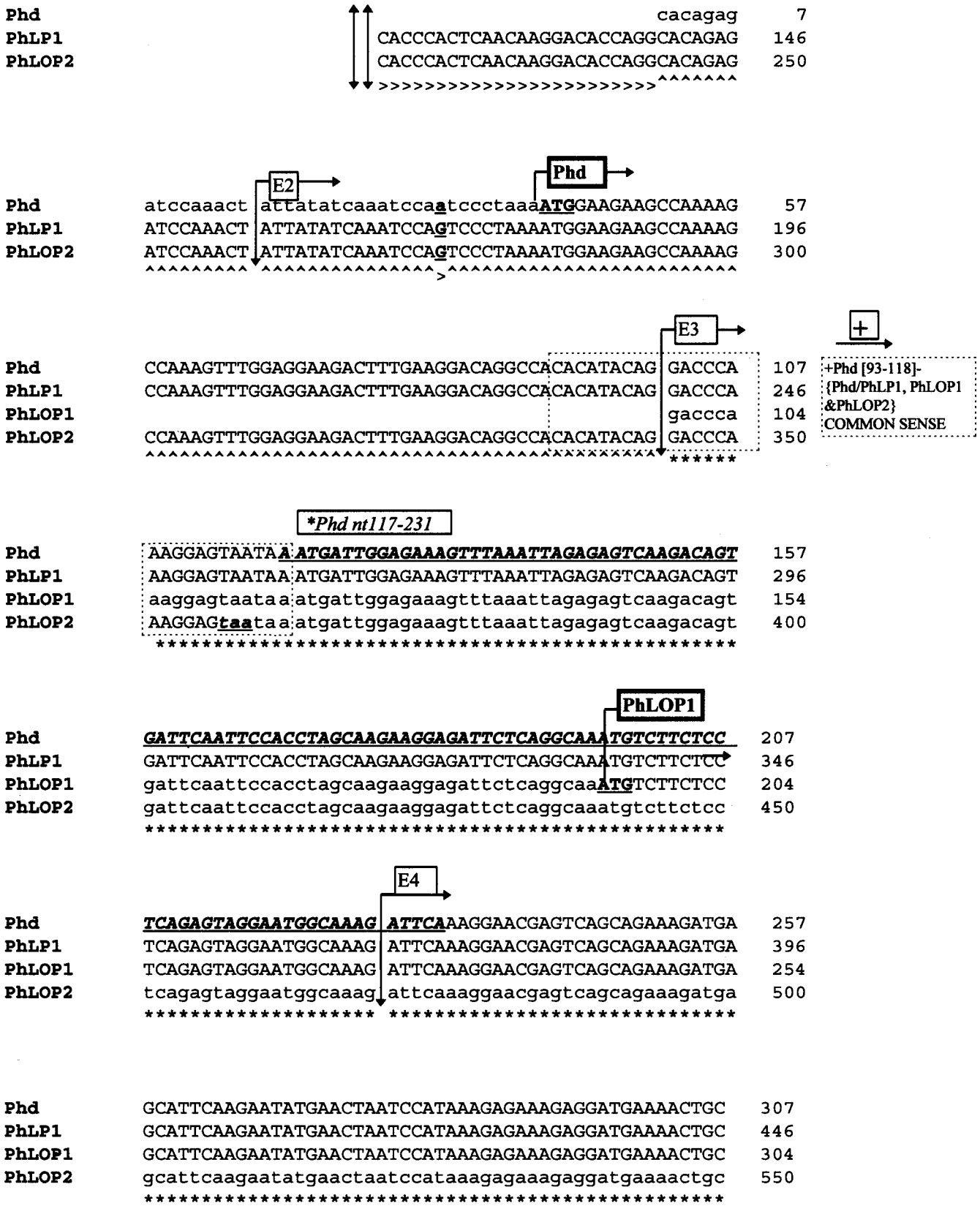


FIGURE 2: (A−D) The 5′-unique nucleotide sequence of PhLP1 (A), PhLOP1 (B), and PhLOP2 (C) and complete nucleotide alignment of the phosducin family (D). The complete nucleotide sequence (lowercase, noncoding regions; and uppercase, coding regions) of each of the phosducin isoforms, PhLP1 (A), PhLOP1 (B), PhLOP2 (C), and Phd (D), with the polyadenylation signal marked by a box preceding the poly-A^+ tail. The predicted translated amino acid of the open reading frame cDNA sequence, without the first and second amino acids, was used in constructs for the GST fusion proteins (see Figure 3). The alignment of the shared regions among the four isoforms was created with the CLUSTAL program. The predicted translation (**ATG**) is indicated with the appropriate isoform boxed and an arrow pointing 3′. The sense (+) and antisense (−) primers are indicated within the sequence and to the right with dotted line boxes, the appropriate name, and nucleotide numbers. Primers identified on the nucleotide sequence are listed in Experimental Procedures: +hrPhd (93−118), +hrPhLP1 (69−98), +hrPhLOP1 (74−96), +hrPhLOP2 (6−35), and -hrPhd isoform (712−690). (A) PhLP1 contains a unique 115 nt sequence with two double-headed arrowheads indicating where it joins the alignment (panel D). The first in-frame ATG begins at nt 69. (B) PhLOP1 contains a unique 98 nt, 5′-noncoding region. The single, double-headed arrow aligns with exon 3 in panel D. PhLOP1 does not have either exon 1 or 2 of the Phd gene present in the PhLOP1 cDNA sequence; however, alternative splicing of the Phd gene is responsible for these 98 nt since an alternative exon is identified within the intron downstream of exon 2 in the Phd gene structure (46). (C) PhLOP2 has 219 nt of unique sequence in the mRNA, including a duplication of nt 117−231 in bold, underlined, and italics (see *Phd nt 117−231, panel D) and nt 192−219 of the 5′-flanking region of Phd (E1, arrow) (*34*). This includes a **T** (nt 219) that shifts the open reading frame of PhLOP2 that is predicted to start at nt 6, substantially reducing the predicted translated amino acid homology of the isoforms (see Figure 3). (D) Note the high degree of nucleotide identity (*) for all isoforms extending 3′ from exon 3 (double-headed arrow), and the nucleotide differences are bold and underlined with three matches indicated by the symbol ∧ and two matches indicated by the symbol >. PhLP1 and PhLOP2 share nucleotide identity from double arrows and from the beginning of the cDNA for Phd and from exon 3 with PhLOP1, except where indicated in bold and underlined. Arrows identify predicted exons (E 1−4) previously identified within the human Phd gene (*34*, *35*). Note that our Phd cDNA (panel D) lacks the published complete 5′-noncoding region.

D


Phd cacagag 7
PhLP1 CACCCACTCAACAAGGACACCAGGCACAGAG 146
PhLOP2 CACCCACTCAACAAGGACACCAGGCACAGAG 250
E2
Phd
Phd atccaaact attatatcaaatccaatccctaaaATGGAAGAAGCCAAAAG 57
PhLP1 ATCCAAACT ATTATATCAAATCCAGTCCCTAAAATGGAAGAAGCCAAAAG 196
PhLOP2 ATCCAAACT ATTATATCAAATCCAGTCCCTAAAATGGAAGAAGCCAAAAG 300
E3
+
Phd CCAAAGTTTGGAGGAAGACTTTGAAGGACAGGCCACACATACAG GACCCA 107
PhLP1 CCAAAGTTTGGAGGAAGACTTTGAAGGACAGGCCACACATACAG GACCCA 246
PhLOP1 gaccca 104
PhLOP2 CCAAAGTTTGGAGGAAGACTTTGAAGGACAGGCCACACATACAG GACCCA 350
+Phd [93-118]-{Phd/PhLP1, PhLOP1 &PhLOP2} COMMON SENSE
*Phd nt117-231
Phd AAGGAGTAATAAATGATTGGAGAAAGTTTAAATTAGAGAGTCAAGACAGT 157
PhLP1 AAGGAGTAATAAATGATTGGAGAAAGTTTAAATTAGAGAGTCAAGACAGT 296
PhLOP1 aaggagtaataaatgattggagaaagtttaaattagagagtcaagacagt 154
PhLOP2 AAGGAGtaataaatgattggagaaagtttaaattagagagtcaagacagt 400
PhLOP1
Phd GATTCAATTCCACCTAGCAAGAAGGAGATTCTCAGGCAAATGTCTTCTCC 207
PhLP1 GATTCAATTCCACCTAGCAAGAAGGAGATTCTCAGGCAAATGTCTTCTCC 346
PhLOP1 gattcaattccacctagcaagaaggagattctcaggcaaATGTCTTCTCC 204
PhLOP2 gattcaattccacctagcaagaaggagattctcaggcaaatgtcttctcc 450
E4
Phd TCAGAGTAGGAATGGCAAAGATTCAAAGGAACGAGTCAGCAGAAAGATGA 257
PhLP1 TCAGAGTAGGAATGGCAAAGATTCAAAGGAACGAGTCAGCAGAAAGATGA 396
PhLOP1 TCAGAGTAGGAATGGCAAAGATTCAAAGGAACGAGTCAGCAGAAAGATGA 254
PhLOP2 tcagagtaggaatggcaaagattcaaaggaacgagtcagcagaaagatga 500
Phd GCATTCAAGAATATGAACTAATCCATAAAGAGAAAGAGGATGAAAACTGC 307
PhLP1 GCATTCAAGAATATGAACTAATCCATAAAGAGAAAGAGGATGAAAACTGC 446
PhLOP1 GCATTCAAGAATATGAACTAATCCATAAAGAGAAAGAGGATGAAAACTGC 304
PhLOP2 gcattcaagaatatgaactaatccataaagagaaagaggatgaaaactgc 550

```
Phd       CTTCGTAAATACCGTAGACAGTGTATGCAGGATATGCACCAGAAGCTCAG  357
PhLP1     CTTCGTAAATACCGTAGACAGTGTATGCAGGATATGCACCAGAAGCTCAG  496
PhLOP1    CTTCGTAAATACCGTAGACAGTGTATGCAGGATATGCACCAGAAGCTGAG  354
PhLOP2    cttcgtaaataccgtagacagtgtatgcaggatatgcaccagaagctgag  600
          **************************************************>**

Phd       TTTTGGGCCTAGATATGGGTTTGTGTATGAGCTGGAAACTGGAAAGCAAT  407
PhLP1     TTTTGGGCCTAGATATGGGTTTGTGTATGAGCTGGAAACTGGAAAGCAAT  546
PhLOP1    TTTTGGGCCTAGATATGGGTTTGTGTATGAGCTGGAAACTGGAAAGCAAT  404
PhLOP2    ttttgggcctagatatgggtttgtgtatgagctggaaactggaaagcaat  650
          **************************************************

Phd       TCCTAGAAACAATTGAAAAGGAACTGAAGATCACCACAATTGTTGTTCAC  457
PhLP1     TCCTAGAAACAATTGAAAAGGAACTGAAGATCACCACAATTGTTGTTCAC  596
PhLOP1    TCCTAGAAACAATTGAAAAGGAACTGAAGATCACCACAATTGTTGTTCAC  454
PhLOP2    tcctagaaacaattgaaaaggaactgaagatcaccacaattgttgttcac  700
          **************************************************

Phd       ATTTATGAAGATGGTATTAAGGGTTGTGATGCCCTAAACAGTAGTTTAAC  507
PhLP1     ATTTATGAAGATGGTATTAAGGGTTGTGATGCTCTAAACAGTAGTTTAAC  646
PhLOP1    ATTTATGAAGATGGTATTAAGGGTTGTGATGCTCTAAACAGTAGTTTAAC  504
PhLOP2    atttatgaagatggtattaagggttgtgatgctctaaacagtagtttaac  750
          **********************************^***************

Phd       ATGCCCTGCAGCAGAATACCCTATAGTTAAGTTTTGTAAAATAAAAGCTT  557
PhLP1     ATGCCTTGCAGCAGAATACCCTATAGTTAAGTTTTGTAAAATAAAAGCTT  696
PhLOP1    ATGCCTTGCAGCAGAATACCCTATAGTTAAGTTTTGTAAAATAAAAGCTT  554
PhLOP2    atgcctTgcagcagaataccctatagttaagttttgtaaaataaaagctt  800
          *****^**********************************************

Phd       CGAATACAGGTGCTGGGGACCGCTTTTCCTTAGATGTACTTCCTACACTG  607
PhLP1     CGAATACAGGTGCTGGGGACCGCTTTTCCTTAGATGTACTTCCTACACTG  746
PhLOP1    CGAATACAGGTGCTGGGGACCGCTTTTCCTTAGATGTACTTCCTACACTG  604
PhLOP2    cgaatacaggtgctggggaccgcttttccttagatgtacttcctacactg  850
          **************************************************

Phd       CTCATCTATAAAGGTGGGGAACTCATAAGCAATTTTATTAGTGTTGCTGA  657
PhLP1     CTCATCTATAAAGGTGGGGAACTCATAAGCAATTTTATTAGTGTTGCTGA  796
PhLOP1    CTCATCTATAAAGGTGGGGAACTCATAAGCAATTTTATTAGTGTTGCTGA  654
PhLOP2    ctcatctataaaggtggggaactcataagcaattttattagtgttgctga  900
          **************************************************

Phd       ACAGTTTGCTGAAGAATTTTTTGCTGGGGATGTGGAGTCTTTCCTAAATG  707
PhLP1     ACAGTTTGCTGAAGAATTTTTTGCTGGGGATGTGGAGTCTTTCCTAAATG  846
PhLOP1    ACAGTTTGCTGAAGAATTTTTTGCTGGGGATGTGGAGTCTTTCCTAAATG  704
PhLOP2    acagtttgctgaagaatttttgctggggatgtggagtctttcctaaatg   950
          **************************************************

Phd       AATATGGGTTACTACCTGAAAGAGAGGTACATGTCCTAGAGCATACCAAA  757
PhLP1     AATATGGGTTACTACCTGAAAGAGAGGTACATGTCCTAGAGCATACCAAA  896
PhLOP1    AATATGGGTTACTACCTGAAAGAGAGGTACATGTCCTAGAGCATACCAAA  754
PhLOP2    aatatgggttactacctgaaagagaggtacatgtcctagagcataccaaa 1000
          **************************************************
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-Phd [712-690]
{Phd/PhLP1/PhLOP1 & 2
COMMON ANTISENSE}

```
Phd       ATAGAAGAAGAAGATGTTGAATGAAGATTCACTATGTCAATATCTCATGT   807
PhLP1     ATAGAAGAAGAAGATGTTGAATGAAGATTCACTATGTCAATATCTCATGT   946
PhLOP1    ATAGAAGAAGAAGATGTTGAATGAAGATTCACTATGTCAATATCTCATGT   804
PhLOP2    atagaagaagaagatgttgaatgaagattcactatgtcaatatctcatgt  1050
          **************************************************

Phd       TTATCCTTTAGGTATTGGATGATGGTTTTGGTAGTATCTATATTGCTTTA   857
PhLP1     TTATCCTTTAGGTATTGGATGATGGTTTTGGTAGTATCTATATTGCTTTA   996
PhLOP1    TTATCCTTTAGGTATTGGATGATGGTTTTGGTAGTATCTATATTGCTTTA   854
PhLOP2    ttatcctttaggtattggatgatggttttggtagtatctatattgcttta  1100
          **************************************************

Phd       GTGAACACAGAGTATGGGCACGGCTATGCTAACTtgacaaaaatgactga   907
PhLP1     GTGAACACAGAGTATGGGCACGGCTATGCTAACTtgacaaaaatgactga  1046
PhLOP1    GTGAACACAGAGTATGGGCACGGCTATGCTAACTtgacaaaaatgactga   904
PhLOP2    gtgaacacagagtatgggcacggctatgctaacttgacaaaaatgactga  1150
          **************************************************

Phd       tgcaacaatcgagttattagcatttcttagtattagttactcaaattgat   957
PhLP1     tgcaacaatcgagttattagcatttcttagtattagttactcaaattgat  1096
PhLOP1    tgcaacaatcgagttattagcatttcttagtattagttactcaaattgat   954
PhLOP2    tgcaacaatcgagttattagcatttcttagtattagttactcaaattgat  1200
          **************************************************

Phd       acaatgcttgactacaaaacaaagctgtcttcagcaacattattagtaga  1007
PhLP1     acaatgcttgactacaaaacagagctgtcttcagcaacattattagtaga  1146
PhLOP1    acaatgcttgactacaaaacgaagctgtcttcagcaacattattagtaga  1004
PhLOP2    acaatgcttgactacaaaacagagctgtcttcagcaacattattagtaga  1250
          ********************^>****************************

Phd       caaagaggatgtggataatattatgacattttcaaaaatccctttcaag  1057
PhLP1     caaagaggatgtggataatattatgacattttcaaaaatccctttcaag  1196
PhLOP1    caaagaggatgtggataatattatgacattttcaaaaatccctttcaag  1054
PhLOP2    caaagaggatgtggataatattatgacattttcgaaaatccctttcaag  1300
          **********************************^***************

Phd       ttatgttttgtctttttactccattttccctcatcactgttattatttg  1107
PhLP1     ttatgttttgtctttttactccattttccctcatcactgttattatttg  1246
PhLOP1    ttatgttttgtctttttactccattttccctcatcactgttattatttg  1104
PhLOP2    ttatgttttgtctttttactccattttccctcatcactgttattatttg  1350
          **************************************************

Phd       gactttcaaattacattattcattataatttctttgtgtaataaaaat  1157
PhLP1     gactttcaaattacattattcattataatttctttgtgtaataaaaat  1296
PhLOP1    gactttcaaattacattattcattataatttctttgtgtaataaaaat  1154
PhLOP2    gactttcaaattacattattcattataatttctttgtgtaataaaaat  1400
          **********************************************

Phd       gaaatctcatgaggaaataaaaaaaaaaaaaaaaaaaaaaaaaa------  1199
PhLP1     gaaatctcatgagaaaataaaaaaaaaaaaaaaaaaaaaaaaa-----  1341
PhLOP1    gaaatctcatgaggaaataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  1204
PhLOP2    gaaatctcatgagaaaataaaaaaaaaaaaaaaaaaaaaa----------  1440
          *************.**************************

Phd       ------------------  1199
PhLP1     ------------------  1341
PhLOP1    aaaaaaaaaaaaaaaaa   1222
PhLOP2    ------------------  1440
```

```
PHD                                                        MEEAKSQSLEEDFEGQAT    18
PHLP1       MPESLDSPTSGRPGVTTHSTRTPGTEIQTIISNPVPKMEEAKSQSLEEDFEGQAT    55
PHLOP1                                                                          0
PHLOP2                                                     MSDKLGRRQTSIQNGQP-    17

                                                                          +
PHD         HTGPKGVINDWRKFKLESQDSDSIPPSKKEILRQMSSPQSRNGKDSKERVSRKMS    73
PHLP1       HTGPKGVINDWRKFKLESQDSDSIPPSKKEILRQMSSPQSRNGKDSKERVSRKMS   110
PHLOP1                                            MSSPQSRNGKDSKERVSRKMS    21
PHLOP2      HDGPR-RLNDWRKFKLESQDSDSIPPSKKEILRQMSSPQSRNGKDSEDEFSR---    68
                                                  ************..  **

PHD         IQEYELIHKEKEDENCLRKYRRQCMQDMHQKLSFGPRYGFVYELETGKQFLETIE   128
PHLP1       IQEYELIHKEKEDENCLRKYRRQCMQDMHQKLSFGPRYGFVYELETGKQFLETIE   165
PHLOP1      IQEYELIHKEKEDENCLRKYRRQCMQDMHQKLSFGPRYGFVYELETGKQFLETIE    76
PHLOP2      -QGFSPTQQGHQAQ----------------------RSKLLYQIQS---------    91
             *... ........                       *  ..*....

PHD         KELKITTIVVHIYEDGIKGCKALNSSLTCPAAEYPIVKFCKIKASNTGAGDRFSL   183
PHLP1       KELKITTIVVHIYEDGIKGCDALNSSLTCLAAEYPIVKFCKIKASNTGAGDRFSL   220
PHLOP1      KELKITTIVVHIYEDGIKGCKALNSSLTCLAAEYPIVKFCKIKASNTGAGDRFSL   131
PHLOP2      --LKWKKPKAKVWRKTLK-----------------------------------     107
              ** ..  .... ...*

PHD         DVLPTLLIYKGGELISNFISVAEQFAEEFFAGDVESFLNEYGLLPEREVHVLEHT   238
PHLP1       DVLPTLLIYKGGELISNFISVAEQFAEEFFAGDVESFLNEYGLLPEREVHVLEHT   275
PHLOP1      DVLPTLLIYKGGELISNFISVAEQFAEEFFAGDVESFLNEYGLLPEREVHVLEHT   186
PHLOP2      ------------------------------------------------DRP     110
                                                                ...

PHD         KIEEEDVE   246
PHLP1       KIEEEDVE   283
PHLOP1      KIEEEDVE   194
PHLOP2      HIQDPK-E   117
            .*.. . *
```

FIGURE 3: Amino acid sequence alignment of phosducin, phosducin-like protein 1, and phosducin-like orphan proteins 1 and 2. The predicted amino acid translations of human retina phosducin and its isoforms were aligned using the PCGENE CLUSTAL (Intelligenetics). Identical residues of all four sequences and similar residues shared among three of the four sequences are marked by stars and periods, respectively. The solid boxed amino acid sequence (TGPKGVINDWR) represents the structural domain for G$\beta\gamma$ binding, and the + identifies the Phd, PKA Ser73 phosphorylation site. The following amino acids are considered similar in this comparison: alanine (A), serine (S), and threonine (T); aspartic acid (D) and glutamic acid (E); asparagine (N) and glutamine (Q); arginine (R) and lysine (K); isoleucine (I), leucine (L), methionine (M), and valine (V); and phenylalanine (F), tryptophan (W), and tyrosine (Y). Cysteine (C), histidine (H), proline (P), and glycine (G) are not considered to be similar to any other amino acid.

(69−98) primer was paired with an antisense −Phd (712−690) primer; Figure 2D]. Moreover, nt 1−115 were identified in BLASTN searches of GenBank databases to be 100% identical to three expressed sequence tags (ESTs, accession numbers U46456, N87272, and N56388), and significantly identical to rat Enigma [accession numbers U48247 and RNU48247, 9.9×10^{-5}, 39 out of 46 (84%) in the range of nt 966−1011 and 54 out of 82 (64%) in the range of nt 984−1065] (*37*, *38*). There is a high degree of homology among the Phd isoforms after exon 3, demonstrating why these isoforms were selected with the cerebral cortex cDNA, PCR probe amplified with +Phd (29−49) paired with −Phd (328−305) during screening by DNA−DNA hybridization of the human retina library.

The sequence of PhLOP1 is identical to those of Phd and PhLP1 from the beginning of published exon 3 (nt 99) to the poly-A^+ tail in exon 4 (nt 1147). There is a predicted start codon at nt 195 (Figure 2D) that extends as an open reading frame (nt 195−888) through the remainder of exon 3 and exon 4. A 5′-flanking sequence extends from nt 1−98 that is novel and from which a specific probe (Figure 2B,D) was developed [+PhLOP1 (74−96) paired with −Phd (712−690)].

PhLOP2 has a methionine at nt 5 (Figure 2C) and an open reading frame of 352 nucleotides (6−356, Figure 2C,D) with exon 2 and 3 encoding unerringly from nt 220 to 356. A premature stop codon (nt 361) is generated as a result of the additional 5′-flanking sequence from exon 1, initiating a

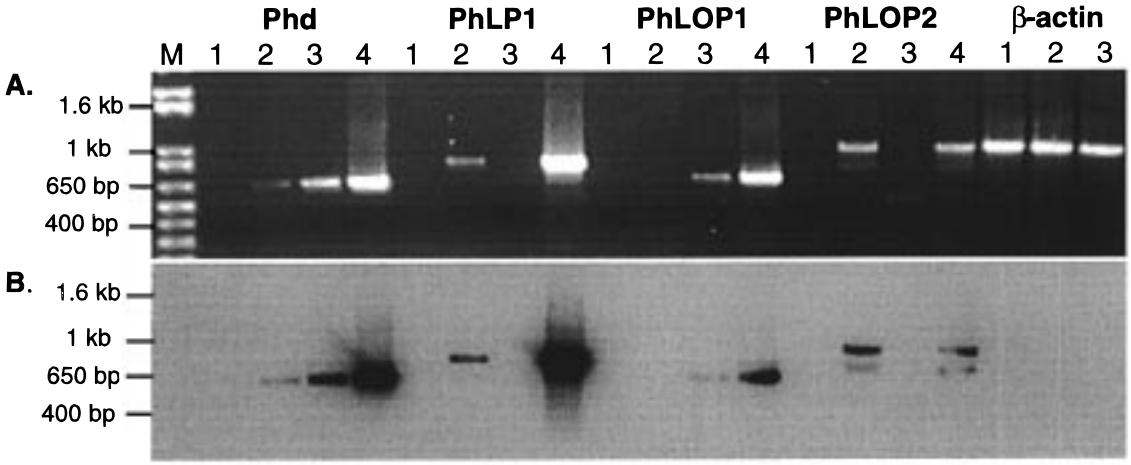

FIGURE 4: Phd isoform expression of specific mRNAs in adult human retina and forskolin-stimulated Y79 retinoblastoma cells. The top and bottom panels represent RT-PCR analysis of the Phd isoforms visualized with 1% agarose gel electrophoresis and a Southern blot analysis of the gel transferred onto nylon membrane and probed with a random primed, radiolabeled [α-P^{32}]dCTP Phd cDNA, respectively. Specific phosducin isoform primers (±) and hybridization conditions are described in Experimental Procedures and Figure 2. Five of 20 μL of the undiluted PCR reaction volume was resolved from each sample. One microliter of first-strand synthesized cDNA was used for each PCR from a reaction volume of 20 μL of transcribed RNA [1 μg of total RNA from each sample of Y79 cells or 0.5 μg from poly-A^+ mRNA (human retina, Clontech)] with the cDNA Cycle Kit using oligo-dT primer (Invitrogen): lane 1, unstimulated Y79 cells; lane 2, forskolin-stimulated Y79 cells; lane 3, human retina; and lane 4, positive cDNA control for each isoform identified above in bold letters, from left to right, Phd, PhLP1, PhLOP1, and PhLOP2. β-Actin in the last panel was a control for the integrity of the cDNA. M [kilobases (kb)] represents molecular mass markers of a 1 kb DNA ladder (Gibco BRL) for determining the size of PCR-amplified fragments: left to right, Phd, 619 nt; PhLP1, 784 nt; PhLOP1, 637 nt; PhLOP2, 951 nt; and β-actin, 963 nt. These data are representative of three independent experiments. Each PCR-amplified product was independently subcloned, sequenced, and verified as the phosducin isoform was targeted and amplified (data not shown).

reading frame shift at nt 219 (**T**), translating a predicted amino acid sequence for PhLOP2 that differs from that of Phd, PhLP1, and PhLOP1. The 5′-region can be divided into three nucleotide regions. That closest to nt 219 (nt 192−219) is identical to the published nucleotide sequence in the 5′-flanking region of Phd exon 1 (*34*). The intermediate region (nt 78−191) is identical to the nucleotide sequence (nt 117−231, bold and underlined) of a region within exon 3 of Phd (Figure 2D, boxed *Phd nt 117−231). The sequence at the 5′-region (nt 1−77) is novel, and from it was generated a distinguishing probe [+PhLOP2 (6−35) paired with −Phd (712−690)] for PhLOP2. The novel sequence at nt 1−77 was identified through database searches to correspond to numerous ESTs that encode a trinucleotide repeat mRNA (accession number L10376) (*39*, *40*).

The amino acid alignment for each isolate is compared to that of Phd (Figure 3). The N-terminal Gβγ binding domain, Phd (amino acids 20−30, TGPKGVINDWR) is boxed with a bold, solid line, and amino acid 73 of Phd is marked by a plus (+) to show the single PKA phosphorylation site in Phd. The predicted masses of each recombinant are as follows: Phd, 246 amino acids, 28 259 Da; PhLP1, 283 amino acids, 32 118 Da; PhLOP1, 194 amino acids, 22 330 Da; and PhLOP2, 117 amino acids, 13 764 Da. When the sequences are aligned and compared with the Clustal analysis program (Intelligenetics PCGene), amino acid identity of all four (*) and similarity of three of the four (.) Phd isoforms is 7.8 and 12.4%, respectively. This is in contrast to a 93% identity at the nucleotide level (Figure 3). Phd and PhLP1 have 99.5% identity between the boundaries defined by amino acids 1 and 246 of Phd.

PhLP1 contains the predicted amino acid sequence for the Gβγ binding site (Figure 3), and it has a serine (amino acid 110) that is equivalent to Ser73 in Phd, the site essential for PKA phosphorylation. PhLOP1 lacks the Gβγ binding site completely, but it does contain a serine (amino acid 21) equivalent to that at amino acid 73 in Phd. PhLOP2 has a diminutive Gβγ binding site, retaining only five of the eleven amino acids that form the proposed site in Phd or PhLP1, and a target for PKA phosphorylation equivalent to Ser73 is absent from PhLOP2.

PhLP1 is a long form of Phd, with differences in predicted amino acids (Figure 3) occurring only in the N terminus. Both PhLOP1 and PhLOP2 are significantly different from Phd and PhLP1. PhLOP1 has a shortened N terminus without alteration to the C terminus. PhLOP2 contains an N terminus of divergent composition and a greatly shortened C terminus. As a result of the additions and deletions, only a 12-amino acid domain (MSSPQSRNGKDS) is shared among all four isoforms (Figure 3).

mRNA Expression of Phosducin Isoforms in Vivo and in Vitro. The expression of mRNAs for each phosducin isoform was observed in either Y79 retinoblastoma cell lines after stimulation with forskolin (Figure 4A,B, lanes 1 and 2 of each panel) or human retina (Figure 4A,B, lane 3 of each panel). In adult human retinas, the rank order of expression was Phd > PhLOP1 while the extent of expression of PhLP1 and PhLOP2 was below the level of detection after one round of amplification. The detected messages for PhLP1 are the expected size and are detected in the stimulated Y79 cells. Unstimulated Y79 cells show virtually no expression of the Phd isoforms, but active expression of actin. Stimulated Y79 retinoblastoma cells upregulate gene expression substantially for Phd, PhLP1, and PhLOP2, but not for PhLOP1. By Northern blot analysis, the mRNA level of expression of the new phosducin isoforms is below detectable levels (data not shown).

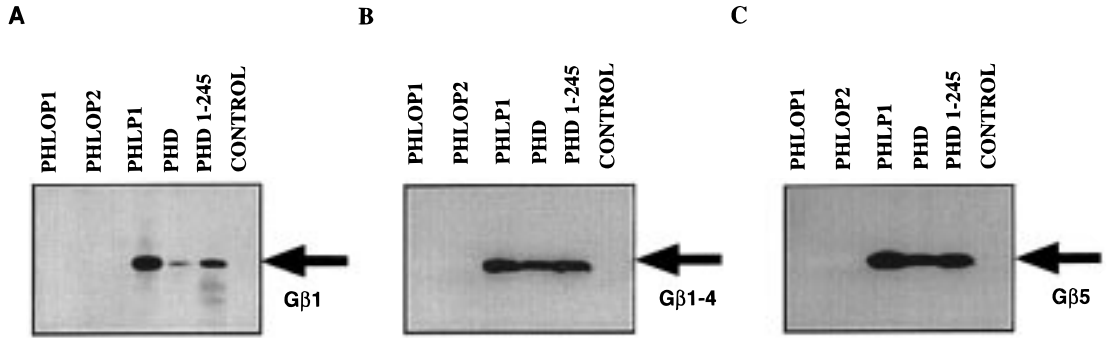

FIGURE 5: (A−C) Immunoblot analysis of protein interactions of GST−phosducin isoforms with either purified transducin G***βγ*** (A) or G***βγ***s (B and C) from the retinal supernatant. GST fusion constructs for each phosducin isoform [Phd, PhLP1, PhLOP1, PhLOP2, brPhd1−245, and GST control (Control)] were expressed, bound to glutathione−Sepharose 4B, and incubated with either purified transducin G$\beta\gamma$ or retinal supernatants. After elution from Sepharose and subjection to SDS−PAGE, proteins were either stained with Coomassie Blue (data not shown) or electrophoretically transferred to PVDF membranes for visualization of Gβs by immunoreactivity and ECL. (A) The GST−Phd fusion proteins interacting with purified transducin, G$\beta\gamma$ (*31*). (B) GST−Phd fusion proteins were incubated with the retinal supernatant and then processed for interaction with antibodies which recognize Gβ1−4 (sc#261). **(**C) Subsequently, the membrane was stripped and incubated with a Gβ5 antisera (*32*). The corresponding arrows indicate the location of the immunoreactive bands: Gβ1, Gβ1−4, or Gβ5. Neither the GST−PhLOP1, PhLOP2, nor GST control interacted significantly with G$\beta\gamma$s identified by antisera used.

Interaction of Phosducin Isoforms with Gβγ. Isoforms of phosducin were prepared as GST fusion proteins. The ability of the GST isoforms to bind the purified $\beta\gamma$ complex of transducin (G$\beta\gamma$) from bovine retinas (Figure 5A) or G$\beta\gamma$ isoforms from retinal supernatants (Figure 5B,C) was analyzed. Purified G$\beta\gamma$ was bound by the GST fusion proteins of Phd, PhLP1, and bovine phosducin (brPhd1−245), but not by the GST fusion proteins of PhLOP1 and PhLOP2 compared to the GST control. When a retinal supernatant was presented to the bead-bound, GST isoforms, a comparable pattern of binding was observed (Figure 5B,C). Antibodies shown to be specific for isoforms 1−4 of Gβ or isoform 5 of Gβ are sequestered above background by the GST fusion proteins of Phd 1−245, Phd, and PhLP1.

When it is considered that G$\beta\gamma$ binding sites may be masked upon attachment of isoforms to the glutathione−Sepharose 4B beads, sucrose density gradient centrifugation was used to evaluate independently the ability of the phosducin isoforms to bind G$\beta\gamma$ (Figure 6). Binding of the GST−Phd isoform ($S_v = 3.8$) to G$\beta\gamma$ creates a complex with increased mass, leading to a transposition to gradient fractions with higher densities ($S_v = 6.2$). GST−PhLP1 shifted, upon incubation with G$\beta\gamma$, from a sedimentation coefficient of 3.8 to 6.2 (Figure 6C), whereas neither GST−PhLOP2 nor a GST control changed position within the gradient, demonstrating a failure to form a complex with purified G$\beta\gamma$ (Figure 6A,B).

GST fusion proteins of Phd, PhLP1, and PhLOP1 were evaluated using surface plasmon resonance technology to determine whether the binding characteristics for G$\beta\gamma$ differed quantitatively between the isoforms (Figure 7). Kinetic studies on BIAcore showed that phosducin and PhLP1 bound G$\beta\gamma$ with comparable kinetics and with similar affinities for G$\beta\gamma$ (Figure 8). The dissociation rate constant was determined to be 5×10^{-4} s^{-1}, which is slightly higher than that identified in a previous report (*27*). This is most likely due to the slight change in the G$\beta\gamma$ preparation procedure. In this work, G$\beta\gamma$ was purified on immobilized Gαo, resulting in higher purity and more uniform labeling of G$\beta\gamma$. The association constant reported here is smaller than that reported earlier, but it was measured at 700−1500 M^{-1} s^{-1} for both Phd and PhLP1. Without detectable binding of G$\beta\gamma$, no kinetics could be obtained for PhLOP1 (Figure 7). In all BIAcore measurements, the two isoforms of phosducin, Phd and PhLP1, are very similar in their binding of G$\beta\gamma$, and in this respect, they appear to be indistinguishable.

DISCUSSION

Phosducin (Phd) binds the $\beta\gamma$ subunits of large G-proteins, modulating through the sequestration of G$\beta\gamma$ the efficacy of receptor-activated signal transduction. Being a cytoplasmic protein, Phd also provides a means for retaining G$\beta\gamma$ in the cytoplasm and for translocation of G$\beta\gamma$ within a cell (*2*, *5*, *14*). Phd competes with the α-subunit of G-proteins for G$\beta\gamma$ that is released upon receptor-activated GTP−GDP exchange, potentially limiting the reassembly of the G-protein complex that is essential for receptor and G-protein interactions (*16*). The crystal structure of the Phd−G$\beta\gamma$ complex verifies that Phd and Tα share overlapping binding sites on G$\beta\gamma$ (*20*).

The Phd protein is divided structurally into an N-terminal domain that is involved mainly in the high-affinity binding of G$\beta\gamma$ and a C-terminal domain that is organizationally, but not functionally, similar to thioredoxin (*20*). Biochemical and structural data indicate that an N-terminal domain of Phd, mainly amino acids 17−105, is required for optimum G$\beta\gamma$ interaction and binding (*27*, *41*, *42*). Still, recent observations suggest that there may be additional G$\beta\gamma$ interactions within the C-terminal domain of Phd and PhLP$_{L/S}$ (*21*, *22*). It was observed that a short peptide from the carboxyl terminal of Phd is able to inhibit G$\beta\gamma$-mediated functions (*22*). Another study found that truncation of the N terminus of PhLP$_L$ did not prevent the binding of a recombinant G$\beta1\gamma2$ protein, although the binding affinity of the truncated protein was decreased significantly (*21*). The data presented in this report heavily favor the proposition that the major capacity for binding G$\beta\gamma$ lies in the N terminus of Phd and most likely within the 11-amino acid domain identified previously (*20*, *27*, *41*, *42*). The binding of G$\beta\gamma$ by Phd and PhLP1 can be predicted from the amino acid sequence within this domain. Moreover, the absence of

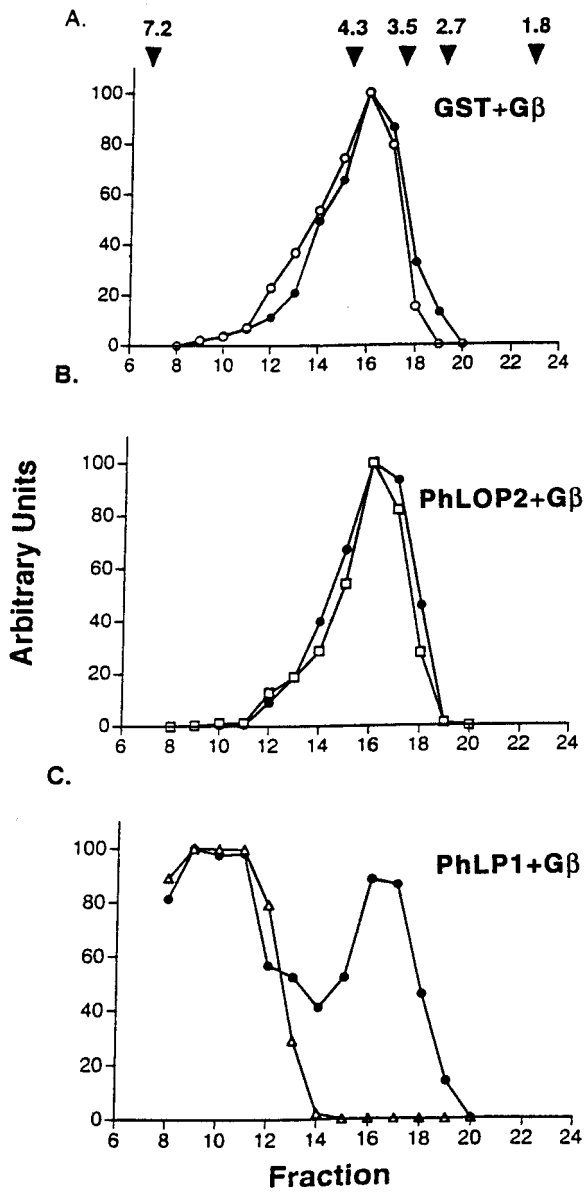

FIGURE 6: Sedimentation analysis of GST−phosducin isoforms binding with G$\beta\gamma$. Purified G$\beta\gamma$1 (●) was incubated with either GST control (A, ○), GST−PhLOP2 (B, □), or GST−PhLP1 (C, △) before centrifugation and analysis of proteins within gradient fractions by 10% SDS−PAGE. Proteins were stained, and the dried SDS gel was quantified for Gβ and Phd isoforms by densitometry. Reference proteins [alcohol dehydrogenase (80 kDa) = 7.3S, bovine serum albumin (67 kDa) = 4.3S, ovalbumin (43 kDa) = 3.53S, carbonic anhydrase (30 kDa) = 2.75S, and cytochrome *c* (12.3 kDa) = 1.8S] were used to establish a gradient sedimentation coefficients profile (displayed at the top of panel A) to which experimental samples were compared. Gradient fractions (*x*-axis) 8−20 were analyzed. Arbitrary density units 0−100 (*y*-axis) are displayed for the GST fusion proteins tested, with 100 units representing the peak density in a gradient fraction. Gβ1 (●) sediments to gradient fraction 14−18 with complexed with G$\beta\gamma$1 alone (A and B), but it descends to a lower position (fraction 8−12) when bound with PhLP1 (△) (C).

fidelity within this domain of PhLOP1 and PhLOP2 also favors this interpretation, particularly since PhLOP1 contains a C terminus that replicates those of Phd and PhLP1. Still, it is too early to know whether G$\beta\gamma$ binding is a function only of the 11-amino acid domain or whether other interactions can affect G$\beta\gamma$ binding. Future studies may show that the intramolecular forces in one domain of Phd can affect

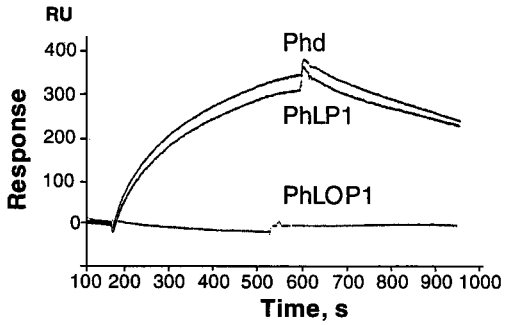

FIGURE 7: BIAcore biosensor analysis of G$\beta\gamma$ binding to GST−phosducin isoforms. The biotinylated G$\beta\gamma$ complex was immobilized on the BIAcore streptavidin-precoated sensor chip as described in Experimental Procedures. Purified recombinant GST−Phd isoforms (Phd, PhLP1, or PhLOP1) were injected across the surface at a concentration of 5 μM and a flow rate of 5 μL/min. The increase of the SPR signal (*y*-axis) is an indication of the complex formation between G$\beta\gamma$ and Phd isoforms during the time of injection (*x*-axis). For Phd and PhLP1, the buffer washout began at 600 s. For PhLOP1, the sample flow was stopped at 550 s. Phd and PhLP1 bound G$\beta\gamma$ with similar affinity and kinetics, whereas PhLOP1 did not bind at all.

G$\beta\gamma$ binding at a distance and that G$\beta\gamma$ binding by Phd or PhLP1 is a function that can be modulated.

A family of phosducin-like proteins was revealed first by Miles and co-workers (*1*) when, in a study of ethanol-induced proteins in cultured neural glioma cells, they found two proteins within the database that were strikingly structurally similar to Phd; according to length, the two proteins were named phosducin-like protein long and short ($PhLP_{L/S}$). When the proposed G$\beta\gamma$ binding site was targeted in a library screen, four informative retinal mRNAs were identified, one being Phd and three others having Phd-like sequences. When the availability of the individual mRNAs was explored in a tissue and cell culture search (Figure 4), each of the isolates was expressed at low levels in either the adult retina or Y79 cells that were stimulated with forskolin. Following sequencing and production of GST fusion proteins, the ability of the individual isoforms to bind G$\beta\gamma$ was investigated. Since the investigation was carried out prior to identification of an 11-amino acid sequence in the N terminus of Phd that seems to be essential for G$\beta\gamma$ binding, it was a surprise to find that only one (PhLP1) of the three isoforms demonstrated a capacity to bind G$\beta\gamma$ (Figures 5−8). We, as others, had equated the phosducin family of proteins with the binding of G$\beta\gamma$, but this view of the family may be too restricted. To accent the divergence of potential function, the three isoforms were renamed; PhLP1 binds G$\beta\gamma$, whereas PhLOP1 and PhLOP2 are orphan proteins that do not bind G$\beta\gamma$.

PhLP1 and Phd differ in only an 115 nt extension at the 5′-end that contains a methionine and an open reading frame. Otherwise, Phd and PhLP1 are identical and, within the criteria utilized in this study, indistinguishable. Phd and PhLP1 contain both the 11-amino acid sequence (TGPKGVINDWR) that is a signature for G$\beta\gamma$ binding and the PKA phosphorylation domain within which a serine (amino acid 73 in Phd) is found to be phosphorylated in the retina upon exposure to darkness and hypophosphorylated upon exposure to light (*13*−*15*). Phosphorylation of the equivalent serine, amino acid 110 in PhLP1 and amino acid 73 in Phd, initiates intramolecular events that diminish the the extent of binding of G$\beta\gamma$ (data not shown). Similarly, it is

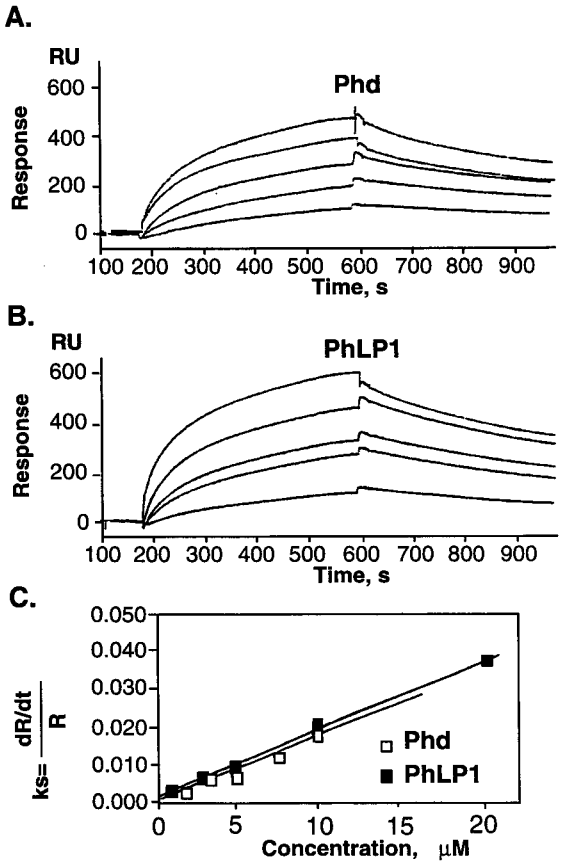

FIGURE 8: Comparison of kinetics of binding of GST−Phd and GST−PhLP1 to immobilized G $\boldsymbol{\beta\gamma}$. (A and B) Overlay plots of binding data recorded using BIAcore during injections of increasing concentrations of Phd isoforms over the surfaces with immobilized biotinylated G$\beta\gamma$. (A) Binding of GST−Phd at 2.0, 3.5, 5.0, 7.5, and 10 mM. Concentrations of GST−PhLP1 were 1.0, 3.0, 5.0, 10, and 20 μM. Between the consecutive injections, the surfaces were regenerated by long (60−120 min) washes by the running buffer. These data are a representative of four independent experiments. Calculation of the kinetic constants for the binding of the Phd isoforms with G$\beta\gamma$. The data were analyzed using BIAEvaluation 2.1 software (BIOCORE, Inc.). The values of k_s (dR/dt vs R, where R is the amount of bound material expressed in refractive units, RU) were plotted as a function of the Phd isoform concentration. The slope of the line is the association rate. The intercept with the y-axis is the dissociation rate. The dissociation rate ($k_d = 1 \times 10^{-4}$ to 1×10^{-3} s^{-1}) determined from the analysis of association kinetics correlated well with the values obtained from the analysis of the dissociation phase, i.e., single-exponential decay of the SPR signal ($k_d = 5 \times 10^{-4}$ s^{-1}) within the first 60 s of beginning of the buffer washout. Phd (white symbols) and PhLP1 (black symbols) bound to G$\beta\gamma$ with nearly identical kinetics.

conceivable that other factors may modulate G$\beta\gamma$ binding, and these forces may arise through still undefined interactions with effector molecules, interacting within the thioredoxin-like carboxyl end of both Phd and PhLP1.

A database search of the nucleotide or amino acid sequences that are shared by ESTs and PhLP1 did not reveal a known function or activity for this domain. Thus, the distinguishing function of PhLP1 is still linked to its ability to bind G$\beta\gamma$ in a modulated manner, but the introduction of another amino-terminal domain within the protein raises images of a broader role for PhLP1 than Phd in cellular activities. Further characterization of PhLP1 will aid in establishing the functional diversity that exists within the phosducin family of proteins.

PhLOP1 is a less abundant Phd isoform compared to Phd that, lacking the predicted G$\beta\gamma$ binding domain, is unable to bind G$\beta\gamma$. In its organization, it is structurally similar to the homologue $PhLP_S$, a proposed truncated, splice variant of $PhLP_L$ (*1*). In related work, we have identified an alternative exon containing the 98 nt sequence from the 5′-noncoding region of PhLOP1 (Figure 2B), downstream of the published exon 2 and within the previously identified intron of the Phd gene, with hallmarks of a promoter (*46*). Since both PhLOP1 and $PhLP_S$ lack the N-terminal region of Phd that is responsible for G$\beta\gamma$ binding, it is unlikely that $PhLP_S$ is able to bind G$\beta\gamma$ to a significant extent. PhLOP1 retains in the N terminus a serine (amino acid 21) equivalent to that phosphorylated by PKA in Phd, but its functional significance is still unclear and open to future investigations.

PhLOP2 is the smallest of the Phd isoforms, and it is unable also to bind G$\beta\gamma$, even though it has partial preservation, including a critical tryptophan, of the 11-amino acid domain that predicts G$\beta\gamma$ binding. Database searches for PhLOP2 identified a unique 77 nt region within the 5′-region of PhLOP2 that is 98% identical to numerous ESTs, plus identity with two trinucleotide repeat cDNAs of unknown function: one from brain, CTG-B33 (L10376), and another from pancreas, CAG-isl7 (*39*, *40*). In PhLOP2, a shift in reading frame occurs within subsequent spliced exons that eliminates at the amino acid level the fidelity of correspondence that exists between the nucleotide sequences of the Phd isoforms. The frame shift also introduces a premature stop codon in PhLOP2, reducing the predicted mass of the protein.

The retinal content of transcribed mRNA for PhLOP2 is below the level of detection (Figure 5), as is that in unstimulated Y79 cells, but forskolin stimulation raises the mRNA content of PhLOP2 to the level of detectability. Thus, forskolin stimulation, and secondarily an increaese in cAMP levels, results in an increased level of expression of PhLOP2 in Y79 cells. The responsiveness to cAMP stimulation suggests either that PhLOP2 participates in cAMP-mediated signal transduction or that it is involved in the response mechanisms that mediate cAMP-initiated activities.

Interestingly, the alternative splicing that creates a frame shift in PhLOP2, mRNA divergence in PhLP1, and exon insertions and/or deletions (PhLOP1 and -2) results in the four Phd isoforms sharing only a single sequence (MSSPQS-RNGKDS) of 12 amino acids when translated (Figures 2 and 3). Several laboratories have noted the presence of multiple mRNAs for phosducin, but it has been difficult to prove whether these arise from multiple genes with distinct tissue-specific promoters for phosducin or a single gene with either multiple promoters or a single promoter with distinct splice variants (*6*, *10*, *34*, *36*, *43*−*46*). A gene for Phd has been localized to chromosome 1 (*6*, *44*−*46*), but gene duplication may have occurred, allowing a linear array of Phd genes on chromosome 1 or a translocation of the duplicated gene to other chromosomes. The four phosducin isoforms, therefore, are products of one or more genes. An analysis of the complete nucleotide sequence alignment of PhLP1 and PhLOP2 reveals distinct sequences 5′ of the published flanking region and 5′ of the predicted transcriptional start site (*34*), including a partial exon duplication (PhLOP2, *Phd nt 117−231) (Figure 2C,D). This latter

finding favors the interpretation that there are at least two genes encoding the Phd isoforms.

Further, the analysis reveals there are only seven mRNA nucleotides that are different in the four isoforms after nt 102 of Phd, the predicted exon−intron 3 junction. The same nucleotide variations have been noted earlier and designated as polymorphisms (*34*, *36*). In this study, the retina cDNA library was constructed from mRNA from several human retinas, making it impossible to exclude this possibility. Undoubtedly, a study of human variants in Phd would reveal some level of polymorphism, but, more importantly, from an attempt to decipher gene structures, the variants are found consistently within the individual isoforms (data not shown), signaling the presence and expression of unique Phd isoforms. In fact, the appropriate selection of Phd isoform primer pairs leads to the selective amplification of distinct cDNAs, verified independently by subcloning the PCR products and sequencing each isoform (data not shown), using either the mRNA from a single human retina or from stimulated retinoblastoma cells (Figure 4). We, and others, are actively developing an analysis of the genes that encode Phd and its isoforms.

Phosducin and its isoforms are widely distributed throughout the body where they may participate in signaling pathways or other cellular activities. The locales at which Phd and its isoforms are expressed reveal that tissue-specific promoters are operating in establishing which cells and tissues are recipients of the Phd isoform. There may be multiple promoters for activating a single Phd gene, but at minimum, there are two genes encoding Phd isoforms. The genes and their expression are linked to cellular dynamics of stimulus and response, with Phd and PhLP1 participating in the modulation of G-protein-based signal transduction and in the regulation of cytoplasmic G$\beta\gamma$ levels through the cell. The Phd orphans, PhLOP1 and PhLOP2, are intriguing, but their function remains an item for future work. In a yeast two-hybrid screen, we have identified a protein−protein interaction of PhLOP1, but not PhLOP2, with a member of the 26S proteasomal complex, SUG1 (*47*). Independently, the identical results were also reported for the PhLPs in an analogous yeast two-hybrid screen (*48*). The PhLPs and PhLOPs show the structural diversity of the Phd family of proteins, and future investigations will undoubtedly link these structural features with divergent functional activities.

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