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9 Centrins, Potential Regulators of Transducin Translocation in Photoreceptor Cells

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Changes in the intracellular Ca^{2+}-concentration regulate the visual signal transduction cascade directly or more often indirectly through Ca^{2+}-binding proteins. In this review, we discuss our 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 photoreceptor cells, centrins are additionally prominent components in the connecting cilium linking the light sensitive outer segment compartment with the biosynthetically active inner segment. Our recent data indicate that Ca^{2+}-activated centrin isoforms generate complexes with the visual heterotrimeric G-protein transducin by binding to its β-subunit. These Ca^{2+}-dependent assemblies of centrin/G-protein complexes are novel aspects of translocation regulations of signaling proteins in sensory cells, and a potential link between molecular trafficking and signal transduction in general.

1. Introduction

Vertebrate photoreceptor cells are highly specialized, polarized neurons, which consist of morphologically and functionally distinct cellular compartments. The light sensitive photoreceptor outer segment is linked

with an inner segment via a modified, non-motile cilium, termed the connecting cilium. The inner segment contains the organelles typical for the metabolism of eukaryotic cells and continues into the perikaryon and the synaptic region. The outer segment contains all components of the visual transduction cascade, which are associated with the stacked membrane disks. Photoexcitation of the visual pigment rhodopsin activates a heterotrimeric G-protein cascade leading to cGMP hydrolysis in the cytoplasm and closure of cGMP-gated channels (CNG channels) localized in the plasma membrane (Heck and Hofmann, 1993; Okada *et al*., 2001). The closure of the CNG channels leads to a drop of the cationic current (carried by Na^+ and Ca^{2+}), resulting in the hyperpolarization of the cell membrane and a decrease in transmitter release from the synaptic terminal (Molday and Kaupp, 2000). The recovery phase of the visual transduction cascade and light adaptation of photoreceptor cells relies on changes in the intracellular Ca^{2+}-concentration, $[Ca^{2+}]_i$. It is well established that changes in $[Ca^{2+}]_i$ affect portions of the phototransduction cascade directly, or more often indirectly through Ca^{2+}-binding proteins (Palczewski *et al*., 2000).

The membranous outer segment disks are continually renewed throughout an animal's lifetime (Young, 1976). Newly synthesized disks are added at the base of the outer segment (Steinberg *et al*., 1980; Usukura and Obata, 1995) whereas disks at the distal tip of the outer segment are phagocytosed by cells of the retinal pigment epithelium (Young, 1976). This permanent turnover requires effective transport mechanisms of outer segment components from the inner segment — the compartment of synthesis — to the outer segment — the compartment of signal transduction (Sung and Tai, 2000). After delivery, some molecules of the outer segments, eg. the membrane proteins for ion transport and channels as well as the visual pigment rhodopsin, stay as permanent residents in the outer segment, whereas other molecules of the signal transduction cascade, eg. arrestin and transducin, exhibit massive light-dependent translocations between the outer segment and inner segment (Brann and Cohen, 1987; Philp *et al*., 1987; Whelan and McGinnis, 1988; Organisciak *et al*., 1991; Pulvermüller *et al*., 2002; Sokolov *et al*., 2002; Wolfrum *et al*., 2002; Peterson *et al*., 2003; Mendez *et al*., 2003). Nevertheless, all intracellular exchanges between these two functional compartments of

photoreceptor cells must occur through the slender connecting cilium. During recent years, an increasing number of proteins have been localized to the connecting cilium, some of which were suggested to play a role in ciliary transport (Schmitt and Wolfrum, 2001; Stohr *et al*., 2003). This list of molecules includes several microtubule and actin-associated molecular motors, which represent good candidates to participate in active molecular translocation through the connecting cilium [eg. myosin VIIa and kinesin II (Liu *et al*., 1997; Liu *et al*., 1999; Marszalek *et al*., 2000; Wolfrum and Schmitt, 2000; Williams, 2002)].

In this review, we discuss the role of centrins in the regulation of light-dependent translocation of the visual heterotrimeric G-protein transducin between the inner and outer segment compartment of photoreceptor cells. The prominent expression of centrins in the connecting cilium of vertebrate photoreceptor cells previously indicated their possible involvement in molecular translocations through the photoreceptor cilium (Wolfrum, 1995; Wolfrum and Salisbury, 1998).

2. What are Centrins? Centrin Genes, Protein Structure and Function

Centrins, also termed 'caltractins,' are highly conserved low molecular weight proteins of a large EF-hand superfamily of Ca^{2+}-binding proteins which includes calmodulin, parvalbumin, troponin C and S100 protein (Salisbury, 1995; Schiebel and Bornens, 1995). Centrins were first described in unicellular green algae where they are associated with the basal apparatus of flagella. In these organisms, centrins participate in Ca^{2+}-dependent and ATP-independent contractions of striated flagellar rootlets (Salisbury *et al*., 1984). Centrins have since been found to be ubiquitously associated with centrioles of basal bodies and centrosomes, as well as mitotic spindle poles in cells from diverse organisms, from yeast to man (Salisbury, 1995; Schiebel and Bornens, 1995).

Over the last decade, centrin genes have been identified in a variety of species from all kingdoms of eukaryotic organisms, protists, fungi, plants, and animals (Baum *et al*., 1986; Baum *et al*., 1988; Huang *et al*., 1988; Lee and Huang, 1993; Errabolu *et al*., 1994; Zhu *et al*., 1995; Levy *et al*., 1996; Madeddu *et al*., 1996; Meng *et al*., 1996; Middendorp *et al*.,

1997; Wottrich, 1998; Gavet *et al*., 2003). Comparisons of amino acid sequences deduced from cDNA clones show that centrins are a highly conserved, distinct subfamily of the EF-hand superfamily of Ca^{2+}-binding proteins. Centrins are small acidic proteins (~ 170 amino acids in length) with an apparent molecular mass of about 20 kDa (Salisbury, 1995; Schiebel and Bornens, 1995). To date, in lower eukaryotes like yeasts or in unicellular green algae, only one centrin gene (*Saccharomyces cerevisiae*: ScCDC31 and *Chlamydomonas reinhardtii*: CrCEN, respectively) has been identified (Baum *et al*., 1986; Baum *et al*., 1988; Huang *et al*., 1988). The recent isolation of a fourth centrin isogene in mouse and rat indicates that in the genome of mammals at least four centrin genes (eg. mouse centrins: MmCen1, MmCen2, MmCen3 and MmCen4) are present (Lee and Huang, 1993; Errabolu *et al*., 1994; Middendorp *et al*., 1997; Gavet *et al*., 2003; Trojan, 2003). Clustal analyses of deduced amino acid sequences of centrins from different organisms reveal several phylogenetic groups within centrins (Fig. 1A). While some protist centrin species cannot be classified to homogeneous groups, most centrins of higher plants, green algae centrins, and all known vertebrate centrin isoforms form phylogenetic groups. In mammals, Cen1p isoforms and Cen2p isoforms are very closely related, showing high amino acid identities of about 90%, whereas sequences of the yeast centrin (ScCdc31p) related vertebrate Cen3p isoforms have only amino acid identities of about 55% to both other isoforms. In the mouse, Cen1p, Cen2p and Cen4p isoforms are more closely related to algal centrin (eg. CrCenp) than to MmCen3p isoform, strongly suggesting two divergent centrin subfamilies (Middendorp *et al*., 1997).

The most characteristic domains of centrins are their four helix-loop-helix EF-hand consensus motifs (Fig. 1B). These potential Ca^{2+}-binding sites define centrins as members of the parvalbumin superfamily of Ca^{2+}-binding proteins (Kretsinger, 1976a; Kretsinger, 1976b; Moncrief *et al*., 1990; Nakayama *et al*., 1992). Protein sequence comparisons between different centrin species reveal that the EF-hand consensus motifs are the most highly conserved domains in various centrin species. Nevertheless, during molecular phylogenesis, some EF-hand motifs in centrins lost their ability to bind Ca^{2+}. In the centrins of the green algae *Chlamydomonas* or *Tetraselmis*, all four EF-hands bind one Ca^{2+}, but

they bind Ca^{2+} with different affinities (two EF-hands bind Ca^{2+} with high and two EF-hands with low affinity) (Coling and Salisbury, 1992; Weber *et al*., 1994). Other green algae possess two or three functional EF-hands whereas mammalian Cen1p and Cen2p molecules bind two Ca^{2+} with their first and the fourth EF-hand and in Cen4p and Cen3p the fourth is the last remaining functional EF-hand motif as it is the case in the yeast centrin ScCdc31p (Salisbury, 1995; Middendorp *et al*., 1997; Wottrich, 1998; Pulvermüller *et al*., 2002; Gavet *et al*., 2003).

As in other EF-hand Ca^{2+}-binding proteins (eg. calmodulin) (Barbato *et al*., 1992; Meador *et al*., 1993) Ca^{2+}-binding to centrins should induce drastic conformation changes in centrin molecules (Salisbury, 1995; Schiebel and Bornens, 1995; Wiech *et al*., 1996; Durussel *et al*., 2000). In contrast to calmodulin, centrin molecules may become more compact upon Ca^{2+}-binding. Ca^{2+}-activated centrins can also form dimers, oligomers and polymers (Wiech *et al*., 1996; Durussel *et al*., 2000) which may be the structural basis for contractile centrin-fiber systems (see above) (Salisbury, 1995). Furthermore, Ca^{2+}-binding to centrins increases the affinity of centrin-binding proteins to centrins (Geier *et al*., 1996; Wiech *et al*., 1996; Durussel *et al*., 2000). Binding of proteins to centrin, recently identified in mammalian retinal photoreceptor cells, also requires Ca^{2+} (see below) (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002; Gießl *et al*., 2004). Thus, centrins may be activated by Ca^{2+}-binding via their EF-hand domains to perform their specific cellular function. To understand Ca^{2+}-induced effects on the molecular function of centrins and their binding characteristics of target proteins, information on protein-binding domains and data from high resolution structural analysis are required.

The most distinctive and variable region of centrins is their amino-terminal subdomain which is unique for small Ca^{2+}-binding proteins. Therefore, it has been suggested to be responsible for some functional diversity among centrin species (Bhattacharya *et al*., 1993; Salisbury, 1995; Wiech *et al*., 1996). Analyses of polymerization properties of centrins indicate that the Ca^{2+}-induced polymerization of centrins is mainly dependent on the amino-terminal domain (Wiech *et al*., 1996). In addition to Ca^{2+}-binding, phosphorylation of centrins regulates their function. In green algae, centrin phosphorylation correlates with centrin-fiber elongation (relaxation) (Salisbury *et al*., 1984; Martindale and Salisbury,

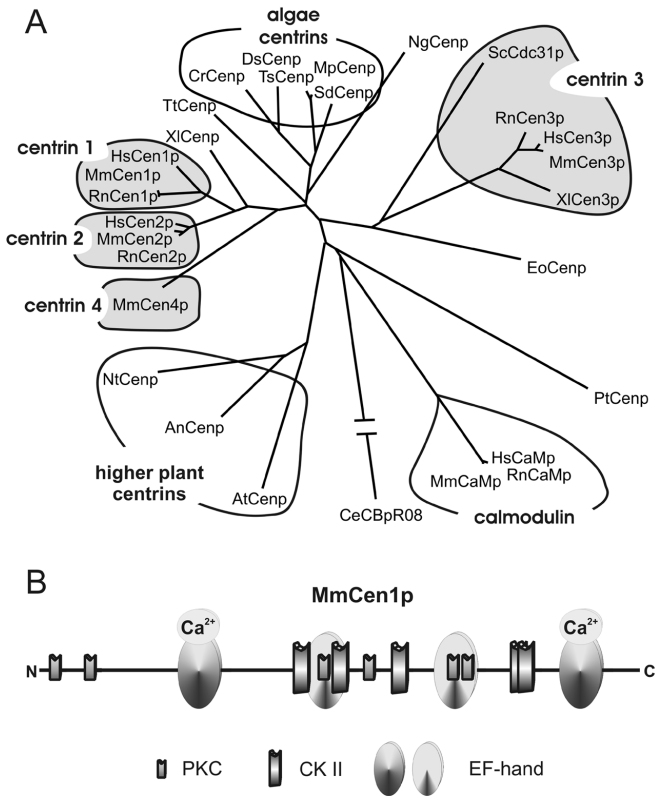

Fig. 1. Comparison of centrin isoforms of diverse species and centrin structural motifs. **(A)** Comparison (used programs: Omiga 2.0, Paup*4.0) of 29 different amino acid sequences of centrins and calmodulins. The phylogram shows a consensus tree which shows the highest frequency of each node of 1000 repetitions. Paup divides the centrins into subgroups of centrin isoforms 1, 2, 3 and 4, algae centrins, higher plant centrins and a group of calmodulin (RnCaMp = rat calmodulin Accession 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, 4p = mouse centrins 1, 2, 3, 4 AN: AAD46390, AAD46391, AAH02162, AAM75880; RnCen1p, 2p, 3p = rat centrins (completed with own data) 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. The tree is not complete. **(B)** Schematic representation of predicted structure of MmCen1p. Centrins bear four EF-hand motifs (EF1–EF4). Sequence analysis of the EF-hand together with experimental data indicate that in mammalian centrins only a subset of EF-hands are enable to bind Ca^{2+}, for example in MmCen1p the EF1 and EF4. In addition to Ca^{2+}-binding, the phosphorylation of centrins may regulate their functions: consensus phosphorylation sites for protein kinase C (PKC) and casein kinase II (CK II) are indicated by symbols in MmCen1p schema.

1990). Although conserved potential phosphorylation sites by protein kinase A (PKA) and $p34^{cdc2}$ kinase are located in the amino-terminal of some centrins (Salisbury, 1995), direct evidence for *in vivo* phosphorylation at the amino-terminus of centrins is missing. Nevertheless, aberrant centrin phosphorylation has been shown under pathogenic conditions in human breast cancer cells that have amplified centrosomes with supernumerary centrioles (Lingle *et al*., 1998). Furthermore, there is some evidence that vertebrate centrins are phosphorylated by PKA at conserved PKA consensus sequences present in the carboxy-terminus of some centrin species. Based on their results, Lutz *et al*. (2001) suggest that the phosphorylation of centrin 2 signals the separation of centrosomes during the prophase of the cell cycle.

Centrin was first described as the major component of the massive striated flagellar rootlets of the unicellular green algae *Tetraselmis striata* (Salisbury *et al*., 1984). In unicellular green alga, centrin containing striated rootlets originate at the basal body apparatus, project into the cell body and extend to the plasma membrane, the nucleus or other organelles (Salisbury, 1989). Later on, centrin-based fiber systems were also described in several other green algae including the algal model system *Chlamydomonas.* In *Chlamydomonas,* centrin is localized in descending fibers which connect the basal body apparatus with the nucleus (Salisbury *et al*., 1987; Schulze *et al*., 1987), in distal fibers which connect both adjacent basal bodies to one another (McFadden *et al*., 1987) and in the

stellate fibers of the transition zone present in the plane between the basal body and the axoneme of the flagella (Sanders and Salisbury, 1989). The green algal centrin fiber systems exhibit Ca^{2+}-triggered contractions which are suggested to be induced by conformation changes in the centrin molecules upon Ca^{2+}-binding (Salisbury *et al*., 1984; Salisbury, 1995; Schiebel and Bornens, 1995). Contraction of stellate fibers in the transition zone may induce microtubule severing and thereby the excision of the flagellum (Sanders and Salisbury, 1989; Sanders and Salisbury, 1994). 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 (Salisbury, 1989) or contributing to the microtubule release from the centrosome, the major microtubule organizing center (MTOC) of higher eukaryotic cells (Schatten, 1994). In the yeast *S. cerevesiae*, centrin (ScCdc31p) is encoded by the CDC31 gene (Fig. 1A). Cdc31p plays an essential role in the cell cycle via regulation of the duplication of the spindle pole body, the yeast MTOC (Schiebel and Bornens, 1995; Geier *et al*., 1996; Wiech *et al*., 1996; Khalfan *et al*., 2000; Ivanovska and Rose, 2001). 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) whose activity probably regulates spindle pole body duplication (Sullivan *et al*., 1998; Khalfan *et al*., 2000).

In vertebrates, centrin proteins are ubiquitously expressed and commonly associated with centrosome-related structures such as spindle poles of dividing cells or centrioles in centrosomes and basal bodies (Salisbury, 1995; Schiebel and Bornens, 1995). As discussed above, in mammals, at least four centrin genes are expressed which cluster to two divergent subgroups (Fig. 1A) (Lee and Huang, 1993; Errabolu *et al*., 1994; Levy *et al*., 1996; Madeddu *et al*., 1996; Meng *et al*., 1996; Middendorp *et al*., 1997; Wottrich, 1998; Gavet *et al*., 2003). 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. 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 centrins in mammalian cells and tissues have been performed with polyclonal and monoclonal antibodies raised against green algae centrins which do not discriminate between the mammalian centrin isoforms. Using these antibodies, centrins were detected in the centrioles of centrosomes and in the pericentriolar matrix (Salisbury *et al*., 1988; Baron and Salisbury, 1991; Baron *et al*., 1992).

Recent studies with antibodies raised against the mammalian Cen3p (or to yeast Ccd31p, respectively) and mouse Cen4p indicate that these antibodies do not show cross-reaction with other centrin isoforms (Middendorp *et al*., 1997; Laoukili *et al*., 2000; Gavet *et al*., 2003; Gießl *et al*., 2004). In contrast, to our knowledge, to date all of the antibodies raised against the close related mammalian Cen1p or Cen2p isoforms do not discriminate between both isoforms (Laoukili *et al*., 2000; Gießl *et al*., 2004). Nevertheless, expression analysis with these anti-centrin antibodies, in combination with comparative RT-PCR experiments (combined reverse transcriptase reaction and polymerase chain reaction), using isoform specific primers demonstrate that the centrin isoforms 2 and 3 are ubiquitously expressed, whereas centrin 1 and centrin 4 expression is restricted to ciliated cells (Wolfrum and Salisbury, 1998; Laoukili *et al*., 2000; Gavet *et al*., 2003; Trojan, 2003; Gießl *et al*., 2004). Subcellular localization studies demonstrate that the proteins Cen1p/Cen2p and Cen3p are localized in the centrioles of centrosomes or basal bodies, respectively (Paoletti *et al*., 1996; Laoukili *et al*., 2000; Gießl *et al*., 2004). Furthermore, Gavet *et al*. (2003) claims that Cen4p expression is restricted to neuronal brain tissue where it is localized in the basal bodies of the ciliary apparatus of ependymal and choroidal ciliated cells (Gavet *et al*., 2003). Based on these studies, it is likely that Cen1p and Cen4p function as centrin isoforms in compartments of cilia and flagella. Functional analyses indicated that ciliary centrins are involved in the beating of cilia. This was confirmed by the identification of a centrin species as a light chain of axonemal inner arm dynein in the ciliate *Tetrahymena* (Laoukili *et al*., 2000; Guerra *et al*., 2003).

The prominent localization of centrins at the centrosomes and basal bodies gave rise to several hypotheses for the function of centrins. In animal interphase cells or in arrested cells of differentiated tissue, the centrosome functions as the major microtubule organizing center (MTOC)

at which microtubules are *de novo* synthesized and the number and polarity of cytoplasmic microtubules is determined. 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 (Schatten, 1994). However, more conclusive evidence was gathered, suggesting 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 microtubule array of the mitotic spindle. While Cen3p, and its yeast relative Cdc31p, participates in centrosome reproduction and duplication (Middendorp *et al*., 2000), Cen2p may play a role in centriole separation preceding centrosome duplication (Lutz *et al*., 2001). Gene silencing experiments using RNA interference in human HeLa cells confirmed a requirement of Cen2p for centrosome reproduction (Salisbury *et al*., 2002).

3. Centrin Isoform Expression and Localization in the Vertebrate Retina

Comparative studies reveal expression of centrins in the retina of species distributed throughout the subphylum of vertebrates (Wolfrum and Salisbury, 1998; Wolfrum *et al*., 2002). In mammals, recently performed RT-PCR analyses with isoform specific primers demonstrate expression of all four known mammalian centrin isoforms in the retina (Fig. 2) (Wolfrum and Salisbury, 1998; Trojan, 2003; Gießl *et al*., 2004). Furthermore, Western blots using antibodies specific for Cen3p, Cen4p and Cen1p/Cen2p, respectively, confirmed these results (Gießl *et al*., 2004). Thus, centrins are probably ancient cytoskeletal proteins in the vertebrate retina indicating this conserved basic function in retinal cells.

As in other cell types of animal tissue, centrins are components of the centrioles of centrosomes and basal bodies in the retinal neurons where they may contribute to centrosomal functions (see above) (Wolfrum and Salisbury, 1998; Wolfrum *et al*., 2002). Nevertheless, in vertebrate retinas, the most prominent anti-centrin immunofluorescence labeling is present in the photoreceptor cell layer (Fig. 3) (Wolfrum, 1992; Wolfrum, 1995; Wolfrum and Salisbury, 1998). Here, centrins, in addition to their basal body localization, are also localized along the entire extension of the

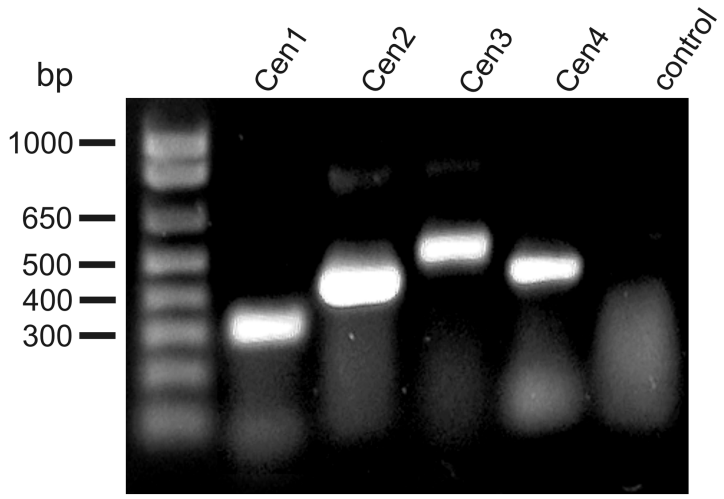

Fig. 2. Expression analysis of centrin isoforms in rat retina by RT-PCR. Mouse centrin specific primer-sets were used to amplify different constructs of centrin isoforms. Total RNA used for all RT-PCR experiments was treated with DNase I to degrade genomic DNA. Control PCR (control) was conducted with DNase I treated RNA without reverse transcriptase to show that no genomic DNA is amplified. Due to the RT-PCR analysis, all four centrin isoforms (RnCen1, RnCen2, RnCen3 and RnCen4) are expressed in rat retina.

connecting cilium of photoreceptor cells (Fig. 3) (Wolfrum, 1995; Wolfrum and Salisbury, 1998). Recently obtained immunoelectron microscopic data indicate that in addition to Cen1p a second centrin isoform, Cen3p is present in the connecting cilium of photoreceptor cells (Fig. 3) (U. Wolfrum and A. Gießl, unpublished data). Further quantification of immunoelectron microscopic labelings revealed that both centrins co-localize in the subciliary domain at the inner face of the ciliary microtubule doublets (Fig. 3D) (Wolfrum and Salisbury, 1998; Pulvermüller *et al.*, 2002; U. Wolfrum and A. Gießl, unpublished data).

The modified connecting cilium of vertebrate photoreceptor cells is the structural equivalent of the extended transition zone present at the base of a common motile cilium (Besharse and Horst, 1990). Therefore, the presence of centrins along the entire extension of the connecting cilium of photoreceptor cells is in agreement with the localization of centrins in the transition zone of motile cilia or the sensory cilia of

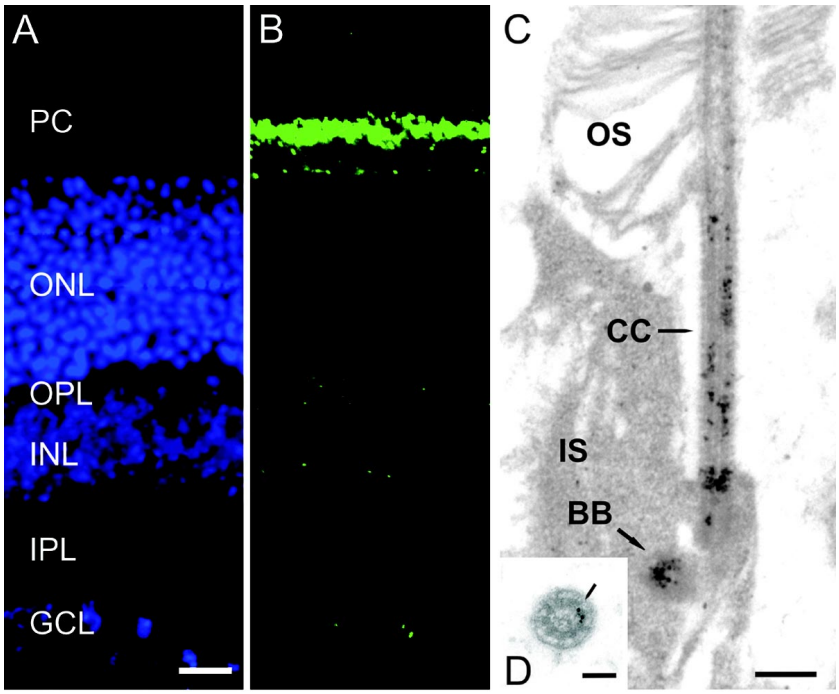

Fig. 3. Localization of centrin in the mammalian retina. **(A)** DAPI-(4′,6-Diamidino-2-phenylindole)-staining of a longitudinal cryosection through the rat retina. Staining of nuclear DNA demonstrates the retinal layers: PC = layer of outer and inner segments of photoreceptor cells; ONL = outer nuclear layer where nuclei of photoreceptors are localized; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GC = ganglion cell layer. **(B)** Indirect anti-centrin 3 immunofluorescence in the retinal cryosection shown in A. Anti-centrin 3 antibodies predominantly react within the photoreceptor cell layer at the joint between the inner and outer segment of the photoreceptor cells. In addition, centrin 3 is detected in dot pairs representing the centriole pairs of centrosomes in the perikarya localized in the inner nuclear layer and the ganglion cell layer. **(C)** Longitudinal ultrathin section of part of a mouse rod photoreceptor cell, illustrating silver enhanced immunogold labeling of centrin 3. Centrin 3 is localized in the non-motile connecting cilium (CC) linking the inner segment (IS) with the light sensitive outer segment (OS). In addition, prominent anti-centrin 3 labeling can be observed in the basal bodies (BB) of the ciliary apparatus in the photoreceptor inner segment. **(D)** Transversal ultrathin section through the connecting cilium of a mouse rod photoreceptor cell, illustrating silver enhanced immunogold labeling of centrin 3. Centrin 3 is localized at the inner surface of the ring of axonemal microtubule pairs. Bars in B = A: 10 µm; C: 250 nm; D: 75 nm.

mammalian olfactory cells (Wolfrum and Salisbury, 1998; Laoukili *et al*., 2000; A. Schmitt and U. Wolfrum, unpublished data). In photoreceptor cells, the connecting cilium links the morphologically and functionally distinct cellular compartments, the light sensitive outer segment and the biosynthetically active inner segment. The connecting cilium serves as an active barrier for membrane components and soluble proteins, regulating the diffusion between the inner and the outer segment of photoreceptor cells (Spencer *et al*., 1988; Besharse and Horst, 1990). It represents the only intracellular bridge between both segments, therefore the entire intracellular exchange between the inner segment and the outer segment is forced to occur through the slender connecting cilium (Besharse and Horst, 1990). The transport of the visual pigment opsin is directed via the membrane of the connecting cilium to its final destination at the base of the outer segment (Liu *et al*., 1999; Wolfrum and Schmitt, 1999; Wolfrum and Schmitt, 2000). There are lines of evidence for an involvement of both actin filament- and microtubule-based molecular motors in the unidirectional ciliary transport of opsin. The membrane attached myosin VIIa may participate as an actin filament associated molecular motor in ciliary transport of rhodopsin (Liu *et al*., 1999; Wolfrum and Schmitt, 1999; Wolfrum and Schmitt, 2000; Wolfrum, 2003; see also chapter 15). Nevertheless, there is also evidence for the contribution of a heterotrimeric kinesin II-motor to the ciliary transport of rhodopsin to the outer segment (Marszalek *et al*., 2000). As in green algae, in photoreceptor cells, kinesin II is part of a microtubule-based intraflagellar transport complex (Rosenbaum *et al*., 1999; Baker *et al*., 2003; see also chapter 5). It might additionally serve in the transport of arrestin to the outer segment (Marszalek *et al*., 2000), which is triggered by light (Philp *et al*., 1987; Whelan and McGinnis, 1988; Organisciak *et al*., 1991; Sokolov *et al*., 2002; Mendez *et al*., 2003; Peterson *et al*., 2003; see also chapter 7). However, cytoskeletal molecules associated with other proteins of the visual transduction cascade (eg. transducin), and which may be 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 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 alignment or

orientation of photoreceptor outer segments was suggested (Wolfrum, 1995). In all cases, centrin-based processes in the cilium should be dependent on and regulated by changes of the free Ca^{2+}-concentration (see above). Our recent results, as discussed below, provide striking evidence for Ca^{2+}-dependent interaction between centrins and the visual G-protein transducin on its pathway through the inner lumen of connecting cilium of mammalian photoreceptor cells (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002; Gießl *et al*., 2004).

4. Centrin-Binding Proteins in Photoreceptor Cells

In the context of the cell, protein function and its regulation is determined by binding proteins to the target protein. Unfortunately, little is known about centrin-binding proteins in vertebrates. In the cytoplasm of arrested *Xenopus* oocytes, XlCenp is sequestered in an inactive state by an interaction with the heat shock proteins HSP70 and HSP90 (Uzawa *et al*., 1995). In yeast 2-hybrid screens, the laminin-binding protein LBP, a component of the extracellular basal lamina, and the cytoplasmic receptor protein tyrosine kinase κ have been identified as proteins interacting with HsCen2p (Paschke, 1997). To our knowledge, to date, none of these putative centrin-binding proteins has an obvious function in the connecting cilium of mammalian photoreceptor cells.

We have recently identified centrin-interacting proteins by Western blot overlay assays of retinal proteins with recombinant expressed MmCen1p (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002). Binding of recombinant MmCen1p to target proteins is restricted to the Ca^{2+}-activated centrin form. This agrees with the Ca^{2+}-dependent increase of the affinity of diverse centrin species to the yeast target protein Kar1p (Schiebel and Bornens, 1995; Geier *et al*., 1996; Wiech *et al*., 1996). Further analysis of other proteins which were identified by the MmCen1p overlay assay, is currently being performed. However, we have already identified the centrin 1-binding protein p37 as the β-subunit of the visual G-protein transducin (G_t) (Fig. 5C) (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002). Our most recent experimental data indicate that not only the isoform Cen1p, but also the three other centrin isoforms specifically interact with β-transducin (Gießl *et al*., 2004).

5. Centrin/Transducin Complex

Transducin (G_t) 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. 4) (Philp *et al*., 1987; Brann and Cohen, 1987; Whelan and McGinnis, 1988; Organisciak *et al*., 1991; Pulvermüller *et al*., 2002; Sokolov *et al*., 2002; Wolfrum *et al*., 2002; Mendez *et al*., 2003). 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, at least the centrin isoforms 1 and 3 are prominent components of the cytoskeleton of the photoreceptor cilium and immunofluorescence double labeling of transducin and centrin indicates that transducin and centrin 1 and 3 co-localize in the connecting cilium (eg. Fig. 4C). Immunoelectron microscopical analysis and the quantification of silver enhanced immunogold decorations reveal that centrin and transducin do not only exist parallel in the cilium, but share also the same subciliary domain, the inner ciliary lumen of the connecting cilium (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002). Their spatial co-distribution indicates that both proteins may physically interact during the exchange of transducin between the photoreceptor segments through the cilium.

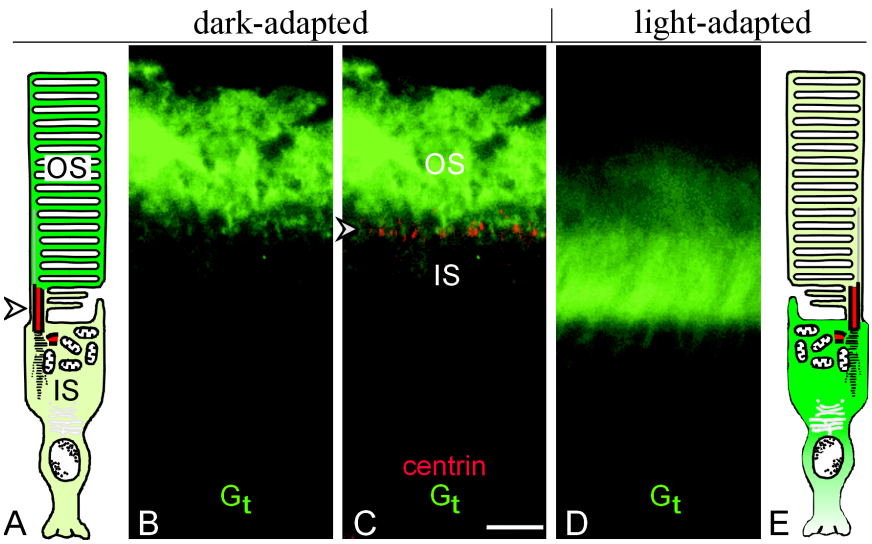

Fig. 4. Light-dependent translocations of transducin in the mammalian retina. (A–C) Dark-adapted mouse retina. (D and E) Light-adapted mouse retina. **(A)** Schematic representation of a dark-adapted rod photoreceptor cell. Green colour indicates G_t distribution. OS: photoreceptor outer segment; IS: photoreceptor inner segment. **(B)** Indirect anti-$G_t\alpha$ immunofluorescence of a longitudinal cryosection through the dark-adapted mouse retina. **(C)** Merged images of B with the anti-centrin 1/2 immunofluorescence (red: Alexa546) concentrated in the connecting cilium between IS and OS of photoreceptor cells. **(D)** Indirect anti-$G_t\alpha$ immunofluorescence in the section through the light-adapted mouse retina. **(E)** Schematic representation of a light-adapted rod photoreceptor cell. Green colour indicates G_t distribution. In dark-adapted photoreceptor cells, the heterotrimeric G_t-complex is predominantly localized in the OS whereas in the light-adapted condition, heterotrimeric G_t-complex is most prominently detected in the IS of photoreceptor cells. Bar: 10 µm.

In our initial studies, we have gathered striking evidence that MmCen1p indeed 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 (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002). An extension of our analyses reveals that not only Cen1p, but also the three additional centrin isoforms, Cen2p to Cen4p, bind with high affinity to transducin (Gießl *et al.*, 2004). *In vitro* assays including

co-immunoprecipitation, GST-pull down, overlay and co-sedimentation assays as well as size exclusion chromatography and kinetic light scattering experiments independently demonstrate that centrins and transducin assemble into protein complexes (see for example Fig. 5) (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002; Gießl *et al*., 2004). Kinetic light scattering studies also indicate that the protein-protein interaction between centrins and transducin is highly specific: the centrin isoforms specifically interact with transducin and do not bind to other molecules of the visual signal transduction cascade, neither to arrestin, rhodopsin, rhodopsin kinase nor to the visual PDE (Fig. 5D). Furthermore, the centrin EF-hand protein relatives, recoverin and calmodulin, which are highly expressed in photoreceptor cells do not exhibit significant affinities to transducin (Pulvermüller *et al*., 2002).

The analysis of GST-pulldown assays, overlay experiments with antibodies specific to transducin subunits and size exclusion chromatography further demonstrate that assembly of centrin/G-protein complexes is mediated by the βγ-complex (Fig. 5C) (Pulvermüller *et al*., 2002; Wolfrum *et al*., 2002; Gießl *et al*., 2004). All protein-protein interaction analyses also show that the assembly of centrin/G-protein complexes is strictly dependent on the Ca^{2+}-concentration. In the case of Cen1p, at least two Ca^{2+}-ions are required for the activation of centrin 1 and the formation of a centrin/G-protein complex (Pulvermüller *et al*., 2002). Further analysis of this complex indicates that at least the activated centrin 1 binds as a homooligomer to the βγ-complex of transducin (Pulvermüller *et al*., 2002).

What is the role of the centrin/G-protein complex in the photoreceptor cell? The spatial co-localization of centrin 1 and 3 with transducin in the lumen of the connecting cilium (see eg. Fig. 3D) emphasizes that in photoreceptor cells, the formation of centrin/G-protein complexes 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 and 3 in the connecting cilium and in turn induce the binding of centrin single molecules or oligomers to transducin passing through the cilia. 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

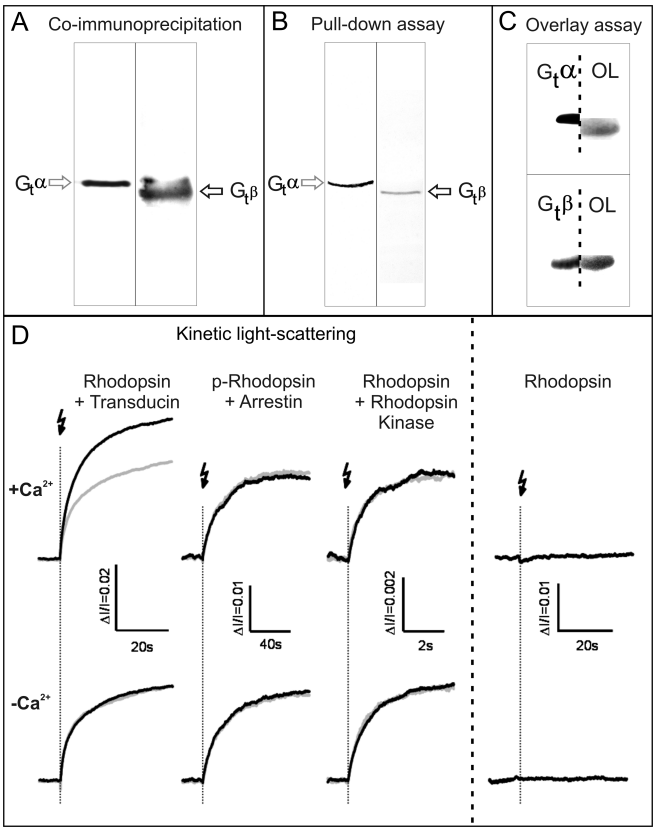

Fig. 5. Proofs of centrin/G-protein complex assembly. **(A)** Co-immunoprecipitation of transducin with centrins from lysed retinal photoreceptor cell fragments. Lanes 1: Western blot analysis with mAb anti-$G_t\alpha$ of an immunoprecipitation with mAb anti-centrin (clone 20H5) from photoreceptor cell fragments of bovine retina. Lane 2: Western blot analysis with polyclonal anti-$G_t\beta$ of an immunoprecipitation with mAb anti-centrin (clone 20H5) from photoreceptor cell fragments of bovine retina. The heterotrimeric G-protein complex, including $G_t\alpha$ and $G_t\beta$, co-immunoprecipitates with centrins. **(B)** GST-centrin pull downs of transducin. Lanes 1: Western blot analysis with mAb anti-$G_t\alpha$ of a pull down with recombinant expressed GST-MmCen1p from bovine retina. Lane 2: Western blot analysis with polyclonal anti-$G_t\beta$ of a pull down with recombinant expressed GST-MmCen1p from bovine retina. The trimeric G-protein complex, including $G_t\alpha$ and $G_t\beta$ is pulled down with GST-centrin. **(C)** 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 $G_t\alpha$ transducin (upper lane

1), and $G_t\beta$ transducin (lower lane 1) and for overlays with recombinantly expressed MmCen1p (OL). The 37 kDa centrin-binding protein is identified by centrin overlays and migrates in the probed SDS-PAGEs at the exact mobility of the $G_t\beta$ subunit. **(D)** Kinetic light-scattering (KLS) binding signals with unphosphorylated or prephosphorylated membranes and transducin (G_t), arrestin, and rhodopsin kinase in the presence (black curves) or absence (gray curves) of MmCen1p. Upper panels represent KLS binding signals (G_t, arrestin or rhodopsin kinase and rhodopsin or p-rhodopsin) in the presence of Ca^{2+} plus/minus MmCen1p and the lower panels KLS binding signals under conditions identical to those in the upper panels, but with EGTA instead of Ca^{2+}. The increase of the binding signal observed by transducin plus MmCen1p indicates binding of Ca^{2+}-centrin to transducin. Absence of the KLS binding signals in the right panel demonstrates that MmCen1p show no light-induced interaction with rhodopsin. Measuring conditions and the used KLS setup are described in Pulvermüller *et al.* (2002).

include the well-studied Ca^{2+}drop within the operating (single quantum detective) range of the rod (Molday and Kaupp, 2000). Recent observations also indicate a Ca^{2+} increase in bright light (rod saturated conditions) (Matthews and Fain, 2001). In any case, the free Ca^{2+} in the connecting cilium should also be affected. 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) (Wolfrum *et al*., 2002). 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 (Philp *et al*., 1987; Whelan and McGinnis, 1988; Organisciak *et al*., 1991; Pulvermüller *et al*., 2002; Sokolov *et al*., 2002; Wolfrum *et al*., 2002; Mendez *et al*., 2003). However, Ca^{2+}-triggered sequential binding of transducin to centrin may although contribute to the transport of transducin through the photoreceptor connecting cilium (Ca^{2+}-gradient hypothesis) (Wolfrum *et al*., 2002).

As previously mentioned, in addition to Ca^{2+}-binding, phosphorylation of centrins regulates the functions of centrins in yeast, green algae and at the centrosome of cultured mammalian cells (Salisbury *et al*., 1984; Martindale and Salisbury, 1990; Saliybury, 1995; Lutz *et al*., 2001). Since the ciliary centrin isoforms bear interesting potential phosphorylation sites (Fig. 1B), we started to investigate the role of phosphorylation of centrins in photoreceptor cell function. The readout of our phosphorylation

assays in explanted rat retinas revealed a drastic increase of the phosphorylation in the immunoprecipitate by anti-pan-centrin (Fig. 6). Thus, our preliminary data strongly indicate that centrins phosphorylation occurs in a light-dependent manner, triggered by the visual signal transduction process (Fig. 6).

The Ca^{2+}-dependent assembly of a G-protein with centrin is a novel aspect of the supply of signaling proteins in sensory cells. Centrins may represent potential molecular linkers between molecular translocations and signal transduction in general.

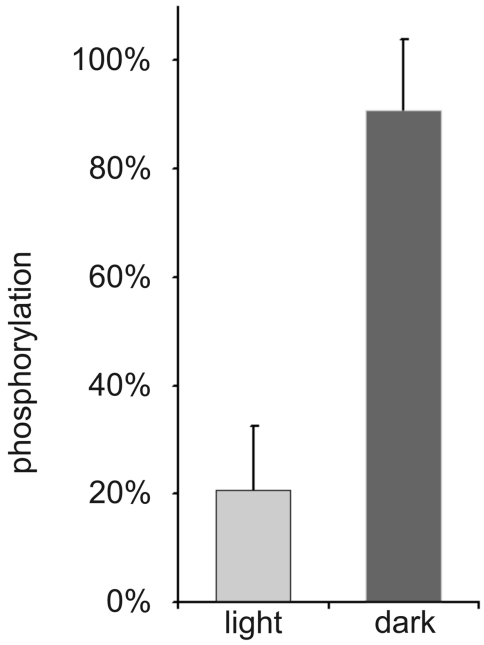

Fig. 6. Light-dependent phosphorylation of centrins in explanted rat retina. Explanted rat retinas were light- and dark-adapted. After incubation with radioactive labeled phosphate ($H_3[^{32}P]O_4$), centrins were immunoprecipitated with the monoclonal anti-pan-centrin antibody (clone 20H5). Subsequently, the radioactivity of immunoprecipitates was analyzed by using a scintillation counter. The amount of incorporated radioactive phosphate into centrins is about three times higher in dark-adapted retinas compared with light-adapted retinas. These results indicate that phosphorylation of centrins occurs in a light-dependent manner.

6. Summary and Conclusions

Centrins are members of a conserved subfamily of EF-hand Ca^{2+}-binding proteins. During the past years, four centrin isogenes were identified and found to be ubiquitously associated with the centrioles of centrosomes or centrosome related structures (eg. basal bodies) in diverse vertebrate cells. All four centrin isoforms are expressed in the neuronal retina of mammals. In photoreceptor cells, they are prominent components of the ciliary apparatus. Several lines of evidence indicate that, in the retina, the centrin isoforms 2 and 4 are expressed at the centrioles of centrosomes and basal bodies of the retinal cells, whereas the centrin isoforms 1 and 3 are additionally localized in the connecting cilium of photoreceptor cells. Improved specific antibodies to centrin isoforms or transfection of the retina with tagged-centrin constructs will provide more liable information on the specific subcellular localization and function of the centrin isoforms in photoreceptor cells. Our recent findings reveal that not only the centrin isoform centrin 1, but also the other three centrin isoforms bind with high affinity to transducin in a strict Ca^{2+}-dependent manner. The results of our current analysis of putative centrin-associated proteins (other than transducin) in the mammalian retina will most probably also gather further insights in the role of centrins in photoreceptor cell function. In the future, we will also continue our analyses of the phosphorylation status of centrins and its correlation with the function(s) of centrin in photoreceptor cells. And finally, the clarification of the structure of centrin isoforms and their protein-binding domains will also enlighten the molecular mechanisms of the diverse functions of centrins in photoreceptors. Functional analysis of centrins may also elucidate the role of transducin and heterotrimeric G-proteins at centrosomal structures of the cell.

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