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The immunolocalization and divergent roles of phosducin and phosducin-like protein in the retina

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Purpose: These investigations were undertaken to compare and contrast the roles of phosducin and phosducin-like protein in the retina.
Methods: Phosducin and phosducin-like protein were compared in an in vitro assay measuring their inhibition of transducin binding to light-activated rhodopsin. The two proteins were localized within the retina by immunoblot analyses and immunocytochemistry using affinity-purified antibodies with high specificity for each of the two homologs. The sensitivity of phosducin-like protein to phosphorylation was probed using in vitro protein kinase reactions.
Results: Phosducin and phosducin-like protein were found to have similar, though not identical, transducin inhibiting activity in vitro. These two proteins were found to be localized dissimilarly within the retina, with spatial overlap limited to the inner segments of the photoreceptors. Phosducin is found exclusively in photoreceptor cells, including the synaptic and nuclear layers, while phosducin-like protein is found throughout the inner retinal layers, most abundantly in the bipolar cells of the inner nuclear layer. Phosducin-like protein is not efficiently phosphorylated by the protein kinases tested, indicating that its regulation differs from that of phosducin.
Conclusions: It appears that phosducin and phosducin-like protein play distinct roles in the retina. While phosducin is likely to be important in feedback regulation of the visual signal, such as in light adaptation, phosducin-like protein probably has little if any function in the phototransduction cascade. Phosducin-like protein may have a role in regulating the processing of visual signals by the neural cells of the inner retina.

The visual signal transduction cascade is a prototypical G protein signaling pathway. Phosducin (Pd) has been identified as a potentially important regulator of this cascade [1,2]. Phosducin binds $G_t\beta\gamma$ with high affinity, competitively excluding the binding of $G_t\alpha$ and formation of the transduction-competent heterotrimer [2-4]. The binding of Pd to $G_t\beta\gamma$ is regulated by protein kinase A (PKA) catalyzed phosphorylation at Ser_{73} of Pd [3,5,6], and its phosphorylation state is light dependent [7]. In the dark, photoreceptor cAMP concentrations rise due to a Ca^{2+}/calmodulin-dependent adenylate cyclase [8], increasing PKA activity, resulting in a concomitant increase in phosphorylation of Pd. Phosphorylated Pd no longer blocks $G_t\alpha$ association with $G_t\beta\gamma$, thus more heterotrimeric transducin (G_t) is available for activation [9]. In the light, cytoplasmic Ca^{2+} and cAMP concentrations decrease, PKA activity drops, and Pd becomes dephosphorylated by cellular phosphatases [10]. Unphosphorylated Pd sequesters $G_t\beta\gamma$ from the $G_t\alpha$ subunit and inhibits further activation by light-activated rhodopsin (Rho*). Thus, Pd seems to play an important role in light and dark adaptation of photoreceptors.

Though there has been a report of Pd expression in varied tissues [11], it is found in abundance only in the retina and the developmentally related pineal gland [12]. However, a homolog termed phosducin-like protein (PhLP) was shown to have a widespread tissue distribution [13]. Phosducin and PhLP are encoded by distinct genes, located on chromosomes 1q [14] and 9 [15] in humans, respectively. Both genes appear to be alternatively spliced generating distinct splice variants [13,16]. Identity between Pd and PhLP is most extensive in the carboxy terminal domain. The amino terminal domain of Pd is seen in the crystal structure of the Pd/$G_t\beta\gamma$ complex to contain most of the specific $G_t\beta\gamma$ binding contacts and to occupy most of the surface to which $G_t\alpha$ binds [4]. Helix 1 of Pd, which is composed of 14 residues, includes seven residues that make specific contacts with $G_t\beta\gamma$. This segment is identical in Pd and PhLP except for one conservative replacement. Though most of the amino acids shown to make specific contacts with $G_t\beta\gamma$, including this helix, are conserved between Pd and PhLP, there is some notable divergence in their amino terminal domains, including a nine residue insertion between helices 1 and 2, and a 38 residue amino terminal extension, both unique to PhLP. Despite these differences, the conservation of key contacting sites allows PhLP to bind $G_t\beta\gamma$ [17]. Both Pd and PhLP have been shown to interact with the SUG1 subunit of the 26S proteasome, which may target them for degradation or possibly suggest a role in transcriptional regulation through SUG1 involvement in the RNA polymerase II holoenzyme [18,19].

In view of the possible functional overlap between PhLP and Pd and the widespread distribution of PhLP, we set out to investigate the specific localization and the comparative roles of these proteins within the retina.

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METHODS

Procedures involving animals followed animal care guidelines of the US Public Health Service.

Assay of Pd/PhLP inhibition of G_t: The pET 15b vector (Novagen, Madison, WI) containing the recombinant rat Pd cDNA with a His_6 tag was a gift from R. Gaudet (Yale University, see [4]). The gene encoding PhLP was cloned by reverse transcription/PCR from rat kidney total mRNA using RNAsin/AMV RT (Promega, Madison, WI), followed by PCR amplification using the primers 5'-CAT GAC AAC CCT GGA TGA CAAG and 5'-TCA ATC TAT TTC TAG ATC GCT GTC TTC with Taq DNA polymerase (Gibco BRL, Gaithersburg, MD). The cDNA was then subcloned into the pET 15b vector using engineered NdeI and BamHI restriction sites, and transformed into BL-21 DE3 cells for protein expression. Recombinant Pd or PhLP was overexpressed in these cells and purified as previously described [4], except that purity was sufficient to obviate the need for the anion-exchange chromotography step. Purified His_6 Pd and PhLP were buffer exchanged into isotonic buffer (20 mM HEPES pH 7.5 with 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) using Centricon concentrators (Millipore, Bedford, MA) and diluted 50% with sterile glycerol to protein concentrations in the range of 1 to 10 mg/ml. When prepared in this manner, His_6 Pd and His_6 PhLP can be stored at -20 °C for up to six months without loss of activity.

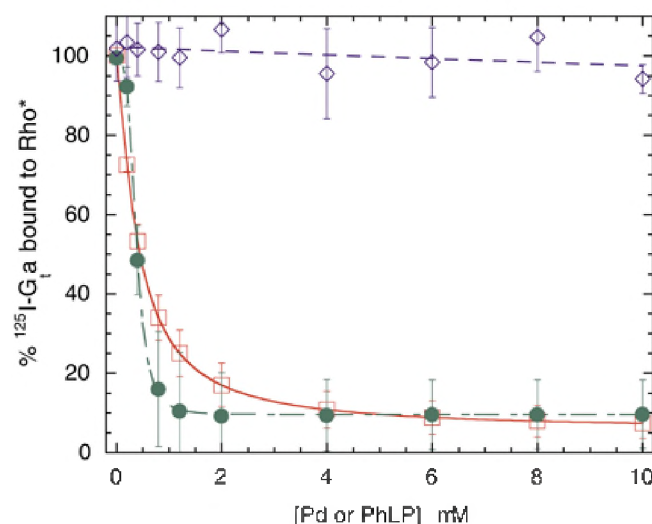

Figure 1. Phosducin (Pd) and PhLP inhibit G_t binding to light-activated rhodopsin (Rho*). Light-induced binding of ^{125}I-$G_t\alpha$ (0.2 µM) and $G_t\beta\gamma$ (0.2 µM) to urea-stripped rod outer segment membranes (1.0 µM Rho) was carried out in the presence of Pd (green, filled circles) or PhLP (red, open squares) at the concentrations indicated (see Methods). Ovalbumin shown as negative control (blue, open diamonds). Error bars represent the first standard deviation of data from three separate experiments. 100% is 0.07-0.1 pmol of ^{125}I-$G_t\alpha$bound/pmol of Rho*. Lines represent non-linear least squares fit of the data to the equation: Fraction bound = $(1 - X)/(1 + [Pd]/K_{1/2}) + X$, where X is the fraction remaining bound at saturating the concentration of Pd and $K_{1/2}$ is the concentration of Pd at which half maximal inhibition occurred. $K_{1/2}$ values were 0.37 µM for Pd and 0.41 µM for PhLP.

Urea-stripped rod outer segment membranes (UROSM), $G_t\alpha$and $G_t\beta\gamma$ were prepared and $G_t\alpha$radioiodinated as previously described [20]. Light-induced binding of $G_t\alpha$and $G_t\beta\gamma$ to UROSM in the presence of varying concentrations of Pd or PhLP was performed as previously described [20]. Briefly, Pd or PhLP was mixed with 0.2 µM $G_t\alpha$, 0.2 µM $G_t\beta\gamma$ and non-illuminated UROSM at 1.0 µM rhodopsin (Rho), and incubated in the dark. The samples were divided in half for light and dark comparison. The light samples were then illuminated for 1 min and all samples were centrifuged to pellet membranes. Supernatant aliquots were counted for ^{125}I and compared to total counts determined from aliquots taken before centrifugation. Bound counts were calculated by subtracting supernatant counts from total counts, and Gα binding to Rho* was determined by subtracting dark counts from light counts.

Phosphorylation of Pd and PhLP: Protein kinase A catalytic subunit (2 units/µl final concentration, purified from bovine heart; Fluka BioChemika, Buchs, Switzerland) was incubated at 37 °C with 0.04 mg/ml recombinant Pd or PhLP in isotonic buffer with 1.5 mM $MgCl_2$ and 1.5 mM ATP, including 0.025 µCi/µl ^{32}P-ATP (NEN Life Science, Boston, MA). Reactions were terminated by addition of 4X SDS sample buffer. Samples were electrophoresed, gels stained with Coomassie and dried. Radioactive protein bands were quantified using a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Protein kinase C from rat brain (Calbiochem, La Jolla, CA; a mixture of isoforms α, β, and γ, used at a final concentration of 0.002 u/µl) was incubated at 37 °C with 0.1 mg/ml recombinant Pd or PhLP in 20 mM Tris pH 7.5 with 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10% (v/v) mixed micelles (mixed micelles: 3% triton X-100, 3.5 mM phosphatidylserine (Avanti Polar-lipids, Alabaster, AL), 1 mM 1,2-didecanoyl-rac-glycerol (Calbiochem), sonicated and vortexed repeatedly), and 10 µM ATP, including 0.025 µCi/µl ^{32}P-ATP. Reactions were stopped, separated, and the phosphorylation quantified as above. Histone type III-S (Sigma, St. Louis, MO) was used at the same concentration as Pd/PhLP as a positive control.

The ε isoform of PKC (Calbiochem; human recombinant, used at 0.003 u/µl) was incubated with 0.1 mg/ml recombinant Pd or PhLP under conditions identical to those described above for the other PKC isoforms except lacking $CaCl_2$. A peptide substrate specific for this isoform (MW 2068, Calbiochem) was used at 7.5 µg/ml final concentration as a positive control.

Affinity purification of antibodies: Polyclonal antibodies were raised against recombinant rat Pd and recombinant rat PhLP (see above), and also against a glutathione-S-transferase (GST) fusion protein incorporating the 50 amino terminal residues of PhLP (which includes the 38 residue amino terminal extension and 12 amino acids that are not homologous to the Pd sequence). This GST fusion protein was created by subcloning the first 150 base pairs of the human PhLP cDNA [21] into the pGEX-4T-3 vector using engineered BamHI and XhoI restriction sites and the PCR primers 5'-AGC CGG ATC CAT GAC CAC CCT TGA TGA TCCG and 5'-ACG CCT CGA GTC AGC CTG CCA GCT CAG CCT CTGC. The

protein was expressed in BL-21 DE3 bacteria, induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (Calbiochem), and harvested as with the recombinant His_6 proteins above. Chromatography was accomplished using glutathione agarose (Sigma), eluting with 20 mM reduced glutathione in 100 mM Tris pH 8.0, on a Biologic chromatography system (BioRad, Hercules,CA). Three milligrams of each of these three proteins (Pd, PhLP, and PhLP N-terminus/GST fusion) were sent to HTI Bioproducts (Ramona, CA) for use as antigens for polyclonal antibody production in rabbits.

Antibodies were affinity purified from the serum. Affinity columns (Affi-Gel 15, BioRad) were prepared as detailed by the manufacturer, cross-linking either recombinant Pd or PhLP. The antisera were prepared for purification by incubating at 56 °C for 30 min and clarified by centrifugation at 10,000 x g for 10 min. The supernatant was loaded on the affinity column which had been pre-equilibrated with 20 mM HEPES pH 7.5, 150 mM NaCl, and 0.2 mM PMSF and washed with several column volumes of this buffer. Non-specifically bound proteins were washed off with a high salt buffer (20 mM HEPES pH 7.5, 1 M NaCl, 0.2 mM PMSF). Specifically bound antibodies were eluted with 20 mM HEPES pH 7.5, 4 M $MgCl_2$, 0.2 mM PMSF. The antibody fraction was buffer exchanged with 100 volumes Q2 Buffer A (20 mM HEPES pH 7.2, 20 mM NaCl, 0.2 mM PMSF) to remove the $MgCl_2$, concentrated to around 100 µl, and further purified with a Q2 Anion Exchange column (BioRad). The antibody eluted during an elution gradient between 0% and 8% Q2 Buffer B (20 mM HEPES pH 7.2, 500 mM NaCl, 0.2 mM PMSF). The resulting antibody peak was collected, concentrated, and the protein concentration determined using the Bradford/Pierce Coomassie method (Pierce, Rockford, IL).

Preparation of extracts: To quantify Pd and PhLP in tissues, bovine intact rod outer segment (IROS) extracts and bovine retinal extracts were used. IROS was prepared by a method based on that of Schnetkampt et al. [22] in the dark using infrared illumination and image converters. Briefly, eyes were removed from cattle freshly killed at a local slaughterhouse and were dark adapted for 1 h at room temperature in HEPES/Ringer's buffer (10 mM HEPES pH 7.5 with 120 mM NaCl, 3.5 mM KCl 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM glucose, 1 mM DTT, 0.2 mM PMSF). For IROS preparation, ten dissected retinas were placed into 10 ml of retinal isolation media (20 mM Tris pH 7.4 with 600 mM sucrose, 10 mM glucose, 1 mM $CaCl_2$, and 5% w/v Ficoll 400), disrupted by repeated passage through a syringe with a 0.4 cm orifice, and the retinal mesh detained by filtration through cheesecloth. The filtrate was then applied to a gradient created by layering 7 ml of retinal isolation media onto an equal volume of 20% (w/w) sucrose, 16% (w/w) Ficoll 400, and the gradient was centrifuged at 27,000 rpm in a Beckman SW27 rotor (Beckman Instruments, Palo Alto, CA) for 1 h. The band containing Rho was removed and diluted with two volumes of retinal isolation media lacking Ficoll, and centrifuged at 1400 x g for 20 min. The pellet was then resuspended in an equal volume of retinal isolation media and stored in the dark at 4 °C. An aliquot was examined under the microscope where it was seen that 60-80% of the visible structures were IROS. An extract was prepared for electrophoresis by diluting IROS 1:10 with 1% SDS and triturating repeatedly through a 23 gauge needle. A retinal extract was also prepared by suspending 50 mg dark adapted bovine retina in 1 ml of 1% SDS, followed by repeated trituration through a 23 gauge needle. Total protein in the extracts was determined using the

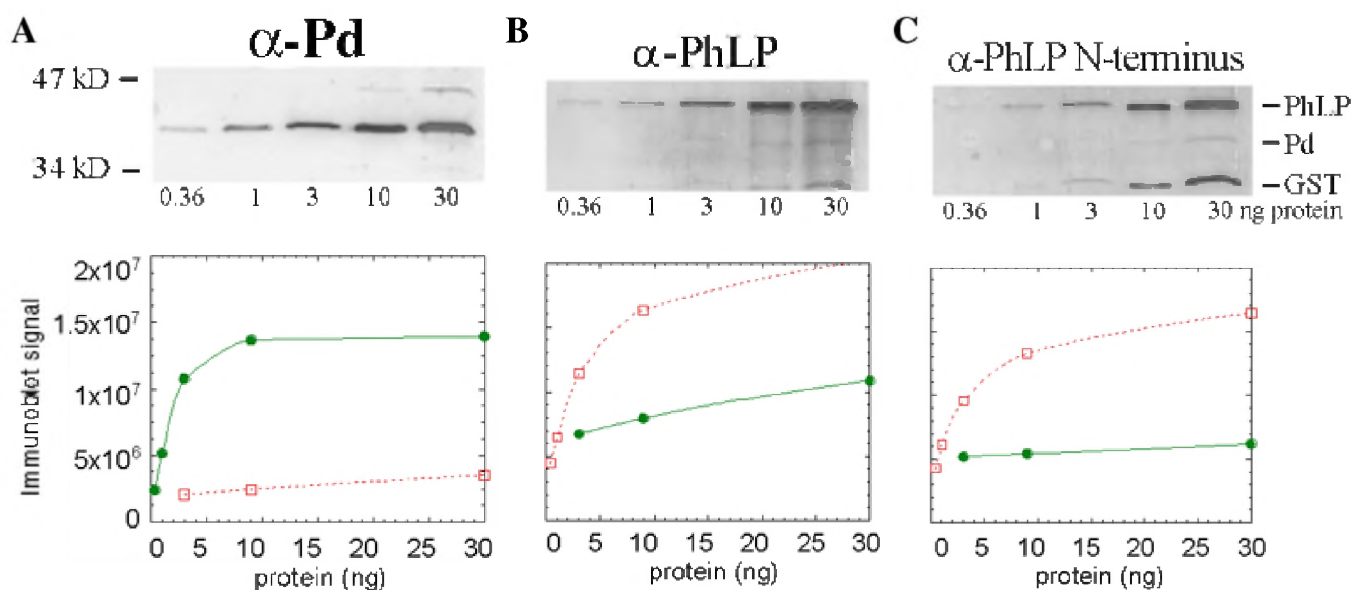

Figure 2. Specificity of the affinity-purified antibodies raised against phosducin and phosducin-like protein. An equal mixture of recombinant PhLP, Pd, and GST were electrophoresed using the amounts of protein indicated at the bottom of **A**-**C** (0.36-30 ng of each protein). Immunoblots were probed with anti-phosducin (**A**), anti-PhLP (**B**), and anti-PhLP N-terminus/GST fusion protein (**C**). See Methods for antibody details. Shown with the blots are graphs of immunoblot signal for Pd (filled circles, solid line interpolated) and PhLP (open squares, dotted line). Specificity was quantified by comparing the slopes of signals in the linear range (first three points). **A**. Anti-phosducin. **B**. Anti-PhLP. **C**. Anti-PhLP N-terminus/GST fusion protein.

BCA assay (Pierce).

Antibody specificity: For determination of antibody specificity, an equal mixture of purified recombinant Pd, PhLP, and GST was prepared and varying amounts (ranging from 0.36 to 30 ng of each protein) were electrophoresed and analyzed by immunoblotting as below. Primary antibodies were used at 1:5000 dilution.

Immunoblotting for quantitation of Pd, PhLP, and Rho: Known amounts of total protein from extracts were separated by SDS-PAGE in 12% gels and transferred to nitrocellulose membranes. Membranes were blocked by incubation for 30-60 min in 50 mM Tris pH 7.6, 150 mM NaCl, 1% tween-20 (TBST) with 5% (w/v) nonfat dry milk. Blots were then incubated overnight at 4 °C in primary antibody (1:1000 to 1:10,000 dilution) in TBST with 5% milk. After two 5 min washes in TBST, blots were incubated for 1 h at room temperature in secondary antibody (goat anti-rabbit IgG, heavy and light chain, peroxidase conjugate, Calbiochem) at 1:1000 dilution in TBST. Blots were then developed with the ECL Plus Western detection system (Amersham Life Science, Arlington Heights, IL) and visualized on the phosphorimager in chemiluminescence mode, for quantitation of immunoblot signals. Concentrations of blotted proteins were determined by comparison with known amounts of purified recombinant protein on the same blot and are reported as the average of duplicate determinations and the standard error of the mean.

For comparison with Rho, bleachable Rho in IROS was determined using the method of Bownds et al. [23]. To calculate the concentration of Rho in retinal extracts, varying amounts of IROS and retinal extract were electrophoresed, blotted, and probed with the R4 anti-rhodopsin antibody (a gift of Dr. D. Takemoto [24]). The known concentration of Rho in the IROS was then used to calculate the the concentration of Rho in the retinal extract.

Immunocytochemistry: Mouse retinal sections were prepared as previously described [25]. Briefly, 12 micron cryostat sections were preincubated in 10% normal goat serum in 0.1 M phosphate buffer pH 7.4 (PB)/0.05% Triton X-100 for 30 min. Sections were incubated 2 h at room temperature in primary antibody (1:100 to 1:250 dilutions in PB/0.05% Triton X-100 for anti-Pd and anti-PhLP antibodies; anti-calretinin antibody (Swant, Switzerland) and anti-calbindin antibody (Sigma) were used at manufacturers' suggested dilutions; anti-recoverin antibody, a gift of Dr. J. F. McGinnis, University of Oklahoma, was used at 1:100 [26]). Following primary incubation and PB washes, sections were incubated with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated goat antirabbit IgG (Vector Laboratories, Burlingame, CA), diluted 1:100 in PB/Triton X-100, for 1 h at room temperature. Double labeling was accomplished by incubation in the first primary followed by incubation in the FITC-conjugated secondary, reblocking with 10% normal goat serum, then incubating in the second primary followed by incubation in the rhodamine-conjugated secondary. Sections were examined and images recorded with an inverted laser scanning confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany). Specificity of signal was determined by preincubation of antibodies with their respective recombinant protein (25 µg/ml) prior to incubation with the tissue section.

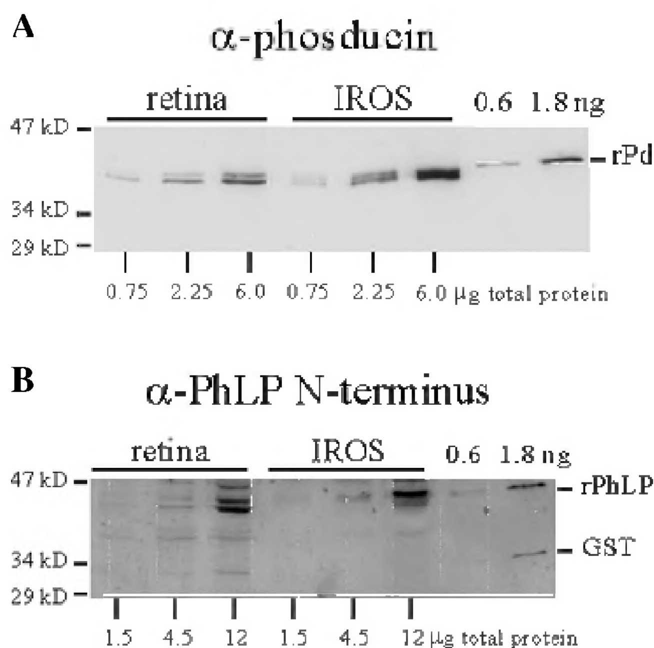

Figure 3. Phosducin and PhLP in extracts of retina and intact rod outer segments (IROS). Bovine retina and IROS were extracted in 1% SDS, diluted to 0.3 mg/ml total protein, and amounts indicated were electrophoresed and blotted (first three lanes, retinal extract; next three lanes, IROS extract). The last two lanes contain 0.6 and 1.8 ng, respectively, of recombinant His_6-Pd and His_6-PhLP. Blots were probed with anti-phosducin (**A**) and anti-PhLP N-terminus/GST fusion protein antibodies (**B**). Quantitation of immunoblot signal from extract dilutions was compared with recombinant proteins to calculate the concentration of Pd and the concentration of PhLP with respect to total protein (see text). **A**. Anti-phosducin antibodies. **B**. Anti-PhLP N-terminus/GST fusion protein antibodies.

RESULTS

Functional analysis of PhLP and Pd in vitro: Since phosducin regulates G protein signals by binding to Gβγ subunits [1-4] and it has been demonstrated that PhLP, like Pd, binds Gβγ subunits of G proteins [17], we wished to examine the possibility that PhLP is capable of regulating the visual signal. We previously reported the development of an in vitro assay for

Figure 4. Determination of the concentration of rhodopsin in retinal extract. Retinal and IROS extracts prepared as described in Figure 3. Total protein loads as indicated were electrophoresed and blotted (first three lanes, retinal extract; next three lanes, IROS extract). Blot was probed with anti-rhodopsin R4 antibody. Immunoblot signal was quantitated and the concentration of Rho in retinal extract was calculated by relating to the concentration of Rho in IROS, determined by photobleaching (see text). Representative of duplicate experiments.

Pd activity based on the role of Pd in regulating the G_t mediated visual signal [3]. This assay measures the ability of Pd to block G_t binding of activated rhodopsin (Rho*). We found that recombinant PhLP was equally effective as recombinant Pd at blocking G_t binding to Rho*, indicating stoichiometric binding of each to Gβγ and blocking of G_t heterotrimer formation (Figure 1). Half-maximal inhibition ($K_{1/2}$) values were 0.37 μM for Pd and 0.41 μM for PhLP. Significantly, these results demonstrate that PhLP and Pd have similar affinity for the $G\beta_1\gamma_1$ G protein isoform that is involved in the visual signal. The results shown in Figure 1 establish a need to investigate whether Pd and PhLP are both present in photoreceptor cells and what overlap or complementarity may exist in their roles.

Specificity of antibodies: To facilitate further comparison between Pd and PhLP, we raised polyclonal antibodies directed against each of these two proteins, as well as an antibody directed against an N-terminal extension that is unique to PhLP. The specificity of these antibodies after affinity purification was tested using immunoblot analyses (Figure 2). Based on the curves shown in Figure 2, the anti-Pd antibody was 57-fold specific for Pd over PhLP. The anti-PhLP antibody bound PhLP 17-fold more avidly than it did Pd. And the anti-PhLP N-terminus antibody exhibited a 53-fold preference for PhLP over Pd. These antibodies were employed in both Western blots and immunocytochemistry to localize Pd and PhLP within the retina.

Pd and PhLP in extracts of retina and IROS: To compare the relative expression levels of Pd and PhLP in whole retina and in dark-adapted rod outer segment preparations, bovine retina and intact rod outer segments (IROS) were extracted with 1% SDS. Protein concentrations were determined and increasing amounts of each were then subjected to SDS-PAGE and blotted along side varying loads of purified recombinant Pd and PhLP. Immunoblot analyses were carried out by probing with the anti-Pd and anti-PhLP N-terminus antibodies (Figure 3). By comparing the immunoblot signal in tissue bands to those of the recombinant proteins, it was determined that retina contains 0.74±0.14 ng Pd/μg total protein, whereas IROS contains 0.43±0.14 ng Pd/μg total protein. Retina was found to express 0.16±0.01 ng PhLP/μg total protein, compared with IROS which expresses 0.14±0.02 ng PhLP/μg total protein. Thus, the molar ratio of Pd:PhLP is 5.6±1.1 in retina and 3.7±1.3 in IROS.

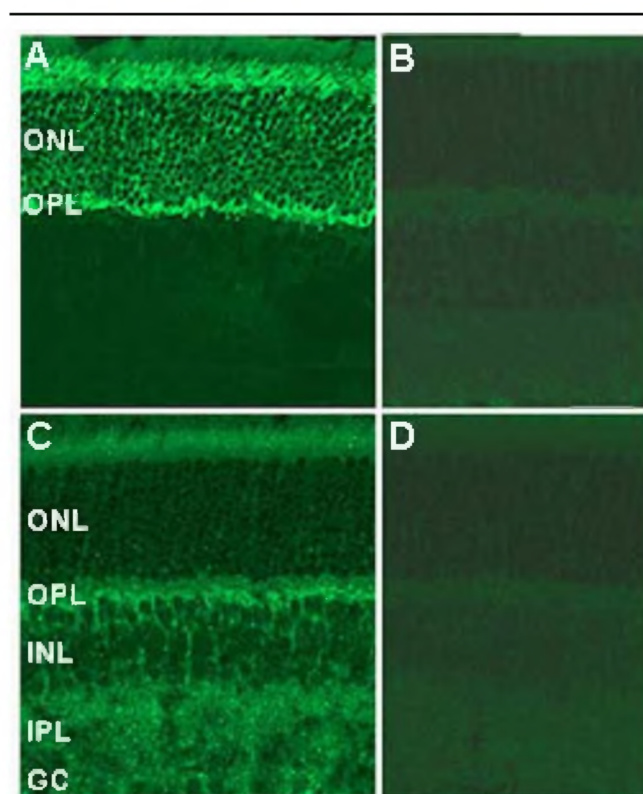

Figure 5. Immunocytochemical localization of Pd and PhLP in mouse retinal sections. **A**. Fluorescein isothiocyanate (FITC) staining indicates the localization of Pd to the photoreceptor cells, including the inner segments (top), outer nuclear layer (ONL), and outermost parts of the outer plexiform layer (OPL). **C**. FITC staining shows localization of PhLP to the region near the OPL, the inner nuclear layer (INL), inner plexiform layer (IPL), and photoreceptor inner segments (top). Specificity of staining was demonstrated by competing away signal with preincubation of the primary antibody with 25 μg/ml recombinant Pd (**B**) or PhLP N-terminus/GST fusion protein (**D**).

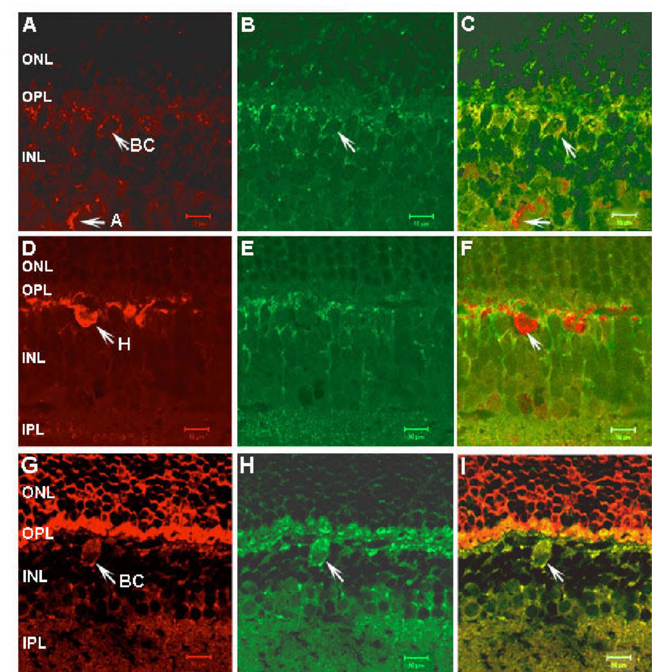

Figure 6. Immunolocalization of PhLP compared with that of calretinin, calbindin and recoverin. **A**. Rhodamine staining indicates localization of calretinin to amacrine cells (arrow) and bipolar cells (arrow). **B**. FITC staining indicates localization of PhLP in the same section (arrow). **C**. Double staining (yellow) indicates colocalization of calretinin and PhLP in bipolar cells but not amacrine cells (arrows). **D**. Rhodamine staining shows localization of calbindin, reported to localize to horizontal cells (arrow). **E**. FITC staining shows localization of PhLP in the same section. **F**. Double staining indicates the lack of significant colocalization of calbindin and PhLP in horizontal cells (arrow). **G**. Rhodamine staining localizes recoverin to photoreceptor cells and bipolar cells (arrow). **H**. FITC staining localizes PhLP in the same section (arrow). **I**. Double staining shows colocalization of recoverin and PhLP in bipolar cells (arrow), but not in photoreceptor cells.

Interestingly, there may be a difference in PhLP isoform expression between retinal extract and IROS extract. PhLP displays an anomalously slow mobility in SDS-PAGE, as does Pd. The apparent MW of recombinant His_6-PhLP is 45 kD, compared to its calculated MW of 36,437. In IROS, a single band of apparent MW 44 kD is detected with the anti-PhLP (not shown) or anti-PhLP N-terminus (Figure 3B) antibodies. However, in retinal extract, another band of apparent MW 42 kD is detected by these antibodies (Figure 3B). Phosducin is also seen as a doublet in both retinal extract and IROS (Figure 3A). The nature of these various isoforms of PhLP and Pd is discussed below.

Ratio of Pd to Rho in IROS and retina: We examined the expression of Pd in relation to Rho in IROS and whole retina. The concentration of Rho in IROS was determined directly by photobleaching [23], and found to be 136±36 ng/µg total protein. As direct determination of the concentration of Rho in retinal extract is not possible, we compared IROS and retinal extracts in immunoblots using an anti-rhodopsin antibody (Figure 4). The concentration of Rho in retina was thus found to be 21.4±4.8 ng/µg total protein. From these Rho expression values and those for Pd expression reported above, ratios of one Pd per 21±6 Rho molecules and one Pd per 228±96 Rho were obtained for intact retina and IROS, respectively.

Immunocytochemical localization: Extracts of IROS are not completely without contamination from other cell types, and loss of soluble proteins such as Pd is possible. Therefore, this comparison with retinal extract offers only a rough estimate of the comparative localization of PhLP and Pd. In order to more thoroughly examine the extent of spatial overlap between these two proteins, we employed the affinity purified antibodies in immunocytochemical analyses of light-adapted mouse retinas. As has been previously reported [27-30], Pd localizes in the retina exclusively to the photoreceptor cells of the retina (Figure 5A). Staining is most pronounced in the inner segments and the synaptic regions where the photoreceptor cells contact the outer plexiform layer (OPL). Phosducin is not found in the inner layers of the retina.

Phosducin-like protein, which has previously been reported to occur in a large number of tissue types [13,21], is found in many cells throughout the retina (Figure 5C). Significantly, though some PhLP is seen in the outer nuclear layer (ONL), it is not found in great abundance in this region, where some Pd is localized. The most intense staining for PhLP is in the inner retinal layers and particularly in the inner nuclear layer (INL) region immediately adjacent to the OPL and in the inner plexiform layer (IPL). This area is populated by a number of different cell types. In order to more precisely define the predominant locations of PhLP, retinal sections were doubly stained with the anti-PhLP N-terminus antibody in conjunction with antibodies against calretinin, calbindin, or recoverin (Figure 6). Calretinin has been localized to amacrine and ganglion cells [31], and is apparently also found in some bipolar cells in the mouse (see Figure 3 of Wassle et al. [31]). Calbindin is found in horizontal cells of the INL [31]. Recoverin is abundant in photoreceptor cells but is also found in bipolar cells [26]. As seen in Figure 6C, there is significant co-localization of PhLP and calretinin near the OPL, indicating that PhLP is found in some abundance in what are likely to be bipolar cells. Calbindin and PhLP are not significantly co-localized, meaning that PhLP is much less abundant in horizontal cells (Figure 6F). Recoverin and PhLP, however show significant co-localization in cells of the INL near the OPL (Figure 6I). Together these results show that PhLP is found in the INL predominantly in bipolar cells and, to a lesser degree, within the inner segments of photoreceptor cells.

A significant question is whether the PhLP signal in the inner segments confirms spatial overlap of PhLP and Pd. Because PhLP and Pd are homologous proteins, there is possible complication from cross-reactivity of antibodies. As stated above, the two antibodies in question display greater than 50 fold preference for their intended targets over the respective homolog. Furthermore, the ratio of Pd to PhLP was determined to be close to one, far from the level of expression that would be required for significant cross-reactivity. To analyze the specificity of the immunocytochemical staining, an-

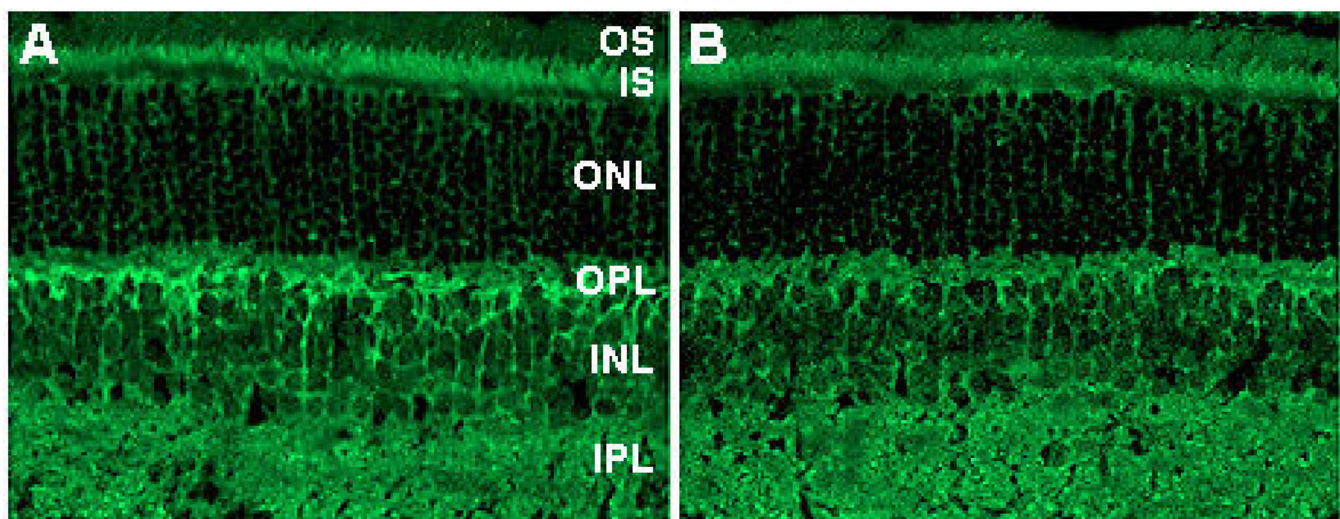

Figure 7. Specificity of PhLP immunolocalization. **A**. Fluorescein isothiocyanate staining shows localization of PhLP using the anti-PhLP N-terminus/GST fusion antibody. **B**. Staining in the same section is somewhat reduced overall but relative intensity of staining in the photoreceptor inner segments (top of panels) is maintained using antibody that has been depleted with 25 µg/ml recombinant Pd.

tibodies where depleted with soluble antigen, either recombinant Pd or recombinant PhLP N-terminus. In both cases, depletion with soluble antigen was seen to completely abolish the staining (Figure 5B,D), indicating that the observed staining results from specific recognition of these antigens. Additionally, we tested the specificity of the PhLP localization by depleting the anti-PhLP N-terminus antibody with recombinant Pd protein prior to staining with it (Figure 7). Though there was some overall decrease in signal, the staining in the inner segments was not reduced any more than that in the inner retinal regions, where Pd is not found. Thus, we conclude that PhLP and Pd are both expressed in inner segments of light-adapted photoreceptors.

Divergence of Pd and PhLP regulation: Phosducin binding of Gβγ is regulated by the phosphorylation of Pd by the cAMP-dependent protein kinase PKA [3,5]. We investigated the suitability of PhLP as a PKA substrate by comparing its phosphorylation in vitro with that of Pd (Figure 8A). Phosducin-like protein was poorly phosphorylated by PKA compared to Pd, the initial rate being 6-fold slower. We observed the phosphorylation of a second site on Pd by PKA, as evidenced by the appearance of another band on SDS-PAGE. The kinetics of phosphorylation at this second site were still somewhat faster than the limited PKA phosphorylation of PhLP that was observed (Figure 8A).

Four consensus sites for protein kinase C (PKC) are found in the sequence of PhLP. We therefore tested the ability of PKC to phosphorylate PhLP in vitro. Compared to a standard PKC substrate, histone H1, PhLP was phosphorylated 250-fold slower by PKC purified from rat brain (Figure 8B). This PKC is predominantly composed of isoforms α, β, and γ. A strong consensus site for PKCε is found at residues $S_{116}GK_{118}$ in PhLP. In vitro phosphorylation of PhLP using this PKC isoform was shown to be 730-fold slower than that of a specific peptide substrate (Figure 8C).

Together these results suggest that neither phosphorylation by PKA nor by PKC α, β, γ, or ε may be sufficiently robust to act as a physiologically important modulator of PhLP.

DISCUSSION

Phosducin and PhLP are highly homologous proteins that have both been shown to bind to the Gβγ heterodimer. Since PhLP is widely distributed, we wished to investigate whether it is expressed in the same cell types within the retina as Pd, and if so whether it plays a redundant or complimentary role to that of Pd in these cells. Though the mRNA encoding PhLP has previously been found in retinal tissue [32], analysis of PhLP protein expression in this tissue had not been performed. Our results show that PhLP, like Pd, binds G_tβγ inhibiting its association with G_tα on Rho*, and that it is expressed in several cell types in the retina, including some expression in the photoreceptor cells.

Phosducin is believed to play an important role in regulation of the visual signal in photoreceptor cells, where it is abundantly expressed [1-3]. We previously reported that Pd was expressed in IROS at a Rho to Pd ratio of 16:1 [33]. This level of expression is similar to that of G_t, which has a Rho to G_t ratio of approximately 10:1 in rods [34-36]. From these data, we concluded that there is sufficient Pd in the rod to bind to a majority of the G_tβγ [33]. In contrast, here we have found a significantly higher Rho to Pd ratio of about 300:1 in IROS. The reason for the discrepancy may be the amount of inner segment that detaches with the outer segment in the bovine IROS preparation, which depends on the force used to shear the rod outer segments from the retina. Since most of the Pd in the rod localizes to the inner segment ([27-30] and Figure 5A), the Rho to Pd ratio will depend on the amount of inner segment in the IROS preparation. To avoid this ambiguity, we determined the ratio of Rho to Pd in the intact retina, in which the rod cells contain their full complement of Pd.

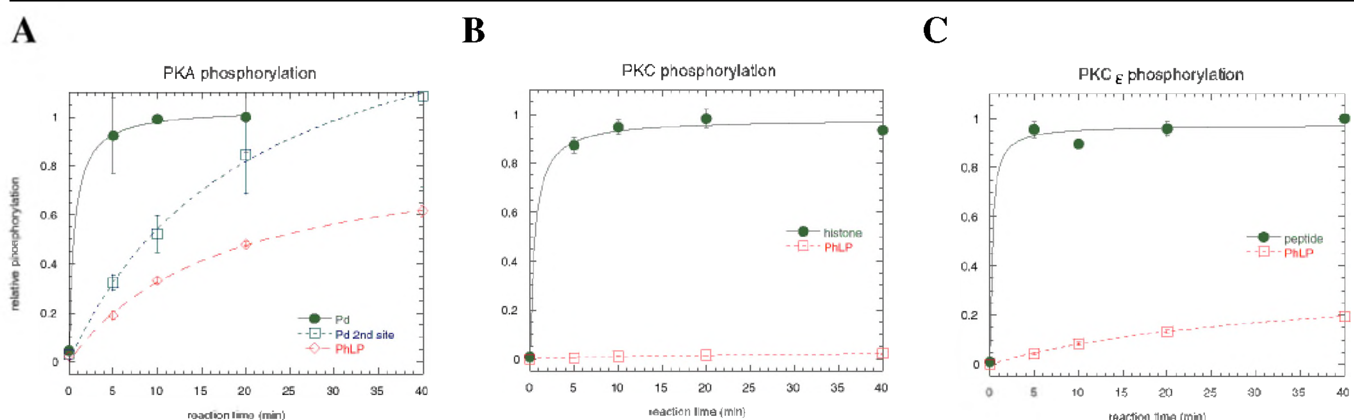

Figure 8. Phosphorylation of PhLP by PKA and PKC isoforms. Phosphorylation of PhLP, Pd, histone and peptide substrates by PKA and PKC isoforms. Phosphorylation conditions are specified in Methods. Error bars represent standard deviation of duplicate or triplicate samples. **A**. Protein kinase A catalytic subunit was used to phosphorylate purified recombinant Pd or PhLP in vitro and the ^{32}P incorporated determined by phosphorimager analysis of SDS gels. The relative amount of phosphorylation is shown for the high-efficiency site that has been shown to be Ser_{73} of Pd (filled circles), for a second less efficient site on Pd which results in the appearance of a second band (open squares), and for PhLP (open diamonds). **B**. Protein kinase C from rat brain (a mixture of isoforms α, β, and γ) was used to phosphorylate a standard substrate (histone, filled circles) and recombinant PhLP (open squares). **C**. Recombinant protein kinase Cε was used to phosphorylate a peptide substrate (filled circles) and recombinant PhLP (open squares).

This ratio was determined to be about 20:1. Since Pd is expressed exclusively in photoreceptor cells (Figure 5A), and primarily in rods over cones [30], this retinal ratio should reflect Pd expression in the rods. Thus, the current data also support the notion that Pd and transducin are expressed at similar levels in intact rods.

The fact that Pd is found primarily in the inner segment raises questions concerning the accessibility of Pd to transducin $\beta\gamma$ in the outer segment. Because it is a soluble, cytosolic protein, Pd may translocate between the inner and outer segment depending on the signaling state of the rod. In fact, immunolocalization experiments in dark and light-adapted retina have shown translocation of Pd in a light-dependent manner, moving from the outer segment in dark-adapted retina to the inner segment and ONL in light-adapted retina [28,29]. Our light-adapted retina data show localization to the inner parts of the photoreceptor cells, in agreement with these reports (see Figure 5A).

Transducin β subunit has been shown to exhibit a similar light-dependent localization to Pd, being found in the synaptic, nuclear, and inner segment regions of photoreceptors in the light and exclusively in the outer segments in the dark [28]. Since Pd is phosphorylated in the dark [7], it presumably is not bound to $G_t\beta\gamma$ in the outer segments. Colocalization of Pd and $G_t\beta\gamma$ in the inner regions of photoreceptor cells in the light is not surprising, since these proteins are known to be associated under these conditions [1]. It is significant that in our light-adapted retinal sections, PhLP is not seen in high abundance in the ONL where the majority of the $G_t\beta\gamma$ signal has been found [28], suggesting that if PhLP does bind $G_t\beta\gamma$ in the photoreceptors, it involves only the limited subpopulation of $G_t\beta\gamma$ that is localized to the inner segments. The sequestration of $G_t\beta\gamma$ away from the outer segments in the light may be important as an additional way of dampening sensitivity, in combination with its inactivation due to Pd binding. The transducin alpha subunit has been localized to the outer segments at night, translocating to the inner segments and ONL during the day [37] in parallel with the light-dependent translocation of both $G_t\beta\gamma$ and Pd. However, it must be recognized that the translocations referred to are deduced from immunocytochemical localizations, and the possibility of light- dependent masking of antibody epitopes must be taken into account in interpreting such results.

In whole retinal extracts, anti-PhLP antibodies detect two bands, only one of which is seen in IROS preparations (Figure 3B). Miles et al. [13] initially reported two different isoforms of PhLP predicted from cDNA's cloned from rat brain. The Miles et al. long form ($PhLP_L$) corresponds to full length PhLP (see genbank entry L15354 for correct N-terminal sequence). These authors also reported a short form ($PhLP_S$) that begins at PhLP residue Met_{78} but is otherwise identical to $PhLP_L$ [13]. The 42 kD band of PhLP that we see in retina cannot be equivalent to $PhLP_S$ because it is recognized by the anti-PhLP N-terminus antibody, which is directed against the first 50 amino acids of PhLP. This 42 kD band does not correspond to any of the forms of Pd family members proposed by Craft et al. [16], again because they would not be recognized with this antibody. Interestingly, the antibody directed against full-length PhLP did not detect any PhLP bands other than the two observed with the anti-PhLP N-terminus antibody (data not shown). The 42 kD band may represent a novel isoform of PhLP or may simply be a proteolytic product of the full length protein produced in the extraction.

Phosducin is seen as a doublet in both retinal and IROS extracts (Figure 3A). We note the appearance of a doublet of Pd upon hyperphosphorylation by PKA in vitro (data not shown). It is possible that the doublet of Pd seen in the in vivo extracts represents two different states of posttranslational modification.

Within the inner retina, we find that PhLP is abundant in bipolar cells (see Figure 6C, I). This finding may be significant in view of the importance of G protein signaling in these cells. Nawy and Jahr [38] showed that glutamate activation of its receptor in depolarizing bipolar cells (also called on-center bipolar cells or ON-bipolar cells) suppresses cGMP-dependent conductance, via signaling that they hypothesized to be very similar to the pathway of photoreceptor light transduction [39]. On-center bipolar cells are excited when the photoreceptor to which they are postsynaptically connected hyperpolarizes, and in turn they stimulate on-center ganglion cells and inhibit off-center ganglion cells. This excitation of the bipolar cell results from the reduction in glutamate that is transmitted across the synapse during the dark current in the photoreceptor. A decrease in glutamate decreases the stimulation of its receptor. This decrease in receptor stimulation has been thought to lead to decreased bipolar PDE activity, accumulation of cGMP, and the opening of cGMP-dependent channels causing depolarization [40]. More recently, however, Nawy [41] has shown that PDE is not involved in salamander bipolar cell signaling. Nonetheless, a G protein pathway seems to be important in bipolar cells, transducing the signal from the glutamate neurotransmitter. Immunocytochemical analyses showed that while transducin is not present in bipolar cells, $G_0\alpha$ is [42,43]. The human metabotropic glutamate receptor type 6 was cloned from retina and found to be expressed in bipolar cells [44]; and this receptor was found to functionally couple to transducin and (more efficiently) to G_0 in an in vitro system [45]. Nawy has recently reported data consistent with the involvement of G_0 but not G_i or G_t in bipolar cell transduction [41]. Thus, PhLP may regulate G_0 activity in this transduction pathway.

In these experiments, we show that while largely disparate in their localization, Pd and PhLP are both found in the inner segments of light adapted photoreceptors (see Figure 5). It is doubtful that one of these dominates in cones and the other in rods, as both seem equally distributed within this layer. von Schantz et al. [30] showed that Pd is found primarily in rods but not in the most abundant cones in ground squirrels. We found that there was not significant colocalization of PhLP and peanut agglutinin, which specifically stains cones (data not shown). Thus we conclude that PhLP and Pd probably do overlap within rod photoreceptors. Importantly, in these light-adapted cells, Pd is found in the nuclear and synaptic region of the cells as well as the inner segments, whereas PhLP is less abundant and more localized to the inner segments.

Where these two proteins do occur together, differences in their regulation would be of great interest. The PKA-dependent regulation of Pd binding to $G_t\beta\gamma$ is significant because it allows feedback regulation of the sensitivity of the visual signal [3,8]. The PhLP sequence $G_{117}KMTLK_{122}$ is homologous to $R_{70}KMS_{73}$, the PKA-phosphorylated regulatory site in Pd, and fits the PKC substrate consensus; however it was not efficiently phosphorylated by the α, β, γ, or ε isoforms of this kinase (see Figure 7B,C). Though residues $K_{89}KLS_{92}$ of PhLP constitute a possible PKA site, we have shown that PKA does not phosphorylate PhLP as efficiently as it does Pd; noting that PhLP is phosphorylated by this enzyme with kinetics even slower than those seen for phosphorylation of a second site (not the Ser_{73} site) in Pd (see Figure 7A). This observation leads us to conclude that the regulatory role of this phosphorylation is questionable. It is possible that PhLP is regulated by phosphorylation by some kinase other than PKA or PKC, or is regulated in some other way. Lazarov et al. [21] report regulation of PhLP mRNA stability by elements contained within the 3' untranslated region, but these do not address the regulation of the translated gene product that we show to coexist with Pd in the same subcellular location within photoreceptors. Further work to determine how PhLP is regulated is necessary before a complete understanding of the divergent roles of Pd and PhLP can be achieved.

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