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Interaction of Phosducin and Phosducin Isoforms with a 26S Proteasomal Subunit, SUG1

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Purpose: Retinal phosducin (Phd) and phosducin-like protein 1 (PhLP1) selectively bind G-protein beta/gamma subunits (Gβγ). Our laboratory has recently identified two phosducin-like orphan proteins (PhLOP1 and PhLOP2) that lack the ability to interact with Gβγ. In search of potential functional protein partner(s) for these phosducin orphans, we examined their protein-protein interactions using a yeast two-hybrid screen.

Methods: A bovine retina yeast expression cDNA library was screened with the GAL4 DNA binding domain (BD) fusion of PhLOP1. Quantitative analysis of the selected positives with PhLOP1 and other Phd isoforms was assessed by growth and beta-galactosidase activity. Further molecular, biochemical, and immunological detection methods utilizing glutathione S-transferase (GST)-Phd isoform fusion proteins and the potential partner were also performed.

Results: A member of the superfamily of putative ATPases was selected in the yeast two hybrid screen. Further characterization identified a direct interaction of this putative ATPase with PhLOP1, as well as Phd and PhLP1, but not with PhLOP2. A database search verified this ATPase as a bovine orthologue of the yeast SUG1 (ySUG1), a putative transcriptional mediator and a subunit of the 26S proteasome complex. Our experiments reveal that the carboxy-terminus of PhLOP1 is essential for the protein-protein interaction with SUG1, but it alone is not sufficient to mediate SUG1 interaction.

Conclusions: Based on these experimental results, Phd, PhLP1 and PhLOP1 have protein-protein interaction with SUG1. PhLOP1, a truncated amino-terminal splice variant of Phd, is the best candidate for the interaction with SUG1 among the four Phd isoforms studied, which suggests a potential function for PhLOP1.

Phosducin (Phd), a 33 kd acidic phosphoprotein [1,2], is abundantly expressed in both photoreceptors and pinealocytes [3-5]. It is phosphorylated at serine 73 by cAMP-dependent protein kinase A (PKA) in a light-dependent manner [6]. Previous work clearly established that retinal Phd plays a role in the guanine nucleotide (G)-protein signaling pathway by competing with G-α subunits for binding to G-βγ subunits [7]. The efficacy of retinal Phd binding to Gβγ is determined in part by its phosphorylation state [7-11]; dephosphorylated Phd tightly binds Gβγ, preventing receptor-mediated Gα reactivation [7,8,12] and blocking the interaction between Gβγ and its effector enzymes [9,10,13,14].

Identification of phosducin-like proteins ($PhLP_L$ and $PhLP_S$) induced by ethanol treatment of a neuronal-glial cell culture [15] and the broader distribution of expression of Phd and PhLP in brain and other tissues [8,16,17], suggests the existence of a superfamily of proteins that structurally and functionally resemble retinal Phd [18,19]. To identify other potential members of this superfamily, our laboratory screened a human retinal cDNA library and identified three new Phd isoforms [20]. One is a phosducin-like protein (PhLP1) that had similar kinetics and binding affinity to Phd; however, two other isoforms had no ability to bind Gβγ, thus were named phosducin-like orphan proteins, PhLOP1 and PhLOP2 [21]. PhLP1 contains the identical coding sequence of Phd, plus an additional 37 amino acid domain at its amino-terminus. PhLOP1 has the complete carboxy-terminal part of Phd but lacks the first 52 amino-terminal residues of Phd. PhLOP2 has only a limited amino acid sequence homology to Phd or the other two isoforms, although its nucleotide sequence has significant homology to Phd [21]. To define the structural and functional attributes of the PhLOPs that lack the ability to interact with the Gβγ, we used the yeast two-hybrid system to search for potential protein-protein interacting partners. With PhLOP1 as a bait, we identified an orthologue of the superfamily of putative ATPases from a bovine retinal cDNA library, bSUG1 (Accession Number AF069053), that has high sequence identity to yeast SUG1 (ySUG1) and examined the interaction of bSUG1 with PhLOP1 and other Phd isoforms.

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METHODS

Yeast strain— The yeast reporter host strain *Saccharomyces cerevisiae* CG-1945 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2, LYS2:: GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA:: GAL4$_{17-mers(x3)}$-CyC1$_{TATA}$-LacZ*) (Clontech Laboratories, Inc., Palo Alto, CA) was grown in YPD (yeast extract / peptone / dextrose) or appropriate selection medium to maintain plasmids. Yeast transformation was done by the lithium acetate method using the YEASTMAKER yeast transformation system (Clontech Laboratories, Inc.)

Plasmid construction— Plasmids were constructed with the GAL4 DNA binding domain (BD) in pBD-GAL4 phagemid vector (Stratagene, La Jolla, CA). The complete coding sequence of human retinal Phd, PhLP1, PhLOP1, PhLOP2 and the truncated N- and C- amino acid domains of PhLOP1 were inserted in-frame into the pBD-GAL4 vector downstream of the binding domain between its *Eco*RI and *Pst*I restriction endonuclease multiple cloning sites. All the inserts subcloned into pBD-GAL4 were obtained by polymerase chain reaction (PCR) technology with the *Eco*RI nucleotide sequence site incorporated into the 5' sense primer and the *Pst*I nucleotide sequence site designed in the 3' antisense primer. A bovine retinal cDNA library (kindly provided by Drs. W. Baehr and A. Surguchev) was fused with GAL4 activation domain (AD) through its N-terminus in the pGAD10 yeast expression vector through its *Eco*RI site (Clontech Laboratories, Inc.). Glutathione S-transferase (GST) fusion proteins of Phd, PhLP1, PhLOP1, and PhLOP2 were made with pGEX-3X vector (Pharmacia Biotech Inc., Piscataway, NJ), as described previously [13,21]. To create a SUG1 with a carboxy-terminal 6 X histidine tag (SUG1-6xHis), the bovine SUG1 (bSUG1) coding region was PCR amplified from one of the full-length cDNAs encoding bSUG1 in pGAD10 vector isolated from bovine retinal cDNA library screening, introducing *Bam* HI restriction endonuclease sites into both the +5' sense and the -3' antisense bSUG1 primers:

+5'-CCGGATCC/ATG/GCG/CTT/GAC/GGA/CCA/GAG/C-3' (sense)

-5'-CGGGATCC/CTT/CCA/TAG/TTT/CTT-3' (antisense, inverse complement)

The PCR fragment was digested with *Bam* HI and ligated in-frame into pQE-12 vector (Qiagen Inc., Santa Clarita, CA). All of the cDNA constructs were completely sequenced with vector and internal primers from both +5' and -3' directions using the ABI PRISM^{TM} Genetic Analyzer model 310 (Perkin Elmer, Foster City, CA) to confirm the correct reading frame and the complete nucleotide sequence.

cDNA Library screening— PhLOP1 in pBD-GAL4 vector was used as a bait to screen a bovine retina cDNA library in the yeast expression vector system. The protein-protein interaction screen was performed with a sequential transformation procedure. The yeast reporter strain, CG-1945, was first transformed with the bait, pBD-GAL4- PhLOP1. Then 100 µg of library plasmids were introduced into the yeast strain expressing the BD-PhLOP1 hybrid protein. Approximately 1.7×10^6 yeast transformants were selected on thirty 15 cm plates with synthetic medium without leucine, tryptophan, and histidine (-Leu-Trp-His) plus 5 mM 3-amino-1,2,4,-triazole (3-AT) (Sigma Chemical Co, St. Louis, MO). After a 15 day incubation at 30 °C, His+ colonies were inoculated in -Leu medium, incubated for 2 days with shaking (250 rpm) at 30 °C, then Leu+Trp- yeast segregants carrying only the AD/ library plasmids were selected as described (Clontech Protocol #PT1020-1). The resulting yeast segregants were grown in -Leu medium and plasmids were isolated. The plasmids were transformed for amplification to *E. coli* SURE 2 electroporation competent cells (Stratagene). These isolated plasmids were cotransformed with the BD-PhLOP1 bait to yeast CG-1945 reporter strain and both *HIS3*and *LacZ* reporter gene expression was checked. The cDNAs for the positive His+/LacZ+ recombinant clones were sequenced.

β-galactosidase (β-gal) assay— For the qualitative assay, yeast transformants were streaked onto -Leu-Trp-His synthetic medium, incubated for 4 days at 30 °C, and transferred to Whatman #1 filter paper (Whatman Inc., Clifton, NJ). The filter was immersed in liquid nitrogen for 15 s, thawed at room temperature, and then placed on top of another Whatman # 1 filter presoaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$, pH 7.0) with 0.27% β-mercaptoethanol (Sigma Chemical Co.) and enzyme substrate 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma Chemical Co.). The filters were incubated at 30 °C until blue color developed, approximately 2 h.

For the quantitative assay, yeast transformants were first plated on -Leu-Trp agar plates, incubated at 30 OC for 4 days, then the colonies were pooled together by scraping into 3 ml of -Leu-Trp-His medium. The yeast cells were diluted and sonicated for 10 s to disperse the cell clusters. The cells were inoculated in 3 ml of -Leu-Trp medium and shaken at 30 °C, 250 rpm until stationary phase (OD_{600} >1). Subsequently, 2 ml of the saturated culture was transferred to 8 ml of YPD medium and incubated at 30 °C with shaking for 4 h. The β-galactosidase activity was determined as described (Matchmaker Library User Manual, Clontech).

Liquid growth assay— Yeast transformants were treated in the identical manner as in the quantitative liquid β-galactosidase assay. After sonication, the cells were counted and seeded at about 1,000 cells/ml into either -Leu-Trp or -Leu-Trp-His media at a final volume of 2 ml. The samples were incubated at 30 °C with shaking for 2 days, then the OD_{600} values were recorded. Data represent the mean ± standard deviation (SD) of a representative experiment done in triplicate and are presented as a percentage of the OD_{600} of yeast grown in selective medium supplemented with histidine [22].

Expression and affinity purification of recombinant proteins— Glutathione S-transferase (GST) fusion proteins of Phd, PhLP1, PhLOP1 and PhLOP2 were made by inserting them in frame into the pGEX-3X vector (Pharmacia Biotech Inc) and expressed in E. coli strain DH5a (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD), induced with iospropyl-b-D-thiogalactoside (IPTG), and purified as previously described [21]. The bSUG1 protein was expressed with a C-terminal 6xHis tag with the QIAexpressionist pQE-12 high expression vector (refer to plasmid construction above). The pQE-12-bSUG1 plasmid was transformed into *E. coli* strain M15[pREP4] (Qiagen Inc.). Expression of bSUG1-6xHis was induced with IPTG at a final concentration of 0.1 mM for 1 h at 30 °C after the OD_{600} reached 0.8. The 6xHis-tagged protein was purified with Ni-NTA resin (Qiagen Inc.) under denaturing conditions with 8 M urea due to the insolubility of the protein under native conditions. Briefly, cells were harvested and resuspended in 30 ml 1X binding buffer

(20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole) (Novagen, Inc., Milwaukee, WI.) supplemented with 8 M urea and 15 units of DNase. The cells were sonicated on ice using six 10 s bursts at 250 W with a 10 s cooling period between each burst. The lysate was incubated on ice for 20 min, followed by 30 min centrifugation at 10,000 X g at 4 °C. The supernatant was applied to Ni-NTA column pre-equilibrated with the sonication buffer (1X binding buffer with 8 M urea). After washing the column 3 times with 10 bed volumes of 1X washing buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 60 mM imidazole) with 8 M urea, the column was further washed with 30 bed volumes of 1X PBS containing 1% Triton X-100, pH 7.4 to remove the urea. The SUG1-6xHis attached Ni-NTA resin was then incubated in the same PBS buffer at room temperature for 30 min for the protein to renature. The renatured protein was used directly for the in vitro binding assay without eluting from the resin [23].

In vitro binding assay— Each GST fusion protein of either Phd, PhLP1, PhLOP1, PhLOP2, or GST control was mixed with 50 µl of bSUG1-6xHis attached Ni-NTA resin in 1X PBS containing 1% Triton X-100, pH 7.4 at a final volume of 250 µl. The mixtures were incubated at room temperature with mild rotation for 30 min, followed by spinning at 13,000 rpm for 1 min. The supernatants were collected for further analysis. The resin was washed 4 times with 500 µl (10X bed volumes) 1X washing buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 60 mM imidazole), bound proteins were eluted with 1X elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1 M imidazole). The supernatants, washes, and eluates were resolved on 10% sodium dodecal sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA) using previously published protocols [21]. The immobilized proteins were detected with anti-GST (Pharmacia Biotech), anti-Phd monoclonal antibody (Mab 1D6, kindly provided by Drs. H. Dua and L. Donoso) and anti-6xHis monoclonal (Clontech laboratories, Inc.) antibodies using an Enhanced Chemiluminescence Kit (Amersham, Arlington Heights, IL).

RESULTS

Identification of a protein that interacts with PhLOP1 as the bovine homologue of ySUG1— A bovine retinal cDNA library in the pGAD10 yeast expression vector was screened for proteins that potentially interact with PhLOP1. The results are summarized in Figure 1. Approximately 1.7 x 10^6 yeast transformants containing both BD-PhLOP1, the bait, and the library plasmids were plated on -Leu-Trp-His + 5 mM 3-AT plates. All *His*3 protein expression of the yeast strain CG-1945 was completely inhibited by 5 mM 3-AT [24,25]. After 15 days of incubation at 30 °C, 68 His+ colonies were isolated. Among these His+ clones, only 7 were pink, robust colonies (>3 mm after one week growth). All the others were pale, small colonies (<2 mm) even after two week growth, but each was clearly evident on the 3-AT inhibited background. In order to select all potential positive clones, including weak and transient interacting proteins, all the His+ clones were processed for further study. After segregating from the BD-PhLOP1 bait, the library plasmids were amplified in *E. coli* cells and retransformed to yeast with the BD-PhLOP1. Only the original 7 clones were confirmed to be positive (His+/LacZ+). These positive clones were sequenced and five of them (clones 1, 3, 6, 7 & 61) had identical nucleotide sequences with the longest open reading frame coding for a 406 amino acid protein. A database search identified this protein as SUG1, a member of a large superfamily of putative ATPases. The deduced amino acid sequence is 100% identical to the recently described human 26S proteasomal regulatory subunit p45 [26], mouse SUG1 (mSUG1) [27] and rat SUG1 (rSUG1) [28]. It is also 99.3% similar to that of the human Trip1 protein, a structural and functional homologue of yeast SUG1 (ySUG1) [29]. Of the five clones obtained, two of them (clones 3 & 7) code for a full-length bSUG1 protein and contain a 5' noncoding region, one (clone 6) lacks the first two amino acids and the other remaining two clones (clones 1 & 61) are identical and lack the first three amino acids (Figure 2). BD-PhLOP1 did not transactivate either *HIS3*or *LacZ* reporter genes above the BD or AD background when expressed alone in CG-1945 (data not shown), indicating that the full-length PhLOP1 protein does not activate transcription on its own when tethered to DNA through a heterologous BD.

Interaction of the Phd isoforms with bSUG1— Phd, PhLP1, and PhLOP2 have amino acid sequence homology to PhLOP1 (Figure 3A). To determine if Phd and the other Phd

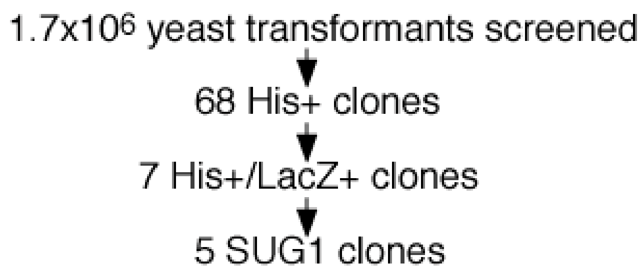

Figure 1. Summary of the yeast two-hybrid library screen. A 100 micrograms of a bovine retinal cDNA library plasmids in yeast expression vector pGAD10 was transformed into yeast strain CG-1945 co-expressing BD-PhLOP1 hybrid protein. One out of 2.5 X 10^4 yeast transformants screened exhibited transactivation of both *HIS3*and *LacZ* reporter genes. Of the 7 positive clones, 5 were characterized and identified as bSUG1.

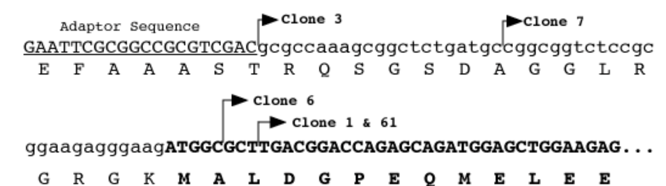

Figure 2. The 5' nucleotide sequences and translation of the bSUG1 cDNAs obtained from the bovine retina yeast expression library screen. The 5' nucleotide noncoding region is shown in lower case letters and the 5' nucleotide coding region is shown in bold upper case letters with the single amino acid residues indicated below. Arrows identify the insert start site of each clone. The 5' nucleotide adaptor sequence, in upper case, is included to establish the correct reading frame coding for the translated SUG1 protein.

isoforms we identified previously [21] also could interact with SUG1, we cotransformed the BD fusion proteins of each isoform in the pBD-GAL4 vector with either the AD vector or AD-SUG1 to the yeast strain CG-1945. None of the BD-Phd isoform hybrid proteins could activate reporter expression when transformed alone or cotransformed with the AD vector (data not shown). Phd and PhLP1, which share the identical C-terminal sequence with PhLOP1, interacted with SUG1; in contrast, PhLOP2, which lacks the C-terminal sequence of Phd, did not interact with SUG1 (Figure 3B). Quantitative analysis revealed that among the four protein isoforms, PhLOP1 had the strongest protein-protein interaction with SUG1 ($p<0.05$) (Figure 4).

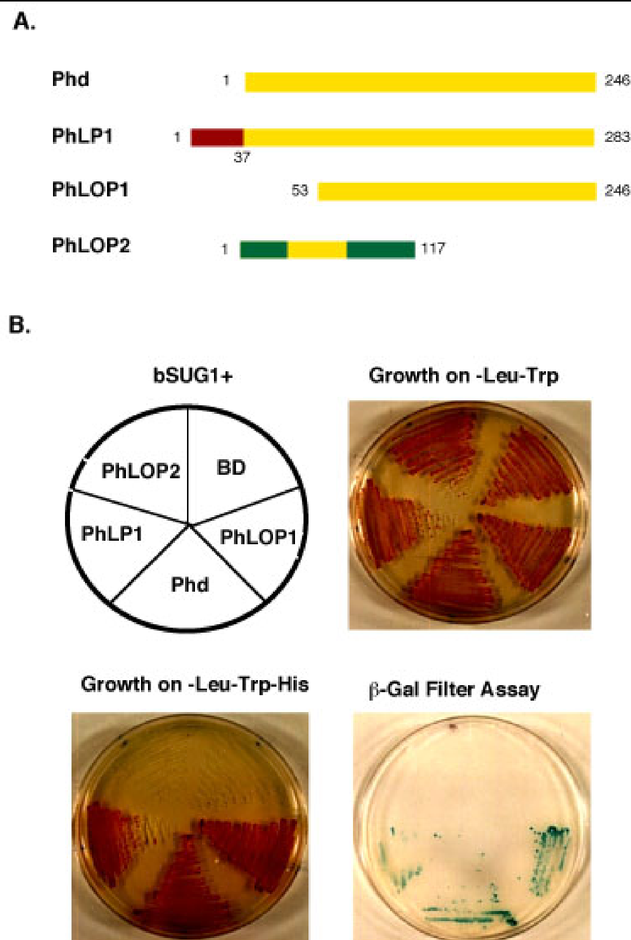

Figure 3. Interaction of Phd isoforms with bSUG1 in yeast. **(A)** Schematic alignment of the domains representing the amino acid sequences of each Phd isoform which was subcloned into the GAL4 BD (not shown in the figure) at their amino-terminus. The identical sequences are indicated with yellow bars. The unique domains for either PhLP1 or PhLOP2 are shown in different colors. **(B)** Assays of *HIS3* and *LacZ* reporter gene expression. The yeast reporter strain CG-1945 was cotransformed with AD-bSUG1 (clone7) and each of the BD-Phd isoforms (top left panel). A pool of colonies from -Leu-Trp plates was streaked onto selective medium with histidine (-Leu-Trp) (top right panel) or without histidine (-Leu-Trp-His) (lower left panel). The plates were incubated at 30 °C for 4 days. Cells grown on -Leu-Trp-His plates were transferred onto a Whatman #1 filter, lysed by rapid freezing in liquid nitrogen and thawing at room temperature. The filter was then placed on top of another a Whatman #1 filter pre-soaked in Z buffer with X-gal and incubated at 30 °C. The positive blue color reaction developed within 2 hours (lower right panel).

Direct association of Phd isoforms with bSUG1 in vitro—In vivo interactions can occur by either direct protein-protein interaction or indirectly through intermediary factors. To identify whether Phd and its isoforms bind SUG1 directly, we purified GST-fusion proteins of Phd and its isoforms and SUG1-6xHis and did an in vitro binding assay. Ni-NTA beads,

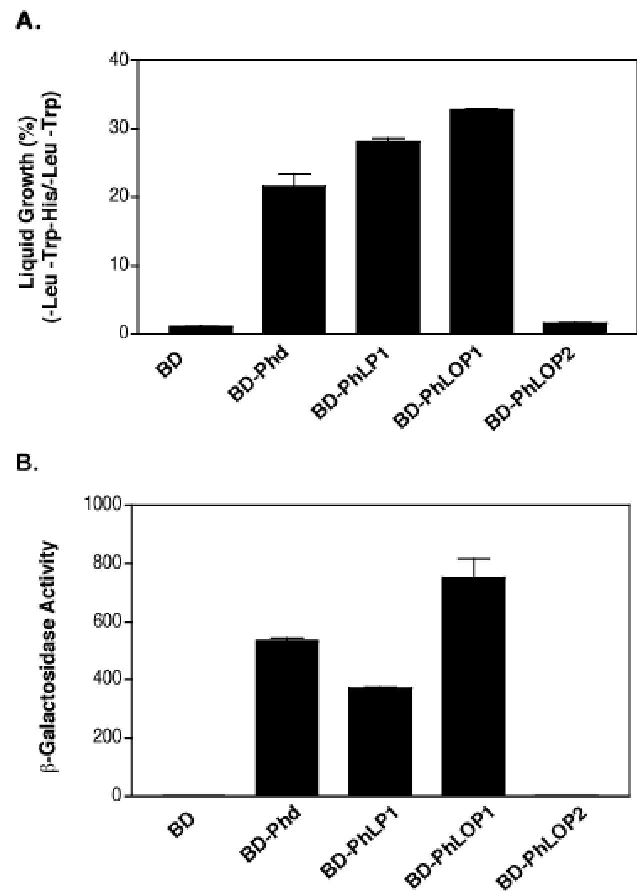

Figure 4. Quantitative analysis of the interaction between each Phd isoform and bSUG1. The yeast reporter strain CG-1945 was cotransformed with AD-bSUG1 and the indicated BD-Phd isoform plasmid. Yeast transformants were plated on -Leu-Trp plates and incubated at 30 °C for 4 days. The colonies were then pooled in -Leu-Trp-His medium and sonicated for 10 sec. **(A)** Graph of data from an experiment in which a pool of colonies was seeded at 1,000 cells/ml into selective media with histidine (-Leu-Trp) or without histidine (-Leu-Trp-His). After incubating liquid cultures at 30 °C for 2 days, growth was measured at OD_{600}. Data represent the mean ± SD of triplicate assays of a representative experiment and is presented as a percentage of the OD_{600} of yeast grown in selective medium supplemented with histidine (-Leu-Trp). Yeast cells cotransformed with SUG1 and PhLOP1 grew faster than with SUG1 and Phd or PhLP1 ($p<0.05$). **(B)** Graph of data from an experiment in which a pool of colonies was collected and prepared for the liquid β-galactosidase reporter assay. Data represent the mean ± SD of two experiments done in triplicate. The β-galactosidase activity is expressed in standard units multiplied by 1,000. The β-galactosidase standard units = 1000 x OD_{420} / time (min) x OD_{600}. Yeast cells cotransformed with SUG1 and PhLOP1 had significantly higher β-galactosidase activity than with SUG1 and Phd or PhLP1 ($p<0.05$).

with or without immobilized SUG1-6xHis, were incubated with each GST-Phd isoform fusion protein or GST control. After extensive washing, bound proteins were eluted with 1 M imidazole. Aliquots of the proteins from the supernatant, washes, and eluant were resolved on SDS-PAGE and transferred to membranes. The membranes were incubated with appropriate antibodies to identify the phosducin isoforms, the GST or 6xHis-tagged proteins. Our results demonstrate that while no proteins were retained by Ni-NTA beads itself without the SUG1-6xHis (Figure 5A), Phd, PhLP1, and PhLOP1 are retained by SUG1 bound Ni-NTA beads; PhLOP2 and GST were not retained by SUG1 (Figure 5B). The above results are consistent with the previous yeast two-hybrid system assay and demonstrate that the interactions between Phd isoforms and SUG1 are a direct protein-protein interaction.

The C-terminus of PhLOP1 is required for functional interaction with SUG1 in yeast— To identify the SUG1 binding region(s), five altered forms of PhLOP1 were fused to the BD in pBD-GAL4 vector (Figure 6A) and examined for protein-protein interaction with either the unfused AD (pGAD10 vector) or the AD-SUG1 in the yeast two-hybrid system. None of the BD fusions of truncated PhLOP1 could activate reporter expression when cotransformed with the unfused AD vector control (data not shown). As evidenced by both the decreased growth rate on -Leu-Trp-His plates (Figure 6A) or the percent growth in liquid media (Figure 6B) and the measurement of the β-galactosidase activity (Figure 6C), the strength of the interaction with SUG1 decreased significantly when 20 amino acids were truncated from the C-terminus of PhLOP1 ($p<0.05$)

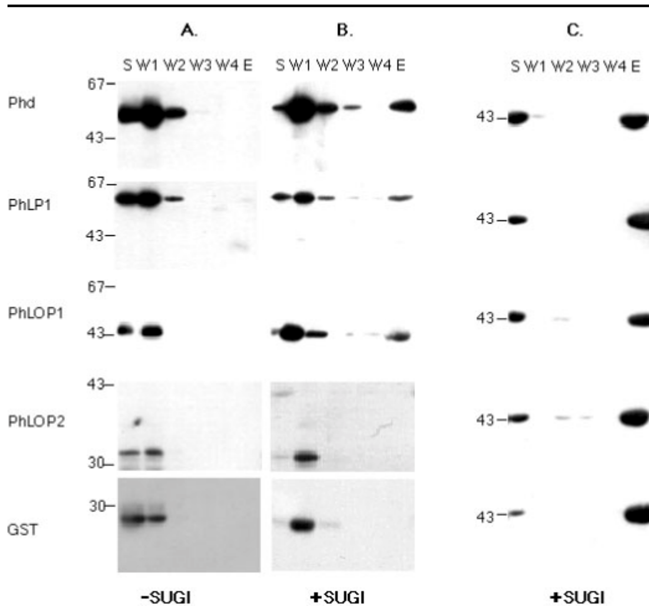

Figure 5. In vitro protein-protein interaction assay with GST-Phd isoform fusion proteins. GST fusion proteins containing the complete coding sequence for either Phd, PhLP1, PhLOP1, PhLOP2, or a GST control were incubated with either Ni-NTA resin without bSUG1-6xHis (-SUG1) or with bSUG1-6xHis attached Ni-NTA resin (+SUG1). After washing four times, the bound proteins were eluted. The supernatants (S), washes (W1-W4) and eluate (E) were subjected to 10% SDS-PAGE, electrophoretic transfer to Immobilon-P, and immunoblot analysis with ECL detection on X-ray film. The numbers represent the standard molecular weight standards loaded with each SDS-PAGE. **(A)** and **(B)** The GST-Phd, GST-PhLP1 and GST-PhLOP1 were detected with Phd monoclonal antibody (Mab 1D6). The GST-PhLOP2 and GST were detected with GST monoclonal antibody. **(C)** The membranes of Figure 5B were stripped and reprobed with 6xHis monoclonal antibody to verify and to detect the presence of the bSUG1-6xHis protein in each supernatant and eluant.

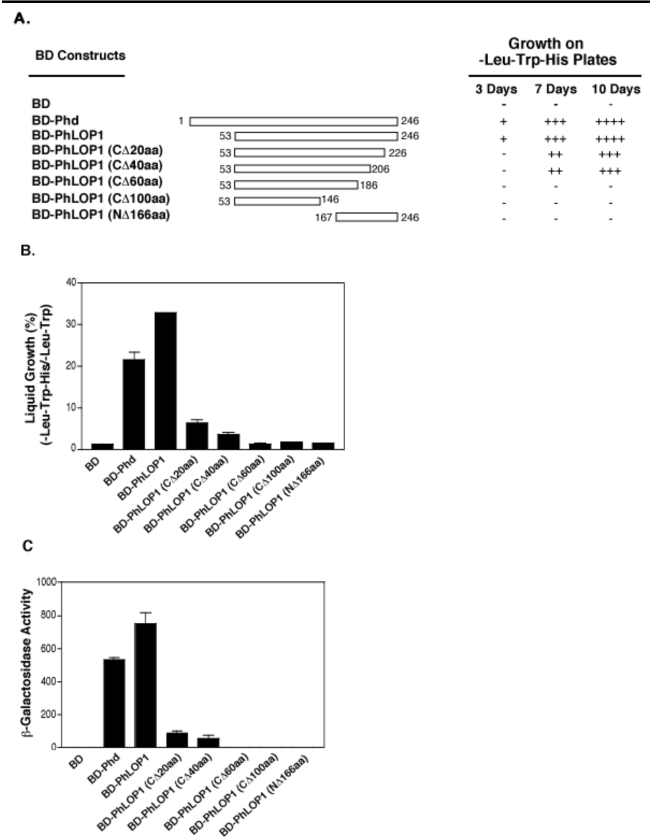

Figure 6. Interaction of PhLOP1 with bSUG1. AD-bSUG1 was cotransformed with the indicated BD-Phd, BD-PhLOP1, or truncated BD-PhLOP1 fusion constructs into yeast CG-1945. The transformants were divided equally and grown in the presence of histidine (-Leu-Trp) or absence of histidine (-Leu-Trp-His) and incubated at 30 °C. **(A)** Schematic alignment representing the BD constructs of Phd (1-246 aa), PhLOP1 (53-246 aa) and truncated PhLOP1s (carboxy terminal deletions: C delta (Δ) 20 aa, 40 aa, 60 aa, 100 aa or amino terminal delection: N Δ 166 aa) and their interaction with bSUG1 by estimating the growth on -Leu-Trp-His plates. (-): Small (<1 mm), pale colonies (background growth because of HIS3 protein “leaky” expression in CG-1945). (+): Small (<1 mm), pink colonies. (++): Medium sized (1-2 mm), pink colonies. (+++): Big (2-3 mm), pink colonies. (++++): Robust (>3 mm), pink colonies. **(B)** The yeast transformants grown on histidine supplemented synthetic (-Leu-Trp) plates were collected after 4 days incubation at 30 °C and a pool of colonies was seeded at 1,000 cells/ml into selective medium with histidine (-Leu-Trp) or without histidine (-Leu-Trp-His). After incubating the cultures for 2 days at 30 °C with shaking, growth was measured at OD_{600}. Data represent the mean ± SD of triplicate assays of a representative experiment and is presented as a percentage of the OD_{600} of yeast grown in selective medium supplemented with histidine (-Leu-Trp). **(C)** A pool of colonies was collected from samples grown on -Leu-Trp plates and prepared for β-galactosidase liquid assay as described in methods. Data represent the mean ± SD of two independent experiments done in triplicate. The β-galactosidase activity is expressed in standard units multiplied by 1,000. The β-galactosidase standard units = 1,000 x OD_{420} / time (min) x OD_{600}.

(Figure 6B, C). When up to 60 amino acids were truncated from the C-terminus, the interaction was completely abolished, implying that the C-terminus of PhLOP1 is the site of SUG1 binding or a site that modulates this binding activity for SUG1. Modulation is suggested since BD-Phd (167-246), which contains the last C-terminal 80 amino acids of Phd, PhLP1, and PhLOP1, shows no interaction with SUG1. This suggests that the C-terminus alone is not sufficient to mediate SUG1 interaction (Figure 6A, B, C).

DISCUSSION

A yeast two hybrid screen was utilized to identify proteins that interact with the Phd isoform, PhLOP1. Five independent clones of a protein, the bovine orthologue of yeast SUG1, named bSUG1, were isolated and their interaction with Phd, PhLP1, PhLOP1, and PhLOP2 was examined in detail.

The ySUG1 was first suggested to be a transcriptional mediator because a mutation of the gene could rescue defects in the GAL4 AD [30]. The suggestion was supported by the finding of ySUG1 in the purified yeast RNA polymerase II holoenzyme [31] and the observation that ySUG1 could directly and specifically bind the acidic ADs of GAL4 and the viral activator, VP16 in vitro, as well as the TATA-binding protein, TBP [32]. Ligand-enhanced interaction between the ligand-dependent transcription activation function 2 (AF-2) domain of nuclear hormone receptors and either Trip1, the human homologue of yeast SUG1 [29,33], or mSUG1 [27] has suggested that mammalian SUG1 may also act as a mediator in ligand-dependent transcriptional activation by nuclear receptors. Functional similarity between yeast and mammalian SUG1 is supported by the ability of Trip1 or mSUG1 to complement the lethal phenotype of loss of ySUG1 [27,29,34].

In addition to the transcriptional mediator function, ySUG1 was recently found in the yeast 26S proteasome, a multi-protein complex that plays a general role in turnover of both short- and long-lived proteins by specifically degrading ubiquitinated proteins [35], and was suggested to regulate the proteasomal activity [36,37]. This suggestion was supported by the observation that the yeast cells harboring a mutant SUG1 accumulated ubiquitinated proteins normally degraded by the 26S proteasome [38]. A role for SUG1 in the regulation of the activity of the 26S proteasome is further supported by the identical amino acid sequence of mSUG1 with human p45 subunit of the PA700 proteasomal regulatory complex [26]. The three amino acid sequence difference between human Trip1 and p45 was suggested to be a sequence error [27] and SUG1 was suggested to be a multi-functional protein associated with distinct cellular protein complexes [28]. Recently, mSUG1 [39], rSUG1 [28], and the *Drosophila* orthologue of ySUG1, called DUG [40], also were found to be associated with the 26S proteasome. The co-localization of mSUG1/FZA-B with c-Fos in the 26S proteasome led to the suggestion that SUG1 might play a role in the regulation of specific transcription by controlling the rate of degradation of transcriptional factors [41]. The identification of SUG2 in the yeast 26S proteasome regulatory subunit and its functional similarity to SUG1 in rescuing GAL4 defects emphasized the paradox of proteasomal proteins having strong effects on transcription [42].

Retinal Phd has a well-studied function in the regulation of visual transduction by sequestering G-protein β/γ subunits [7-10,12-14]. Our recent work discovered an isoform, PhLOP1, which is distinct from Phd, but is also expressed in retina. Unlike Phd, PhLOP1 fails to bind G-protein β/γ subunits, leaving its physiological function currently unknown [21]. In this report, we discovered that Phd, PhLP1, and PhLOP1 interact with SUG1 both in the yeast two-hybrid assay and in the in vitro binding assay. Since SUG1 has been shown to be a subunit of the 26S proteasome, which is a universal multi-protein complex whose existence in lens [43,44], retinal pigment epithelial cells [45] and retinal photoreceptors [46] has been verified, the interaction between SUG1 and Phd isoforms may have an additional important physiological function. Because unphosphorylated Phd tightly binds Gβγ and PhLOP1 has a stronger protein-protein interaction with SUG1 than the other Phd isoforms, PhLOP1 may be the major isoform that SUG1 interacts with under physiological conditions and it may have other cellular signaling functions involved in proteosomal "reverse chaperoning."

Taken together, the association of the Phd isoforms with SUG1 suggests that SUG1 might play a role in regulating intracellular signaling pathways through targeting regulatory proteins in the pathway for proteasomal degradation or it may suggest that Phd and its isoforms, like most of the other SUG1 interacting proteins, are potential transcriptional factors. We have found an internal transcriptional activation domain in these SUG1 interacting Phd isoforms that is normally masked by the full-length protein (unpublished observation). Further studies are ongoing to address the issues regarding the significance of the protein-protein interactions between SUG1 and Phd isoforms.

Note: After this paper was submitted, other investigators also found that the phosducin-like proteins, $PhLP_L$ and $PhLP_S$ both interacted with mouse SUG1 in a similar yeast two-hybrid screen and that inhibition of proteasome function led to accumulation of high molecular weight, ubiquitin-immunoreactive protein precipitated by PhLP antiserum [47]. These data support our first assumption that SUG1 might target proteins in the Phd family for degradation. It is also possible that both Phd and $PhLP_{L/S}$ isoforms are transcriptional regulators.

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