A proline-rich domain in the gamma subunit of phosphodiesterase 6 mediates interaction with SH3-containing proteins

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Phosphodiesterase 6 (PDE6) is the primary effector of phototransduction in vertebrate photoreceptors [1]. This enzyme was first characterized in retinal rods as being composed of two catalytic subunits, α (88 kDa) and β (84 kDa), each interacting with a smaller (11 kDa) γ regulatory subunit [2-4]. The heterotetramer αβγ2 represents the inactive state of the enzyme that prevails in dark-adapted rods. Upon illumination, photolyzed rhodopsin activates the GTP-binding protein transducin, which in turn activates PDE6 by chelating its γ subunits and releasing the catalytic core αβ. cGMP hydrolysis by active PDE6 results in closure of cGMP gated channels in the plasma membrane and hyperpolarization of the cell (1, for review). Cone photoreceptors express a distinct set of phototransduction proteins, including cone-specific opsins [5], cone-transducin [6] and cone-PDE6. The latter is composed of a dimer of αγ′ catalytic subunits (94 kDa) and of cone-specific (13-kDa) γ regulatory subunits [7-9]. By shuttling between transducin and the PDE6 catalytic core, the γ subunits of PDE6 (Pγ) link the activity of the effector to the activation state of the rhodopsin/transducin complex and therefore play a central role in the forward flow of information of the phototransduction cascade. However, according to recent studies, Pγ appears to have additional functions, both in photoreceptors and in non-photosensitive tissues. One of these functions was discovered as a result of an effort to identify the GTPase activating proteins of heterotrimeric G-proteins. It was observed that the GTPase activity of αβ-transducin is increased by RGS9, a retina-specific member of the RGS (regulator of G-protein signaling) protein family and that Pγ is required for optimal effect of RGS9 [10,11]. Another function of Pγ was identified in the guinea pig lung, where a Pγ-like immunoreactive protein was reported [12], followed by RT-PCR detection of Pγ mRNA [13]. In this tissue, Pγ appears to prevent phosphorylation and activation of lung PDE5 by protein kinase A [13]. More recently, Pγ was immunodetected in HEK293 cells where its overexpression increased MAP kinase activity [14]. Based on these informations, we scanned the Pγ sequence for motifs that might be relevant to its ability to interact with multiple effector proteins. In doing so, we identified a proline-rich region at positions 20 to 28 of Pγ...
(PVTPRGKPP), that might mediate interaction with SH3 domains. This prompted us to initiate a two-hybrid screen in order to identify SH3-containing partners of Pγ.

**METHODS**

**Animals:** Rats (Wistar) were from Janvier (Le Genest-Saint-Isle, France). 13 day old rat embryos (E13) were obtained from timed-pregnancies.

Isolation of total and polyA+ RNA from rat tissues: Total RNA was isolated as described by Bothwell et al. [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 6000xg 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. PolyA+ RNA was purified from total RNA with Dynabeads oligo-dT25 (Dynal), following the manufacturer’s instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) on total RNA: Reverse transcription was performed on Dynabeads oligo-dT25 (Dynal). Total RNA (3 μg) was denatured (70 °C for 2 min) then mixed with 10^7 beads in a final volume of 20 μl containing 0.5 mM dNTP, 200 U reverse transcriptase (MMLV-RT, Promega) and the manufacturer’s buffer.

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name of oligonucleotide or oligonucleotide pair</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Py-rod S #1</td>
<td>5'-ATGAACCTGGAGACCCACGCC-3'</td>
<td>RT-PCR and 3'-RACE</td>
</tr>
<tr>
<td>Py-rod AS #1</td>
<td>5'-GATGATCCATACCTGGGGCC-3'</td>
<td>RT-PCR and 5'-RACE</td>
</tr>
<tr>
<td>Py-rod S #2</td>
<td>5'-AGTTATCTGTCTCCACGCCG-3'</td>
<td>3'-RACE</td>
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<tr>
<td>Py-rod AS #2</td>
<td>5'-GTTATCTGTCTCCACGCCG-3'</td>
<td>5'-RACE</td>
</tr>
<tr>
<td>Py-cone S</td>
<td>5'-CCTCCACTACCAACCCAGGACC-3'</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Py-cone AS</td>
<td>5'-TCAGATGATCCCAAACTGGAGC-3'</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Anchor-poly-dG</td>
<td>5'-CUACUAUAUAGGACCCCCCGGTTAGTTACTCCACAGG-3'</td>
<td>3'-RACE</td>
</tr>
<tr>
<td>Poly-dT-(Eco-Not)</td>
<td>5'-TTCTAGAATTTCACACCAGGCCGCT-3'</td>
<td>3'-RACE</td>
</tr>
<tr>
<td>Eco-Not</td>
<td>5'-TTCTAGAATTTCACACCAGGCCGCT-3'</td>
<td>3'-RACE</td>
</tr>
<tr>
<td>EcoRI-Py-rod S</td>
<td>5'-AAAGAATCTGATAGCCACCCACCCAGC-3'</td>
<td>Construction of pBTM116-Pγ</td>
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<tr>
<td>BamHI-Py-rod AS</td>
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<tr>
<td>NcoI-CIP4 S</td>
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<td>Construction of pACT2-CIP4</td>
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<tr>
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<td>pACT2 S</td>
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<td>PCR of pACT2 inserts</td>
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<tr>
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<td>Mutation of proline</td>
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<td>5'-CGGGTGATGGGAGGCGCCGTC-3'</td>
<td>5'-Py to alanine</td>
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<tr>
<td>Py (P23A)</td>
<td>5'-GTGAGGGGAGGAGGCACCGC-3'</td>
<td>20 of Py-rod to alanine</td>
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<td>Py (P27A)</td>
<td>5'-GCCAGGAAAAGGACTCCTAATAATTAG-3'</td>
<td>23 of Py-rod to alanine</td>
</tr>
<tr>
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<td>20 and 23 of Py-rod to alanines</td>
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<tr>
<td>Py (P23,27A)</td>
<td>5'-GTGAGGGGAGGAGGCACCGC-3'</td>
<td>23 and 27 of Py-rod to alanines</td>
</tr>
<tr>
<td>Py (P42A)</td>
<td>5'-TTCAGAAGGCAAAACGCCGACAATGAGG-3'</td>
<td>42 of Py-rod to alanine</td>
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<td>FBP17 (W94A)</td>
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<td>Mutation of proline</td>
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<tr>
<td>FBP17 (P108L)</td>
<td>5'-GACAAAGGGGAGGAGGCGCCGTC-3'</td>
<td>Mutation of proline</td>
</tr>
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</table>

The oligonucleotides used for RT-PCR, 5'-RACE, 3'-RACE, plasmid construction, and site-directed mutagenesis are listed. The abbreviated names are those found in the text with “S” for sense and “AS” for antisense.
A control reaction without reverse transcriptase was run in parallel for each tissue. The RT reaction was carried out for 2 h at 42 °C. First-strand cDNA was purified (90 °C 3 min, then two rinses in 100 µl distilled water) and resuspended in distilled water/glycerol: v/v. PCR reactions were performed on 1/20th of a reverse transcriptase reaction, in 50 µl containing 50 pmol of each primer, 0.2 mM dNTP, 1.5 U of Taq DNA polymerase (Pharmacia) and the manufacturer’s buffer. The PCR reaction was: 95 °C for 50 s, (Tm-2 °C) for 50 s, 72 °C for 30 s; 30 or 35 cycles. The reaction products were analyzed by electrophoresis (1.5% agarose gel containing 0.7 µg/ml ethidium bromide) and visualized under UV light. The primers used for amplification of the PDE6 subunits were as follows: Pγ-rod sense and antisense primers (Pγ-rod S Number 1 and Pγ-rod AS Number 1, see Table 1) were designed after the rat Pγ-rod sequence [16]. Pγ-cone sense and antisense primers (Pγ-cone S and Pγ-cone AS, see Table 1) were chosen in conserved regions of human and bovine Pγ-cone sequences [9,17].

**5’-RACE:** Total RNA (10 µg), obtained either from adult rat cerebellum or from the extracephalic moiety of rat embryo (E13), was reverse transcribed with Dynabeads oligo-dT25 (Dynal) and dC-tailed. PCR amplification was performed with a the Pγ-rod antisense specific primer (Pγ-rod AS Number 1, see Table 1) and an anchor-poly-dG primer (see Table 1) for the dC-tail. The PCR reaction was then reamplified with a nested Pγ-rod antisense primer (Pγ-rod AS Number 2, see Table 1) and the anchor-poly-dG primer. The nested PCR product was gel-purified, TA-cloned in pGEM-Te vector (Promega), and sequenced.

**3’-RACE:** Total RNA (9 µg) from the extracephalic moiety of rat embryos (E13) was reverse transcribed with poly-dT-Eco-Not primer (see Table 1). The cDNA template was then submitted to a semi-nested PCR protocol: first reaction with the primer pair Pγ-rod S Number 2 / Eco-Not (see Table 1), second reaction (on 1/50th of the first reaction) with the primer pair Pγ-rod S Number 1 / Eco-Not (see Table 1). The PCR product was gel-purified, TA-cloned in pGEM-Te vector (Promega) and sequenced.

**DNA sequencing:** Plasmid DNA preparations (Wizard Plus SV Minipreps, Promega) and gel-purified PCR products (QIA Quick Gel Extraction, Qiagen) were sequenced using Big Dye reagent (Applied Biosystems) and analyzed by a sequencing company (Genome Express, Grenoble, France).

**Ribonuclease Protection Assay (RPA):** A pGEM-Te clone containing the entire coding region of rat Pγ-rod (261 bases) was PstI-linearized and used as a DNA template to generate a [UTP-35S]-labeled antisense riboprobe (351 bases), using the “MAXI-script in vitro Transcription” kit (Ambion). Hybridization of the probe (8 pg, 1.6x10⁴ cpm) with target RNA and subsequent RNase A/T1 digestion (37 °C, 30 min) were performed with the “HybSpeed RPA” kit (Ambion). Samples were electrophoresed through a 6% polyacrylamide gel containing 6 M urea. The gel was dried and exposed to a PhosphoImager screen (Storm 820, Molecular Dynamics).

**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 (Clontech), together with the yeast reporter strain L40 (MATa, trp1, leu2, his3, ade2, LYS2::lexA-HIS3, URA3::lexA-lacZ) was used. Yeast were grown on YNB (0.16% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% (NH₄)₂SO₄, 1% glucose) solid or liquid medium, supplemented
with adenine and specific amino acids (50 µg/ml).

Two-hybrid screening: As bait, we used the first 65 amino acids of the Pγ-rod (Pγ-rod1-65). The 22 C-terminal amino acids were deleted because they conferred artefactual transcriptional activity to the lexA fusion protein. The Pγ-rod1-65 cDNA sequence was generated by PCR using a Pγ-rod sense primer (EcoRI-Pγ-rod S, see Table 1), that introduced an EcoRI site at the 5’-end, and a Pγ-rod antisense primer (BamHI-Pγ-rod AS, see Table 1), that introduced a BamHI site preceded by a termination codon. Pγ-rod1-65 was cloned as an EcoRI-BamHI fragment into pBTM116, in frame with the lexA DNA-binding domain (lexADB), and sequenced. Yeast strain L40 containing the pBTM116-Pγ-rod1-65 bait was transformed with a rat brain cDNA library in pACT2 vector (Clontech), as described [18]. Approximately 10^6 transformants were plated on (YNB+ade) medium selecting for protein-protein interaction. His+ colonies were re-streaked on (YNB+ade+his) medium selecting for the presence of bait and library plasmids, and used to generate crude plasmid DNA preparations as described [19]. These were used to PCR amplify pACT2 inserts, using primers of the pACT2 vector on both sides of the cloning site (PACT2 S and AS, see Table 1). PCR products were gel-purified and directly sequenced. To eliminate false positives, candidate clones were re-examined in a two-hybrid assay with the neutral bait pBTM116-ERG19 (ERG19: mevalonate diphosphate decarboxylase of S. cerevisiae) [20].

Construction of the pACT2-CIP4 vector: We used the C-terminal half (amino acids 293-547) of the rat CIP4 protein (GenBank Accession Number AB006914). The corresponding cDNA was obtained from rat cerebellum by RT-PCR, using a CIP4 sense primer (NcoI-CIP4 S, see Table 1) that introduced an NcoI site at the 5’-end, and a CIP4 antisense primer (XhoI-CIP4 AS, see Table 1), that introduced a XhoI site at the 3’-end. CIP4 (amino acids 293-547) was cloned as a NcoI-XhoI fragment into pACT2, in frame with the gal4 activation domain (gal4AD), and sequenced.

Site-directed mutagenesis: Specific amino acids were mutated within the Pγ-rod sequence of the pBTM116-Pγ-rod1-65 construct or the formin-binding protein 17 (FBP17) sequence of the pACT2-FBP17 construct, using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. Briefly, two complementary oligonucleotide primers encoding the mutation were used to extend both strands of the entire plasmid during temperature cycling, by means of Pfu DNA polymerase. Parental DNA was digested with DpnI endonuclease (target sequence 5’-Gm6A TC-3’), while the unmethylated PCR-amplified plasmid could not be restricted by DpnI. Supercompetent cells (Epicurian coli(r), Stratagene) were transformed with the PCR-generated plasmid DNA and plated. Several clones were se-

Figure 2. Mutations of the Pγ-rod proline-rich domain interrupt binding to FBP17. A: Scheme of the proline mutations within the Pγ-rod bait. B: The indicated proline mutants of Pγ-rod (as lexAADBD fusions) were transformed into L40 yeast expressing FBP17 (as gal4AD fusion) and plated as triplicate drops on medium lacking histidine. Identical results were obtained with 3 to 5 independent clones of each mutant. Positive control corresponds to wild-type (WT) Pγ-rod. Negative control corresponds to the neutral bait ERG19 (ERG19). C: Protein extracts from the cotransformants described above were analyzed by western blot to verify that wild type and mutated forms of Pγ-rod were correctly expressed. The lexAADBD-Pγ-rod fusion proteins were detected with anti-lexA antibody as described in “Experimental procedures”.

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quenced to confirm correct mutagenesis. The mutagenic oligonucleotide pairs are listed in Table 1, with the RT-PCR, 5’-RACE, 3’-RACE, and construction oligos.

**Two-hybrid interaction assay:** Yeast cotransformed with the appropriate pACT2 and pBTM116 constructs were grown for 3 days at 27°C on medium selecting for the presence of both plasmids (YNB+ade+his). Three independent yeast colonies were then resuspended in water and drops were loaded on medium selecting for protein-protein interaction (YNB+ade). Plates were incubated for 3 to 4 days at 27°C.

**Western blot detection of fusion proteins expressed in yeast:** Yeast cotransformed with the appropriate pACT2-FBP17 (wild-type or mutant) and pBTM116-Pγ-rod1-65 (wild-type or mutant) constructs were grown in 20 ml of (YNB+ade+his), until OD600 = 2, then centrifuged. Pellets were resuspended in 100 μl of sample buffer [21], briefly sonicated and re-centrifuged. A 20 μl aliquot of the supernatant was fractionated on SDS-PAGE (15% acrylamide) and blotted on a nitrocellulose membrane. The lexADB-Pγ-rod fusion protein was detected by probing the membrane with mouse anti-lexA antibody (Clontech), followed by goat anti-mouse peroxidase conjugate (Amersham). The gal4AD-FBP17 fusion protein was detected using mouse anti-hemagglutinin antibody (Boehringer), followed by goat anti-mouse peroxidase conjugate (Amersham). Immunoreactions were revealed with the ECL reagent (Amersham). Kodak X-ray film exposure was for 1 min.

**GST pull-down:** *In vitro* translation of FBP17: A cDNA fragment encoding the SH3 domain of FBP17 downstream of an initiation codon and a HA tag was obtained by BglII and XhoI digestion of the FBP17 clone isolated in the two-hybrid assay. The fragment was subcloned in PcDNA3 (Invitrogen) under the T7 promoter. This construct was used as template in a Transcription/translation reaction with the TNT kit (Promega) in presence of 35S-Methionine. The corresponding 35S labeled protein was analysed by SDS PAGE and autoradiography and migrated at the expected size of 17 kDa. GST-Pγ fusion protein: the coding sequence of Pγ-rod was amplified by PCR using the primers BamHI 5’Pγ and Xho3’ Pγ. After amplification, the PCR fragment was digested with the enzymes BamHI and XhoI and inserted in the pGEX4T2 vector (Pharmacia Biotech) to produce the Glutathione S transferase (GST)-Pγ fusion protein. After production in E. coli Bl21 strain, the GST-Pγ fusion protein was bound to glutathione-sepharose beads following the manufacturer’s instructions (Pharmacia Biotech). GST pull down: 35S labeled SH3 domain of FBP17 (1/4 of a TNT reaction) was incubated with either GST-Pγ or GST bound to 50 μl of glutathione beads at 4°C for 72 h in the following

![Image](http://www.molvis.org/molvis/v9/a57)

**Figure 3. Mutations in FBP17 SH3 domain interrupt binding to Pγ-rod.** A: Sequence of the FBP17 clone with the SH3 domain in lowercase letters: tryptophane-94 and proline-108 residues, mutated respectively into alanine (W94A mutant) and leucine (P108L mutant), are in red. B: The indicated mutants of FBP17 (as gal4AD fusions) were transformed into L40 yeast expressing the lexADBBD-Pγ-rod bait and plated as triplicate drops on medium lacking histidine. Positive control corresponds to wild-type FBP17. Results were confirmed on three independent clones for each mutation. C: Protein extracts from the cotransformants described above were analyzed by western blot to verify that wild type and mutated forms of FBP17 were correctly expressed. The gal4AD-FBP17 fusion proteins were detected with anti-hemagglutinin antibody as described in “Experimental procedures”.
buffer: 10 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X100. After 3 washes in the same buffer, the samples were separated by SDS PAGE and autoradiographed.

**Western blot detection of Pγ:** Tissues were homogenized (10 strokes in a teflon/glass potter) in buffer A (0.25 mM sucrose, 10 mM TRIS, HCl pH 7.4, 1 mM EDTA, Protease inhibitor cocktail from Sigma, as described in 12). The final concentration of protein was measured (BioRad protein assay) and brought to 10 mg/ml. This extract was centrifuged at 14,000x g for 10 min at 4 °C and the supernatant was centrifuged again at 100,000g for 20 min at 4 °C. The final pellet was resuspended in Laemmli buffer and run on a SDS PAGE (15% acrylamide). The samples were then transferred overnight on a nitrocellulose sheet. After a blocking step in PBS, tween 0.1%, BSA 5% (room temperature 1 h), the membrane was incubated, first with an antibody directed against the C-terminal region of bovine Pγ (antibody 9710, dilution 1:15,000, obtained from Dr. R. Cote, University of New Hampshire, Durham, NH) and then with an antibody directed against the N-terminal extremity of Pγ-rod (PA1-723, Affinity Bioreagents, dilution 1:500). Immunoreactions were revealed with the ECL reagent, as above. Recombinant Pγ-rod (gift of Dr. R. Cote, University of New Hampshire, Durham, NH) was used as a positive control.

**RESULTS**

**Isolation of FBP17, an SH3-containing partner of Pγ-rod:** To identify protein partners of Pγ-rod, a rat brain cDNA library in pACT2 was submitted to a yeast two-hybrid screen, using pBTM116-Pγ-rod1-65 construct as a bait. A screen of 10^6 yeast transformants was performed and His+ colonies were further characterized by sequencing the pACT2 inserts. Two identical clones appeared of special interest because they consisted essentially of the SH3 (Src homology 3) domain of rat formin-binding protein 17 (97.5% identity with mouse FBP17, GenBank Accession Number U40751). The specificity of the interaction between FBP17 and the Pγ-rod was verified using a neutral bait (Figure 1). FBP17 is a protein of extensive tissue-distribution, whose interaction with sorting nexin 2 suggests a possible role in the intracellular trafficking of receptor tyrosine kinases [22]. Based on this information and on the recently-introduced notion that Pγ-rod regulates MAP kinase activity in HEK293 cells [14], we decided to further investigate the structural basis and specificity of Pγ-rod interaction with SH3 domains.

**In vitro interaction of Pγ-rod with the SH3 domain of FBP17:** To validate the two hybrid screen, we tested the in vitro binding of Pγ-rod to 35 S-labeled FBP17. As illustrated in Figure 1, the GST-Pγ-rod fusion protein bound the SH3 domain of FBP17. The specificity of the interaction was con-
firmed by the fact that the GST protein alone was unable to bind FBP17 SH3 domain (Figure 1).

**Mutational analysis of the Py-rod-FBP17 interaction:** To further investigate the structural determinants of the Py-rod-FBP17 interaction, mutants of both proteins were constructed and tested in the yeast two-hybrid system. Five single amino acid mutants and two double mutants of Py-rod were generated by exchanging proline to alanine (P5A, P20A, P23A, P27A, P42A, P20, 23A, P23, 27A), and tested for two-hybrid interaction with FBP17. As illustrated in Figure 2, the P5A and P42A mutations, located outside the proline-rich region of Py-rod, did not affect yeast growth on medium lacking histidine. In contrast, all mutations affecting the proline-rich domain of Py-rod (P20A, P23A, P27A, P20, 23A, and P23, 27A) resulted in a much slower yeast growth, indicating a severe impairment of the interaction with FBP17 (Figure 2). We verified that wild-type and mutated forms of Py-rod had similar expression levels in yeast (Figure 2). Based on information concerning the essential amino acids of SH3 domains [23], two single amino acid mutants of FBP17 were generated (W94A and P108L, see Figure 3). As illustrated in Figure 3, these mutations severely impaired the interaction with Py-rod.

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**Figure 5.** RT-PCR, RPA and western blot analyses of Py-rod expression in adult and embryonic rat tissues. **A:** Total RNA (3 µg) from the indicated adult rat tissues was reverse transcribed, followed by 35 cycles PCR amplifications with Py-rod primers. Test reactions (+) were run in parallel with control reactions lacking reverse transcriptase (-). The reaction products (1/5 of the reaction) were analyzed by electrophoresis and visualized under UV light. Standard: 100 bp ladder (Promega). **B:** Total RNA (3 µg from trunk or limb buds of E13 rat embryos or 0.3 µg from adult rat pineal) was reverse transcribed, followed by 30 cycles PCR amplification with Py-rod primers. Test reactions (+), controls without reverse transcriptase (-). The reaction products (1/5 of the reaction) were analyzed as above. **C:** Aliquots of target RNA from the indicated tissues were hybridized with the Py-rod 35S-riboprobe and subjected to digestion with RNase A/T1. Samples were electrophoresed through 6% acrylamide / 6 M urea gel. The gel was dried and exposed 3 days to a PhosphoImager screen. Lane 1: polyA+ RNA (15 µg) from rat embryonic body (trunk+limb buds). Lane 2: total RNA (1 µg) from adult rat retina. Lane 3: yeast total RNA (50 µg). Lane 4: yeast total RNA (50 µg) without RNase. **D:** Protein extracts prepared as described in Experimental Procedures, were analyzed by SDS-PAGE and western blot (retina 20 µg, pineal 20 µg, embryonic trunk 20 µg, limb buds 20 µg, recombinant Py 50 ng). The membranes were incubated with an antibody directed against the c-terminal region of Py-rod (1:15,000) and with an antibody directed against its N-terminal region (1:500). Immunoreactions were developed with peroxidase-coupled anti-rabbit (1:3000) and the enhanced chemiluminescence detection kit (Amersham).
Similar expression levels of wild-type and mutated forms of FBP17 were verified on western blot (Figure 3).

Interaction of Pγ-rod with CIP4, another SH3-containing protein: A homology search revealed that the SH3 domain of rat FBP17 was highly homologous (63% identity, 84% similarity) to the carboxy terminal SH3 domain of the Cdc42-interacting protein 4 (Figure 4), a protein involved in cytoskeleton dynamics [24]. To investigate the binding of CIP4 to Pγ-rod, the C-terminal half (amino acids 293-547) of the rat CIP4 sequence, containing the SH3 domain, was cloned in pACT2 and tested in the two-hybrid system with pBTM116-Pγ-rod(1-65) as a bait. Growth of cotransformant yeast on selective medium lacking histidine confirmed the interaction between Pγ-rod and CIP4 (Figure 4). The specificity of this interaction was verified using the neutral bait pBTM116-ERG19 (Figure 4). As judged by slower yeast growth, CIP4 appeared to be a weaker interacting partner for Pγ-rod than was FBP17. As expected for a polyproline-SH3 interaction, the mutations P20A, P23A and P27A in Pγ-rod severely impaired the interaction with CIP4 (Figure 4).

Detection of Pγ-rod mRNA and protein in embryonic and adult rat tissues: The ability of Pγ to interact with SH3-containing proteins would strengthen the notion that its function is not limited to regulating the light-sensitive phosphodi-
contains a domain highly homologous to the region of PDE6 and its interaction with PDE6. Indeed, the alignment of vertebrate species studied so far, comprising mouse, bovine, and chicken [30], showed that Pγ-rod mRNA was differentially detected in a number of adult rat tissues after 35 amplification cycles (Figure 5). Relatively strong signals were obtained in the cerebellum and the Pγ-rod transcript of this tissue was further characterized by 5'RACE and sequencing. A complete reading frame was obtained (Figure 6) and the 5'end of this mRNA was 7 bases longer than the one previously described in the pineal gland [16]. Embryonic tissues gave stronger RT-PCR signals than adult tissues and the reaction product was still easily detectable after 30 amplification cycles (Figure 5). The Pγ-rod mRNA levels present in the extracephalic moiety of 13 days embryo were sufficient for detection by RNase protection assay (Figure 5), but not by northern blot. Correct splicing of this transcript was verified by 5'RACE and 3'RACE: a complete Pγ-rod cDNA was obtained which differed from that of the rat pineal [16] by 142 additional bases in the 5'-untranslated region (Figure 6). A result suggesting that transcription of Pγ-rod can be initiated from different promoters in a tissue-specific or age-related manner. At the protein level, very low amounts of Pγ could be detected in limb buds of the rat embryo (Figure 5), but not in the embryonic trunk, nor in any adult tissue other than retina and pineal. The detection of Pγ on western blots of embryonic tissue required the sequential use of anti-N terminal and anti-C terminal antibodies (Figure 5). The Pγ-immunoreactive protein had a molecular weight of 11 kDa in embryonic limb buds, adult retina and pineal gland, similar to recombinant Pγ-rod (Figure 5).

**DISCUSSION**

Using yeast two-hybrid and GST pull down assays, we observed that Pγ-rod has the ability to interact with SH3-containing proteins. Previous studies had already shown that, in addition to its inhibitory action on retinal PDE6, Pγ-rod prevented phosphorylation of lung PDE5 by protein kinase A [13]. The effect of Pγ-rod on PDE5 appeared to involve similar structural determinants as its interaction with PDE6. Indeed, the same region of Pγ-rod (amino acids 24-45) was identified in both interactions [13,25,26] and it was observed that PDE5 contains a domain highly homologous to the region of PDE6 (Pxx81-540) that interacts with Pγ-rod [25]. In contrast, our study describes a new type of interaction of Pγ-rod, one involving a proline-rich domain, previously uncharacterized in this protein. This new molecular function would include Pγ-rod in the growing number of proteins implicated in SH3 interactions and might be relevant to the recently described effect of Pγ on MAP kinase activity [14]. It should be noted that the proline-rich domain is present in Pγ-rod and Pγ-cone of all vertebrate species studied so far, comprising mouse, bovine, human [9,17,27-29] and chicken [30]. Indeed, the alignment of Pγ-rod and Pγ-cone across species reveals divergent N-terminal sequences, followed by a long stretch of very high sequence identity (94%), starting precisely at the proline-rich region and extending to the C-terminus. This would appear to add weight to the functional relevance of the proline motif, because the structural determinants of the canonical function of the Pγ-rod and Pγ-cone (i.e., regulation of PDE6) have all been identified downstream of this motif [13,26,31-34]. Our two-hybrid screen revealed an interaction between Pγ-rod and FBP17 that was validated by GST pull down and further characterized by mutational analysis. The proline domain of Pγ-rod (P20VTPRKGPP) conforms to the class II consensus, with a basic residue (K25, underlined) at position +2 from the “core” PxxP motif (boldface letters). Point mutation analysis revealed that the core motif prolines (P20 and P23) were essential for efficient binding to FBP17. In addition, we found that the downstream proline (P27) was also required for the interaction. This finding is consistent with previous reports indicating that additional residues flanking the minimal proline consensus are important determinants of SH3 binding affinity and selectivity [35,36]. Reciprocally, point mutations of W94 or P108 residues within the SH3 domain of FBP17 strongly impaired the interaction with the Pγ-rod. These residues, highly conserved among SH3 domains, were previously shown to build the hydrophobic patch required for proline binding [37,38]. Due to their conserved nature, W94 and P108 are likely to bind the core PxxP motif of Pγ-rod, while less conserved SH3 residues, yet to be defined, may contact the downstream P27.

Collectively, our mutagenesis data provide evidence that the FBP17-Pγ-rod interaction is specific and relies on bona fide SH3-proline binding.

The SH3-binding function of Pγ-rod is further supported by its interaction with CIP4, the closest homologue of FBP17 found in gene banks (63% identity, 84% similarity in the SH3 domain). This result also disclosed some specificity in the Pγ-rod-FBP17 interaction because CIP4 clearly was a weaker interacting partner for Pγ-rod, in spite of its high sequence homology with FBP17. Further studies should therefore aim at a more complete description of the spectrum of SH3 partners of the Pγ-rod, using the SH3 sequences available in gene banks. This information would also help identify the cellular functions that may be affected by Pγ-rod-SH3 interactions. In a recent study, FBP17 was shown to interact with Sorting Nexin 2, a protein involved in EGF receptor recycling [22], while in another study, Pγ was shown to regulate thrombin- and EGF-dependent activation of MAP kinase [14]. The proline motif of Pγ described herein might provide a rationale to understand how this protein affects receptor internalization-dependent MAP kinase activity, because this signaling pathway involves a number of SH3-containing proteins [39]. Studies are underway to identify other SH3 partners of Pγ that may be relevant to this function.

The detection and characterization of Pγ-rod mRNA in a number of rat tissues extends previous reports on the presence of this transcript in non-photosensitive tissues of rodents and in HEK293 human embryonic kidney cell line [12-14,16]. Interrogation of EST databases also indicated the presence of Pγ-rod mRNA in rat placenta and in human uterus tumor. Our study provides further information by showing that the 5' ends of Pγ-rod transcripts in adult cerebellum and in embryonic...
We wish to thank Mrs F. Chevalier, Mrs N. Girard and Mr. G. interaction with SH3 domains. Identify new functions of this protein, possibly related to its onset of electroretinographic responses, on postnatal day 13. This would suggest that Pγ receptors by postnatal day 10 [40]. This would suggest that Pγ KO mice generated previously failed to develop normal photoreceptor differentiation, even before the expression of cGMP phosphodiesterase from bovine rod outer segments. Proc Natl Acad Sci U S A 1990; 87:293-7.


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