Chapter 10

CENTRINS, A NOVEL GROUP OF CA^{2+}-BINDING PROTEINS IN VERTEBRATE PHOTORECEPTOR CELLS

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Uwe Wolfrum,[1] Andreas Gießl,[1] Alexander Pulvermüller[2]

ABSTRACT

Changes in the intracellular Ca^{2+}-concentration affect the visual signal transduction cascade directly or more often indirectly through Ca^{2+}-binding proteins. Here we review recent findings on centrins in photoreceptor cells of the mammalian retina. Centrins are members of a highly conserved subgroup of the EF-hand superfamily of Ca^{2+}-binding proteins commonly associated with centrosome-related structures. In vertebrate photoreceptor cells, centrins are also prominent components in the connecting cilium linking the light sensitive outer segment with the biosynthetically active inner segment compartment. Recent findings demonstrate that Ca^{2+}-activated centrin forms a complex with the visual G-protein transducin in photoreceptor cells. This Ca^{2+}-dependent assembly of G-proteins with centrin is a novel aspect of the supply of signaling proteins in sensory cells, and a potential link between molecular translocations and signal transduction in general.

INTRODUCTION

Vertebrate rod and cone photoreceptor cells are highly specialized, polarized neurons, which consist of morphologically and functionally distinct cellular compartments (see Fig. 1E, 5D). The light sensitive photoreceptor outer segment is linked with an inner segment via a modified, non-motile cilium, the so-called connecting cilium. The inner segment contains the organelles typical for the metabolism of a

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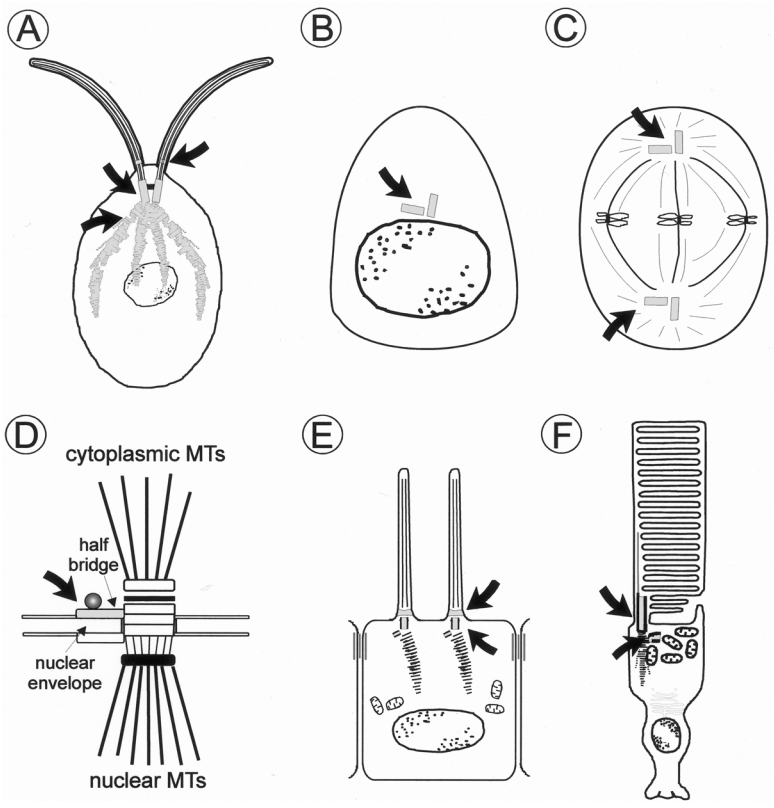

Figure 1. Localization of centrin in diverse cell types. Schematic diagrams of A unicellular green algae (e.g., *Chlamydomonas reinhardtii*); B animal cell in G_1 or G_0 phase (e.g., retinal non-photoreceptor cells, cells of the retinal pigment epithelium) and C in metaphase; D spindle pole body of the yeast *Saccharomyces cerevisiae*, MTs = microtubules; E ciliated epidermal cell; F vertebrate photoreceptor cell. Centrin cellular localization is coloured and indicated by arrows. In the yeast, cdc31p (yeast centrin) is associated with the half bridge of the spindle pole body which accts as the major microtubule organizing centre (MTOC). Centrin is also commonly found at the MTOC, the centrosome of animal cells and at the centrosome-related basal bodies of ciliated cells. In cilia, centrin is also a component of the transition zone which links the basal body region with the axoneme.

eukaryotic cell and continues into the perikaryon and the synaptic region where the electrical signal generated in the photoreceptor cell is transmitted to secondary neurons of the neuronal retina. The outer segment contains all components of the visual transduction cascade (see below) which are arranged disconnected from the plasma membrane bound to hundreds of stacked membrane discs. These membraneous discs are continually renewed throughout lifetime. Newly synthesized membrane is added at the base of the outer segment by the expansion of the plasma membrane[1] or by incorporation of vesicular structures into nascent disc membranes,[2] whereas discs at

the distal tip of the outer segment are phagocyted by the cells of the retinal pigment epithelium.[3]

At the outer segment disk membrane, photoexcitation of the visual pigment rhodopsin activates a heterotrimeric G protein cascade leading to cGMP hydrolysis in the cytoplasm and closing of cGMP-gated channels (CNG channels) in the plasma membrane.[4,5] By its rapid lateral diffusion in the membrane, a single molecule of activated rhodopsin (Rho*) can successively activate hundreds of copies of the tissue-specific G-protein (G_t, transducin, composed of an $G_t\alpha$-subunit bearing the guanine nucleotide binding site with GDP attached and an undissociable $\beta\gamma$-complex), thus amplifying the light signal. The activated, GTP-binding α-subunits holds the effector, a cGMP specific phosphodiesterase (PDE), in an enzymatically active form before GTP hydrolysis terminates the interaction and the active state of the PDE. As long as active PDE is present, it decreases the cGMP concentration resulting in closure of the CNG channels and a drop of the cationic current through the channels, which is mainly carried by Na^+ and Ca^{2+}. This hyperpolarizes the cell membrane thus providing the neuronal signal by decreasing transmitter release from the synaptic terminal.

The recovery phase of the phototransduction cascade and the adjustment to background light (light adaptation) of photoreceptor cells rely on changes in the intracellular Ca^{2+}-concentration, $[Ca^{2+}]_i$. It is well established that changes in $[Ca^{2+}]_i$ affects portions of the visual transduction cascade directly or more often indirectly through Ca^{2+}-binding proteins.[6] As a consequence of photoabsorption the efflux of Ca^{2+} (via a light-insensitive plasma membrane Na/Ca-K-exchanger, termed NCKX) exceeds the influx, resulting in $[Ca^{2+}]_i$ decrease, which in turn increases the sensitivity of the cGMP-gated channel to cGMP and accelerates the recovery of the dark current by the release of the Ca^{2+}-binding protein calmodulin (CaM) from the β-subunit of the CNG channel (chapter 18 of the present book).[7,8] Lowering of $[Ca^{2+}]_i$ also stimulates the production of cGMP through activation of a photoreceptor-specific particulate guanylate cyclase (GC).[9] The feedback is mediated by one or more Ca^{2+}-binding proteins, termed guanylate cyclase-activating proteins (GCAPs) or GCAP-like proteins (GLPs) described in detail in other chapters of the present book.[6] Besides this well-established role of Ca^{2+} in restoring the dark level of cGMP, yet another mechanism is discussed in the literature, which is thought to act at the level of the activated receptor. It is mediated by another Ca^{2+}-binding protein, recoverin, and affects the phosphorylation of rhodopsin by rhodopsin kinase and thus the quench of light-activated rhodopsin.[10] Furthermore, other Ca^{2+}-binding proteins may also regulate the light insensitive NCKX-exchanger.[6]

The Ca^{2+}-binding proteins involved in the regulation of phototransduction described above are all members of the large EF-hand superfamily of Ca^{2+}-binding proteins which includes besides calmodulin, parvalbumin, troponin C and S100 Ca^{2+}-binding proteins, but also the highly conserved proteins of the centrin subgroup.[11,12] We have recently also identified members of the centrin subgroup as structural proteins in vertebrate retinas.[13-15] The prominent localization of centrin in cytoskeleton of the connecting cilium of vertebrate photoreceptor cells indicated a

role in the intracellular transport between the inner segment and the outer segment of the photoreceptor cell. In view of the importance of Ca^{2+}-binding proteins in the regulation of photoreceptor function, centrin´s strategic localization and the small knowledge on centrins in the field will present the most recent information on the centrin subgroup of Ca^{2+}-binding proteins. Beside examining the role of centrins in photoreceptor function, we will also provide new insights linkages between the signal transduction cascade with the cytoskeleton.

WHAT ARE CENTRINS?

Centrins, also termed "caltractins", are highly conserved low molecular weight proteins of a subfamily of EF-hand Ca^{2+}-binding proteins.[11,12] The first centrin was discovered as the major component of striated flagellar rootlets associated with the basal bodies of unicellular green algae where it participates in Ca^{2+}-dependent and ATP-independent rootlet contractions.[16] Centrins have since been found to be ubiquitously associated with centrioles of basal bodies and centrosomes, and mitotic spindle poles in cells from diverse organisms, including yeast, ciliates, green algae, higher plants, invertebrates, and vertebrates (Fig. 1).[11,12]

CENTRIN GENES AND MOLECULAR STRUCTURE OF CENTRIN PROTEINS

Cloning efforts in recent years have resulted in the identification of centrin genes in a variety of species from all kingdoms of eukaryotic organisms, protists, fungi, plants, and animals.[17-27] Analyses of amino acid sequences deduced from the cDNA clones demonstrates that centrins are a highly conserved, yet distinct subfamily of the EF-hand superfamily of Ca^{2+}-binding proteins (Fig. 2). Centrins are acid proteins, about 170 amino acids in length, which is in good agreement with their apparent molecular mass of about 20 kDa.[11,12] To date, in lower eukaryotes like the yeast *Saccharomyces cerevisiae* or the unicellular green algae *Chlamydomonas reinhardtii* only one centrin gene (ScCDC31 and CrCEN, respectively) has been identified, whereas in the genome of vertebrates at least three centrin genes (e.g., HsCEN1, HsCEN2, and HsCEN3) are present.[17-19,21,22,26] Clustal analyses of deduced amino acid sequences of centrins from different organisms reveal several phylogenetic groups of centrins (Fig. 3). While some protist centrin species can not be grouped into homogeneous groups, most centrins of higher plants, green algae centrins, and all three known vertebrate centrin isoforms form a phylogenetic group. In vertebrates, Cen1p isofoms and Cen2p isoforms are very close related showing amino acid identities of about 80 percent to 90 percent, whereas sequences of the yeast centrin (ScCdc31p) related vertebrate Cen3p isoforms have only amino acid identities of about 55 percent to both other isoforms. Interestingly, in vertebrate species Cen1p and Cen2p isoforms are closer related to algal centrin (e.g., CrCenp) than to Cen3p isoform of the same species, strongly suggesting two divergent subfamilies.[26]

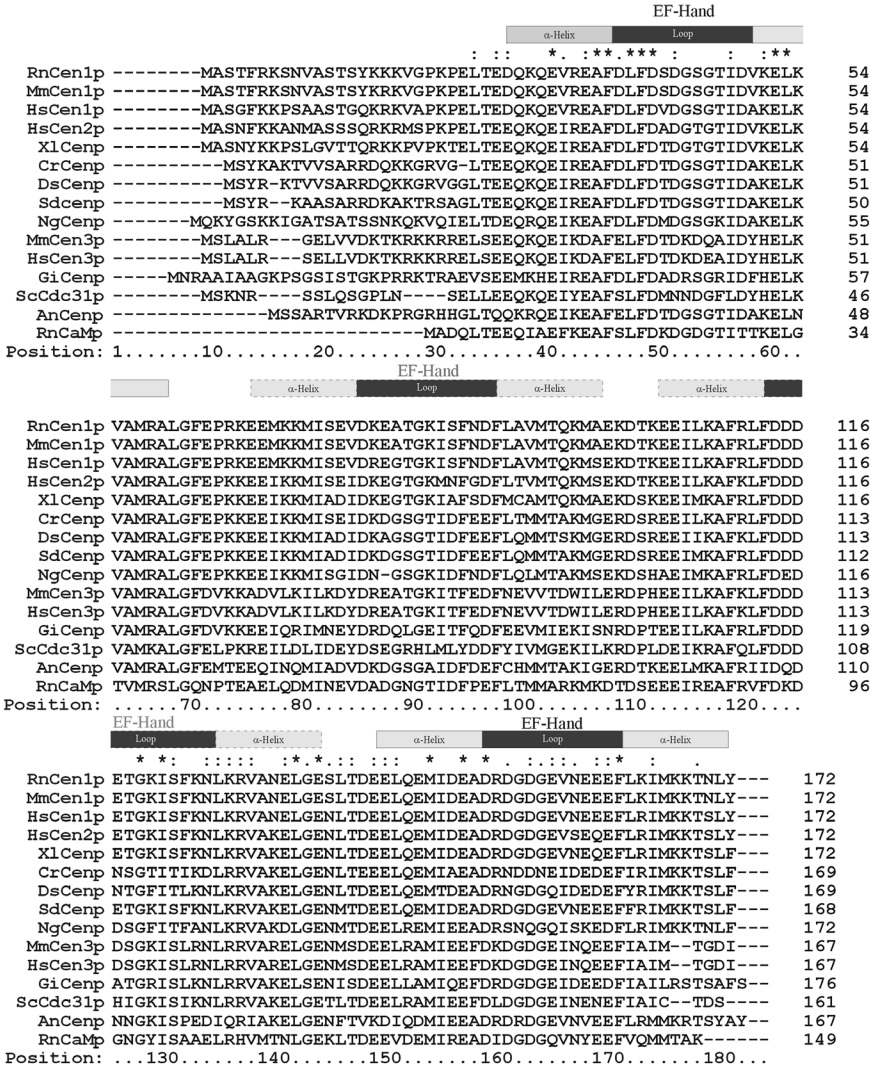

Figure 2. Protein alignment of centrin isoforms and calmodulin from diverse species. ClustalX alignment of 15 different amino acid sequences of centrin species and rat calmodulin. (RnCaMp = rat calmodulin Accession Number (AN): CAA32120; NgCenp = *Naegleria gruberi* centrin AN: AAA75032; DsCenp = *Dunaliella salina* centrin AN: AAB67855; HsCen1p, 2p, 3p = human centrins 1, 2, 3 AN: AAC27343, AAH13873, AAH05383; MmCen1p, 3p = mouse centrins 1, 3 AN: AAD46390, AAH02162; RnCen1p, = rat centrins AN AAK20385, AnCenp = *Atriplex nummularia* centrin AN: P41210; CrCenp = *Chlamydomonas reinhardtii* centrin AN: CAA41039; SdCenp = *Scherffelia dubia* centrin AN: CAA49153; ScCdc31p = *Saccharomyces cerevisiae* ("yeast centrin") AN: P06704; XlCenp = *Xenopus laevis* centrin AN: AAA79194; GiCenp = *Giardia intestinalis* centrin AN: AAB05594). EF-hand domains are indicated as a block above the sequence alignment. EF-hands are composed of an a-helix and a loop. Note, that the EF-hands 2 and 3 of most centrins appear most probably non-functional.

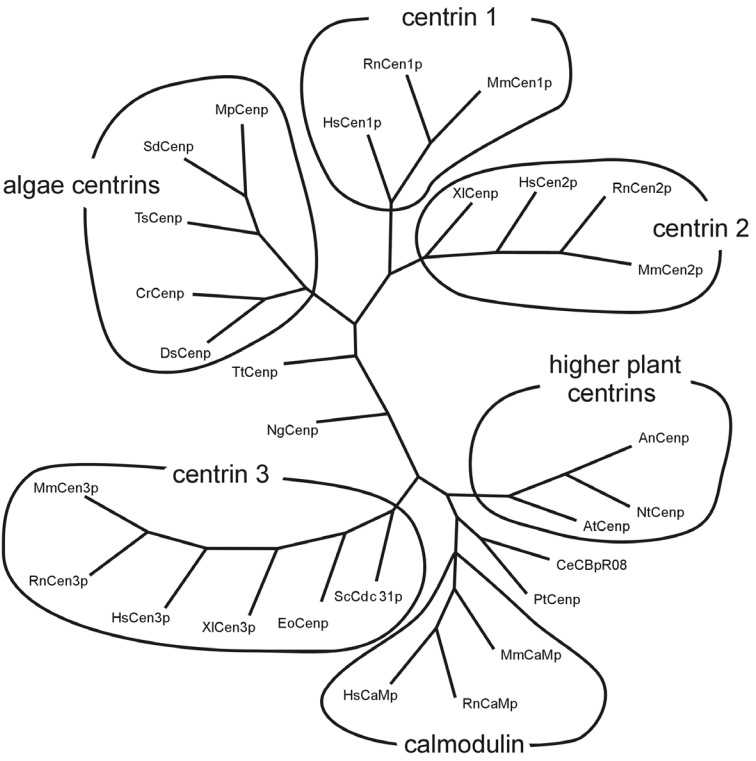

Figure 3. Comparison of centrin isoforms of diverse species. Comparison (using programs: Omiga 2.0, Genedoc 2.5.006 and phylip) of 28 different amino acid sequences of centrins and calmodulins. The phylogram shows a consensus tree which shows the highest frequency of each node of 1000 repetitions. Phylip divids the centrins into subgroups of centrin isoforms 1, 2, 3 , algae centrins, higher plant centrins and a group of calmodulin (RnCaMp = rat calmodulin Accsession Number (AN): CAA32120; MmCaMp = mouse calmodulin AN: NP_033920; HsCaMp = human calmodulin AN: BAA08302; NgCenp = *Naegleria gruberi* centrin AN: AAA75032; XlCenp = *Xenopus laevis* centrin AN: AAA79194; XlCenp3 = *Xenopus laevis* centrin 3 AN AAG30507; PtCenp = *Paramecium tetrauelia* centrin AN: AAB188752; DsCenp = *Dunaliella salina* centrin AN: AAB67855; HsCen1p, 2p, 3p = human centrins 1, 2, 3 AN: AAC27343, AAH13873, AAH05383; MmCen1p, 2p, 3p = mouse centrins 1, 2, 3 AN: AAD46390, AAD46391, AAH02162; RnCen1p, 2p, 3p = rat centrins AN AAK20385, AAK20386, AAK83217; AtCenp = *Arabidopsis thaliana* centrin AN: CAB16762, AnCenp = *Atriplex nummularia* centrin AN: P41210; NtCenp = *Nicotiana tabacum* centrin AN AAF07221; CrCenp = *Chlamydomonas reinhardtii* centrin AN CAA41039; SdCenp = *Scherffelia dubia* centrin AN CAA49153; MpCenp = *Micromonas pusilla* centrin AN CAA58718; EoCenp = *Euplotes octocarinatus* centrin AN CAB40791; TsCenp = *Tetraselmis striata* centrin AN P43646; ScCdc31p = *Saccharomyces cerevisiae* AN P06704; CeCBpR08 = *Caenorhabditis elegans* AN P30644; TtCenp = *Tetrahymena thermophila* AN AAF66602. Tree is not complete.

As members of the parvalbumin superfamily of Ca^{2+}-binding proteins, centrins contain four helix-loop-helix EF-hands consensus domains which may each bind one Ca^{2+}.[28-31] Protein sequence comparisons between different centrin species

reveal that the EF-hand consensus motifs are the most highly conserved domains (Fig. 2). Further phylogenetic analyses indicate that the EF-hand domains arose from two-fold duplication of an ancestral EF-hand motif.[32] However, during molecular phylogenesis EF-hand motifs in centrins lost their ability to bind Ca^{2+}. Binding studies indicate that in the green algae *Chlamydomonas* CrCenp and *Tetraselmis* TsCenp all four EF-hands bind a Ca^{2+}, two EF-hands bind Ca^{2+} with high affinity and two EF-hands bind Ca^{2+} with low affinity,[33,34] whereas other green algae possess two or three functional EF-hands.[27] Sequence analysis of vertebrate centrin isoforms suggests that Cen1p and Cen2p molecules bind two Ca^{2+} with their first and the fourth EF-hand and in Cen3p the fourth is the last remaining functional EF-hand motif as it is the case in the yeast centrin ScCdc31p.[11,26,27,35] There are several lines of evidence that Ca^{2+}-binding to centrins induces drastic conformation changes in centrin molecules [11,12,36,37] as previously demonstrated for the related EF-hand protein calmodulin.[38-40] In contrast to calmodulin, centrin molecules become more compact upon Ca^{2+}-binding and Ca^{2+}-activated centrins form dimers and oligomers.[36,37] In polymerization assays, Ca^{2+}-binding induces even centrin polymers, not only with green algae centrins but also with mammalian centrin 1,[36] which may be the structural basis for contractile centrin-fiber systems (see above). Furthermore, Ca^{2+}-binding to centrins increases the affinity of centrin-binding proteins to centrins [36,37,41] which we recently also demonstrated in mammalian photoreceptor cells (Gießl et al, in preparation).[35] To understand Ca^{2+}-induced conformation changes in centrins and binding characteristics of target proteins of centrins, data from high resolution structural analysis are required.

The amino-terminal subdomain of centrins is unique for small Ca^{2+}-binding proteins, unlike those found in, e.g., calmodulin or GCAPs. It is also the most distinctive and variable region of centrins and it has been suggested to be responsible for some functional diversity among centrin species.[11,32,36] Studies on the polymerization properties of centrins indicate that the Ca^{2+}-induced polymerization of centrins, e.g., the formation of contractile centrin-fibers in green algae, is mainly dependent on the amino-terminal domain.[36] In the green algae, it has been demonstrated that centrin phosphorylation correlates with centrin-fiber elongation (relaxation).[16,42] Although conserved potential sites for phosphorylation by protein kinase A (PKA) and $p34^{cdc2}$ kinase are located in the amino-terminal of centrins,[11] direct evidence for in vivo phosphorylation at the amino-terminus of centrins is missing. However, aberrant centrin phosphorylation has been shown under pathogenic conditions in human breast cancer cells that have amplified centrosomes containing supernumerary centrioles.[43] Furthermore, recent studies by Lutz and coworkers[44] indicate that vertebrate centrins are phosphorylated by PKA at conserved PKA consensus sequences present in the carboxy-terminal of centrin molecules. These results suggest that centrin phosphorylation in centrioles signals the separation of centrosomes during the prophase of the cell cycle.

CENTRIN´S CELLULAR LOCALIZATION AND FUNCTION

Centrin was first described as the major component of the massive striated flagellar rootlets of the Prasinophocean unicellular green alga *Tetraselmis striata*.[16] Centrin containing striated rootlets are commonly found in unicellular green algae.[45] They originate near the centrioles of the the basal body apparatus, project into the cytoplasm of the cell body and extend to the plasma membrane, the nucleus or other organelles. In the algal model system *Chlamydomonas*, descending centrin-based fibers connect the basal body apparatus with the nucleus (Fig. 1A).[46,47] In addition to these descending fibers, in *Chlamydomonas* at least two other fiber systems contain centrin: the distal fibers which connect both adjacent basal bodies to one another[48] and the stellate fibers in the transition zone in the plane between the basal body an the axoneme of the flagella.[49] In green algae, all of these centrin fibers have in common that they contract in response on an increase of the intracellular Ca^{2+}-concentration, $[Ca^{2+}]_i$. Most interestingly Ca^{2+}-triggered contraction of centrin fibers of the transition zone may induce microtubule severing and thereby the excision of the flagellum.[49,50] Present microtubule severing mediated by Ca^{2+}-activated centrin may be a more wide spread phenomenon proceeding the massive reorganization of the microtubule cytoskeleton during cell migration[51] or contributing to the microtubule released from the centrosome, the major microtubule organizing center (MTOC) of higher eukaryotic cells.[52]

Major contributions to evaluate the function of centrins in the cell cycle are provided by intensive studies on yeast centrin.[12,36,41,53,54] In baker's yeast *S. cerevesiae*, centrin encoded by the CDC31 gene functions in the duplication of the spindle pole body, the structural equivalent of the centrosome in higher eukaryotic cells. During the first steps of the yeast spindle pole body duplication the binding of Cdc31p to Kar1p is required. Furthermore, Cdc31p specifically interacts with other yeast proteins including an essential kinase (Kic1p) which activity probably regulates the spindle pole body duplication.[53,55]

In vertebrates, centrin proteins are ubiquitously expressed commonly associated with centrosome-related structures such as spindle poles of dividing cells or centrioles in centrosomes and basal bodies.[11,12] As discussed above, in mammals at least three centrin genes are expressed which may cluster to two divergent subfamilies.[26] As a consequence of the isoform diversity the three mammalian centrin isoforms may also exhibit differences in their subcellular localization as well as in their cellular function. Unfortunately, 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 the centrin in mammalian cells and tissues have been performed with polyclonal and monoclonal antibodies raised against green algae centrins which do not discriminate between the centrin isforms. Using these antibodies, centrins were detected in the centrioles of centrosomes and in the pericentriolar matrix.[56-58] Further immunological experiments show that antibodies to yeast Ccd31p or mammalian Cen3p react exclusively with Cen3p[26,59] whereas, to our knowledge, to date all of the antibodies raised against the close related mammalian

Cen1p or Cen2p isoforms react with both isoforms[59] (Gießl et al in preparation). Nevertheless, recent studies by immunoelectron microscopy demonstrate that Cen1p/Cen2p and Cen3p are localized in the central lumen of the centrioles of centrosomes and basal bodies.[59,60] In human ciliated tracheal cells, immunoelectron microscopy reveals that the isoform Cen3p is exclusively a core component of the basal body centriole, antibodies to Cen1p/Cen2p additionally decorate epitopes in transition zone of motile cilia.[59] Furthermore, comparative RT-PCR experiments (combined reverse transcriptase reaction and polymerase chain reaction) using isoform specific primers demonstrate that CEN2 is ubiquitously expressed, whereas CEN1 expression is restricted to ciliated cells.[15,59] Thus, it is likely that Cen1p functions as a centrin isoform in compartments of cilia and flagella. Functional analyses indicate that ciliary centrins are involved in the beating of cilia which is controlled by the intraciliary Ca^{2+}-concentration.[59]

The prominent localization of centrins at the centrosomes and basal bodies gave the rise for several hypothesis of the function of centrins. In interphase cells or in arrested cells of differentiated tissue, the centrosome functions as the major microtublule organizing center determining the number and polarity of cytoplasmic microtubules. Polymerization of novel microtubules at the centrosome is preceded by the de novo nuclation of microtubules in the pericentriolar matrix that surrounds and connects the centriole pair of an individual centrosome. It has been suggested that centrins are involved in the microtubule severing which should occur to release de novo synthesized microtubules from the pericentriolar origin.[52] However, more liable evidence was gathered that centrins may play important, but probably distinct roles at the centrosome during the cell cycle. Once in the cell cycle, the centrosome is duplicated to give rise to two spindle poles that organize the microtubules array of the mitotic spindle. While Cen3p, as its yeast relative Cdc31p, participates in centrosome reproduction and duplication,[61] Cen1p/Cen2p may play a role in centriole separation preceding centrosome duplication.[44]

CENTRINS IN THE VERTEBRATE RETINA

RT-PCR studies with centrin isoform specific primers reveal that all three centrin isoforms are expressed in the mammalian retina, which has been confirmed by Western blot analysis using antibodies specific for Cen3p and Cen1p/Cen2p, respectively[15] (A. Gießl, A. Schmitt, and U. Wolfrum, unpublished results). Further studies showed that centrins are expressed in the retina of species distributed throughout the subphylum of vertebrates (Fig. 4). Thus, centrins are probably ancient cytoskeletal proteins in the vertebrate retina indicating this conserved basic function in retinal cells.

Immunocytochemical studies demonstrate that centrins are concentrated in the cells of the vertebrate retina in two basically distinct structural domains (Fig. 5). As in other cell types of animals, centrins are components of the centrioles of centrosomes and basal bodies in the retinal neurons contributing to centrosome function discussed above. However, in all of our studies on numerous different vertebrate

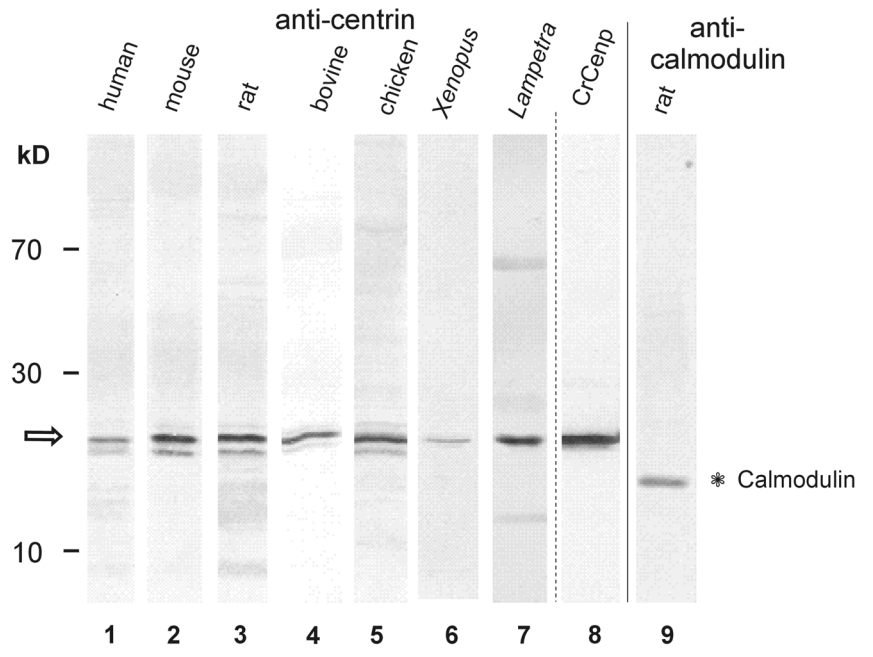

Figure 4. Western blot analysis reveals centrin expression in retina of various vertebrate species. Lane 1—Lane 8: Anti-centrin (mAb clone 20H5) Western blots. Lane 9: Anti- calmodulin Western blot of rat retina. Lane 1: human retina. Lane 2: mouse retina. Lane 3: rat retina. lane 4: bovine retina. Lane 5: chicken retina. Lane 6: *Xenopus* retina. Lane 7: *Lampetra* retina. Lane 8: Bacterially expressed *Chlamydomonas* centrin. Anti-centrin antibodies detect bands at about the predicted molecular weight of 20 kDa (arrow) and do not crossreact with the calmodulin migrating at 17 kDa. Note: in some lanes (e.g., lane 5) several bands around 20 kDa are anti-centrin positive. These bands do neither represent different centrin isoforms nor different Ca^{2+}-binding status of centrin. The higher bands most probably resemble phosphorylated centrn[43,44] and some lower bands may result from proteolytic digestion.

species, indirect anti-centrin immunofluorescene was most prominent in photoreceptor cell layer (Fig. 5).[13-15]

CENTRIN FUNCTIONS AS A CYTOSKELETAL COMPONENT THE CONNECTING CILIUM OF THE PHOTORECEPTOR CELL

Higher magnification of anti-Cen1p/Cen2p stained cryosections through vertebrate retinas shows that centrins are localized at the photoreceptor layer at the joint between the photoreceptor inner segment and outer segment (Fig. 5). Analysis of immunolabled isolated photoreceptor fragments reveals that centrins are not only present in the basal body, but also localized along the entire longitudinal extension

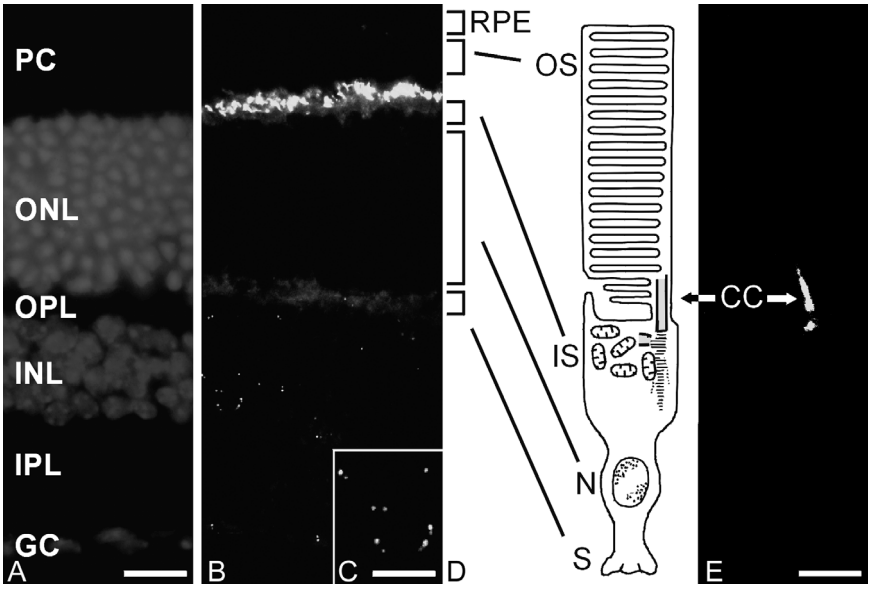

Figure 5. Localization of centrin in the mammalian retina and in photoreceptor fragments by indirect immunofluorescence. A DAPI-staining of a longitudinal cryosection through the rat retina. Staining of nuclei DNA demonstrates the retinal layers: PC = layer of outer and inner segments of photoreceptor cells; ONL = outer nuclear layer where nuclei of photoreceptors are localized; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = Inner plexiform layer; GC = ganglian cell layer. B Indirect anti-centrin immunofluorescence in the cryosection through rat retina. Anti-centrin antibodies predominantly react within the photoreceptor cell layer at the joint between the inner and out segment of the photoreceptors. In addition, indirect immunofluorescence is present in dot pairs in the inner nuclear layer and ganglion cell layer. C Higher magnification of immunofluorescent staining with antibodies against centrin in the inner nuclear layer of the section shown in figure B. Centrin is present in the centrioles of the centrosomes present in the perikaryon of retinal neurons. Note that as a rule one centriole of a single centrosome shows brighter anti-centrin immunofluorescence. D Schematic representation of a mammalian rod photoreceptor cell. The light sensitive outer segment (OS) is linked via the non-motile connecting cilium (CC) with the inner segment (IS) where the protein synthesis machinery is localized. N = nuclear region; S = synaptic region. Centrin localization is indicated by the green color of centrin in the PRC. E Indirect anti-centrin immunofluorescence of a photoreceptor fragment of the rat retina analysed by confocal laser scanning microscopy. RPE = rod pigment epithelium. The figure shows the a labeling of the connecting cilium and the basal body.
Bars in B = A: 20 μm; C: 7 μm, E: 2 μm.

of the connecting cilium (Fig. 5E).[14,15] Precise subcellular localization by immunoelectron microscopy and the quantification of silver-enhanced immunogold labeling show that centrin is localized in the subciliary domain of the inner face of the microtubule doublets of the connecting cilium of rod and cone photoreceptor cells (Fig. 6).[35] As in other ciliated cells, in photoreceptor cells the centrin decorated by immunolabeling in the connecting cilium most likely resembles the centrin 1 isoform.

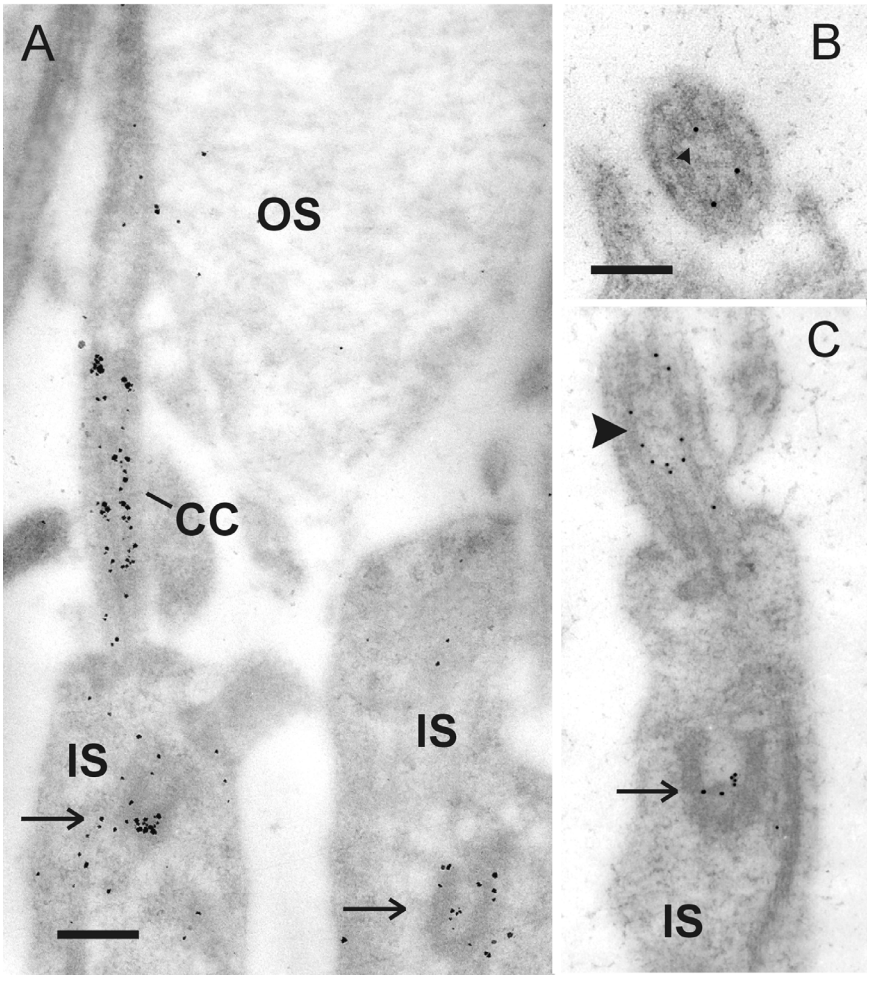

Figure 6. Immunoelectron microscopic localization of centrin in the connecting cilium of rod photoreceptor cells. A Silver-enhanced immunogold labeling of centrin in a longitudinal section of parts of rat rod photoreceptor cell. Centrin labeling is exclusively localized in the connecting cilium (CC) and the basal body complex (arrow) in the inner segment (IS) of photoreceptors. B Transversal section through the connecting cilium reveals that centrin is localized in the sub-ciliary domain of the ciliary lumen encircled by axonemal microtubule doublets. C Slightly tangential section through the apical part of rat rod photoreceptor cell inner segment. Centrin antibodies react in the connecting cilium at the inner surface of the axonemal microtubule doublets (arrowhead). The arrow indicates basal body labeling.
Bars: A: 265 nm, B, C: 175 nm

The modified connecting cilium of vertebrate photoreceptor cells is the structurally equivalent of an extended transition zone present at the base of a common motile cilium.[62] Therefore, the presence of centrin (most probably centrin 1) along

the entire extension of the connecting cilium of photoreceptor cells is in agreement with the centrin localization in the transition zone of motile cilia or the sensory cilia of mammalian olfactory cells.[59] In photoreceptor cells, the connecting cilium links the morphological and functional distinct cellular compartments of the light sensitive outer segment with the biosynthetically active inner segment. The connecting cilium serves as an active barrier for membrane components and soluble proteins regulating free diffusion between the inner and the outer segment of photoreceptor cells.[62,63] Since it is also the only intracellular bridge between both segments, intracellular exchanges between the inner segment and the outer segment are forced to occur through the slender connecting cilium.[62] Recently, we and others have shown that the visual pigment opsin is translocated to its final destination at the base of the photoreceptor outer segment along the membrane of the connecting cilium.[64-66] Actin filament-based and microtubule-associated transport processes seem to be involved in the unidirectional ciliary transport of opsin: The membrane associated molecular motor protein myosin VIIa has been shown to participate in ciliary transport of rhodopsin.[64-66] Marszalek and co-workers[67] gathered indications by a genetic approach that the microtubule-based heterotrimeric kinesin II-motor might be additionally involved in ciliary transport of rhodopsin but also of arrestin. However, cytoskeletal molecules associated with other proteins of the visual transduction cascade and which, therefore, are probably involved in the ciliary translocation of these proteins, have not yet been identified. The prominent localization of centrin in the connecting cilium of photoreceptor cells obviously indicates a specific role of centrin in the function of the photoreceptor cilium. Besides its possible role in ciliary transport, an involvement of centrin in retinomotor movement and in the photoreceptor outer segment alignment or orientation has been discussed.[14] If any of these processes are based on the centrin system of the cilium they should be dependent on and regulated by changes of the intracellular Ca^{2+}-concentration. Our recent results, as discussed below provide striking evidence for Ca^{2+}-dependent interaction between centrin 1 and the visual G-protein transducin on its pathway through the inner lumen of connecting cilium of mammalian photoreceptor cells.[35]

CENTRIN-INTERACTING PROTEINS IN MAMMALIAN PHOTORECEPTOR CELLS

In the context of the cell, protein function and its regulation is determined by the binding proteins to the target protein. Unfortunately, little is know about centrin-binding proteins in mammalian photoreceptor cells. To evaluate centrin functions in vertebrates, in other experimental systems, different strategies for the identification of centrin-associated or centrin-interacting proteins were applied. Analysis of proteins in co-immunoprecipitations performed with antibodies against algae centrin has revealed centrin interaction with the heat shock proteins HSP70 and HSP90 in cytoplasm of arrested *Xenopus* oocytes.[68] The centrin/HSP-complex may sequestrate centrin in a non-active form until Ca^{2+}-activation of the oocyte causes the dissociation of the complex making centrin available for subsequent centrosome

assembly. In yeast 2-hybrid screens the laminin-binding protein (LBP) of the basal lamina and the cytoplasmic receptor protein tyrosine kinase k have been identified as proteins interacting with HsCen2p, the ubiquitously expressed centrin isoform.[69] Although, there is no specific experimental evidence, none of the proteins identified as centrin-binding proteins has an obvious function in the connecting cilium of the photoreceptor cells. Nevertheless, Western blot overlay assays of retinal proteins with recombinant expressed MmCen1p indicate the presence of several centrin 1-binding proteins in the mammalian retina (Fig. 7). However, only centrin 1 in its Ca^{2+}-activated form interacts with the several polypeptides. A Ca^{2+}-dependent increase of the affinity of centrin to target proteins is known from binding of the yeast centrin Cdc31p to Kar1p[12] which has been confirmed in in vitro binding studies of diverse recombinant expressed centrin species to the yeast target protein.[36,41] Further analysis of the proteins identified by the MmCen1p overlay assay are currently performed. However, the centrin 1-binding protein p37 has been already identified as the β-subunit of the visual G-protein transducin (G_t) (Fig. 8,9B).

CENTRIN/TRANSDUCIN COMPLEX

Recently, evidence was provided that MmCen1p interacts with the visual G-protein transducin with high affinity, and thereby form functional protein-protein complexes in photoreceptor cells in a Ca^{2+}-dependent manner.[35] Transducin is the tissue-specific G-protein of the visual signal transduction cascade of the photoreceptor cells in the vertebrate retina (see also introduction). Upon light-activation, rhodopsin (Rho*) activates hundreds of G-protein molecules and the light signal is amplified. This receptor-G-protein interaction requires the intact G_t holoprotein, composed of an α-subunit bearing the guanine nucleotide binding site with GDP bound and an undissociable βγ-complex, and initiates the intermolecular transduction of the light signal by catalyzing the exchange of GDP for GTP in the α-subunit of the G-protein. Activated, GTP-binding α-subunits are free to couple to the effector, a cGMP specific phosphodiesterase (PDE).

In vertebrate photoreceptor cells the subcellular localization of transducin is modulated by light: in the dark G_t is highly concentrated in outer segments while in light, the majority of G_t is translocated and abundantly localized in the inner segment and the cell body of photoreceptor cells (Fig. 8).[35,70,71] Light-induced exchanges and movements of the cytoplasmic components between the photoreceptor segments have to occur through the connecting cilium, since the slender cilium serves as the only intracellular linkage between both photoreceptor compartments. As described above, centrin 1 is a prominent component of the cytoskeleton of the non-motile motified cilium and immunufluorscence double labelings of tranducin and centrin indicate that transducin and centrin 1 co-localize in the connecting cilium (Fig. 8C). Further immunoelectron microscopical analysis and the quantification of silver enhanced immunogold decorations reveal that centrin and transducin are not only exist parallel in the cilium, but share also the same subciliary domain, the inner ciliary lumen of the connecting cilium.[35] Their spatial co-distribution indicate that both

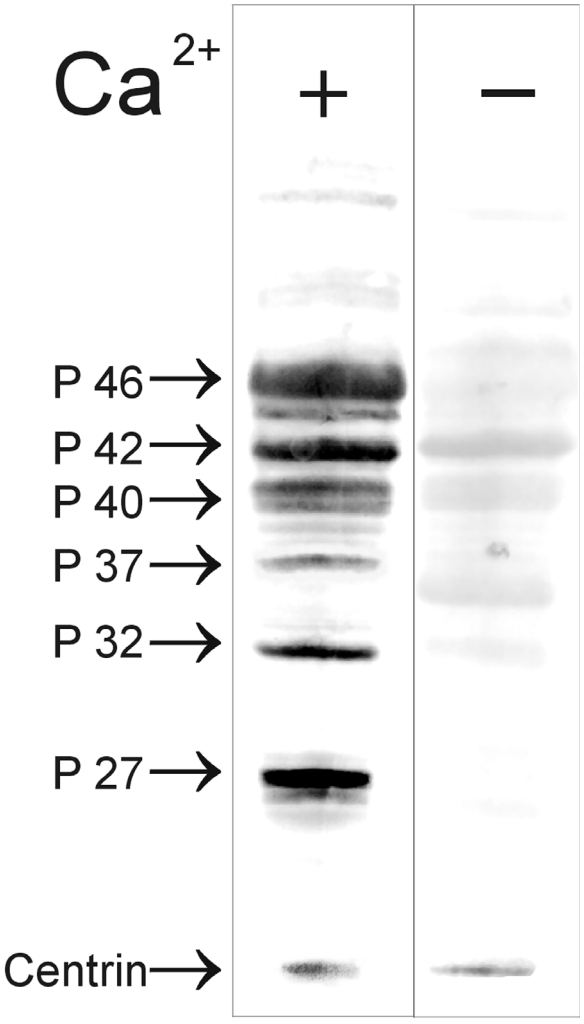

Figure 7. Analysis of centrin blot overlays with bovine retinal proteins. Western blot of retinal proteins overlay with recombinant expressed MmCen1p (67 µg/ml). Bound centrin was detected in a 2nd step by immunolabeling with anti-centrin antibodies (mAb clone 20H5). Lane 1: Centrin overlay assay in the presence of Ca^{2+} (1 µM $CaCl_2$). Lane 2: Centrin overlay assay in the absence of Ca^{2+} (6 mM EGTA). MmCen1p interaction with retinal proteins is dependent on the presence of Ca^{2+}. In the absence of Ca^{2+} MmCen1p-binding was dramatically reduced. Centrin interacting proteins are named according to their molecular weight (P 27, P 32, P 37, P 40, P 42, P46).

proteins may physically interact during the exchange of transducin between the photoreceptor segments through the cilium.

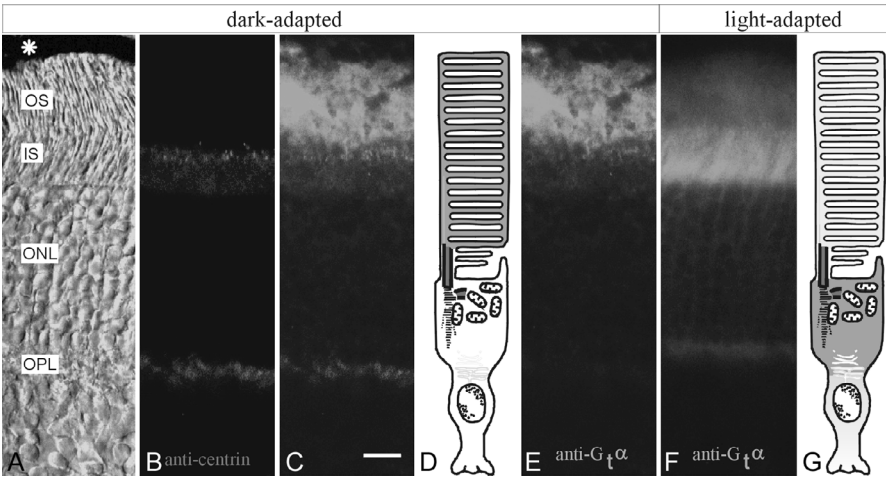

Figure 8. Light-dependent translocations of transducin in the mammalian retina. A-E dark-adapted mouse retina. F, G light adapted retina. A DIC-image of a cryosection through mouse retina. Asterisk indicates retinal pigment epithelium, OS: photoreceptor outer segment; IS: photoreceptor inner segment, ONL: outer nuclear layer, OPL: outer plexiform layer. B anti-centrin immunofluorescence (Alexa,546) is concentrated in the connecting cilium between IS and OS of photoreceptor cells. C Merged images of B and E suggest partial co-localization of $G_t\alpha$ and centrin in the joint between both photoreceptor segments. D Schematic representation of a dark- adapted rod photoreceptor cell. Green colour indicates $G_t\alpha$ distribution. E Indirect anti-$G_t\alpha$ immunofluorescence in the double labeled cryosection through the dark-adapted mouse retina shown in A-C. F Indirect anti-$G_t\alpha$ immunofluorescence in the section through the light-adapted mouse retina. G Schematic representation of a light-adapted rod photoreceptor cell. Green colour indicates $G_t\alpha$ distribution. In dark adapted photoreceptor cells, $G_t\alpha$ is predominantly localized in the OS where as in the light-adapted condition $G_t\alpha$ is most prominent stained in the IS of photoreceptor cells.
Bar: 10 μm

Recently, we have gathered striking evidence that centrin 1 and transducin indeed interact with high affinity.[35] In vitro assays including co-immunoprecipitation, overlay and co-sedimentation assays as well as size exclusion chromatography and kinetic light scattering experiments independently demonstrate that centrin 1 binds with high affinity to transducin (Fig. 9).[35] Our studies also show that the protein-protein interaction centrin 1 and transducin is highly specific: centrin 1 specifically interacts with transducin and does not bind to other components of the visual signal transduction cascade (e.g., arrestin, rhodopsin, rhodopsin kinase, PDE). The centrin relatives recoverin and calmodulin do not show significant affinities to transducin. The analyses of MmCen1p overlay assays with antibodies specific to transducin subunites and size exclusion chromatographies further demonstrate that assembly of centrin 1/G-protein complex is mediated by the βγ-complex (see also Fig. 9B, D-G). Our data also reveal that the assembly of the centrin 1/G-protein protein complex is strictly dependent on the Ca^{2+}-concentration and that at least two Ca^{2+}-ions are required for the activation of centrin 1 necessary for the formation of

centrin 1/G-protein complex. Moreover, further analysis indicates that activated centrin 1 binds as a homooligomer to the βγ-complex of transducin.[35]

What is the role of the centrin 1/G-protein complex in the photorecptor cell? Current working hypotheses of the centrin 1/G-protein complex function in the photorecptor cell are summarized in the cartoon in Figure 10. The spatial co-localization of centrin 1 and transducin in the lumen of the connecting cilium emphasizes that in photoreceptor cells, the formation of centrin 1/G-protein complex should occur in this ciliary compartment. An increase of the intracellular Ca^{2+}-concentration in the photoreceptor cell should cause the activation of centrin 1 in the connecting cilium and in turn induce the binding of centrin 1 oligomers to transducin passing through the ciliary. As a consequence of the assembly of centrin/transducin complexes the movement of transducin should be effected. In photoreceptors, light modulated changes of free Ca^{2+} in the outer segment which include the well-studied the Ca^{2+}-drop within the operating (single quantum detective) range of the rod[10] and recent observations of Ca^{2+} increase in bright light (rod saturated conditions)[72] should also effect the free Ca^{2+} in the connecting cilium. In the cilium the assembly of centrin 1/G-protein complexes may contribute to a Ca^{2+}-induced barrier for further exchange of transducin between the photoreceptor inner and outer segment (barrier hypothesis Fig. 10B). A drop of Ca^{2+} should induce the disassembly of the complex, thus providing a necessary condition for the light-modulated exchange of transducin between the inner and the outer segment of photoreceptor cells described above.[35,70,71] However, Ca^{2+} triggered sequential binding of transducin to centrin 1 may although contribute to the transport of transducin though the photoreceptor connecting cilium (Ca^{2+}-gradient hypothesis Fig. 10C). The Ca^{2+}-dependent assembly of a G-protein with centrin is a novel aspect of the supply of signaling proteins in sensory cells, and a potential link between molecular translocations and signal transduction in general.

CONCLUSION

Centrins are members of a conserved subfamily of EF-hand Ca^{2+}-binding proteins. During the past years, 3 centrin isoforms have been found to be ubiquitously associated with the centrioles of centrosomes or centrosome related structures in diverse vertebrate cells. Our work on centrins in photoreceptor cells has revealed that centrins are prominent components of the ciliary apparatus of photoreceptor cells. Although several lines of evidence indicate defined spatial distributions of the known centrin isoforms, the differential localization of centrin isoforms by using isoform specific antibodies or by the transfection of the retina with tagged-centrin constructs should provide more liable information on the specific localization and function of the centrin isoforms in photoreceptor cells. Our recent findings reveal that the centrin isoform centrin 1 binds with high affinity to transducin in a strict Ca^{2+}-dependent manner. Additional experimental efforts are necessary to resolve the question whether transducin binding is restricted to centrin 1. If so, what are the functions of the other centrin isoforms in photoreceptor cells? The results of the

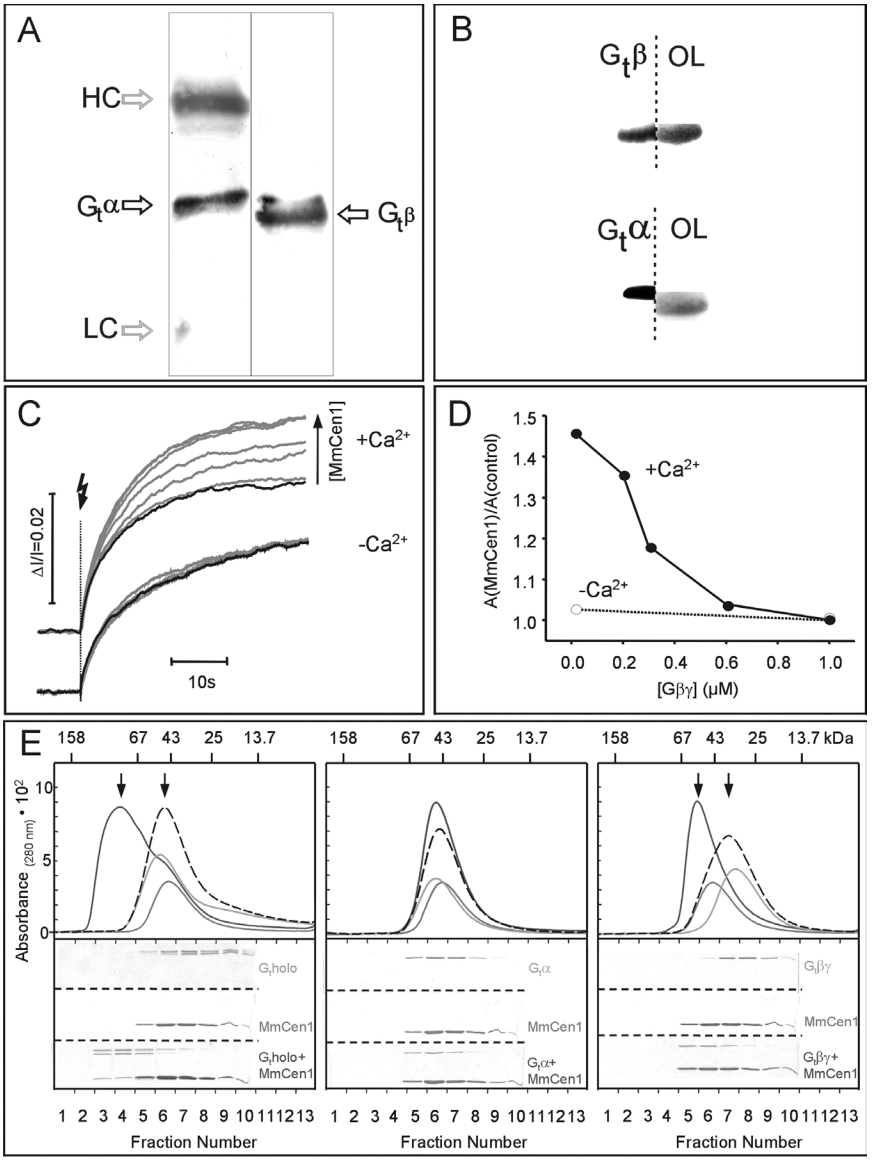

Figure 9. Calcium dependent assembly of a centrin1/G-protein complex. A) Co-Immunoprecipitation of transducin with centrin from lysed retinal photoreceptor cell fragments.
Lanes 1: Western blot analysis with mAb anti-G_ta of an immunoprecipitation with mAb anti-centrin (clone 20H5) from photoreceptor cell fragments of bovine retina. (Upper and lower bands in lane 1 correspond to the heavy (HC) and light chains (LC) of mouse antibodies.) Lane 2: Western blot analysis with polyclonal anti-$G_t\beta$ of anti-centrin of an immunoprecipitation with mAb anti-centrin (clone 20H5) from photoreceptor cell fragments of bovine retina. $G_t\alpha$ and $G_t\beta$ co-immunoprecipitate with centrin. The upper and lower bands in lane 1 correspond to the heavy (HC) and light chains (LC) of the mouse antibodies.

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B)Combined Western blot-overlay analysis identifies retinal centrin-interacting protein P37 as $G_t\beta$ subunit of transducin.

For specific determination of the centrin binding protein Western blotted lanes were cut in half and parallel processed for immunolabeling with subunit specific antibodies against Gtβ (upper lane 1), and Gtα transducin (lower lane 1) and for overlays with recombinantly expressed MmCen1p (67 μg/ml) (OL). The 37 kDa centrin-binding protein (P37 in Fig. 7) is identified by centrin overlays had the exact mobility as the $G_t\beta$ subunit.

C) Calcium-dependent enhancement of kinetic light-scattering (KLS) G_t-binding signal in the presence of MmCen1p. Upper panel represent KLS binding signals (3 μM rhodopsin, 0.5 μM G_t) in the presence of calcium, and 0 (control, black curve), 0.6, 1.2, 2.5, 3.6, 5, 7.3, and 10 μM MmCen1p (gray curves), respectively. Lower panel represent KLS binding signals under the same conditions as in the upper panel, but with EGTA instead of calcium. Experimental conditions were 50 mM BTP, pH 7.5 containing 80 mM NaCl, 5 mM $MgCl_2$ and either 100 μM $CaCl_2$ or 1 mM EGTA at 20°C, sample volume of 300 μL, and cuvette path length of 1 cm; 32% rhodopsin was photolyzed per flash (500±20 nm).

D) Competition between $G_t\beta\gamma$-subunit and G_t for binding to MmCen1p. Calcium dependent inhibition of the MmCen1p enhanced amplitude of flash-induced KLS G_t-binding signals by the βγ-subunit of G_t. The KLS assay was carried out as described in (A). Experiments were performed with the bg-subunit of G_t. Data points represent the normalized amplitude of the MmCen1 dependent enhancement of the G_t-binding signal (AMmCen1) divided to the control G_t-binding signal without added MmCen1 (control). Filled and empty circles indicate the results obtained from experiments with calcium and with EGTA, respectively.

E) Calcium-dependent interaction of MmCen1p with G_t and its subunits analyzed by size-exclusion chromotography and SDS-PAGE.

Upper panels represent elution profiles of MmCen1p alone (L), G_t or its subunits alone (—-) and the mixture of MmCen1p with G_t or its subunits (æ) in the presence of calcium. The gray dotted lines are the calculated superpositions of the respective single component profiles (MmCen1p plus G_t or its subunits) yielding the predicted profiles for the mixture of the two non-interacting components. Arrows indicate the shift of the formed complexes. In the lower panel the SDS-PAGE analysis of the fractions of the size-exclusion chromatography is shown. Interaction of MmCen1p with the transducin holoprotein is shown in 1^{st} panel with the $G_t\alpha$-subunit in 2^{nd} panel and with the $G_t\beta\gamma$-subunit in 3^{rd} in the presence of calcium.

Experimental conditions: 10 μg of MmCen1p and 10 μg of $G_t$holo (or G_t subunits) were incubated in 50 mM BTP, pH 7.0 containing 80 mM NaCl, 1 mM $MgCl_2$ and 100 μM $CaCl_2$ for 5 min at room temperature, loaded on a Superose TM 12 column (using the Smart System, Pharmacia Biotech. Inc., flow rate, 40 μL/min) equilibrated with the same buffer, eluted by monitoring the absorbance at 280 nm and subsequent analyzed by SDS-PAGE. Note: G_t holoprotein elutes at an apparently lower MW, as compared to its subunits.[73]

current analysis of putative centrin-associated proteins (other than transducin) in the mammalian retina will most probably also provide further insights in the role of centrins in photoreceptor cell function. In the future, we will also address the question whether Ca^{2+}-activation of centrins is the only post-translational modification regulating the function(s) of centrin in photoreceptor cells. And finally, the clarification of the structure of centrin isoforms will also enlighten the molecular mechanisms of the diverse functions of centrins in photoreceptors.

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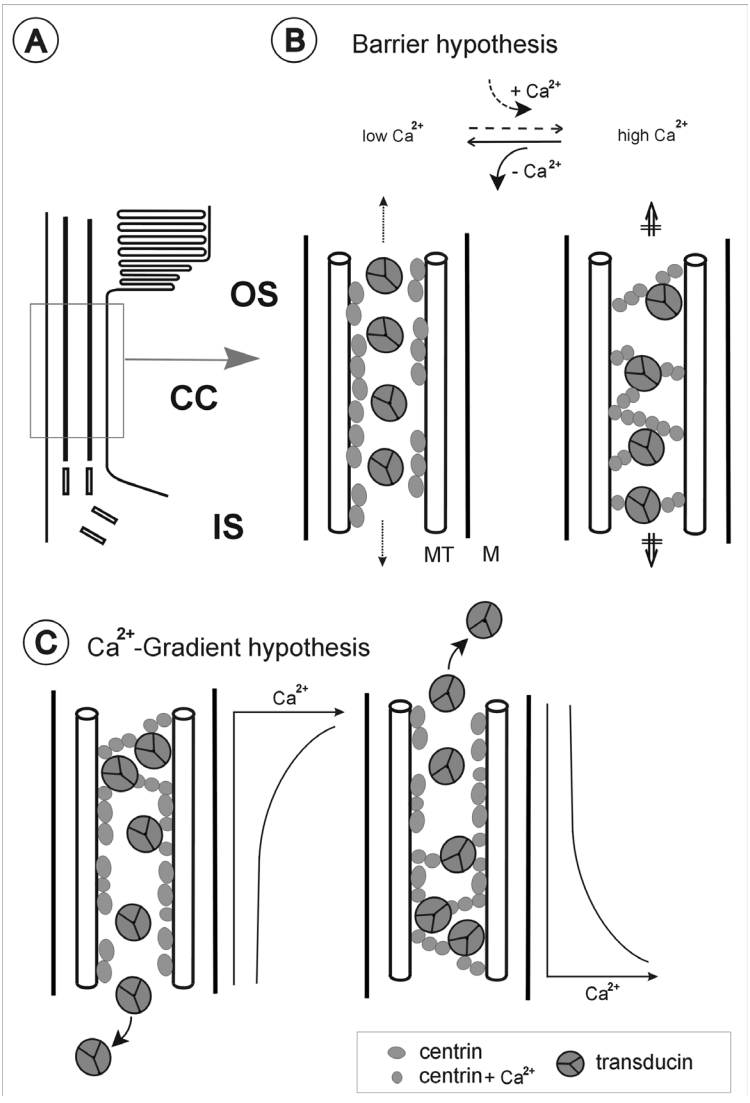

Figure 10. Models for Ca^{2+}-dependent centrin-transducin assembly in the connecting cilium of vertebrate photoreceptor cell. A) Schematic representation of a part of a rod photoreceptor cell shows the linkage between the outer segment (OS) and inner segment (IS) by the connecting cilium (CC). (B and C) Enlargement of CC indicated in Figure A. B) Barrier hypothesis: under low free Ca^{2+} centrin is not activated and transducin floats through the inner lumen of the connecting cilium. If free Ca^{2+} increases in the outer segment and, in the cilium, centrin is activated by Ca^{2+} which induces Ca^{2+}-centrin-transducin complex assembly and centrin fiber contraction. Thus transducin is trapped in the connecting cilium and a barrier between inner and outer segment raises. C) Ca^{2+}-gradient hypothesis: transducin may bind to centrin 1 dependent on free Ca^{2+} concentration actually present in the ciliary domains. A putative Ca^{2+}-gradient along the ciliary lumen may cause sequential assembly of the centrin 1-transducin complex and the release of transducin from the complex.

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