Differential Expression and Interaction with the Visual G-protein Transducin of Centrin Isoforms in Mammalian Photoreceptor Cells*

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Photoisomerization of rhodopsin activates a heterotrimeric G-protein cascade leading to closure of cGMP-gated channels and hyperpolarization of photoreceptor cells. Massive translocation of the visual G-protein transducin, Gt, between subcellular compartments contributes to long term adaptation of photoreceptor cells. Ca²⁺-triggered assembly of a centrin-transducin complex in the connecting ciliobranchial cusp of photoreceptor cells may regulate these transducin translocations. Here we demonstrate expression of all four known, closely related centrin isoforms in the mammalian retina. Interaction assays revealed binding potential of the four centrin isoforms to Gtβγ heterodimers. High affinity binding to Gtβγ and subcellular localization of the centrin isoforms Cen1 and Cen2 in the connecting ciliobranchial cusp indicated that these isoforms contribute to the centrin-transducin complex and potentially participate in the regulation of transducin translocation through the photoreceptor cilia. Binding of Cen2 and Cen4 to Gtβγ of non-visual G-proteins may additionally regulate G-proteins involved in centriole and basal body functions.

Vertebrate rod and cone photoreceptor cells are highly polarized neurons that consist of morphologically and functionally distinct cellular compartments. Light-sensitive outer segments are linked via a non-motile connecting ciliobranchial cusp with inner segments that contain the organelles typical for the metabolism of eukaryotic cells (see Fig. 6A). The outer segments are characterized by specialized disklike membranes where one of the best studied examples of a G-protein transduction cascade is arranged (1, 2). Photoexcitation leads to photoisomerization of the visual pigment rhodopsin (Rh*), which catalyzes GDP/GTP exchange on the heterotrimeric holo G-protein transducin (Gt, hol). This releases the α-subunit of transducin (Gt, α), which in turn activates a phosphodiesterase, catalyzing cGMP hydrolysis in the cytoplasm and closure of cGMP-gated channels localized in the plasma membrane (2, 3). The closure of these channels leads to a drop of the circulating cationic current, resulting in the hyperpolarization of the cell membrane (4). The recovery phase of the enzymatic machinery of visual transduction and rapid light adaptation of photoreceptor cells (time scale of seconds) rely on a feedback mechanism. This depends on changes in the intracellular Ca²⁺ concentration [Ca²⁺], affecting the phototransduction cascade through Ca²⁺-binding proteins (5). However, massive bidirectional translocation of transducin or other proteins between functional compartments of photoreceptor cells can also contribute to a much slower adaptation of rod photoreceptor cells (6, 7).

Light-induced exchanges of signal cascade components were first noted about a decade ago (8–10) and are currently of prominent interest in the field (e.g. Hardie (11), and see current review by Giessl et al. (12)); upon illumination, 80% of Gt,α and Gtβγ move in minutes from the outer segment to the inner segment and the cell body of rod photoreceptor cells. A recent study indicates that binding of the photoreceptor-specific protein phosducin to Gtβγ is not essential for this movement but facilitates light-driven Gtβγ translocation to the inner segment (7). The G-protein subunits return to the outer segments in the dark in a more leisurely time course of hours. So far, it is not known whether any intracellular exchange between the inner and outer segmental compartments of photoreceptor cells should occur through the slender non-motile connecting ciliobranchial cusp (13), and this represents a suitable domain for potential regulation of intersegmental molecular exchange (14). Our initial studies revealed that transducin is translocated through the photoreceptor connecting ciliobranchial cusp and further indicated that the Ca²⁺-induced assembly of a protein-protein complex of the G-protein transducin and the cytoskeletal protein centrin 1 regulates ciliary G-protein translocation (12, 15, 16). The assembly of this centrin 1-G-protein complex strictly depends on Ca²⁺ and is mediated by the Gtβγ complex.

Centrin 1 is a member of the centrin protein family, a subfamily of the parvalbumin superfamily of Ca²⁺-binding proteins (17, 18). Centrins were first described in unicellular green algae and are encoded by a family of at least 12 genes. Centrin 1 is highly conserved among species and is expressed in a variety of tissues, including photoreceptor cells, where it is thought to play a role in the regulation of cGMP-gated channels and other intracellular signal transduction pathways (19, 20). Centrin 1 is known to interact with a variety of cellular proteins, including G-proteins, kinases, and cytoskeletal elements, and to participate in a variety of cellular processes, including cell motility, mitosis, and cytokinesis (21–23).

Centrin isoforms 1–4; Cen1–4, centrin isoforms 1–4; Cen1p–4p, centrin isoform 1–4 proteins; pMmC1–4: polyclonal antibody against mouse centrin 1–4; BTP, 1-beta-tris(hydroxymethyl)methylammoniumpropane; GST, glutathione S-transferase; RT, reverse transcription; GTPyS, guanosine 5’-O-(thio)triphosphate; CHAPS, 3-[[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.
alge where they form filamentous structures that contract in response to an increase of [Ca\(^{2+}\)]\(_{i}\) (17–19). In vertebrates, centrins are ubiquitously expressed and commonly associated with centrosome-related structures such as spindle poles of dividing cells or cilia in centrosomes and basal bodies (17, 18). At least four different centrin genes are expressed in mammals (20–27). As a consequence of the isoform diversity in the mammalian genome, the four mammalian centrins should exhibit differences in their subcellular localization as well as in their cellular function. Little is known about the specific subcellular localization of the different centrin isoforms in diverse cell types and tissues. Most studies on the localization of centrins in mammalian cells and tissues have been performed with polyclonal and monoclonal antibodies raised against green alga centrins that do not discriminate between the mammalian centrin isoforms. Using these antibodies, centrins were detected in the centrioles of centrosomes and in the pericentriolar matrix (28–30). In previous studies on the mammalian retina, centrins were localized in two basically distinct subcellular domains (12, 14, 16). As in other animal cells, centrins are components of centrosomes and basal bodies in retinal neurons but were also found to be present in the connecting cilium of photoreceptor cells (14–16, 32). Although our recent studies provided evidence that isoform Cen1 is localized in the connecting cilium, a ciliary expression of other centrin isoforms remains elusive (16).

Here we show by glutathione S-transferase (GST) pull-down assays, size exclusion chromatography, and kinetic light-scattering experiments that all four centrin isoforms bind to the G\(_{\beta}\gamma\) complex with different affinities. In the present study, we also demonstrated retinal expression of the four centrin isoforms. Furthermore we were able to show for the first time that the centrins are co-expressed in the same cell type, particularly in highly specialized photoreceptor cells. Nevertheless there they are localized in different subcellular domains. The localization of the centrin isoforms Cen1 to Cen3 in the photoreceptor connecting cilium suggests that these centrins can be part of the centrin-transducin complex. In contrast, the centriolar localization of centrin isoforms 2–4 indicates an additional function of these centrin isoforms.

**EXPERIMENTAL PROCEDURES**

**Animals and Tissue Preparation**—All experiments described herein conformed to the standards set by the Association for Research in Vision and Ophthalmology as to the care and use of animals in research. Adult Sprague-Dawley albino rats and C57BL/6J mice were maintained on a 12:12-h light/dark cycle with lights on at 6 a.m. with food and water ad libitum. After sacrifice of the animals in CO\(_2\) as anesthetics were removed through a slit in the cornea prior to fixation and embedding for microscopy or further molecular biological and biochemical analysis. Bovine eyes were obtained from the local slaughter houses and were kept on ice in the dark until further processing.

**Antibodies**—Affinity-purified polyclonal rabbit antibodies against the \(\alpha\)- and \(\beta\)-subunit of G-proteins were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and a second affinity-purified polyclonal rabbit antibody against the \(\beta\)-subunit of G-proteins (7–20) was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody against centrin (clone 20H5) and a monoclonal antibody against HsCen2p (clone hCetn2.4) have been characterized previously (32). Polyclonal antisera from rabbit or goat against recombinantly expressed polyclonal rabbit antibodies against the \(\alpha\)- and \(\beta\)-subunit of G-proteins (7–20) were purchased from Molecular Probes (Eugene, OR). Monoclonal antibody against centrin (clone 20H5) and a monoclonal antibody against HsCen2p (clone hCetn2.4) was isolated from frozen dark-adapted bovine retinas according to Ref. 3. Subunits were further purified on Blue Sepharose (1 ml of Hitrap Blue, Amersham Biosciences) using a salt gradient (15). G\(_{\text{holo}}\), G\(_{\alpha}\), and G\(_{\beta}\gamma\) concentrations were determined by Bradford assay (37) using bovine serum albumin as the standard. The amount of intact, activable G\(_{\alpha}\) was determined precisely by fluorometric titration with GTP•S (38).

**SDS-PAGE and Western Blot**—For Western blots, isolated retinas or GST pull-down complexes were homogenized and placed in SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8) containing 10% glycerol, 2% SDS, 5% mercaptoethanol, 1 mM EDTA, and 0.025% bromphenol blue). Proteins were separated by SDS-PAGE (34) using 15% polyacrylamide gels, transferred electrophotically to polyvinylidene difluoride membranes (Bio-Rad), and probed with primary and secondary antibodies (32).
reaction the samples were treated for 15 min at 75 °C. Poly(dT)-primed cDNA synthesis (reverse transcriptase reaction) was performed using the Invitrogen cDNA Cycle™ kit and 5 µg of total RNA according to the directions. In control preparations, total RNA (DNase-treated or un-treated) was amplified by PCR without prior reverse transcriptase reactions using the MmCen1 primers. PCR was performed in a volume of 50 µl using 2.5 µl of prepared cDNA according to directions and 0.25 µg of each primer/reaction. Cycling conditions were 39 cycles at 94 °C for 1 min, 59 °C for 30 s, and 72 °C for 3 min followed by a 10-min 72 °C extension. PCR product lengths were determined on 0.8% agarose gels. As DNA markers, 5′-labeled PstI-digested lambda DNA (Invitrogen) was used. Sequencing of PCR products was performed by Centerprise (Mainz, Germany). For sequence comparisons and oligonucleotide generation the computer program Oligo™ Version 2.0 (Oxford Molecular Ltd., Oxford, UK) was used.

**Peptide Preadsorption of pMmC Centrin Antibodies with Recombinant Centrins—**To increase antibody specificity, the polyclonal antibodies pMmC1 to pMmC4 were preincubated with the appropriate recombinant centrin isoform proteins. For this purpose centrin isoform proteins were immobilized on polyvinylidene difluoride membranes (Bio-Rad) and incubated with the affinity-purified antibodies for 12 h at 4 °C in blocking solution (0.5% cold-water fish gelatin (Sigma) plus 0.1% ovalbumin (Sigma) in PBS). The following protein amounts were used: pMmC1: 200 µg of MmCen1, 150 µg of MmCen3, and 150 µg of MmCen4; pMmC2: 400 µg of MmCen1, 300 µg of MmCen3, and 200 µg of MmCen4; pMmC3: 100 µg of MmCen1 and 30 µg of MmCen4; pMmC4: 300 µg of MmCen1, 200 µg of MmCen2, and 400 µg of MmCen3. The supernatants containing the “preabsorbed” antibodies were subsequently used in Western blots or immunocytochemical experiments, respectively.

**Fluorescence Staining of Retinal Cryosections—**Immunofluorescence studies were essentially performed as described previously (15, 43). Briefly eyes from adult mice were prefixed in 4% paraformaldehyde in PBS for 1 h at room temperature, washed, soaked with 30% sucrose in PBS overnight, and cryofixed in melting isopentane. Cryosections were placed on poly-l-lysine-precoated coverslips (44, 45). Specimens were incubated with 50 mM NH₄Cl and 0.1% Tween 20 in PBS overnight, and cryofixed in melting isopentane. Cryosections were processed for immunoelectron microscopy as described previously (43). To immobilize the transducin subunits were pulled down by GST alone. All GST-centrins pulled down Gα from bovine retina extracts but not the purified GβGγ heterodimers (A and C). These results indicated that all four centrin isoforms bind to heterotrimeric GβGγ via Gβγ.

**GST-Centrin Pull-downs of transducin.** GST fusion proteins were incubated with bovine retina extracts (A and C) or biochemically purified subunits of transducin, Gα or GβGγ, respectively (B and D). Western blot analysis with anti-Gα (A and B) or anti-GβGγ antibodies (C and D) is shown. In bovine retina extracts the antibodies detected bands specific for the G-protein subunits (first lanes in A and C). None of the transducin subunits were pulled down by GST alone. All GST-centrin pull-downs Gα from bovine retina extracts but not the purified GβGγ heterodimers (A and C) or anti-Gα and anti-Gβ antibodies (C and D) is shown. In bovine retina extracts the antibodies detected bands specific for the G-protein subunits (first lanes in A and C). None of the transducin subunits were pulled down by GST alone. All GST-centrin pull-downs Gα from bovine retina extracts but not the purified GβGγ heterodimers (A and C). These results indicated that all four centrin isoforms bind to heterotrimeric GβGγ via Gβγ.

**Western Blot Analyses—**Western blot analyses of the GST-centrin-co-precipitations with antibodies against Gα or GβGγ respectively, revealed that the undissociable GβGγ was present in all co-precipitations, while Gα was not found in any of the reactions (Fig. 1B). This demonstrated that all centrin isoforms interact with the GβGγ as an isolated heterodimer or with GβGγ within the heterotrimer of transducin (GβGγholo = GαβGγ).
Size Exclusion Chromatography—To further validate our pull-down results, binding of purified G\textsubscript{holo} and its subunits (G\textsubscript{α} and G\textsubscript{βγ}) to the recombinant mouse centrin isoforms (MmCen1 to MmCen4) was investigated by size exclusion chromatography and SDS-PAGE/colorimetry. The elution profiles obtained with elution buffer containing 100 μM CaCl\textsubscript{2} are shown for centrin isoforms alone (red), G\textsubscript{α} or its subunits alone (green), and the mixture of centrin isoforms with G\textsubscript{α} or its subunits (blue) in the upper panels. The dotted lines are the calculated superpositions of the respective single component profiles yielding the predicted profiles for the mixture of the two non-interacting components. SDS-PAGE analysis of the fractions of the size exclusion chromatography is shown in the lower panels. Interactions of centrin isoforms (each 10 μg) with G\textsubscript{holo} (10 μg) (A–D), with G\textsubscript{α} (10 μg) (E–H) and with G\textsubscript{βγ} (10 μg) (I–L) in the presence of 100 μM CaCl\textsubscript{2} are shown. Note that 1) the lower amplitude of the MmCen2 peak absorption compared with the other centrin isoforms is due to the fact that only one aromatic amino acid is present in this molecule and 2) G\textsubscript{holo} elutes at an apparently lower molecular weight compared with its subunits (15).
assayed by the transition of soluble Gt (Gt,sol) to the membrane (40, 41). Moreover, this assay is applicable to any soluble protein that interacts with Rh* (40) and can be used as a tool to analyze changes in the amount/or molecular weight of transducin when it interacts with centrin isoforms. All recombinant mouse centrin isoforms (MmCen1 to MmCen4) resulted in an amplitude increase of the binding signal in a Ca$^{2+}$-dependent manner (an example is given in the inset of Fig. 3D). The increase of amplitude was significantly lower or was even not observed in the absence of free Ca$^{2+}$ (1 mM EGTA) for all tested centrin isoforms. Titration of the light-scattering Gt binding signals with the different centrin isoforms are shown in Fig. 3. The analysis of the titration curves of MmCen1, MmCen2, and MmCen4 revealed in the presence of free Ca$^{2+}$ that the effective concentrations of half-maximal binding (EC$_{50}$) are in the range of 1.8–2.9 μM (Table I and Fig. 3, A, B, and D). In contrast, the EC$_{50}$ for MmCen3 was about 5 times higher than for the other centrin isoforms (Table I and Fig. 3C). This difference indicates a significant lower affinity between MmCen3 and the transducin holoprotein. In addition to the lower affinity, the titration curve of the MmCen3-transducin interaction is consistent with a model in which each Gt holoprotein binds a monomer of MmCen3 (calculated with Hill equation with the parameters shown in Table I).

**Expression of Centrin Isoforms in Photoreceptor Cells**—To assess the relevance of transducin binding to the centrin isoforms in photoreceptor cell function, we analyzed the expression of the four centrin isoforms in the mouse retina. To address the question which of the four known centrin isoforms are expressed in the mammalian retina we first performed RT-PCR. Total RNA was extracted from isolated mouse or rat retinas, and after reverse transcription centrin cDNAs were amplified by PCR using centrin isoform-specific primer sets. Total RNA was extracted from isolated mouse or rat retinas, and after reverse transcription centrin cDNAs were amplified by PCR using centrin isoform-specific primer sets. Subsequently the identities of the amplified PCR products were confirmed by DNA sequencing. The present RT-PCR analysis revealed co-expression of all four centrin isoform mRNA in the adult mouse (Fig. 4) and rat retina (data not shown).

**Influence of centrin isoform concentration on calcium-dependent enhancement of the Gt binding signals probed by kinetic light scattering**

| Centrin isoform | Calculated fit parameter using the Hill equation$^a$ | EC$_{50}$$^d$ |
|-----------------|---------------------------------|----------------|
| MmCen1          | 0.47 ± 0.02                     | 2.9 ± 0.2      |
| MmCen2          | 0.48 ± 0.02                     | 1.8 ± 0.2      |
| MmCen3          | 0.87 ± 0.37                     | 11.1 ± 10.8   |
| MmCen4          | 0.69 ± 0.08                     | 2.8 ± 0.7      |

$^a$ Calculated EC$_{50}$ (Eq. 1) = \left(A/(MmCen^n)\times\left[(MmCen^n+EC_{50}^n)\right]^{-1}+1\right)

$^b$ Hill coefficient.

$^d$ Effective concentrations of half-maximal binding in μM.
centrin polypeptides previously used as antigens for immunization. All centrin antibodies detected their centrin isoform and also cross-reacted with one (pMmC3) or more of the complementary other three centrin isoforms (Fig. 5, A–D, left panel). Since centrin isoforms are very closely related (12, 16) and the cross-reactivities between antibodies generated against centrins with other centrin family members were frequently reported in previous studies (e.g. Refs. 14, 16, and 47), these results were not surprising. To minimize or even avoid these cross-reactivities, we preadsorbed antibodies raised against centrins with the polypeptides of the three other centrin isoforms prior to our expression analyses. Following this approach, we were able to discriminate between the proteins of all four centrin isoforms (Fig. 5, A–D, right panel). Preadsorption of the antibodies pMmC1 to pMmC4 with the appropriate recombinant centrin proteins abolished cross-reactivity with any nonspecific centrin isoform. Subsequent Western blot analyses of proteins extracted from bovine and mouse retinas with the preadsorbed anti-centrin antibodies revealed that the proteins of all four centrin isoforms (Cen1p to Cen4p) were expressed in bovine (Fig. 5E) and mouse retinas (data not shown).

Subcellular Localization of the Complex Partners Transducin and Centrin Isoforms in Photoreceptor Cells—We studied subcellular distribution of transducin in light- and dark-adapted retinas of mice. Previous immunofluorescence studies have shown that Gt is predominantly found in the photoreceptor outer segments in dark-adapted retinas, while after light adaptation Gt moves into the inner segment of photoreceptor cells (e.g. Refs. 6, 10, and 15). The present silver-enhanced immunogold labeling confirmed in principle these overall distributions under both illumination conditions shown by indirect immunofluorescence (Fig. 6). Nevertheless the resolution of immunoelectron microscopy revealed that during light adaptation Gt accumulated in the connecting cilium of photoreceptor cells (Fig. 6A). In contrast, minor Gt labeling was present in the cilium of dark-adapted cells (Fig. 6B). In dark-adapted rods in the absence of Gt molecules in the inner segment cytoplasm, G-protein staining was also obvious in the centriole of the basal bodies localized at the base of the photoreceptor cilium (Fig. 6B).

Now we addressed the subcellular localization of the centrin isoforms in retinal photoreceptor cells. Although in previous immunocytochemical studies the centrin antibodies used were not always isoform-specific, centrin isoforms 1, 3, and 4 were suggested to localize in cilia or their basal bodies, respectively (12, 14, 27, 47). To prove the differential expression of centrin isoforms, immunocytochemical experiments with the preadsorbed antibodies pMmC1 to pMmC4 were performed in retinal cryosections. Untreated affinity-purified antibodies pMmC1 to

![Fig. 4. Expression analysis of centrin isoforms in mouse retina by RT-PCR. Mouse centrin-specific primer sets were used to amplify different constructs of centrin isoforms. Total RNA used for all RT-PCR experiments was treated with DNase I to degrade genomic DNA. Control PCR (control) was conducted with DNase I-treated RNA lacking reverse transcriptase to demonstrate that no genomic DNA is amplified. All four centrin isoforms (MmCen1 to MmCen4) are expressed in the mouse retina.](image-url)

![Fig. 5. Validation of centrin isoform antibody specificity and expression analysis of bovine retina by Western blots. Affinity-purified antibodies raised against centrin isoforms (pMmC1 to pMmC4) cross-react with recombinant centrin isoforms (Cen1p to Cen4p) in Western blots (A–D, left panels). These cross-reactivities were abolished by preadsorption of centrin pMmC antibodies with recombinant centrin polypeptides (see “Experimental Procedures”) (A–D, right panels). In Western blot analysis of bovine retinal extract with preadsorbed antibodies pMmC1, pMmC2, and pMmC3 single specific bands at 20 kDa, the molecular mass of the Cen1, Cen2, and Cen3, were detected (E). With preadsorbed pMmC4 two fade bands at about 15 (arrow B) and 19 (arrow A) kDa were recognized; these bands represent bovine Cen4 and a shorter splice variant of Cen4 identified previously in other mammalian species (27).](image-url)
pMmC4 reacted in addition to the connecting cilium with basal bodies and centrosomes (data not shown). In contrast, indirect immunofluorescence of pMmC1 preadsorbed with the recombinant centrin isoforms 2–4 was only present in the connecting cilium of photoreceptor cells (Fig. 7, A and A'). Thus, Cen1p expression in the retina was restricted to the connecting cilium. Indirect immunofluorescence of pMmC2 and pMmC3 revealed the localization of Cen2p and Cen3p in the basal body complex as well as in the connecting cilium (Fig. 7, B and C and B' and C'). In contrast, labeling with the preadsorbed pMmC4 antibody showed no ciliary staining but a basal body labeling (Fig. 7, D and D'). The ciliary localization of MmCen3 was confirmed by immunoelectron microscopic analysis with the preadsorbed pMmC3 antibody (Fig. 8A). In the connecting cilium, MmCen3 was localized at the inner surface of the axonemal microtubule doublets in exactly the same ciliary subdomain where we previously found the co-localization of MmCen1/MmCen2 with transducin (15). Our present immunocytochemical studies further indicated the localization of the centrin proteins MmCen2 and MmCen3 in centrosomes of non-photoreceptor retinal cells, whereas MmCen1 and MmCen4 were not detectable there (Fig. 7, A–D and A'–D'). The differential localization of the centrin isoforms in mammalian photoreceptor cells and in non-specialized cells is summarized in the diagrams shown in Fig. 8, B and C.

**DISCUSSION**

This study was designed to analyze the interaction of transducin Gt, the heterotrimeric G-protein of the visual signal transduction cascade, with all four known mammalian centrin isoforms. We have demonstrated previously that the centrin isoform 1 binds with high affinity and specificity to Gt in a strictly Ca$^{2+}$-dependent manner (15, 16). Here we show that all four centrin isoforms are differentially expressed in mammalian photoreceptor cells and interact with transducin.

The present protein-protein interaction experiments demonstrate transducin binding to all four centrin isoforms. Centrin-GST pull-down assays and size exclusion chromatography reveal that the undissociable Gt$\beta\gamma$ dimer interacts with all centrin isoforms as an isolated heterodimer or within the heterotrimeric Gt-holo. As demonstrated for centrin 1 by blot overlay assays in our initial studies all centrins interact via the Gt$\beta\gamma$ dimer of transducin (15, 16). Furthermore our protein-protein interaction assays show that the assembly of all centrin-transducin complexes is strictly dependent on Ca$^{2+}$. Previous biophysical analyses of centrins have indicated that the binding of Ca$^{2+}$ via their EF-hands induces conformational changes of the molecules resulting in activation of centrins (18, 27, 48, 49). In diverse unicellular green algae, Ca$^{2+}$-activated centrins are responsible for the formation and contraction of centrin-containing nanofibers (17, 18). Although a recent study showed Ca$^{2+}$-independent binding of centrin to a target protein (50), in most cases the activation of centrins by Ca$^{2+}$ is necessary for the interaction with their binding partners (16, 18). In the yeast Saccharomyces cerevisiae, Ca$^{2+}$ facilitates binding of Cdc31p, the yeast centrin homologue, and of the human centrin isoforms 1 and 2 to Kar1p, which is necessary for division of the yeast cell (48, 51, 52). In our previous centrin blot overlay assays, the binding of recombinant MmCen1 to all detected interacting proteins was Ca$^{2+}$-dependent (15, 16). Here we show that not only the binding of centrin 1 to transducin but also of the other three centrin isoforms is triggered by Ca$^{2+}$.

Although all centrin isoforms interact with the Gt$\beta\gamma$ in a Ca$^{2+}$-dependent manner, we observed remarkable differences between the binding capacity of the centrin isoforms in particular between the isoform 3 and the other three isoforms. Our kinetic light-scattering experiments revealed that centrin 3 binds with a 5 times lower affinity to Gt$\beta\gamma$ (EC$_{50}$ is 5 times higher) then the centrin isoforms 1, 2, and 4. However, the EC$_{50}$ for centrin 3 binding is at least 2–3 times higher than the EC$_{50}$ of calmodulin, previously used in a control experiment as a well known EF-hand Ca$^{2+}$-binding protein that is related to centrin protein family members (15). Furthermore the comparison of the obtained Hill coefficient suggested that the centrins 1, 2, and 4 interact with Gt$\beta\gamma$ as oligomers, while the centrin
isoform 3 binds as a monomer in these assays. These findings confirm the ability of oligomerization, even of polymerization, of centrins previously demonstrated (48). In green algae, centrins are the major component of the contractile fibers of ciliary rootlets (19, 53).

In vitro studies with purified centrins indicate that centrins can polymerize to large polymeric structures induced by slowly increasing the Ca\(^{2+}\) concentration (48). Future studies will be necessary to prove whether the centrin isoforms, found to co-localize in subcellular domains of photoreceptor cells, may also form heteromeres.

In our initial studies, we discussed that the Ca\(^{2+}\)-induced assembly of a G-protein-centrin complex regulates the ciliary G-protein translocation in retinal photoreceptor cells (12, 15, 16). The accumulation of G\(_i\) observed in the present study in the ciliary domain of rod photoreceptor cells after light adaptation strongly suggests that the G\(_i\)-centrin complex is induced by light. In the connecting cilium previous quantification of silver-enhanced immunogold labeling demonstrated the co-localization of centrins with G\(_i\) at the inner surface of the axonemal microtubule doublets, a specific ciliary subdomain (12, 15). The present immunoelectron microscopic analysis further supports the hypothesis that centrin-G\(_i\) complexes assemble in the connecting cilium. As a consequence of the formation of this complex, the mobility of G\(_i\) through the slender cilium should decrease (barrier hypothesis discussed in Wolfrum et al. (16)) representing the foundation for the accumulation of G\(_i\) in the
ciliary compartment of rod photoreceptor cells. In the connecting cilium, the assembly of the centrin-G-protein complex is modulated by light and the ciliary free Ca2+ concentration. Changes of free Ca2+ in the photoreceptor within the operating (single quantum detector) range of the rod have been well studied (4). In rod operating range, a dramatic Ca2+ drop occurs in the outer segment after light activation of the visual cascade. However, recently a Ca2+ increase was observed in rod photoreceptor cells in bright light and rod-saturated conditions (54) that might be the source of Ca2+ for the induction of centrin/G-protein binding in the cilium.

Which centrin of the four closely related isoforms is responsible for the interaction with transducin in mammalian rods? This study has demonstrated mRNA and protein expression of four centrin isoforms in the mammalian retina, and investigations of others have found that centrin isoforms are expressed in different cellular systems (14, 27, 47). Here we show for the first time co-expression of all four centrin species in one and the same cell type. In retinal photoreceptor cells, each of the four centrin isoforms is present and should have the potential to participate in the regulation of transducin translocation through the photoreceptor cilium. The isoform-specific immunolabelings also reveal differential subcellular localizations of the isoforms in the photoreceptors. Only a subset of centrin isoforms, namely the isoforms 1, 2, and 3, are localized in the connecting cilium of photoreceptor cells. In contrast, centrin 4 is exclusively localized in the basal body at the base of the connecting cilium, leaving centrin isoforms 1, 2, and 3 as potential regulators of transducin translocation. Because centrin 3 has much lower affinity to transducin than the three other centrin isoforms, centrin 1 and 2 remain as the predominant candidates for Ca2+-dependent regulation of transducin translocation.

As already mentioned centrins are not only concentrated in the connecting cilium of rod cells but are also present at the centrioles of centrosomes and basal bodies (e.g., Figs. 7 and 8). Might centrins in non-ciliary localizations also interact with heterotrimeric G-proteins? Interestingly there is growing evidence for functions of heterotrimeric G-proteins at centrioles present in centrosomes and spindle poles. In recent years G-protein signaling independent of membrane-integral G-protein-coupled receptors has been the focus of intensive research (for reviews, see Refs. 55–57). In embryos of Drosophila melanogaster and Caenorhabditis elegans subunits of heterotrimeric G-proteins act as effectors in the organization of asymmetric spindles (56, 57). The mitotic spindles are organized at spindle poles, which originate from centriole duplication in the G1 phase of mitosis. Recent “knock-down” studies with small interfering RNA revealed that in human cultured cells centrin 2 is required for centriole duplication (31). In photoreceptor cells, the centrin isoforms 2, 3, and 4 are also concentrated at the centrioles. The present immunoelectron microscopy revealed that heterotrimeric G-proteins co-localize with the three centrins at photoreceptor centrioles, and upon a local increase of free Ca2+ centrin-G-protein complexes most probably also assemble there. These findings further support the hypothesis that centrins might be the molecular linkers between G-protein signaling and the function of spindle poles, centrosomes, and basal bodies in organizing cellular processes.

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