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Centrins, gatekeepers for the light-dependent translocation of transducin through the photoreceptor cell connecting cilium

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Abstract

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 the retina, centrins are also prominent components of the photoreceptor cell ciliary apparatus. Centrin isoforms are differentially localized at the basal body and in the lumen of the connecting cilium. All molecular exchanges between the inner and outer segments occur through this narrow connecting cilium. Ca^{2+}-activated centrin isoforms bind to the visual heterotrimeric G-protein transducin via an interaction with the βγ-subunit. Ca^{2+}-dependent assemblies of centrin/G-protein complexes may regulate the transducin movement through the connecting cilium. Formation of this complex represents a novel mechanism in regulation of translocation of signaling proteins in sensory cells, as well as a potential link between molecular trafficking and signal transduction in general.

Keywords: Centrin; Transducin; Photoreceptors; Light-dependent translocation; Connecting cilium

1. Introduction

Vertebrate photoreceptor cells are highly polarized neurons that are optimized for the detection of light. The cells offer morphological and functional arrangements in several compartments. The light absorbing molecules are concentrated in the outer segment at the apical end of the cell and are separated from the synaptic connection at the basal end by the nucleus and an inner segment which contains the typical energy producing and protein synthesizing components of an eukaryotic cell. All proteins synthesized in the inner segment but destined for the outer segment must pass through a narrow non-motile connecting cilium linking the outer segment to the inner segment. The outer segment contains all components of the visual transduction cascade, which are associated with the stacked membrane disks. In rod photoreceptors, photoexcitation of the visual pigment rhodopsin responses over transducin and the phosphodiesterase in a rapid hydrolysis of cGMP, the second messenger of the phototransduction, and hyperpolarization of the cell. Rod photoreceptors are able to retain light responsiveness at different levels of illumination, a process known as light adaptation that requires feedback mechanisms to control the sensitivity and gain of the phototransduction cascade. The majority of these mechanisms operate on a fast timescale using Ca^{2+} as an internal messenger (Howes et al., 2002; Krizaj, Demarco, Johnson, Strehler, & Copenhagen, 2002).

The non-motile cilium in rod photoreceptor cells serves as a bottle neck through which some phototransduction molecules pass in a bidirectional manner (Brann & Cohen, 1987; Broekhuyse, Tolhuizen, Janssen, & Winkens, 1985; Gießl et al., 2004a; Mangini & Pepperberg, 1988; McGinnis, Matsumoto, Whelan, & Cao, 2002; Philp, Chang, & Long, 1987; Pulvermüller et al., 2002; Sokolov et al., 2002; Whelan & McGinnis, 1988). For arrestin and transducin, the direction of this translocation was reversible and depen-

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dent on the lighting environment to which the animal was exposed. Both proteins are localized in opposite compartments and were moving in opposite directions when light conditions changed. Nevertheless, all intracellular exchanges between these two functional compartments of photoreceptor cells must occur through the slender connecting cilium. In the last years, an increasing number of proteins has been identified at the connecting cilium (Schmitt & Wolfrum, 2001). This list of molecules includes several cytoskeletal molecular motors, which represent good candidates in active molecule transport through the connecting cilium (Liu, Udovichenko, Brown, Steel, & Williams, 1999; Liu, Vansant, Udovichenko, Wolfrum, & Williams, 1997; Marszalek et al., 2000; Williams, 2002; Wolfrum, Bode, Tai, Sung, & Schmitt, 2000). But also a transport via diffusion of the two proteins arrestin and transducin has been discussed during the last few years (Elias, Sezate, Cao, & McGinnis, 2004; Kerov et al., 2005; Nair et al., 2005; Sokolov et al., 2002). Here, we discuss the role of centrins in the regulation of the light-dependent translocation of the visual heterotrimeric G-protein transducin between the inner and outer segments of photoreceptor cells. The localization of centrins in the connecting cilium of photoreceptor cells previously suggested their possible involvement in molecular translocations of proteins through the photoreceptor cilium (Wolfrum & Salisbury, 1995; Wolfrum & Salisbury, 1998).

2. Centrins – protein structure and function

Centrins are highly conserved phospho-proteins belonging to a large EF-hand superfamily of Ca^{2+}-binding proteins which include the second messenger calmodulin, parvalbumin, troponin C and S100 (Salisbury, 1995; Schiebel & Bornens, 1995). Like calmodulin, centrins contain four EF-hand motifs and are small proteins with a molecular weight of approximately 20 kDa and 170 amino acids. Centrins were first described in unicellular green algae where they are associated with the basal apparatus of flagella (Salisbury, Baron, Surek, & Melkonian, 1984; Salisbury & Floyd, 1978). Green algal centrins form super fine filaments where they participate in Ca^{2+}-dependent and ATP-independent rootlet contractions (Salisbury et al., 1984; Schiebel & Bornens, 1995). As is the case with other EF-hand Ca^{2+}-binding proteins, (e.g., calmodulin) Ca^{2+} binding to centrins is thought to induce conformational changes in centrin molecules (Barbato, Ikura, Kay, Pastor, & Bax, 1992; Durussel, Blouquit, Middendorp, Craescu, & Cox, 2000; Meador, Means, & Quiocho, 1993; Salisbury, 1995; Schiebel & Bornens, 1995; Wiech et al., 1996). Furthermore, the affinity of centrins to interacting target proteins is increased by bound Ca^{2+} (Durussel et al., 2000; Geier, Wiech, & Schiebel, 1996; Gießl et al., 2004a; Pulvermüller et al., 2002; Wiech et al., 1996). Centrin functions are not only regulated by Ca^{2+} but also by phosphorylation (Lutz, Lingle, McCormick, Greenwood, & Salisbury, 2001; Martindale & Salisbury, 1990; Meyn et al., 2006; Salisbury et al., 1984; Wottrich, 1998). All mouse centrin isoform amino acid sequences contain several predicted phosphorylation sites for protein kinase A, protein kinase C and protein kinase CK2. The kinases may regulate centrin functions in mammalian cells (Lingle, Lutz, Ingle, Maihle, & Salisbury, 1998; Lutz et al., 2001; Salisbury, 1995).

Expression analyses with anti-centrin antibodies, in combination with comparative RT-PCR experiments demonstrate that the centrin isoforms 2 and 3 are ubiquitously expressed, whereas centrin 1 and 4 expression is restricted to ciliated cells (Gavet, Alvarez, Gaspar, & Bornens, 2003; Gießl et al., 2004a; Laoukili et al., 2000; Wolfrum & Salisbury, 1998). In eukaryotic cells centrins are always associated with centrioles of centrosomes or basal bodies. In ciliary cells centrins are components of the transition zone and the basal body complex of the cilia. Based on these studies it is likely that centrin 1 and 4 function as centrin isoforms in compartments of cilia and flagella (Gavet et al., 2003; Gießl et al., 2004a; Guerra, Wada, Leick, Bell, & Satir, 2003; Laoukili et al., 2000), whereas centrin 2 and 3 may play a role in the cell cycle in centrosome reproduction and duplication (Lutz et al., 2001; Middendorp et al., 2000; Salisbury, Suino, Busby, & Springett, 2002).

3. Expression of centrin isoforms in the vertebrate retina

Comparative studies have revealed the expression of centrins in the retina of species distributed throughout the subphylum of vertebrates (Wolfrum, 1995; Wolfrum, 1998; Wolfrum, Gießl, & Pulvermüller, 2002; Wolfrum & Salisbury, 1995). In rodents, RT-PCR analyses with specific primers demonstrate expression of all four known mammalian isoforms on mRNA level in the retina (Gießl et al., 2004a; Gießl, Trojan, Pulvermüller, & Wolfrum, 2004b; Wolfrum & Salisbury, 1998). Furthermore Western blot analyses with specific antibodies against the four centrin isoforms confirmed these results (Gießl et al., 2004a). In the vertebrate retina, centrins are not only expressed at centrosomes of all retinal neurons and ganglia cells but also in the ciliary apparatus of rod and cone photoreceptor cells (Fig. 1) (Gießl et al., 2004b; Pulvermüller et al., 2002; Wolfrum, 1992; Wolfrum, 1995; Wolfrum et al., 2002; Wolfrum & Salisbury, 1998). Double labeling experiments with antibodies against centrin and rootletin, which is a prominent component of the ciliary rootlets (Yang, Adamian, & Li, 2006; Yang et al., 2005; Yang et al., 2002), excluded the localization of centrins in the ciliary rootlets (Fig. 1), where centrins were previously described in sensory cells of insects (Wolfrum, 1991; Wolfrum, 1997). Using isoform specific antibodies we recently showed that the four centrin isoforms are differentially localized in the ciliary apparatus of retinal photoreceptor cells (Gießl et al., 2004a). Our findings are summarized in Fig. 2: centrin isoforms 1–3 are localized in the connecting cilium of photoreceptor cells. Immunoelectron microscopy revealed a subciliary localization of these centrin isoforms at the inner surface of the

axonemal microtubule doublets (Gießl et al., 2004a; Pulvermüller et al., 2002), indicating a specific role of these centrins in the functions of the connecting cilium. While centrin 1 localization is restricted to the photoreceptor cilium, centrin 2 and 3 are also found in the basal body complex. Centrin 4 is only found at basal bodies which confirmed previous results on the centrin 4 expression in the brain (Gavet et al., 2003; Gießl et al., 2004a).

4. Identification of β-transducin as a centrin-binding protein in photoreceptor cells

In the cellular context, protein function is often predestinated by interaction partners. Unfortunately, little is known about centrin-binding proteins in vertebrate cells (Gießl et al., 2004a; Schiebel & Bornens, 1995; Wolfrum et al., 2002). In yeast, several Cdc31p (centrin 3 homolog) binding proteins like Karp1, Mps3, Kic1p and Sfi1p were identified (Biggins & Rose, 1994; Byers, 1981; Geier et al., 1996; Jaspersen, Giddings, & Winey, 2002; Kilmartin, 2003; Paoletti et al., 2003; Rose & Fink, 1987; Spang, Courtney, Grein, Matzner, & Schiebel, 1995; Sullivan, Biggins, & Rose, 1998; Vallen, Ho, Winey, & Rose, 1994). Without any experimental evidence homologs of Sfi1p were suggested as binding partners of filamentous centrin bundles in mammalian cells (Salisbury, 2004). In arrested *Xenopus* oocytes XlCenp (similar to MmCen1p and MmCen2p) revealed an interaction with the heat shock proteins HSP70 and HSP90 in the cytoplasm (Uzawa, Grams, Madden, Toft, & Salisbury, 1995). Other binding-partners of HsCen2p identified in yeast two-hybrid screens (e.g., laminin-binding protein and protein tyrosin kinase k) (Paschke & Ludgate, 1997) have no obvious function in the connecting cilium of mammalian photoreceptor cells (Gießl et al., 2004a). Western blot overlay assays of retinal proteins with recombinantly expressed MmCen1p demonstrated numerous putative centrin interacting partners in the retina (Pulvermüller et al., 2002; Wolfrum et al., 2002). Binding of MmCen1p to target proteins of the retina extract is restricted to the Ca^{2+} activated centrin form. This agrees with the Ca^{2+}-dependent increase of the binding affinity of diverse centrins to the yeast target protein Karp1, which is localized in yeast at the spindle pole body (SPB) and is responsible for SPB duplication (Antoniacci, Kenna, Uetz, Fields, & Skibbens, 2004; Geier et al., 1996; Schiebel & Bornens, 1995; Wiech et al., 1996). So far, we identified one of the "centrin-positive" proteins in the retina extract: the β-subunit of the visual G-protein transducin (Pulvermüller et al., 2002; Wolfrum et al., 2002).

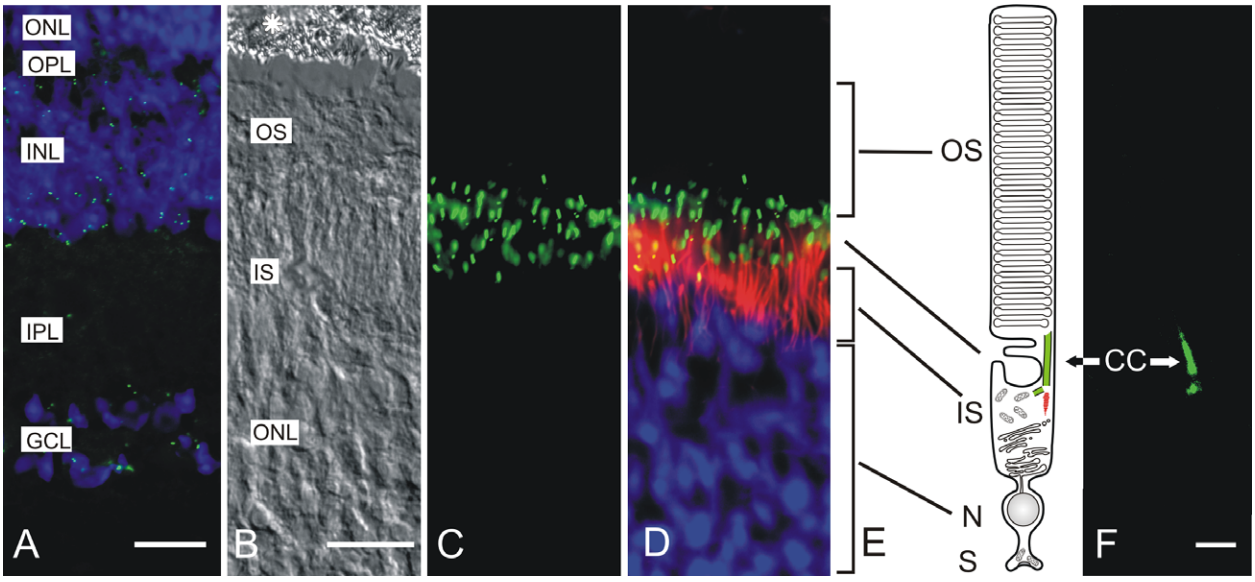

Fig. 1. Localization of centrin 2 in the mammalian retina. (A) Indirect anti-centrin 2 immunofluorescence (green) in a longitudinal cryosection through the rat retina. DAPI-(4′,6-diamididino-2-phenylindole) stains the nuclear DNA (blue), which demonstrates the retinal layers: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Anti-centrin 2 antibody reacts in dot pairs representing the centriole pairs of centrosomes in the perikarya localized in the inner nuclear and in the ganglion cell layer. (B) Differential interference contrast image of the same cryosection shown in A with higher magnification and more focus on the photoreceptor cells divided into the outer segment (OS), inner segment (IS) and the outer nuclear layer (ONL). The asterisk indicates retinal pigment epithelium. (C) Subcellular immunolocalization of centrin 2 (green) stains predominantly the joint between the inner and the outer segment of the photoreceptor cells. (D) Merged image of C and two not shown pictures. Staining of the connecting cilium (CC) with the centrin 2 antibody (green, C). Immunolocalization of rootletin (red), an other protein of the photoreceptor cells, which is localized at the rootlets of the connecting cilium. Present double labeling revealed that centrin is not localized at the ciliary rootlets in photoreceptor cells. DAPI stains the nuclear DNA (blue) in the outer nuclear layer. (E) 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 and rootletin localization are colored with green and red. (F) Indirect anti-centrin immunolabeling of a photoreceptor cell fragment of rat retina analyzed by confocal laser scanning microscopy. The green labeling shows centrin at the connecting cilium and the basal body in the photoreceptor cell. Bars in: A = 13 μm; B = 8.2 μm; F = 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

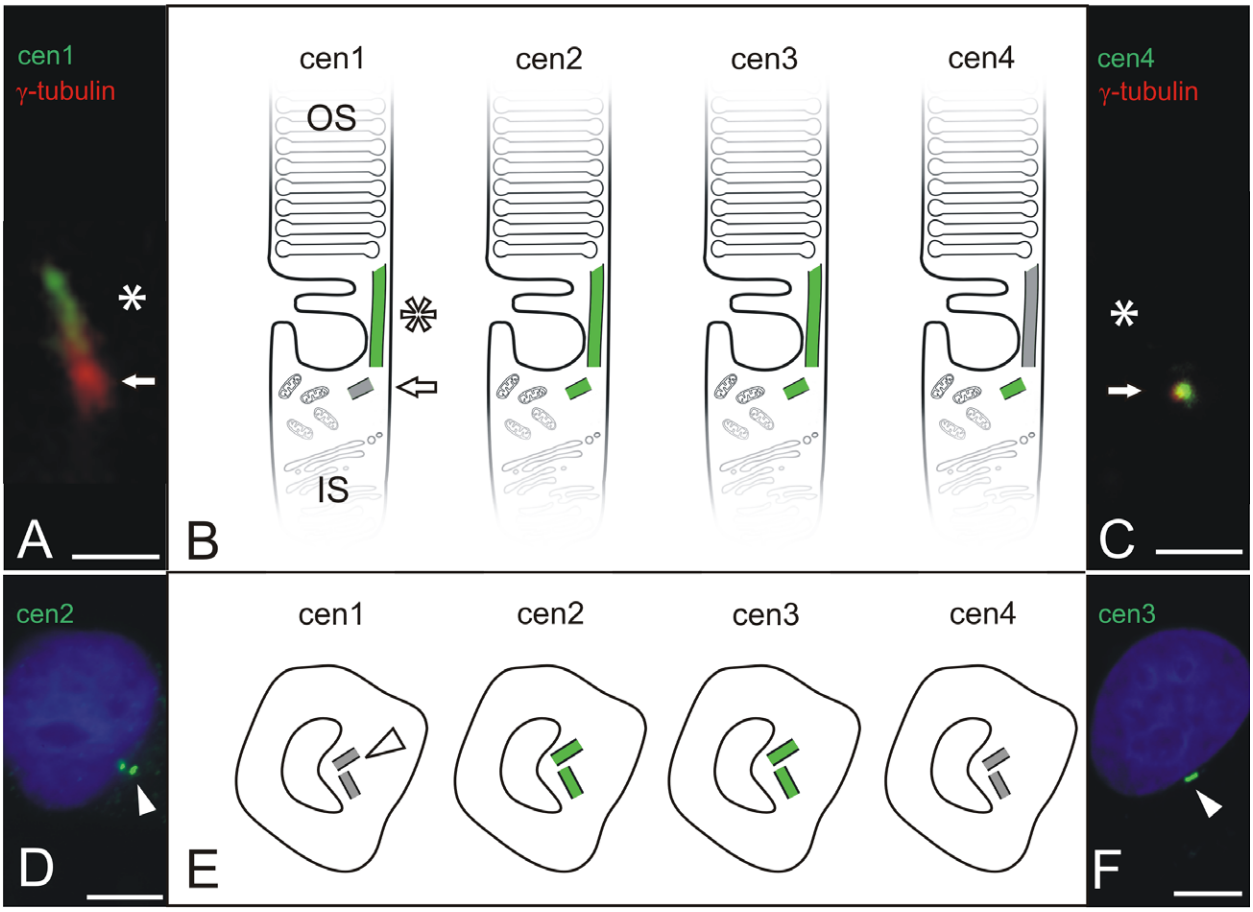

Fig. 2. Subcellular localization of centrin isoforms in the mouse retina and schematic illustrations of the centrin isoform localization in photoreceptor cells and non-photoreceptor cells. High magnification of indirect anti-centrin immunofluorescence (green) of the photoreceptor connecting cilium (asterisk), its basal body (arrow) and centrioles of centrosomes in non-photoreceptor-cells. (A) Preadsorbed centrin 1 antibodies exclusively stains the connecting cilium, which appears as a green strip (asterisk) but not the basal body which is marked by anti-γ-tubulin staining (red, arrow). (B) Schematic illustration of the differential localization of the four distinct centrin isoforms in specialized vertebrate photoreceptor cells. Centrin 1, centrin 2 and centrin 3 are localized in the connecting cilium (asterisk). Centrin 2 and centrin 3 are additionally present in the basal body (arrow). Cen4 is restricted to the basal body (arrow). (C) Preadsorbed centrin 4 antibodies exclusively stains the basal body which is double marked by anti-γ-tubulin staining (red, arrow). (D and F) Preadsorbed centrin 2 and centrin 3 specific antibodies (green, arrowhead) react in dot pairs representing the centriole pairs in non-photoreceptor-cells. DAPI-(4′,6-diamididino-2-phenylindole) stains the nuclear DNA (blue). (E) Schematic illustration of the differential localization of the four centrin isoforms in non-specialized eukaryotic cells. Centrin 2 and centrin 3 are localized in centrioles of centrosomes, while centrin 1 and centrin 4 are not associated with centrosomes (arrowhead). Bars in: A and C = 1 μm; D and F = 4.1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

5. Centrin/transducin complexes may regulate light-dependent transducin translocations

Based on our knowledge of the differential expression of all four centrin isoforms in rodent photoreceptor cells (Gießl et al., 2004a; Gießl et al., 2004b), we addressed whether the Ca^{2+}-dependent assembly of the centrin/transducin complex is restricted to the centrin isoform 1 or if this interaction also occurs between transducin and other centrin isoforms. To evaluate the binding of all centrin isoforms to transducin, we deployed several independent but complementary interaction assays (summarized in Table 1). Applying these techniques we demonstrated that not only centrin 1, but also the three other centrin isoforms, centrin 2, 3, and 4, bind with high affinity to transducin (Gießl et al., 2004a; Gießl et al., 2004b; Pulvermüller et al., 2002; Wolfrum et al., 2002). Kinetic light scattering experiments further indicated the specificity of this protein–protein interaction: on the one hand, centrins did not bind to other molecules of the visual signal transduction cascade, neither with arrestin, rhodopsin and rhodopsin-kinase nor with the visual phosphodiesterase (Gießl et al., 2004b; Pulvermüller et al., 2002). On the other hand, the EF-hand proteins recoverin and calmodulin, which are highly expressed in the photoreceptor cells, did not feature significant affinities to transducin (Gießl et al., 2004b; Pulvermüller et al., 2002; Wolfrum et al., 2002).

In conclusion, all analyses indicate that the assembly of the centrin/transducin complexes is mediated by the βγ-subunits of transducin with high specificity (Gießl et al., 2004a; Gießl et al., 2004b; Pulvermüller et al., 2002; Wolfrum et al., 2002). These protein–protein interactions are

Table 1
Summery of all interaction and colocalization analyses of transducin and all centrin isoforms

Binding assays	Ca^{2+}	$G_t$holo	$G_t\alpha$	$G_t\beta\gamma$	Centrin isoforms	Affinity	Ref.
Overlay	+	np	–	+	1	np	B
GST-pull down	nd	+	–	+	1, 2, 3, 4	np	C
IP	+	+	–	–	1, 2, 3, 4	np	A, B
Size-exclusion	+	+	nd	nd	1, 2, 3, 4	np	A, C
Centrifugation	+	+	nd	nd	1	np	A
KLS	+	+	nd	nd	1, 2, 3, 4	Cen1p +++ Cen2p +++ Cen3p + Cen4p +++	A, D
Colocalization	nd	+	+	+	1, 2, 3, 4	np	A, B, C, D

The summery of all binding studies reveals a Ca^{2+}-dependent interaction of all centrin isoforms with the heterotrimeric G-protein transducin via its βγ-subunit. Also, immunoelectron microscopy and indirect immunofluorescence analyses demonstrate, that centrins and transducin are concentrated in the same subcellular compartment of the connecting cilium of rod photoreceptor cells, at the inner surface of the photoreceptor axoneme (nd, not detected; np, analysis not possible; GST, gluthadione-*S*-transferase; IP, immunoprecepitation; KLS, kinetic light scattering; A, Pulvermüller et al., 2002; B, Wolfrum et al., 2002; C, Gießl et al., 2004a; D, Gießl et al., 2004b).

strictly dependent on the Ca^{2+}-concentration in the assays. Titrations of the centrin isoforms in kinetic light scattering experiments in the presence of Ca^{2+} showed differences in the affinity of the centrin isoforms to transducin. Centrin 3 indicated a significantly lower affinity to the transducin holo-protein than the other centrin isoforms (Gießl et al., 2004a). Also a titration curve of centrin 3-transducin interaction announced that each transducin binds a monomer of centrin 3 in contrast to the other isoforms, which probably bind to the G-protein as homooligomers (Gießl et al., 2004a).

What are the functions of the centrin/G-protein complexes in the photoreceptor cell? The spatial colocalization of centrin 1, 2 and 3 with transducin in the inner lumen of the connecting cilium (Fig. 3) provides evidence that in photoreceptor cells, the formation of centrin/G-protein complexes should occur in this subciliary compartment (Pulvermüller et al., 2002). The high affinities of centrin 1 and 2 to transducin indicate that they are the most important candidates for this Ca^{2+}-dependent protein–protein interaction. In photoreceptor cells, light modulated changes of free Ca^{2+} in the outer segment are described. It is well accepted that in operating range of single quantum detection of rods, a dramatic Ca^{2+} drop occurs in the outer segment after light activation of the visual cascade (Molday & Kaupp, 2000). However, Matthews, Fain and coworkers (Matthews & Fain, 2001; Matthews & Fain, 2002; Woodruff, Lem, & Fain, 2004; Cilluffo, Matthews, Brockerhoff, & Fain, 2004) recently observed an increase of the free Ca^{2+} in bright light under rod-saturated conditions. If light-induced changes in the free Ca^{2+} in the outer segment are transmitted into the connecting cilium at all, actually the later condition may reflect the scenario of the transducin localization in the inner segment and the induction of the assembly of centrin/G-protein complexes in the cilium. However, the presence of Ca^{2+}-ATPases (PMCAs) in the plasma membrane of the connecting cilium also supports an outer segment-independent Ca^{2+}-homeostasis in the connecting cilium (Krizaj et al., 2002; Krizaj, Liu, & Copenhagen, 2004). Anyway, the Ca^{2+}-induced assembly of centrin1/2/G-protein complexes may contribute to a barrier for further light-dependent exchanges of transducin between the two photoreceptor cell compartments, the inner and outer segment (Fig. 4) (Wolfrum et al., 2002).

Recent investigations on the role of centrin phosphorylation in photoreceptor cells indicate that the formation of centrin/G-protein complexes may not only be triggered by Ca^{2+} ions but that it may also be modulated by light-dependent phosphorylation of centrins (Gießl et al., 2004b; Trojan et al., in preparation) (Fig. 4).

6. Summary and conclusions

Centrins are members of a conserved subfamily of EF-hand Ca^{2+}-binding proteins. During the past years, four centrin isoforms were identified and found to be associated with the centrioles of centrosomes or centrosome-related structures 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, differentially localized in the connecting cilium and at the basal bodies. Centrin isoforms bind with high affinity to transducin in a strictly Ca^{2+}-dependent manner. The centrin isoforms 1 and 2 may regulate the light-dependent translocation of transducin through the lumen of the connecting cilium. 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. Ongoing studies indicate that the function of the centrin isoforms is also regulated by their phosphorylation. Results of our current analysis of putative centrin-associated proteins (other than transducin) in the mammalian retina will gather further insights into the role of centrins in photoreceptor cell function.

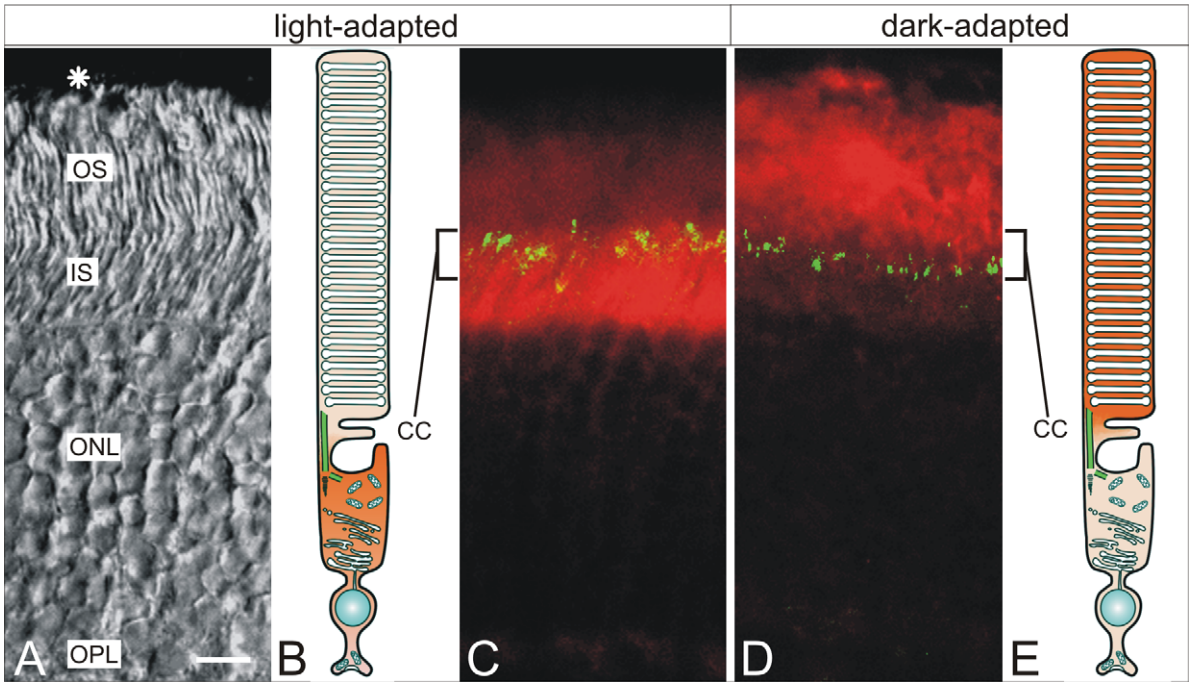

Fig. 3. Light-dependent translocation of transducin in murine retina. (A–C) Light-adapted mouse retina. (D and E) Dark-adapted mouse retina. (A) Differential interference contrast image of a longitudinal cryosection of photoreceptor cells divided into the outer segment (OS), inner segment (IS), the outer nuclear layer (ONL) and the outer plexiform layer (OPL). The asterisk indicates retinal pigment epithelium. (B) Schematic representation of a light-adapted rod photoreceptor cell. Red color indicates transducin and green color centrin distribution. (C) Indirect anti-transducin (red) and anti-centrin (green) immunofluorescence of light-adapted retina in (A). Transducin is predominantly localized in the inner segment and in the perikaryon containing the nucleus in the outer nuclear layer down to the synaptic terminal in the outer plexiform layer. Only minor anti-transducin immunolabeling is present in the outer segment. Green double immunofluorescence of the pan-centrin monoclonal antibody (clone 20H5), which detects all four centrin isoforms, is present in the basal body and in the connecting cilium of the photoreceptor cell. (D) Double labeling of a dark-adapted retinal cryosection of transducin (red) and centrin (green). Transducin is predominantly localized in the outer segment of the dark-adapted retina, whereas centrin (green) stays in the cilium complex. (E) Schematic representation of a dark-adapted rod photoreceptor cell. Red color indicates the transducin distribution and green color the localization of centrin in the connecting cilium. Bars in: A = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

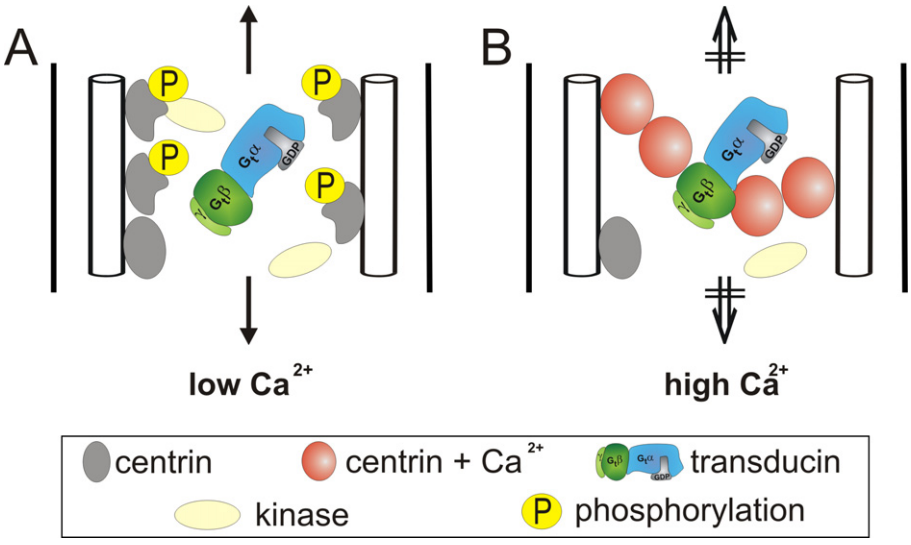

Fig. 4. Extended barrier hypothesis of Ca^{2+}-dependent centrin-transducin assembly in the connecting cilium of vertebrate photoreceptor cells (Gießl et al., 2004a, 2004b). (A) Under low free Ca^{2+}-concentrations centrin is phosphorylated and not active, so transducin can float through the inner lumen of the connecting cilium. (B) If free Ca^{2+} increases in the cilium, centrin is dephosphorylated and activated by Ca^{2+} which induces the centrin-transducin complex assembly. Later complex may either contain the $G_t$holo or only $G_t\beta\gamma$ subunits.

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