PHOSDUCIN-LIKE PROTEIN 1 (PhLP1) AND PHOSDUCIN-LIKE ORPHAN PROTEIN 1 (PhLOP1)

Chromosomal Localization to 1q25.3 and Genomic Structure Reveal Alternative Splicing of the Human Phosducin Gene

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SUMMARY

Phosducin (Phd) is a soluble phosphoprotein, selectively binding to the beta-gamma ($\beta\gamma$) complex of G-proteins, playing a role in intracellular signaling processes. We have molecularly identified 3 Phd isoforms expressed in the human retina. Based on their ability or inability to interact in our assay system with G$\beta\gamma$ proteins, we named them Phosducin-Like Protein 1 (PhLP1) or Phosducin-Like Orphan Proteins (PhLOP1 & PhLOP2), respectively. To further study these Phd isoforms and their potential involvement in normal and abnormal visual processes through genetic alterations, we identified and characterized an 85 kb P1-PhLP human genomic clone that contains PhLP1 and PhLOP1. With fluorescence *in situ* hybridization (FISH) to human metaphase chromosomes, this P1-PhLP genomic clone was mapped to human chromosome 1q25.3. Previous work identified a gene locus for phosducin (PDC) to 1q25–q31.1 by somatic cell hybridization and *in situ* hybridization. Within the P1-PhLP genomic clone,

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the previously characterized complete PDC gene was identified; furthermore, the 5′-flanking region and a potential promoter region of PhLOP1 was identified between Exon 2 and Exon 3 of the PDC gene. Initial transfection experiments with luciferase activity reporter PhLOP1 constructs, ranging in size from 386 to 852 basepairs (bp), suggest that no tissue specific retinal promoter activity is contained in this 5′-flanking region of the PhLOP1 when compared to the high retinal specific promoter activity observed for a 123 bp construct for Interphotoreceptor Retinoid-Binding Protein (IRBP). Alternatively, a repressor element is still present and preventing promoter activity in the PhLOP1 constructs. These results correspond to the low levels of mRNA of the PhLOP1 observed in the retina compared to Phd. These data suggest that both PhLP1 and PhLOP1 are created through alternative splicing of the Phd gene and that a single PDC gene at 1q25.3 is responsible for these three retinal isoforms.

1. INTRODUCTION

Heterotrimeric G-proteins with their alpha (α), beta (β), and gamma (γ)-subunits play a major role in transmembrane G-protein receptor coupled (GPRC) signaling processes.[1,2] Phosducin (Phd) binds to the G-protein βγ complex and prevents the reassociation of Gα.[3–5] Originally identified in retina and pineal gland,[6–9] Phd, phosducin-like protein ($PhLP_{Long(L)/Short(S)}$, PhLP1), and phosducin-like orphan protein (PhLOP1 and PhLOP2) isoforms are expressed at low levels in other tissues examined.[10–17] PhLPs, like Phd, regulate Gβγ signaling; however, without the amino terminal domain, the binding and affinity for Gβγ decreases, suggesting alternative physiological functions for the truncated, orphan Phd isoforms.[18]

To investigate the Phd isoforms in the human retina, we identified three variants of Phd from a human retina cDNA library, named PhLP1, PhLOP1, and PhLOP2, respectively (Accession #AF076463, AF076464, and AF076465).[18] One Phd isoform, PhLP1, has the complete Phd sequence plus an additional amino terminal domain of 37 predicted amino acids. Like Phd, *in vitro* studies with recombinant PhLP1 and Gβγ proteins reveal similar Gβγ binding capacity. PhLOP1 has the carboxy terminal part of Phd; however, it lacks the first 52 amino acid terminal residues of Phd. PhLOP2 is also truncated, sharing limited amino acid sequence identity to the other isoforms, but its nucleotide sequence identity is significantly homologous with the other three isoforms. In our reconstitution assays with glutathione S-transferase/phosducin isoform fusion proteins, both PhLOP1 and PhLOP2 fail to bind to Gβγ because each lacks the major 11 amino acid domain within the amino terminus, which by crystallography analysis reveals structural interaction with Gβ.[19] In search of other functional partners for the PhLOP1 isoform, we discovered through a yeast 2-hybrid screen of a retinal cDNA library a protein-protein interaction with a proteasomal 26S ATPase protein, SUG1.[20]

2. METHODS

2.1. Fluorescent *In Situ* Hybridization

The human genomic P1-PhLP clone was labeled with digoxigenin dUTP by nick translation and combined with sheared human DNA, hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes from a male

donor in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. For two color experiments the detection was the same except for the inclusion of avidin Texas red.

2.2. Restriction Endonuclease Enzyme Digestions and Southern Blot Analysis

The P1-PhLP genomic DNA was digested with each of the following enzymes: *Hin*DIII, *Eco*RI, *Bam*HI, *Xba*I, *Cla*I / *Sma*I, and *Cla*I / *Sac*I (Boehringer Mannheim and New England BioLabs) and electrophoresed on an agarose gel. Typically, 5 µg of DNA was digested with 10 units of the enzyme in appropriate buffer in a final volume of 20 µl. After 4 hr incubation, the digestions were applied onto a 0.65% agarose gel (12 × 20 × 0.6 cm) in 1x TBE (89 mM Tris base, 89 mM Boric Acid, 2 mM EDTA) and electrophoresed at 80 V for 12 hrs. The DNA bands were transferred onto Hybond-N+ nucleic acid transfer membrane (Amersham). The transferred DNA was subsequently cross-linked to the membrane by UV irradiation. The membrane was incubated at least 4 hr at 42°C in 50% deionized formamide, 1x Denhardt's (100x is 10 g of Ficoll 400, 10 g of polyvinylpyrolidone, and 10 g of Bovine Serum Albumin), 2X SSC (20X SSC is 0.3 M Sodium Citrate; 3 M NaCl, pH 7.0), 1% SDS, 25 M NaH_2PO_4, 0.01 mg/ml of Salmon Sperm DNA. The total cDNA encoding PhLOP1 and Phd were isolated by gel purification after double enzyme *Xba*I/*Xho*I digestion of the original λMAX clones.[18] The probes were labeled with 100 µCi of α-[P^{32}]-dCTP by random priming, using the DNA Labeling System (Amersham). Hybridization was performed in 50% formamide buffer at 42°C overnight after prehybridization for 4 hr. The membrane was then washed 2 times for 20 min at room temperature (2X SSC, 0.1% SDS). The final wash was done for 20 min at 62°C (0.2X SSC, 0.5% SDS). The membrane was briefly dried and exposed to X-ray film or phosphoimager for 12 hrs.

2.3. DNA Sequence Analysis

DNA sequence analysis was performed by automated DNA sequencing with an ABI-310 Genetic Analyzer (Applied Biosystem/Perkin-Elmer), according to the manufacturer's recommendation. Both vector and Phd isoform primers were used to obtain DNA sequence information.

2.4. Cell Culture and Transfections

WERI retinoblastoma (Rb-1) cells (ATCC) were grown in RPMI 1640 containing Glutamax (Gibco, BRL), penicillin, streptomycin, and 10% fetal bovine serum (inactivated) in a 5% CO_2 humidified atmosphere. Transfection was performed using Superfect (Qiagen) in accordance to the manufacturer's recommendation. Cells seeded on a 3 cm plate (10^6 in 2.0 ml), were co-transfected with 1.7 µg test plasmids (PhLOP1/IRBP/CMV constructs) and 0.8 µg pGL3 control plasmid as an internal control for transfection efficiency, using 8 ml of Superfect in serum free medium to a total 60 µl volume.

Cells were collected 40 h later, washed twice with phosphate buffered saline, resuspended in 200 µl of PLB buffer (Promega) and lysed by two cycles of freezing and

thawing. *Renilla* and firefly activities were assayed on the same 20 µl cell extract, using the Dual Kit Luciferase Assay (Promega). *Renilla* luciferase activity was normalized to the corresponding firefly luciferase activity for each sample. The data obtained represent the average ± S.E.M. of at least three independent experiments, each performed in duplicate.

3. RESULTS

3.1. Chromosomal Localization of a P1-PhLP Genomic Clone

To characterize the chromosomal localization and the genomic structure of these human phosducin isoforms, we isolated a genomic DNA clone (P1-PhLP) from a human P1 genomic library with a radiolabeled cDNA probe for the human retinal PhLP1, synthesized by polymerase chain reaction (PCR) using vector primers (λMAX vector, sense and antisense primers, +Gal4 and -T7, respectively), as previously described.[18] The purified PCR product was used for the human P1 genomic library hybridization screen using previously published protocols[5] (Genome Systems Inc. St. Louis, MO). The result of the screen yielded a positive genomic DNA clone, P1-PhLP (F322), about 85 kilobases long. F322 was subsequently used for fluorescence *in situ* hybridization (FISH). The initial experiment resulted in specific labeling of the long arm of a group A chromosome. A second experiment was conducted in which a biotin labeled probe which is specific for the heterochromatic region of chromosome 1 was cohybridized with clone P1-PhLP. This experiment resulted in the specific labeling of the heterochromatin in red and the long arm in green of chromosome 1 (data not shown). Measurement of 10 specifically hybridized chromosomes 1 demonstrated that P1-PhLP is located at a position, which is 42% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome arm 1q, an area that corresponds to band 1q25.3 (Fig. 1). A total of 80% metaphase cells were analyzed with 68 exhibiting specific labeling. Confirming the previously report that the human phosducin gene was mapped to chromosome 1q25–1q32.1,[23–25] we have further narrowed the location to 1q25.3 and mapped the PhLP1 isoform to this identical chromosomal region.

3.2. Genomic Structural Relationship of Phd, PhLP1, and PhLOP1

To study the genomic structural relationship among the retinal Phd isoforms in P1-PhLP genomic clone, we analyzed the restriction endonuclease digestions of the P1 DNA by Southern blot hybridization and compared the fragment pattern obtained with cDNA probes for either Phd, PhLP1, or PhLOP1 isoform. The P1-PhLP genomic DNA was digested with each of the following enzymes: *Hin*DIII, *Eco*RI, *BamH*I, *Xba*I, *Cla*I / *Sma*I, and *Cla*I / *Sac*I (Boehringer Mannheim and New England BioLabs), electrophoresed on an agarose gel, transferred to membrane, hybridized with each appropriately labeled cDNA encoding each Phd isoform, washed and exposed to a phosphoimager. The results on this study are shown in Fig. 2. As expected because of a high degree of nucleotide sequence identity among the three isoforms, shared, common fragments are hybridized across all three isoforms; however, unique bands hybridize with either the labeled Phd or PhLP1 probes. The fragments present in Phd but not PhLOP1 are indicated with a diamond to the left side of the fragments (Fig. 2,

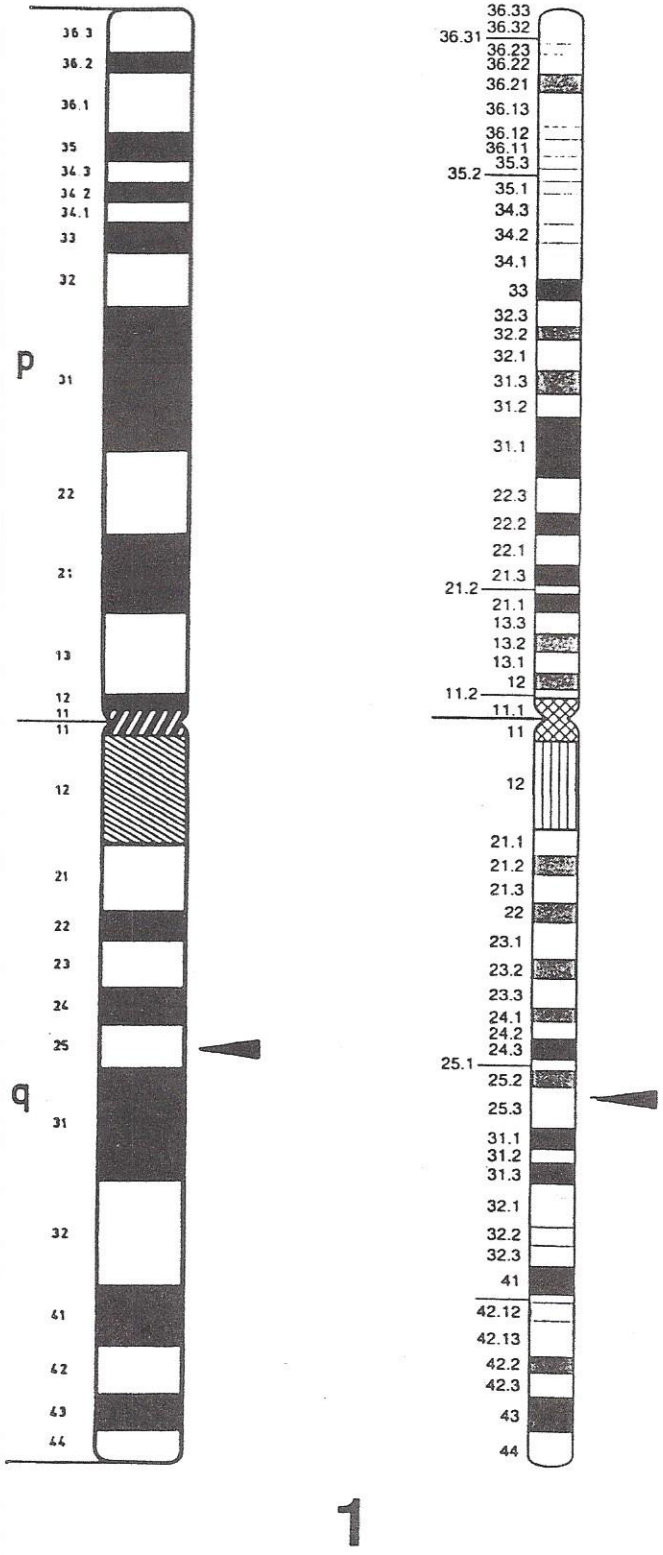

Figure 1. Ideogram illustrating the chromosomal position of P1-PhLP1 at 1q25.3 from the International System for Human Cytogenetic Nomenclature 1995. The pointers identify the location of the fluorescent *in situ* hybridization (FISH) results with the P1-PhLP, 85 kb genomic clone (F322), which maps to human 1q25.3 band, which refines the location of the PDC gene (1q25–1q31).[21,22]

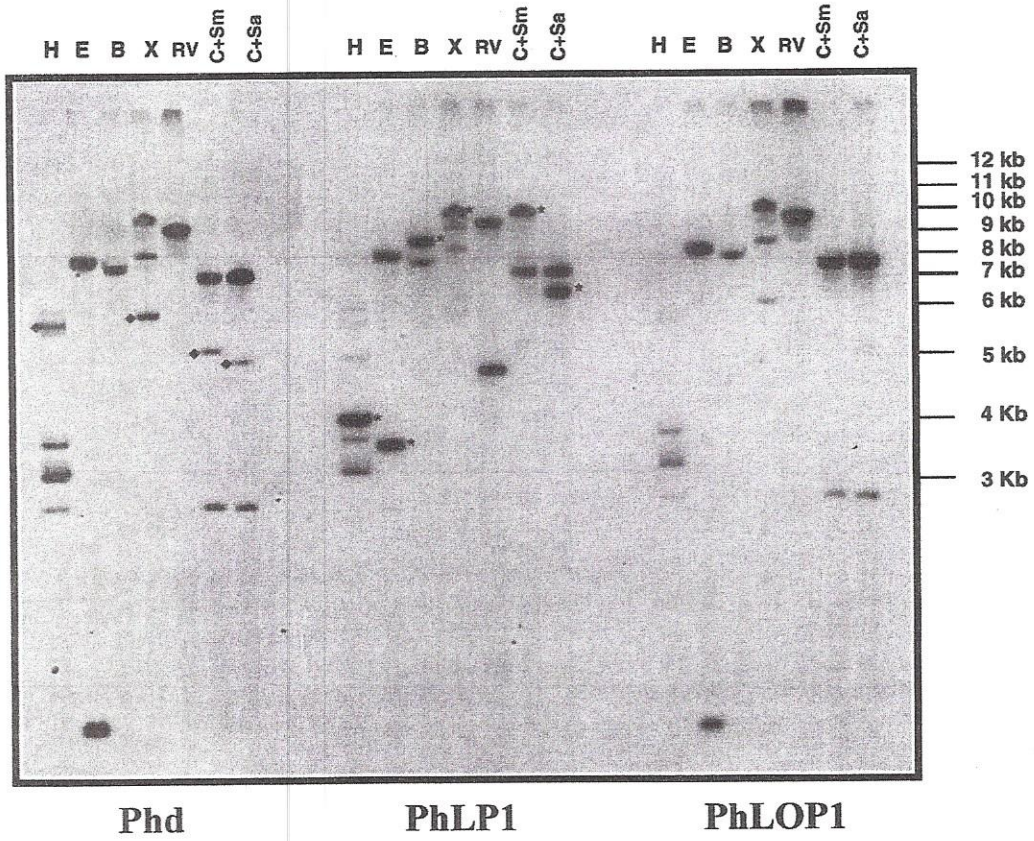

Figure 2. Southern hybridization of restriction endonuclease digestions of the P1-PhLP genomic clone (F322) used in the FISH with either a complete cDNA encoding human retina Phd, PhLP1 or PhLOP1 with digested P1-PhLP1 genomic DNA. P1-PhLP genomic DNA was digested with specific restriction endonucleases, electrophoresed in triplicate through 0.65% agarose gels, transferred and hybridized with random primed, radiolabeled cDNA for each isoform, and processed before exposure to a phosphoimager overnight. Listed from left to right, Phd, PhLP1, and PhLOP1. The restriction endonucleases indicated across the top are abbreviated: H, *Hind* III; E, *Eco*R I; X, *Xba* I; RV, *Eco*R V; C + Sm, *Cla* I/*Sma* I; C + Sa, *Cla* I/*Sac* I. The molecular weight marker sizes are indicated on the right. Details of the procedures are in the Methods section. Note the diamond markers on the left side fragments identify the bands unique to Phd hybridization not shared with either PhLP1 or PhLOP1; the stars on the right side fragments identify the bands unique to PhLP1 not shared with either Phd or PhLOP1.

left panel), while those present in PhLP1 but not Phd or PhLOP1 are indicated with a star to the right side of the fragments (Fig. 2, middle panel).

Based on Southern blot hybridization and the gel analysis of selected restriction enzyme sites, several P1-PhLP genomic DNA fragments were selected for subcloning into pBluescript IISK+ (Stratagene). P1/*BamH* I-11.5 kb, P1/*Cla* I-12.6 kb, P1/*Sma* I-12.3 kb, and P1/*Sma* I-8.3 kb DNA fragments were subcloned. These fragments were further confirmed by PCR using PCR primers that identify the entire PDC gene structure, including the coding, noncoding, and 5′-flanking region previously published.[23–25] The schematic diagram of the predicted P1-PhLP gene structure, its restriction map and the location of the subcloned genomic fragments are shown in Fig. 3.

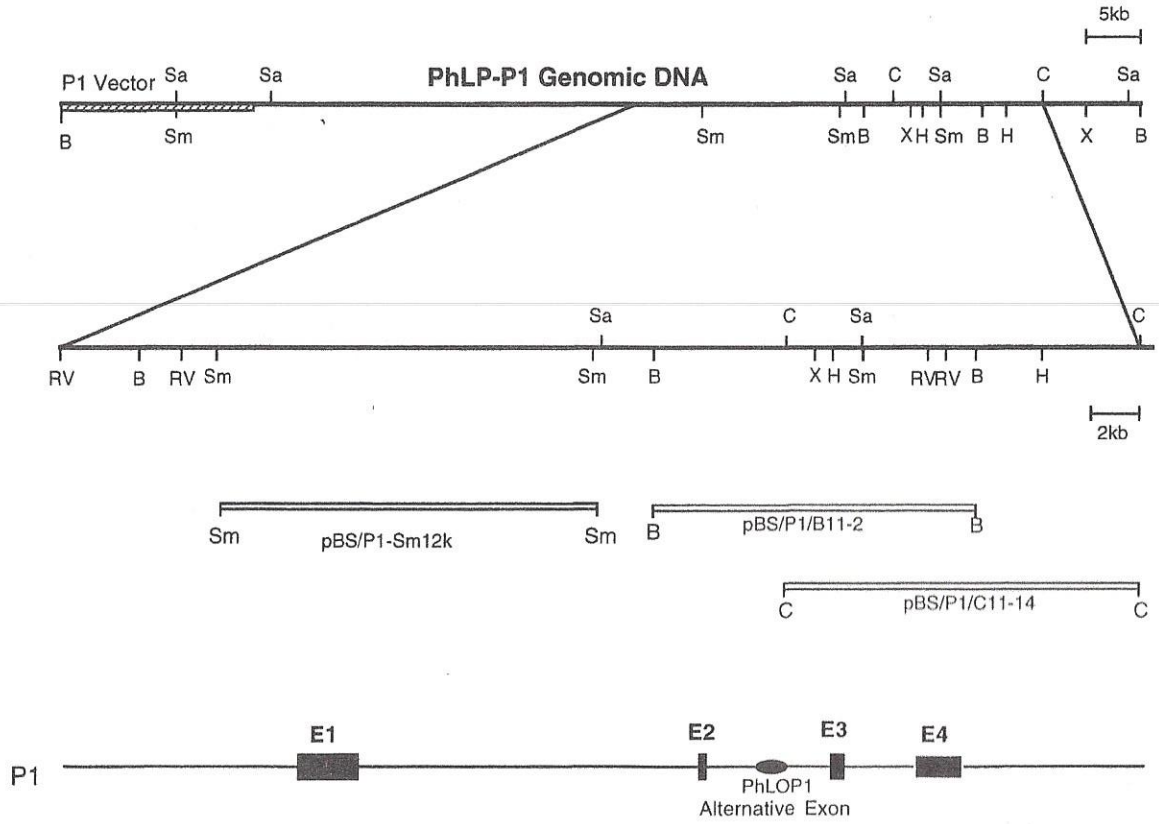

Figure 3. Diagram of Phd gene structure, including the alternative exon for PhLOP1 and the restriction map of the P1-PhLP genomic clone, including the position of the subclones. The top line represents the entire P1-PhLP (F322) genomic DNA fragment of approximately 85 kb in length, 5 kb indicator length above the line. Based on the Southern blot hybridization results of the cDNA of the Phd isoforms (Fig. 2), fragments that cover the Phd cDNA were selected, subcloned into pBluescript (pBS), and analyzed further: *Sma* I/12.3 kb = pBS/P1-Sm12k, *Bam*H I/12 kb = pBS/P1/B11-2, and *Cla* I/12.5 kb = pBS/P1/C11–14. The enzyme abbreviations are identical to Fig. 2. The lower line, 2 kb indicator length above the subclones, represents the exon (E)/intron locations of the PhLP gene verified by sequence analysis in the subcloned fragments, E1–E4 of the previously identified Phd gene,[23–25] including the alternative exon for PhLOP1 located within the intron between E2/E3.

3.3. Identification of an Alternative Exon and 5′-Flanking Region for PhLOP1

DNA sequence analysis using internal primers in the Phd coding region and the P1-PhLP genomic subclones as templates, the sequence was analyzed across each predicted exon/intron junction.[23–25] Sequence analysis of the P1/*BamH*I-11.5 kb subclone revealed that the 103 bp 5′-noncoding sequence that is unique for PhLOP1 is located within the previously identified intron 2 of the phosducin gene[23–25] (Fig. 4). The splicing site donor/acceptor sequences were confirmed by Gene Finder Programs (Baylor College of Medicine; BCM Search Launcher: Gene Feature Searches, http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html). Between the Phd exon 2 and the PhLOP1 splicing donor site there are 678 bp, from the splicing acceptor site to the previously identified Phd exon 3 is approximately 1200 bp.

Although the 5′ flanking region sequence of Phd is documented,[23–25] promoter activity for the Phd gene has not been observed (data not shown). To explore whether or not the 5′ flanking region isolated for PhLOP1 has the potential for promoter activity to drive the transcription of this mRNA for PhLOP1 in the retina, several fragments around the PhLOP1 splicing sites were subcloned into pGL-3 (Firefly Luciferase as reporter, Promega), pRL-null (Renilla Luciferase as reporter, Promega).[26] Four constructs ranging in size from 386 to 852 bp in length were transfected into WERI retinoblastoma cells with SuperFect method (Qiagen) or Calcium Phosphate method, as previously described.[26] Similar to our observations of the Phd 5′ flanking region, no promoter activity was detected from the constructs prepared compared to the well established promoter activity of the 124 bp minimal promoter gene construct from the Interphotoreceptor Retinoid-binding protein, used as a retinal specific transcription activation control[27] (Fig. 5).

4. DISCUSSION

Phosducin and its isoforms are widely distributed in body tissues where they participate in signal transduction pathways.[3–9] Phd, PhLP1, and $PhLP_L$ possess an 11 amino acid conserved epitope domain (TGPKGVINDWR) that controls the high affinity binding of Gβγ, implicating these isoforms in the G-protein signaling pathway.[9–11,18] Several reports also suggest that even without this domain, the carboxy terminal domain is still capable of limited interaction with Gβγ.[14–16] In our experiments, the phosducin-like orphan proteins (PhLOPs) fail to bind Gβγ,[18] suggesting that the PhLOP isoforms may participate in other signaling pathways related to the carboxy terminal domain, such as the proteasomal ubiquination pathway through the ATPase, SUG1, that was recently identified.[20]

The mRNA of the Phd isoforms is expressed in either human retina or forskolin-stimulated Y79 retinoblastoma cells, verifying their expression independently of the cDNAs for each that was isolated through a human retina library screen.[18] The present data demonstrates that Phd and at least two isoforms, PhLP1 and PhLOP1, all map to 1q25.3 and that alternative spicing is responsible for the truncated isoform, PhLOP1. Although no retinal specific promoter activity is currently measured from constructs of the 5′-flanking region of PhLOP1 with WERI retinoblastoma cells (Fig. 5), we have observed significant reporter activity of the 852 bp fragment in a non-retinal cell line (data not shown). Because low mRNA levels of PhLOP1 are only detectable by

Exon1/Intron1:

***TTCCTCCTATAG**
****GAGATCCAAACT**gtgagtccacaagtagt

Exon2/Intron2:

CCACACATACAGgcaagcaaagctagtagaagaaa

E-PhLOP1/Intron3:

ATTGAAGAAAAGgttggtaaattcatttataa

Exon3/Intron4:

GTCAGCAGAAAGgtaagataatacaaaaagca

Intron1/Exon2:

tttctttgttcctcctatag**ATTATATCAAAT**

Intron2/E-PHLOP1:

acaagagtaccagccaaaat**GTGTGGAGATTT**

Intron3/Exon3:

*cattccag**GACCCAAAGGAG**

Intron4/Exon4:

tctatcattcttattctcag**ATGAGCATTCAA**

Figure 4. Splicing junctions and the exon/ intron nucleotide boundary sequences of Phd and PhLOP1. The lower line from Fig. 3 is identical. The nucleotide sequence at each exon/intron boundary is listed. The asterisks *, ** indicate verification of previously published boundaries from reference 23 and 24, respectively.

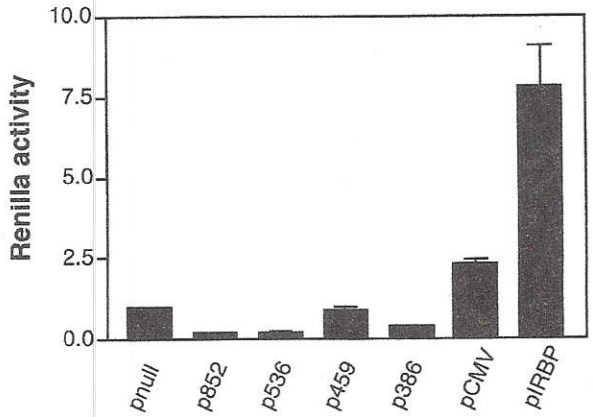

Figure 5. Reporter gene expression driven by fragments of decreasing size of the human PhLOP1 and the 123 bp IRPB promoter (pIRBP) in WERI retinoblastoma cells. *Renilla* luciferase expression driven by the CMV promoter (pCMV), an internal control; pnull, construct without promoter inserts, decreasing base-pair (bp) lengths (852–386 bp) of the PhLOP1 5′-flanking region (p852, p536, p459, p386) and the pIRBP (123 bp) promoter construct is a retina tissue specific control. Values are representative of at least three transfection experiments, each performed in duplicate. The *Renilla* luciferase activity arbitrary units are on the y axis (0.0–10.0) and the constructs used are on the x axis.

RT-PCT in the retina sample, perhaps an inhibitory element is blocking higher levels of tissue specific expression.[18] Additional studies are necessary to resolve this issue.

One plausible explanation for the alternative PhLOP1 splice variant would be the existing splice site at the PDC exon 2/intron 2 junction. The donor site at this junction is GC, not the predicted GT (Fig. 4), while all other donor/acceptor sites follow the predicted GT/AG rule, including the new donor site for the PhLOP1 exon. Altered splicing sites can lead to aberrant RNA splicing and is frequently associated with human disease, such as amyotrophic lateral sclerosis.[28] Although not common, an aberrant splicing database was established recently to track these altered variants (Web site: http://www/imcb.oaka-u.ac.jp/nakai/asdb.html), including "cryptic" splice sites that lead to exon skipping, which accounts for 50% of the genetic mutations listed.[29] It is possible that an altered splicing mechanism and exon skipping could result in an improper balance of the retinal phosducin isoforms, which could ultimately effect the Gβγ binding capacity and normal function of Phd.

In summary, the data presented suggests that both PhLP1 and PhLOP1 are created through alternative splicing of the PDC gene, and the P1-PhLP genomic fragment maps within the same 1q25–31.1 chromosomal region as the PDC gene.[21,22] At present, no visual genetic defect is associated with the 1q25.3 chromosomal region. The unique hybridizing fragment pattern and P1-PhLP subclones for Phd, PhLP1, and PhLOP1 provide important clues to solve the complex genomic structure and to identify the promoter(s) of the Phd gene. Our P1-PhLP1 genomic DNA covers all the previously identified exons and introns of Phd and two isoforms, suggesting that only one gene with alternative promoters codes for these three isoforms.

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