The regulatory subunit of PDE6 interacts with PACSIN in photoreceptors

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Purpose: In photoreceptors, phosphodiesterase 6 (PDE6) is regulated in response to light, due to the shuttling of a regulatory subunit (PDE6γ) between the catalytic subunits of PDE6 and the activated form of transducin. We showed previously that PDE6γ is able to interact with the Src-homology type 3 (SH3) domain of formin-binding protein 17 (FBP17), a protein involved in membrane receptor endocytosis. FBP17 was not detected in rat retina. Therefore, we looked for other SH3 domain-containing proteins that might interact with PDE6γ in rat photoreceptors.

Methods: Several SH3 domains highly homologous to this domain of FBP17 were found by structural alignment. Yeast two-hybrid system and GST pull-downs were used to test interaction of PDE6γ with these putative partners. Expression patterns in rat retina and the SH3 containing candidates were also determined by immunohistochemistry and western blotting. GST pull-downs and co-immunoprecipitations were then used to test in vivo interaction with PDE6γ in rat retina extracts. Colocalization and light translocation of PDE6γ and one of its partner were studied by confocal microscopy.

Results: PDE6γ interacts in vitro with a number of SH3 domains. These interactions involve a polyproline motif located between amino acids 20 and 28 of PDE6γ. Several of the putative partners of PDE6γ are expressed in photoreceptor cells and might therefore interact in vivo with PDE6γ. Our results show that only PACSIN, a protein implicated in endocytosis, was found to interact with PDE6γ in rat retina extracts. The colocalization of the two proteins occurs in photoreceptor inner segments and synapses and is greatly enhanced upon illumination of the retina.

Conclusions: PDE6γ function is mostly documented in the regulation of phototransduction. Our results provide evidence that in vitro PDE6γ has a broad pattern of SH3 containing partners expressed in photoreceptors. PDE6γ interaction with PACSIN points to a possible role of PDE6γ in endocytosis. Further studies will be needed to understand the exact role of PDE6γ-PACSIN interactions in photoreceptors. The description of this new function of PDE6γ might help to understand the molecular mechanism of the severe retinal degeneration observed in PDE6γ knockout mice.

Phosphodiesterase 6 is the primary effector of phototransduction in vertebrate photoreceptors [1,2]. This enzyme is composed of two catalytic subunits, PDE6α (88 kDa) and PDE6β (84 kDa) and two inhibitory subunits, PDE6γ (11 kDa). In photoreceptor outer segments, photolyzed rhodopsin activates the GTP-binding protein transducin, which in turn activates PDE6 by chelating its PDE6γ subunits [1]. The PDE6γ domains that mediate its interactions with the catalytic subunits of PDE6 and with the GTP-binding subunit of transducin have been mapped. They are comprised of an internal basic region (amino acids 24-45), a stretch of 10-20 aa upstream of position 76 and the C-terminal end of the protein (aa 77-87) [3-9]. In a recent study [10], we observed that PDE6γ also contains a functional polyproline motif (aa 20-28) that mediated its interaction with the Src homology type 3 (SH3) domain of formin-binding protein 17 (FBP17), a protein that is involved in the functional network linking endocytosis, cytoskeleton dynamics and MAP kinase signaling [11], through its interactions with sorting nexin 2 and with dynamin [12,13]. Our results fell in good agreement with other studies indicating that, when overexpressed in HEK293 cells, PDE6γ interfered with MAP kinase signalling and co-precipitated with the endocytosis-related protein, dynamin [14]. These observations called for further studies aimed at identifying the SH3-containing partners of endogenously-expressed PDE6γ. Although several studies have reported the presence of PDE6γ protein and/or mRNA in non-photosensitive tissues or cell lines [10,14], expression levels appeared to be quite low and ill-adapted for the identification of PDE6γ-associating proteins. In contrast, PDE6γ is naturally expressed at high levels in photoreceptors, which makes them an obvious site in which to examine the in vivo interactions of PDE6γ with SH3-containing proteins. In the present work, we investigate the ability of PDE6γ to associate with a variety of SH3-containing proteins, functionally related to endocytosis and to receptor tyrosine kinase signalling. As PACSIN was found to interact with PDE6γ in vivo and to colocalize with it in photoreceptors inner segments and synaptic pedicles, our interest progressively focused on this protein of the endocytosis machinery.

METHODS

Antibodies: The antibodies used in this study were, a mouse monoclonal anti-PACSIN (anti-syndapin I; Transduction Laboratories, Lexington, KY), a mouse monoclonal anti-AMPHIPHYSIN (Bramp2; Transduction Laboratories), a...
mouse monoclonal anti-Grb2 (Santa Cruz, Santa Cruz, CA), a rabbit polyclonal anti-P13K (P85-P13K; Upstate, Inc., Charlotteville, VA), a rabbit polyclonal anti-Src (c-Src; Santa Cruz, Inc., Santa Cruz, CA), a mouse monoclonal anti-transducin (anti-GaT-1 subunit; Merck, Darmstadt, Germany). PDE6γ-9710 affinity purified antibody directed against the C-terminal part of PDE6γ, and anti-PDE6α affinity purified antibody directed against the N-terminal part of PDE6α, were kind gifts of Dr. Rick Cote (University of New Hampshire, Durham, NH). FBP17 anti-serum was a generous gift of Dr. Arndt Borkhardt (Children’s University Hospital, Giessen, Germany).

Animals: Rats (Wistar) were from Janvier (Le Genest-Saint-Islé, France). Animals were sacrificed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Immunohistochemistry: Eyes were dissected from 4 week old rats in the middle of the day and fixed in Bouin’s fluid overnight. After dehydration in 95% ethanol, 100% ethanol, and butanol, the eyes were embedded in paraffin. Tissue sections (5 µm) were rehydrated and blocked in PBS, 1% BSA for 1 h and in 1% normal goat serum for 1 h. After an incubation in 10 mM citrate pH 3 for 30 min (antigen retrieval), endogenous peroxidases were inhibited by an incubation in 0.3% H2O2 in PBS for 1 h at room temperature. After an overnight incubation at 4 °C with the primary antibody (either PDE6γ-9710, 1:10,000, anti-PDE6α, 1:20,000, anti-PACSIN 1:150, anti-Src 1:100, anti-AMPHIPHYSIN 1:500, anti-P85-P13K 1:500, anti-transducin-Gat 1:500, or anti-Grb2 1:100), sections were washed in PBS three times and incubated with the secondary antibody (biotinylated goat anti-rabbit antibody or biotinylated horse anti-mouse antibody, 1:200) for 1 h at room temperature. After three PBS washes, reactions were revealed using a Vectastain kit following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA).

Adsorption of PDE6γ and PACSIN antibodies: 300 µl of a 1:10,000 PDE6γ 9710 antibody dilution were incubated with GST-PDE6γ (100 fold excess) spotted on a nitrocellulose membrane. A 1:250 dilution of anti-PACSIN antibody was incubated on the 52 kDa PACSIN band of a rat cerebellum western blot (35 µg protein). After overnight incubation with membranes, adsorbed antibodies were centrifuged and incubated with retina sections. The immunohistochemistry protocol was performed as above.

Isolation of total RNA from rat tissues: Total RNA was isolated using LiCl-urea [15]. Tissues were sonicated 30 s in ice-cold 3 M LiCl/6 M urea. After standing overnight at 4 °C, total RNA was obtained by centrifugation at 6,000 x g for 30 min. Pellets were resuspended in TE/SDS buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8, 0.5% sodium dodecylsulfate), washed by phenol/chloroform extraction and RNA was precipitated with ethanol. The precipitates were resuspended in sterile water and the RNA concentration was determined spectrophotometrically.

Random reverse transcription: cDNA was obtained from total RNA by random reverse transcription. Total RNA (1 µg) was mixed with OD30 hexamers and the volume was brought to 5 µl with H2O. The RNA sample was denatured 5 min at 95 °C and ice cooled for 2 min. 5 µl reverse transcription mix (250 mM Tris HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) was added with reverse transcriptase (200 U, M-MLV R; Promega, Madison, WI). Control reactions were run in parallel without reverse transcriptase. Samples were incubated 90 min at 37 °C.

DNA sequencing: Plasmid DNA preparations (Wizard Plus SV Miniprep; Promega) and gel-purified PCR products (QIA Quick Gel Extraction; Qiagen, Valencia, CA) were sequenced using Big Dye reagent (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 310.

Plasmids, strains, and media used in the two-hybrid system: A LexA-based two-hybrid system containing pBTM116 as bait plasmid and pACT2 as library plasmid (Clontec, San Jose, CA), together with the yeast reporter strain L40 (MATa, trpl1, leu2, his3, ade2, LYS2:lexA-HIS3, URA3:lexA-lacZ) was used. Yeast were grown on YNB (0.16% yeast nitrogen base without amino acids or ammonium sulfate (Difco, Spars, MD), 0.5% (NH4)2SO4, 1% glucose) solid or liquid medium, supplemented with adenine and specific amino acids (50 µg/ml).

Two-hybrid assay: The yeast two-hybrid assay has been described previously [10]. Briefly, we used as a bait PDE6γ-rod first 65 amino acids in frame with the LexA DNA binding domain. Yeast strain L40 containing the pBTM116-PDE6γ-rod (1-65) bait was transformed with cDNAs encoding different SH3 domains cloned as an EcoRI-Xhol fragment in vector pACT2, in frame with the gal4 transcription activating domain (gal4AD; Figure 1). We used cDNAs encoding SH3 domains of PACSIN 2 (aa 291-556), AMPHIPHYSIN 2 (aa 255-589), Grb2 (aa 1-204), P85-P13K (aa 1-234), and Src (aa 71-234). These cDNAs were obtained from rat retina by RT-PCR.

Directed mutagenesis of PDE6γ: The PDE6γ mutant (P20, 23A) was generated as previously described [10] using the Quick-Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA).

Western blot: Rats were killed in the middle of the day. Two retinas from animals were sonicated in Bramp buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, Triton X-100 1% [16]).

Retina extracts were homogenized in Laemmli sample buffer (2.5 µg protein/µl) and 20 µg were analyzed by SDS-PAGE. For PACSIN expression analysis, different tissues, retina, pineal, cerebellum, blood, and trachea, were homogenized in Laemmli buffer and 20 µg protein for each tissue were loaded and analyzed by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and probed with the suitable antibody (PDE6γ-9710 affinity purified antibody 1:10,000; anti-PDE6α 1:20,000; anti-PACSIN 1:250; anti-Src 1:100; anti-AMPHIPHYSIN 1:500; anti-P85-P13K 1:500; anti-Grb2 1:500). Antibody-antigen complexes were detected using 1:10,000 dilution anti-rabbit IgG conjugated to horseradish peroxidase and ECL reagent (Amershams Biosciences, Buckinghamshire, England). Immunoblots were then exposed to Kodak X-ray film (Eastman Kodak Company, Rochester, NY).
**GST pull-downs:** The GST pull-down assay that we used was described previously [10]. For in vitro translated products: cDNA fragments encoding PACSIN, AMPHIPHYSIN, Grb2, P85-P13K, and Src SH3 domains were cloned downstream of an initiation codon in pCDNA 3.1 under control of the T7 promoter. Constructs were used as template in an in vitro transcription-translation reaction with a TNT kit (Promega) in presence of 35S-methionine. Corresponding 35S-labeled proteins were analyzed by SDS PAGE and autoradiography. For rat retina extracts: two retinas from 4 week-old rat were sonicated 30 s on ice in 500 µl of Bramph buffer. PMSF (2 mM) and Protease inhibitor cocktail (Sigma, St. Louis, MO) were added. Extract (250 µl) was incubated overnight at 4 °C with 100 µl of GST or GST-PDE6γ beads (50% slurry in Bramph buffer). Complexes were collected by a brief centrifugation and washed three times with Bramph buffer. Samples were then subjected to SDS-PAGE and western blotting.

**Co-immunoprecipitation:** Retinas of one-month-old rats were dissected and kept frozen at -80 °C. After thawing, two retinas were sonicated in Bramph buffer at final protein concentration of 20 µg/µl. For immunoprecipitation, 5 µg of antibodies and 100 µl of protein A slurry were added to 400 µg retinal protein. After incubation overnight at 4 °C, protein A beads were washed 5 times with 500 µl of Bramph buffer and resuspended in Laemmli buffer. Samples were analyzed by SDS-PAGE and western blotting.

**Immunofluorescence:** Preparation of retina sections was the same as described above except for the secondary antibody which was replaced by a goat anti-mouse antibody AlexaFluor 488 and a goat anti-rabbit AlexaFluor 568 (Molecular Probes, Eugene, OR). Slides were mounted with Fluoprep (Biomérieux, Marcy l’Etoile, France). Fluorescence-stained sections were examined with a spectral confocal station FV 1000 installed on an inverted microscope IX-81 (Olympus, Tokyo, Japan). Image analysis (intensity profiles) was performed with Visilog 6.2 software (Noesis, Les Ulis, France).

**Expression and immunolocalization of PDE6γ and PACSIN in dark and light adapted retinas:** Three week-old rats were raised under a 12 H light/dark cycle (50 lux). For light adaptation, rats were kept in a room with 500 lux ambient light for 6 h and killed. Eyes were not dilated and rats moved freely in the cage, as previously described for studies of a light/dark cycle [17].

For the dark condition, after 12 h in the dark, rats were maintained for 6 h in the dark and killed under dim red light. PDE6γ and PACSIN detections are described in the immunofluorescence and western blot sections above.

**RESULTS**

In silico identification of a subset of SH3 domains structurally related to FBP17: To identify proteins that might share with FBP17 the ability to interact with PDE6γ, we proceeded to screen the “conserved domain database” [18], with a structural alignment program, using the SH3 domain of FBP17 as a query. The closest neighbors of FBP17 identified by this procedure had well established functions in endocytosis or MAP kinase signalling: Src, Grb2, P85-P13K, AMPHIPHYSIN, PACSIN (Figure 1). Key amino acids (W and P) previously shown to play a role in PDE6γ-FBP17 interaction were conserved in all these SH3 domains (Figure 1).

AMPHIPHYSIN and PACSIN have well established functions in endocytosis [19,20]. Src and Grb2 play a central role in linking receptor tyrosine kinases to MAP kinase signalling pathway [21]. P85-P13K is the regulatory subunit of PI3K that phosphorylates inositol lipids and participates in the regulation of the actin skeleton [22].

In vitro interactions between PDE6γ and selected SH3 domains: The ability of this in silico-identified subset of SH3 domains to interact with PDE6γ was tested in a yeast two-hybrid system. As illustrated in Figure 2A, PDE6γ interacted strongly with PACSIN and AMPHIPHYSIN SH3 domains (as shown in Figure 1).
strongly as with FBP17 SH3 domain, used as a positive control. PDE6γ interactions with Grb2 and P85-PI3K SH3 domains were of weaker intensity (Figure 2A). All interactions appeared to be specific because they did not occur with an irrelevant protein (ERG19, Figure 2A). Double mutation in the polyproline motif of PDE6γ (P20, 23A) disrupted interactions with PACSIN, Src, and P85-PI3K and decreased interactions with P85-PI3K, Grb2, and AMPHIPHYSIN (Figure 2B). A biochemical verification of interactions was performed by GST pull-down. As illustrated in Figure 2C, a GST-PDE6γ column efficiently bound the in vitro-synthesized SH3 domains of PACSIN, P85-PI3K, Grb2, and FBP17. In contrast, the SH3 domain of AMPHIPHYSIN did not bind the GST-PDE6γ column. Our attempt to produce the SH3 domain of Src by in vitro transcription and translation remained unsuccessful, which precluded the verification of its interaction with PDE6γ by GST pull-down.

Expression of SH3-containing partners and PDE6 subunits in the rat retina: Next, we examined which of the SH3 containing proteins identified above were expressed in rat retina and in which cell type. With the exception of FBP17, all the proteins were detected at their expected molecular weights, on western blots of retinal proteins (Figure 3). Immunohistochemical labeling failed to detect FBP17 and Grb2 in rat retina. In contrast, Src, P85-PI3K, AMPHIPHYSIN, and PACSIN gave clear-cut immunohistochemical labeling in specific layers of the retina (Figure 3). All of them were detected in the inner plexiform layer (a region of synaptic contacts between bipolar, amacrine, and ganglion cells), in the outer plexiform layer (a region of synaptic contacts between photoreceptors, bipolar cells, and horizontal cells). Immunohistochemical staining for PACSIN, AMPHIPHYSIN, and P85-PI3K were of high intensity and extended to the inner segments layer, which left no doubt of their presence in photoreceptors (Figure 3). Moreover, the Src labeling was also detected in photoreceptor outer segments. PDE6γ immunohistochemical detection produced the expected high-intensity staining of photoreceptor outer and inner segments and more surprisingly, a clear-cut labeling of the outer plexiform layer (Figure 3). PDE6γ immunostaining was obtained with an affinity-purified antibody that was highly specific by western blotting (Figure 3), and it was abolished by preadsorption of the antibody with GST-PDE6γ protein (Figure 3). Synaptic localization was not observed for PDE6 catalytic subunits (Figure 3). PACSIN immunostaining specificity in rat retina was confirmed by an adsorption protocol, which severely reduced the immunostaining on sections of retinal tissue (Figure 3).

Ex vivo and in vivo interactions of PDE6γ with PACSIN: As PACSIN, AMPHIPHYSIN, Src, and P85-PI3K could be detected in photoreceptors, PDE6γ interaction with native forms of these proteins was examined by GST pull-down from retina homogenates. As illustrated in Figure 4A, a GST-PDE6γ column efficiently extracted PACSIN. Moreover, in vivo interaction between PDE6γ and PACSIN could be detected by co-immunoprecipitation (Figure 4B) on light-adapted retina. Immunoprecipitations with antibodies directed against all the

![Figure 2. PDE6γ in vitro interaction with SH3 domains.](http://www.molvis.org/molvis/v11/a125/)

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Figure 3. Immunodetection of SH3 domain-containing proteins and PDE6 subunits in the rat retina. Immunohistochemistry: eyes obtained in the middle of the day were fixed in Bouin’s fixative and embedded in paraffin. Tissue sections (5 µm) were incubated overnight at 4 °C with the indicated antibodies (anti-AMPHIPHYSIN 1:500; anti-Src 1:100; anti-Grb2 1:100; anti-P85-PI3K 1:500; anti-PACSIN 1:250; control: anti-PACSIN 1:250 preadsorbed on rat cerebellum extracts; anti-PDE6α 1:20,000; anti-PDE6γ 1:10,000; control: anti-PDE6γ 1:10,000 preadsorbed on GST-PDE6γ fusion protein), then 1 h with a biotinylated secondary antibody (1:200). Immunoreactions were detected with the ABC complex and nickel-diaminobenzidin (the scale bar represents 50 µm). Western blots: retinas obtained in the middle of the day were homogenized in Laemmli sample buffer and analyzed by immunoblotting with the indicated antibodies, at the same concentrations as above, using the ECL method.
other putative partners failed to precipitate PDE6γ. These results confirmed the formation of complexes between PDE6γ and native PACSIN in vivo. As shown in Figure 4C, PACSIN is detected, by western blot analysis, not only in retina but also in cerebellum and to a lower level in pineal.

**Immunolocalization and expression of PDE6γ and PACSIN in dark and light adapted retinas:** Under dark conditions, PDE6γ immunoreactivity was mainly detected in photoreceptor outer segments and the signal in inner segments was about 50% that of outer segments, but only a very weak signal was observed in synapses (Figure 5A). PACSIN was localized in photoreceptor inner segments and synapses (Figure 5A). Thus, in dark conditions, only a minor fraction of PDE6γ and PACSIN colocalized in photoreceptor inner segments and synapses (Figure 5A).

In light-adapted retinas, PDE6γ labeling was observed mainly in photoreceptors inner segments but also in synapses (Figure 5A). PACSIN subcellular localization did not change upon retina illumination (Figure 5A). PDE6γ and PACSIN expression level did not vary between dark and light conditions as shown by immunoblot analysis (Figure 5B). As a result, PDE6γ and PACSIN colocalization in photoreceptors inner segments and synapses was considerably increased in response to light (Figure 5A). PDE6γ translocation from outer segments to inner segments and basal pedicles was confirmed by measuring fluorescence intensity in the photoreceptor layer (Figure 5C).

One possible PDE6γ partner, Gαε, the Transducin alpha subunit, was also analyzed as a control in this experiment (Figure 5D). As shown previously [23-27], Gαε translocates from outer segments to inner segments and synapses upon retina illumination (Figure 5D).

**DISCUSSION**

In the present study, we gathered evidence that PACSIN (a well-established dynamin-binding protein) [28], might inter-
act with PDE6γ in photoreceptors. Based on co-localization of PDE6γ and PACSIN, this interaction would appear to take place in inner segments and in synaptic terminals of photoreceptors in light. Together, our data would appear to place PDE6γ in the endocytosis protein network.

PDE6γ was originally identified in photoreceptor outer segments, as the regulatory subunit of the light-activated phosphodiesterase PDE6 [29-31]. By shuttling between catalytic subunits of PDE6 and the GTPase subunit of transducin, PDE6γ plays a pivotal role in the phototransduction cascade [1].

More recently, several studies have reported the presence of different splice variants and transcription start site variants of PDE6γ mRNA in non-photosensitive tissues and cell lines, as well as low levels of western blot-detectable PDE6γ protein [10,14,32,33]. In addition, PDE6γ was shown to interact with SH3-containing proteins [10] and, when overexpressed in HEK cells, was shown to regulate the endocytosis-MAP kinase activity and to co-precipitate with dynamin [14]. This prompted us to identify SH3-containing proteins that could interact with PDE6γ in photoreceptors.

In the present study, the identification of potential PDE6γ-interacting proteins was guided by three-dimensional alignment with the FBP17 SH3 domain, which efficiently binds the polyproline motif of PDE6γ [10]. The predictive value of this alignment was confirmed in our experiments, as all the closest neighbors of FBP17 interacted with PDE6γ in a yeast two-hybrid assay.

Confirmation of interactions by in vitro GST pull-down was obtained for PACSIN, P85-PI3K, and Grb2. Together, yeast two-hybrid and in vitro GST pull-down assays strongly confirmed and extended the SH3-associating function. However, only PDE6γ-PACSIN interaction could be observed in vivo. For some of the SH3-containing proteins expressed at

Figure 5. Expression and immunolocalization of PDE6γ and PACSIN in dark and light adapted retinas. A: PDE6γ and PACSIN were detected in cross-sections of dark- or light-adapted retinas by immunofluorescence staining. Eyes were fixed in Bouin’s fixative and embedded in paraffin. Tissue sections (12 µm) were incubated overnight at 4 °C with a mix of anti-PDE6γ (1:10,000) and anti-PACSIN (1:250) antibodies, then for 1 h with a mix of Alexa488-labeled goat anti-rabbit IgG (1:200, green fluorescence for PDE6γ) and Alexa568-labeled goat anti-mouse IgG (1:200, red fluorescence for PACSIN). Upon retina illumination, PDE6γ translocates to photoreceptor inner segments and synapses. PACSIN is localized in inner segments and synapses in both conditions. PDE6γ-PACSIN colocalization in inner segments and synapses layers of photoreceptors increased in light conditions (scale bar indicates 40 µm).

B: Western blot analysis of PDE6γ and PACSIN expression under dark and light conditions: Extracts of light or dark adapted retinas were homogenized in Laemmli sample buffer (3 µg protein/µl) and 60 µg were analyzed by SDS-PAGE and western blot. Ponceau red staining of the western blots was used to verify equal protein loading. C: Fluorescence intensity of PDE6γ labeling in different layers of dark- and light-adapted retinas. PDE6γ translocates from outer to inner segments and outer plexiform layer upon retina illumination. D: Immunofluorescence detection of transducin, GaT, in photoreceptors of dark- and light-adapted retinas as a control (the scale bar represents 40 µm). Retinal layers are abbreviated as follows: OS represents outer segments; IS represents inner segments; ONL represents outer nuclear layer; and OPL represents outer plexiform layer.
low level in the retina (Grb2 and Src) this lack of in vivo interaction with PDE6γ might reflect a limit of sensitivity of the technique. Nevertheless, Src and Grb2 have been described previously in outer segments of rat photoreceptors [34,35]. Regarding P85-PI3K and AMPHIPHYSIN, the apparent lack of in vivo interaction with PDE6γ was observed despite strong in vitro interactions and fairly high expression levels in photoreceptors. This would indicate, that native forms of the proteins impose a higher level of specificity on the PDE6γ-SH3 domain interaction. One possible explanation to these observations is that a nonphosphorylated form of PDE6γ was used in in vitro assays, whereas native PDE6γ is a substrate for phosphorylation by different protein kinases present in rod photoreceptors [14,36-38]. PDE6γ is phosphorylated on Threonine 22 and Threonine 35 [39], and one of these residues (Threonine 22) is located in the proline rich motif (aa 20-28) of PDE6γ. Therefore, PDE6γ phosphorylation might modify its ability to interact with SH3 domains, as was previously shown for its interaction with transducin [39].

Only PACSIN met the final criterion of co-localization and co-immunoprecipitation with PDE6γ. Colocalization of the two proteins in photoreceptor inner segments and synapses dramatically increases in light by a massive translocation of PDE6γ in to these compartments. Translocation of phototransduction proteins has been described in the retina and has been attributed to a light adaptation mechanism. Transducin translocates from outer to inner segments upon retina illumination [23-27]. Conversely, arrestin is detected massively in inner segments under dark condition and moves towards outer segments in light [25]. Recently, recoverin was shown to be the third phototransduction protein known to translocate [40]. No previous study has shown the light induced translocation of PDE6γ [41]. In our study, we observed that a large proportion of PDE6γ translocates and that PDE6γ is found down to photoreceptors synapses. In this compartment, PDE6γ could modify transmission of visual information downstream from photoreceptors. It may be interesting to note that PDE6γ undergoes a light-dependent translocation in the same direction as α-transducin [23-27]. As light activates a-transducin interaction with PDE6γ, both proteins could translocate as a complex.

Increased PDE6γ-PACSIN colocalization under light condition suggests that their interaction could be light-regulated, but we were not able to confirm this hypothesis by co-immunoprecipitation. PACSIN has been shown to be involved in endocytosis by interacting with dynamin. It belongs to the syndapin/PACSIN/FAP52 family [28]. FAP52, one of the PACSIN homologues, is also a filamin partner and participates in cytoskeleton organization [42]. Thus, by interacting with PACSIN, PDE6γ could interfere with the endocytosis pathway or cytoskeleton organization in photoreceptors. It should be noted that PACSIN, AMPHIPHYSIN, and FBP17 all share the ability to interact with dynamin [13,43,44], which adds weight to the hypothesis of a functional link between PDE6γ and the endocytosis machinery. PDE6γ-PACSIN interaction also provides a rationale to the previously reported co-immunoprecipitation of PDE6γ with dynamin [14]. A direct interaction between PDE6γ and dynamin would appear unlikely, because they both contain a polyproline motif. Instead, they might associate through a dimer of PACSIN, as this protein was previously shown to dimerize [42]. Another observation in favor of a link between PDE6γ and the endocytosis machinery is its previously, reported interaction with visual arrestin [45]. Another form of arrestin-β arrestin-is known to act as an adaptor in the formation of clathrin-coated pits [19]. A possible β arrestin interaction with PDE6γ would deserve further study because it might constitute another link between PDE6γ and endocytosis.

Endocytosis in photoreceptors occurs in several compartments such as inner segments and synapses [46,47]. The photoreceptor ribbon synapse is specialized for sustained release of neurotransmitter in the dark, with light transiently decreasing the amplitude of the release. This tonic synapse is the site of intense endocytosis [46]. Accordingly, specific proteins appear to be required at this synapse, as previously illustrated by the detection of a photoreceptor specific form of AMPHIPHYSIN [44]. The PDE6γ-PACSIN interaction may be another characteristic of the ribbon synapse machinery. Interestingly, the nrc mutant in zebrafish, lacking synaptoplakin I, a well-known PACSIN partner, presents unanchored ribbons at the photoreceptor synapse, a decreased number of synaptic vesicles, and an abnormal synaptic transmission in cone photoreceptors [48].

These observations, together with the data presented herein, suggest that PDE6γ knockout mice [49] should be re-examined, with special attention to photoreceptor synapse formation. Previous studies in mice lacking the PDE6γ subunit have indicated that photoreceptors develop abnormal outer segments by postnatal day 10, and then undergo a complete degeneration in less than two weeks [49]. However, there has been no report on photoreceptor synapse formation in these animals. A developmental study is underway to examine whether PDE6γ knockout mice present signs of ultrastructural disorganization of the ribbon synapse that might be reminiscent of the situation described in the zebrafish nrc mutant [48].

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