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Cyclic Nucleotide-Gated Channels Require Ankyrin-G for Transport to the Sensory Cilium of Rod Photoreceptors

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Abstract

Cyclic nucleotide-gated channels localize exclusively to the plasma membrane of photosensitive outer segments of rod photoreceptors where they generate the electrical response to light. Here we found that targeting of cyclic nucleotide-gated channels to the rod outer segment required their interaction with ankyrin-G. Ankyrin-G localized exclusively to rod outer segments, coimmunoprecipitated with the cyclic nucleotide-gated channel, and bound to the C-terminal domain of the β1-subunit. Ankyrin-G depletion in neonatal mouse retinas markedly reduced cyclic nucleotide-gated channel expression. Transgenic expression of cyclic nucleotide-gated channel β-subunit mutants in *Xenopus* rods showed that ankyrin-G binding was necessary and sufficient for targeting of the β1-subunit to outer segments. Thus ankyrin-G is required for transport of cyclic nucleotide-gated channels to the plasma membrane of rod outer segments.

Cyclic nucleotide gated (CNG) channels initiate the electrical responses to light in photoreceptors and to chemical stimuli in olfactory neurons (1). CNG channels are segregated to sensory cilia, where visual and olfactory signal transduction takes place. This precise intracellular localization is dependent on the channel's β-subunit (CNG-β1) in both classes of neurons (2-4). However, the molecular mechanism(s) of CNG channel targeting to the plasma membrane of sensory cilia, where this channel normally functions, are unclear.

Ankyrin-G is a versatile membrane adaptor involved in the formation and maintenance of diverse specialized membrane domains (5-9). Ankyrin-G is localized exclusively to rod outer segments (ROS), where it was found along with CNG channels which have been localized to the ROS plasma membrane (10) (Fig. 1A, B). In contrast, the plasma membrane of the inner segment was lined with ankyrin-B, which is required for the coordinated expression of the Na/K ATPase, Na/Ca exchanger, and beta-2 spectrin ((11); Fig. 1A). Localization of ankyrin-G to the plasma membrane was evident in isolated mouse ROS, but was better demonstrated in frog ROS, which are 3-4-fold larger in diameter (Fig. 1B). Ankyrin-G also localized in the olfactory sensory cilia and the principal piece of sperm flagella, together with CNG-β1 (4, 12) (fig. S1). When we treated isolated bovine ROS with a cleavable cross-linker, solubilized them in 0.1% SDS, and immunoprecipitated using anti-ankyrin or anti-CNG-β1 antibodies, reciprocal CNG-β1/ankyrin-G co-immunoprecipitation was observed (Fig. 1C). The interaction with ankyrin-G was specific, because CNG-β1 was not precipitated by non-immune or anti-ankyrin-B antibodies, and the major ROS-specific protein, rhodopsin, was not precipitated in either case (Fig. 1C).

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The CNG channel binding to ankyrin-G was further evaluated using a HEK 293 cell-based assay for detecting ankyrin-membrane protein interactions (13). In this assay, over-expressed exogenous ankyrin-G fused to the C-terminus of green fluorescent protein (GFP; ankyrin-G-GFP) normally localized to the cytoplasm, is recruited to the plasma membrane upon co-expression of ankyrin binding partners such as neurofascin. CNG-α1 expressed in HEK 293 cells localized to the plasma membrane but did not recruit ankyrin-G-GFP (Fig. 1D), whereas CNG-β1 failed to localize to the plasma membrane of these cells when expressed by itself (fig. S2). Co-expression of CNG-α and CNG-β does yield functional heterotetrameric channels in the plasma membrane of HEK 293 cells (14). Such co-expression also resulted in efficient plasma membrane recruitment of ankyrin-G-GFP (Fig. 1E; complete recruitment was observed in 65% cells co-expressing the three proteins, n = 50). Ankyrin-B-GFP was not recruited to the plasma membrane under similar conditions (Fig. 1E), indicating that CNG-β1 interacts with ankyrin-G.

We next sought to evaluate whether ankyrin-G is required for localization of CNG channels to ROS *in vivo*, using shRNA to knockdown ankyrin-G expression in neonatal mouse retinas. We injected a mixture of a plasmid encoding shRNA targeting mouse ankyrin-G in 10-fold excess over a plasmid encoding GFP into the eyes of newborn pups followed by electroporation (15). Under these conditions, rods expressing GFP are typically co-transfected with the shRNA plasmid. Two weeks post-injection, photoreceptors transfected with control shRNA expressed GFP, displayed normal morphology and were robustly immunostained with ankyrin-G (Fig. 2). In contrast, photoreceptors transfected with ankyrin-G shRNA (GFP-positive) displayed a major reduction in the ankyrin G immunofluorescence in ROS (20-30% of control, based on immunofluorescence intensity of samples on the same slide, fig. S3) and their ROS were significantly shortened (average length of 4.7 μm vs. 15.5 μm in control rods; n=25; compare rhodopsin labeled sections). The immunofluorescence levels of both CNG-β1 and CNG-α1 were also markedly reduced, to a degree comparable with the ankyrin-G reduction (Fig. 2,fig. S3). In contrast, rhodopsin levels estimated by fluorescence intensities of samples on the same slide were similar between control and ankyrin-G shRNA expressing ROS (Fig. 2,fig. S3). The shortened ROS phenotype in ankyrin-G-depleted retina was more severe than reported for mice lacking the CNG-β1 subunit (2). Thus, ankyrin-G plays role(s) in either assembly and/or maintenance of ROS in addition to localization of the CNG channel. This result is similar to the requirement of ankyrin-G for biogenesis of the lateral membrane in cultured columnar epithelial cells (16).

The reduction in CNG channel expression in ankyrin-G-depleted rods could be explained by a requirement for ankyrin-G in targeting the channel to ROS from the ER or Golgi located in the inner segment. Indeed, ankyrin-G is required for both post-Golgi transport and immobilization of its binding partner E-cadherin in epithelial cells (6). To test this hypothesis, we needed to identify CNG-β1 mutants lacking ankyrin-G-binding. We first determined if ankyrin-G bound to either the N- or C-terminal cytoplasmic domain of CNG-β1 (Fig. 3A). Ankyrin-G–GFP was co-expressed with protein constructs in which the ankyrin-binding domain of neurofascin was replaced with either the entire cytoplasmic N- or C-terminus of human CNG-β1 (NF-CNG-βN (amino acids 1-654) and NF-CNG-βC (amino acids 1041-1251), respectively; Fig. 3A). Ankyrin-G interacted only with the C-terminal domain of CNG-β1, both in the HEK 293-based plasma membrane recruitment assay (fig. S4) and co-immunoprecipitation experiments (Fig. 3B). Immunoprecipitation experiments in HEK cells were performed in the absence of a crosslinking reagent.

A truncation of the C-terminal 28 residues of CNG-β1 is associated with retinitis pigmentosa (RP; hCNG-βΔ28, Fig. 3A (17)). Indeed, neurofascin fused to CNG-β1 C-terminal domain bearing this deletion failed either to recruit or to co-immunoprecipitate ankyrin-G-GFP (fig. S2A, Fig. 3B). Additional deletion mutagenesis (hCNG-βC1243 and hCNG-βC1236, Fig. 3C)

narrowed the interaction site to a 7-amino acid stretch in this region (underlined, Fig 3C, fig. S4B) and alanine-scanning mutagenesis revealed that the highly conserved residues I1237 and L1238 were essential for ankyrin-G binding (fig. S4B and Fig. 3C). Co-expressed CNG-α1 with mutant CNG-β1 (RP deletion or IL1237AA mutation) in HEK 293 cells abolished ankyrin-G binding without affecting the normal CNG-α1 and CNG-β1 association (Fig. 3D).

To test whether ankyrin-G binding was required for delivery of the channel to outer segments, human CNG-β1, wild type or mutants unable to interact with ankyrin-G, were expressed in the rods of transgenic *Xenopus laevis* (18). We used a specific antibody against human CNG-β1 (19) to distinguish it from the endogenous *Xenopus* CNG-β1 (Fig. 4). Wild type human CNG-β1 (WT) was found in ROS in a distinctive pattern consistent with its plasma membrane localization (Fig. 4). In marked contrast, both the RP (Δ28) and IL1237AA mutants were confined to perinuclear sites within rod cell bodies and were completely absent from ROS (Fig. 4).

We next tested if an ankyrin-G-binding site from an unrelated protein was sufficient for targeting CNG-β1 to ROS. The native site in CNG-β1 required for interaction with ankyrin-G was substituted with 14-amino acids from beta-dystroglycan which binds ankyrin-G directly (5), and has little sequence similarity with the CNG-β1 motif (Fig. 3C). The CNG-β1/dystroglycan (CNG β-DAG) chimera associated with ankyrin-G when co-expressed with CNG α in HEK 293 cells (fig. S5). When expressed in transgenic *Xenopus*, this chimera was targeted to the ROS plasma membrane, however, the mutant CNG β-DAG IIF/AAA chimera lacking the ankyrin-binding site (fig. S5; (5)) was retained in the photoreceptor cell body (Fig. 4). Because there is no retrograde movement of membrane proteins from ROS back into the cell body (20), we conclude that ankyrin-G-binding is both necessary and sufficient for trafficking CNG-β1 to the outer segment. The ankyrin-G pathway could intersect with the microtubule motor Kif17/osm3, which is found in ROS (21) and is required for ciliary transport of olfactory CNG channels expressed in MDCK cells (3). Another question relates to the specific beta spectrin partner of ankyrin-G in ROS. Ankyrins partner with beta-spectrins in performing their scaffolding roles in the membrane cytoskeleton and in mediating post-Golgi transport through interactions with phospholipids and motor proteins (22-25). Beta-2 spectrin found in inner segments(26) is reduced in ankyrin-B (+/−) retina (11) and thus is a likely partner for ankyrin-B there. Beta-4 spectrin is associated with ankyrin-G in axon initial segments, and is present in rod inner and outer segments (fig. S4) making it a plausible ankyrin-G partner in ROS.

Ankyrin-G accomplishes two critical functions in photoreceptors: it is required for transport of CNG-beta1 from its site of synthesis and the assembly and/or maintenance of ROS. This resembles the role of ankyrin-G in axon initial segments where it binds to and coordinates the localization of three proteins required for the initiation and regulation of action potentials (Nav1.6, KCNQ2/3 channels, and 186 kDa neurofascin) (27,28). Without ankyrin-G, axon initial segments lose these proteins and express dendritic markers (29). In epithelial cells ankyrin-G is required both for targeting E-cadherin to the plasma membrane and for biogenesis of the lateral membrane (6,16). We hypothesize that, in addition to targeting the CNG channel, ankyrin-G can interact with other ROS membrane proteins as well as proteins required for their ROS trafficking and these interactions are essential for ROS morphogenesis. A conserved ankyrin-G-based mechanism may thus be shared by photoreceptors, neurons, and epithelial cells that accomplishes both the targeting of membrane-spanning proteins to specialized plasma membrane domains as well as assembly and/or maintenance of these domains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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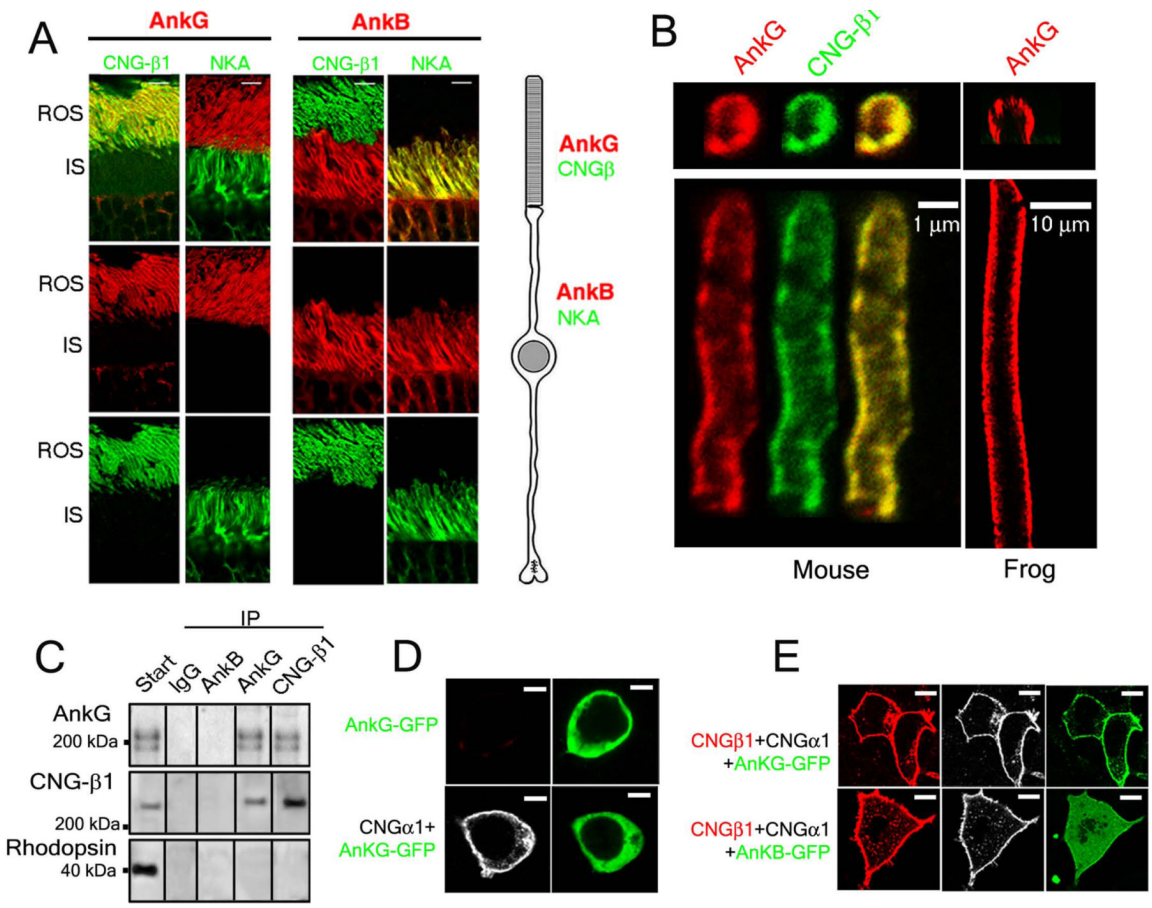

Fig 1. Ankyrin-G is restricted to photoreceptor outer segments and binds the rod CNG channel

(A) Co-localization of ankyrin-G (AnkG, red, left 2 columns) with CNG channel (CNG-β1, green) in ROS and co-localization of ankyrin-B (AnkB, red, right 2 columns) with NKA (green) in inner segments (IS). A schematic of a rod cell is shown to the right. (B) Ankyrin-G (red) localizes to the plasma membrane of isolated mouse and frog ROS labeled with anti-CNG-β1 antibody (green). ROS tangential sections are shown in upper panels and longitudinal sections in lower panels. (C) Co-immunoprecipitation of ankyrin-G with CNG-β1 channels from bovine ROS extracts. Antibodies used for precipitations are indicated on the top, antibodies used for protein detection are indicated on the left. (D) CNG-α1 (white) alone does not recruit ankyrin-G-GFP (green) to the plasma membrane of HEK 293 cells. (E) Ankyrin-G-GFP (green) is recruited to the plasma membrane of HEK 293 cells co-expressing CNG-β1 (red) and CNG-α1 (white). Scale bars are: 5 μm in A, 10 μm in D and E.

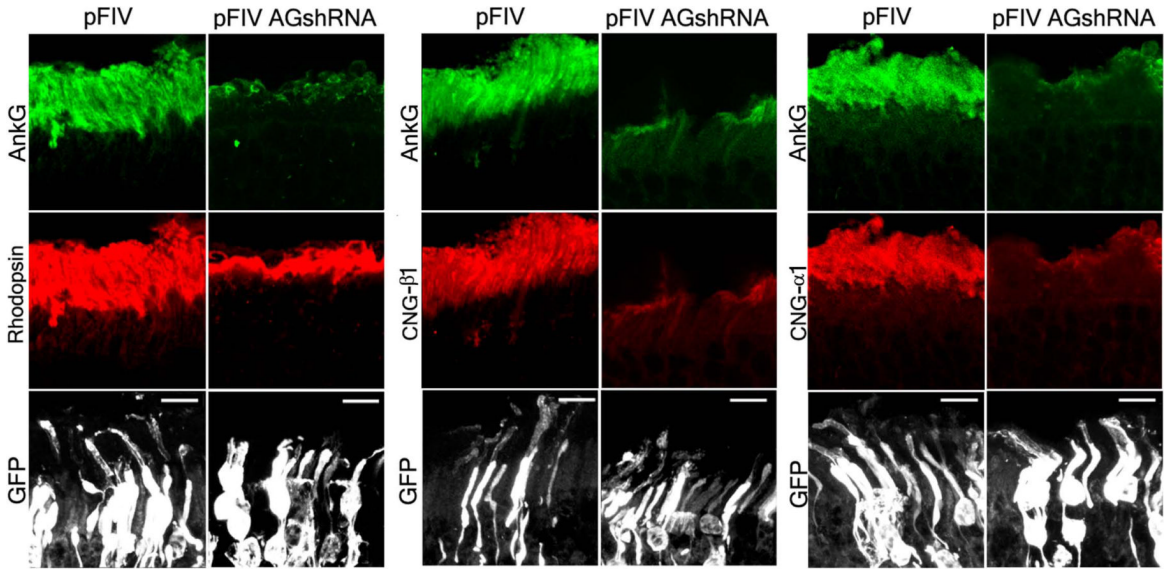

Fig 2. Ankyrin-G is required for ROS morphogenesis

Retinas of newborn mice were electroporated with either ankyrin-G shRNA or control pFIV (3μg/μl) plasmid, each mixed with pCAGGS-GFP (0.3μg/μl). Ankyrin-G (AnkG) staining is shown in green. The staining of rhodopsin (left), CNG-β1 (center) and CNG-α1 (right) is shown in red. GFP staining is shown in white. Scale bars are 10 μm.

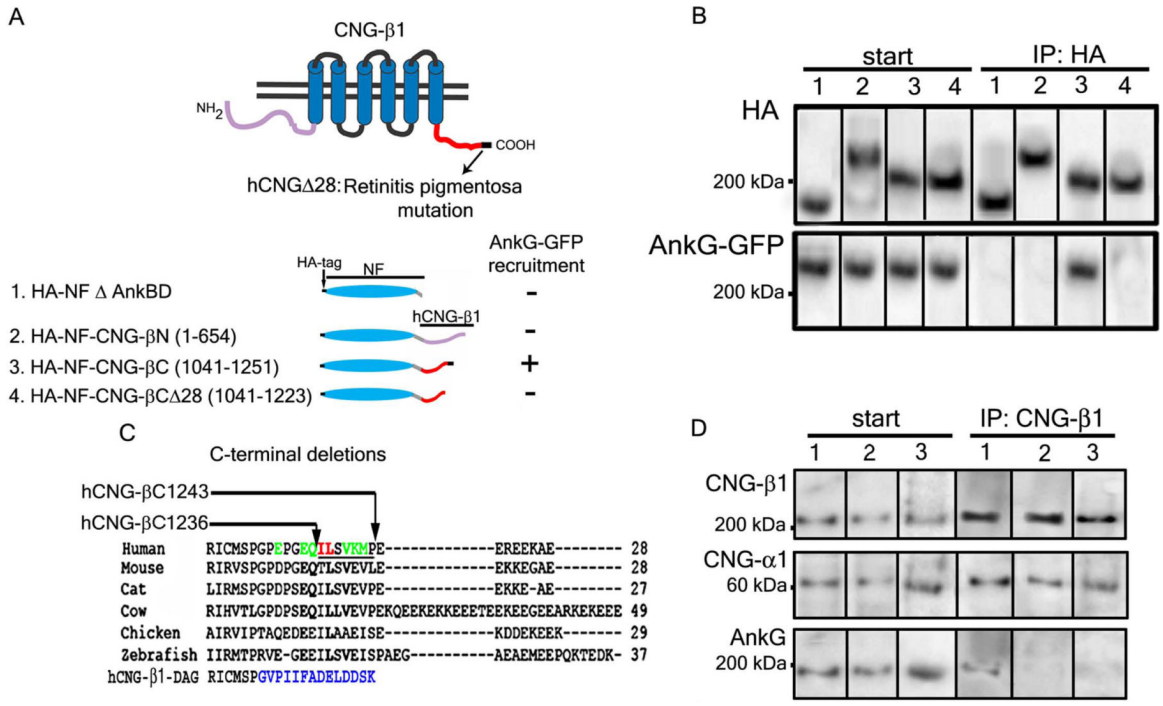

Fig 3. The ankyrin-G binding site resides in a C-terminal motif of CNG-β1

(A) Schematic diagrams of rod CNG-β1 (top) and HA-tagged neurofascin (HA-NF) chimeras with CNG-β1 (bottom). Number within brackets indicate the amino acids stretches of the CNG-β1 polypeptide that were fused to neurofascin. The abilities of chimeras to recruit ankyrin-G-GFP to plasma membrane of HEK 293 cells is indicated by + or - (AnkBD, ankyrin-binding domain). (B) Ankyrin-G-GFP was co-expressed in HEK 293 cells with the chimeras shown in panel A, cells were lysed and proteins were immunoprecipitated by anti-HA antibodies. Immuno blots of samples from the starting material (left) and precipitated proteins (right) were probed with anti-HA or anti-GFP antibodies. Lane numbers correspond to the numbered chimeras in panel A. (C) Sequence of the 28 C-terminal amino acids of human CNG-β1 and homologous regions from other vertebrates. Arrows indicate sites of C-terminal deletions hCNG-βC1243 and hCNG-βC1236 used to identify residues critical for ankyrin-G binding. Colored residues were mutated to alanine with those in red being critical for ankyrin-G binding and those in green being neutral. The human CNG-β1/β-dystroglycan chimera (hCNGβ-DAG) is shown at the bottom with the dystroglycan sequences marked in blue. (D) CNG-β1 (lane 1) CNG-β^{Δ}28 (lane 2) and CNG-β1 IL1237AA (lane 3) were coexpressed with CNG-α1 in HEK 293 cells and immunoprecipitated using the anti-CNG-β1 antibodies. Each CNG-β1 mutant normally co-precipitated with CNG-α1, but failed to bind endogenous ankyrin-G. Starting material is shown on the left and immunoprecipitates on the right.

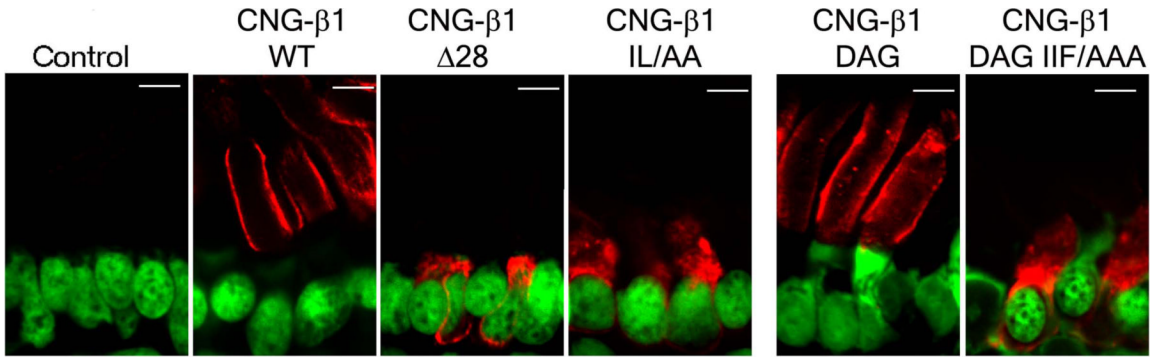

Fig 4. Ankyrin-G binding is necessary and sufficient for CNG-β1 transport to ROS of transgenic *Xenopus*

Retina sections in each panel are stained with anti-CNG-β1 antibody (red) and TOTO-3 to label the nuclei. Non-transgenic tadpole control on the left demonstrates that this antibody does not recognize the endogenous channel. Other panels depict the localization of wildtype (WT) human CNG-β1 or its mutants (indicated above each panel; see results for abbreviations). Scale bar 5 μm.