



In vitro interaction of tubulin with the photoreceptor cGMP phosphodiesterase γ -subunit

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ABSTRACT

The α and β tubulins compose the microtubule cytoskeleton which is involved in many cellular processes such as vesicular transport. The photoreceptor cells in the retina are neurons specialized for phototransduction. Here we report a novel interaction between tubulin and the photoreceptor cGMP phosphodiesterase (PDE6) gamma subunit (PDE γ). The specificity and molecular details of the PDE γ :tubulin interaction were analyzed through the experiments of pull down, microtubule co-sedimentation, and NMR spectroscopy. The tubulin-interacting site was identified to be in the PDE γ C-terminal I67–G85 region, and the interaction interface appeared to be distinct from those with the other PDE γ targets in phototransduction. We also observed that PDE γ interacted with tubulin in a GTP-dependent manner. Our findings offer implications for non-phototransduction role(s) of PDE γ in the photoreceptor neurons.

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Microtubules, a major component of the cytoskeleton, participate in numerous cellular processes ranging from cell division, organelle positioning, intracellular transport, to neuronal differentiation [5]. They usually consist of laterally associated protofilaments, each made up of α/β -tubulin dimers that are able to self-assemble in a GTP-dependent way. There are a large number of tubulin/microtubule-interacting proteins that do not necessarily share sequence homology or structural similarity. While many of them regulate microtubule stability/dynamics, some undertake intracellular protein transport such as kinesins and dyneins. Noticeably, many of the microtubule-associated proteins contain intrinsically disordered domains, for example, MAP2 and tau [4], doublecortin and RP1 [12], TPPP/p25 [14], α -synuclein [21], and stathmin [19]. In this study, we have identified another intrinsically disordered tubulin/microtubule-interacting protein, the γ -subunit (PDE γ) of the photoreceptor cGMP phosphodiesterase (PDE6) [8].

The rod photoreceptor PDE6 is composed of two similar catalytic subunits (PDE $\alpha\beta$) and two identical inhibitory PDE γ subunits. PDE γ is a small protein of 87 amino acids containing two distinct functional domains: the positively charged central domain (residues G19–G49) and a negatively charged but relatively hydrophobic C-terminal domain (T62–I87) (for review see [8]). The

canonical function of PDE γ in phototransduction has been well defined. PDE γ keeps PDE6 inactive in the dark but regulates the turn-on as well as turn-off of visual signaling upon photoresponse, *via* interactions with PDE $\alpha\beta$, the transducin α subunit (G α t) and the regulator of G protein signaling (RGS9-1). Crystal structures revealed distinct PDE γ C-terminal interactions with the chimeric PDE5/6 catalytic domain [2], G α t and the RGS9-1 catalytic core [16]. An NMR study indicated that some structural elements for interacting with these targets were preconfigured in the free PDE γ molecules in solution although PDE γ was overall disordered [18].

Consistent with the feature of intrinsically disordered proteins that they can interact with distinct partners to achieve various functions, increasing evidence implicates non-phototransduction PDE γ targets [8]. A recent study reported that the N-terminal proline rich region of PDE γ interacts with PACSIN, suggesting a possible role of PDE γ in endocytosis in the photoreceptor cell [10]. We were therefore motivated to find additional PDE γ -interacting proteins in the retinal photoreceptors.

Bovine and mouse retinal homogenates were prepared according to the method described previously [6]. Biotinylated PDE γ (Btn-PDE γ) was generated by covalently linking maleimide-PEO₂-biotin (Pierce) to the single cysteine placed at the PDE γ N-terminus. The peptide Btn-Bz30 containing a PDE γ N-terminal polycationic sequence (BtnG19–Q32) was custom-synthesized at the University of Wisconsin Peptide Synthesis Facility. Btn-CytC (cytochrome C) and Btn-BSA were prepared by first reacting maleimide-PEO₂-biotin with pure proteins and then removing the unreacted biotin molecules with a G-50 spin column (Amersham).

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Pull down from retinal homogenates was performed using the streptavidin beads (Pierce) pre-bound with Btn-PDE γ . The beads were first incubated with retinal homogenates for 1 h at 4°C in buffer A (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM MgSO₄, 50 μ M AlCl₃, 30 mM NaF, and 50 μ M GDP), and then washed 3 \times with the same buffer. Proteins on the beads were eluted using the SDS/DTT sample buffer and resolved on a 15% SDS gel. In the control, equal amounts of retina homogenates were incubated with the streptavidin beads with no Btn-PDE γ bound.

Western blotting was performed with antibody dilutions as follows: anti-PDE α (Affinity Bioreagents), 1000-fold; anti-tubulin- α (Sigma–Aldrich), 10,000-fold; anti-G α t (Affinity Bioreagents), 5000-fold.

For pulling down microtubules, purified bovine brain tubulin (Cytoskeleton, purity >99%) was used. Tubulin polymerization was performed according to the manufacturer's instruction, and Taxol was added to stabilize microtubules. For each pull down reaction, 5 μ g of microtubules was added to 1 μ l of streptavidin beads with 2 μ g Btn-PDE γ pre-bound. Following incubation of the reactions for 30 min at room temperature in the BST01 buffer (80 mM PIPES, pH 7.0, 1 mM EGTA, 1 mM MgCl₂) supplemented with AlF₄[−]–GDP, the beads were washed 3 \times with 50 μ l of buffer each time.

Microtubule co-sedimentation assays were performed according to the published method [22] with minor modifications. In each reaction, 20 μ g of microtubules was incubated with 2 μ g of PDE γ for 30 min at room temperature in Buffer A. Microtubules were pelleted by centrifugation at 20,000 \times g for 30 min at room temperature, and then washed twice by repeating centrifugation and removal of the supernatants. The protein pellets were resolved on a SDS gel.

NMR analysis of the tubulin–PDE γ interactions was performed based on the published methods [12,18]. ¹⁵N-labeled PDE γ was prepared as previously described [18]. The NMR experiments were performed at 20°C using a 600-MHz Varian Inova spectrometer equipped with ¹H, ¹⁵N, and ¹³C triple-resonance cryogenic probe at the National Magnetic Resonance Facility at Madison, WI (NMR-FAM). [¹H,¹⁵N]-HSQC (heteronuclear single quantum correlation) spectra were first collected for 30 μ M ¹⁵N-labeled free PDE γ in the buffer containing 10 mM PIPES, 1 mM EGTA, 1 mM Mg²⁺, 90% H₂O/10%D₂O, pH 6.2. The data collection was repeated under the same experimental conditions following addition of 10 μ M tubulin dimer and 1 mM GTP or GDP.

We have observed a novel PDE γ :tubulin interaction. This finding resulted from the effort to identify additional PDE γ targets in the photoreceptor cells through pull down experiments using Btn-PDE γ (Fig. 1A–E).

The major pull down from bovine retinal homogenates appeared as a prominent band of ~55 kDa (A, lane 3; C, lane 2). N-terminal micro-sequencing of this band resulted in two sequences: MRE-CISIH and MREIVHIQ. Blast search indicated that these sequences match the bovine tubulin α and β subunits, respectively. Apparently, this 55 kDa band contained both α and β -tubulin because these two subunits form a constitutive heterodimer [5].

The specificity of the observed tubulin pull down is manifested by the following facts: (1) There was no prominent tubulin pull down in the control, in which no Btn-PDE γ was bound on the beads (A, lane 2; B, lane 4; C, lane 1). (2) Tubulin was not pulled down by Btn-30Bz (lane 3 in C), indicating that tubulin was pulled down by a specific PDE γ sequence other than the N-terminal polycationic G19–Q32 region. In contrast, arrestin in photoreceptor cells is reported to bind microtubules with its positively charged surface [9]. (3) Tubulin was not pulled down by Btn-CytC either (lane 4 in C), which like PDE γ is also a positively charged protein with an isoelectric point at pH 9.25. This result together with the lack of pull down by Btn-30Bz argues against the possibility that tubulin was pulled down merely due to a nonspecific charge effect. (4) While tubu-

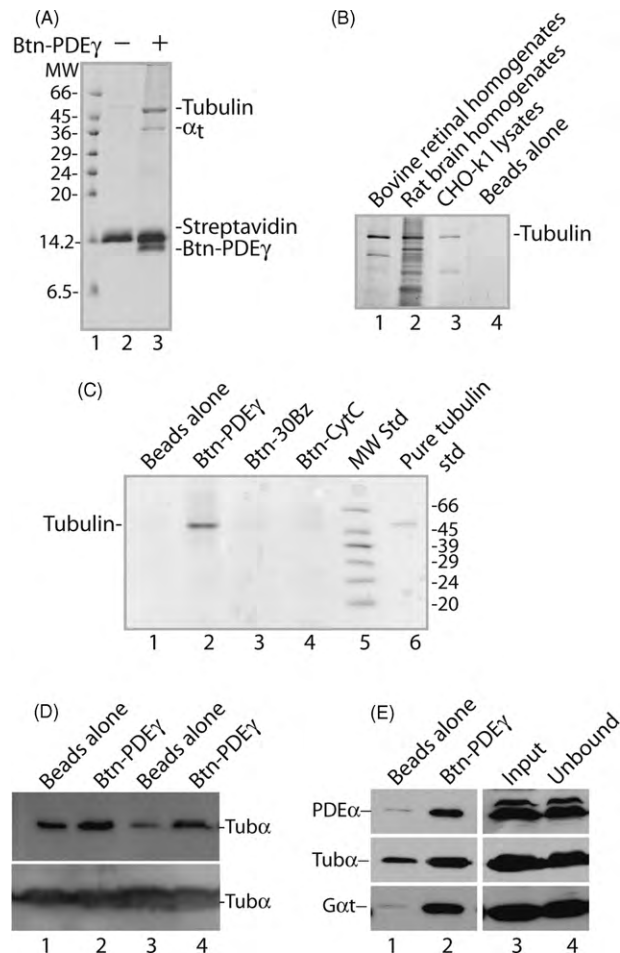


Fig. 1. Specific pull down of tubulin by PDE γ from bovine retinal homogenates. Shown are Coomassie-stained SDS gels (A–C) and Western blots (D, E), each is a representative of 2–4 similar experiments. In each reaction pull down from 100 μ g retinal homogenates was performed using 2 μ g (or otherwise stated) of Btn-PDE γ immobilized on 1 μ l streptavidin beads. (A) The tubulin pull down by Btn-PDE γ from retinal homogenates appears as a prominent band of ~55 kDa (lane 3). Lane 2 is the control with no Btn-PDE γ . MW, molecular weight. (B) Tubulin was pulled down by Btn-PDE γ from bovine retinal homogenates (lane 1), rat brain homogenates (lane 2), as well as CHO-k1 cell lysates (lane 3). The gel was silver-stained using the Pierce SilverSNAP Stain kit II. (C) Btn-PDE γ on the streptavidin beads pulled down tubulin specifically from retinal homogenates (lane 2), as compared to the beads with no Btn-PDE γ (lane 1), the beads bound with Btn-30Bz (lane 3), and the beads bound with Btn-CytC (lane 4). (D) Btn-PDE γ specifically pulled down tubulin from both bovine (see lanes 1 and 2) and mouse (see lanes 3 and 4) retinal homogenates, as revealed by immunoblotting. The lower panel shows unbound tubulin in the supernatants. (E) Immunoblotting of PDE α , tubulin- α (tub α), and transducin- α (G α t) in the pull down from bovine retinal homogenates. For each reaction 6 μ g of Btn-PDE γ and 2 μ l of streptavidin beads were used. Lane 1 is the Btn-PDE γ minus control; lane 2 is pull down on the Btn-PDE γ beads; lane 3 is the homogenates used for each reaction; lane 4 is the unbound protein in the supernatant. Semi-quantitation of tubulin pull down relative to input (100%, lane 3): 6.7%, 34.4%, and 58.2% in lanes 1, 2, and 4, respectively.

lin is abundant in the retina, actin, another abundant cytoskeleton protein (42 kDa), was not efficiently pulled down (Lane 3 in A). (5) PDE α and G α t, however, were both pulled down, as confirmed by Western blotting (Fig. 1E). This result served as a positive control because PDE α and G α t are well-documented PDE γ -interacting proteins [8]. It is unlikely that PDE γ pulled down tubulin indirectly by binding to G α t because G α t does not interact with tubulin although G α s, G α i and G α q do interact [3]. (6) In addition, Btn-PDE γ also readily pulled down tubulin from mouse retinal homogenates (Fig. 1D, lane 4), rat brain homogenates and CHO-k1 cell lysates (Fig. 1B), further supporting a specific PDE γ –tubulin interaction.

We then further confirmed the specific PDE γ :tubulin interaction using pure proteins. As seen in Fig. 2A, in comparison to the background control (lane 4), Btn-PDE γ readily pulled down microtubules which were prepared with the pure bovine brain tubulin (lane 2). Btn-BSA which is irrelevant to PDE γ , however, pulled down microtubules only to the background level (lane 6). Btn-CytC did not pull down microtubules efficiently either (lane 5). This is in agreement with the data obtained with retinal homogenates (Fig. 1C, lane 4), further eliminating a possibility of nonspecific charge effect in the observed PDE γ :tubulin interaction. Remarkably, when added as competitors, the C-terminal PDE γ peptide PDE γ_{62-87} but not the N-terminal peptide PDE γ_{1-61} , significantly reduced microtubule pull down (Fig. 2B and lane 3 in A). This is also in agreement with the observation that the PDE γ central peptide Btn-30Bz was not able to pull down tubulin from retinal homogenates (Fig. 1C, lane 3), suggesting that the PDE γ C-terminal domain was responsible for the observed PDE γ -tubulin interaction. Thus the PDE γ domain-dependence of this interaction shows an additional layer of specificity.

One useful feature of tubulin is that the polymerized form (microtubules) can be readily pelleted down by centrifugation, and appears as visible glassy pellet at the bottom of the microcentrifuge tube (Cytoskeleton Inc.). Thus the normally formed microtubule matrix is distinguishable from the opaque appearance of possible protein precipitations. Taking advantage of this property, we have further characterized the specificity of the PDE γ -tubulin interaction by co-sedimentation assays. Consistent with the Btn-PDE γ pull down results (Fig. 2A), PDE γ co-sedimented

with microtubules (Fig. 2C, lanes 2 and 3); CytC however, did not (lanes 4 and 5). PDE γ itself as a highly soluble protein was not pelleted in the absence of microtubules (lane 1).

These pull down and co-sedimentation experiments together indicated that tubulin interacted with PDE γ specifically, probably with its C-terminal domain.

Since tubulin is a GTP/GDP binding protein, we examined a possible GTP dependence of the PDE γ :tubulin interaction by pull down assays and NMR spectroscopy. As shown in Fig. 2D, while Btn-PDE γ pulled down tubulin 1.6-fold over the background in the presence of GDP, the pull down was 3.2-fold over the background in the presence of GTP, indicating a GTP dependence of the PDE γ :tubulin interaction.

This feature was also revealed by NMR spectroscopy, a method that can provide molecular details in PDE γ interactions [18]. Identification of residues that exhibit a large change in NMR chemical shift or peak intensity upon complex formation offers an informative approach for mapping the protein-protein interaction interface [12,18]. We have therefore performed the NMR titrations on full-length ^{15}N -labeled PDE γ with pure tubulin (Fig. 3). The ^1H , ^{15}N HSQC spectra were collected for free PDE γ (black), PDE γ in the presence of tubulin and GTP (blue), as well as PDE γ in the presence of tubulin and GDP (red). The NMR peaks were assigned based on the published results [18]. The residues of PDE γ in contact with tubulin were identified by the analysis of signal broadening.

Inspection of the NMR spectra indicated that, whereas most of the NMR peaks retained nearly identical intensities upon addition of tubulin in the presence of GTP, some PDE γ residues experienced

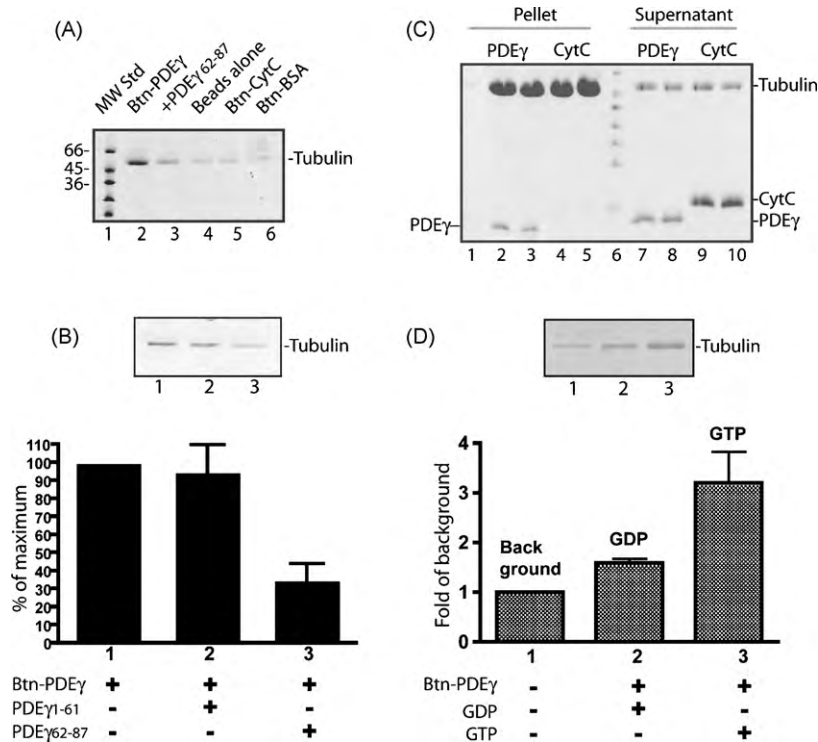


Fig. 2. Specific PDE γ :tubulin interaction revealed by assays using pure proteins. Streptavidin beads pull down assays (A, B, D) or microtubule co-sedimentation assay (C) were performed using pure tubulin proteins. (A) Btn-PDE γ pulled down microtubules specifically (lane 2) as compared with various controls including the beads with no Btn-PDE γ bound (lane 4), the beads with Btn-CytC bound (lane 5), and the beads with Btn-BSA bound (lane 6). In lane 3, the PDE γ C-terminal peptide PDE γ_{62-87} in a 20-fold molar excess over Btn-PDE γ was added. (B) Microtubules pull down by Btn-PDE γ on the streptavidin beads (lane 1) was reduced in the presence of PDE γ_{62-87} (lane 2) but not PDE γ_{1-61} (lane 3) in a 20-fold molar excess. Pull down was quantitated as percentage of the Coomassie staining in the absence of PDE γ peptides (lane 1). Error bar is a standard deviation from 3 separated experiments. Student's *t*-tests indicate that the difference between bars 2 and 1 is insignificant ($p > 0.5$); whereas the difference between bars 3 and 1 is very significant ($p < 0.1$). (C) Co-sedimented proteins are shown in lanes 2–5; the supernatants of the corresponding conditions are shown in lanes 7–10. Each condition is duplicates except lane 1, which is the control of PDE γ pellet from the reaction with no microtubules. (D) GTP dependence of tubulin pull down by Btn-PDE γ -beads. Tubulin (9 μg) was incubated with beads alone (lane 1) or beads pre-bound with 1 μg of Btn-PDE γ for 1 h at room temperature in the buffer containing 20 mM HEPES, pH 7.4, 120 mM NaCl, and 5 mM MgCl_2 , in the presence of 2 mM GDP (lane 2) or 2 mM GTP (lane 3). Pull down was quantitated as fold of the Coomassie staining in the control (lane 1). Error bar is a standard deviation of 2 separate experiments. The difference between the GDP condition and the GTP condition is significant ($p = 0.207$).

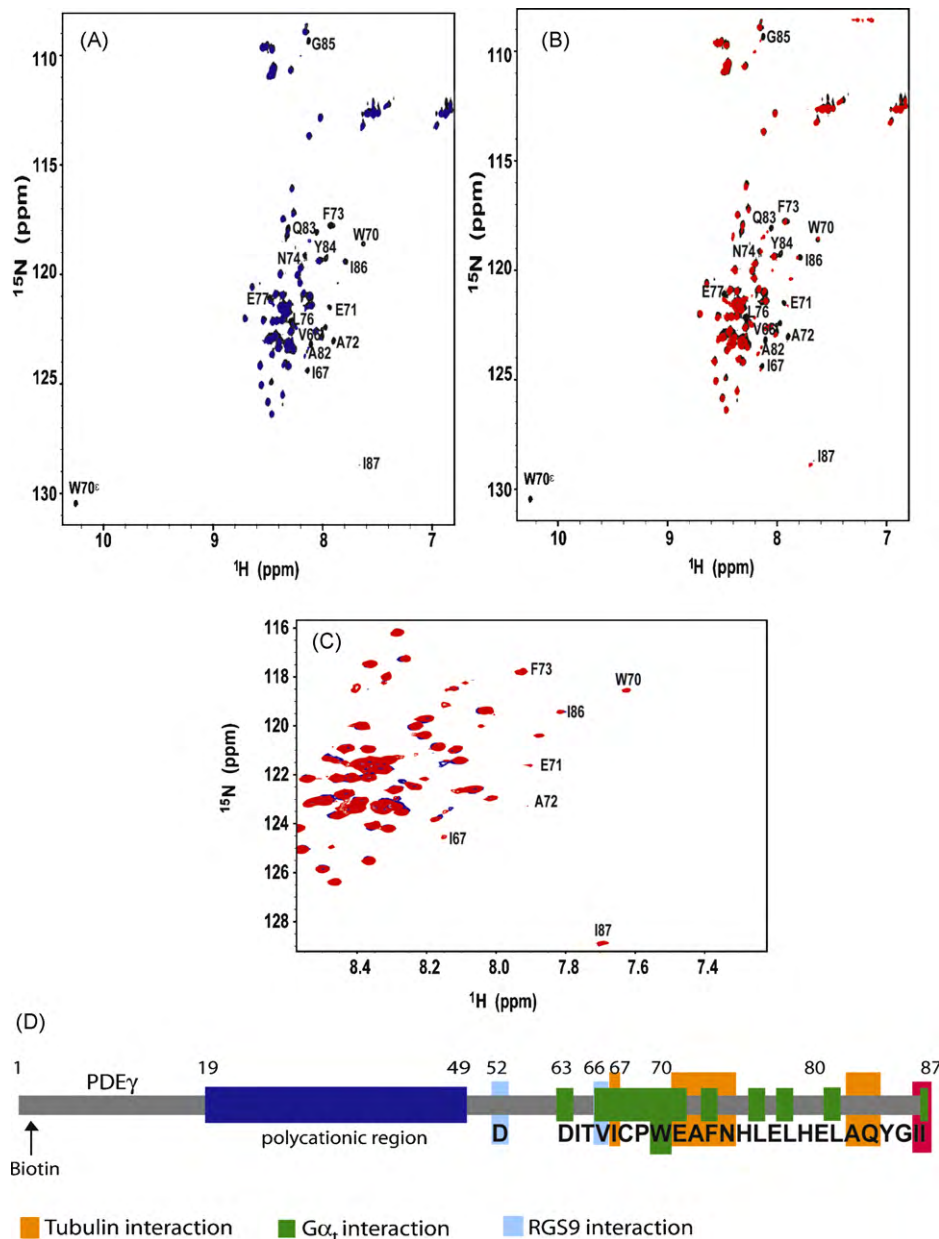


Fig. 3. NMR HSQC spectra indicating the PDE γ :tubulin interaction. The [^1H , ^{15}N] HSQC spectra were collected on 30 μM ^{15}N -labeled PDE γ , in the absence (black) or presence of 10 μM tubulin dimer in the buffer containing 10 mM PIPES, 1 mM EGTA, 1 mM Mg^{2+} , 90% $\text{H}_2\text{O}/10\%\text{D}_2\text{O}$, pH 6.2, including either 1 mM GTP (blue) or 1 mM GDP (red). (A) Overlay of the HSQC spectra of free ^{15}N -PDE γ (black) and ^{15}N -PDE γ in the presence of tubulin-GTP (blue). The cross peaks corresponding to the indole group of W70 is denoted as W70^c. (B) Overlay of the HSQC spectra of free ^{15}N -PDE γ (black) and ^{15}N -PDE γ in the presence of tubulin-GDP (red). (C) Overlay of the HSQC spectra of ^{15}N -PDE γ + tubulin-GTP (blue) and ^{15}N -PDE γ + tubulin-GDP (red). (D) A diagram of PDE γ interactions with different partners. The PDE γ residues that showed most significant interactions with tubulin-GTP are highlighted in orange. The polycationic region is shown in blue. I86 and I87 are labeled red. The $\text{G}\alpha_t$ -interacting (green) and RGS9-interacting (cyan) residues are shown based on the crystal structure including the C-terminal half of the PDE γ molecule [16]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

significant signal loss (Fig. 3A). When mapped to the sequence of PDE γ , these residues were located to the PDE γ C-terminal I67–G85 region. This is in accord with the observation that the PDE γ C-terminal peptide PDE γ_{62-87} but not the N-terminal peptide PDE γ_{1-61} eliminated the tubulin pull down (Fig. 2B). Residues that displayed most prominent signal broadening included I67, E71, A72, F73, N74, A82, and Q83. Interestingly, these residues are not critically important for either the $\text{G}\alpha_t$ GTPase stimulation [17] or PDE $\alpha\beta$ inhibition [7]. By contrast, residues I86 and I87, which are key for the inhibitory PDE γ interaction with the PDE6 catalytic domain [2,7], exhibited minimum signal broadening. Meanwhile, the indole group of W70, which is critical for binding of PDE γ with $\text{G}\alpha_t$ [16,17], was also unaffected by the presence of tubulin. Thus

this NMR study suggests that the PDE γ :tubulin interaction is distinguished from the established PDE γ C-terminal interactions with its visual signaling targets [2,7,16] (Fig. 3D).

Furthermore, the GDP-bound tubulin appeared to impose less line broadening effect than the GTP-bound tubulin (Fig. 3A–C), confirming a GTP dependence of the PDE γ :tubulin interaction observed through pull down assays (Fig. 2D).

In this study we have identified a novel PDE γ interaction with tubulin/microtubules. This interaction was determined to be specific for the PDE γ C-terminus, and was enhanced in the presence of GTP. The tubulin-interacting interface, however, was distinct from the PDE γ interaction with either transducin, the PDE6 catalytic domain, or the RGS9-1 catalytic core. The uniqueness of

the PDE γ :tubulin interaction may provide useful clues for possible non-phototransduction PDE γ functions in the photoreceptor cells.

An important feature of the photoreceptor cell is that all the phototransduction proteins are synthesized in the inner segment, and must be transported through the narrow connecting cilium into the outer segment, the default destination, to perform their functions [1]. Microtubules constitute the “railway” for this inter-segmental transport, with the minus ends anchored in the basal body [15] and the plus ends extended either into the inner segment or through the connecting cilium into the outer segment (Fig. S1). The mechanism underlying transport inside the inner segment, from the ER (endoplasmic reticulum) to the basal body, is largely unknown. Recent studies using mouse models led to a hypothesis that PDE6 and other membrane proteins transport along microtubules on cargo vesicles, which are moved from the ER region to the basal body most likely by the minus end-directed dynein II [11].

Our results showed that the PDE γ :tubulin interaction is GTP-dependent (Figs. 2D and 3) and is differential from the PDE $\alpha\beta$ interaction (Fig. 3). We thus speculate that through the interaction with GTP-tubulin, PDE γ may help load the PDE6-carrying vesicles to microtubules and thus facilitate PDE6 transport from ER to the basal body (see the diagram in Fig. S1). Since fatty acids are attached to the PDE6 α and β C-termini by a post-translational process on the ER [11], the PDE6 $\alpha\beta\gamma\gamma$ heterotetramer may be assembled and then tethered to the membrane of carrier vesicles in the ER region. The PDE6-laden vesicles can then be “docked” through the PDE γ :tubulin interaction to nearby microtubule plus ends where GTP-bound tubulins are accessible [5]. Thus temporary immobilization of the PDE6 vesicle to microtubules should facilitate its efficient recognition by dyneins, probably also with the assistance of the dynactin complex. In this case, PDE γ could reasonably bind tubulin and PDE $\alpha\beta$ simultaneously, because it is the PDE γ polycationic region rather than the tubulin-interacting C-terminal domain (Figs. 2B and 3) that provides most of the binding strength with PDE $\alpha\beta$ [13]. In addition, the key PDE6-inhibiting residues did not interact with tubulin (Fig. 3), suggesting that tubulin may not compete with the PDE6 catalytic domain for interaction with the PDE γ C-terminus. Once GTP-tubulin is converted into GDP-tubulin by its GTPase activity [5], PDE γ dissociates from microtubules and the PDE6-carrying vesicles can now be moved along microtubules toward the connecting cilium by the motor proteins.

Microtubule dynamics is a highly regulated event that plays a critical role in neurite extension. The microtubule plus end is stabilized by the loading of GTP-tubulin subunits [5]. Therefore, in a scenario alternative to a possible PDE γ role in PDE6 trafficking, PDE γ which recognizes GTP-tubulin (Figs. 2D and 3) may modulate microtubule dynamics and thus regulate the morphogenesis of the photoreceptor synaptic terminal.

In either case, the novel interaction between the PDE γ C-terminus and tubulin that we have observed may provide important implications for understanding the degenerative retinal diseases caused by the lack of PDE γ [20]. Further investigations are warranted to unravel possible cellular functions of the PDE γ :tubulin interaction in the photoreceptor neurons. Distribution of the PDE6 α and β subunits in the retinal outer segment of the PDE γ -knockout mouse should be determined. In the absence of PDE γ , PDE6 may not be able to transport normally from the inner segment to the outer segment, which may partially explain the abnormal development of the rod outer segment prior to a rapid retinal degeneration in the PDE γ -knockout mouse [20]. In addition, the morphology of the rod photoreceptor synaptic terminal

in the PDE γ -knockout mouse should also be examined. A lack of possible regulation of microtubule dynamics by PDE γ could lead to abnormal synapse formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2010.07.044.

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