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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 (PDEγ). The specificity and molecular details of the PDEγ: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 PDEγ C-terminal I67-G85 region, and the interaction interface appeared to be distinct from those with the other PDEγ targets in phototransduction. We also observed that PDEγ interacted with tubulin in a GTP-dependent manner. Our findings offer implications for non-phototransduction role(s) of PDEγ in the photoreceptor neurons.

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 (PDEγ) of the photoreceptor cGMP phosphodiesterase (PDE6) [8].

The rod photoreceptor PDE6 is composed of two similar catalytic subunits (PDEαβ) and two identical inhibitory PDEγ subunits. PDEγ 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 PDEγ in phototransduction has been well defined. PDEγ keeps PDE6 inactive in the dark but regulates the turn-on as well as turn-off of visual signaling upon photoresponse, *via* interactions with PDEαβ, the transducin α subunit (Gαt) and the regulator of G protein signaling (RGS9-1). Crystal structures revealed distinct PDEγ C-terminal interactions with the chimeric PDE5/6 catalytic domain [2], Gαt 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 PDEγ molecules in solution although PDEγ 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 PDEγ targets [8]. A recent study reported that the N-terminal proline rich region of PDEγ interacts with PACSIN, suggesting a possible role of PDEγ in endocytosis in the photoreceptor cell [10]. We were therefore motivated to find additional PDEγ-interacting proteins in the retinal photoreceptors.

Bovine and mouse retinal homogenates were prepared according to the method described previously [6]. Biotinylated PDEγ (Btn-PDEγ) was generated by covalently linking maleimide-PEO_2-biotin (Pierce) to the single cysteine placed at the PDEγ N-terminus. The peptide Btn-Bz30 containing a PDEγ N-terminal polycationic sequence (BtnG_{19}-Q_{32}) was custom-synthesized at the University of Wisconsin Peptide Synthesis Facility. Btn-CytC (cytochrome C) and Btn-BSA were prepared by first reacting maleimide–PEO_2–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-PDEγ. 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 $MgSO_4$, 50 μM $AlCl_3$, 30 mM NaF, and 50 μM GDP), and then washed 3× 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-PDEγ bound.

Western blotting was performed with antibody dilutions as follows: anti-PDEα (Affinity Bioreagents), 1000-fold; anti-tubulin-α (Sigma–Aldrich), 10,000-fold; anti-Gαt (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 μg of microtubules was added to 1 μl of streptavidin beads with 2 μg Btn-PDEγ 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 $MgCl_2$) supplemented with AlF_4^--GDP, the beads were washed 3× with 50 μl of buffer each time.

Microtubule co-sedimentation assays were performed according to the published method [22] with minor modifications. In each reaction, 20 μg of microtubules was incubated with 2 μg of PDEγ for 30 min at room temperature in Buffer A. Microtubules were pelleted by centrifugation at 20,000 × *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–PDEγ interactions was performed based on the published methods [12,18]. ^{15}N-labeled PDEγ was prepared as previously described [18]. The NMR experiments were performed at 20 °C using a 600-MHz Varian Inova spectrometer equipped with ^{1}H, ^{15}N, and ^{13}C triple-resonance cryogenic probe at the National Magnetic Resonance Facility at Madison, WI (NMRFAM). [^{1}H,^{15}N]-HSQC (heteronuclear single quantum correlation) spectra were first collected for 30 μM ^{15}N-labeled free PDEγ in the buffer containing 10 mM PIPES, 1 mM EGTA, 1 mM Mg^{2+}, 90% H_2O/10%D_2O, pH 6.2. The data collection was repeated under the same experimental conditions following addition of 10 μM tubulin dimer and 1 mM GTP or GDP.

We have observed a novel PDEγ:tubulin interaction. This finding resulted from the effort to identify additional PDEγ targets in the photoreceptor cells through pull down experiments using Btn-PDEγ (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: MRECISIH 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-PDEγ 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 PDEγ 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 PDEγ 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 tubulin is abundant in the retina, actin, another abundant cytoskeleton protein (42 kDa), was not efficiently pulled down (Lane 3 in A). (5) PDEαβ and Gαt, however, were both pulled down, as confirmed by Western blotting (Fig. 1E). This result served as a positive control because PDEαβ and Gαt are well-documented PDEγ-interacting proteins [8]. It is unlikely that PDEγ pulled down tubulin indirectly by binding to Gαt because Gαt does not interact with tubulin although Gαs, Gαi and Gαq do interact [3]. (6) In addition, Btn-PDEγ 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 PDEγ–tubulin interaction.

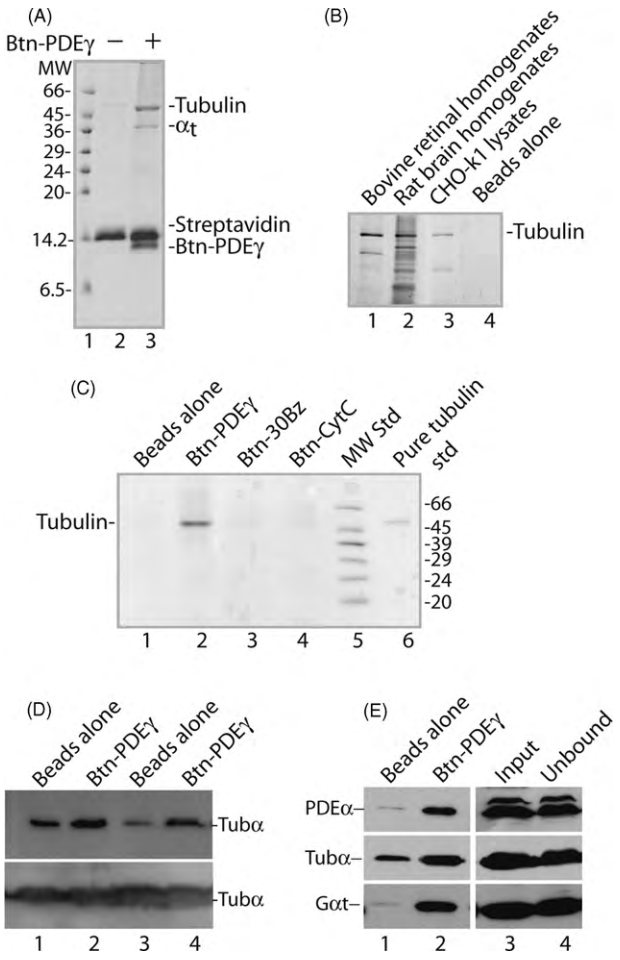

Fig. 1. Specific pull down of tubulin by PDEγ from bovine retinal homogenates. Shown are Coomassie-stained SDS gels (A–C) and Western blots (D, E), each is a representative of 2–4 similar experiments. In each reaction pull down from 100 μg retinal homogenates was performed using 2 μg (or otherwise stated) of Btn-PDEγ immobilized on 1 μl streptavidin beads. (A) The tubulin pull down by Btn-PDEγ from retinal homogenates appears as a prominent band of ~55 kDa (lane 3). Lane 2 is the control with no Btn-PDEγ. MW, molecular weight. (B) Tubulin was pulled down by Btn-PDEγ 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-PDEγ on the streptavidin beads pulled down tubulin specifically from retinal homogenates (lane 2), as compared to the beads with no Btn-PDEγ (lane 1), the beads bound with Btn-30Bz (lane 3), and the beads bound with Btn-CytC (lane 4). (D) Btn-PDEγ specifically pulled down tubulin from both bovine (see lanes 1and 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 PDEα, tubulin-α (tubα), and transducin-α (Gαt) in the pull down from bovine retinal homogenates. For each reaction 6 μg of Btn-PDEγ and 2 μl of streptavidin beads were used. Lane 1 is the Btn-PDEγ minus control; lane 2 is pull down on the Btn-PDEγ 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.