Charakterisierung funktioneller Domänen von Centrin-Isoformen

Dissertation
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Dekan:

1. Berichterstatter:

2. Berichterstatterin:

Tag der mündlichen Prüfung:

Wer noch staunen kann,
wird auf Schritt und Tritt belohnt.

(Oskar Kokoschka)

Anmerkungen

Die vorliegende Arbeit ist kumulativ gestaltet und besteht im Kern aus den drei Publikationen Trojan *et al*., 2008 (Dissertation Publikation I), Thissen *et al*., eingereicht (Dissertation Publikation II) und Trojan *et al*., 2008a (Dissertation Publikation III). Anzumerken ist, dass sich die Dissertation Publikation II im Begutachtungsprozess bei der Zeitschrift „FEBS letters“ befindet und somit als Manuskript beigefügt ist. Die beiden anderen Publikationen liegen seit Januar 2008 als elektronische Versionen bei den Zeitschriften „BBA Molecular Cell Research“ (Dissertation Publikation I) und „Progress in Retinal and Eye Research“ (Dissertation Publikation III) vor. Die Ergebnisse aller drei Publikationen sind im Abschnitt „Zusammenfassung der Ergebnisse“ dargestellt. Alle weiteren Publikationen, zu denen im Rahmen dieser Arbeit Beiträge geleistet werden konnten, sind im Text mit einem Stern (*) hervorgehoben. Eine detaillierte Übersicht der geleisteten Beiträge zu jeder Publikation wird im Anhang gegeben.

Veröffentlichungen und Kongressbeiträge

Teile der vorliegenden Dissertation wurden auf internationalen Kongressen vorgestellt und in folgenden Zeitschriften publiziert:

Publikationen

Trojan P, Krauss N, Choe HW, Gießl A, Pulvermüller A, Wolfrum U (2008a) Centrins in retinal photoreceptor cells: Regulators in the connecting cilium. Prog Retin Eye Res. 2008 Jan 31; Epub ahead of print.

Thissen MC, **Trojan P**, Kriegelstein J, Wolfrum U, Klumpp S Dephosphorylation of centrins by protein phosphatase 2Ça and 2Cß. FEBS letters eingereicht

Trojan P, Rausch S, Gießl A, Klemm C, Krause E, Pulvermüller A, Wolfrum U (2008) Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein. Biochim Biophys Acta. 2008 Jan 17; Epub ahead of print.

Giessl A, **Trojan P**, Rausch S, Pulvermüller A, Wolfrum U (2006) Centrins, gatekeepers for the light-dependent translocation of transducin through the photoreceptor cell connecting cilium.Vision Res. Dec;46 (27):4502-9.

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Kongressbeiträge

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Trojan P, Rausch S, Thissen MC, Gießl A, Wünschig D, Klumpp S, Pulvermüller A, Wolfrum U (2006) Light-dependent CK2 mediated phosphorylation of centrinsregulates the formation of centrin/transducin complexes in photoreceptor cells. 6th Annual Meeting of the Interdisciplinary Science Network Molecular & Cellular Neurobiology, Mainz p13

Thissen M**, Trojan P**, Giessl A, Wolfrum U, Krieglstein J, Klumpp S. (2006) Protein phosphatase 2C dephosphorylates centrins, International Symposium on Neuroprotection and Neurodegenration, Münster

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Danke!

Für meine Familie….

Inhaltsverzeichnis

Abbildungsverzeichnis

1. Einleitung

1.1. Photorezeptorzellen und Photorezeption in der Retina von Vertebraten

Der Sehprozess wird durch die Detektion eines Lichtsignals durch die Photorezeptorzellen in dem äußeren Bereich der Retina von Vertebraten ausgelöst (Abb. 1A). Grundsätzlich lassen sich zwei unterschiedliche Typen von Photorezeptoren in der Retina von Vertebraten unterscheiden. Zum einen die Stäbchen für das Sehen bei schwacher Beleuchtung (skotopisches Sehen), zum anderen die Zapfen, die für das Farbsehen (photopisches Sehen) verantwortlich sind.

Die Stäbchen und Zapfen der Vertebraten sind in höchstem Maße polarisierte und spezialisierte Nervenzellen. Sie sind aus morphologisch und funktionell unterschiedlichen subzellulären Kompartimenten aufgebaut (Abb. 1B). Das lichtsensitive Außensegment der Photorezeptoren ist über ein modifiziertes nicht-motiles Cilium, dem so genannten Verbindungscilium, mit dem Innensegment verbunden (Abb. 1C).

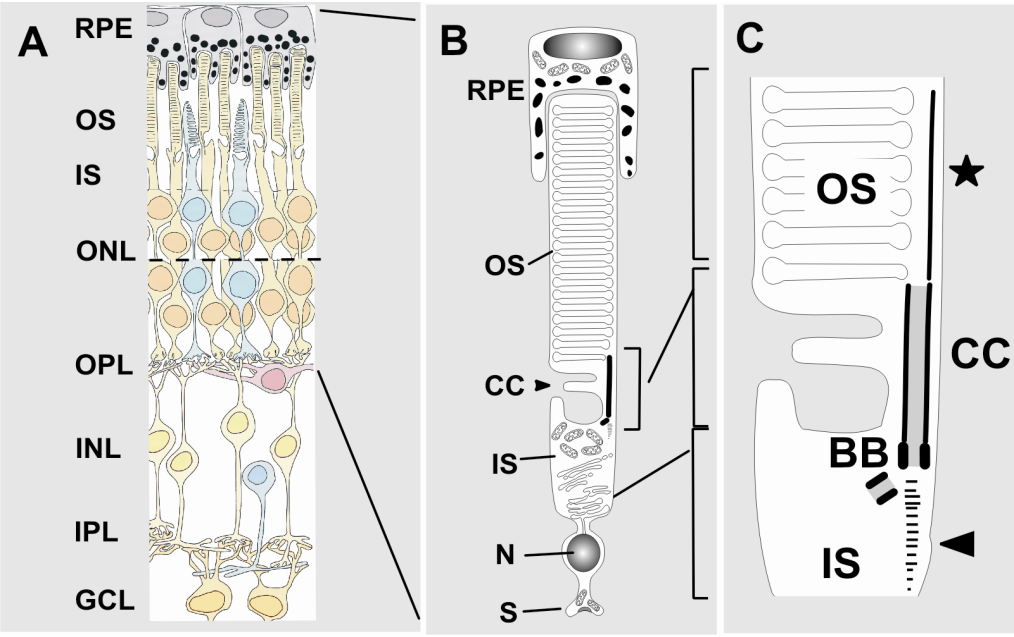

Abb.1: Schematische Darstellung der Retina und einer Stäbchen-Photorezeptorzelle von Vertebraten. (A) Schematische Darstellung eines Längsschnitts durch die Retina von Vertebraten. Die Außen- und Innensegmente (OS und IS) der Photorezeptoren bilden die äußerste Schicht der Retina (Photorezeptorzellschicht). Die apikalen Bereiche der OS werden von den Zellen des retinalen Pigmentepithels (RPE) umschlossen. Die Zellkerne der Photorezeptoren bilden die äußere Körnerschicht (ONL). Die äußere plexiforme Schicht (OPL) wird von den Synapsen der Photorezeptoren und nachgeschalteten Neuronen, wie Bipolarzellen und Horizontalzellen, gebildet. Die Perikaryen dieser nachgeschalteten Neurone sind in der inneren Körnerschicht (INL) lokalisiert,

auf die die innere plexiforme Schicht (IPL) folgt. Die Ganglienzellen (GCL) leiten die eingehenden Signale über den *Nervus opticus* ins Gehirn weiter. **(B)** Schematische Darstellung einer Stäbchen-Photorezeptorzelle von Vertebraten. Die Photorezeptorzelle besteht aus einem lichtsensitiven OS, das über ein spezialisiertes, unbewegliches Cilium, dem so genannten Verbindungscilium (CC), mit dem IS verbunden ist. Am äußersten Ende des OS schließt sich das RPE an. Die Zellen des RPE umschließen den distalen Bereich der OS mit ihren microvillären Fortsätzen. **(C)** Schematische Darstellung des ciliären Apparates von Photorezeptorzellen. Der ciliäre Apparat besteht aus dem CC, das eine verlängerte Übergangszone motiler Cilien darstellt, und dem Basalkörperkomplex (BB). Ciliäre axonemale Mikrotubuli erstrecken sich als Verlängerung des CC in das OS der Photorezeptoren (Stern). Die Cilienwurzel erstreckt sich vom Basalkörperkomplex in das IS der Photorezeptorzelle (Pfeilspitze). (nach Dissertation Publikation III).

Das Innensegment der Photorezeptoren beherbergt alle Zellorganellen, die für den Stoffwechsel eukaryotischer Zellen notwendig sind. Zusätzlich umfasst es den Zellkern, an den sich die Synapse anschließt. Das Außensegment ist aus mehreren hundert Membranstapeln, den so genannten Diskmembranen, aufgebaut, an denen die visuelle Signaltransduktionskaskade stattfindet (Abb. 2). Die visuelle Signaltransduktion ist eine typische G-Protein gekoppelte Signaltransduktion. Sie beginnt mit der Aktivierung des Sieben-Transmembran-Rezeptors Rhodopsin durch Photonen. Das Rhodopsin ist an das nachgeschaltete, visuelle heterotrimere G-Protein Transducin gekoppelt. Durch die Aktivierung von Rhodopsin kommt es, durch den Austausch von GDP durch GTP, zur Aktivierung von Transducin (Abb. 2) (Fung *et al.*, 1981; Heck und Hofmann, 2001). Das heterotrimere G-Protein dissoziiert in die α- und die βγ-Untereinheiten, wobei die GTP-gebundene α-Untereinheit weitere, regulatorische Proteine, wie die cGMP-Phosphodiesterase, stimuliert. Dieses Enzym hydrolysiert cGMP zu 5`GMP und induziert dadurch den Verschluss cGMP-abhängiger Kanäle in der Plasmamembran der Photorezeptorzellen (Heck und Hofmann, 1993; Okada *et al.*, 2002). Durch das Verschließen dieser Kanäle sinkt die cytoplasmatische Konzentration von Kationen (hauptsächlich Na^+ und Ca^{2+}) was zur Hyperpolarisation der Plasmamembran führt. Die Hyperpolarisation senkt die Ausschüttung des Neurotransmitters Glutamat an der Synapse der Photorezeptorzelle (Molday und Kaupp, 2000).

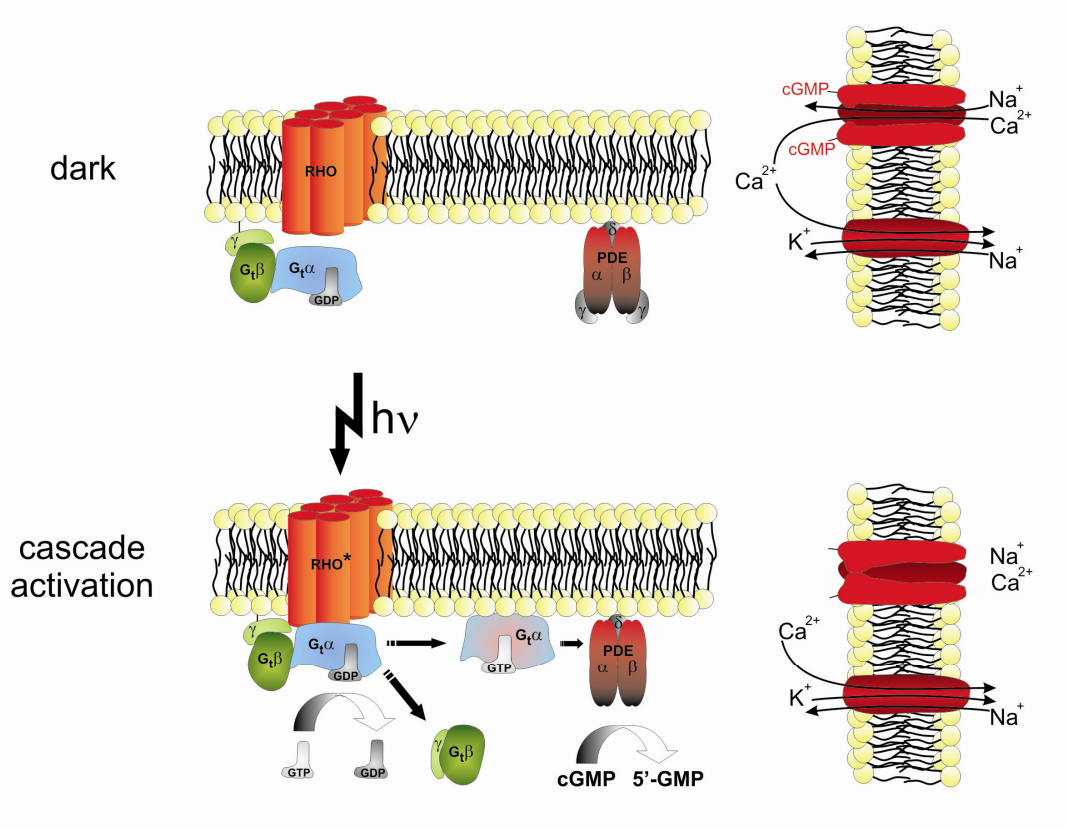

Abb. 2: Schematische Darstellung der visuellen Signaltransduktionskaskade in den Stäbchen der Vertebraten. Die visuelle Signaltransduktionskaskade ist an den Diskmembranen der Außensegmente lokalisiert. Im Dunkeln (dark) sind die Proteine der Signaltransduktionskaskade inaktiv. Ionenkanäle in der Plasmamembran der Photorezetorzelle sind durch die Bindung von zyklischem GMP (cGMP) permeabel für Na^+ und Ca^{2+}. Dadurch bleibt die Plasmamembran der Photorezeptoren depolarisiert. Trifft ein Photon (hν) auf den Sieben-Transmembran-Rezeptor Rhodopsin (RHO) wird dieser aktiviert (RHO*) (cascade activation). Dies führt zur Bindung des visuellen heterotrimeren G-Proteins Transducin (G_t), das aus den drei Untereinheiten $G_t\alpha$, $G_t\beta$ und $G_t\gamma$ aufgebaut ist. Die Bindung von Transducin an RHO* führt zum Austausch von GDP durch GTP an $G_t\alpha$. Durch die Aktivierung von G_t dissoziiert $G_t\alpha$ von $G_t\beta/\gamma$ und aktiviert die Phosphodiesterase (PDE) durch die Abspaltung von deren inhibitorischen γ-Untereinheiten. Die PDE hydrolysiert cGMP zu 5`-GMP und induziert dadurch den Verschluss der cGMP-abhängigen Ionenkanäle in der Plasmamembran der Photorezeptoren. Dies führt zum Abfall der intrazellulären Ca^{2+}-Konzentration und zur Hyperpolarisation der Photorezeptorzellen (Verändert nach (Gießl, 2004)).

Die Diskmembranen des Außensegments unterliegen permanenter Erneuerung. Neu gebildete Membranen werden an der Basis der Außensegmente hinzugefügt. Am distalen Ende der Außensegmente werden die Diskmembranen abgeschnürt und von den Zellen des retinalen Pigmentepithels phagozytiert und anschließend abgebaut (Young, 1976). Um einen solchen permanenten Durchsatz der Membranen aufrecht zu erhalten, sind sehr effiziente Transportmechanismen aus dem Innensegment, dem Ort der Proteinbiosynthese, ins Außensegment, dem Ort ihrer Funktion, notwendig (Sung und Tai, 2000). Nach dem Transport vom Innen- ins Außensegment bleiben die meisten Proteine dauerhaft an ihrem Bestimmungsort. Dazu zählen beispielsweise Ionenkanäle oder das visuelle Sehpigment

Rhodopsin. Im Gegensatz dazu existieren Proteine, wie zum Beispiel Transducin oder Arrestin, deren Lokalisation vom Belichtungszustand der Photorezeptorzelle abhängt. Diese Proteine werden lichtabhängig zwischen dem Außen- und Innensegment hin und her transportiert (Dissertation Publikation III, Fig 9). Solche intrazellulären Translokationen wurden erstmals vor 20 Jahren beobachtet und stellen seit einigen Jahren ein Feld sehr intensiver Forschungen dar (Brann und Cohen, 1987; Philp *et al.*, 1987; Whelan *und* Mcginnis, 1988; Organisciak *et al.*, 1991; Mcginnis *et al.*, 2002; Pulvermüller *et al.*, 2002; Sokolov *et al.*, 2002; Mendez *et al.*, 2003; Sokolov *et al.*, 2004; Reidel *et al.*, 2008). Diese differenziellen, lichtabhängigen Translokationen von Bestandteilen der visuellen Signaltransduktion dienen nach vorliegenden Erkenntnissen einer langsamen, aber nachhaltigen Adaption der Photorezeptorzellen (Sokolov *et al.*, 2002; Hardie, 2003; Sokolov *et al.*, 2004; Frechter und Minke, 2006; Gießl *et al.*, 2006*). Leider ist bislang wenig über die molekularen und zellulären Mechanismen dieser intrazellulären Transportprozesse bekannt (Chen *et al.*, 2005; Calvert *et al.*, 2006; Strissel *et al.*, 2005; Reidel *et al.*, 2008). Generell sind zwei verschiedene Mechanismen für solche Translokationen denkbar. Zum einen die Diffusion von Molekülen, zum anderen aber der aktive Transport mittels molekularer Motoren entlang von Cytoskelettelementen (Reidel *et al.*, 2008). Die aktuelle Literatur sieht die molekulare Diffusion als treibende Kraft dieses intrasegmentalen Austauschs von Transducin und Arrestin an (Calvert *et al.*, 2006). Zudem gibt es Hinweise, dass die Diffusion von Transducin durch die Bindung von regulatorischen Proteinen im Verbindungscilium beeinflusst wird (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2006*; Roepman und Wolfrum, 2007). Allerdings konnte auch gezeigt werden, dass ein intaktes Mikrotubuli- und Aktincytoskelett für die Translokationen von Arrestin und Transducin während der Dunkeladaption essentiell ist (Peterson *et al.*, 2005; Reidel *et al.*, 2008). Diese aktuellen Forschungsergebnisse deuten auf unterschiedliche molekulare Mechanismen bei der Translokation von Transducin und Arrestin während der Hell- und Dunkeladaption der Photorezeptorzellen hin.

Unabhängig davon, welche der Hypothesen zutreffend ist, stellt das Verbindungscilium die einzige cytoplasmatische Verbindung zwischen dem Innen- und dem Außensegment der Photorezeptorzelle dar (Abb. 1 B und C). Aus diesem Grund müssen alle Transportprozesse zwischen diesen beiden subzellulären Kompartimenten durch das Verbindungscilium stattfinden. Bei der Passage durch das Verbindungscilium könnten regulatorische Proteine die lichtabhängige Translokation beeinflussen (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2006*). Während der Passage durch das

Verbindungscilium colokalisiert das visuelle G-Protein Transducin mit den Proteinen der Centrin-Familie (Abb. 3). Diese Ca^{2+}-bindenden Proteine sind in der Lage, Komplexe mit dem visuellen G-Protein Transducin zu bilden (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*). Durch ihre prominente Lokalisation im Verbindungscilium der Photorezeptorzellen stellen sie möglicherweise eine Ca^{2+}-regulierte Barriere für das visuelle G-Protein Transducin bei dessen lichtabhängiger Translokation zwischen dem Außen- und Innensegment dar (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Die Analyse der Regulation der Interaktion von Centrinen mit dem visuellen G-Protein Transducin stand im Zentrum der vorliegenden Arbeit.

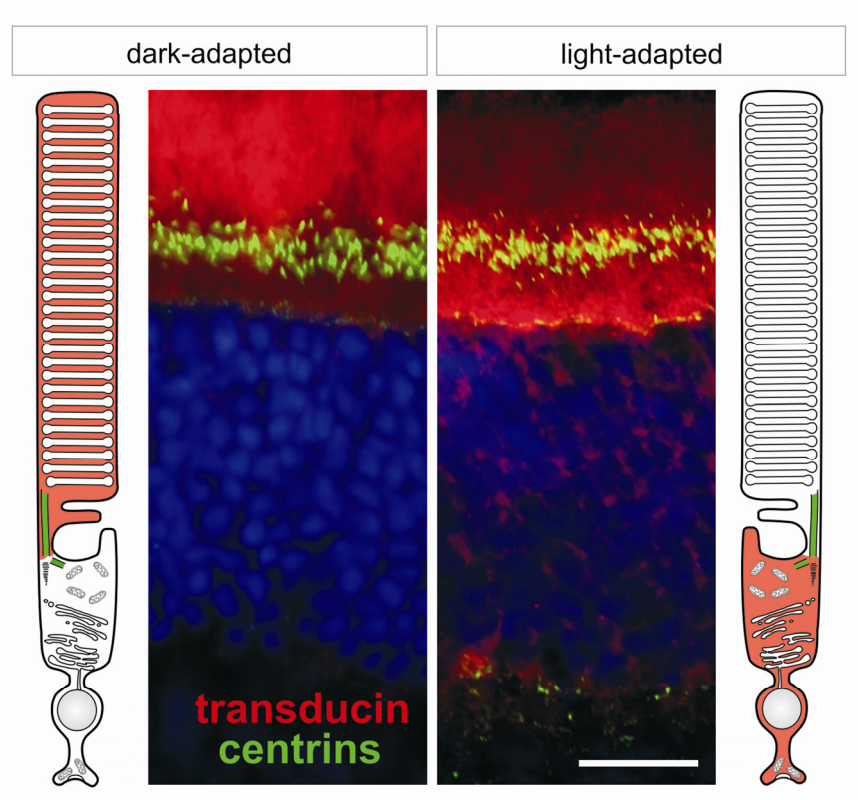

Abb. 3: Lichtabhängige Translokation von Transducin in den Photorezeptorzellen von Vertebraten. Immunhistochemische Analyse der subzellulären Lokalisation von Transducin (rot) und Centrinen (grün) in den Photorezeptorzellen von Ratten. In Dunkel adaptierten Photorezeptorzellen (dark-adapted) befinden sich ~80% des Transducins im Außensegment. Die Centrine sind am Verbindungscilium lokalisiert, das die einzige cytoplasmatische Verbindung zwischen Außen- und Innensegment darstellt. Während der Helladaption (light-adapted) translozirt Transducin ins Innensegment der Photorezeptorzelle. Diese intrazelluläre Translokation findet durch das Verbindungscilium statt, wo dann beide Proteine colokalisieren. Größenbalken: 13 µm.

1.2. Centrine

Obwohl der aktuelle Stand der Forschung über Centrine in dem Übersichtsartikel Trojan *et al.*, 2008a (Dissertation Publiaktion III) ausführlich zusammengefasst ist, sollen hier in aller Kürze die Centrine nochmals vorgestellt werden. Centrine, die in der Literatur früher auch als Caltractine bezeichnet wurden, sind Ca^{2+}-bindende Phosphoproteine mit einer Länge von ~170 aa und einem Molekulargewicht von ~20 kDa (Dissertation Publikation III) (Salisbury, 1995; Schiebel und Bornens, 1995; Gießl *et al.*, 2004b*). Auf Grund ihrer Struktur sind die Centrine in die große Familie der Ca^{2+}-bindenden Proteine mit EF-Händen einzuordnen, zu der ebenfalls die Proteine Parvalbumin, Troponin C und der sehr gut charakterisierte Ca^{2+}-Sensor Calmodulin gehören (Kretsinger und Nockolds, 1973; Kretsinger, 1976; Nakayama und Kretsinger, 1994; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*; Roepman und Wolfrum, 2007). Erstmals wurden Centrine als Bestandteile der Basalkörper und Flagellenwurzeln von Flagellen der einzelligen Grünalgen *Tertaselmis striata* beschrieben (Salisbury *et al.*, 1984). Centrine sind in den Grünalgen an der Ca^{2+}-abhängigen, aber ATP-unabhängien Kontraktion der Flagellenwurzeln beteiligt (Salisbury *et al.*, 1984). Mittlerweile wurden Centrine als Bestandteile von Centrosomen und Basalkörpern aller analysierten eukaryotischen Organismen identifiziert (Salisbury, 1995; Schiebel und Bornens, 1995; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Centrine sind zwar in fast allen Organismen des eukaryotischen Reichs, von der Hefe bis zum Menschen, beschrieben worden, besitzen aber keine homologen Proteine in den Prokaryoten (Dissertation Publikation III) (Gießl *et al.*, 2004b*; Salisbury, 2007). Vergleiche der Aminosäure- und cDNA-Sequenzen bekannter Centrine aus unterschiedlichsten Organismen innerhalb der Eukaryoten zeigen, dass es sich bei diesen Proteinen um eine sehr hoch konservierte Proteinfamilie handelt (Abb. 4) (Dissertation Publikation III) (Gießl *et al.*, 2004b*; Salisbury, 2007).

Trotz ihrer hohen Konservierung gibt es deutliche Unterschiede in der Anzahl der Centrin-Isoformen in den verschiedenen Organismen. In niederen Organismen wie der Bäckerhefe *Saccharomyces cerevisae* ist bislang nur das Gen *CDC31* bekannt, welches für das Centrin-homologe Protein Cdc31p codiert (Baum *et al.*, 1986; Baum *et al.*, 1988). Ebenfalls nur ein Centrin codierendes Gen (*VFL2*) wurde bislang in Grünalgen beschrieben (Wright *et al.*, 1985). Allerdings weisen Proteomanalysen auf die Existenz drei verschiedener Centrine in Grünalgen hin (Keller *et al.*, 2005). Bis zu 35 verschiedene Centrin-Isoformen konnten in dem ciliären Einzeller *Paramecium tetraurelia* nachgewiesen werden (Madeddu *et al.*, 1996; Gogendeau *et al.*, 2008). In Säugetieren konnten bislang bis zu vier Gene identifiziert werden die für Centrine codieren. So besitzen die Rodentia *Mus musculus* und

Rattus norvegicus jeweils vier Centrin Gene (*MmCetn1-4* und *RnCetn1-4*), wohingegen beim Menschen nur drei funktionale Centrin-codierende Gene bekannt sind (*HsCETN1-3*) (Dissertation Publikation III) (Lee und Huang, 1993; Middendorp *et al.*, 1997; Gavet *et al.*, 2003; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*). Phylogenetische Analysen der Aminosäuresequenzen der Centrin-Isoformen in höheren Organismen zeigen - trotz ihrer hohen Konservierung - eine deutliche Einteilung in zwei unterschiedliche Gruppen. Dabei sind die Centrin-Isoformen 1, 2 und 4 sehr nah miteinander verwandt und ähneln dem Centrin der Grünalgen (Abb. 4). Die zweite Gruppe repräsentiert die Isoform 3, die dem Cdc31p der Hefe ähnelt, was auf verschiedene evolutionäre Ursprünge hindeutet (Dissertation Publikation III) (Gießl *et al.*, 2004b*; Salisbury, 2007).

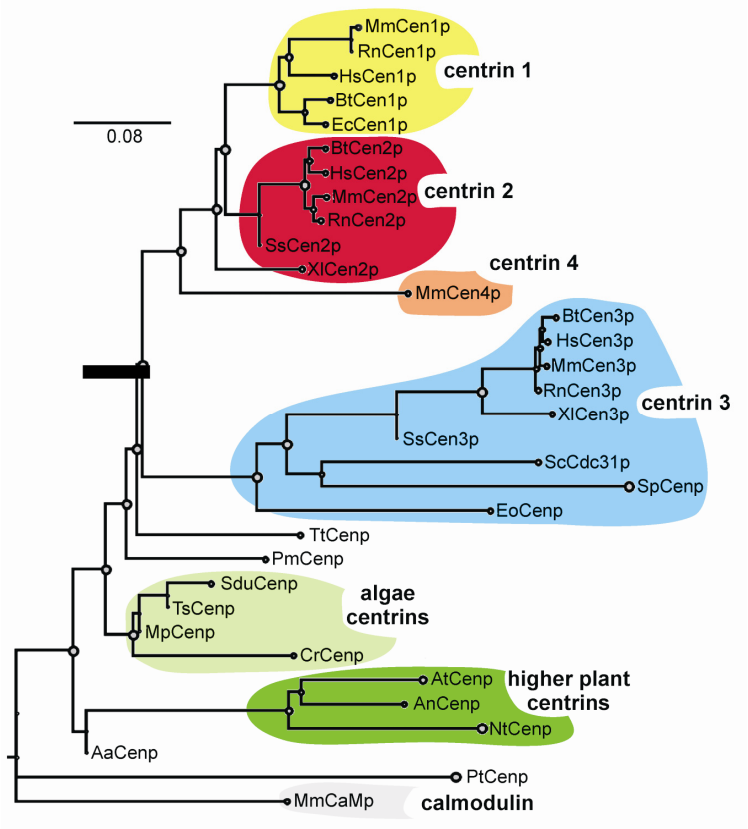

Abb. 4: Phylogenetische Verwandtschaft von Centrinen aus verschiedenen Organismen. Vergleich des Verwandtschaftsgrads der Aminosäure-Sequenzen 33 verschiedener Centrine und von Calmodulin der Maus mittels der Software Genious3.0 pro (Biomatters Ltd.). Die Centrine lassen sich phylogenetisch in unterschiedliche Untergruppen einteilen. Die Centrine der Vertebraten 1-4, die Centrine der Algen (algae centrins), die Centrine höherer Pflanzen (higher plant centrins) und Calmodulin. (MmCaMp= *Mus musculus* calmodulin AN: CAA43674; XlCenp2p, 3p = *Xenopus laevis* centrin 2, 3 AN: BC054948, AAG30507; PtCenp = *Paramecium tetraurelia* centrin AN: AAB188752; BtCen1p–3p = *Bos taurus* centrins 1–3 AN:NP001072974, NP001033604, AAI20178; SsCen2p, 3p = *Sus scrofa* centrins 2, 3 AN: AAY33861, AAY67906; EcCen1p = *Elaphodus cephalophus* centrin1 AN: ABP57024; HsCen1p–3p = *Homo sapiens* centrins 1–3 AN: AAH29515, AAH13873, AAH05383; MmCen1p–4p = *Mus musculus* centrins 1–4 AN: NP031619, NP062278, NP031710, NP665824; RnCen1p–3p = *Rattus norvegicus* centrins (completed with own data) AN: AAK20385, AAK20386, AAK83217; AtCenp = *Arabidopsis thaliana* centrin AN:CAA08773, AnCenp = *Atriplex nummularia* centrin AN: P41210; NtCenp = *Nicotiana tabacum* centrin AN: AAF07221; CrCenp =

Chlamydomonas reinhardtii centrin (Vfl2p) AN: EDO98562; SduCenp = *Scherffelia dubia* centrin AN: Q06827; MpCenp = *Micromonas pusilla* centrin AN: CAA58718;EoCenp = *Euplotes octocarinatus* centrin AN: CAB40791; TsCenp = *Tetraselmis striata* centrin AN: P43646; ScCdc31p = *Saccharomyces cerevisiae* AN:CAA52609; SpCenp = *Schizosaccharomyces pombe* centrin AN: CAA20670; PmCenp = *Prorocentrum minimum* centrin AN: ABI14404; Aa-Cenp = *Acetabularia acetabulum* centrin AN: AAM00015; TtCenp = *Tetrahymena thermophila* AN: AAF66602). Der Baum ist nicht vollständig (AN:accession number). (Verändert nach Dissertation Publikation III).

Expressionsanalysen der einzelnen Centrine in Säugetieren ergaben, dass die Centrin-Isoformen 2 und 3 (Cen2p und Cen3p) ubiquitär in allen somatischen Zellen exprimiert werden. Frühere Arbeiten unserer Arbeitsgruppe zeigten, dass die Centrin-Isoform 1 (Cen1p) ausschließlich in ciliären Zelltypen exprimiert wird (Wolfrum und Salisbury, 1998; Gießl *et al.*, 2004a*; Gießl *et al.*, 2006*). Die Isoform 4 (Cen4p) wurde bislang nur in ciliären Zellen des *Plexus choroideus* im Gehirn und in ciliären Sinneszellen detektiert. (Gavet *et al.*, 2003; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Analysen der subzellulären Lokalisation von Centrinen zeigten, dass Cen2p und Cen3p an den Centriolen der Centrosomen, den Basalkörpern von Cilien und in der periciliären Matrix von Centrosomen detektierbar sind. Im Gegensatz zu diesen Isoformen konnten wir in früheren Arbeiten zeigen, dass Cen1p nur in der Übergangszone von Cilien lokalisiert ist (Gießl *et al.*, 2004a*; Gießl *et al.*, 2006*). Die Lokalisation von Cen4p andererseits beschränkt sich auf den Basalkörper von Cilien (Gavet *et al.*, 2003; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*).

Aufgrund ihrer prominenten Lokalisation an den Centrosomen jeder somatischen Zelle bezogen sich in der Vergangenheit funktionelle Analysen der Centrine meistens auf deren Aufgaben an diesem Zellorganell. Während des Zellzyklus wird das Centrosom einmal dupliziert. Anschließend wandern die Centrosomen auseinander und bilden die, für die Zellteilung notwendigen, Spindelpole. Für Cen3p wird eine Rolle bei der Duplikation der Centrosomen während der G2 Phase des Zellzyklus diskutiert (Middendorp *et al.*, 2000). Die andere ubiquitäre Isoform Cen2p ist wahrscheinlich in die Trennung der neu gebildeten Centrosomen nach der Duplikation involviert (Lutz *et al.*, 2001; Salisbury *et al.*, 2002). Im Folgenden soll näher erläutert werden, wie die bekannten zellulären Funktionen der Centrine reguliert werden. Dabei stehen Phosphorylierungen und Ca^{2+}-Bindung als Hauptregulationsmechanismen im Mittelpunkt.

1.2.1. Regulationsmechanismen der Centrine

Centrine gehören zu der großen Protein-Familie der Ca^{2+}-bindenden Proteine mit EF-Hand Motiven (Dissertation Publikation III) (Schiebel und Bornens, 1995; Gießl *et al.*, 2004b*). Diese funktionellen Strukturen dienen ihnen zur Bindung von Ca^{2+}-Ionen. Obwohl die EF-

Hand Motive hoch konserviert sind, haben sie in den Centrinen teilweise die Fähigkeit zur Ca^{2+}-Bindung verloren (Dissertation Publikation III) (Wolfrum *et al.*, 2002; Gießl *et al.*, 2004b*). In Grünalgen sind aber noch alle vier EF-Hand Motive zur Ca^{2+}-Bindung fähig und dienen der Grünalge als Ca^{2+}-Sensor (Sanders und Salisbury, 1994; Schiebel und Bornens, 1995). In allen bislang analysierten Organismen müssen Centrine für nahezu alle Protein-Protein Interaktionen durch die Bindung von Ca^{2+} aktiviert werden. (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*). So ist die Ca^{2+}-Bindung an humanes Cen2p beispielsweise für die Bildung von Homodimeren zweier Centrine notwendig (Durussel *et al.*, 2000; Tourbez *et al.*, 2004). Eine weitere, durch Ca^{2+}-regulierte Funktion der Centrin-Isoformen stellt die Bindung an heterotrimere G-Proteine dar. In vorangegangenen Arbeiten konnten wir zeigen, dass alle Centrin-Isoformen der Vertebraten mit dem visuellen heterotrimeren G-Protein Transducin interagieren (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Die Bildung des Centrin/Transducin Komplexes ist strikt Ca^{2+}-abhängig (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Durch diese Interaktion regulieren die ciliären Centrine Cen1p-3p möglicherweise die lichtabhängige Translokation des visuellen G-Proteins Transducin durch das Verbindungscilium der Photorezeptorzellen (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*).

Neben der Ca^{2+}-Bindung an den EF-Hand Motiven sind Phosphorylierungen der zweite Hauptregulationsmechanismus der Centrine (Dissertation Publikation III). Phosphorylierungen repräsentieren die häufigste posttranslationale Modifikation von Proteinen (Ubersax und Ferrell, Jr., 2007). Phosphorylierungen sind in nahezu jeden zellulären Prozess involviert, wie beispielsweise Stoffwechsel, Zellteilung, Entwicklung, Proliferation, Bewegung, Muskelkontraktion, Membrantransport sowie intrazelluläre Transportprozesse (Manning *et al.*, 2002b; Manning *et al.*, 2002a). Phosphorylierungen werden durch Protein Kinasen katalysiert. Sie übertragen das γ-Phosphat von ATP auf spezifische Aminosäurereste der Proteine (Ubersax und Ferrell, Jr., 2007). In Eukaryoten handelt es sich dabei meist um Serin-, Threonin- oder Tyrosinreste. Allerdings können auch Histidinreste durch Phosphorylierungen modifiziert werden (Boyer *et al.*, 1962; Zetterqvist, 1967; Klumpp und Krieglstein, 2005). Wie wichtig Phosphorylierungen als Regulationsmechanismus von Proteinen sind, repräsentiert die Tatsache, dass das menschliche Genom über 500 Gene besitzt, die für Protein Kinasen kodieren (Arena *et al.*, 2005). Trotz der großen Bandbreite an verschiedenen Substraten sind sich die meisten eukaroytischen Protein Kinasen strukturell sehr ähnlich (Hanks *et al.*, 1988; Hanks und Hunter, 1995). Sie besitzen

normalerweise eine ~250 Aminosäure große katalytische Domäne, in der ATP gebunden werden kann (Ubersax und Ferrell, Jr., 2007). Durch die Bindung des Substrats an die Protein Kinase kommt es zur Übertragung des γ-Phosphatrests von ATP auf das Substrat. Phosphorylierungen stellen zumeist reversible Modifikationen dar (Ubersax und Ferrell, Jr., 2007). Um einen kovalent gebundenen Phosphatrest von einer Aminosäure zu entfernen, sind Protein Phosphatasen notwendig. Diese Gegenspieler der Protein Kinasen können Phosphatreste an Aminosäuren hydrolysieren und heben somit die Phosphorylierung auf (Mumby und Walter, 1993). Bislang hängt das Wissen über Regulationsmechanismen von Protein Phosphatasen allerdings noch deutlich hinter dem der Protein Kinasen zurück (Ubersax und Ferrell, Jr., 2007).

Centrine besitzen eine Mehrzahl möglicher Phosphorylierungsstellen und wurden bereits mehrfach als Substrat für Protein Kinasen beschrieben (Dissertation Publikation III) (Salisbury *et al.*, 1984; Martindale und Salisbury, 1990; Lingle *et al.*, 1998; Lutz *et al.*, 2001). So konnte gezeigt werden, dass Cen2p während des Zellzyklus in menschlichen Kulturzellen phosphoryliert wird (Lutz *et al.*, 2001). Die Phosphorylierung von Cen2p wird durch die Protein Kinase A vermittelt und findet während des G1/S Übergangs des Zellzyklus statt (Lutz *et al.*, 2001). Zusätzlich zur normalen Phosphorylierung während des Zellzyklus werden Centrine während des entarteten Zellzyklus in Brusttumorzellen hyperphosphoryliert (Lingle *et al.*, 1998). Diese Zellen besitzen vergrößerte Centrosomen mit mehreren Centriolen (Lingle *et al.*, 1998). Allerdings konnte bislang keine dieser Arbeiten Protein Phosphatasen identifizieren, die in der Lage sind, Centrine zu dephosphorylieren. In Grünalgen konnten Phosphorylierungen der Centrine als Gegenspieler der Ca^{2+}-Bindung an die Centrine beschrieben werden (Sanders und Salisbury, 1989; Sanders und Salisbury, 1994; Schiebel und Bornens, 1995). Die Ca^{2+}-Bindung der Centrine führt zu ATP-unabhängigen Kontraktionen der Flagellenwurzeln (Sanders und Salisbury, 1989; Sanders und Salisbury, 1994; Schiebel und Bornens, 1995). Zur Entspannung dieser Kontraktionen müssen die Centrine phosphoryliert werden (Salisbury *et al.*, 1984; Martindale und Salisbury, 1990). Erst kürzlich konnte gezeigt werden, dass die Protein Kinase A für diese Phosphorylierungen der Centrine in Grünalgen verantwortlich ist (Meyn *et al.*, 2006). Eine der zentralen Fragestellungen der vorliegenden Arbeit war, ob die Funktion der Centrine in den Photorezeptorzellen der Vertebraten ebenfalls antagonistisch durch reversible Phosphorylierungen und Ca^{2+}-Bindung reguliert wird.

1.2.2. Die Rolle der Centrine beim molekularen ciliären Transport in Photorezeptorzellen

Centrine sind in der Retina von Vertebraten - wie in allen somatischen Zelltypen - Bestandteile von Centrosomen (Wolfrum und Salisbury, 1998; Wolfrum *et al.*, 2002). Zusätzlich sind Centrine aber auch im Verbindungscilium und dem Basalkörperkomplex von Stäbchen und Zapfen lokalisiert (Wolfrum, 1992; Wolfrum und Salisbury, 1998; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Die Photorezeptorzellen der Vertebraten repräsentieren bislang den einzigen Zelltyp, in dem alle vier Centrin-Isoformen parallel exprimiert werden. Analysen der subzellulären Lokalisation der einzelnen Centrin-Isoformen am Verbindungscilium und Basalkörperkomplex ergaben allerdings, dass die Centrine Isoform-spezifische Lokalisationen aufweisen (Abb. 5). Die Isoformen Cen2p und Cen3p sind im Verbindungscilium und am Basalkörper lokalisiert. Im Gegensatz dazu beschränkt sich die Lokalisation von Cen1p auf das Verbindungscilium und die von Cen4p auf den Basalkörper (Abb. 5) (Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Das Verbindungscilium ist die einzige cytoplasmatische Verbindung zwischen dem Außen- und dem Innensegment der Photorezeptoren.

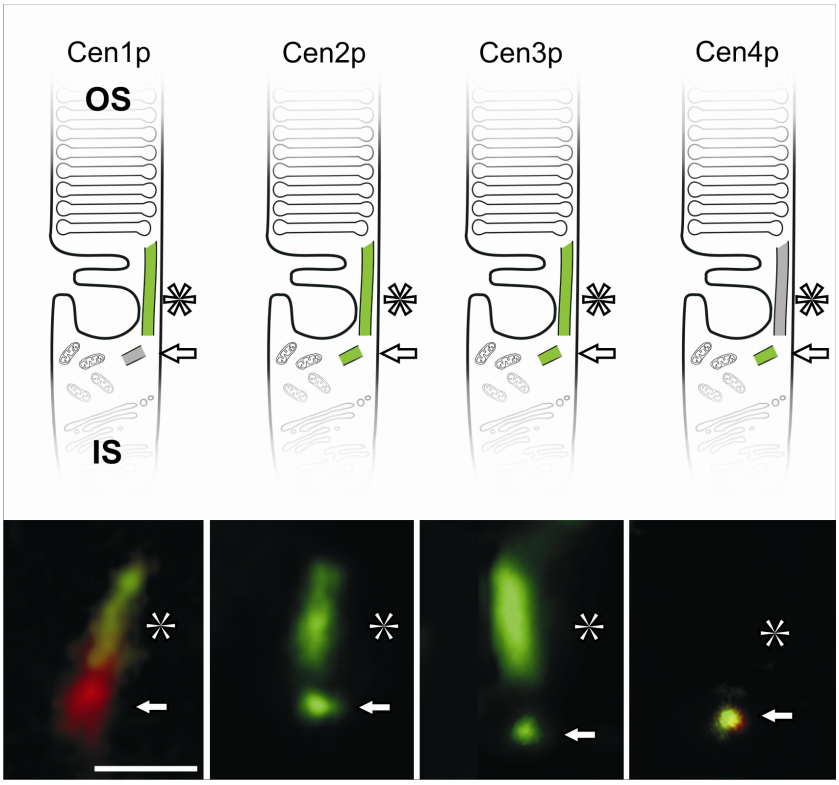

Abb. 5: Subzelluläre Lokalisation der Centrin-Isoformen am Verbindungscilium der Photorezeptorzellen von Vertebraten. Schematische Darstellung (oberer Bereich) und dazu gehörende indirekte Immunfluoreszenz (unterer Bereich) der Isoform-spezifischen Lokalisation von

Cen1p-Cen4p am Verbindungscilium (Stern) und am Basalkörper (Pfeil) der Photorezeptorzelle. Indirekte Immunfluoreszenz mit Antikörpern gegen die einzelnen Centrin-Isoformen (grün) und gegen γ-Tubulin (rot) zeigen die Lokalisation von Cen1p, Cen2p und Cen3p im Verbindungscilium. Zusätzlich sind Cen2p und Cen3p am Basalkörper lokalisiert. Cen4p ist nur am Basalkörper detektierbar, wo Cen1p hingegen nicht lokalisiert ist. Größenbalken: 1 µm (nach Dissertation Publikation III).

Wie bereits unter 1.1 dargestellt, translociert das visuelle G-Protein Transducin lichtabhängig durch das Verbindungscilium zwischen dem Außen- und Innensegment der Photorezeptorzellen (Abb. 3). So verlagern sich ~ 80% des Transducins während der Helladaption ins Innensegment der Photorezeptoren und bei Dunkeladaption in entgegen gesetzter Richtung (Calvert *et al.*, 2006). Solche bidirektionalen Transportvorgänge dürften durch regulatorische Bindeproteine kontrolliert werden (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2006*; Roepman und Wolfrum, 2007). In früheren Arbeiten konnten wir zeigen, dass alle Centrine mit dem visuellen G-Protein Transducin interagieren können (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Die subzelluläre Colokalisation der ciliären Centrine mit Transducin im Verbindungscilium der Photorezeptorzellen ermöglicht die Regulation des adaptiven, lichtabhängigen Transports des visuellen G-Proteins zwischen dem Außen- und dem Innensegment (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Zur Bildung der Centrin/Transducin Komplexe müssen die Centrine durch die Bindung von Ca^{2+} aktiviert werden (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Biochemische Analysen der Centrin/Transducin Interaktion zeigten, dass Centrine die β/γ-Untereinheit ($G_t\beta\gamma$) nicht jedoch die α-Untereinheit ($G_t\alpha$) von Transducin binden können. Zudem zeigen die Centrin-Isoformen unterschiedlich hohe Bindungsaffinitäten zu Transducin, wobei Cen3p die geringste Affinität besitzt (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Diese differenziellen Bindeaffinitäten der Centrin-Isoformen für das visuelle G-Protein deuten auf unterschiedliche Funktionen und Regulationen dieser Interaktion hin. Neben der Ca^{2+}-Bindung stellen Phosphorylierungen den wichtigsten Regulationsmechanismus der Centrine in anderen zellulären Kontexten dar. Daraus ergibt sich die Frage, ob die Komplexbildung der Centrine mit dem visuellen G-Protein ebenfalls durch Protein Kinasen und durch Protein Phosphatasen regulierbar ist.

1.3. Zielsetzung der Arbeit

Vorausgegangene Arbeiten unserer Arbeitsgruppe zeigten, dass alle vier Centrin-Isoformen differenziell am Verbindungscilium der Photorezeptorzellen lokalisiert sind (Wolfrum und Salisbury, 1998; Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Durch den Einsatz biochemischer und biophysikalischer Analysen gelang es, die β/γ-Untereinheiten des visuellen G-Proteins Transducin als direkten Interaktionspartner aller Centrin-Isoformen zu identifizieren (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Zur Bildung der Centrin/Transducin Komplexe müssen die Centrin-Isoformen durch die Bindung von Ca^{2+} aktiviert werden (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Obwohl alle vier Centrin-Isoformen direkt mit dem visuellen G-Protein interagieren können, sind die einzelnen Affinitäten der Isoformen zu Transducin sehr unterschiedlich. Die Isoformen Cen1p, Cen2p und Cen4p binden Transducin mit sehr hohen Affinitäten, wogegen Cen3p eine deutlich reduzierte Bindungsstärke besitzt (Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Neben der Bindung von Ca^{2+} stellen Phosphorylierungen den wichtigsten regulatorischen Mechanismus der Centrine dar. So konnten vorangegangene Arbeiten belegen, dass Centrine während des Zellzyklus von humanen Zellkulturzellen durch die Protein Kinase A phosphoryliert werden (Lutz *et al.*, 2001). In Grünalgen wurde gezeigt, dass die Phosphorylierung der Centrine antagonistisch zur Ca^{2+}-Bindung der Centrine wirkt (Martindale und Salisbury, 1990; Lutz *et al.*, 2001). Darüber hinaus verändert die Phosphorylierung von Centrinen der Grünalgen *in vitro* ihre Bindungseigenschaften zu anderen Proteinen (Meyn *et al.*, 2006).

In der vorliegenden Arbeit sollte analysiert werden, ob die Funktionen der Centrin-Isoformen in den Photorezeptorzellen von Vertebraten ebenfalls durch Phosphorylierungen reguliert werden. Dies sollte durch folgende Zielsetzungen erreicht werden:

a) Analyse lichtabhängiger Phosphorylierungen der Centrine in der Retina von Vertebraten und Identifikation der verantwortlichen Protein Kinasen

b) Identifikation von Phosphatasen, die für die Dephosphorylierung der Centrine relevant sind

c) Die Rolle des Phosphorylierungs- und Dephosphorylierungszyklus von Centrinen bei der Kontrolle von Centrin/G-Protein Komplexen

Zu a) Analyse lichtabhängiger Phosphorylierungen der Centrine in der Retina von Vertebraten und Identifikation der verantwortlichen Protein Kinasen

Zu Beginn der vorliegenden Arbeit stand die Etablierung und Weiterentwicklung der experimentellen Ansätze zur lichtabhängigen Phosphorylierung der Centrine im Vordergrund. Dabei konnte auf erste Ergebnisse aus meiner Diplomarbeit zurückgegriffen werden. Zur Identifizierung von Protein Kinasen, die für die lichtabhängige Phosphorylierung der Centrine verantwortlich sind, wurden spezifische Kinase Inhibitoren verwendet. Anschließend wurde die Phosphorylierung der Centrine *in vitro* charakterisiert, um Aminosäurereste zu identifizieren, die als Ziele für die beobachteten Phosphorylierungen dienen. Zu diesem Zweck wurden Deletionskonstrukte der einzelnen Centrin-Isoformen hergestellt. Diese Deletionsfragmente fanden Einsatz in den *in vitro* Phosphorylierungen. Zur genauen Identifikation der Phosphorylierungsstellen wurden die Deletionskonstrukte nach der Phosphorylierung enzymatisch verdaut und von unseren Kooperationspartnern in Berlin massenspektrometrisch analysiert. Des Weiteren wurden rekombinante Centrine hergestellt, in denen mögliche Phosphorylierungsstellen durch Alanine ersetzt wurden.

Zu b) Identifikation von Phosphatasen, die für die Dephosphorylierung der Centrine relevant sind

Phosphorylierungen als posttranslationale Modifikation von Proteinen stellen zumeist keine Einbahnstraße dar. Aus diesem Grund ist nicht nur das detaillierte Verständnis der phosphorylierenden Protein Kinasen sondern auch ihres Gegenspielers, der dephosphorylierenden Protein Phosphatasen, unerlässlich. Mit Hilfe unserer Kooperationspartner im Labor von Prof. Dr. Susanne Klumpp wurden Protein Phosphatasen identifiziert, die CK2-phosphorylierte Centrine dephosphorylieren können. Weiterführend wurde die Dephosphorylierung der Centrine durch diese Protein Phosphatasen biochemisch charakterisiert. Diese Analysen ermöglichten Einblicke in mögliche zelluläre Regulationsmechanismen der Dephosphorylierung. So spielt es für den zellulären Kontext eine wesentliche Rolle, ob die Dephosphorylierung durch Kationen oder Fettsäuren beschleunigt werden kann oder inhibiert wird. Des Weiteren wurde die subzelluläre Lokalisation der Protein Phosphatasen mittels licht- und elektronenmikroskopischen Methoden analysiert.

Zu c) Die Rolle des Phosphorylierungs- und Dephosphorylierungszyklus von Centrinen bei der Kontrolle von Centrin/G-Protein Komplexen

Bisherige Analysen zur Funktion der Centrine in den Photorezeptorzellen von Vertebraten zeigten, dass sie in der Lage sind, direkt mit dem visuellen G-Protein Transducin zu interagieren (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Die Bildung des Centrin/Transducin-Komplexes wurde zuvor bereits als Ca^{2+}-abhängig beschrieben (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Weiterführend wurde nun analysiert, ob die Phosphorylierungen der Centrine Einflüsse auf die Bindungsaffinitäten zum visuellen G-Protein Transducin haben. Die Geschwindigkeit der Komplexbildung konnte mit Hilfe der kinetischen Lichtstreuung gemessen werden. Diese Methode ist durch unsere Kooperationspartner in Berlin seit langem etabliert und wurde zu diesem Zweck genutzt. Durch den Einsatz hoch auflösender Licht- und Elektronenmikroskopie wurde analysiert, ob die Centrine mit der Kinase und der Phosphatase im gleichen subzellulären Kompartiment der Photorezeptorzellen colokalisiert sind. Die räumliche Nähe dieser Proteine stellt die Grundvoraussetzung für spezifische Enzym-Substrat Reaktionen dar.

Durch die Beantwortung der beschriebenen Fragestellungen konnte in der vorliegenden Arbeit gezeigt werden, dass die Centrine in den Photorezeptoren der Vertebraten lichtabhängig phosphoryliert werden. Die Centrine werden durch die Protein Kinase CK2 phosphoryliert und durch Protein Phosphatasen der 2C Familie dephosphoryliert. Durch die lichtabhängige Phosphorylierung verringert sich die Bindungsaffinität der Centrine zum visuellen G-Protein Transducin. Das Mikrotubuli-Cytoskelett des Verbindungsciliums dient dabei als Plattform für die lichtabhängige Phosphorylierung und Dephosphorylierung der Centrine. Die beschriebene, lichtabhängige und reversible Phosphorylierung der Centrine dürfte an der Regulation der lichtabhängigen intrazellulären Translokation des visuellen G-Proteins Transducin zwischen dem Innen- und Außensegment der Photorezeptorzellen beteiligt sein.

2. Publikationen

2.1 Trojan P, Rausch S, Gieβl A, Klemm C, Krause E, Pulvermüller A, Wolfrum U (2008) Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein. Biochim Biophys Acta. 2008 Jan 17; Epub ahead of print.

2.2 Thissen MC, Trojan P, Kriegelstein J, Wolfrum U, Klumpp S Dephosphorylation of centrins by protein phosphatase 2Cα and 2Cβ. FEBS letters eingereicht

2.3 Trojan P, Krauss N, Choe HW, Gießl A, Pulvermüller A, Wolfrum U (2008a) Centrins in retinal photoreceptor cells: Regulators in the connecting cilium. Prog Retin Eye Res. 2008 Jan 31; Epub ahead of print.

Dissertation Publikation I

2.1 Trojan P, Rausch S, Gießl A, Klemm C, Krause E, Pulvermüller A, Wolfrum U (2008) Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein. Biochim Biophys Acta. 2008 Jan 17; Epub ahead of print.

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Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein

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Abstract

Centrins are Ca^{2+}-binding EF-hand proteins. All four known centrin isoforms are expressed in the ciliary apparatus of photoreceptor cells. Cen1p and Cen2p bind to the visual G-protein transducin in a strictly Ca^{2+}-dependent way, which is thought to regulate light driven movements of transducin between photoreceptor cell compartments. These relatively slow motile processes represent a novel paradigm in light adaptation of photoreceptor cells.

Here we validated specific phosphorylation as a novel regulator of centrins in photoreceptors. Centrins were differentially phosphorylated during photoreceptor dark adaptation. Inhibitor treatments revealed protein kinase CK2 as the major protein kinase mediating phosphorylation of Cen1p, Cen2p and Cen4p, but not Cen3p, at a specific target sequence. CK2 and ciliary centrins co-localize in the photoreceptor cilium. Direct binding of CK2 and centrins to ciliary microtubules may spatially integrate the enzyme–substrate specificity in the cilium. Kinetic light-scattering assays revealed decreased binding affinities of phosphorylated centrins to transducin. Furthermore, we show that this decrease is based on the reduction of Ca^{2+}-binding affinities of centrins. Present data describe a novel regulatory mechanism of reciprocal regulation of stimulus dependent distribution of signaling molecules.

Keywords: Cytoskeleton; Ca^{2+}-binding proteins; Molecular translocation; Heterotrimeric G-protein; Signal transduction; Vision

1. Introduction

In cellular biology, phosphorylation is one of the most important regulatory mechanisms for the proper function of proteins. These post-translational modifications are highly regulated processes mediated by hundreds of different protein kinases phosphorylating about 30% of all cellular proteins [1]. In mammalian retinal photoreceptor cells, multiple protein kinases have been previously documented, e.g. G-protein coupled receptor kinase 1 (GRK1, rhodopsin kinase) in rods [2], the related GRK7 (iodopsin kinase) in cones [3], PKC [4], PKA [5] and protein kinase CK2 (formerly known as casein kinase 2) [6], which are highly specific for target molecules. A more complete set of kinases present in photoreceptor cells was obtained by a recent proteomic analysis [7].

Cone and rod photoreceptor cells are highly specialized polarized neurons. They consist of an inner segment containing the organelles typical for eukaryotic cells and an outer segment specialized for single photon uptake. For the light perception the outer segments possess one of the best characterized prototypical G-protein coupled receptor signaling pathways, the visual signal transduction cascade. Photons activate the G-protein coupled receptor rhodopsin which in turn activates the heterotrimeric

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visual G-protein transducin (G_t). Activated transducin then activates the downstream effector phosphodiesterase, which hydrolyzes cGMP to 5′GMP. This leads to the closure of cGMP dependent Ca^{2+}-channels in the plasma membrane of the photoreceptors leading to a drop of the intracellular Ca^{2+}-concentration and the hyperpolarization of the cells (for reviews see [8–10]). The recovery phase of the enzymatic machinery of visual signal transduction and rapid light adaptation of photoreceptor cells rely on a feedback mechanism. This depends on changes in the intracellular Ca^{2+}-concentration, affecting the phototransduction cascade through Ca^{2+}-binding proteins [11]. While this well known Ca^{2+}-triggered rapid adaptation process works on a time scale of subseconds, a much slower adaptation of rod photoreceptor cells is based on the light driven bidirectional translocation of transduction cascade components between the functional compartments of photoreceptor cells [12]. Upon illumination, 80% of G_t-protein subunits move within minutes from the outer segment to the inner segment and the cell body of rod photoreceptor cells [13]. In the dark they return to the outer segments in a more leisurely time course of hours. This inter segmental exchange between the inner and outer segmental compartments occurs through the slender non-motile connecting cilium [14,15]. Our initial studies indicated a regulation of the G_t translocation through the connecting cilium by centrins [14,16–18]. The basis of the regulation is the binding of centrins to the non-dissociable $G_t\beta\gamma$-subunit which occurs in a strictly Ca^{2+}-dependent manner [14,16–19]. However, the function of centrins is not only regulated by Ca^{2+}-binding but also by phosphorylation [20–22].

The present study was designed to analyze phosphorylation of centrins and its contribution to the regulation of centrin functions in vertebrate photoreceptor cells. Centrins are members of the parvalbumin superfamily of Ca^{2+}-binding phosphoproteins [23,24]. Centrins were first identified as components of contractile fibers of flagellar rootlets in unicellular green algae [20,21,23,24]. In vertebrates, centrins are commonly associated with centriole-related structures such as spindle poles of dividing cells or centrioles of centrosomes and basal bodies [19,23,24]. At least four different centrin genes (*Cetn1–4*) are expressed in mammals (reviewed in [17–19]). Our previous studies showed the expression of all four known centrin isoforms (Cen1p–4p) in mammalian retinal photoreceptor cells. Although all four isoforms are components of the ciliary complex of photoreceptor cells they exhibit differential localizations in the diverse subciliary compartments [16,18]. Cen1p–3p are localized at the connecting cilium. Cen2p and Cen3p were additionally found at the basal body of the photoreceptor where Cen4p was exclusively located.

In addition to the binding of Ca^{2+} [17,19,23], centrins are known to be regulated by phosphorylation in other cellular settings [20–22]. Phosphorylation of centrins was first described in green algae where the relaxation of the Ca^{2+}-induced contraction of the flagellar rootlets was triggered by phosphorylation of centrins [20,21]. This PKA-mediated phosphorylation modulates the structure and the biochemical activity of centrins by altering the binding affinity to other proteins like Kar1p in yeast [25]. In higher eukaryotic cells, phosphorylation of centrins, in particular the ubiquitously expressed Cen2p, by PKA plays an important role for the duplication of centrioles during the cell cycle [22].

Here we analyze the role of phosphorylation for the function of centrin isoforms in vertebrate photoreceptor cells. We show that centrins are light-dependently phosphorylated and identified protein kinase CK2 as being responsible for the specific phosphorylation of centrins. We demonstrate here, that CK2 and centrins co-localize in the inner lumen of the connecting cilium. Present *in vitro* microtubule binding assays further indicate direct binding of CK2 and Cen1p–3p to the microtubules in the connecting cilium. In addition, we demonstrate that CK2-mediated phosphorylation of Cen1p, Cen2p and Cen4p leads to reduced binding affinities for the heterotrimeric G-protein transducin. Assembling all results, our data point towards light-dependent CK2-mediated phosphorylation of centrins as a novel regulatory mechanism during the adaptive translocation of transducin between the outer and inner segments of mammalian photoreceptor cells.

2. Materials and methods

2.1. Animals and tissue preparation

All experiments described herein conform to the statement by the Association for Research in Vision and Ophthalmology (ARVO) as to the care and use of animals in research. Adult Sprague–Dawley albino rats and C57BL76 mice were maintained on a 12/12 h light/dark cycle with lights on at 6 a.m. and with food and water *ad libitum*. After sacrifice of the animals in CO_2, retinas were removed as described [15]. Bovine eyes used were obtained from the local slaughter houses and were kept on ice in the dark until further processing.

2.2. Recombinant expression of centrin isoforms

Subcloning of murine *Cetn1–4* cDNAs into the pGEX-4T3 expression vector (GE Healthcare, München, Germany) and protein expression (Cen1p–4p) and purification were performed as described [14].

2.3. Primer used for generation of centrin fragments

All forward primers contain a BamH1 restriction site and all reverse primers were generated with an XhoI site. The following primers were used to generate Cen1p and Cen2p fragments. Cen1pΔN-term, the forward primer Cen1pΔN-term forward (5′-GTA CGG ATC CCA AGA AGT TCG GGA AGC CTT T-3′) and the reverse primer Cen1p holo reverse (5′-GCG GCT CGA GTT AAT AAA GGT TGG TCT TTT T-3′). Cen1pΔEF1, the forward primer Cen1pΔEF1 forward (5′-GTA CGG ATC CAA GGA AGA GAT GAA GAA AAT G-3′) and the reverse primer Cen1p holo reverse (5′-GCG GCT CGA GTT AAT AAA GGT TGG TCT TTT T-3′). Cen1pΔEF12, the forward primer Cen1pΔEF12 forward (5′-GTA CGG ATC CAC CAA AGA GGA AAT CCT GAA G-3′) and the reverse primer Cen1p holo reverse (5′-GCG GCT CGA GTT AAT AAA GGT TGG TCT TTT T-3′). Cen1pΔEF12, the forward primer Cen1pΔEF12 forward (5′-GTA CGG ATC CAC CAA AGA GGA AAT CCT GAA G-3′) and the reverse primer Cen1p holo reverse (5′-GCG GCT CGA GTT AAT AAA GGT TGG TCT TTT T-3′). Cen1pΔEF34, the forward primer Cen1p holo forward (5′-GTA CGG ATC CAT GGC GTC CAC CTT CAG GAA G-3′) and the reverse primer Cen1pΔEF34 reverse (5′-GCG GCT CGA GTT AAT CTT TCT CGG CCA TCT T-3′). Cen2pΔN-term, the forward primer Cen2pΔN-term forward (5′-GTA CGG ATC CCA GGA AAT CCG GGA AGC TTT T-3′) and the reverse primer Cen2p holo reverse (5′-GCG GCT CGA GTT AAT AGA GGC TGG TCT TTT TCA T-3′). Cen2pΔEF1, the forward primer Cen2pΔEF1

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forward (5′-GTA CGG ATC CAA AGA AGA AAT TAA GAA AAT G-3′) and the reverse primer Cen2p holo reverse (5′-GCG GCT CGA GTT AAT AGA GGC TGG TCT TTT TCA T-3′). Cen2pΔEF12, the forward primer Cen2pΔEF12 forward (5′-GTA CGG ATC CAC TAA AGA AGA AAT CCT GAA A-3′) and the reverse primer Cen2p holo reverse (5′-GCG GCT CGA GTT AAT AGA GGC TGG TCT TTT TCA T-3′). Cen2pΔEF34, the forward primer holo forward (5′-GTA CGG ATC CAT GGC CTC TAA TTT TAA GAA G-3′) and the reverse primer Cen2pΔEF34 reverse (5′-GCG GCT CGA GTT AAG TGT CTT TCT CAG ACA T-3′).

The potential CK2 phosphorylation sites S^{136} and T^{138} in murine Cen1p were mutated to alanin (AGC→GCG and ACA→GCG, respectively) using the *QuikChange Site-Directed Mutagenesis Kit* (Stratagene, La Jolla, CA). Mutagenic primers used in this study were as follows: Cen1p-S136A, the forward primer C1-S136A-F (5′-C AAT GAG CTG GGG GAA **GCG** CTC ACA GAC GAG GAG C-3′), the reverse primer C1-S136A-R (5′-G CTC CTC GTC TGT GAG **CGC** TTC CCC CAG CTC ATT G-3′); and Cen1p-T138A, the forward primer C1-T138A-F (5′-CTG GGG GAA AGC CTC **GCG** GAC GAG GAG CTG CAG-3′), the reverse primer C1-T138A-R (5′-CTG CAG CTC CTC GTC **CGC** GAG GCT TTC CCC CAG-3′). The primers were designed with PrimerX (http://bioinformatics.org;). The plasmids pGEX-Cen1-S136A and pGEX-Cen1-T138A, carrying the mutated *Cetn1* cDNA, were transformed into *Escherichia coli* strain BL21 DE3 and the proteins were expressed and purified as previously described [14].

2.4. In vitro phosphorylation of centrin fragments

Equal amounts of recombinant GST-tagged centrin fragments and native GST were coupled to glutathione sepharose beads (GE Healthcare) in the presence of NETN buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet NP-40). Beads were washed 2 times in NETN buffer and 2 times in phosphorylation buffer (20 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA). For *in vitro* phosphorylation beads were incubated for 12 h at 30 °C in phosphorylation buffer in the presence of 100 U of recombinant protein kinase CK2 (Calbiochem, Darmstadt, Germany) and 23 μCi of [γ^{32}P]ATP (GE Healthcare). Non-radioactive experiments were performed in a similar fashion however, 100 nmol ATP were used (Sigma, Deisenhofen, Germany). Beads were washed twice using phosphorylation buffer. GST-centrin fragments and GST alone were eluted from the beads by incubation for 20–30 min at 25 °C in elution buffer (50 mM Tris–HCl (pH 8.0), 15 mM glutathione, 11 mM C12E10 (Sigma)). Radioactivity of the supernatant was analyzed using a scintillation counter. Non-radioactive supernatant was analyzed by SDS-PAGE.

Phosphorylated Cen1p–4p for kinetic light-scattering analysis (KLS) were prepared using 4 mg/ml of centrins in 1 ml phosphorylation buffer with 400 μM ATP and 500 U CK2, incubated for 2 h at 30 °C. Non-phosphorylated controls were prepared the same way without adding kinase. All samples were finally purified from kinase and nucleotides by ion exchange chromatography on a MonoQ HR 5/5 column (GE Healthcare) using a linear gradient from 0–200 mM NaCl over 10 ml and a flat linear gradient 200–400 mM NaCl over 30 ml in 20 mM BTP buffer at pH 8.5 and 4 °C. Phosphorylated proteins were identified by in-gel staining using the Pro-Q® Diamond Phosphoprotein Gel Stain Kit (Molecular Probes, Leiden, Netherlands).

2.5. Phosphorylation of recombinant centrins by bovine retina extracts

Five explanted bovine retinas were transferred to phosphate-free DMEM medium (Sigma-Aldrich) and adapted for 2 h at 4 °C in light (1000 lx) or in complete darkness. After centrifugation (2 min, 4000 ×*g*, 4 °C) retinas were homogenized in preheated lyses buffer [14]. 500 μl of retina extracts were incubated with 12 μCi of [γ^{32}P]ATP (GE Healthcare) and 100 μg of recombinant murine Cen1p–4p respectively for 3 h at 4 °C. Immunoprecipitations using pan-centrin antibody 20H5 were performed as described [14]. Radioactivity

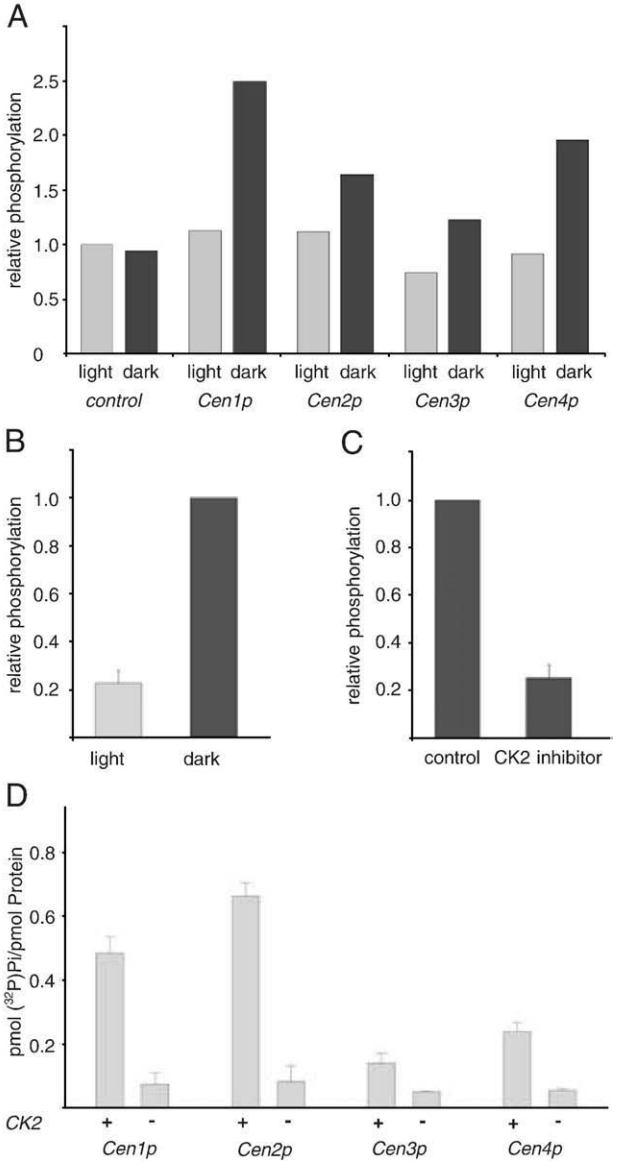

Fig. 1. Light-dependent phosphorylation of murine centrin isoforms. (A), *In vitro* phosphorylation of recombinant murine Cen1p–4p. Light adapted (grey bars) or dark adapted (black bars) bovine retina lysates were incubated with Cen1p–4p respectively in the presence of [γ-^{32}P]ATP. After immunoprecipitation of the centrins radioactivity incorporation was analyzed using a scintillation counter. Recombinant murine Cen1p, Cen2p and Cen4p exhibit higher radioactivity incorporation from dark adapted retina lysate compared to light adapted ones and to control experiments without recombinant centrins (n=5). In contrast, Cen3p is phosphorylated neither from dark nor from light adapted retinas. (B), *Ex vivo* phosphorylation of endogenous centrins from rat retinas. Explanted light or dark adapted rat retinas were incubated with radioactive phosphate and immunoprecipitated for endogenous centrins. Radioactivity incorporation was analyzed using a scintillation counter. Radioactivity was 80% reduced in centrins from light adapted retinas compared to centrins from dark adapted retinas (n=3). (C), CK2 is involved in the *ex vivo* phosphorylation of endogenous centrins. *Ex vivo* phosphorylation of endogenous centrins from dark adapted rat retinas was performed using a specific inhibitor for CK2. Radioactivity incorporation was reduced by DRB (5,6-dichlorobenzimidazole riboside) to 25% compared to untreated samples (n=3). (D), Quantitative *in vitro* phosphorylation of recombinant murine Cen1p–4p with protein kinase CK2. CK2 strongly phosphorylates Cen1p and Cen2p. In contrast, Cen4p is only a weak substrate for CK2 and Cen3p shows nearly no phosphorylation. Note that the amount of incorporated phosphate into Cen1p–4p molecules is always lower than one. This shows clearly that all centrins are phosphorylated by protein kinase CK2 at only one position.

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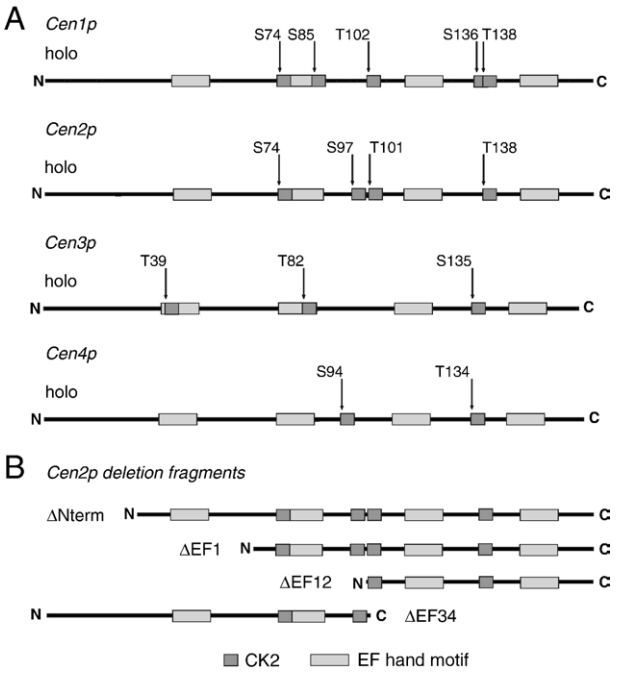

Fig. 2. Identifications of putative target amino acids for CK2 in murine Cen1p–4p. (A), Schematic representation of murine Cen1p–4p proteins. Amino acid sequences have been screened for typical CK2 target sequences (S/T-X-X-D/E). Murine Cen1p contains five such motifs at serines 74 (S^{74}), 85 (S^{85}), 136 (S^{136}) and threonines 102 (T^{102}), 138 (T^{138}). In Cen2p two serines (S^{74} and S^{97}) and two threonines (T^{101} and T^{138}) are putative targets for CK2-mediated phosphorylation. Cen3p just comprises three residues for CK2 phosphorylation (T^{39}, T^{82} and S^{135}). Cen4p only contains two putative CK2 phosphorylation sites (S^{94} and T^{134}). (B), Schematic representations of Cen2p deletion fragments for further analysis of CK2-mediated phosphorylation. Deletion constructs for Cen1p and Cen3p where subcloned accordingly. Deletion fragments lack the N-terminal domain (ΔN-term), EF-hand motif 1 (ΔEF1), both N-terminal EF-hands (ΔEF12) and the C-terminal domain including the EF-hand motifs 3 and 4 (ΔEF34).

incorporation of immunoprecipitated centrins was analyzed using a scintillation counter and autoradiography.

2.6. Ex vivo phosphorylation of endogenous centrins

Adult Sprague–Dawley albino rats were light and dark adapted for 12 h respectively. After sacrifice of the animals in CO_2, entire retinas were removed through a slit in the cornea. Explanted retinas were cultured in phosphate-free DMEM medium for 2.5 h in the presence of $H_3[^{32}P]O_4$ in light (1000 lx) or dark. Subsequently, retinas were washed in preheated lysis buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 10 mM NaF; 20 mM β-glycerophosphate; 1% sodium dodecyl sulfate [SDS]; 5% NP-40; 5% deoxycholicacid) and homogenized [14]. Immunoprecipitations were performed and radioactivity incorporation was analyzed using a scintillation counter and autoradiography.

2.7. Membrane and protein preparations

Preparations of rod outer segments, hypotonically stripped disk membranes by the Ficoll floating procedure are described by Giessl et al. [16]. Rhodopsin concentration was determined from its absorption at 500 nm using $\varepsilon_{500} - 40{,}000\ M^{-1}\ cm^{-1}$ [26]. Membrane suspensions were either kept on ice and used within 4 days without any loss of activity or stored at −80 °C until use. Preparation of transducin and the isolation of its subunits were previously described [14]. Transducin and $G_t\beta\gamma$ concentrations were determined by the method of Bradford using bovine serum albumin as a standard. Concentration of intact $G_t\alpha$ was determined precisely by fluorometric titration with guanosine 5′-3-*O*-(thio)triphosphate.

2.8. Kinetic light-scattering (KLS)

Changes in intensities of scattered near-infrared light were measured as described before [27]. Light-induced binding of soluble proteins to activated, membrane bound rhodopsin leads to an increase of the size of the scattering particle and a concomitant increase of the intensity of scattered light. The light-scattering change is proportional to the gain of mass and depends on the measuring conditions and the experimental setup [27,28]. Light-scattering changes at an angular range of 16±2° were monitored at 820 nm in a 10 mm cuvette. Samples containing rhodopsin, transducin and centrin isoforms (phosphorylated and unphosphorylated) were prepared in 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 [14]. The scattering signal was induced by a 500±20 nm flash that photolyzed 32% of rhodopsin in the sample. The recorded traces for light-scattering binding signals were corrected by the control N-signal (the light-scattering reflection of rhodopsin photoactivation without added proteins) [29].

2.9. Determination of free Ca^{2+}

The free Ca^{2+}-concentration was set using an EGTA buffer system (20 mM BTP, pH 7.65, 100 mM NaCl, 2.6 mM $MgCl_2$ and 50 μM EGTA). The original $CaCl_2$ concentration in the saline buffer was measured spectrophotometrically and the free Ca^{2+}-concentration was calculated [14].

2.10. Enzymatic in-gel digestion of phosphorylated Cen1p–4p for mass spectrometry

Protein bands were excised, washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The gel pieces were swollen in 5 μl of 5 mM ammonium bicarbonate containing 200 ng of AspN (sequencing grade, Roche Diagnostics, Mannheim, Germany). 5 μl of 5 mM ammonium bicarbonate were added to keep the gel pieces moist during enzymatic cleavage (37 °C, 12 h). Peptides were extracted by adding 10 μl of 0.5% trifluoroacetic acid in acetonitrile. The separated supernatant was dried under vacuum and redissolved in 6 μl of 2% formic acid in acetonitrile–water (3:7, v/v) prior to the selective binding of the phosphopeptides onto a titanium dioxide column.

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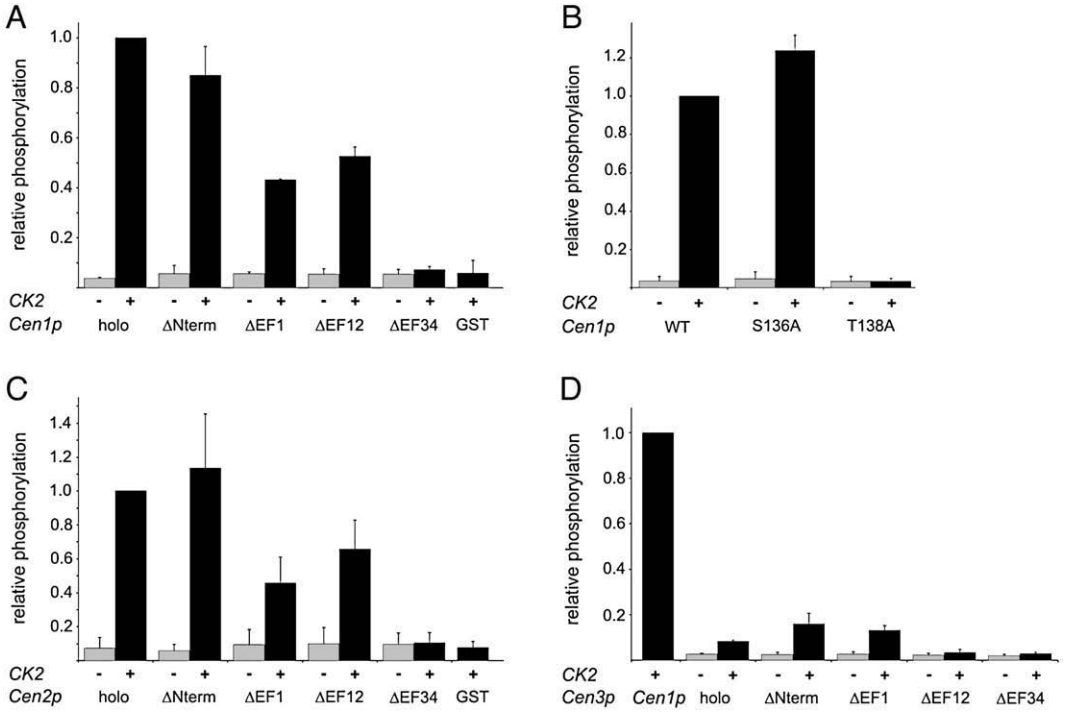

Fig. 3. Identification of functional phosphorylation sites for CK2-mediated phosphorylation in the murine ciliary Cen1p–3p. Recombinant murine Cen1p–3p holo proteins and deletion fragments were incubated with [γ^{32}P]ATP in the presence (dark bars) or absence (light bars) of CK2. Incorporated radioactivity was determined relative to holo proteins and control experiments were performed using GST alone as phosphorylation target. (A), CK2 phosphorylates holo Cen1p and fragment ΔN-term to nearly the same amount. Fragments ΔEF1 and ΔEF12 are less phosphorylated compared to the holo protein. In contrast fragment ΔEF34 is not phosphorylated by CK2. (B), To identify the target amino acid for CK2 phosphorylation in Cen1p, S^{136} and T^{138} were mutated to alanine (S136A and T138A). Phosphorylation was measured relative to the non-mutated holo protein (WT). When T^{138} was mutated, Cen1p is no longer a target for CK2-mediated phosphorylation. (C), Cen2p fragments can only serve as targets for CK2 when they contain the C-terminal domain, indicating S^{137} as the target for CK2 phosphorylation. (D), CK2 does not phosphorylate Cen3p fragments *in vitro*. Cen3p phosphorylation is normalized to phosphorylation of Cen1p holo protein. Cen3p holo only shows 8% phosphorylation compared to Cen1p. Fragments ΔN-term and ΔEF1 are 16% and 13% phosphorylated respectively. ΔEF12 and ΔEF34 show no phosphorylation.

2.11. Selective binding of phosphorylated peptides onto a titanium dioxide column

Separation of phosphorylated peptides was performed by selective binding on titanium dioxide (Sachtleben Chemie GmbH, Duisburg) as previously described [30]. The experiments were performed on a LC system (Ultimate, Dionex, Idstein, Germany) using a capillary titanium dioxide column (5 μm, 100 Å; 5 mm × 300 μm i.d.). 5 μl of the sample were injected at a flow rate of 1.5 μl/min. After washing with 20 μl of 2% formic acid in acetonitrile–water (3:7, v/v) the phosphopeptides were eluted with 20 μl of 100 mM ammonium bicarbonate at a flow rate of 0.8 μl/min. The eluate was dried in a vacuum centrifuge and redissolved in 6 μl of 0.1% trifluoroacetic acid in acetonitrile–water (1:9, v/v) prior to the analysis by LC-MS/MS.

2.12. Mass spectrometry

Tandem MS experiments were performed on a quadrupol orthogonal acceleration time-of-flight mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a Z-spray nanoelectrospray source. A CapLC liquid chromatography system (Waters, Milford, MA, USA) was used to deliver the peptide solution to the electrospray source. Peptides were separated using a capillary column (PepMap C18, 3 μm, 100 Å, 150 mm × 75 μm i.d., Dionex, Idstein, Germany) and an eluent flow rate of 200 nl/min. Mobile phase A was 0.1% formic acid in acetonitrile–water (3:97, v/v) and B was 0.1% formic acid in acetonitrile–water (8:2, v/v). Runs were performed using a gradient of 4–65% B in 60 min. To perform MS/MS experiments, automatic function switching (survey scanning) was employed. The MS survey range was m/z 300–1990 and the scan duration was 1.0 s. The collision gas was argon. The processed MS/MS spectra (MassLynx version 4.0 software) were compared with the theoretical fragment ions of AspN fragments of Cen1p and Cen2p.

2.13. Microtubule binding of centrin isoforms and protein kinase CK2

To analyze the binding of centrins and CK2 to microtubules the microtubule binding protein spin down assay kit was used (Cytoskeleton Inc., Denver). Microtubules were polymerized according to users manual and incubated with 1 μg of Cen1p–3p or CK2 at room temperature for 30 min. After centrifugation at 100,000 ×*g* for 45 min, supernatants and pellets were analyzed by Western blotting as described [16].

2.14. Antibodies

Affinity-purified polyclonal rabbit and monoclonal mouse antibodies against protein kinase CK2 were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal pan-centrin antibody (clone 20H5) and polyclonal rabbit antibody against murine centrins have been previously described [16]. Alexa-coupled secondary antibodies for indirect immunofluorescence were obtained from Molecular probes (Leiden, Netherlands). Nanogold-coupled secondary antibodies for immunoelectron microscopy were obtained from Nanoprobes (Yaphank, NY).

2.15. Immunohistochemistry

Eyes of adult mice were cryofixed in melting isopentane, cryosectioned and treated as described previously [16]. Mounted retinal sections were examined

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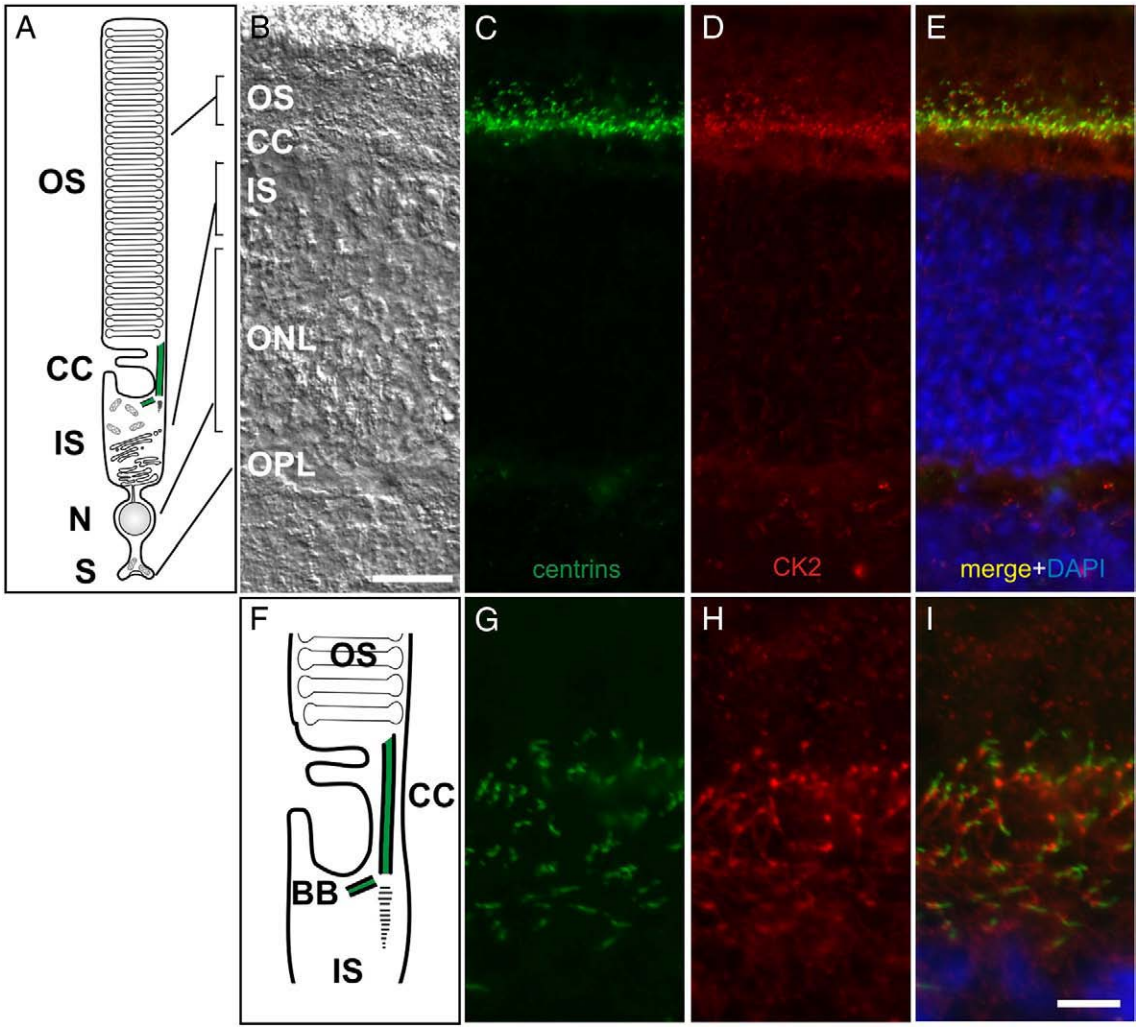

Fig. 4. Subcellular localizations of centrins and CK2 in murine retinas. (A), Schematic representation of a vertebrate photoreceptor cell. The photoreceptors are composed of the light sensitive outer segment (OS) which is linked via the connecting cilium (CC, green) to the inner segment (IS). The nuclei (N) of the photoreceptors form the outer nuclear layer (ONL, B), whereas the synapses (S) of the photoreceptors form the outer plexiform layer (OPL, B). (B), Differential interference contrast image of a cryosection through a murine retina. (C), Indirect anti-centrin immunostaining. Anti-centrin staining is predominantly located at the CC which joints the IS and the OS. (D), Indirect anti-CK2 immunostaining is predominantly found at the joint between the inner and outer segments and at the synapses of the photoreceptor cells. (E), Merged images of (C) and (D) with additional DAPI staining of the nuclei. Anti-centrin and anti-CK2 fluorescence are partially co-localized at the ciliary apparatus of the photoreceptors. (G–I), Higher magnification of the connecting cilium and the basal body complex. (F), Schematic representation of the ciliary apparatus of a vertebrate photoreceptor cell. The ciliary apparatus (green) is composed of the connecting cilium (CC) and the basal body complex (BB). (G), Staining for centrins displays a typical semicolon-like staining pattern of the CC and the BB. (H), Anti-CK2 staining reveals localization mainly at the ciliary rootlet, but also staining of the CC. (I), Merged image of pictures (G) and (H) indicates co-localization of centrins and CK2 at the basal part of the connecting cilium. CK2 is additionally localized in a point-like structure at the joint between the connecting cilium and the basal body complex. Bars, 12 μm (B–E) and 3 μm (G–I).

with a Leica DMRP microscope. Images were obtained with a Hamamatsu Orca ER CCD camera (Hamamatsu City, Japan) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

2.16. Immunoelectron microscopy

Fixation, embedding and further handling of mouse retinal samples for immunoelectron microscopy were performed as described [31]. Monoclonal antibodies from mouse directed against CK2 and a pan-centrin antibody raised in rabbit were applied to ultrathin sections through LRWhite-embedded mouse retina. Nanogold-labeling was silver-enhanced according to [32]. After counterstaining with 2% aqueous uranyl acetate (Sigma, Germany), sections were analyzed in a FEI Tecnai 12 BioTwin transmission electron microscope (FEI, Eindhoven The Netherlands), imaged with a SCCD SIS MegaView III camera (Münster, Germany) and digital images were processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

3. Results

3.1. Light-dependent phosphorylation of centrins in retinal photoreceptor cells

The functions of centrins are known to be not only regulated by Ca^{2+} but also by phosphorylation [21,22]. Due to the prominent localization of centrins in the connecting cilium of vertebrate photoreceptor cells we wanted to analyze whether centrins are differentially phosphorylated under varying light

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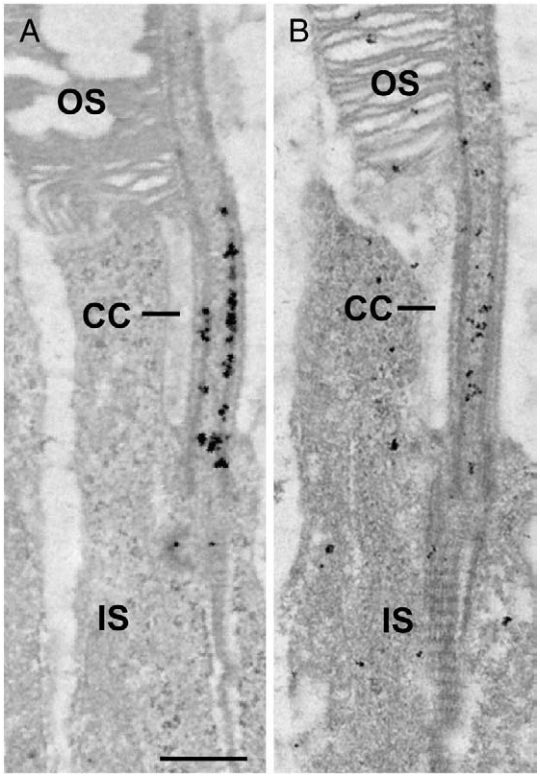

Fig. 5. Immunoelectron microscopic localization of centrins and protein kinase CK2 in a mouse photoreceptor cell. (A and B), longitudinal ultrathin sections through part of mouse rod photoreceptor cells. (A), Silver-enhanced immunogold labeling by pan-centrin antibody. Centrins are localized at the inner surface of the connecting cilium (CC) linking the inner segment (IS) and the outer segment (OS) of the photoreceptor cell. (B), Silver-enhanced immunogold labeling by CK2 antibody. Protein kinase CK2 shows localization, similar to centrins, at the inner surface of the CC. Bar, 500 nm.

conditions. For this purpose, we incubated light or dark adapted bovine retina extracts with recombinant murine Cen1p–4p in the presence of [γ^{32}P]ATP. After immunoprecipitation of the centrins with a pan-centrin antibody which precipitates all four centrin isoforms, the radioactivity of the supernatant was analyzed. The phosphorylation of centrins was increased after incubation with dark adapted retina extracts compared to the treatment with light adapted extracts (Fig. 1A). Nevertheless, there were substantial quantitative differences in the phosphorylation of the different isoforms: phosphorylation of recombinant Cen1p was increased by 130% in the dark adapted retinas compared to the light adapted ones. Phosphorylation of Cen4p was 100% increased, whereas phosphorylation of Cen2p and Cen3p was increased by 50% using dark adapted retina lysates (Fig. 1A).

In a next series of experiments we evaluated the light-dependent phosphorylation of endogenous centrins in rat photoreceptor cells. In this *ex vivo* approach retinas were isolated from light or dark adapted rats, explanted and cultured for 2.5 h in culture medium in the presence of $H_3[^{32}P]O_4$ in the antipodal light situation. Subsequently, centrins were immunoprecipitated by pan-centrin antibody and the radioactivity was analyzed. Radioactivity incorporation was 77% lower in centrins immunoprecipitated from light adapted retinas compared to those from dark adapted retinas (Fig. 1B). To identify protein kinases responsible for the phosphorylation of centrins, specific kinase inhibitors were applied to the described *ex vivo* approach. Only weak effects were obtained by the use of inhibitors for PKA and PKC (data not shown). In contrast, the implementation of 5,6-dichlorobenzimidazole riboside (DRB) as a specific inhibitor for protein kinase CK2 strongly reduced the phosphorylation of endogenous centrins in dark adapted retinas (Fig. 1C).

Subsequently, CK2 was evaluated as the potential protein kinase for the light-dependent centrin phosphorylation in mammalian photoreceptor cells. For this purpose, recombinant murine Cen1p–4p were phosphorylated by CK2 *in vitro* (Fig. 1D). CK2 strongly phosphorylated murine Cen1p and Cen2p, whereas Cen3p and Cen4p were only poor targets for CK2-mediated *in vitro* phosphorylation (Fig. 1D). Interestingly, the amount of incorporated phosphate indicated that CK2 phosphorylates all four centrins only at one single residue *in vitro* (Fig. 1D). Additional *in vitro* phosphorylation assays revealed completion of the CK2-mediated phosphorylation of centrins in the range of a few minutes (data not shown).

3.2. Determination of specific CK2 phosphorylation sites in ciliary centrin isoforms

Amino acid sequence analysis of murine Cen1p–4p for motifs, able to serve as target sequences for CK2-mediated phosphorylation, revealed several putative consensus sequences (Fig. 2). Cen1p contains five consensus sequences (S/T-X-X-D/E) [33,34] recognizable by CK2 (Fig. 2A, upper part). Cen2p possesses four target sequences for CK2 (Fig. 2A, middle). In Cen3p, only three such consensus motifs for CK2-mediated phosphorylation were found (Fig. 2A, lower part). Cen4p contains two target sequences (Fig. 2A, lowest part). To identify the functional CK2 consensus sequences in the centrin isoforms we established deletion fragments for ciliary Cen1p–3p (Fig. 2B, e.g. Cen2p).

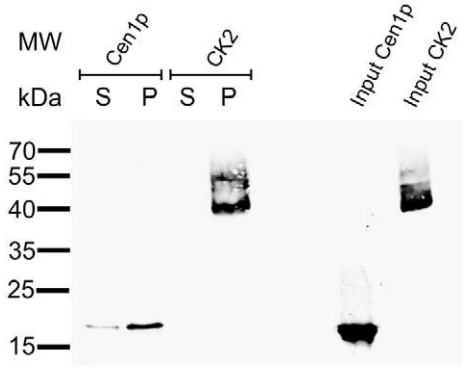

Fig. 6. Direct bindings of Cen1p and CK2 to microtubules. *In vitro* spin down assays were performed to demonstrate binding of centrins and CK2 to microtubules and analyzed by specific antibodies to Cen1p and CK2 in Western blots. This analysis of the supernatant (S) and the pellet (P) reveal direct binding of Cen1p as well as CK2 to microtubules. Input Cen1p/Input CK2=75% of deployed protein.

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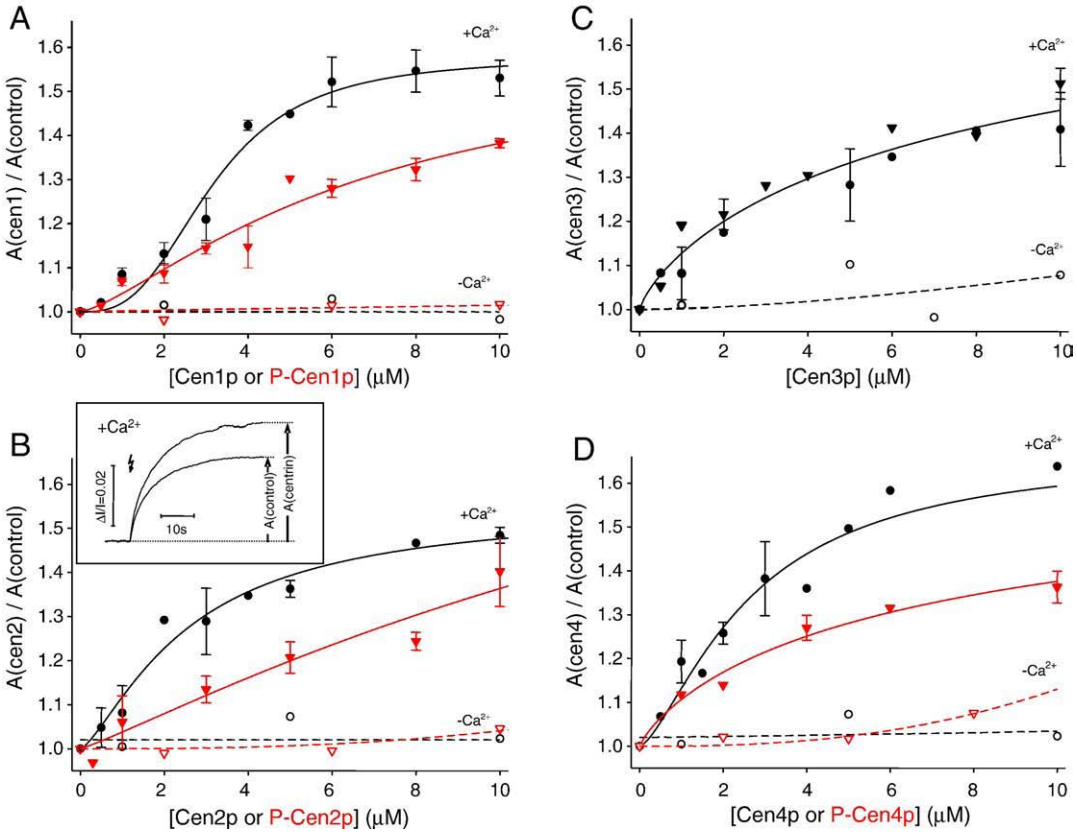

Fig. 7. Effect of centrin isoform phosphorylation on the G_t-binding signal analyzed by kinetic light-scattering. Shown is the dependence of the amplitude of flash-induced kinetic light-scattering G_t-binding signals on the concentration of Cen1p (A), Cen2p (B), Cen3p (C) and Cen4p (D), respectively. In each panel red triangles represent the phosphorylated and circles the unphosphorylated centrin isoforms. Note: Cen3p is not phosphorylated. In all traces the centrin-dependent enhancement of the G_t-binding signals is normalized to the amplitude of the G_t-binding signal without added centrin (*control*), in the presence of 100 μM $CaCl_2$ (*filled symbols*) or 1 mM EGTA (*open symbols*). Data points were fitted using the Hill equation with the parameters shown in Table 1. The inset in (B) shows exemplarily kinetic light-scattering binding signals (0.5 μM G_t, 3 μM rhodopsin) without and with Cen1p (10 μM). Measuring conditions were as described under "Materials and methods". The error bars display the S.D. for $n=3$ to 4.

These deletion fragments and the holo proteins of Cen1p–3p were recombinantly expressed and phosphorylated by CK2 to determine the specific target residues (Fig. 3). These assays revealed strong phosphorylation of the Cen1p holo protein and the ΔN-term fragment which lacks the N-terminal domain (Fig. 3A). Fragments ΔEF1 and ΔEF12 showed reduced phosphorylation compared to the holo protein. In contrast, CK2 was no longer able to phosphorylate the fragment ΔEF34 which lacks EF-hand motifs 3 and 4 (Fig. 3A). The same was true for Cen2p fragments where CK2 was not able to phosphorylate the fragment lacking EF-hands 3 and 4 (Fig. 3C). However, Cen3p was identified as a very weak substrate for CK2-mediated *in vitro* phosphorylation (Fig. 3D). Cen3p holo protein showed 8% phosphorylation compared to Cen1p holo protein (Fig. 3D). All Cen3p deletion fragments did not exhibit significant phosphorylation by CK2 (Fig. 3D). These data indicate strong evidence for isoform specific phosphorylation of Cen1p and Cen2p by CK2 in the C-terminal domain.

ESI tandem mass spectrometry analysis (ESI-MS) of CK2 phosphorylated murine Cen1p–4p clearly identified residue T^{138} in Cen2p and T^{134} in Cen4p as the target sites for CK2-mediated phosphorylation (Supplementary material Fig. S1B and S1D, respectively). If any phosphorylation was detected in Cen3p it was found at residue S^{135} (Supplementary material Fig. S1C). Unfortunately, we were not able to discriminate between S^{136} and T^{138} as the phosphorylated residues in Cen1p by mass spectrometry (Supplementary material Fig. S1A). To solve this problem S^{136} and T^{138} were changed independently from serine and threonine to alanine by site directed mutagenesis. The following phosphorylation experiments revealed that the mutant

Table 1
Influence of centrin isoform phosphorylation on Ca^{2+}-dependent enhancement of G_t-binding signals probed by kinetic light-scattering

Centrin isoform	Calculated fit parameter using the Hill equation [a]		
	A [b]	n [c]	EC_{50} [d]
Cen1p	0.58±0.04	2.9±0.7	3.2±0.3
P-Cen1p	0.60±0.31	1.4±0.5	6.8±5.3
Cen2p	0.55±0.08	1.4±0.3	2.5±0.7
P-Cen2p	1.21±0.21	1.1±0.3	21.2±5.2
Cen3p	0.89±0.62	0.8±0.3	9.5±6.1
Cen4p	0.68±0.07	1.5±0.3	2.6±0.5
P-Cen4p	0.67±0.05	0.8±0.1	7.4±1.7

[a] $f=(A\cdot[\text{Cenp}]^n)/([\text{Cenp}]^n+EC_{50}^n)+1$.
[b] Maximum Cenp-dependent enhancement of the G_t-binding signal.
[c] Hill coefficient.
[d] Effective concentrations of half maximal binding in μM.

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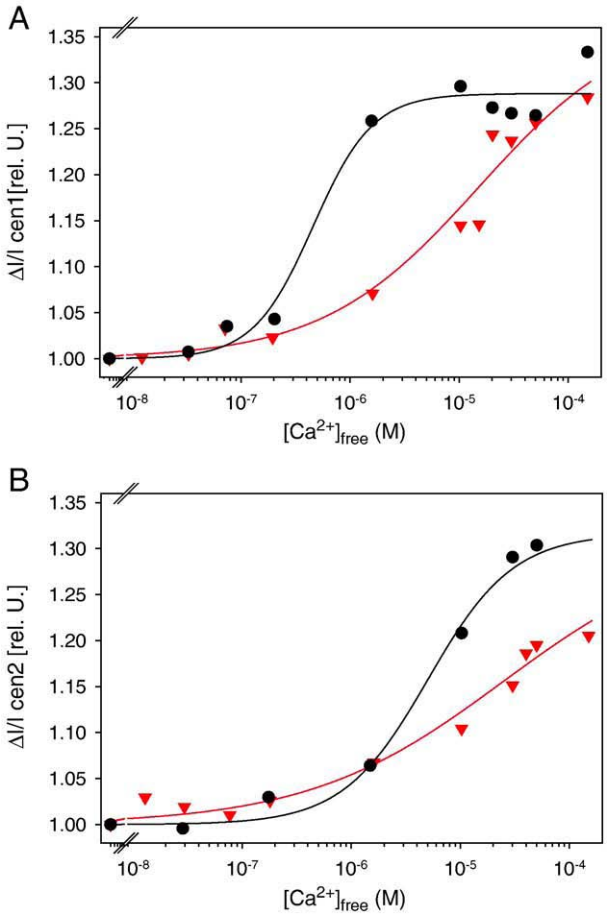

Fig. 8. Calcium titration of the centrin enhanced KLS G_t-binding signal. Amplitudes of KLS G_t-binding signals for (A) p-Cen1p and Cen1p and (B) p-Cen2p and Cen2p are shown as a function of free Ca^{2+} concentration in solution (grey triangles with dotted lines represent phosphorylated and black circles unphosphorylated centrins). Measurement conditions and calculation of the Ca^{2+} concentration were as described under "Materials and methods". Data points were fitted using the Hill equation $f=(A\cdot[Ca^{2+}]^n)/([Ca^{2+}]^n+EC_{50}^n)+1$, yielding the results shown in Table 2 (see description of parameters in Table 1).

Cen1p-S136A was still a target for phosphorylation by CK2 while the mutant Cen1p-T138A was no longer phosphorylated (Fig. 3B). This proved T^{138} as the target site for CK2-mediated phosphorylation in Cen1p.

3.3. Subcellular localization of centrins and protein kinase CK2 in murine retinas

To get more insights into the function of CK2-mediated phosphorylation of centrins in mammalian photoreceptor cells, we analyzed the subcellular localization of both proteins in murine retinas by using indirect immunofluorescence and immunoelectron microscopy. Centrins were predominantly localized at the ciliary apparatus of the photoreceptor cells consisting of the connecting cilium and the basal body (Fig. 4C). The protein kinase CK2 staining was largely confined between the outer and inner segments and at the synapses of photoreceptor cells (Fig. 4D). Higher resolution of the ciliary apparatus revealed partial co-localization of centrins (Fig. 4G and I) with CK2 (Fig. 4H and I) in the basal part of the connecting cilium of the photoreceptor cells. In conclusion, our analyses by indirect immunofluorescence suggested a ciliary co-localization of protein kinase CK2 with centrins.

We next sought to define the spatial distribution of CK2 in the ciliary region more precisely by performing immunoelectron microscopy. The present silver-enhanced immunogold labeling of centrins (Fig. 5A) as well as of CK2 (Fig. 5B) revealed localization of both proteins in the connecting cilium of mammalian photoreceptor cells.

3.4. Binding of CK2 and centrins to microtubules

The subciliary co-localization of CK2 and Cen1p–3p at the axonemal microtubules of the connecting cilium prompted us to analyze whether CK2 and the ciliary centrins directly bind to microtubules (Fig. 6). For this purpose, we used recombinant CK2 and Cen1p–3p in microtubule spin down assays (e.g. [35]). In this assay proteins, directly binding to microtubules, are recovered after centrifugation in the pellet whereas non-binding proteins stay in the supernatant. In these spin down assays, we recovered Cen1p–3p isoforms and CK2 mainly in the pellet (Fig. 6, Cen2p and Cen3p data not shown). These data confirmed CK2-binding to microtubules as recently indicated [36,37] and revealed centrins as microtubule binding proteins for the first time.

3.5. Influence of centrin phosphorylation on the centrin–transducin interaction

To analyze the interaction between phosphorylated and non-phosphorylated murine Cen1p–4p with transducin, centrin isoforms were phosphorylated by protein kinase CK2 and their binding affinity for transducin was analyzed using the kinetic light-scattering monitor [28]. The latter assay is based on the following: The gain of mass of a membrane, when transducin is bound from the solution, produces large and readily measurable changes in near-infrared light-scattering. Our measuring conditions (2 μM rhodopsin, 0.5 μM G_t) represent a diluted system where about 40% of transducin is in solution [31,47]. Bleaching excess rhodopsin (Rh*) induces the transition of soluble G_t ($G_t$sol) to the membrane in the absence of GTP which gives rise to an increase in scattered light ("binding signal") [38,39].

Table 2
Influence of centrin isoform phosphorylation on Ca^{2+} affinity probed by kinetic light-scattering G_t-binding signals using constant centrin concentrations

Centrin isoform	Calculated fit parameter using the Hill equation[a]		
	A[b]	n[c]	EC_{50}[d]
Cen1p	0.29±0.01	1.6±0.4	0.5±0.2
P-Cen1p	0.37±0.08	0.6±0.2	15.1±12.3
Cen2p	0.32±0.09	1.1±0.2	5.1±1.1
P-Cen2p	0.31±0.09	0.5±0.1	24.8±31.7

[a] $f=(A\cdot[Ca^{2+}]^n)/([Ca^{2+}]^n+EC_{50}^n)+1$.
[b] Maximum $[Ca^{2+}]$-dependent centrin induced enhancement of the G_t-binding signal.
[c] Hill coefficient.
[d] Effective concentrations of half maximal binding in μM.

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The assay is applicable to any soluble protein that interacts with Rh* [28] and can also serve to analyze changes in the amount and/or molecular weight of transducin when it interacts with all known centrin isoforms [16]. Addition of phosphorylated centrin isoforms (P-Cen1p, P-Cen2p and P-Cen4p) also led to an enhanced amplitude of the binding signal when Ca^{2+} was present (an example is given in Fig. 7B, inset) whereas in the absence of free Ca^{2+} the increase of the amplitude was significantly lower. To determine the effective concentrations of half maximal binding (EC_{50}) of the interaction between phosphorylated and non-phosphorylated centrin isoforms with transducin, we employed the procedure described previously [14]. At a constant concentration of Rh* and transducin and varying centrin concentrations, the enhanced amplitude of the G_t-binding signal increased and reached saturation (Fig. 7). The analysis of the titration curves revealed that the EC_{50} values of the phosphorylated P-Cen1p, P-Cen2p and P-Cen4p isoforms were significantly higher than for the non-phosphorylated centrin isoforms (for details see Table 1 and Fig. 7). In contrast, Cen3p was not phosphorylated by CK2 and therefore, no change of the EC_{50} value was observed (Fig. 7C). These differences (especially for Cen2p) indicate significantly lower affinities between transducin holo protein and the phosphorylated centrins, than for unphosphorylated centrins. The calculated Hill coefficient of P-Cen1p and P-Cen2p are lower than for the unphosphorylated forms. But since the coefficient is still larger than 1, it cannot be excluded that phosphorylated centrins also bind to G_t as oligomers (calculated Hill coefficient $n \geq 1$, see Table 1 and Fig. 7).

3.6. Lower affinity of phosphorylated centrins to transducin is caused by decreased Ca^{2+}-sensitivity

CK2-mediated phosphorylation of Cen1p and Cen2p at T^{138} in the C-terminal domain can differentially affect their binding to target proteins. The introduction of a phosphate group close to the target binding site may cause steric or electrostatic hindrance of target binding to the C-terminus of Cen1p and Cen2p. However, the phosphate group of the centrins may also alter the ability of the EF-hands 3 and 4 to bind Ca^{2+}-ions.

To address this question we performed a Ca^{2+}-titration in the kinetic light-scattering assay using fixed concentrations of Rh*, transducin and centrin while varying the concentration of free Ca^{2+} (Fig. 8). Fitting the plots using the Hill equation yields the data, shown in Table 2, for the cooperativity of Ca^{2+}-binding and the effective concentrations for half maximal binding. Data are shown for Cen1p compared to P-Cen1p and for Cen2p compared to P-Cen2p. The Hill coefficient for Cen1p is $n = 1.6 \pm 0.4$, which is in good agreement with our earlier data ($n = 1.7 \pm 0.2$; [17]). For P-Cen1p we found a value of $n = 0.6 \pm 0.2$, which indicated that less Ca^{2+}-binding sites were occupied when the centrin molecules were phosphorylated. The same effect was observed for Cen2p ($n = 1.1 \pm 0.2$) and P-Cen2p ($n = 0.5 \pm 0.1$). Further analysis of the titration curves revealed that the EC_{50} values for Ca^{2+}-binding were significantly higher for P-Cen1p and P-Cen2p (for details see Table 2 and Fig. 8). This indicated a significantly lower Ca^{2+}-affinity of P-Cen1p (30 times lower) and P-Cen2p (5 times lower) compared to the non-phosphorylated centrins.

4. Discussion

We and others have previously shown that the functions of centrins are regulated by the modulation of the intracellular concentration of free Ca^{2+} [17,19,23,24]. In photoreceptor cells of the mammalian retina, Ca^{2+}-ions trigger the binding of centrin isoforms to the visual G-protein transducin [14,16,17,19]. However, there is evidence from other systems that functions of centrins can be also regulated by phosphorylation [20–22,25]. Here we report that centrins are phosphorylated in dark adapted photoreceptor cells of the mammalian retina. In contrast to previous studies, where protein kinase A (PKA) is responsible for the modification of centrins, we identified CK2 as the major kinase phosphorylating a specific site in Cen1p, Cen2p and Cen4p under a well defined physiological condition of vertebrate photoreceptor cells.

4.1. Centrins as targets for CK2-mediated phosphorylation

Our present studies highlight centrins as novel targets for protein kinase CK2 in mature vertebrate photoreceptor cells. We verified these phosphorylations *in vitro* by using deletion fragments of Cen1p–4p and point mutations in combination with LC-ESI tandem mass spectrometry analyses. Murine Cen1p, Cen2p and Cen4p were phosphorylated only at a single residue (Cen1p and Cen2p at T^{138} and Cen4p at T^{134}), whereas Cen3p was not a target for the CK2-mediated phosphorylation. The identified target residues for these phosphorylations are located between the Ca^{2+}-binding EF-hands 3 and 4 in the centrin molecules. They represent typical CK2 phosphorylation sites with the consensus sequence S/T-X-X-D/E [33,34,40]. Comparative amino acid analyzes of Cen1p, Cen2p and Cen4p revealed these identified target sequences to be 100% conserved for Cen1p and Cen2p (E^{132}LGESLTDEELQE^{144}) and with only two residues changed in Cen4p (E^{128}LGESLTEDELQE^{140}). The alignment of amino acid sequences of centrins from all vertebrate species analyzed (Supplementary material Fig. S2) revealed conserved target sequences throughout these species (Supplementary material Table S1). These data indicate that CK2-mediated phosphorylation in these sites is a well conserved and common regulatory mechanism for Cen1p, Cen2p and Cen4p. In contrast, in Cen3p the corresponding sequence (E^{129}LGENMSDEELRA^{141}) is modified downstream by a basic arginine (R^{140}) which might inhibit CK2 since it requires acidic clusters for proper function [33,41]. The displayed differences between known mammalian centrins go along with the fact that they belong to two different phylogenic subgroups of centrins. One group contains Cen1p, Cen2p and Cen4p and the other group is related to the yeast centrin Cdc31 where Cen3p is situated [17,42].

4.2. Light-dependent modulation of CK2-mediated phosphorylation of centrins

Based on the current knowledge on the modulation of CK2 activity, there are mainly two possible alternatives for the regulation of the phosphorylation status of centrins: i.) An

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upstream signaling cascade regulates the activity of CK2 and in turn the phosphorylation of centrins. ii.) CK2 is constantly active and the phosphorylation status of centrins is modulated by centrin dephosphorylation mediated by the kinase counterpart, namely protein phosphatases.

In parallel to the present study, we have recently identified protein phosphatases 2Cα and β (PP2Cα and β) as the phosphatases responsible for the hydrolysis of the phosphate at CK2 phosphorylation sites in Cen1p, Cen2p and Cen4p with extraordinary efficiency in the photoreceptor cilium (Thissen et al., in preparation). Since the activity of PP2Cα and β is inhibited by Ca^{2+}-ions and stimulated either by Mg^{2+}-ions or by certain unsaturated long chain fatty acids [43,44] all three features may modulate the phosphorylation of CK2 sites in centrins. In photoreceptor cells, no physiological changes in the free Mg^{2+}-concentration were observed [45] and the reported alterations of unsaturated long chain fatty acids in photoreceptor membranes are too slow for regulatory processes by PP2C-mediated dephosphorylation of centrins [46,47]. Ca^{2+}-ions seem to have slight inhibitory effects on both PP2Cβ and CK2 (Thissen et al., in preparation) and may therefore also not be relevant for the regulation of the phosphorylation status of centrins.

An alternative scenario is that the phosphorylation status of centrins is regulated by changes of the CK2 activity. A most recent study on the Wnt/β-catenin signaling pathway indicated the activation of CK2 triggered by activated α-subunits of G_o or G_q, respectively [48]. Since transducin α-subunit $G_t\alpha$ is closely related to G_o [49], the light triggered activation of G_t and subsequent release of the α-subunit may also modulate the CK2 activity in photoreceptor cells. In addition, an alternative for regulation of CK2 activity by centrins depending on their Ca^{2+}-binding status cannot be excluded.

4.3. CK2 as a component of the photoreceptor ciliary complex

Recent proteome analysis of the photoreceptor ciliary complex confirmed the presence of CK2 in the ciliary fraction of photoreceptor cells [7], which is in agreement with previous biochemical analyses of the axonemal fraction of bovine photoreceptor cells [6]. In a most recent study, we have shown that CK2 directly interacts with lebercilin, a resident protein of the connecting cilium essential for photoreceptor cell function [50]. Here we demonstrated for the first time a CK2 localization *in situ* in the connecting cilium of photoreceptor cells, which correlates with the transition zone of prototypic cilia [15,51]. Thus, CK2 co-localizes with its substrates Cen1p and Cen2p in the connecting cilium. Present microtubule binding assays revealed the direct binding of Cen1p and CK2 to microtubules indicating the parallel anchorage of the ciliary centrins and CK2 at the microtubules of the photoreceptor connecting cilium. This close molecular proximity is necessary since we have found no stable complex formation of Cen1p and CK2. The subciliary co-localization of substrates, the protein kinase complex, and the phosphatases should provide further specificity, sensitivity and speed to the enzyme–substrate reactions.

4.4. Ciliary impact and role of CK2-mediated centrin phosphorylation on light induced transducin translocation

One aspect of a CK2-mediated phosphorylation of ciliary proteins was derived from a study that proved its participation in transport processes of cilia [52]. In the latter study, CK2 phosphorylation of PACS-1 is essential for localization of PACS-1 and nephrocystin to the transition zone of renal cilia. This indicated CK2-dependent transport processes as a novel pathway of targeting proteins to the transition zone of cilia [52]; which is homologous to the connecting cilium of photoreceptor cells [15]. Comparable CK2-driven transport processes are likely located in photoreceptor cells since nephrocystin also is a component of the connecting cilium [53].

In addition to targeting proteins to the transition zone of cilia, CK2 was shown to participate in the regulation of Ca^{2+}-homeostasis in primary cilia of *Caenorhabditis elegans* [54]. In vertebrate photoreceptor cells, the ciliary Cen1p and Cen2p have been shown to bind to the heterotrimeric visual G-protein transducin (G_t) in a strictly Ca^{2+}-dependent manner [14,16–19]. Here we demonstrated that phosphorylation of murine Cen1p and Cen2p leads to decreases in the binding affinities for G_t.

As shown by Meyn et al. [25] for *Chlamydomonas reinhardtii* centrin (CrCenp), the addition of a phosphate group has a strong steric influence on surrounding amino acids. In our case, this should lead to conformational changes in the C-terminal domain which may lead to reduced Ca^{2+}-sensitivity and/or binding affinity of interacting partner proteins. The CK2-mediated phosphorylation is targeted to one specific threonine residue T^{138} of Cen1p and Cen2p that is located at the loop joining the two Ca^{2+}-binding EF-hand motifs 3 and 4 [55,56]. Recent structural data indicate that a part of the N-terminal extension of "MmCen1p-L" [56] which probably mimics $G_t\beta\gamma$ interaction with Cen1p introduces a slight distortion of the second helix of EF-hand 3 [56]. This brings K^{127} into close proximity of D^{139}, next to the phosphorylation site, likely forming a saltbridge. A phosphate at position T^{138} of Cen1p may inhibit the saltbridge formation by steric hindrance of D^{139} or it might form a saltbridge to D^{139} by itself.

Our present results obtained by kinetic light-scattering experiments with varying concentrations of free Ca^{2+} strongly support the idea of a lowered Ca^{2+}-sensitivity, caused by phosphate induced conformational changes. The Hill coefficient was about 2.5-fold higher for the unphosphorylated Cen1p and two-fold higher for unphosphorylated Cen2p compared to the phosphorylated forms, which indicates that less Ca^{2+} is bound and not all possible Ca^{2+}-binding sites are occupied. In addition to that, the EC_{50} values for Ca^{2+}-binding to P-Cen1p and P-Cen2p are about 30-fold and 5-fold lower, respectively. From these data we conclude that the addition of a phosphate in position T^{138} of Cen1p and Cen2p influences the structure in a way that, most likely, EF-hand 3 is not able to bind Ca^{2+} anymore and the overall Ca^{2+}-affinity is reduced significantly. A similar change in Ca^{2+}-sensitivity was demonstrated for the closely related prototypical Ca^{2+}-sensor calmodulin [57].

The presented results indicate an antagonistic function of Ca^{2+}-binding and CK2-mediated phosphorylation on the formation of

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centrin/G-protein complexes. Antagonistic relationships between Ca^{2+}-binding and phosphorylation in centrin functions were previously described in green algae [20,21]. There, Ca^{2+}-binding to centrins induces contractions of centrin fibers whereas the phosphorylation of centrins is necessary for the fiber relaxation.

In conclusion, present data demonstrate a drastic increase in the phosphorylation of Cen1p, Cen2p and Cen4p during dark adaptation of photoreceptor cells. This phosphorylation lowers the Ca^{2+} binding affinity of centrins and consequently leads to reduced binding affinity for the visual G-protein transducin to the ciliary centrins. Therefore, the flow of transducin through the connecting cilium is not impeded by binding to the ciliary centrins in dark adapted photoreceptor cells. After illumination, the dephosphorylation of centrins increases their affinity to Ca^{2+} and propagates the assembly of centrin/transducin complexes. The interplay of phosphorylation and dephosphorylation of ciliary centrins together with the modulation of local ciliary Ca^{2+}-concentrations may regulate the translocation of transducin through the connecting cilium of photoreceptor cells. The reported novel stimulus dependent reversal regulation of centrin/G-protein complex formation by protein kinase CK2 might also have implications in other cellular processes, e.g. on the function of centrosomes, by recruiting regulatory G-proteins at special time points during the cell cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.01.006.

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Glossary

CK2: protein kinase CK2 (formally known as Casein Kinase 2)
PKA: protein kinase A
PKC: protein kinase C
G_t*:* retinal G-protein transducin
Cetn1–4: murine centrin genes 1–4
Cen1p–4p: murine centrin isoforms 1–4
P-Cen1p–4p: CK2-phosphorylated centrin isoforms 1–4
GST: glutathione S-transferase
cGMP: cyclic guanosine monophosphate
5′GMP: guanosine monophosphate
$[\gamma^{32}P]ATP$*:* radioactive gamma-labeled adenosintriphosphate
$H_3[^{32}P]O_4$*:* radioactive labeled inorganic phosphate
H_3PO_4*:* inorganic phosphate
NMR: nuclear magnetic resonance
EDTA: diaminoethanetetraacetic acid
Nonidet NP-40: nonylphenylpolyethylene glycol
C12E10: polyoxyethylene 10 lauryl ether;
amino acids are indicated with one-letter code

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Dissertation Publikation II

2.2 Thissen MC, Trojan P, Kriegelstein J, Wolfrum U, Klumpp S Dephosphorylation of centrins by protein phosphatase 2Cα and 2Cβ. FEBS letters eingereicht

Dephosphorylation of centrins by protein phosphatase 2Cα and 2Cβ

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Running title
Dephosphorylation of centrins by PP2C

Key words
Centrin; Dephosphorylation; Protein kinase CK2, Protein phosphatase type 2C; Signal transduction

Abbreviations
BAD, Bcl-X_L/Bcl-2 associated death promoter; Cen, centrins; Cen1-4, centrin isoforms 1-4; CK2, protein kinase CK2 (formerly known as casein kinase 2); GST, glutathione S-transferase; P-BAD, phosphorylated BAD protein; P-Cen, phosphorylated centrin; PP, serine/threonine protein phosphatase; TBB, 4,5,6,7 tetrabromobenzimidazole.

Summary

Centrins (Cen) are Ca^{2+}-binding proteins. Their physiological functions are not only regulated by free Ca^{2+} but also by reversible phosphorylation. Corresponding kinases have been identified. Knowledge on phosphatases for dephosphorylation of centrins, however, is still missing.

In the present study, we identified protein phosphatases dephosphorylating centrins previously phosphorylated by protein kinase CK2. Considering the importance and the role of centrins in photoreceptor cells, various phosphatases known to be present in the retina were tested: PP1, PP2A, PP2B, PP2C, PP5 and alkaline phosphatase. PP2Cα and PP2Cβ were capable to dephosphorylate P-Thr^{138}-Cen1 most efficiently. PP2Cδ was inactive and the other retinal phosphatases also had much less or no effect. Similar results were observed for Cen2 and Cen4. Cen3 was not a substrate for CK2. We subsequently characterized PP2Cβ-mediated dephosphorylation of P-Thr^{138}-Cen1 in more detail and extended our studies to investigate the subcellular distribution of PP2Cβ and Cen. Indirect immunofluorescence and immunoelectron microscopy revealed co-localization of retinal centrin isoforms, protein kinase CK2 and PP2Cβ within the photoreceptor connecting cilium suggesting functional interplay of these proteins *in vivo* there.

Introduction

Reversible phosphorylation is one of the most important regulatory events for protein function and cellular processes. Members of the protein phosphatase 2C (PP2C) family are Mg^{2+}/Mn^{2+}-dependent serine/threonine phosphatases and insensitive to inhibition by okadaic acid [1-4]. At least 17 distinct PP2C human paralogues have been found. PP2C enzymes are not evolutionary related to the serine/threonine phosphatases type-1, 2A, and 2B which are all multisubunit proteins. Several PP2Cs are involved in regulation and inactivation of stress activated protein kinases. Other PP2C isozymes are associated with eukaryotic cell cycle progression via inactivation of cyclin dependent kinases. PP2C enzymes are mainly soluble and display broad substrate specificity, however, dephosphorylation of P-Thr is preferred. The discovery of PP2Cα and PP2Cβ goes back to the 1980s [5,6]. The prototype 42 and 45 kDa proteins share 75% amino acid sequence identity [7]. For the α-isoenzyme two splice variants (36 and 42 kDa) were found and six splice variants for PP2Cβ (42-55 kDa). Their activity can be stimulated by certain unsaturated fatty acids [8] and inhibited by Ca^{2+}-ions [4,9,10].

In the present study we focus on the regulation of the function of centrins by phosphorylation and dephosphorylation. In vertebrates, four centrin isofoms (Cen1 to Cen4) are known. They are ubiquitously expressed and associated with centrioles of centrosomes and centrosome-related structures such as spindle poles of dividing cells [11-14]. The effect of centrins is triggered by Ca^{2+}-binding and by phosphorylation[15-17]. In mammalian cells, phosphorylation of Cen2 is a necessary checkpoint for the duplication of the centrioles during the cell cycle [15]. In the highly specialized vertebrate photoreceptor cells, centrins are components of the ciliary apparatus localized in the connecting cilium and their basal bodies [11,18,19]. There, the Cen1 and Cen2 interact with the visual heterotrimeric G-protein transducin in a strict Ca^{2+}-dependent way [11,18,20]. The formation of the latter complex is thought to play an essential role in the regulation of the light-dependent movements of G-protein through the connecting cilium [11,14,18]. However, the function of centrins is not

only modulated by free Ca^{2+}, but also by site specific phosphorylation: in green algae and in cultured human cell lines centrins are phosphorylated by protein kinase A [15,16]. Recently, we have shown that CK2 is the major enzyme which phosphorylates centrins in photoreceptor cells of mammalian retinas [17,18]. In fully differentiated photoreceptor cells, CK2 phosphorylates Cen1 and Cen2 localized within the connecting cilium during dark adaptation. Since the phosphorylation of the ciliary centrins drastically reduces the binding to transducin, it is suggested that the light-dependent translocation of transducin through the cilium is further regulated by CK2 phosphorylation and by the phosphatase involved.

The present study was designed to identify protein phosphatases that serve as counterparts for the CK2-mediated light-dependent phosphorylation of centrins in mammalian photoreceptor cells. We found that Ser/Thr protein phosphatases PP2Cα and PP2Cβ quite effectively dephosphorylate P-Cen1, P-Cen2 and P-Cen4. In contrast, five other representatives of the most common phosphatases present in the retina and the δ-isozyme of PP2C led to much less or no dephosphorylation of P-centrins. Immunohistochemical analysis of the murine retina using indirect immunofluorescence and immunoelectron microscopy indicated colocalization of PP2Cβ and centrins within the ciliary apparatus of photoreceptor cells. Together, these findings support a role for PP2Cα and PP2Cβ to influence the phosphorylation status of centrins thus regulating the light-dependent exchange of G-proteins between the outer segment and the inner segment of retinal photoreceptor cells.

Results

Phosphorylation of centrins by CK2

Phosphorylation of Cen1 is critical for its complex assembly with transducin [17]. It was shown that Cen1 can be phosphorylated at Thr^{138} by CK2. Here we set out to further characterize phosphorylation of centrins by CK2.

Purified recombinant Cen1 could be phosphorylated *in vitro* by CK2 using ATP as phosphate source within a few minutes only (Fig. 1A). Phosphorylation of Cen1 by CK2 was not detectable in the presence of 100 μM of the CK2-inhibitor TBB (Fig. 1B, left panel). Guanine nucleotides are playing a uniquely important role in the retina and for vision [21]. Indeed, phosphorylation of Cen1 by CK2 worked equally well using GTP as phosphate source instead of ATP (Fig. 1C).

Four isoforms of centrins have been identified in rodents [11,18]. Thr^{138} of Cen1 is conserved in Cen2 (Thr^{138}) and Cen4 (Thr^{134}) whereas Cen3 (Ser^{135}) carries a serine residue instead. As expected from the amino acid sequence identity, Cen2 and Cen4 also could be phosphorylated by CK2 (Fig. 1D). A variety of proteins is phosphorylated by CK2 at serine residues (for review see [22]). Cen3, however, was not a substrate of CK2 (Fig. 1D). Coomassie staining was used in parallel to verify equal protein loading (Fig. 1E).

Identification of the phosphatases hydrolyzing P-centrins

Cen1 was phosphorylated by CK2 at Thr^{138} as described in the Experimental Procedures and the reaction terminated either by removal of unincorporated ATP or by addition of the CK2 inhibitor TBB. Our search for phosphatases acting on P-Cen1 included PP1, PP2A, PP2B, PP2Cβ and PP5. Those protein phosphatases represent the ´classical´ and most abundant Ser/Thr-phosphatases acting on a variety of substrates, respectively [23]. Unspecific alkaline phosphatase was also tested. All these phosphatases examined here for potential dephosphorylation of P-Cen1 are present in the retina [24-27]. The CK2-inhibitor TBB used

to prevent ongoing phosphorylation upon incubation with the phosphatases had no effect on the phosphatase activities as exemplified for PP2Cβ (Fig. 1B, right panel).

Surprisingly, among the 6 phosphatases tested here PP2Cβ was most efficiently dephosphorylating P-Cen1 (Fig. 2A). All the other phosphatases tested had no or much less effect (Fig. 2A). This unexpected selectivity prompted us to run the dephosporylation of P-BAD as an extra control. For that purpose BAD was phosphorylated at Thr^{117} by CK2 [28]. Dephosporylation of P-BAD was run in parallel and identical to the experiments dealing with the putative dephosphorylation of P-Cen1. In analogy to what is known for the majority of phosphorylation sites in any protein, our *in vitro* studies revealed that P-Thr^{117}-BAD more or less could be hydrolyzed by all the phosphatases tested (Fig. 2B). This was in sharp contrast to the results obtained with phosphatases acting on P-Cen1 (Fig. 2A versus 2B). This unexpected result - strongest dephosphorylation of P-Cen1 by PP2Cβ (Fig. 2A) - was also observed for P-Cen2 and P-Cen4 (data not shown).

Characterization of dephosphorylation of P-Cen1 by PP2Cβ

Dephosphorylation of P-Cen1 by PP2Cβ was studied in more detail. An increasing amount of PP2Cβ protein resulted in enhanced dephosphorylation (Fig. 3A). PP2C enzymes are characterized by their requirement for Mg^{2+}- or Mn^{2+}-cations for activity [29]. In line with that, dephosphorylation of P-Cen1 by PP2Cβ increased upon addition of Mg^{2+}-ions (Fig. 3B). In contrast, Ca^{2+}-ions are known to inhibit PP2C activity in a variety of organisms and tissues [4,10]. Increasing the Ca^{2+}-ion concentration reduced dephosphorylation of P-Cen1 by PP2Cβ (Fig. 3C). Unsaturated long chain fatty acids are inhibiting PP2C activity from plants [30] but activate PP2Cα and PP2Cβ in vertebrates [8]. Oleic acid (18:1) was capable of stimulating dephosphorylation of P-Cen1 by PP2Cβ (Fig. 3D). A similar stimulating effect was observed with docosahexaenoic acid (22:6) (data not shown).

PP2Cα and PP2Cβ are known for more than 30 years. Properties of PP2Cα and PP2Cβ are indistinguishable except for different substrate specificities towards proteins of the MAPK-pathway [24,28]. Localization and function of the other PP2C-isozymes are quite distinct. Dephosphorylation of P-Cen1 was detectable not only with PP2Cβ as shown before but also with PP2Cα (Fig. 3E). In contrast, P-Thr^{138}-Cen1 could not be hydrolyzed by PP2Cδ (Fig. 3E).

Co-localization of PP2Cβ and centrins in the connecting cilium of photoreceptor cells

The remarkable selectivity for dephosphorylation of P-Thr^{138}-Cen1 by PP2Cα and PP2Cβ *in vitro* supports the idea of *in vivo* relevance. To evaluate whether P-Cen might be substrates for PP2Cβ in retinal photoreceptor cells we performed immunohistochemical analyses by light- and electron microscopy.

Indirect immunofluorescent experiments of cryosections through the mouse retina revealed the brightest anti-PP2Cβ immunofluorescence in the connecting cilium of photoreceptor cells (Fig. 4C). Previous studies demonstrated that Cen1, Cen2 and Cen3 are localized in the connecting cilium of photoreceptor cells [11,19]. Therefore, we further evaluated the potential ciliary co-localization of PP2Cβ and centrins. Cen3 is an established marker protein for the connecting cilium [11, 14, 17, 18, 20, 36, 44]. Accordingly, we focused on Cen3 for localization studies. Merged images of immunofluorescence double staining with anti-PP2Cβ and anti-Cen3 antibodies revealed co-localization of both proteins in the ciliary apparatus of photoreceptor cells (Fig. 4D). High magnification of these double stainings in the ciliary complex of photoreceptor cells further supported co-localization of PP2Cβ and Cen3 in the connecting cilium and the basal body complex (Fig. 4G-I).

Immunoelectron microscopy was performed to determine the subcellular distribution of PP2Cβ more precisely (Fig. 5). In post-embedding labelling experiments, we stained ultrathin LRWhite sections with antibodies against PP2Cβ and against Cen3 in parallel. Electron microscopic analysis of anti-centrin silver-enhanced immunogold labelling confirmed previous results with clear restriction of Cen3 to the connecting cilium (Fig. 5A) [11,18,19]. Silver-enhanced immunogold labelling of PP2Cβ was present in the outer segment, the inner segment and in the connecting cilium of photoreceptor cells (Fig. 5B). To approve the subcellular detection of PP2Cβ in photoreceptor cells we also applied the pre-embedding labelling method for immunoelectron microscopy analysis [31]. The latter application has the advantage that the antibody labelling takes place in weakly fixed and not dehydrated whole mounts of the mouse retina. It revealed a more prominent staining of PP2Cβ in the photoreceptor cilium, yet also in the photoreceptor inner segment (Fig. 5C). In comparison to the post-embedding experiments, the staining for PP2Cβ was much weaker in the outer segment (Fig. 5C). Taken together, our immunocytochemical analyses supported subcellular co-localization of Cen3 and the PP2Cβ in the connecting cilium of photoreceptor cells.

Discussion

There is overwhelming evidence that reversible phosphorylation of proteins is the major mechanism for regulation of a broad spectrum of fundamental cellular processes. This study was designed to analyze phosphorylation and dephosphorylation of centrins as a regulatory mechanism in mammalian photoreceptor cells. It has been recently shown that the mammalian members of the EF-containing Ca^{2+}-binding protein family of centrins are phosphorylated by protein kinase CK2 in dark adapted photoreceptor cells [17]. In particular, CK2 phosphorylates specific sites at the C-terminal region between EF-hands 3 and 4 of the murine centrin isoforms Cen1, Cen2 and Cen4 (Thr^{138}, Thr^{138}, and Thr^{134}, respectively). In contrast, Cen3 was not a target for CK2. Here we demonstrate that CK2 can use GTP with the same efficiency as ATP for phosphorylation of centrins. It was known that both nucleotides are present in the inner- and outer segments of photoreceptor cells [45].

The human genome encodes several hundred protein kinases. CK2 was first described 50 years ago. Its extraordinary pleiotropy is documented by a still growing list of currently >300 protein substrates [22]. One of the most remarkable features of CK2 is the fact that its kinase activity is detectable in cells and tissue even in the absence of stimulation or addition of cofactors. CK2 is constitutively active and has been considered unregulated [22,32]. Phosphorylation of centrins by CK2 occurs during dark adaptation in photoreceptor cells of the mammalian retina. It reciprocally regulates the Ca^{2+}-mediated binding of centrins to the βγ-subunit of the visual heterotrimeric visual G-protein transducin [11,17,20]. If CK2 is constantly active in photoreceptor cilia, as seen in most systems studied so far, the identity and regulation of a phosphatase responsible for dephosphorylation of CK2-mediated centrin phosphorylation might be crucial for the biological effect of centrins.

Accordingly, in the present study, we addressed the important question which phosphatase is capable of dephosphorylating P-Thr^{138}-Cen1. All the most abundant retinal phosphatases were tested: PP1, PP2A, PP2B, PP2Cα and PP2Cβ, PP5 and alkaline phosphatase [24-27].

Our results were most striking: PP2Cα and PP2Cβ most efficiently hydrolyzed P-Cen1, all other phosphatases tested had no or much less effect. This unexpected finding was verified using P-Thr^{117}-BAD, phosphorylated by CK2, for control [28]. As expected, P-BAD was dephosphorylated by all those phosphatases which is in sharp contrast to the specific dephosphorylation of centrins by PP2Cα and PP2Cβ.

Further studies in the future are necessary to establish which kind of regulation applies to switch on and off PP2Cα and PP2Cβ in the retina. The following regulatory mechanisms are currently discussed for PP2Cα and PP2Cβ: inhibition by Ca^{2+}-ions [4,9,10], and activation by certain unsaturated long chain fatty acids [29], e.g. docosahexanoic acid or oleic acid. The dependence on light for regulation of the phosphorylation/dephosphorylation cycle of centrins in photoreceptor cells supports the idea that the activity of a potential regulator is triggered by a light modulated second messenger. The Ca^{2+}-concentration changes depending on light. This cation might serve as a negative regulator for PP2Cα and PP2Cβ. Determination of the activity of PP2Cα and PP2Cβ in light adapted and dark adapted retinas might be worth while studying, although difficult to perform because of a huge background activity of all other phosphatases present and because of the lack of an inhibitor for PP2C. Alternatively, PP2Cα and PP2Cβ might be activated by omega-3 fatty acids like docosahexanoic acid (DHA). Recent work showed that high concentrations of DHA activate PP2Cβ [29]. The amount of DHA in the plasma membrane of photoreceptor cells is known to alter in response to light [33,34]. However, changes of the lipid membrane take place on a time scale of days and weeks and might be too slow for the adaptive processes occurring within hours to minutes underlined by PP2C-mediated dephosphorylation of P-centrins.

Cellular and subcellular localization of molecules often provide key information for their functions. Our present immunohistochemical analysis revealed that indirect immunofluorescence of PP2Cβ was predominantly present in the connecting cilium of

photoreceptor cells of murine retinas. Thus, PP2Cβ co-localizes not only with its P-centrin substrates, but also with CK2, the protein kinase responsible for phosphorylation of centrins [11,17].

For fine regulation of their function many proteins are phosphorylated at several distinct sites. Knowledge on the reversible phosphorylation of centrins currently comprises PKA at Ser^{167} [15,16,35], PKC [15], Cdc2 [15] and CK2 [36]. This report is the first focusing on phosphatases acting on P-centrins. Because of the unexpected potency of PP2Cα and PP2Cβ to dephosphorylate CK2-mediated P-Cen1, we briefly checked whether PP2Cα and PP2Cβ might also dephosphorylate P-Cen1 after phosphorylation by PKA. This was not the case (data not shown). Therefore, we conclude that if there is crosstalk and hierarchy among the two phosphorylation sites identified in centrin proteins, PP2Cα and PP2Cβ are playing a most decisive role. Overall, dephosphorylation of P-centrins by PP2Cα and PP2Cβ should increase the affinity of centrins to $G_t\beta\gamma$ and finally reduce transport of the G-protein transducin through the connecting cilium.

Experimental procedures

Chemicals and proteins

Protein kinase CK2 was a gift from Lorenzo A. Pinna (Padova, Italy), protein phosphatase type 5 was provided by Joachim Neumann (Halle, Germany), and the expression vector containing protein phosphatase type 2Cδ cDNA by Shinri Tamura (Sendai, Japan). Protein phosphatase type 1 was purchased from Calbiochem. Protein phosphatases type 2A and type 2B were obtained from Upstate. Alkaline phosphatase was from Sigma. BAD, PP2Cα and PP2Cβ were expressed and purified as described, respectively [24,37]. Subcloning of murine Cen1–4 cDNAs into the pGEX-4T3 expression vector (GE Healthcare), protein expression (Cen1–4) and purification was performed as described [17,20]. Polyclonal antibodies specific

against PP2Cβ were generated and purified as reported [24]. Monoclonal PP2Cβ antibodies were kindly provided by Sara Lavi (Tel Aviv, Israel). Polyclonal antibodies from rabbit against recombinant mouse Cen1 to Cen4 were previously described [11]. Fluorescent secondary antibodies labeled with AlexaFluor® 488 or AlexaFluor® 546 were purchased from Molecular Probes. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from GE Healthcare. Mowiol 4.88 was obtained from Hoechst. Protein was determined by Lowry [38] using bovine serum albumin as a standard.

Phosphorylation of centrins and BAD

GST-Centrins (0.2 µg) or GST-BAD (0.6 µg) were incubated in 30 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.2 µg CK2, 0.06% 2-mercaptoethanol, 1 mM EGTA, and 100 µM ATP including 1 µCi [γ-^{32}P]ATP in a volume of 10 µl for 15 min at 37 °C. Then unincorporated ATP was removed by centri-SEP® spin colums. Alternatively, phosphorylation reactions were stopped by addition of 100 µM of the CK2 inhibitor TBB (4,5,6,7 tetrabromobenzimidazole). Dephosphorylation reactions followed immediately.

Dephosphorylation of P-centrins and P-BAD

Upscale to a 10-fold phosphorylation reaction volume (100 µl) was feasible to allow 9 dephosphorylation reactions from one substrate preparation. Phosphorylated proteins were incubated with 0.16 µg PP1 or 0.05 µg PP2A or 1.3 µg PP2B or 0.08-0.8 µg PP2Cα or 0.08-1.5 µg PP2Cβ or 0.08-0.8 µg PP2Cδ or 0.8 µg PP5 or 1.5 µg alkaline phosphatase in a total volume of 15 µl, respectively. Incubations contained a 10 µl aliquot of the completed phosphorylation reaction plus 5 µl 50 mM Tris/HCl, pH 7.5, 1% glycerol, 0.1% 2-mercaptoethanol and an additional 5 mM $MnCl_2$ for PP1, PP2A, and PP2Cδ; or 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 2 µg calmodulin for PP2B; or 1 mM $MgCl_2$ for PP2Cα and PP2Cβ; or 100

µM oleic acid for PP5. Alkaline phosphatase assays contained 50 mM Tris/HCl, pH 7.9 and 1 mM $MgCl_2$. Reactions were stopped after 30 min at 37 °C by adding 5 µl sample buffer (130 mM Tris/HCl, pH 6.8, 10% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.06% bromphenol blue). Proteins were separated on 15% SDS-PAGE minigels and radiolabeling was detected by autoradiography and phosphoimager.

Animals

C57 BL/6J mice were maintained on a cycle of light (200 lux) and darkness (12 h, respectively), with food and water ad libitum. All experiments described herein conform to the statement of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Vision and Ophthalmic Research and the national and institutional guidelines for animal care.

Immunohistochemistry

Eyes of adult mice were cryofixed in melting isopentane, cryosectioned and treated as described [11,39,40]. Mounted retinal sections were examined with a Leica DMRP microscope. Images were obtained with a Hamamatsu Orca ER CCD camera (Hamamatsu City, Japan) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

Immunoelectronmicroscopy

Fixation, embedding and further handling of mouse retinal samples for immunoelectron microscopy were performed as described [11,41]. Monoclonal antibodies from mouse directed against PP2Cβ [42] and anti-centrin antibodies raised in rabbit [11] were applied to ultrathin sections through LRWhite-embedded mouse retina. Nanogold-labeling was silver-enhanced according to [43]. Preembedding labelling was performed as described [31,44]. Vibratome sections through mouse retinas were stained by anti-lebercilin and visualized by

appropriate 2nd antibodies (Vectastain ABC-Kit, Vector, England). After fixation with 0.5% OsO_4 specimen were embedded in araldite. Ultrathin sections were sliced and counterstained with 2% aqueous uranyl acetate (Sigma, Germany). Sections were analyzed in a FEI Tecnai 12 BioTwin transmission electron microscope (Eindhoven, The Netherlands), imaged with a SCCD SIS MegaView III camera (Münster, Germany) and digital images were processed with Adobe Photoshop.

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Figure legends

Fig. 1. Characterization of phosphorylation of centrins by CK2. Centrins (0.2 μg, respectively) were phosphorylated by CK2 (0.2 μg) using [γ-^{32}P]ATP as phosphate source as described in Experimental Procedures. (A-D) Autoradiograms. A-C, Cen1 as a substrate for CK2. (A) Time dependence. (B) Effect of the CK2-inhibitor TBB (4,5,6,7 tetrabromobenzimidazole). The inhibitor was present either in the phosphorylation reaction (left panel) or added after phosphorylation prior to and present upon dephosphorylation by PP2Cβ (right panel). (C) Phosphorylation with GTP (1 μCi [γ-^{32}P]GTP and 100 μM GTP) in comparison to that with ATP. (D) Phosphorylation of centrin isoforms (0.2 μg, respectively) by CK2. (E) Coomassie protein stain of centrin isoforms (0.2 μg, respectively).

Fig. 2. Dephosphorylation of P-centrin 1 and P-BAD. (A) Incubation of P-Thr^{138}-Cen1 (0.2 μg) with phosphatases as indicated. (B) Incubation of P-Thr^{117}-BAD (0.6 μg) with phosphatases. The amount of a phosphatase added for the dephosphorylation reactions was the same in (A) and (B) (0.16 μg PP1, 0.05 μg PP2A, 1.3μg PP2B, 1.5 μg PP2Cβ, 0.8 μg PP5, or 1.5μg alkaline phosphatase). PP2Cβ is most efficient in dephosphorylating P-Cen1 phosphorylated by CK2. The BAD protein - also phosphorylated by CK2 - was run for control to verify activeness of the phosphatases.

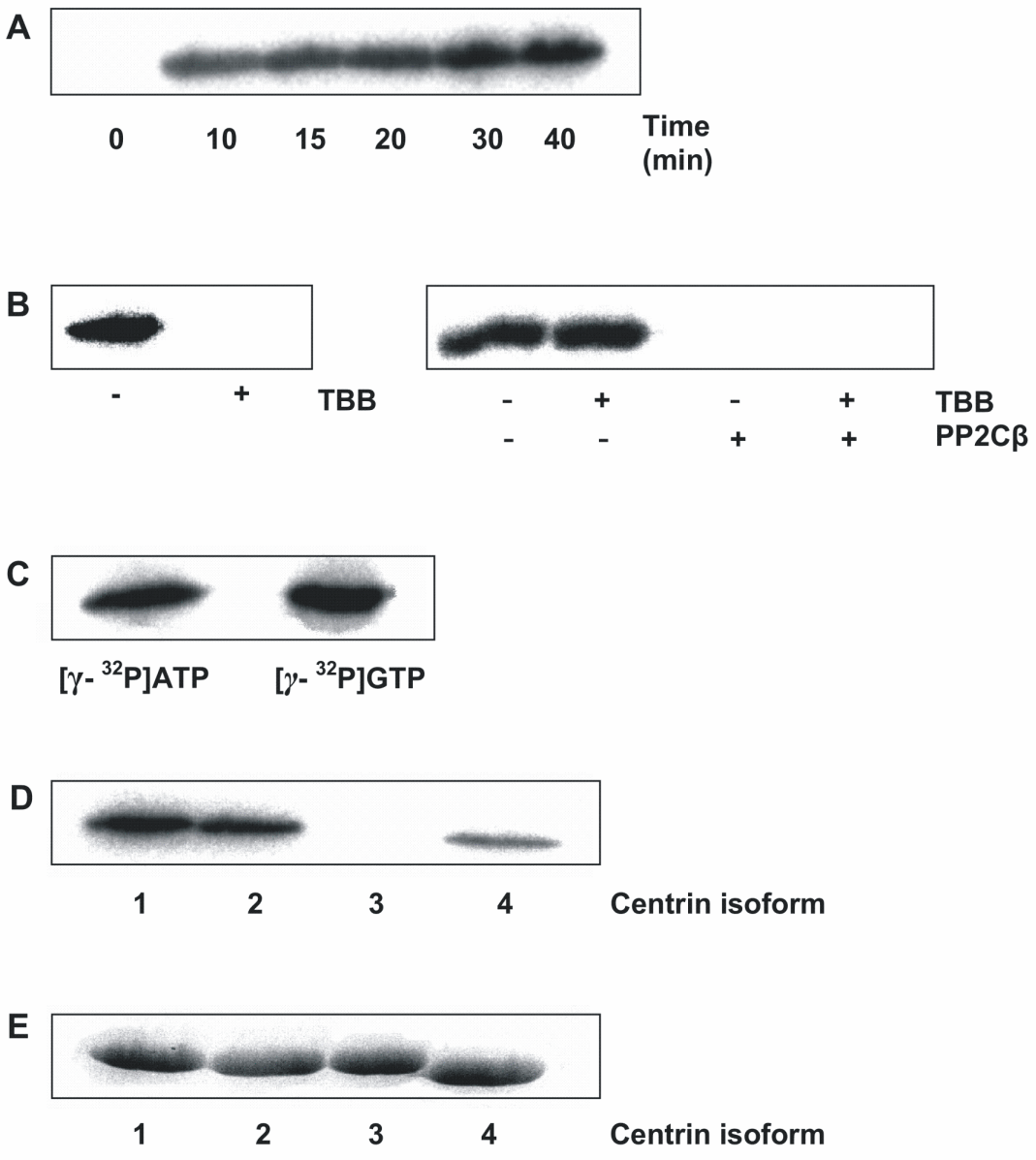
Fig. 3. Characterization of dephosphorylation of P-centrin 1 by PP2C. Cen1 (0.2 μg) was phosphorylated by CK2 (0.2 μg) and [γ-^{32}P]ATP. (A-D) Dephosphorylation by 0.08 μg PP2Cβ performed in the presence of 1 mM Mg^{2+} unless indicated otherwise. (A) Protein dependence. (B) Requirement for Mg^{2+}-ions for activity. (C) Inhibition by Ca^{2+}-ions. (D) Stimulation by oleic acid. (E) Effect of PP2C isozymes α, β and δ on P-Thr^{138}-Cen1.

Fig. 4. Subcellular localization of centrins and PP2Cβ in murine retinas. (A) Schematic representation of a vertebrate photoreceptor cell. A photoreceptor is composed of the light sensitive outer segment (OS) which is linked via the connecting cilium (CC, red) to the inner segment (IS). Nuclei (N) of photoreceptors form the outer nuclear layer (ONL, Fig. 4*B*), whereas the synapsis (S) of photoreceptors form the outer plexiform layer (OPL, Fig. 4*B*). (B) Differtial interference contrast image of a cryosection through a murine retina. (C) Indirect immunofluorescence staining by anti-Cen3 antibodies shows localization of Cen3 at the ciliary apparatus of photoreceptor cells where all ciliary centrins (Cen1 to Cen3) are localized [11]. (D) Indirect immunofluorescence by PP2Cβ specific antibodies reveals staining of IS and the ciliary apparatus. (E) Merge images of (C) and (D) with additional DAPI staining of the nuclei. Cen 3 and PP2Cβ fluorescence are co-localized at the ciliary apparatus of photoreceptors. (F-I) High resolution immunoflurescence analysis of the localization of centrins and PP2Cβ at the ciliary apparatus. (F) Schematic representation of the CC and the basal body (BB) localized at the linkage of the OS with the IS. (G) Anti-Cen3 antibodies react with the CC and the BB. (H) Anti-PP2Cβ antibody staining reveals PP2Cβ localization at the CC as well as a prominent localization at the BB. (I) Merge image of pictures (G) and (H) show co-localization of Cen3 and PP2Cβ in the CC and at the BB. Bars 8.2 µm (B-E) and 0.5 µm (G-I)

Fig. 5. Immunoelectron microscopic localization of Cen3 and PP2Cβ in the ciliary complex of mouse photoreceptor cells. (A-C) Longitudinal ultrathin sections of a mouse rod photoreceptor cell. (A) Silver-enhanced immunogold labeled by Cen3 antibody. Centrins are localized at the inner surface of the connecting cilium (*asterisk*) and the basal body (BB). (B) Silver-enhanced immunogold labeled by PP2Cβ antibody. PP2Cβ shows similar localization to centrins at the inner surface of the connecting cilium (CC) but is additional localized to OS

as well as to the IS. (C, D) Pre-embedding labelling of PP2Cβ. PP2Cβ is located in the CC (*asterisk*) and at the BB. In addition, IS and OS are weakly stained. Bar 500 nm.

Fig. 1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15
20
30
40
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-
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TBB
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PP2Cβ
C
[γ- 32P]ATP
[γ- 32P]GTP
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Centrin isoform
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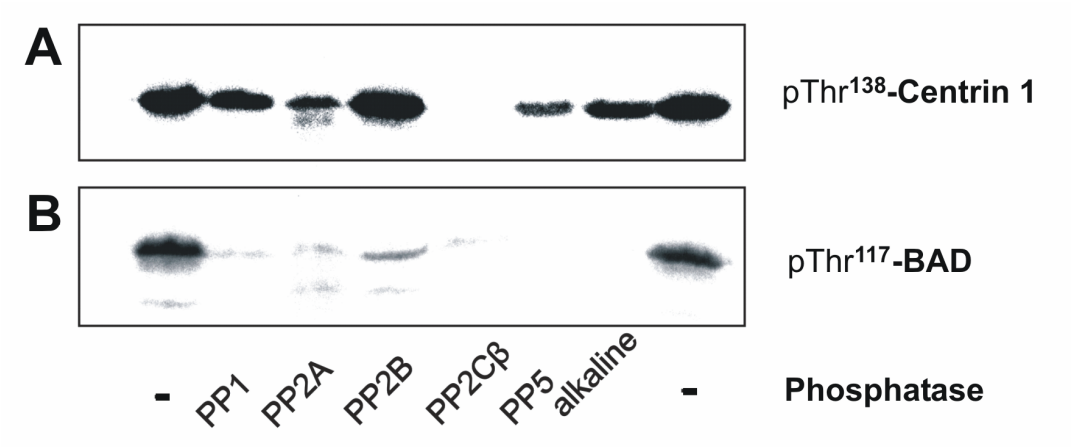
Centrin isoform

Fig. 2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pThr138-Centrin 1
B
pThr117-BAD
-
PP1
PP2A
PP2B
PP2Cβ
PP5
alkaline
-
Phosphatase

Fig. 3

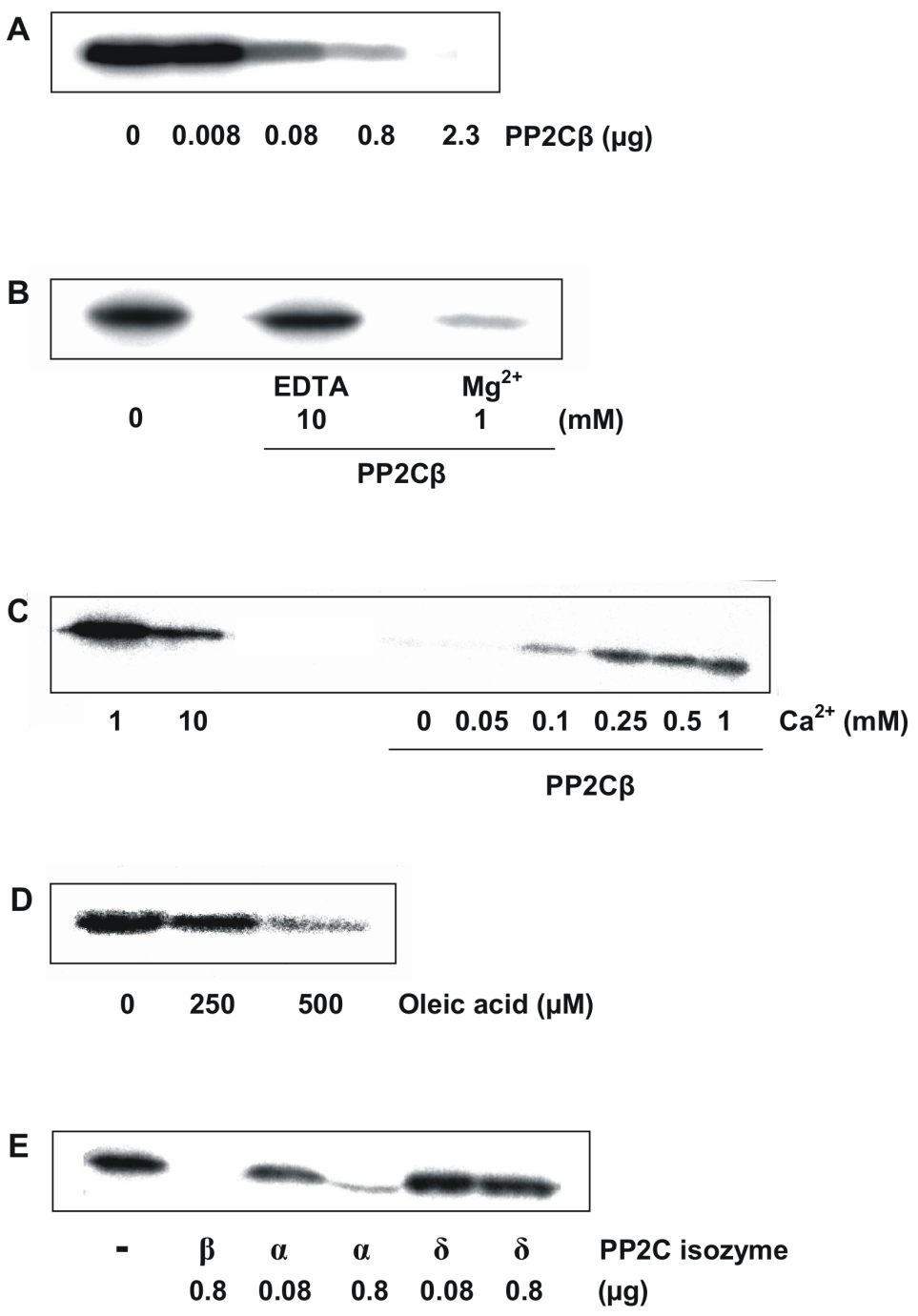
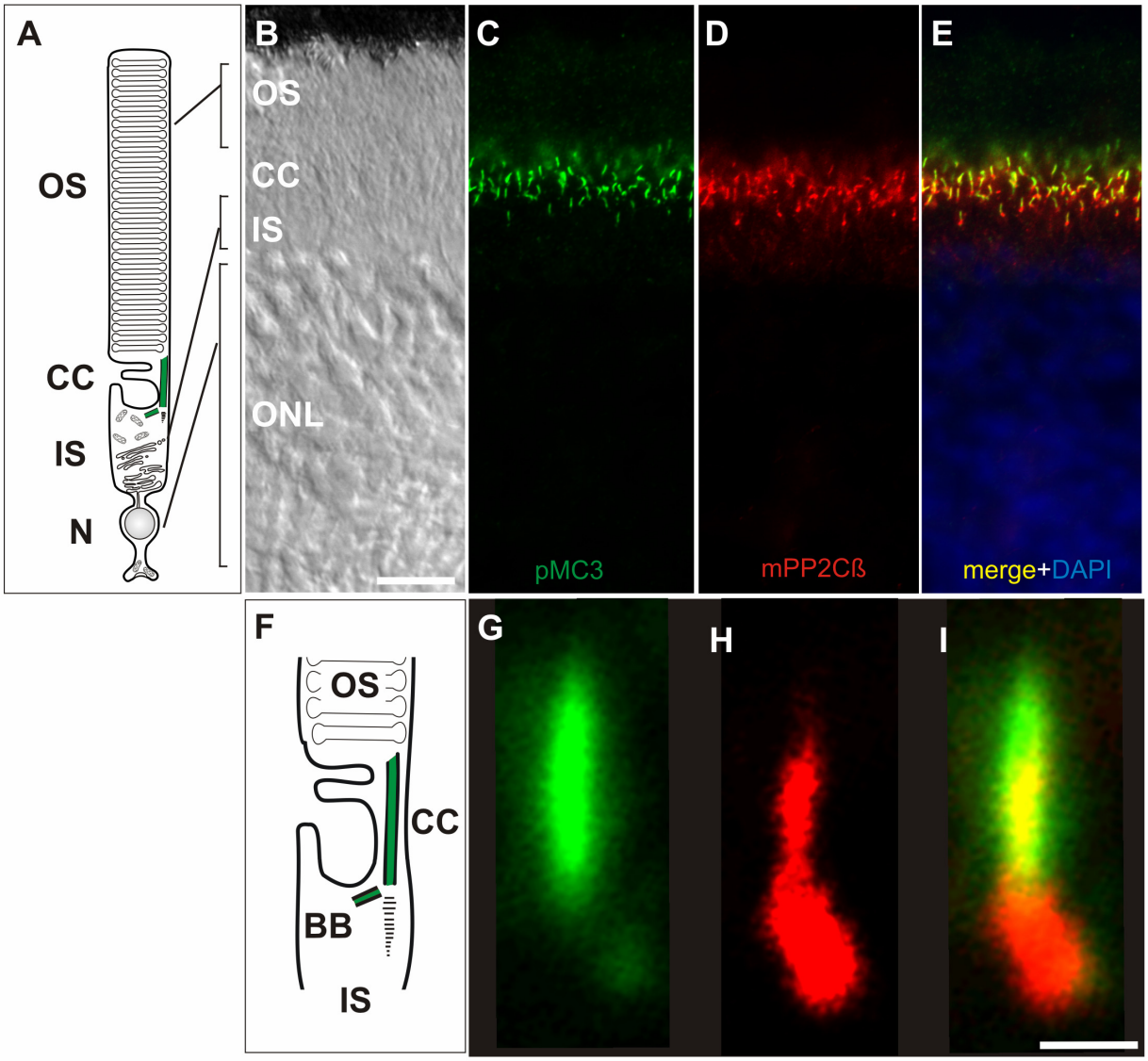

A
0 0.008 0.08 0.8 2.3 PP2Cβ (μg)
B
EDTA
Mg2+
0
10
1
(mM)
PP2Cβ
C
1 10
0 0.05 0.1 0.25 0.5 1
Ca2+ (mM)
PP2Cβ
D
0 250 500 Oleic acid (μM)
E
- β α α δ δ PP2C isozyme
0.8 0.08 0.8 0.08 0.8 (μg)

Fig. 4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OS
CC
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N
B
OS
CC
IS
ONL
C
pMC3
D
mPP2Cß
E
merge+DAPI
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OS
CC
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H
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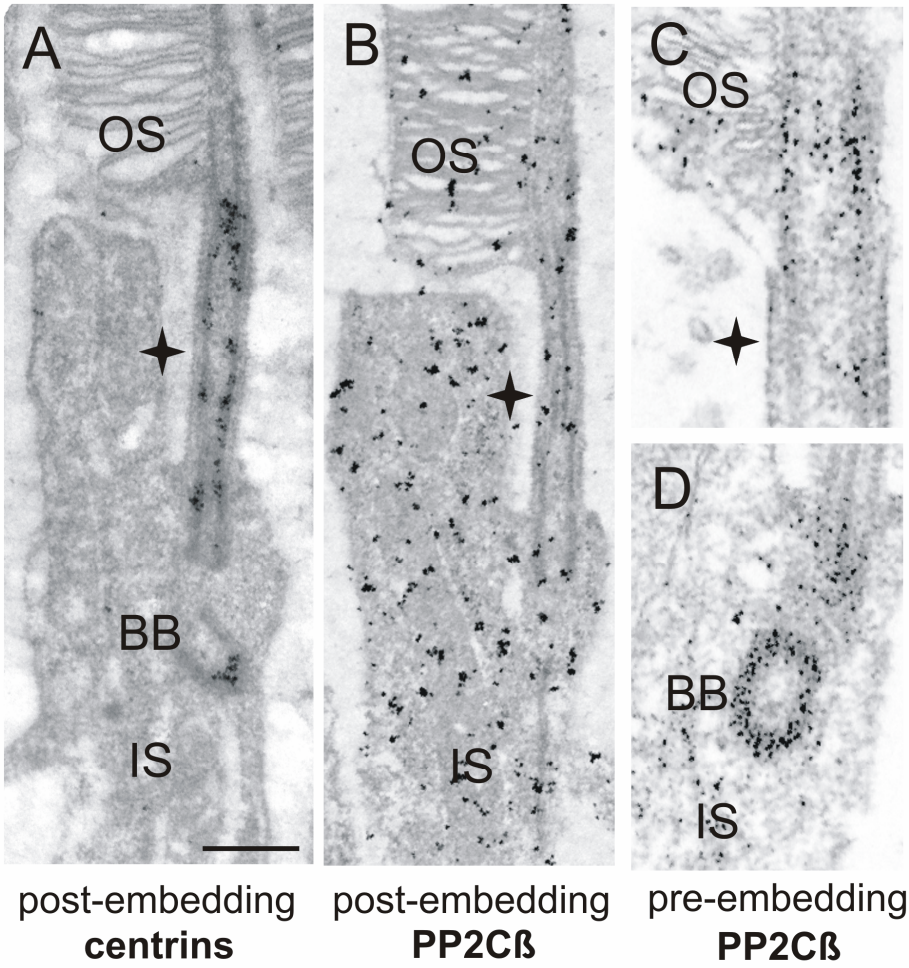
Fig. 5



post-embedding **centrins** | post-embedding **PP2Cß** | pre-embedding **PP2Cß**

Dissertation Publikation III

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Centrins in retinal photoreceptor cells: Regulators in the connecting cilium

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Abstract

Changes in the intracellular Ca^{2+} concentration regulate the visual signal transduction cascade directly or more often indirectly through Ca^{2+}-binding proteins. Here we focus on centrins, which are members of a highly conserved subgroup of the EF-hand superfamily of Ca^{2+}-binding proteins in photoreceptor cells of the vertebrate retina. Centrins are commonly associated with centrosome-related structures. In mammalian retinal photoreceptor cells, four centrin isoforms are expressed as prominent components in the connecting cilium linking the light-sensitive outer segment compartment with the metabolically active inner segment compartment. Our data indicate that Ca^{2+}-activated centrin isoforms assemble into protein complexes with the visual heterotrimeric G-protein transducin. This interaction of centrins with transducin is mediated by binding to the $\beta\gamma$-dimer of the heterotrimeric G-protein. More recent findings show that these interactions of centrins with transducin are reciprocally regulated via site-specific phosphorylations mediated by the protein kinase CK2. The assembly of centrin/G-protein complexes is a novel aspect of translocation regulation of signalling proteins in sensory cells, and represents a potential link between molecular trafficking and signal transduction in general.

Keywords: Ca^{2+}-binding proteins; Centrins; Retina; Photoreceptor cells; Transducin; Light-dependent translocation

Contents

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1. Introduction

Cone and rod photoreceptor cells are highly polarized specialized neurons, which consist of morphologically and functionally distinct cellular compartments (Fig. 1). The light-sensitive photoreceptor outer segment is linked with an inner segment via a modified, non-motile cilium, termed connecting cilium (Fig. 1). 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 and is arranged as hundreds of stacked membrane disks. Signal transduction in vertebrate rod cells starts with the light-induced formation of active rhodopsin (Rh*), which interacts with the visual G-protein transducin (G_t). G_t activation by Rh* represents a key amplification step in the cascade, in which a single Rh* can catalyze the activation of hundreds of G_t molecules (Fung and Stryer, 1980; Heck and Hofmann, 2001). Thereby, Rh* interacts with the GDP-bound form of the intact G_t holoprotein ($G_t\alpha$-GDP-$G_t\beta\gamma$ or $G_t$holo), and initiates the light-dependent transduction process by triggering the rapid exchange of bound GDP for GTP on the α-subunit ($G_t\alpha$). This is followed very rapidly by the dissociation of the $G_t\alpha$-GTP (or G*) from the Rh* as well as from the membrane bound $\beta\gamma$-heterodimeric subunit ($G_t\beta\gamma$). Activated $G_t\alpha$-GTP stimulates the activity of its effector enzyme, the cGMP phosphodiesterase (PDE), also known as PDE6 (Beavo, 1995), which in turn hydrolyzes cGMP to 5′-GMP, leading to the closure of cGMP-gated channels (CNG channels) localized in the plasma membrane (Heck and Hofmann, 1993; Okada et al., 2002). The closure of 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 the decrease of transmitter release from the synaptic terminal (Molday and Kaupp, 2000). The recovery phase of the visual transduction cascade and light adaptation of photoreceptor cells rely on changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. It is well established that changes in $[Ca^{2+}]_i$ affect portions of the phototransduction cascade indirectly through Ca^{2+}-binding proteins (Palczewski et al., 2000).

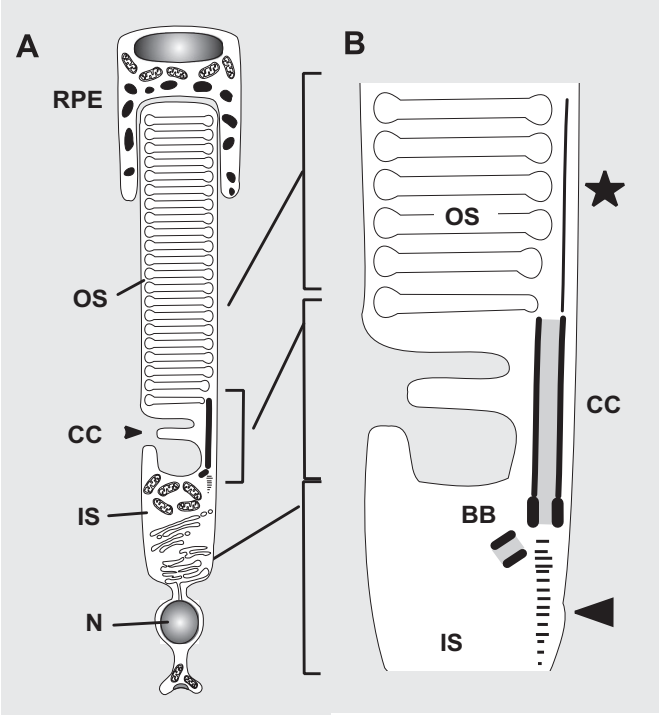

Fig. 1. Schematic representation of a vertebrate rod photoreceptor cell. (A) The photoreceptor cell is composed of a light-sensitive outer segment (OS) which is linked via a specialized non-motile cilium, the connecting cilium (CC) with the inner segment (IS). The OS tips are attached to the cells of the retinal pigment epithelium (RPE). The IS contains all organelles necessary for biosynthesis of the cell. (B) Schematic representation of the ciliary apparatus of a photoreceptor cell. The ciliary apparatus is composed of the CC, which represents an elongated transition zone, and the basal body complex (BB). Ciliary axonemal microtubules (*asterisk*) project from the CC into the OS. The ciliary rootlet (*arrowhead*) project from the basal body complex into the IS of the photoreceptor cell. Centrin localizations in the CC and at the BB are indicated in grey.

The membranous outer segment disks which contain the signal transduction machinery are continually renewed throughout lifetime (Young, 1976). Newly synthesized disks are added at the base of the outer segment (Steinberg et al., 1980; Usukura and Obata, 1996) 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 mechanisms for the transport of newly synthesized components from cell organelles of biosynthesis in the inner segment, to the outer segment, the cell compartment of signal transduction (Sung and Tai, 2000). After delivery, some molecules of the outer segments, e.g. membrane proteins (ion transporters, channels, as well as the visual pigment rhodopsin) stay there permanently, whereas other molecules of the signal transduction cascade, e.g. arrestin and transducin, exhibit massive light-dependent reversible translocations between

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photoreceptor compartments (Brann and Cohen, 1987; Philp et al., 1987; Whelan and McGinnis, 1988; Organisciak et al., 1991; McGinnis et al., 2002; Pulvermüller et al., 2002; Sokolov et al., 2002, 2004; Wolfrum et al., 2002; Mendez et al., 2003; Peterson et al., 2003). These bidirectional translocations of components of the transduction cascade are thought to contribute to slow but long lasting adaptation of photoreceptor cells (Sokolov et al., 2002, 2004; Hardie, 2003; Frechter and Minke, 2006).

All intracellular exchanges between these two functional compartments of vertebrate photoreceptor cells occur through the slender connecting cilium, which is the only cytoplasmic bridge between the inner segment and the outer segment (Fig. 1). During recent years, an increasing number of proteins and protein complexes have been identified at the ciliary apparatus of vertebrate photoreceptor cells, which is composed of the connecting cilium and the basal body complex (Liu et al., 2007; Roepman and Wolfrum, 2007). The identified molecules of the cilium were very often suggested to play a role in the ciliary transport (Schmitt and Wolfrum, 2001; Stohr et al., 2003; Liu et al., 2007; Roepman and Wolfrum, 2007). The list of molecules identified in the photoreceptor cilium contains proteins from different classes and families, but also includes molecular motors associated with microtubules and actin filaments and represent good candidates for the active molecular transport through the connecting cilium (e.g. myosin VIIa and kinesin II) (Liu et al., 1997, 1999; Marszalek et al., 2000; Wolfrum and Schmitt, 2000; Williams, 2002; Luby-Phelps et al., 2007). In addition, all four known centrin isoforms are also found in the ciliary apparatus of rodent photoreceptor cells (Gießl et al., 2004a, b, 2006).

The present review deals with the current view of structure, expression, subcellular localization and function of centrins. In particular, we focus on these aspects of the small Ca^{2+}-binding centrins in the vertebrate retina. The prominent localization of centrin isoforms is described in the connecting cilium of the photoreceptor cell and the putative role of centrin/transducin protein complexes in the regulation of transducin movements through the cilium is discussed.

2. Centrin genes and protein structures

2.1. Centrin genes and proteins in their phylogenic context

Centrins, also termed "caltractins", are highly conserved low molecular weight phospho-proteins (Salisbury, 1995; Schiebel and Bornens, 1995; Gießl et al., 2004a). They belong to the large EF-hand superfamily of Ca^{2+}-binding proteins which includes parvalbumin, troponin C, the S100 protein and the well-known Ca^{2+}-sensor calmodulin (Kretsinger and Nockolds, 1973; Kretsinger, 1976; Persechini et al., 1989; Nakayama and Kretsinger, 1994). Centrins were first described in unicellular green algae, where they are associated with the basal apparatus of flagella (Salisbury et al., 1984). In these protists, centrins are thought to participate in Ca^{2+}-dependent contractions of striated flagellar rootlets (Salisbury et al., 1984). More recently, centrins have been found to be ubiquitously associated with centrioles of basal bodies and centrosomes, as well as mitotic spindle poles in cells from diverse eukaryotic organisms, from yeast to man (Salisbury, 1995; Schiebel and Bornens, 1995). The centrin protein family is one class of about 350 "eukaryotic signature proteins" (ESPs) that occur in all eukaryotic cells but have no significant homology to proteins in archaea and bacteria (Hartman and Fedorov, 2002; Salisbury, 2007). These ESPs define an ancient class of proteins that might be uniquely critical for the structure and function of the eukaryotic cell in general (Hartman and Fedorov, 2002).

Over the last two decades, centrin genes were described in a large variety of species from all kingdoms of eukaryotic organisms, protists, fungi, plants and animals (Huang et al., 1988a; Baum et al., 1986, 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; Daunderer et al., 2001; Gavet et al., 2003; Guerra et al., 2003; Correa et al., 2004; Lemullois et al., 2004; Ribichich and Gomes, 2005; Nagamune and Sibley, 2006; Boutet et al., 2008). Comparisons of amino acid sequences deduced from cDNA clones certainly show that centrins are highly conserved, and yet distinct members of a subfamily of the EF-hand superfamily of Ca^{2+}-binding proteins, also termed the parvalbumin or troponin C superfamily (Fig. 2) (Kretsinger and Nockolds, 1973; Kretsinger, 1976; Persechini et al., 1989; Nakayama and Kretsinger, 1994). Centrins are small acidic proteins (~170 amino acids in length; apparent molecular mass ~20 kDa) (Salisbury, 1995; Schiebel and Bornens, 1995).

To date, in lower eukaryotes like yeast, only one centrin gene (e.g. *Saccharomyces cerevisiae*: *ScCDC31*) has been identified (Baum et al., 1986, 1988). In the unicellular algae *Chlamydomonas reinhardtii*, database searches also provide one centrin gene listed as *CrCEN* or *VFL2*. However, in the proteomic analysis of isolated *Chlamydomonas* centrioles, Keller et al. (2005) identified two other centrins related to mammalian Cen2p and 3p in addition to the previously found centrin (CrCenp/Vfl2p) (Keller et al., 2005). Whereas for lower vertebrate species, an incomplete (most probably) set of one to two centrin genes are deposited in databases, in mammals up to four centrin genes have been described (Fig. 2A) (Friedberg, 2006). In the rodents *Mus musculus* and *Rattus norvegicus* four centrin genes (*MmCetn1–4*; *RnCetn1–4*) were identified (Lee and Huang, 1993; Middendorp et al., 1997; Gavet et al., 2003; Trojan, 2003). In the human genome, three centrin genes are present (*HsCETN1–3*). A predicted fourth centrin gene is found on chromosome 4 (accession number: XR_015512), but the potential gene transcript encodes a very short, 98 amino acids long peptide (Gießl, 2004). It is doubtful whether this transcript will exist as a functional polypeptide in the cell (Gießl, 2004).

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Cluster analyses of the deduced amino acid sequences of the diverse centrins of different organisms reveal several phylogenetic groups within the centrin protein family (Fig. 2). While some centrins of protists cannot be classified to homogeneous groups, most centrins of higher plants, green algae and all vertebrate centrin isoforms form phylogenetic groups. In mammals, Cen1p, 2p and 4p isoforms are very closely related exhibiting high amino acid identities (Table 1). In contrast, sequences of vertebrate Cen3p isoforms, related to the yeast centrin (ScCdc31p), only have high amino acid identities among each other (Table 1, Fig. 2). In mammals, Cen1p, 2p and 4p isoforms are closer related to algal centrin (e.g. *Chlamydomonas* CrCenp/Vfl2p) than to the mammalian Cen3p isoform. This strongly suggests two divergent centrin subfamilies (Middendorp et al., 1997): one centrin subfamily grouped around the mammalian Cen1p, 2p and 4p and another centrin subfamily related to yeast centrin Cdc31p. Since the separation of both centrin subfamilies is already implemented in the unicellular green algae (see above; Keller et al. 2005), this division was a very early event in the molecular evolution of eukaryotes.

2.2. Primary domain structure of centrins

Analyses of the primary structures of centrins demonstrate that the most characteristic domains are the four helix–loop–helix EF-hand consensus motifs (Fig. 2C). These potential Ca^{2+}-binding sites define centrins as members of the superfamily of EF-hand Ca^{2+}-binding

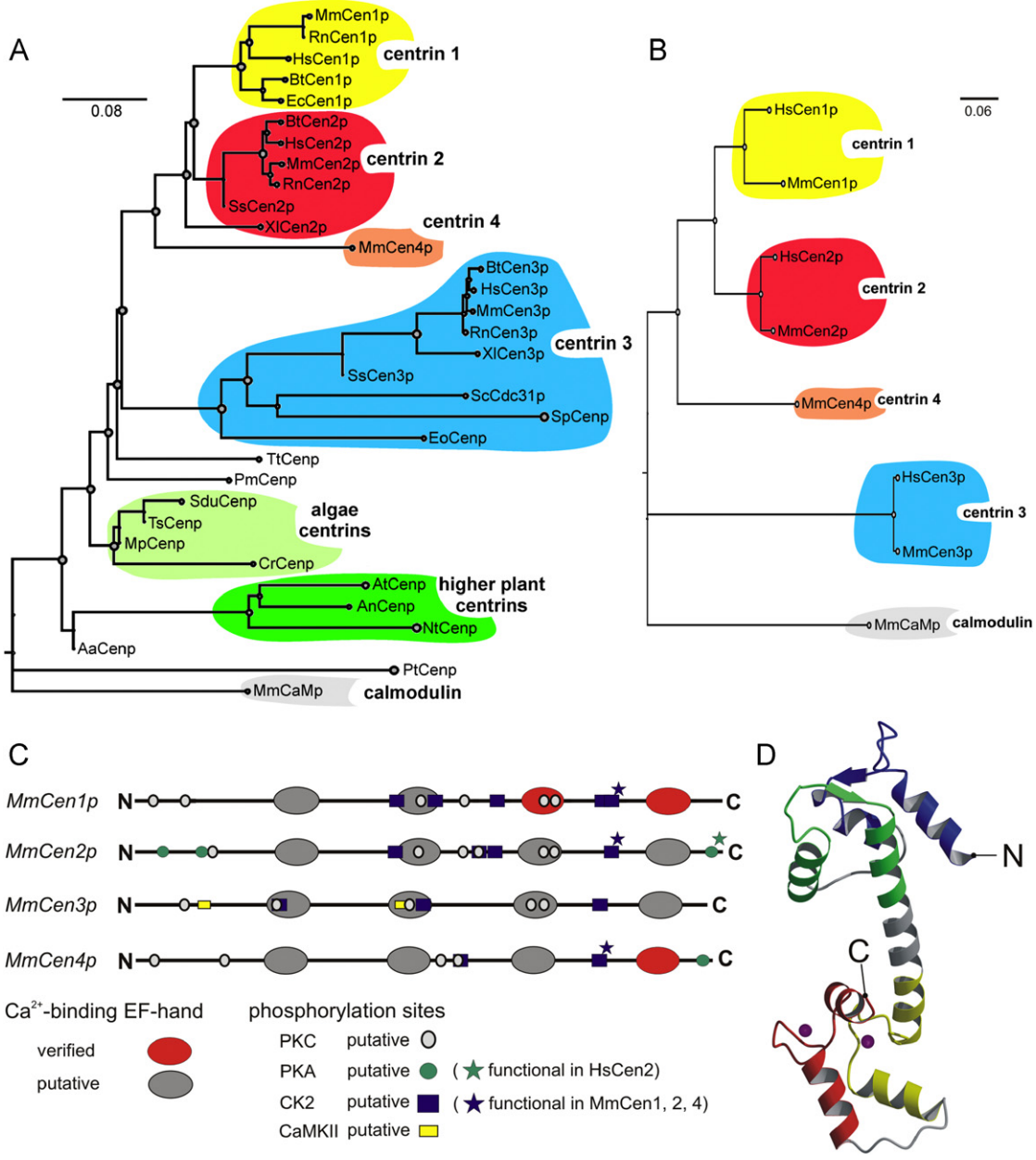

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Table 1
Identity of human and murine centrin isoforms

	CAA43674 MmCaMp	NP_004057 HsCen1p	P41208 HsCen2p	NP_004356 HsCen3p	NP_031619 MmCen1p	NP_062278 MmCen2p	NP_031710 MmCen3p	NP_665824 MmCen4p	Protein
MmCalm1 X61432		52	50	40	51	50	40	46	MmCaMp genbank:CAA43674
HsCETN1 NM_004066	*58*		83	53	90	84	53	69	HsCen1p genbank:NP_004057
HsCETN2 BC013873	*57*	*76*		52	81	95	52	69	HsCen2p genbank:P41208
HsCETN3 NM_004365	*51*	*59*	*60*		52	52	98	49	HsCen3p genbank:NP_004356
MmCetn1 NM_007593	*57*	*84*	*77*	*58*		81	52	67	MmCen1p genbank:NP_031619
MmCetn2 NM_019405	*57*	*76*	*90*	*60*	*78*		52	70	MmCen2p genbank:NP_062278
MmCetn3 NM_007684	*49*	*59*	*60*	*89*	*59*	*59*		49	MmCen3p genbank:NP_031710
MmCetn4 NM_145825	*56*	*66*	*68*	*58*	*67*	*69*	*58*		MmCen4p genbank:NP_665824
Coding sequence	*MmCalm1 X61432*	*HsCETN1 NM_004066*	*HsCETN2 BC013873*	*HsCETN3 NM_004365*	*MmCetn1 NM_007593*	*MmCetn2 NM_019405*	*MmCetn3 NM_007684*	*MmCetn4 NM_145825*	

Summary of the percentage identities of human (HsCen1p–3p) and murine (MmCen1p–4p) centrin proteins (non-italicized). In addition murine calmodulin (MmCaMp) was analyzed. The percentage identity of the coding sequences of the human and murine centrin genes (*HsCETN1–3* and *MmCetn1–4*) are summarized in italics. In addition the coding sequence of the murine calmodulin gene was analyzed (*MmCalm1*). For all analyzed proteins (non-italicized) and coding nucleotide sequences (italicized) the accession numbers are indicated. All sequences were analyzed using the Omiga2.0 software (Oxford molecular Ltd.).

proteins (Kretsinger, 1976; Moncrief et al., 1990; Nakayama et al., 1992). Beside the EF-hand motifs, the primary structures of centrins exhibit several putative phosphorylation sites for protein kinases A, C, CK2 and CaMKII (Fig. 2C). Very recent work identified site- and isoform-specific phosphorylation of murine centrins by CK2 (Trojan et al., 2008). Comparison of the centrin sequences reveals only small differences between the isoforms

Fig. 2. Phylogenetic relationship of centrins and molecular structure of centrins. (A) Phylogenetic relationship of centrins from diverse organisms. Comparison of 33 different amino acid sequences of centrins and murine calmodulin. The genetic consensus tree shows the highest frequency of each node of 1000 repetitions. Geneious 3.0 pro software (Biomatters Ltd) divides the centrins into subgroups: vertebrate centrin isoforms 1–4, algae centrins and higher plant centrins and an out grouped subgroup of calmodulin (MmCaMp = *Mus musculus* calmodulin AN: CAA43674; XlCenp2p, 3p = *Xenopus laevis* centrin 2, 3 AN: BC054948, AAG30507; PtCenp = *Paramecium tetraurelia* centrin AN: AAB188752; BtCen1p–3p = *Bos taurus* centrins 1–3 AN: NP001072974, NP001033604, AAI20178; SsCen2p, 3p = *Sus scrofa* centrins 2, 3 AN: AAY33861, AAY67906; EcCen1p = *Elaphodus cephalophus* centrin 1 AN: ABP57024; HsCen1p–3p = *Homo sapiens* centrins 1–3 AN: see (C); MmCen1p–4p = *Mus musculus* centrins 1–4 AN: see (C); RnCen1p–3p = *Rattus norvegicus* centrins (completed with own data) AN: AAK20385, AAK20386, AAK83217; AtCenp = *Arabidopsis thaliana* centrin AN: CAA08773, AnCenp = *Atriplex nummularia* centrin AN: P41210; NtCenp = *Nicotiana tabacum* centrin AN: AAF07221; CrCenp = *Chlamydomonas reinhardtii* centrin (Vfl2p) AN: EDO98562; SduCenp = *Scherffelia dubia* centrin AN: Q06827; MpCenp = *Micromonas pusilla* centrin AN: CAA58718; EoCenp = *Euplotes octocarinatus* centrin AN: CAB40791; TsCenp = *Tetraselmis striata* centrin AN: P43646; ScCdc31p = *Saccharomyces cerevisiae* AN: CAA52609; SpCenp = *Schizosaccharomyces pombe* centrin AN: CAA20670; PmCenp = *Prorocentrum minimum* centrin AN: ABI14404; AaCenp = *Acetabularia acetabulum* centrin AN: AAM00015; TtCenp = *Tetrahymena thermophila* AN: AAF66602). The tree is not complete (AN: accession number). (B) Phylogenetic tree of four mouse and three human centrins. Genious 3.0 pro separates mammalian centrins into the subgroups 1–4, thereby the Cen1p, 2p and 4p cluster closer to each other (tree isolated to mouse calmodulin). (C) Schematic representation of functional domains in murine centrins. Murine centrin isoforms 1–4 (MmCen1p–4p) possess four EF-hand motifs (*ovals*). Functional EF-hands are indicated by *red ovals*. In MmCen1p EF-hands III and IV bind Ca^{2+}, whereas MmCen4p possesses only one functional EF-hand (EF-hand IV). Unfortunately, no data are available for MmCen2p and 3p. All centrins contain a variety of putative phosphorylation sites for protein kinases A, C, CK2 and CaMKII. In MmCen1p six putative PKC phosphorylation sites are predicted (*grey small ovals*). Only one of five putative CK2 phosphorylation sites (*blue squares*) is functional (*blue star*). MmCen2p contains three putative PKA phosphorylation sites (*green ovals*). One of these sites is functional in HsCen2p (*green star*). For MmCen2p no experimental data are available. Six putative PKC sites are present as well (*grey small ovals*). Like in MmCen1p one CK2 phosphorylation site is functional (*blue star*) and three are inactive (*blue squares*). In contrast, MmCen3p contains no functional CK2 site but three inactive ones (*blue squares*). In addition, there are five putative PKC sites (*grey small ovals*) and two putative CaMKII phosphorylation sites (*yellow rectangles*). MmCen4p contains one active CK2 site (*blue star*) and one is inactive. In addition, four putative PKC sites are present. (D) Crystal structure of MmCen1p. The structure of MmCen1p-L (Park et al., 2006, in prep.), shown as ribbon representation. Large parts of the N-terminal sequence and a small portion at the C-terminus are not visible in the electron density map. The structural model shown here comprises residues Asp28 (indicated by 'N') to Lys167 (indicated by 'C', numbering refers to wild-type MmCen1p). EF-hand motifs I–IV are coloured blue, green, yellow and red, respectively. The N-terminal half of the molecule adopts a typical 'closed' conformation, whereas the C-terminal half is in an 'open' conformation. Ca^{2+} ions (*spheres*) are bound to the EF-hands III and IV; EF-hand I is only partially occupied by Ca^{2+}.

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(Table 1) (Gießl et al., 2004a; Salisbury, 2007). In addition to the EF-hand motifs, the most conserved region is the C-terminal half of the centrins, in particular the short C-terminal sequence (-KKTSLY). This sequence could be responsible for general features of centrins, like the positioning at centrosomal structures (e.g. centrosomes, basal bodies or transition zones of cilia). In contrast to the conserved C-terminal domain, the N-terminus, especially the first 20 amino acids, represent the most variable region of the centrin sequences (Hart et al., 1999, 2001; Salisbury, 2007). Therefore, this region has been suggested to be responsible for some functional diversity among centrin species (Bhattacharya et al., 1993; Salisbury, 1995; Wiech et al., 1996; Wolfrum et al., 2002; Gießl et al., 2004b; Yang et al., 2006b).

2.2.1. Ca^{2+}-binding at EF-hand motifs of centrins

The most prominent characteristic of the centrin protein family are the four EF-hand motifs which posses the potential capacity for Ca^{2+}-binding (Fig. 2C). Therefore, it is not surprising that the function of centrins is accompanied by binding of Ca^{2+} ions. As a prerequisite, centrins need to be activated by bound Ca^{2+} for the interaction with most of its known interacting partners (details see in Sections 3.1 and 5) (Schiebel and Bornens, 1995; Wiech et al., 1996; Durussel et al., 2000; Pulvermüller et al., 2002; Gießl et al., 2004b, 2006; Hu et al., 2004; Cox et al., 2005). In green algae, centrins serve as Ca^{2+} sensors at the contractile flagellar rootlets by recognizing the increase of the intracellular Ca^{2+} concentration (Sanders and Salisbury, 1989, 1994; Schiebel and Bornens, 1995). This binding of Ca^{2+} leads to an ATP-independent contraction of Ca affinity centrin-containing fibres of the flagellar rootlets (Sanders and Salisbury, 1994; Schiebel and Bornens, 1995).

Although the four EF-hand motifs of centrins are highly conserved, the Ca^{2+} affinities between the different EF-hands in centrins are not identical. In the green algae *Chlamydomonas*, centrin molecules bind two Ca^{2+} ions with high affinity at the N-terminal domain and two with low affinity at the C-terminal domain (Weber et al., 1994; Durussel et al., 2000). The N-terminus of *Chlamydomonas* centrin serves as a Ca^{2+} sensor and strengthens protein–protein interactions, like the complex formation with Sfi1p (Sheehan et al., 2006). In contrast to *Chlamydomonas* centrin where all EF-hands bind Ca^{2+}, in higher eukaryotic cells some EF-hand motifs lost the ability to bind Ca^{2+}. In HsCen2p, for example, Ca^{2+}-binding is only mediated via the EF-hand motif IV in the C-terminal domain (Durussel et al., 2000). This Ca^{2+}-binding induces conformational changes which lead to the exposure of hydrophobic surfaces and therefore supporting the formation of homodimers (Durussel et al., 2000; Tourbez et al., 2004). This functional EF-hand IV of HsCen2p is strictly Ca^{2+}-specific and does not bind other cations like Mg^{2+} (Durussel et al., 2000; Cox et al., 2005). Such mixed cation-binding sites were identified in HsCen3p and indicate isoform-specific regulations of the EF-hands. HsCen3p belongs to the second subfamily of centrins, related to yeast Cdc31p, and contains three functional EF-hands (Cox et al., 2005). However, two of them show only low Ca^{2+} affinity and one has a rather unspecific, but high affinity to both cations Ca^{2+} and Mg^{2+} (Cox et al., 2005). Preliminary data on MmCen1p indicate extraordinary high Ca^{2+} affinity of EF-hands in the C-terminal domain of the molecule (~1000 times higher than human calmodulin) (Black et al., 2006; Park et al., 2006). Although all centrins contain the same distribution of EF-hand motifs the regulation is highly selective since the binding properties differ between species and between isoforms in one species. Due to the fact that most known homomeric and heteromeric protein–protein interactions of centrins are Ca^{2+}-triggered, the Ca^{2+}-binding represents the most important, but not the only, molecular regulatory mechanism of centrins.

2.2.2. Site-specific phosphorylation of centrins

Besides Ca^{2+}-binding, phosphorylation represents a second principle modification of centrin molecules which regulates the functions of centrins in yeast, green algae and in mammalian cells (Salisbury et al., 1984; Martindale and Salisbury, 1990; Salisbury, 1995; Lutz et al., 2001; Gießl et al., 2004b; Trojan et al., 2008). In unicellular green algae, centrins are the major components of contractile fibres at the basal bodies. These fibres contract upon an increase of intracellular Ca^{2+}. For fibre relaxation, centrins have to be phosphorylated at the C-terminal domain (Salisbury et al., 1984; Martindale and Salisbury, 1990). In the green algae *Chlamydomonas*, protein kinase A (PKA) phosphorylates a centrin species at Ser167 *in vitro* (Meyn et al., 2006). The identified target sequence is located in the C-terminus, the highest conserved region of centrins. In human HeLa cells, phosphorylation of this conserved PKA phosphorylation site occurs in HsCen2p during the cell cycle at the G1/S transition (Lutz et al., 2001). Interestingly, centrins are hyperphosphorylated during the abnormal cell cycle of breast cancer cells obtained from human patients (Lingle et al., 1998).

Recently, we have identified CK2 as the protein kinase which phosphorylates murine centrin isoforms with high specificity in fully differentiated retinal photoreceptor cells (for details see Section 5.2) (Trojan et al., 2008). CK2 phosphorylation is centrin isoform specific. Only Cen1p, 2p and 4p, but not Cen3p are phosphorylated by CK2 at a specific site. Furthermore, CK2-mediated phosphorylation occurs in the dark and strongly reduces the binding affinities of centrins for target interactor proteins like G-proteins. The latter effect is probably due to the reduction of centrin Ca^{2+} affinity induced by phosphorylation.

In conclusion, regulatory modifications of centrins include two major events: Ca^{2+}-binding on the one hand and phosphorylation on the other hand. Comparisons of high-resolution structures in centrins under different Ca^{2+} concentration and phosphorylation states may provide more insights in these mechanisms.

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Table 2
Domain and full-length centrin structures from different sources determined by either NMR spectroscopy or X-ray crystallography

Structure	Reference	Method	PDB code	Deposition
C-HsCen2p[a]	Matei et al. (2003)	NMR	1M39	2003
C-CrCenp/Kar1p[b]	Hu and Chazin (2003)	NMR	1OQP	2003
C-HsCen2p/XPCp[c]	Yang et al. (2006a)	NMR	2A4J	2005
N-CrCenp[d]	Sheehan et al. (2006)	NMR	2AMI	2006
N-HsCen2p	Yang et al. (2006b)	NMR	1ZMZ	2006
HsCen2p/XPCp	Thompson et al. (2006)	X-ray	2GGM	2006
Cdc31p/Sfi1p[e] (Ca^{2+} bound)	Li, S. et al. (2006)	X-ray	2DOQ	2006
Cdc31p/Sfi1p (Ca^{2+} free)	Li, S. et al. (2006)	X-ray	2GV5	2006
HsCen2p/XPCp	Charbonnier et al. (2007)	X-ray	2OBH	2007
MmCen1p-L[f]	Park and Pulvermüller (unpublished)	X-ray	–	–

[a]'C-' indicates the C-terminal half of a centrin containing EF-hand motifs III and IV.
[b]Kar1p is a peptide derived from the cell division control protein Kar1p.
[c]XPC is a peptide derived from *Xeroderma pigmentosum* group C protein.
[d]'N-' indicates the N-terminal half of a centrin containing EF-hand motifs I and II.
[e]Sfi1p is a protein from the half bridge attached to the spindle pole body.
[f]MmCen1p-L is an N-terminally extended mouse Cen1p (Park et al., 2006, 2005).

2.3. *High-resolution molecular structure of centrins and their complexes*

Detailed protein structures at high-resolution based on nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography provide more insights into the molecular composition and function of molecules. High-resolution structural data on centrins from different organisms are available from solution studies by NMR spectroscopy and from X-ray crystallography (Table 2). These studies were carried out either on individual C- or N-terminal domains, or on full-length proteins, and the proteins were either in their unbound states or in complexes with target proteins.

As a result of these analyses, centrins are known to form dumbbell-like structures, which closely resemble those of the other proteins belonging to the calmodulin-parvalbumin superfamily (Fig. 2D). Pairs of the first and second EF-hands and of the third and the fourth EF-hands form compact structures in the N- and C-terminal halves, respectively, which are connected by a long central α-helix (Fig. 2D). Typically an 'open' conformation is reported for the C-terminal domains and a 'closed' conformation is found for the N-terminal domains of the centrin molecules. 'Closed' conformations are usually adopted by the Ca^{2+}-free domains of calmodulin-parvalbumin-type proteins, whereas Ca^{2+}-binding triggers a switch to the 'open' conformation as was shown in detail for troponin C by X-ray crystallography (Herzberg and James, 1985; Sundaralingam et al., 1985; Satyshur et al., 1994; Houdusse et al., 1997; Strynadka et al., 1997). Correspondingly, in the two crystal structures of the HsCen2p–XCPp complex (Table 2; for functional details see Section 5), the N-terminal domains are Ca^{2+}-free and each of the EF-hand motifs III and IV in the C-terminal domain has a Ca^{2+} ion bound (Thompson et al., 2006; Charbonnier et al., 2007). In agreement with a relatively low Ca^{2+} affinity of EF-hand III this site is occupied by Ca^{2+} in only 70% of the HsCen2p molecules in the crystal (Charbonnier et al., 2007). Surprisingly, a typical 'open' conformation is reported for the C-terminal domains of centrin molecules even in the Ca^{2+}-free Cdc31p/Sfi1p complex. In contrast, in the Ca^{2+}-bound Cdc31p/Sfi1p complex and in MmCen1p-L[2] Ca^{2+} ions are bound to the EF-hand I and both centrins adopt a 'closed' conformation (Li, S. et al., 2006; Park et al., 2006). Assuming that the Ca^{2+} affinities of the N-terminal domains of HsCen2p (Yang et al., 2006a) and Cdc31p are comparably low, the observed binding of Ca^{2+} to EF-hand I in one of the Cdc31p/Sfi1p complex structures is a result of the unphysiologically high Ca^{2+} concentration (0.1 M) used for crystallization (Li, S. et al., 2006). The fact that Ca^{2+} binding does not change the conformation was also shown by NMR spectroscopy for the N-terminal domain of HsCen2p (Yang et al., 2006a), but, on a structural level, the reason for this unusual behaviour of an EF-hand protein remains to be explained. Clearly different from the majority of centrins, which have been structurally investigated, the N-terminal domain of CrCenp adopts an 'open' conformation in the presence of Ca^{2+} and is proposed to act as a Ca^{2+} sensor (Sheehan et al., 2006).

Analyses of polymerization properties of centrins indicate that their Ca^{2+}-induced polymerization is mainly dependent on the N-terminal subdomain (Wiech et al., 1996). Studies on HsCen2p suggest that polymerization is facilitated by intermolecular interactions of the N- and the C-terminal subdomains (Yang et al., 2006a). NMR spectroscopy revealed the N-terminal subdomains to be of irregular and dynamic structure in solution (Yang et al., 2006a). These regions were also not visible in the electron

[2]N-terminally extended MmCen1p containing the additional GSPGISGGGGGIRLRAPLRSQLLWR peptide sequence.

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density maps of centrin crystals (Li, S. et al., 2006; Park et al., 2006; Thompson et al., 2006).

Taken together, the X-ray crystal structures of centrins available to date do not indicate that these proteins undergo substantial conformational changes upon Ca^{2+}-binding. This is in contrast to earlier observations made by circular dichroism-spectrometry (Wiech et al., 1996). However, the certain Ca^{2+} dependency of centrin functions might be related to subtle effects of submolecular structural rearrangements, modulation of flexibility or reduction of conformational heterogeneity resulting from Ca^{2+}-binding (Matei et al., 2003; Yang et al., 2006a; Charbonnier et al., 2007). Nevertheless, the reason for this unusual behaviour of an EF-hand protein remains to be explained on a structural level.

3. Subcellular localization and cellular function of centrins

3.1. Centrins as ubiquitous components of centrosomes, spindle poles and basal bodies

The first centrin protein was described as the major component of the massive striated flagellar rootlets of *Tetraselmis striata* (Salisbury et al., 1984). In these unicellular green algae, centrins-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). Subsequently, centrin-based fibre systems were also described in several other green algae including the algal model system, the "green yeast" *Chlamydomonas rheinhardtii*. In *Chlamydomonas*, centrins are found in descending fibres which connect the basal body apparatus with the nucleus (Salisbury et al., 1987; Schulze et al., 1987), in distal fibres which connect both adjacent basal bodies to one another (McFadden et al., 1987) and in the stellate fibres of the transition zone present in the plane between the basal body and the axoneme of the flagella (Sanders and Salisbury, 1989) (Fig. 3A). The green algal centrin fibre systems exhibit Ca^{2+}-triggered contractions (Salisbury et al., 1984; Salisbury, 1995; Schiebel and Bornens, 1995). Contractions of these stellate fibres are thought to induce microtubule severing in the transition zone and thereby the excision of the flagellum (Sanders and Salisbury, 1989, 1994). Microtubule severing mediated by Ca^{2+}-activated centrin was discussed as 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 centre (MTOC) of higher eukaryotic cells (Schatten, 1994). Nevertheless, the microtubule severing properties of centrin polymers are still under debate. There is reliable evidence that the AAA+ ATPases katanin may mediate axonemal severing during *Chlamydomonas* deflagellation (Lohret et al., 1998; Karabay et al., 2004; Baas et al., 2005).

However, centrins are not only found in nanofibres associated with cilia or basal bodies, but also as molecular components of the ciliary axonem and basal bodies (Huang et al., 1988a, b; Piperno et al., 1992; Baron et al., 1995; Guerra et al., 2003). There, centrins are required for basal body/centriole duplication which occurs during cell division (Geimer and Melkonian, 2004). Furthermore, the analysis of centrins in the free-living ciliate *Tetrahymena* provides evidence for a requirement of a centrin isoform for structural integrity of pre-existing centrioles (Stemm-Wolf et al., 2005).

In the yeast *Saccharomyces cerevesiae*, centrin is encoded by the *CDC31* gene. The yeast centrin orthologue protein, Cdc31p is a component of the half bridge of the spindle pole body (SBP) (Spang et al., 1993), the centrosome/spindle pole homologue in yeast (Fig. 3B). Cdc31p plays an essential role in the cell cycle via regulation of the duplication of the SBP in mitosis and meiosis II (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 yeast SPB duplication, binding of Cdc31p to the protein Kar1p is required. In addition, Cdc31p specifically interacts with other yeast proteins including an essential protein kinase (Kic1p) whose activity probably regulates SPB duplication (Sullivan et al., 1998; Khalfan et al., 2000). Furthermore, during SPB duplication Cdc31p recruits Mps3p to the half bridge (Jaspersen et al., 2002). Mps3p is involved in sister-chromatid telomere cohesion (Antoniacci and Skibbens, 2006) and meiotic bouquet formation in *S. cerevisiae* (Conrad et al., 2007). Further observations indicated a role of Cdc31p in the nuclear mRNA export machinery in yeast (Fischer et al., 2004).

In vertebrates, centrin proteins are ubiquitously expressed and commonly associated with centrosome-related structures such as spindle poles of dividing cells or centrioles of centrosomes and basal bodies (Fig. 5) (Salisbury, 1995, 2007; Schiebel and Bornens, 1995; Wolfrum et al., 2002; Gießl et al., 2004b, 2006). As discussed above, the vertebrate centrins cluster to two divergent subgroups (Cen1p, 2p, 4p and Cdc31p/Cen3p) (Fig. 2A and B). This phylogenic and evolutionary diversity may also become manifest in differences in the cellular function and/or subcellular localization of the centrin isoforms. Unfortunately, little is known about the specific subcellular localizations and specialized functions of the different centrin isoforms and their specific function in diverse cell types and tissues. Many localization and biochemical studies in mammalian cells and tissues were performed with polyclonal and monoclonal antibodies generated against green algae centrins which do not discriminate between the mammalian centrin isoforms (e.g. Wolfrum and Salisbury, 1995, 1998; Laoukili et al., 2000; Pulvermüller et al., 2002; Gießl et al., 2004b). Unfortunately, such non-discriminatory antibodies are continually in use (Chang et al., 2006; Tsang et al., 2006) and are unhelpful to solve fundamental questions on specific molecular and cellular functions of centrin isoforms. Nevertheless, using

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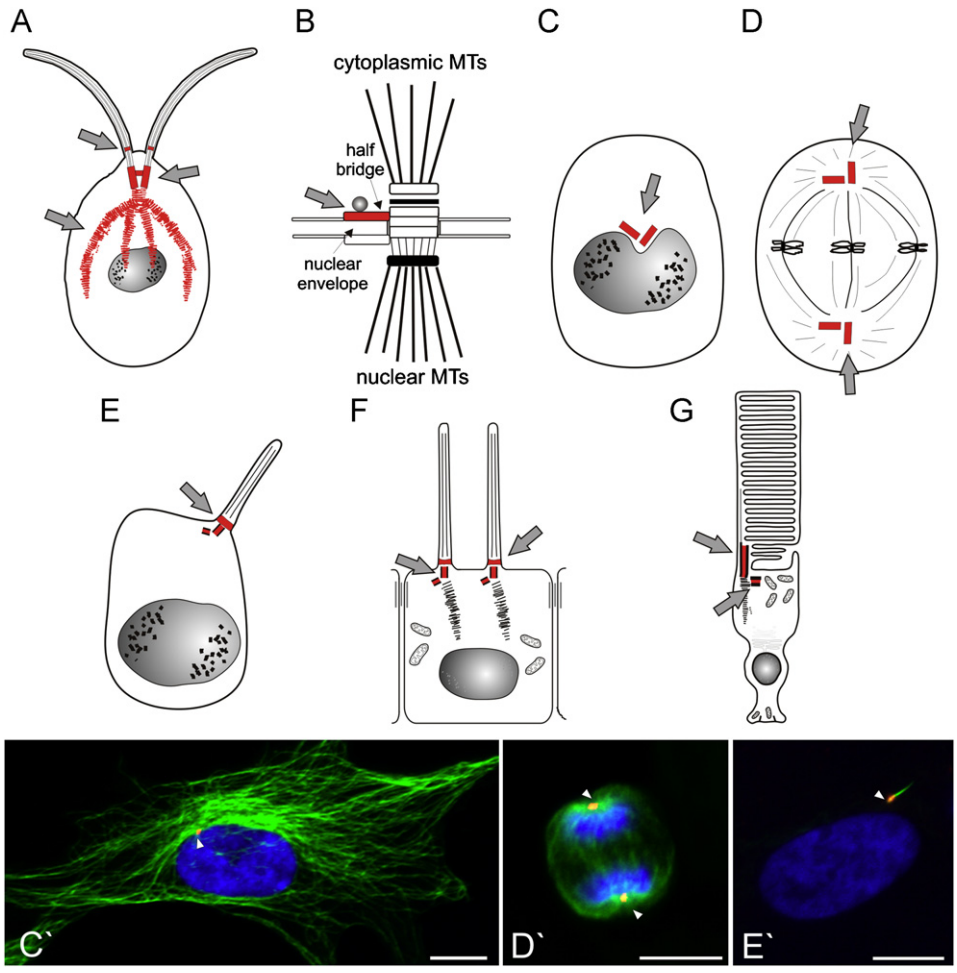

Fig. 3. Subcellular localization of centrins in different cell types. (A–G) Schematic representation of centrin localizations in various cell types is indicated in red and by arrows. (A) In unicellular green algae (e.g., *Chlamydomonas reinhardtii*), centrins are located at transition zones of flagella, basal bodies, distal connecting fibres and in nuclear-basal-body connectors. (B) The yeast centrin Cdc31p is localized at the half bridge of the spindle pole body, the major microtubule organizing centre (MTOC) in yeast. (C–E) Centrin localization in different stages of cultured animal cells. Centrins are localized in centrioles of centrosomes during interphase (C), the spindle poles in mitotic cells (D) and in the transition zone and in basal bodies of primary cilia in cell cycle-arrested cells (E). (F) In multi-ciliated epithelial cells, centrins are found in the transition zone and in basal bodies of cilia. (G) In the vertebrate photoreceptor cell, centrins are located in the connecting cilium (= elongated transition zone) and in the basal bodies of the ciliary apparatus. (C′–E′) Subcellular localization of centrins in cultured hTERT-RPE1 cells. Double immunofluorescence staining with antibodies against centrin isoform 3 (red) and anti-α-tubulin (green) of cells during interphase (C′) and in mitosis (D′). Cen3p is localized at the centrosome (*arrowhead*) during interphase (C′) and at the mitotic spindles of dividing cells (D′). (E′) Double immunofluorescence staining of anti-Cen3p (red) and acetylated-tubulin (green) in primary cilia of serum starved cells. Cen3p is exclusively located at the base of the primary cilium, the transition zone and the basal body (*arrowhead*). In contrast, acetylated-tubulin is found in microtubules throughout the entire length of the primary cilium. Nuclear DNA is counterstained with DAPI (4′,6-diamididino-2-phenylindole). Bars: 6 μm.

these antibodies, centrins were detected in centrioles and the pericentriolar matrix (PCM) of centrosomes and basal bodies (Salisbury et al., 1988; Baron et al., 1991, 1994). Furthermore, centrin was found to be localized in pericentriolar satellites which are interconnected and linked to the PCM by a network of centrin-containing nanofibres (Baron et al., 1992, 1994) and, in stably transfected cultured cells, green fluorescent protein (GFP) tagged-Cen1p was identified among the first proteins to localize at sites of newly forming centrioles (La Terra et al., 2005).

So far only a few studies have been performed with isoform-specific probes for mammalian centrin isoforms (Wolfrum and Salisbury, 1998; Salisbury et al., 2002; Gavet et al., 2003; Gießl et al., 2004a). In these analyses, centrin isoform-specific primers were applied in comparative combined reverse transcriptase reaction and polymerase chain reaction (RT-PCR) experiments and/or isoform-specific antibodies were successfully used. In summary, these expression analyses provide the following view: centrin isoforms Cen2p and 3p are ubiquitously expressed in all somatic cells; Cen1p is expressed in male germ cells and ciliated cells; Cen4p expression is restricted to ependymal and choroidal ciliated cells of the brain and ciliated sensory cells (Wolfrum and Salisbury, 1998; Laoukili et al., 2000; Gavet et al., 2003; Trojan, 2003; Gießl et al., 2004a). Cen2p and 3p are localized in the proximal portion of the centrioles in centrosomes, in the basal bodies of cilia and flagella as well as in the periciliary

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matrix surrounding the centrioles (Paoletti et al., 1996; Laoukili et al., 2000; Gießl et al., 2004a). In the course of mitosis, both, Cen2p and 3p, appear and stay in the spindle poles. In contrast to Cen2p and 3p, Cen1p was mapped to the transition zone of cilia and Cen4p was found in the basal body of ciliated neurons and sensory cells (Gavet et al., 2003; Gießl et al., 2004a).

The prominent localization of centrins at the centrosomes and basal bodies gave rise to several hypotheses regarding the cellular functions of centrins. In animal interphase cells or in arrested cells of differentiated tissue, the centrosome functions as the major MTOC (Fig. 3) (Bettencourt-Dias and Glover, 2007). At the MTOC, microtubules are *de novo* synthesized; 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 reliable evidence was gathered for important, but probably distinct roles of centrins at the centrosome during the cell cycle. The centrosome is duplicated once during the cell cycle to give rise to two spindle poles that organize the microtubule array of the mitotic spindle (Fig. 3D and D′). Like its close relative, the yeast Cdc31p, Cen3p may participate in centrosome reproduction and duplication during G2 of the interphase of mitosis (Middendorp et al., 2000). Cen2p seems to play a specific role in centriole separation preceding centrosome duplication (Lutz et al., 2001). Gene silencing experiments using RNA interference in human HeLa cells confirmed the requirement of Cen2p for correct centrosome duplication and for proper cytokinesis (Salisbury et al., 2002).

Nevertheless, centrins are not only expressed in the centrioles during the *de novo* formation of basal bodies or during the prearrangement and execution of centriol duplication, but also at basal bodies and centrioles and centrosomes in interphase G1 or in fully differentiated cells (G0) (Fig. 3E and E′). However, little is known about the function of centrins in the latter cell stages. In G1 and G0 cells, vertebrate centrins are probably required for structural integrity of pre-existing centrioles as shown for centrins in the ciliate *Tetrahymena* (Stemm-Wolf et al., 2005; Salisbury, 2007). Furthermore, centrins may contribute to membrane-independent G-protein signalling at the centrosomes and the basal body apparatus of ciliated cells (Gießl et al., 2004b). In the highly specialized, fully differentiated photoreceptor cells of the vertebrate retina binding of centrins to the visual G-protein may regulate the translocation of transducin through the connecting cilium (see below, Section 5.1).

4. Centrins in the vertebrate retina

4.1. Centrin expression in the vertebrate retina

Comparative studies demonstrate expression of centrins in the retina of species distributed throughout the subphylum of vertebrates (Fig. 4) (Wolfrum and Salisbury, 1998; Wolfrum et al., 2002). In mammals, RT-PCR analyses with isoform-specific primers demonstrate expression of all four known mammalian centrin isoforms in the retina (Wolfrum and Salisbury, 1998; Trojan, 2003; Gießl et al., 2004a). The RT-PCR results were confirmed in protein expression studies by Western blot analyses using specific antibody probes for specific centrin isoforms (Gießl et al., 2004a).

4.2. Subcellular localization of centrins in retinal cells — in particular in photoreceptor cells

As in other cell types of animal tissues, centrins are common components of centrioles of the centrosomes and of basal body apparatuses in neurons of the vertebrate retina (Figs. 3 and 6) (Wolfrum and Salisbury, 1998; Wolfrum et al., 2002). Furthermore, centrins were found in the connecting cilium of rod and cone photoreceptor cells in all vertebrate species investigated so far (Fig. 4) (Wolfrum, 1992; Wolfrum and Salisbury, 1995, 1998; Schmitt and Wolfrum, 2001; Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a, b, 2006). Our detailed analysis of the diverse centrins in the mouse retina revealed differential expression and subcellular distribution of centrin isoforms (Figs. 5 and 6) (Gießl et al., 2004a, b, 2006): Cen2p and 3p are expressed in all cell types of the retina and the associated cells of the retinal pigment epithelium. As in other cell types, Cen2p and 3p are localized at the centrosomes of non-photoreceptor retinal neurons. In rod and cone photoreceptor cells, subcellular localization of Cen2p and 3p is found in the basal body and the connecting cilium. In contrast, the expression of Cen1p and 4p in the retina is restricted to photoreceptor cells. Cen1p and 4p are localized in the connecting cilium or in the basal body of rod and cone photoreceptor cells, respectively (Fig. 6). In conclusion, it is worth noting that rod and cone photoreceptor cells of the mammalian retina are the only cell types known so far that express all four centrins in parallel; three isoforms (Cen1p–3p) in the cilium and three in the basal body (Cen2p–4p) (Fig. 6).

High-resolution immunofluorescence techniques and immunoelectron microscopy enabled assignment of centrins to organelle substructures in retinal cells. As in centrioles of other cell types, centrins are found in the apical part of the centrioles. In the connecting cilium of photoreceptor cells, the ‘‘ciliary centrins’’, Cen1p–3p are localized along the entire extension of the connecting cilium (Figs. 5 and 6) (Wolfrum and Salisbury, 1995; Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a, b, 2006). Therefore, antibodies raised against centrins are frequently used as molecular markers not only for centrioles (e.g. Nagasato and Motomura, 2004; La Terra et al., 2005; Dahm et al., 2007), but also for the connecting cilium (e.g. Liu et al., 1997; den Hollander et al., 2007; Overlack et al., 2008; Maerker et al., 2008). Immunoelectron microscopy data demonstrate that the

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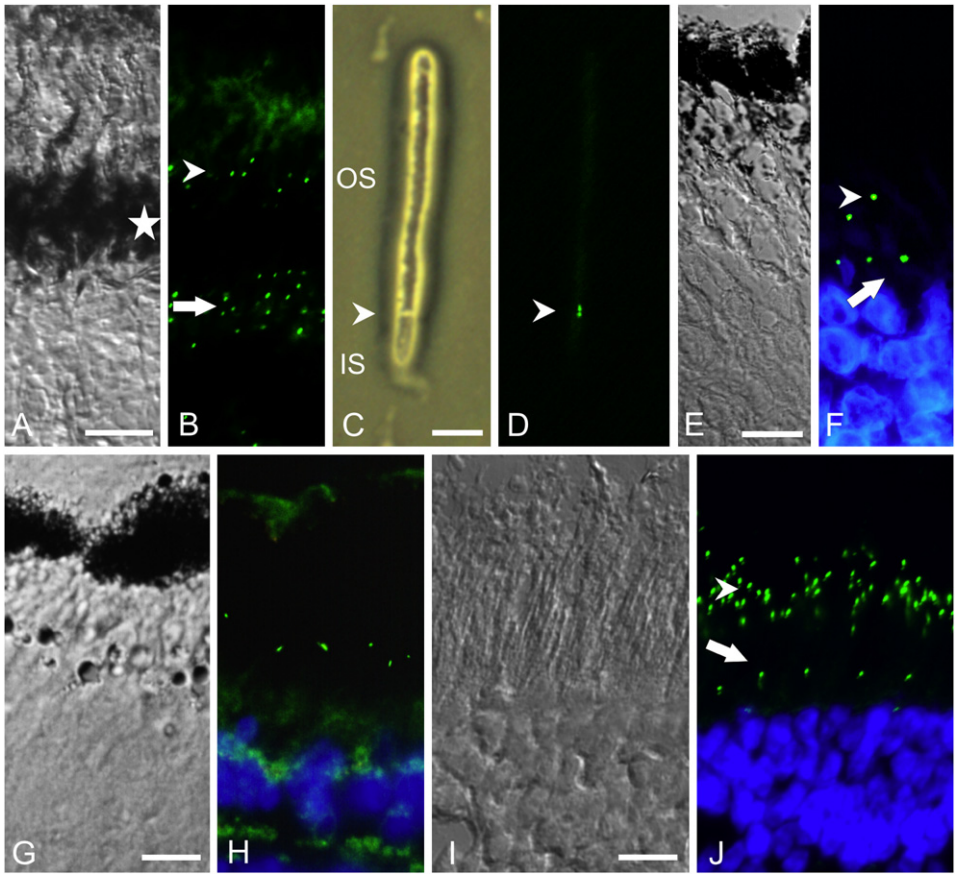

Fig. 4. Localization of centrins in vertebrate photoreceptor cells. (A–J) Immunofluorescence localizations of centrins in photoreceptor cells of species from different vertebrate classes. (A, C, E, G and I) Differential interference contrast images. (B, D, F, H and J) Indirect immunofluorescence staining with anti pan-centrin antibodies (green) and DAPI DNA staining (blue). Centrins are predominantly located in the ciliary apparatus, composed of the connecting cilium and the basal body. (A, B) Cryosection of a light-adapted fish retina (*Danio rerio*). Centrins are localized at the joint between the inner and outer segment of rods (*arrowhead*) and cones (*arrow*). Note: fish photoreceptor cells and RPE cells exhibit light-dependent retinomotor movement. In light-adapted retinas, melanin granules in the long microvilli-like extensions of the retinal pigment epithelium cells (*asterisk*) are located between the layer of rod and cone photoreceptor cells. (C, D) In an isolated rod photoreceptor cell of the teleost *Lepomis cyanellus*, centrins are stained in the short connecting cilium (green upper dot) and the basal body (green lower dot) (*arrowhead*). (E, F) In amphibian photoreceptor cells (*Ambystoma mexicanum*) centrin antibodies label connecting cilia in rods (*arrowhead*) and cones (*arrow*). (G, H) In chicken (*Gallus gallus*), pan-centrin antibodies label the ciliary apparatus of photoreceptor cells. (I, J) In the pig *Sus scrofa* (mammal), centrins are localized in the cilary apparatus of rod (*arrowhead*) and cone (*arrow*) photoreceptor cells.

"ciliary centrins" co-localize at the inner surface of the microtubule doublets of the connecting cilium (Fig. 5H; Wolfrum and Salisbury, 1998; Pulvermüller et al., 2002; U. Wolfrum and A. Gießl, unpublished data). Our recent observation of a direct binding of Cen1p to microtubules further supports this attachment to the ciliary microtubule pairs (Trojan et al., 2008).

The modified connecting cilium of vertebrate photoreceptor cells is the structural equivalent of an extended transition zone present at the base of a common motile cilium (Besharse and Horst, 1990; Liu et al., 2007; Roepman and Wolfrum, 2007). The presence of centrins along the entire extension of the connecting cilium is in agreement with the localization of centrins in the transition zone of motile cilia or the sensory cilia of mammalian olfactory cells (Wolfrum and Salisbury, 1998; Laoukili et al., 2000). The prominent localization of "ciliary centrins" in the connecting cilium certainly indicates a specific role of centrins in the function of the photoreceptor cilium. At the joint between the outer and the inner segment of the photoreceptor cell "ciliary centrins" may participate in the alignment of the photoreceptor outer segment (Wolfrum and Salisbury, 1995). In mammals, variation of the alignment angle of each outer segment is thought to achieve optimal light infiltration in each photoreceptor outer segment (Enoch, 1981). In addition, "ciliary centrins" may contribute to the massive molecular transport through the connecting cilium (Wolfrum and Salisbury, 1995). They may also contribute to the barrier for soluble proteins which is thought to be established in the connecting cilium for the regulation of molecular diffusion between the inner and the outer segment of photoreceptor cells (Spencer et al., 1988; Besharse and Horst, 1990; Wolfrum and Salisbury, 1998). In any case, centrin-based processes in the connecting cilium should be dependent on regulatory changes of the free Ca^{2+}

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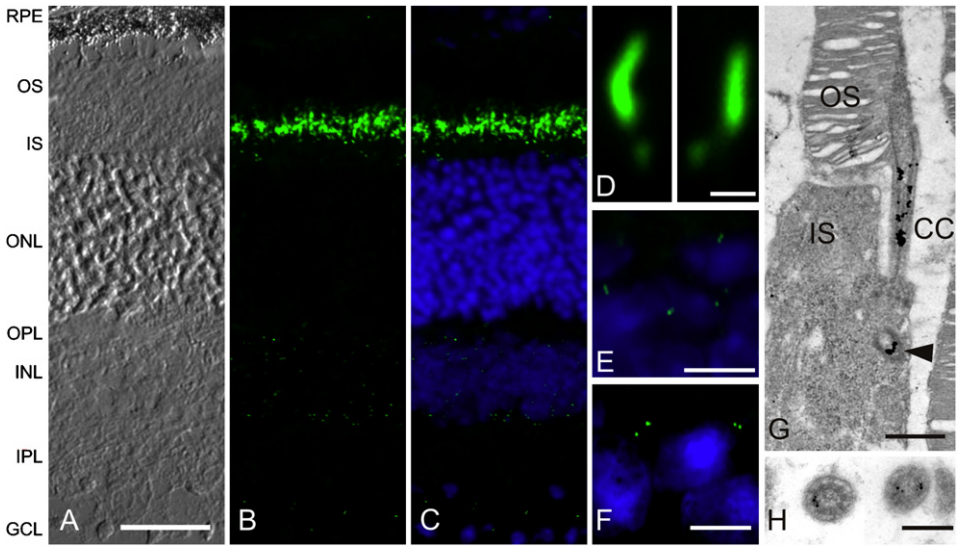

Fig. 5. Subcellular localization of centrins in the mouse retina. (A–F) Longitudinal cryosections through a murine retina. (A) Differential interference contrast image reveals the different layers of the retina. Light-sensitive outer segments (OS) are linked with inner segments (IS) of rod photoreceptor cells. Nuclei of photoreceptor cells are located in the outer nuclear layer (ONL), whereas synapses of photoreceptor cells and following neurons form the outer plexiform layer (OPL). Inner nuclear layer (INL) is composed of the nuclei of these neuronal cells. Their synapses in the inner plexiform layer (IPL) are linked to ganglion cells (GCL). RPE: retinal pigment epithelium. (B–F) Indirect immunofluorescence analysis of anti-pan-centrin. Centrins are mainly located in the ciliary apparatus, at the joint between the OS and the IS of photoreceptor cells (B–D). In addition, centrins are found at centrosomes in cells of the INL (B, C and E) and the GCL (B, C and F). (G and H) Immunoelectron microscopy analysis of ultrathin sections through parts of mouse rod photoreceptor cells. (G) Silver-enhanced immunogold labelling with antibodies specific for Cen3p reveals Cen3p localization in the connecting cilium (CC) and the basal body of a longitudinal sectioned rod cell. (H) Silver enhanced immunogold labelling of cross-sections through connecting cilia reveals localization of Cen3p at the inner surface of ciliary microtubule duplets. Bars: A–C: 20 μm; D and G: 0.5 μm; E and F: 4 μm; H: 150 nm.

concentration and/or the phosphorylation of centrin molecules (see Sections 2.2 and 5.2). Our recent results provide striking evidences for Ca^{2+}-dependent interaction between centrins and the visual G-protein transducin on its pathway through the inner lumen of the connecting cilium of mammalian photoreceptor cells (for details see Section 5.2) (Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a).

5. Centrin-binding proteins in vertebrates

In the cellular context, proteins are in permanent crosstalk with interacting partner molecules throughout their entire molecular lifespan. Interacting molecules control, modify, protect proteins and guarantee the proper cellular function of the partners. Therefore, knowledge of specific interacting partners is a necessary prerequisite to solve the *in vivo* function of proteins. Unfortunately, in vertebrates, only few centrin-binding proteins are presently known. Numerous centrin-binding proteins have been genetically identified in yeast (see also Section 3.1). However, for most of theses proteins, which bind to Cdc31p, e.g. Kar1p, no vertebrate homologues have been identified so far.

In vertebrates, the first protein complexes containing centrins were isolated from the cytoplasm of arrested *Xenopus* oocytes. Centrins (XlCen2p and/or XlCen3p) are sequestrated in an inactive state by the interaction with the heat shock proteins HSP70 and HSP90 (Uzawa et al., 1995). In the mammalian retina, preliminary data indicate that HSP70/90 centrin complexes can also be assembled (U. Wolfrum, unpublished data).

In vertebrates, centrins were found in protein complexes containing centrosomal and ciliary proteins, e.g. CEP290 and CP110 (Chang et al., 2006; Tsang et al., 2006). CP110 and centrins were shown to interact not directly but functionally during cytokinesis (Tsang et al., 2006). The complex containing CEP290 and centrins is thought to be involved in the molecular transport through the connecting cilium of retinal photoreceptor cells and therefore being essential for photoreceptor maintenance and survival (Chang et al., 2006). Mutations of genes encoding molecules in this complex lead to several forms of photoreceptor degeneration and are causes for several forms of inherited blindness (Chang et al., 2006; Sayer et al., 2006). Furthermore, they are involved in Joubert syndrome, including retinal degeneration, *nephronophthisis* and cerebellar defects. Unfortunately, in these studies it remains elusive which centrin isoforms are integrated into the complexes containing CP110 and CEP290.

Proteins which interact directly with centrins were identified in yeast 2-hybrid screens (Paschke, 1997). Using human centrin 2 (HsCen2p) as a bait construct, the laminin-binding protein LBP, a component of the extracellular basal lamina, and the cytoplasmic receptor protein tyrosine kinase κ were identified as putative interaction partners (Paschke, 1997). To our knowledge, to date none of these putative centrin-binding proteins has an obvious

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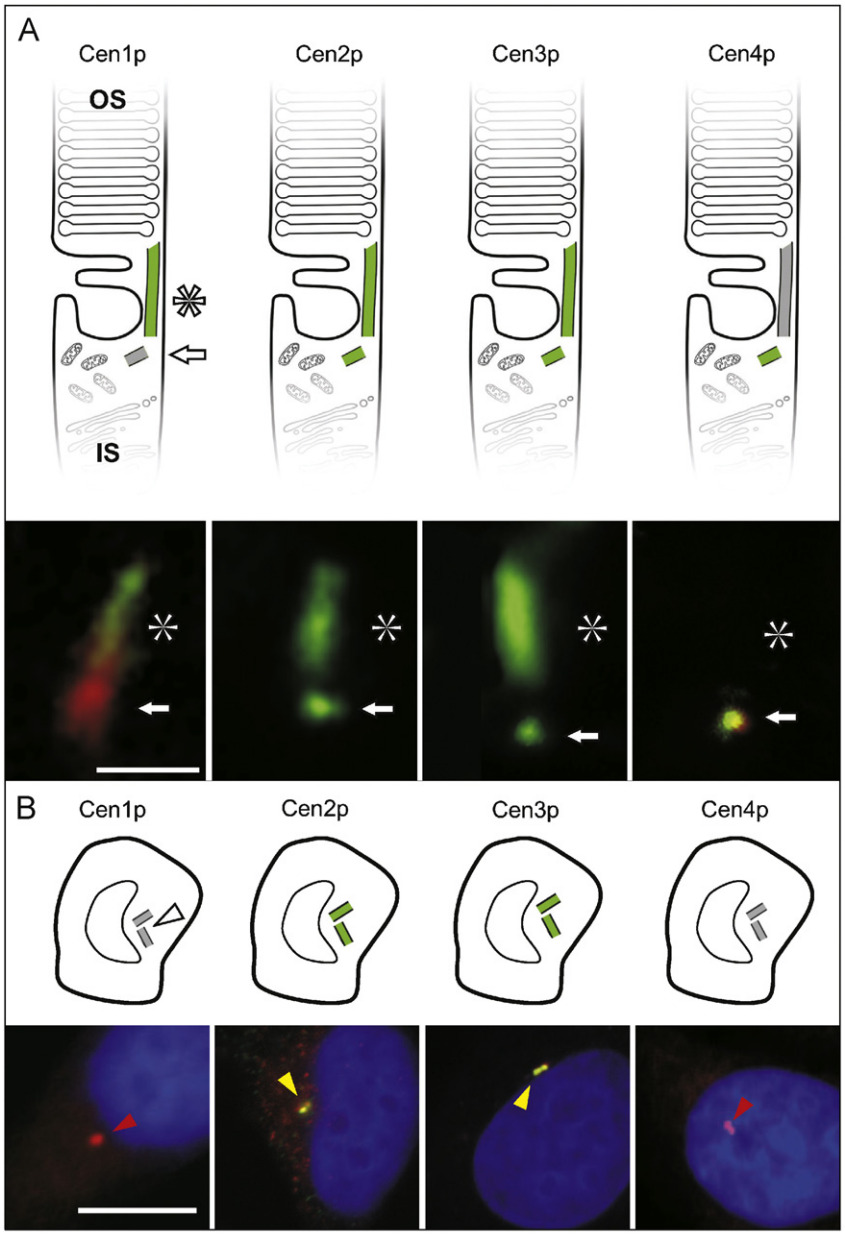

Fig. 6. Differential localization of centrin isoforms in the ciliary apparatus of vertebrate photoreceptor cells and at centrosomes of neuronal cells of the outer nuclear layer. (A) Schematic representation (upper part) and corresponding indirect immunofluorescence (lower part) of the isoform-specific localization of Cen1p–4p in the connecting cilium (*asterisk*) and at the basal body (*arrow*). Indirect immunofluorescence using centrin isoform-specific antibodies (green) with anti-γ-tubulin staining (red) reveal localization of Cen1p–3p in the connecting cilium. In addition, Cen2p and 3p are localized at the basal body, where Cen4p is exclusively localized and no Cen1p is detectable. (B) Cen2p and 3p-specific antibodies (green, *arrowhead*) react in dot pairs representing the centriole pairs in non-photoreceptor cells. DAPI stains the nuclear DNA (blue) (lower part). Schematic illustration of the differential localization of the four centrin isoforms in non-specialized eukaryotic cells (upper part). Cen2p and 3p are localized in centrioles of centrosomes, while no Cen1p and 4p is detectable (*arrowhead*). Bars: A: 1 μm; B: 8.2 μm.

function in the connecting cilium of mammalian photoreceptor cells. In cultured human HeLa cells, another interacting partner of HsCen2p was identified. HsCen2p was shown to interact directly with the *Xeroderma pigmentosum* group C protein (XPCp), which is responsible for nucleotide excision repair of genomic DNA (Araki et al., 2001; Charbonnier et al., 2006). The HsCen2p–XPCp complex seems to translocate from the centrosome and the cytoplasm into the nucleus after exposure of cells to UV-irradiation (Charbonnier et al., 2007). This process is thought to connect the functions of the centrosome and its integrative sensing with the XPCp-mediated DNA excision repair. Similar mechanisms are also conceivable for the function of centrins and XPCp in the photoreceptor cells to prevent damages after bright light irradiation.

A novel directly interacting partner of centrins was identified in yeast and termed Sfi1p (Kilmartin, 2003). Interestingly, Sfi1p was captured in a screen for

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Ca^{2+}-independent centrin interaction partners. This protein contains multiple conserved centrin-binding repeats and was discussed as a structural scaffolding protein forming Ca^{2+}-sensitive contractile fibres (Kilmartin, 2003; Li, X. et al., 2006). Mutations in SFI1 gene lead to drastic spindle pole defects in budding yeast indicating a role during the duplication of the MTOC and during mitotic spindle assembly (Kilmartin, 2003; Li, X. et al., 2006; Anderson et al., 2007). Sfi1p-like proteins containing centrin-binding repeats are conserved from yeast to humans (Kilmartin, 2003; Salisbury, 2004; Li, X. et al., 2006; Gogendeau et al., 2007). However, no clear evidence for an interaction of centrins with Sfi1p-like proteins and a functional role of such protein complexes has been described in higher eukaryotes so far.

5.1. Centrin-binding proteins in mammalian photoreceptor cells

Little is known about the expression and function of centrin-binding proteins like XPCp-protein or Sfi1p in the retina. Unpublished data obtained by RT-PCR indicate that Sfi1p is expressed in the murine retina (Ph. Trojan and U. Wolfrum, unpublished). Unfortunately, a further evaluation of the Sfi1p function in retinal photoreceptor cells is lacking.

A screen for centrin-interacting proteins in the retina by far Western blot analyses of retinal proteins overlayed with recombinant expressed MmCen1p revealed several polypeptide bands (Pulvermüller et al., 2002; Wolfrum et al., 2002). In these assays, binding of recombinant MmCen1p to target proteins was restricted to Ca^{2+}-activated centrin. An increase of affinity to a centrin target protein has been previously described for the binding of diverse centrin species to the yeast target protein Kar1p (Schiebel and Bornens, 1995; Geier et al., 1996; Wiech et al., 1996).

5.2. Molecular characteristics of centrin/transducin complexes

Analyses of proteins which were identified by our far Western blot screens of retinal centrin-binding proteins revealed the MmCen1p-binding protein p37 as the β-subunit of the visual G-protein transducin (G_t) (Fig. 7) (Pulvermüller et al., 2002; Wolfrum et al., 2002). Transducin (G_t) certainly plays a central role in the activation process of the visual signal transduction cascade in the vertebrate retina (Fung and Stryer, 1980; Heck and Hofmann, 1993, 2001) (see also Section 1). In recent years, we focused on the molecular and functional characterization of centrin/transducin complexes in photoreceptor cells of the mammalian retina.

In our initial studies, we demonstrated that Cen1p interacts with the visual G-protein transducin with high affinity, and thereby forms functional protein complexes in a Ca^{2+}-dependent manner (Fig. 7) (Pulvermüller et al., 2002; Wolfrum et al., 2002). Based on our knowledge of the differential expression of all four centrin isoforms (Cen1p–4p) in rodent photoreceptor cells (Gießl et al., 2004a, b), we addressed the question whether the Ca^{2+}-dependent assembly of centrin/transducin complexes also occurs between transducin and other centrin isoforms, namely Cen2p–4p. Applying independent but complementary interaction assays including co-immunoprecipitation,

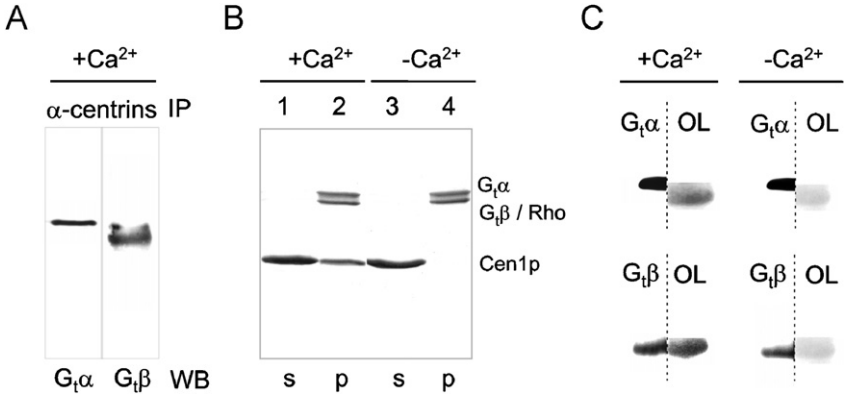

Fig. 7. Ca^{2+}-dependent assembly of MmCen1p/transducin complex. (A) Co-immunoprecipitation of transducin with Ca^{2+}-activated centrins from bovine retina lysates. Left lane: Western blot analysis with monoclonal antibodies against α-subunit of transducin ($G_t\alpha$) of an immunoprecipitation with monoclonal pan-centrin antibody (clone 20H5). Right lane: Western blot analysis with polyclonal antibodies against β-subunit of transducin ($G_t\beta$). The heterotrimeric G-protein complex (including $G_t\alpha/G_t\beta\gamma$) co-immunoprecipitates with MmCen1p in the presence of Ca^{2+}. (B) Membrane binding of the centrin/transducin complex. In the assay, centrin/transducin complexes are formed and bind to rhodopsin (Rh)-containing membranes of bovine photoreceptor cells. The pellets (p) and supernatants (s) were analysed using SDS-PAGE. (C) Combined far Western blot overlay analysis identifies retinal centrin-interacting protein p37 as $G_t\beta$ subunit of transducin. Left panel shows that in the presence of 1 mM $CaCl_2$, MmCen1p binds to a 37 kDa protein, which appears intensely labelled. Right panel shows that in the absence of Ca^{2+} (6 mM EGTA), MmCen1p binding is dramatically reduced. For specific determination of the centrin-binding protein Western blotted lanes were cut in half and processed in parallel for Western blotting with subunit-specific antibodies against $G_t\alpha$ (upper lanes), and $G_t\beta$ (lower lanes) and for overlays with recombinant expressed MmCen1p (OL). The 37 kDa centrin-binding protein is identified by centrin overlays and migrates in the probed SDS-PAGE at the exact mobility of the $G_t\beta$ subunit. (Adapted from Pulvermüller et al., 2002).

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GST-pull down, overlay and co-sedimentation assays as well as size exclusion chromatography and kinetic light-scattering experiments, we have shown that not only Cen1p, but also the three other centrin isoforms, Cen2p–4p, bind with high affinity to transducin (Pulvermüller et al., 2002; Gießl et al., 2004a, b, 2006). Further analyses using kinetic light-scattering experiments (see description in Fig. 8D) indicate that the centrin/transducin interactions are highly specific: centrin-related EF-hand proteins, calmodulin and recoverin, which are highly expressed in photoreceptor cells do not show any detectable Ca^{2+}-dependent interaction with transducin (Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004b). In addition, centrins do not bind to any other molecule of the visual transduction cascade, neither to arrestin, rhodopsin-kinase or rhodopsin, nor do they influence the activity of the cGMP PDE (Fig. 8) (Pulvermüller et al., 2002; Gießl et al., 2004b).

Analyses with our set of complementary protein–protein interaction assays further demonstrate that assembly of centrin/transducin complexes is mediated by the $\beta\gamma$-heterodimer (Fig. 7) (Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a). Later studies also revealed a strict dependence of the assembly of these complexes on the free Ca^{2+} concentration. 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. Cen3p has a significantly lower affinity to the transducin holoprotein than the other centrin isoforms (Gießl et al., 2004a). In the case of Cen1p and 2p, at least two Ca^{2+} ions are required for the activation of these centrin isoforms and for centrin/transducin complex formation (Pulvermüller et al., 2002; Trojan et al., 2008). Further analyses of these complexes indicate that Cen1p, 2p and 4p bind as homooligomers to the $G_t\beta\gamma$-heterodimer, in contrast to Cen3p which binds as

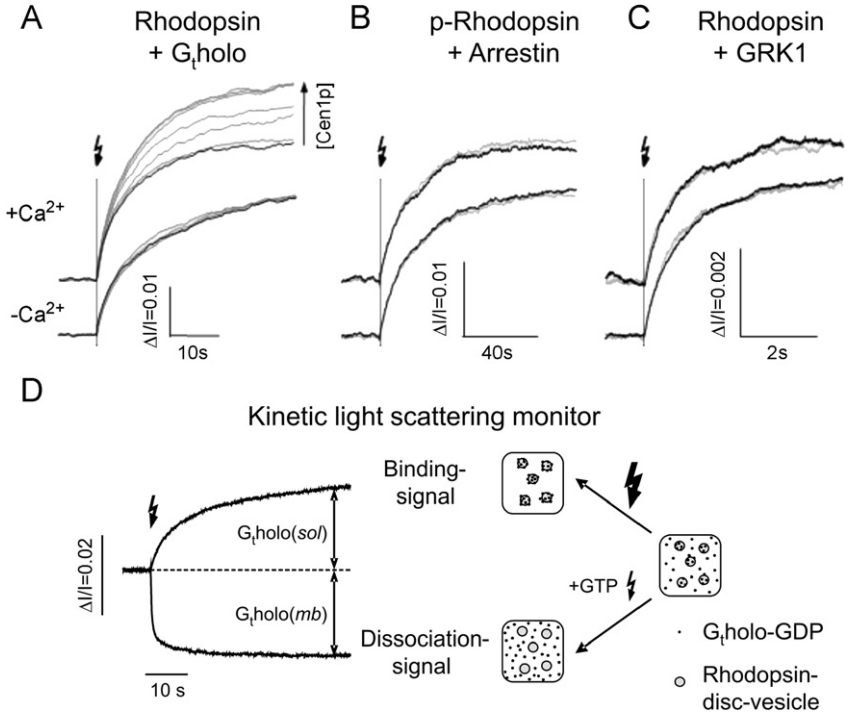

Fig. 8. Light-scattering setup to analyse the centrin/transducin assembly. (A) Ca^{2+}-dependent enhancement of kinetic light-scattering (KLS) binding signal with unphosphorylated membranes and transducin ($G_t$holo) in the presence of Cen1p. Upper panel represents KLS binding signals (3 μm rhodopsin, 0.5 μm $G_t$holo) in the presence of Ca^{2+}, and 0 (control, black curve), 0.6, 1.2, 2.5, 3.6, 5, 7.3, and 10 μm MmCen1p (grey curves), respectively. Lower panel represents KLS binding signals under the same conditions as in the upper panel, but with EGTA instead of Ca^{2+}. (B, C) KLS binding signal with unphosphorylated or prephosphorylated membranes and arrestin and rhodopsin kinase (GRK1) in the presence (grey curves) or absence (black curves) of MmCen1p. Upper panels represent KLS binding signals (0.5 μm arrestin or GRK1 and 3 μm rhodopsin) in the presence of Ca^{2+} plus/minus MmCen1p and the lower panels KLS binding signals in the absence of Ca^{2+}. 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) Real-time monitoring of rhodopsin-transducin complex-formation by KLS. Shown is the time course of normalized light-scattering intensity originated from rhodopsin-containing disk vesicles. Left panel represents an example of KLS binding and dissociation signals. Reactions were triggered by flash photolysis of rhodopsin with a green (500 ± 20 nm) flash, attenuated by appropriate neutral density filters. The flash intensity is quantified photometrically by the amount of rhodopsin bleached and expressed as the mole fraction of photoexcited rhodopsin (Rh*/Rh). KLS binding signals (Rh*/Rh = 32%) were corrected by a reference signal (N-signal) measured on a sample without added protein as described by (Pulvermüller et al., 1993). KLS dissociation signals were recorded with a 0.5–5 ms dwell time of the A/D converter (Nicolet 400, Madison, WI) in the presence of 1 mM GTP and with catalytic amounts of flash activated rhodopsin (Rh*/Rh = 0.5%). To suppress base-line activation, 2.5 mM NH_2OH was added to the sample. The KLS binding signal is interpreted as a gain of protein mass bound to the disk membranes and the KLS dissociation signal as loss of protein mass from the disk vesicle (Heck et al., 2000). The right panel illustrates light-induced mass changes of the scattering membranous particles causing the binding and dissociation signal, respectively.

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a monomer to each $G_t\beta\gamma$ (Pulvermüller et al., 2002; Gießl et al., 2004a).

5.2.1. Functions of centrin/G-protein complexes in mammalian photoreceptor cells

At first view the spatial distribution of centrins (present in the connecting cilium and the basal bodies) and transducin (associated with the visual signal transduction machinery in the outer segment) should exclude any molecular interaction between centrins and transducin in photoreceptor cells. However, the visual G-protein transducin (G_t) is not a permanent resident of the outer segment. It undergoes light-dependent reversible bidirectional translocation in vertebrate photoreceptor cells (Fig. 9) (Brann and Cohen, 1987; Philp et al., 1987; Whelan and McGinnis, 1988). In the dark, G_t is highly concentrated in the rod outer segment, while under bright light conditions, the majority of G_t is translocated into the inner segment, the cell body, and even to the synapse of photoreceptor cells (Fig. 9) (Organisciak et al., 1991; Pulvermüller et al., 2002; Sokolov et al., 2002; Calvert et al., 2006). Interestingly, the clearance of transducin from the outer segment is completed in a few minutes after illumination and is much faster than transducin movements back into the outer segment (Sokolov et al., 2002; Calvert et al., 2006) indicating that different cellular mechanisms are involved (Peterson et al., 2003). Nevertheless, the bidirectional translocations of transducin are thought to contribute to slow but long lasting adaptation of rod photoreceptor cells (Sokolov et al., 2002, 2004; Hardie, 2003; Frechter and Minke, 2006).

Since any intracellular exchange between the inner and outer segmental compartments of photoreceptor cells occurs through the slender non-motile connecting cilium (Besharse and Horst, 1990; Roepman and Wolfrum, 2007) the bidirectional translocation of transducin should take this intracellular route (Fig. 9). Indeed, the power of immunoelectron microscopy provided us with the insight that transducin travels through the connecting cilium on its way between the two photoreceptor cell compartments (Pulvermüller et al., 2002). The slender, closely defined connecting cilium represents not only a track for the molecular translocation but also a suitable domain for regulation of intersegmental molecular exchange (Spencer et al., 1988; Besharse and Horst, 1990; Wolfrum and Salisbury, 1998), and the subcellular compartmentalization of the binding of transducin to the ciliary centrins.

As described in Section 4, centrins are present in the ciliary apparatus (connecting cilium plus basal body complex) of photoreceptor cells of all vertebrate retinas investigated. Our extensive analyses have shown that the four mammalian centrin isoforms (Cen1p–4p) are differentially localized in the ciliary apparatus of retinal photoreceptor cells (see Fig. 6) (Gießl et al., 2004a, 2006). These studies revealed the localization of the centrin isoforms Cen1p–3p in the connecting cilium of photoreceptor cells. Double-immunofluorescence analyses using

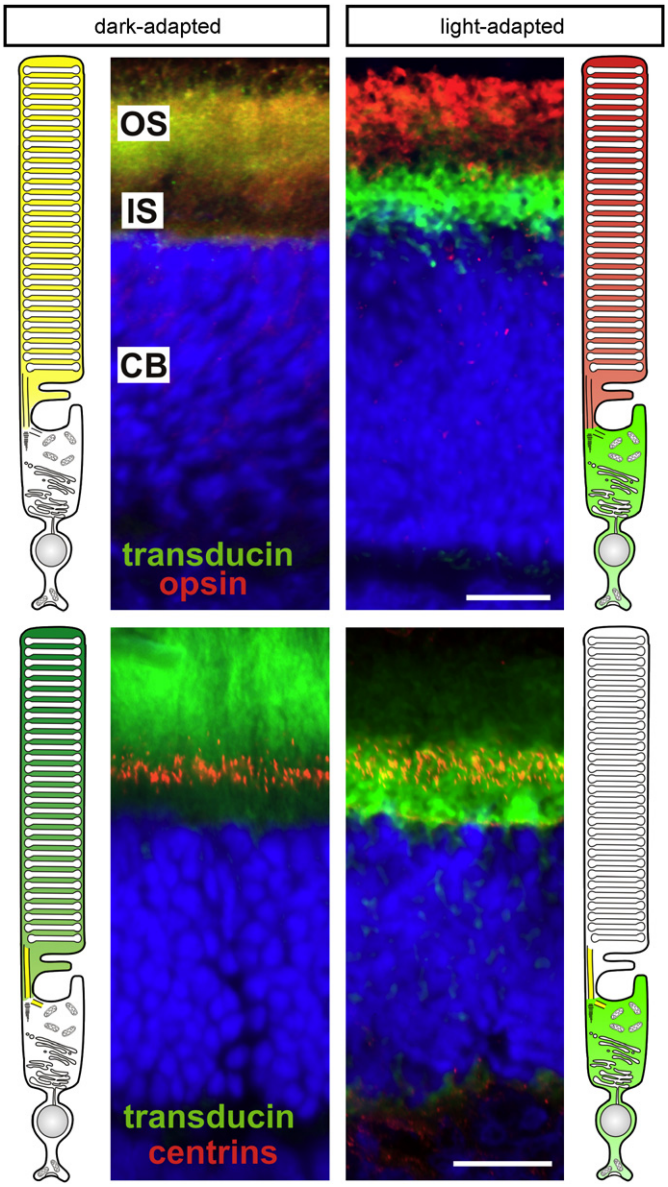

Fig. 9. Immunofluorescence localization of opsin, transducin and centrins in dark- and light-adapted mouse retinas. Upper panel: triple labelled dark-adapted (left) and light-adapted (right) mouse retina. Transducin (green) is localized to the outer segment (OS) of dark-adapted photoreceptor cells and moves into the inner segment (IS) and cell body (CB) during light adaptation. Opsin (red) stays in the outer segment during both conditions. Lower panel: Triple labelled dark-adapted (left) and light-adapted (right) mouse retina. Transducin (green) is localized as described for the upper panel. Centrins (red) are stained in the connecting cilium and basal bodies at the joint between the outer segment and the inner segment of dark- and light-adapted photoreceptor cells. Transducin passes centrins during its passage through the connecting cilium. Bars: 13.1 μm.

antibodies against centrins and transducin subunits show co-localization of the ciliary centrins in the photoreceptor connecting cilium (Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a, b, 2006). Quantitative analyses of silver-enhanced immunogold labelling revealed that ciliary centrins and transducin share the same

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subciliary domain, at the inner surface of the microtubule doublets of the connecting cilium (Pulvermüller et al., 2002; Wolfrum et al., 2002; Gießl et al., 2004a). This spatial subciliary co-localization is consistent with the hypothesis that the ciliary centrins Cen1p–3p and transducin physically interact in the ciliary compartment of vertebrate photoreceptor cells. A changeover of assembly and disassembly of centrin/transducin complexes may regulate the diffusion of transducin through the connecting cilium.

5.2.1.1. Regulation of centrin/G-protein complex assembly in photoreceptor cells. As described above in Section 5.2.1, the binding of centrins to transducin is strictly dependent on Ca^{2+} activation of centrins. In photoreceptor cells, the activation of the ciliary centrins Cen1p and 2p should be triggered by an increase of the free Ca^{2+} concentration in the connecting cilium. There are several alternative mechanisms how the local intraciliary free Ca^{2+} concentration is modulated: Firstly, the intraciliary Ca^{2+} concentration may be affected by light-modulated changes of free Ca^{2+} in the outer segment. Light induces a decrease of Ca^{2+} in the outer segment which occurs within the operating (single quantum detective) range of the rod (Molday and Kaupp, 2000). This would cause an inactivation of centrins in the cilium upon outer segment illumination. However, recent observations also indicate a Ca^{2+} increase in bright light (rod saturated conditions) which would activate the ciliary centrins (Matthews and Fain, 2001; Leung et al., 2007). In any case, we cannot estimate which one of these opposite light-modulated changes in the free Ca^{2+} concentration in the outer segment may regulate the affinity of centrins to transducin. Finally, there is some indication for the presence of a local modulation of the Ca^{2+} homeostasis in the connecting cilium that is independent from the outer segment (Krizaj et al., 2002; Gallagher et al., 2006): Ca^{2+} fluxes through TRPP2 (polycystin-2) channels and the plasma membrane Ca^{2+}-ATPases (PMCA), recently identified in the connecting cilium, may modulate the intraciliary free Ca^{2+} homeostasis. Whatever process leads to an increase of the intraciliary Ca^{2+} concentration, Ca^{2+} activates the ciliary centrins Cen1p and 2p and promotes the assembly of centrin/G-protein complexes.

In the cilium, the Ca^{2+}-triggered assembly of centrin/G-protein complexes may contribute to a barrier for further exchange of transducin between the photoreceptor inner and outer segment (barrier hypothesis) (Wolfrum et al., 2002). A drop of free Ca^{2+} in the connecting cilium should induce the disassembly of the complex, thus providing the necessary condition for the 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, 2004; Wolfrum et al., 2002; Mendez et al., 2003; McGinnis, 2004). However, Ca^{2+}-triggered sequential binding of transducin to centrins may contribute to the transport of transducin through the photoreceptor connecting cilium (Ca^{2+}-gradient hypothesis) (Wolfrum et al., 2002).

5.2.1.2. Regulation of centrin/G-protein complex assembly by centrin phosphorylation. Centrin functions are not only regulated by Ca^{2+}-binding but also by site-specific phosphorylation (see Section 2.2.1). This is certainly also the case in photoreceptor cells of the mature mammalian retina. Our most recently obtained data indicate antagonistic regulation of the formation of centrin/transducin complexes by Ca^{2+}-binding and CK2-mediated phosphorylation in retinal photoreceptor cells (Trojan et al., 2008). In *ex vivo* phosphorylation assays in explanted rat retinas, we found a drastic increase of centrin phosphorylation during dark-adaptation (Fig. 10A). This phosphorylation of centrins turned out to be highly specific for CK2, since it is completely inhibited by specific protein kinase CK2-inhibitors (Fig. 10A). This CK2-mediated phosphorylation of centrins is highly specific for the ciliary centrins Cen1p and 2p (Fig. 10B) (Trojan et al., 2008) and is abolished by dephosphorylation mediated by specific isoforms of the protein phosphatase PP2C (Thissen et al., in prep.). Furthermore, we provide evidence for a reduced Ca^{2+} affinity of phosphorylated centrins causing a reduction of their affinity to transducin (Trojan et al., 2008). In conclusion, CK2-mediated phosphorylation of centrins lowers the ability for the assembly of centrin/transducin complexes in the connecting cilium of dark-adapted photoreceptor cells. An extension of the previous displayed barrier hypothesis on the function of centrins in the connecting cilium of photoreceptor cells is shown in Fig. 11. The light-triggered signalling pathway that targets the antagonistic regulation of the centrin/transducin complex assembly remains elusive. The phosphorylation of centrins can be modulated via illumination either by the reduction of the CK2 activity or indirectly by an activation of PP2C in light. In our opinion, there is no doubt that the formation of centrin/transducin complexes is physiologically regulated and seems to play an important role for photoreceptor cell function.

6. Summary and conclusions

Centrins are members of a conserved subfamily of EF-hand Ca^{2+}-binding proteins commonly associated with the function of centrioles and centrosomes in the cell cycle. In the fully differentiated photoreceptor cells of the mammalian retina, four centrin isoforms are differentially expressed in the ciliary apparatus of photoreceptor cells. In the connecting cilium, ciliary centrins are localized at a subcellular compartment strategically favourable for the regulation of molecular exchange between the inner segment and the outer segment of photoreceptor cells. The unconventional search for centrin-binding proteins in the mammalian retina by far Western blot analyses resulting in identification of transducin as an interacting partner, together with complementary-independent

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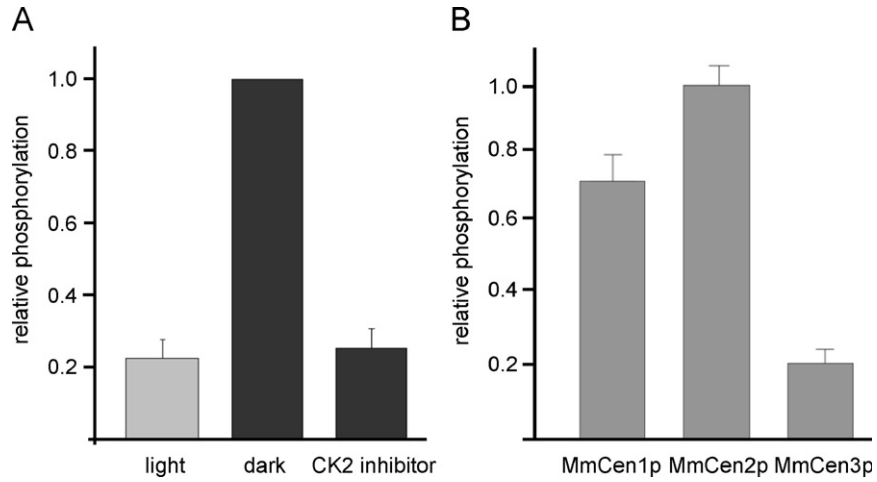

Fig. 10. *In vitro* and *ex vivo* phosphorylation of centrins. (A) Light-dependent *ex vivo* phosphorylation of endogenous centrins. Rats were light (grey bar) or dark (black bars) adapted. After sacrifice of the animals, the retinas were explanted and cultured in the presence of radioactive labelled phosphate. Centrins were immunoprecipitated from these retinas using a monoclonal pan-centrin antibody (clone 20H5) and the radioactive incorporation was analysed. Centrins were five times higher phosphorylated from dark-adapted retinas (dark) compared to centrins from light-adapted ones (light). This light-dependent phosphorylation could be inhibited by the specific protein kinase CK2-inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) in dark-adapted retinas. (B) CK2-mediated *in vitro* phosphorylation of ciliary centrins MmCen1p–3p. Recombinant MmCen1p–3p were phosphorylated by CK2 in the presence of radioactive ATP. Radioactive incorporation was analysed and the highest radioactive incorporation was set as 1 (MmCen2p). CK2-mediated phosphorylation is isoform specific, since only MmCen1p and 2p but not MmCen3p could be phosphorylated *in vitro*.

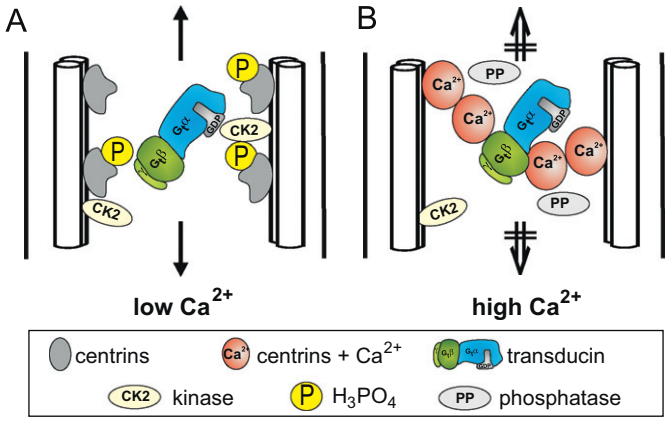

Fig. 11. Model on Ca^{2+}-dependent centrin/transducin complex assembly in the connecting cilium of vertebrate photoreceptor cells. (A) Under low free Ca^{2+} concentrations centrins are phosphorylated by protein kinase CK2 and not active, so transducin floats through the inner lumen of the connecting cilium. (B) If free Ca^{2+} increases in the cilium, centrins are dephosphorylated and activated by Ca^{2+} inducing the assembly of centrin/transducin complexes.

validation strategies supplies reasonable evidence for a specific function of centrins in photoreceptor cells. The molecular characterization of centrin/transducin complexes provides us valuable insights in the highly regulated interplay between the two partner proteins and their putative role in the regulation of light-dependent inter-compartmental exchange of the visual G-protein transducin. Moreover, a physiological relevant interaction between G-proteins and centrins can be judged as a novel aspect of the potential link of signal transduction and molecular translocation and should support insights into the supply of signalling proteins in general.

7. Future directions

Ongoing research utilizing improved isoform-specific antibodies to centrins and tagged-centrin constructs will provide more reliable information on the specific sub-cellular targeting and localization of centrin isoforms in their cellular environment. These results will not only provide better insights in photoreceptor function but also lead to a better understanding of centrins' role in the control of centriole duplication during the cell cycle in general. Since centrins additionally play an essential role in the basal body duplication during ciliogenesis and this aspect is crucial in differentiation of photoreceptor cells from progenitor cells, efforts to analyse the role of centrins during developmental stages of the retina will be very helpful. Further analyses of properties of the centrin/transducin complexes are currently being addressed to understand specific reciprocal-binding sites establishing the specific interaction between centrin isoforms and the $\beta\gamma$-subunit of the G-protein. High-resolution structure analyses of crystals of centrins in specific physiologic stages and centrin/transducin complexes co-crystals by NMR-spectroscopy and X-ray crystallography will help to elucidate the submolecular characteristics of centrin functions and the centrin/G-protein interaction. Future studies will be well worthwhile on the *in vivo* validation of current knowledge on centrin/transducin complexes, obtained by complementary and independent *in vitro* assays. Chasing further potential centrin-binding partners in the retina will certainly deepen our knowledge on the role of centrins in photoreceptor cell function. The establishment of knock out models deficient in centrin isoforms will provide valuable insights into the biological role of centrins.

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However, because of the fundamental roles of most centrin isoforms in the cell cycle, where they are essential for the proper embryonic development all known attempts of the generation of centrin knock out mice have failed due to lethal defects in early developmental stages. Ongoing research utilizing conditional knock out mice will overcome the latter problems. An alternative strategy that substitutes the time and resource-consuming knock out approaches would be the future application of RNAi technologies *in vivo* or *ex vivo* in organotypic retina cultures. For this purpose, improved gene transfer technologies for the application of centrin probes in the living animal or in organotypic retina cultures are currently being tested. Functional analysis of centrins in retinal photoreceptor cells will provide further insights in the cellular and molecular function of centrins in general and may also elucidate the role of transducin and heterotrimeric G-proteins at the centrosome of the eukaryotic cell.

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3. Zusammenfassung der Ergebnisse

In der vorliegenden Arbeit sollten neuartige Regulationsmechanismen der Centrine in der Retina von Vertebraten identifiziert werden. Durch die Kombination biochemischer, zellbiologischer, biophysikalischer und mikroskopischer Techniken war es möglich, signalabhängige, reversible Phosphorylierungen von Centrinen zu beschreiben. Die lichtabhängige Phosphorylierung der Centrine wird durch die Protein Kinase CK2 vermittelt und durch Protein Phosphatasen der 2C Familie aufgehoben. Diese posttranslationalen Modifikationen dienen der Regulation der Bindung von Centrinen an die βγ-Untereinheiten des visuellen G-Proteins Transducin. Diese Komplexbildung dürfte bei der lichtabhängigen, adaptiven Translokation von Transducin zwischen dem Außen- und Innensegment der Photorezeptorzellen stattfinden.

3.1. Centrine werden in der Retina von Vertebraten lichtabhängig durch die Protein Kinase CK2 phosphoryliert

Im Rahmen dieser Arbeit wurde die lichtabhängige Phosphorylierung durch die Protein Kinase CK2 (CK2) erstmals als Regulationsmechanismus der Centrine in Photorezeptorzellen von Vertebraten beschrieben (Dissertation Publikation I). Die Phosphorylierung der Centrin-Isoformen wurde mittels neu entwickelter Versuchsansätze analysiert. Dazu wurden rekombinante Centrin-Isoformen in der Gegenwart von radioaktiv markiertem ATP mit Lysaten aus hell- beziehungsweise dunkeladaptierten Rinderretinae inkubiert. Nach Immunpräzipitationen der Centrine wurde der radioaktive Einbau in die Centrin-Isoformen gemessen. Um die lichtabhängige Phosphorylierung endogener Centrine zu bestimmen, wurden *ex vivo* Versuchsansätze etabliert. Für diesen Zweck wurden lebende Ratten hell- beziehungsweise dunkeladaptiert. Nach der Explantation wurden die Retinae der Ratten in Kulturmedium mit radioaktivem Phosphat kultiviert. Anschließend wurden die endogenen Centrine immunpräzipitiert und die inkorporierte Radioaktivität gemessen. Diese *in vitro* und *ex vivo* Versuchsansätze zeigten unabhängig voneinander, dass die Centrine während der Dunkeladaption der Retina von Vertebraten phosphoryliert werden (Dissertation Publikation I Fig. 1a, b, Dissertation Publikation III Fig. 10a).

Alle Centrin-Isoformen besitzen mehrere putative Phosphorylierungsstellen für die Protein Kinasen A (PKA), die Protein Kinase C (PKC), die Calmodulin-abhängige Protein Kinase II (CaMKII) und die Protein Kinase CK2 (CK2). Zur Identifizierung der für diese lichtabhängige Phosphorylierung verantwortlichen Kinasen wurden spezifische Kinase-

Inhibitoren in den *ex vivo* Experimenten appliziert. Dadurch konnte die CK2 als maßgebliche Kinase für die lichtabhängige Phosphorylierung endogener Centrine in explantierten Retinae identifiziert werden (Dissertation Publikation I Fig. 1c, Dissertation Publikation III Fig. 10a). Weiterführende *in vitro* Phosphorylierungsversuche mit rekombinanter CK2 und bakteriell exprimierten Centrin-Isoformen der Maus ergaben, dass nicht alle Centrin-Isoformen gleichermaßen phosphoryliert werden. Die Centrin-Isoformen 1 und 2 (Cen1p und Cen2p) erwiesen sich gleichermaßen als beste Substrate für die CK2-vermittelte Phosphorylierung. Im Gegensatz dazu wurde die Centrin-Isoform 3 (Cen3p) nicht - und die Isoform 4 (Cen4p) nur schwach - von der CK2 phosphoryliert. Diese Ergebnisse verdeutlichen, dass die CK2-vermittelte Phosphorylierung der Centrine isoformspezifisch ist (Dissertation Publikation I Fig. 1d, Dissertation Publikation II Fig. 1a, d, Dissertation Publikation III Fig. 10b). Die *in vitro* Phosphorylierung von Cen1p durch die CK2 ist außergewöhnlich schnell und bereits nach wenigen Minuten nahezu komplett abgeschlossen (Dissertation Publikation II, Fig. 1a). Interessanterweise ist die CK2 in der Lage, sowohl ATP als auch GTP als Phosphatquelle für die Phosphorylierung von Cen1p umzusetzen (Dissertation Publikation II Fig. 1c).

Die Analysen der Primärstrukturen von Cen1p-4p ergaben, dass alle Isoformen eine Mehrzahl putativer Phosphorylierungsstellen für die CK2 besitzen (Dissertation Publikation I Fig. 2, III Fig. 2c). Die quantitative Analyse der inkorporierten Radioaktivität nach der Phosphorylierung der Centrine durch CK2 ergab, dass tatsächlich nur eines dieser möglichen Motive funktional ist (Dissertation Publikation I Fig. 1d). Zur Identifizierung der für die CK2-vermittelten Phosphorylierung relevanten Aminosäuresequenzen wurden Deletionskonstrukte der Centrin-Isoformen generiert und in den *in vitro* Phosphorylierungsversuchen eingesetzt (Dissertation Publikation I Fig. 2b). Da in Cen1p zwei CK2 Phosphorylierungsstellen sehr dicht beieinander liegen, war es zusätzlich notwendig, Punktmutationen in diesen beiden Aminosäureresten zu setzten. Durch die Kombination dieser Methoden gelang es, eine hoch konservierte Aminosäuresequenz (E^{132}LGESLTDEELQE^{144}) zwischen den EF-Hand Motiven III und IV der Centrin-Isoformen als spezifische Zielsequenz für die CK2-vermittelte Phosphorylierung zu identifizieren (Dissertation Publikation I Fig. S2, Dissertation Publikation III Fig. 2). Die Isoform Cen3p besitzt diese Zielsequenz nicht und ist darum kein Substrat für die CK2-vermittelte Phosphorylierung.

Eine wesentliche Voraussetzung für spezifische *in vivo* Enzym-Substrat Reaktionen in Geweben ist die räumliche Nähe der Reaktionspartner. Darum wurde die subzelluläre Lokalisation der Centrine und der Protein Kinase CK2 in der Retina von Vertebraten analysiert. Mit Hilfe hoch auflösender licht- und elektronenmikroskopischer Techniken

konnten die Centrine mit der CK2 im gleichen subzellulären Kompartiment der Photorezeptoren, dem Verbindungscilium, visualisiert werden (Dissertation Publikation I Fig. 4, 5). Mittels biochemischer Interaktionsstudien, wie Immunpräzipitationen oder GST-Pull down Assays, konnte allerdings keine stabile Komplexbildung zwischen den Centrinen und der CK2 nachgewiesen werden.

Da Mikrotubuli das charakteristische Cytoskelettelement des Verbindungsciliums sind, wurde analysiert, ob die Centrine und die CK2 direkt mit dem Mikrotubuli-Cytoskelett interagieren können. Dadurch könnten die Mikrotubuli des Verbindungsciliums die notwendige räumliche Nähe zwischen den Centrinen und der Protein Kinase CK2 herstellen. Mittels Zentrifugationsassays konnte gezeigt werden, dass sowohl die CK2 als auch Cen1p in der Lage sind, direkt mit Mikrotubuli zu interagieren (Dissertation Publikation I Fig. 6). Die Mikrotubuli des Verbindungsciliums stellen somit möglicherweise die Plattform dar, an der die räumliche Nähe zwischen Enzym und Substrat hergestellt wird.

Um den Einfluss der CK2-vermittelten Phosphorylierung der Centrine auf deren Bindungsaffinitäten zu Transducin zu messen, wurden biophysikalische Methoden angewandt. Durch kinetische Lichtstreuung zeigte sich, dass die CK2-vermittelte Phosphorylierung der Centrine die Bindungsaffinität zum visuellen G-Protein Transducin verringert. Dieser Effekt beruht auf einer reduzierten Ca^{2+}-Sensitivität der phosphorylierten Centrine (Dissertation Publikation I Fig. 7, 8).

Die beschriebenen lichtabhängigen Phosphorylierungen der Centrine stellen einen neuartigen Regulationsmechanismus bei der Bindung zum visuellen G-Protein Transducin dar. Da die Phosphorylierung von Proteinen meistens eine reversible Proteinmodifikation darstellt, stand die Identifikation und Charakterisierung von Protein Phosphatasen im Mittelpunkt der weiteren Analysen.

3.2. Die Protein Phosphatasen 2Cα und 2Cβ als Gegenspieler der CK2-vermittelten Phosphorylierung der Centrine

Weiterführende Analysen zur Dephosphorylierung der Centrine wurden mit unseren Kooperationspartnern in Münster im Labor von Frau Prof. Dr. Susanne Klumpp durchgeführt. Dadurch konnten die Protein Phosphatasen 2Cα und 2Cβ (PP2Cα und PP2Cβ) als Gegenspieler der CK2-vermittelten Phosphorylierung von Centrinen identifiziert werden (Dissertation Publikation II Fig. 1e und 3e). Dabei ist die Dephosphorylierung der Centrine durch die PP2Cβ in hohem Maße spezifisch. Andere Phosphatasen, die bereits in der Retina beschrieben wurden (z. B. PP1, PP2A, alkalische Phosphatase), sind nicht in der Lage, CK2-

phosphorylierte Centrine zu dephosphorylieren (Dissertation Publikation II Fig. 2a). Des Weiteren wurde die Dephosphorylierung mittels biochemischer Versuchsansätze charakterisiert. Dabei konnte gezeigt werden, dass die PP2C-vermittelte Dephosphorylierung der Centrine durch Mg^{2+} und Ölsäure aktiviert wird (Dissertation Publikation II Fig 3b und 3d). Im Gegensatz dazu wirken hohe Ca^{2+}-Konzentrationen inhibitorisch auf die Funktion der PP2Cβ (Dissertation Publikation II Fig 3c). Um zu überprüfen, ob die bisher beschriebenen Dephosphorylierungen der Centrine durch die PP2Cs relevant für die Funktion in der Retina von Vertebraten sind, wurde die zelluläre Lokalisation der PP2Cβ in diesem Gewebe analysiert. Mit Hilfe immunhistochemischer und immunelektronenmikroskopischer Arbeiten konnte die Colokalisation der PP2Cβ mit den Centrinen im Verbindungscilium und am Basalkörperkomplex der Photorezeptorzellen visualisiert werden (Dissertation Publikation II Fig. 4, 5).

Somit gelang es in der vorliegenden Arbeit erstmals, Protein Phosphatasen zu identifizieren, die in der Lage sind, Centrine zu dephosphorylieren. Die Protein Phosphatasen 2Cα und 2Cβ sind in höchstem Maße spezifische Gegenspieler der CK2-vermittelten Phosphorylierungen der Centrine. Die subzelluläre Colokalisation der PP2Cβ mit den Centrinen ermöglicht eine spezifische Enzym-Substrat Reaktion im Verbindungscilium der Photorezeptorzellen.

Zusammenfassend veranschaulichen die vorgestellten Ergebnisse, dass die Centrine im zellulären Kontext der Photorezeptoren nicht nur durch die Bindung von Ca^{2+} reguliert werden, sondern auch durch lichtabhängige, CK2-vermittelte Phosphorylierung. Die Phosphorylierung der Centrine verringert die Ca^{2+}-Affinität der Centrine und wirkt somit antagonistisch zur Ca^{2+}-Aktivierung. Die CK2-vermittelte Phosphorylierung der Centrine stellt einen reversiblen Vorgang dar, der durch die Protein Phosphatasen der 2C Familie aufgehoben werden kann. Die entgegen gesetzte Regulation der Centrine durch Ca^{2+}-Bindung und reversible Phosphorylierung hat Einfluss auf die Bindungseigenschaften der Centrine zum visuellen heterotrimeren G-Protein Transducin und dürfte an der Regulation der lichtabhängigen Translokation von Transducin zwischen den Kompartimenten der Photorezeptorzellen beteiligt sein.

4. Zusammenfassende Diskussion

Die vorliegende Dissertation beschreibt zellbiologische und molekulare Regulationsmechanismen von Centrinen in der Retina, speziell den Photorezeptorzellen, von Vertebraten. Zum Verständnis der Funktion von Proteinen ist es essentiell, die Mechanismen aufzuschlüsseln, die deren zelluläre Aufgaben regulieren und kontrollieren. In der Regel ist die zelluläre Funktion eines Proteins keine starre Einbahnstrasse, sondern muss flexibel sein, um sich auf wechselnde physiologische Zustände einzustellen. Aus diesem Grund ist es notwendig, dass ein Protein durch mehrere verschiedenartige Prozesse auf molekularer Ebene modifiziert wird, um mehrschichtige zelluläre Aufgaben zu meistern. Die Identifizierung neuartiger Regulationsmechanismen und deren Auswirkungen auf die Funktion der Centrine im zellulären Kontext der Photorezeptorzellen stellte die wichtigste Fragestellung der vorliegenden Arbeit dar.

Im Folgenden sollen die Ergebnisse der beiden Hauptpublikationen Trojan *et al.* 2008 (Dissertation Publikation I) und Thissen *et al.* eingereicht (Dissertation Publikation II), ebenso wie die Daten aus dem Übersichtsartikel Trojan *et al.* 2008a (Dissertation Publikation III) insgesamt diskutiert werden.

4.1. Reversible Phosphorylierung als Regulationsmechanismus der Centrine

Die zelluläre Funktion von Proteinen kann durch so genannte „second messenger“ Moleküle, wie Calcium (Ca^{2+}), Stickstoffmonoxid (NO) oder zyklisches Adenosinmonophosphat (cAMP) reguliert werden. Diese kleinen Moleküle binden passiv an spezielle Aminosäuremotive in ihren Zielproteinen. Beispielsweise erfolgt die Bindung von Ca^{2+}-Ionen an Proteine oftmals durch Komplexbildung an den EF-Hand Motiven. Neben der Bindung von „second messenger“ Molekülen sind kovalente Modifikationen von Proteinen die wichtigsten regulatorischen Elemente. Die häufigste kovalente Veränderung von Proteinen stellen Phosphorylierungen dar. Diese werden durch Protein Kinasen vermittelt und können mit Hilfe von Protein Phosphatasen rückgängig gemacht werden (Ubersax und Ferrell, Jr., 2007). Bei bisherigen Analysen zu den Funktionen von Centrinen in den Photorezeptorzellen der Vertebraten stand die Regulation über den „second messenger“ Ca^{2+} im Mittelpunkt (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). In der vorliegenden Arbeit konnte gezeigt werden, dass die Phosphorylierung durch die Protein Kinase CK2 (CK2) und die Dephosphorylierung durch PP2Cα/β einen weiteren essentiellen Regulationsmechanismus der

Centrine in den Photorezeptorzellen der Vertebraten darstellt (Dissertation Publikationen I und II).

Analysen der Primärstrukturen der Centrin-Isoformen wiesen zwei generell unterschiedliche Arten funktioneller Bereiche auf. Alle Centrine besitzen vier EF-Hand Motive, die der Bindung von Ca^{2+} dienen können (Dissertation Publikation I, Dissertation Publikation III) (Kretsinger, 1976; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Die Bindung von Ca^{2+} an die EF-Hand Motive stellt die Grundvoraussetzung für nahezu alle bislang bekannten Protein Interaktionen der Centrine dar (Dissertation Publikation III, 2.2.1.1) (Schiebel und Bornens, 1995; Wiech *et al.*, 1996; Durussel *et al.*, 2000; Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Hu *et al.*, 2004; Cox *et al.*, 2005; Gießl *et al.*, 2006*). So konnten wir zeigen, dass alle Centrin-Isoformen in den Photorezeptorzellen von Vertebraten Ca^{2+}-abhängig mit dem visuellen G-Protein Transducin interagieren (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Die bislang einzige bekannte Ca^{2+}-unabhängige Protein Interaktion gehen Centrine mit dem Gerüstprotein Sfi1p ein (Kilmartin, 2003). Das Gerüstprotein Sfi1p wurde erstmals in Hefen beschrieben und ist in der Lage, mehrere Centrin-Moleküle zu binden (Kilmartin, 2003). Mittlerweile wurde Sfi1p auch in höheren Organismen, wie beispielsweise dem Menschen, nachgewiesen (Salisbury, 2004). Durch erste Expressionsanalysen mittels RT-PCR konnten wir zeigen, dass Sfi1p auch in der Retina von Mäusen exprimiert wird (P. Trojan und U. Wolfrum, unveröffentlicht).

Neben den EF-Hand Motiven besitzen alle Centrin-Isoformen eine Vielzahl möglicher Phosphorylierungsstellen als weitere strukturelle Merkmale (Dissertation Publikation I, III). Dabei handelt es sich um Zielsequenzen für die Protein Kinasen A (PKA), C (PKC), CK2 und die Calmodulin-abhängige Kinase II (CaMKII) (Dissertation Publikation III, Fig. 2c).

In der vorliegenden Arbeit wurde erstmals ein lichtabhängiger Phosphorylierungs-Dephosphorylierungs-Zyklus für die Centrine in der Retina von Vertebraten beschrieben (Dissertation Publikationen I, II und III). Die Phosphorylierung der Centrine durch die CK2 ist Isoform-spezifisch (Dissertation Publikation I und II). Cen1p und Cen2p werden gleichermaßen gut von der CK2 phosphoryliert, wogegen Cen4p nur schwach phosphoryliert wird, und Cen3p kein Substrat für die CK2 darstellt (Dissertation Publikation I und II). Die Unterschiede in der Phosphorylierung der Centrin-Isoformen durch die CK2 verdeutlichen die Existenz zweier phylogenetischer Untergruppen der Centrine. Zum einen die Gruppe um Cen1p, Cen2p und Cen4p, zum underen Cen3p, das näher mit dem Centrin der Hefen Cdc31p verwandt ist als mit den underen drei Isoformen (Abb. 4) (Dissertation Publikation III, 2.1) (Gießl *et al.*, 2004b*; Salisbury, 2007).

Während sich frühere Arbeiten ausschließlich mit der Phosphorylierung von Centrinen beschäftigten (Lingle *et al.*, 1998; Lutz *et al.*, 2001; Meyn *et al.*, 2006), konnten hier erstmals auch Phosphatasen identifiziert werden, die für die Dephosphorylierung von Centrinen verantwortlich sind (Dissertation Publikation II und III). CK2-phosphorylierte Centrine können durch die Protein Phosphatasen 2Cα und 2Cβ (PP2Cα und PP2Cβ) dephosphoryliert werden (Dissertation Publikation II). Die Dephosphorylierung der Centrine durch PP2Cα und PP2Cβ ist ausgesprochen spezifisch. Ein vergleichbarer Regulationsmechanismus durch die CK2 und die PP2Cs wurde bereits für das Apotose-auslösende Protein Bcl-Xt/Bcl-2 associated death promotor (BAD) beschrieben (Klumpp *et al.*, 2004). Dabei konnten unsere Kooperationspartner in Münster zeigen, dass im zellulären Kontext die Phosphorylierung von BAD durch die CK2 essentiell für das Überleben neuronaler Zellen ist (Klumpp *et al.*, 2004). Allerdings ist diese Reaktion wesentlich unspezifischer, da die Phosphatasen PP1 und PP2A ebenfalls in der Lage sind, BAD zu dephosphorylieren (Klumpp *et al.*, 2004). Im Gegensatz dazu konnten die PP1 und PP2A und weitere analysierte Phosphatasen, die bereits in der Retina von Vertebraten beschrieben wurden (REIS, 1954; Zhao und Sancar, 1997; Selke *et al.*, 1998), Centrine nicht dephosphorylieren (Dissertation Publikation II). Dies verdeutlicht die hohe Spezifität der Dephosphorylierung der Centrin-Isoformen 1, 2 und 4. Da mit den Photorezeptorzellen der Vertebraten nun ein zweites neuronales System beschrieben wurde, in dem Proteine durch die CK2 phosphoryliert und durch PP2Cs dephosphoryliert werden, stellt dies möglicherweise einen grundlegenden Regulationsmechanismus in neuronalen Zellen dar.

4.2. Die Rolle des lichtabhängigen Phosphorylierungszyklus der Centrine in den Photorezeptorzellen der Vertebraten

Die Photorezeptorzellen der Retina von Vertebraten sind hoch spezialisierte und polarisierte Neurone (Wolfrum *et al.*, 2002; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Da ihre Hauptaufgabe in der Detektion von Licht besteht, verwundert es nicht, dass viele Prozesse durch die bestehenden Lichtverhältnisse reguliert werden. So translozieren Komponenten der Signaltransduktionskaskade, wie beispielsweise das visuelle G-Protein Transducin, lichtabhängig zwischen dem Innen- und Außensegment der Photorezeptorzellen (Abb.3) (Brann und Cohen, 1987; Mcginnis *et al.*, 2002; Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Während der Helladaption der Stäbchen verlagert sich innerhalb weniger Minuten ~80% des visuellen G-Proteins Transducin vom Außen- ins Innensegment. Während der Dunkeladaption befindet sich Transducin im Außensegment der Photorezeptoren (Dissertation Publikation III) (Sokolov *et al.*, 2002;

Reidel *et al.*, 2008). Solche intrazellulären Transportvorgänge müssen durch die einzige cytoplasmatische Verbindung zwischen den Innen- und Außensegmenten, dem Verbindungscilium, erfolgen (Dissertation Publikation III) (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Roepman und Wolfrum, 2007). Diese dünne Brücke zwischen den Kompartimenten der Photorezeptorzelle eignet sich auf Grund ihrer Morphologie als Kontrollpunkt für den intrazellulären Austausch von Proteinen (Roepman und Wolfrum, 2007). Bislang ist nur wenig über die genauen molekularen Mechanismen dieser intrazellulären Transportprozesse bekannt (Chen *et al.*, 2005; Calvert *et al.*, 2006; Strissel *et al.*, 2005; Reidel *et al.*, 2008). Bisherige Analysen unserer Arbeitgruppe zeigten, dass alle vier Centrin-Isoformen mit dem visuellen G-Protein Transducin direkt interagieren können und differentiell am Verbindungscilium lokalisiert sind (Dissertation Publikation III) (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*). Darum dürfte die Translokation von Transducin durch das Verbindungscilium durch die Bindung an Centrine reguliert werden (Dissertation Publikation III) (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*).

In der vorliegenden Arbeit konnte gezeigt werden, dass der lichtabhängige Phosphorylierungszyklus der Centrine in der Retina der Vertebraten die Bildung des Centrin/Transducin Komplexes regulieren könnte (Dissertation Publikation I, II und III). Während der Dunkeladaption der Photorezeptorzellen phosphoryliert die CK2 die ciliären Cen1p und Cen2p, sowie Cen4p, dessen Lokalisation auf den Basalköperkomplex beschränkt ist. Im Gegensatz dazu wird das ciliäre Cen3p nicht lichtabhängig phosphoryliert (Dissertation Publikation I, II und III). Die lichtabhängige Phosphorylierung der Centrine verringert die Bindungsaffinität für das visuelle G-Protein Transducin (Dissertation Publikation I und III). Bisherige Analysen der Centrin/Transducin Interaktion zeigten, dass diese Interaktion strikt Ca^{2+}-abhängig ist (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*). Für die Aktivierung der ciliären Centrine Cen1p und Cen2p ist die Bindung von zwei Ca^{2+}-Ionen an den EF-Hand Motiven III und IV notwendig, um mit Transducin interagieren zu können (Dissertation Publikation I und III) (Pulvermüller *et al.*, 2002). Wie in Dissertation Publikation I experimentell gezeigt, ist die Phosphorylierungsstelle in Cen1p und Cen2p direkt zwischen diesen beiden EF-Hand Motiven lokalisiert. Durch die CK2-vermittelte Phosphorylierung der Centrine kommt es möglicherweise zu Konformationsänderungen in den EF-Händen III und IV. Diese räumlichen Veränderungen führen zu verringerten Ca^{2+}-Affinitäten der Centrine und folglich zu einer geringeren Affinität zum visuellen G-Protein (Dissertation Publikation I). Eine entsprechende Regulation der Ca^{2+}-Sensitivität wurde für

den intrazellulären Ca^{2+}-Sensor Calmodulin beschrieben (Bildl *et al.*, 2004). In Nervenzellen wird Calmodulin von der CK2 phosphoryliert und durch die Protein Phosphatase 2A dephosphoryliert, wodurch Ca^{2+}-abhängige Kalium Kanäle in der Plasmamembran reguliert werden (Bildl *et al.*, 2004).

In der vorliegenden Arbeit konnte gezeigt werden, dass die Bildung des Centrine/Transducin Komplexes nicht nur durch die Ca^{2+}-Bindung reguliert wird, sondern ebenfalls durch reversible Phosphorylierungen. Während der lichtabhängigen Passage des visuellen G-Proteins durch das Verbindungscilium der Photorezeptorzellen könnte es zu direkten Interaktionen mit den ciliären Centrinen kommen. Diese Wechselwirkungen dürften an der Regulation der Translokation von Transducin durch das Verbindungscilium beteiligt sein (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Gießl *et al.*, 2004a*; Gießl *et al.*, 2004b*; Gießl *et al.*, 2006*). Die Daten der Dissertation Publikation I zeigen, dass die Centrine während der Dunkeladaption von der CK2 phosphoryliert werden. Dadurch verringert sich die Ca^{2+}-Affinität der Centrine, und die Interaktion mit Transducin wird verhindert.

Bisher ist nur sehr wenig über die molekularen Mechanismen der lichtabhängigen Translokation des visuellen G-Proteins Transducin bekannt (Calvert *et al.*, 2006; Reidel *et al.*, 2008). Aktuelle Arbeiten favorisieren die molekulare Diffusion als treibende Kraft bei der Translokation von Transducin während der Helladaption (Calvert *et al.*, 2006). Die Centrine im Verbindungscilium sind während der Helladaption dephosphoryliert und können Ca^{2+}-abhängig mit Transducin interagieren (Dissertation Publikation I und III). Die Bildung des Centrin/Transducin Komplexes reguliert möglicherweise die Diffusion von Transducin vom Außen- ins Innensegment während der Passage durch das Verbindungscilium. Alternativ könnten die Centrine als molekulare Barriere dienen, die den passiven Rückfluss von Transducin zurück ins Außensegment verhindert. Die Centrine des Verbindungsciliums wären dann für den Verbleib des visuellen G-Proteins im Innensegment während der Helladaption verantwortlich. Im Gegensatz zur Helladaption läuft die Translokation von Transducin während der Dunkeladaption sehr viel langsamer ab (Sokolov *et al.*, 2002; Elias *et al.*, 2004). Dies deutet auf unterschiedliche regulatorische Mechanismen für den intrazellulären Transport von Transducin hin. Die Centrine des Verbindungsciliums werden während der Dunkeladaption von der CK2 phosphoryliert. Diese Phosphorylierung verhindert die Interaktion mit dem visuellen G-Protein bei dessen Translokation vom Innen- ins Außensegment der Photorezeptorzellen. Neueste Studien zur Translokation von Transducin zeigen, dass Cytoskelettelemente während der Dunkeladaption für diesen Prozess notwendig

sind (Reidel *et al.*, 2008). So ist es denkbar, dass die Rückführung von Transducin ins Außensegment underen Regulationsmechanismen unterliegt, da Centrine durch die Phosphorylierung für die Interaktion mit dem visuellen G-Protein inaktiviert sind.

Weiterführende experimentelle Ansätze zur Rolle der Centrine bei der lichtabhängigen Translokation von Transducin könnten mit Hilfe der organotypischen Retinakultur durchgeführt werden. Diese konnte im Labor von Prof. Dr. Uwe Wolfrum etabliert werden. Die organotypische Retinakultur könnte mit GFP-markierten Centrin-Isoformen mit Punktmutationen in funktionellen Bereichen - wie der CK2 Phosphorylierungsstelle - transfiziert werden. Anschließende lichtmikroskopische Analysen der Translokation des visuellen G-Proteins bei der Hell- und Dunkeladaption der Retinakultur würde, die Rolle der Centrine bei diesem Prozess verdeutlichen. Alternativ ist es denkbar, die lichtabhängige Phosphorylierung der Centrine durch die Zugabe eines CK2-Inhibitors in der organotypischen Retinakultur zu unterdrücken. Des Weiteren könnte die Funktionsweise der Centrine in den Photorezeptorzellen mittels RNAi analysiert werden. Durch den Einsatz spezifischer doppelsträngiger RNA Moleküle könnte der posttranskriptionale Abbau der einzelnen Centrin-Isoformen in der Retinakultur induziert werden. Dadurch könnte die Rolle der einzelnen Centrin-Isoformen bei der adaptiven Translokation von Transducin in den Photorezeptorzellen studiert werden.

Für die Regulation des lichtabhängig variierenden Phosphorylierungsstatus der Centrine kommen zwei unterschiedliche Mechanismen in Frage. Zum einen könnte die Protein Kinase CK2 durch einen übergeordneten Signalweg in den Photorezeptorzellen aktiviert werden. Zum underen ist es denkbar, dass die CK2 immer aktiv ist, jedoch die Aktivität ihres Gegenspieler, der PP2Cα und PP2Cβ, während der Helladaption erhöht ist. Nahezu alle früheren Arbeiten stellten die Protein Kinase CK2 als weitestgehend unreguliert und permanent aktiv dar (Übersichtsartikel (Guerra *et al.*, 1999; Pinna, 2002)). Neuere Analysen zeigen jedoch, dass die CK2 in die Wnt/β-catenin Signaltransduktion involviert ist und von α-Untereinheiten der G-Protein-Familien G_q und G_o aktiviert werden kann (Gao und Wang, 2006). Die α-Untereinheit des visuellen G-Proteins Transducin der Photorezeptorzellen gehört zu der Familie der G_o-G-Proteine (Simon *et al.*, 1991). Somit ist es denkbar, dass die Lichtaktivierung von Transducin und die darauf folgende Dissoziation der Untereinheiten Veränderungen der Kinase Aktivität der CK2 nach sich zieht (Dissertation Publikation I). Sollte die CK2 allerdings konstitutiv aktiv sein, könnten Veränderungen des Phosphorylierungsstatus der Centrine alternativ durch die Regulation der Protein Phosphatasen erreicht werden.

Die Funktion der PP2Cs ist strikt Mg^{2+}-abhängig und kann durch ungesättigte Fettsäuren, wie Docosahexaensäure oder Ölsäure, stimuliert werden (Wang *et al.*, 1995; Klumpp *et al.*, 1998). Die intrazelluläre Mg^{2+}-Konzentration in den Photorezeptorzellen von Vertebraten ist nach heutigem Stand als weitestgehend konstant zu erachten und kann somit als Regulationsmechanismus der PP2Cs nahezu ausgeschlossen werden (Dissertation Publikation I und II) (Chen *et al.*, 2003). Die PP2Cs könnte jedoch auch durch Veränderungen der Lipid-Zusammensetzung der Plasmamembranen stimuliert werden und dadurch die Centrine dephosphorylieren. Wie in Dissertation Publikation I dargestellt, finden lichtabhängige Veränderungen der Zellmembranen zu langsam statt, um die Dephosphorylierung der Centrine zu regulieren (Penn und underson, 1987; underson und Penn, 2004). Allerdings existieren Hinweise, dass die Erhöhung der Ca^{2+}-Konzentration die Funktion der PP2Cs herabsetzt (Dissertation Publikation II) (Pato und Kerc, 1991; Wang *et al.*, 1995; Leube *et al.*, 1998). Daher ist es denkbar, dass hohe Ca^{2+}-Konzentrationen im Verbindungscilium zur Inhibition der Phosphatasen PP2Cα und PP2Cβ führen und folglich zur Erhöhung der Phosphorylierung der Centrine beitragen.

Die Protein Phosphatase PP2Cβ ist mit der Protein Kinase CK2 und den ciliären Centrinen Cen1p, Cen2p und Cen3p im Verbindungscilium colokalisiert. (Dissertation Publikation I, II und III) (Gießl *et al.*, 2004a*; Gießl *et al.*, 2006*). Da in der vorliegenden Arbeit keine stabile Komplexbildung der Centrine mit der Kinase oder der Phosphatase nachweisbar waren, stellen möglicherweise die Mikrotubuli des Verbindungsciliums die Plattform für den supramolekularen Funktionskomlex aus Enzymen und Substraten dar. Durch biochemische Versuchsansätze konnte hier erstmals die direkte Bindung der ciliären Centrine an das Mikrotubuli-Cytoskelett nachgewiesen werden (Dissertation Publikation I). Des Weiteren konnte die Protein Kinase CK2 als Mikrotubuli-bindendes Protein (MAP) bestätigt werden (Dissertation Publikation I) (Serrano *et al.*, 1987; Faust und Montenarh, 2000; Lim *et al.*, 2004; Canton und Litchfield, 2006). Eine direkte Interaktion mit dem Mikrotubuli-Cytoskelett wurde ebenfalls für Protein Phosphatasen der 2C Familie beschrieben (Grothe *et al.*, 1998). Somit dürfte die direkte Bindung dieser Proteine an die Mikrotubuli des Verbindungsciliums die räumliche Nähe zwischen den Reaktionspartnern schaffen, die notwendig ist, um den lichtabhängigen Phosphorylierungs-Dephosphorylierungs-Zyklus der Centrine zu gewährleisten.

4.3. Zusammenfassung der Funktion der Centrine im Verbindungscilium der Photorezeptorzellen von Vertebraten

Die Funktion der Centrine im Verbindungscilium der Photorezeptorzellen wird antagonistisch durch die Bindung von Ca^{2+} und durch lichtabhängige reversible Phosphorylierungen reguliert (Abb. 4) (Dissertation Publikation III, Fig. 11). Bislang stellt die Bindung des visuellen G-Proteins Transducin während der lichtabhängigen Translokation die bekannte Hauptaufgabe der Centrine in den Photorezeptorzellen dar. Während der Passage von Transducin durch das Verbindungscilium kommt es zur Interaktion mit den ciliären Centrinen (Abb. 6).

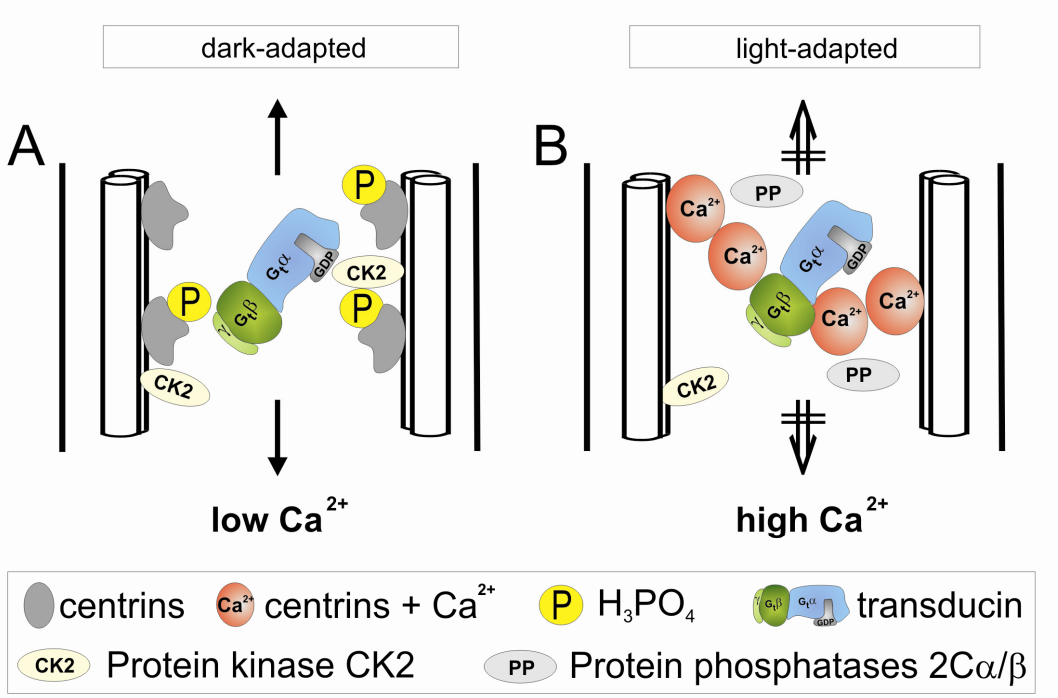

Abb. 6: Modell der Bildung des Centrin/Transducin Komplexes im Verbindungscilium der Photorezeptorzellen von Vertebraten. (A) Bei einer niedrigen intrazellulären Ca^{2+}-Konzentration werden die Centrine durch die Protein Kinase CK2 phosphoryliert. Das visuelle heterotrimere G-Protein Transducin kann durch das innere Lumen des Verbindungsciliums translozieren. Die präsentierten Daten deuten darauf hin, dass dies während der Dunkeladaption (dark-adapted) der Photorezeptorzellen der Fall ist. **(B)** Durch die Erhöhung der Ca^{2+}-Konzentration kommt es zur Dephosphorylierung der Centrine durch die Protein Phosphatase 2Cβ. Dies führt zur Bildung des Centrin/Transducin Komplexes, der die Passage von Transducin verhindert. Dieser physiologische Zustand dürfte während der Helladaption (light-adapted) vorliegen (verändert nach Dissertation Publikation III).

Unter niedrigen Ca^{2+}-Konzentration im Verbindungscilium werden die Centrine durch die CK2 phosphoryliert und somit die Interaktion mit Transducin verhindert. Das visuelle G-Protein kann durch das Verbindungscilium translozieren. Die hier präsentierten

Phosphorylierungsexperimente deuten darauf hin, dass dies während der Dunkeladaption der Photorezeptorzellen der Fall ist. Bei der Helladaption kommt es zur Dephosphorylierung der Centrine durch die PP2Cα und PP2Cβ, was zur Ca^{2+}-Bindung der Centrine führt. Diese Ca^{2+}-Bindung ermöglicht die direkte Interaktion der Centrine mit dem visuellen G-Protein und verhindert so die Translokation von Transducin durch das Verbindungscilium der Photorezeptorzellen (Abb. 6).

Mehrere Arbeiten zeigten bereits, dass das Verbindungscilium der Photorezeptorzellen morphologisch einer verlängerten Übergangszone („transition zone“) motiler beziehungsweise primärer Cilien entspricht (Rohlich, 1975; Besharse und Horst, 1990; Schmitt und Wolfrum, 2001; Roepman und Wolfrum, 2007). Der hier erstmals beschriebene Reaktionskomplex aus Mikrotubuli-assoziierten Centrinen, CK2 und der Protein Phosphatasen der 2C Familie könnte somit einen generellen Regulationsmechanismus an Übergangszonen von Cilien im Allgemeinen repräsentieren.

4.4. Funktion der Centrine an Übergangszonen primärer Cilien

Die Centrin-Isoformen Cen2p und Cen3p sind in allen somatischen Zelltypen während des gesamten Zellzyklus mit dem Centrosom oder davon abgeleiteten Strukturen assoziiert (Dissertation Publikation III). Alle Zellen bilden während der G0 Phase des Zellzyklus primäre Cilien, die sensorische Organellen darstellen und deren Übergangszone homolog zum Verbindungscilium der Photorezeptorzellen ist (Roepman und Wolfrum, 2007). Die Centrine konnten bereits mittels immuncytochemischer Analysen an der Übergangszone primärer Cilien in Kulturzellen nachgewiesen werden (Abb. 7). Neben der prominenten Lokalisation der Centrine an der Übergangszone dieser Zellorganellen, konnten aktuell auch funktionelle Aspekte der CK2-vermittelten Phosphorylierung an primären Cilien aufgedeckt werden.

Aktuelle Arbeiten zur Funktion der CK2-vermittelten Phosphorylierung in Epithelzellen der Lunge zeigten, dass diese Phosphorylierung für den gerichteten Transport von Proteinen an die Übergangszone des Ciliums notwendig ist (Schermer *et al.*, 2005). Es ist denkbar, dass die CK2-vermittelte Phosphorylierung der Centrine ebenfalls dem Transport an die Übergangszone primärer Cilien oder an das Verbindungscilium der Photorezeptoren dient. Daher wäre es lohnenswert, in weiterführenden Experimenten zu überprüfen, ob die CK2-vermittelte Phosphorylierung ebenfalls der Zielsteuerung der Centrine dient. Dazu könnten Kulturzellen mit GFP-Centrinen transfiziert werden, die durch Punktmutationen nicht mehr durch die CK2 phosphorylierbar sind. Der anschließende Entzug von Serum würde in diesen Zellen die Bildung eines primären Ciliums induzieren. Mittels lichtmikroskopischer Analysen

der GFP-Markierung könnte die subzelluläre Lokalisation der Centrine analysiert werden, um festzustellen, ob die Zielsteuerung an die Übergangszone der Cilien beeinträchtigt ist.

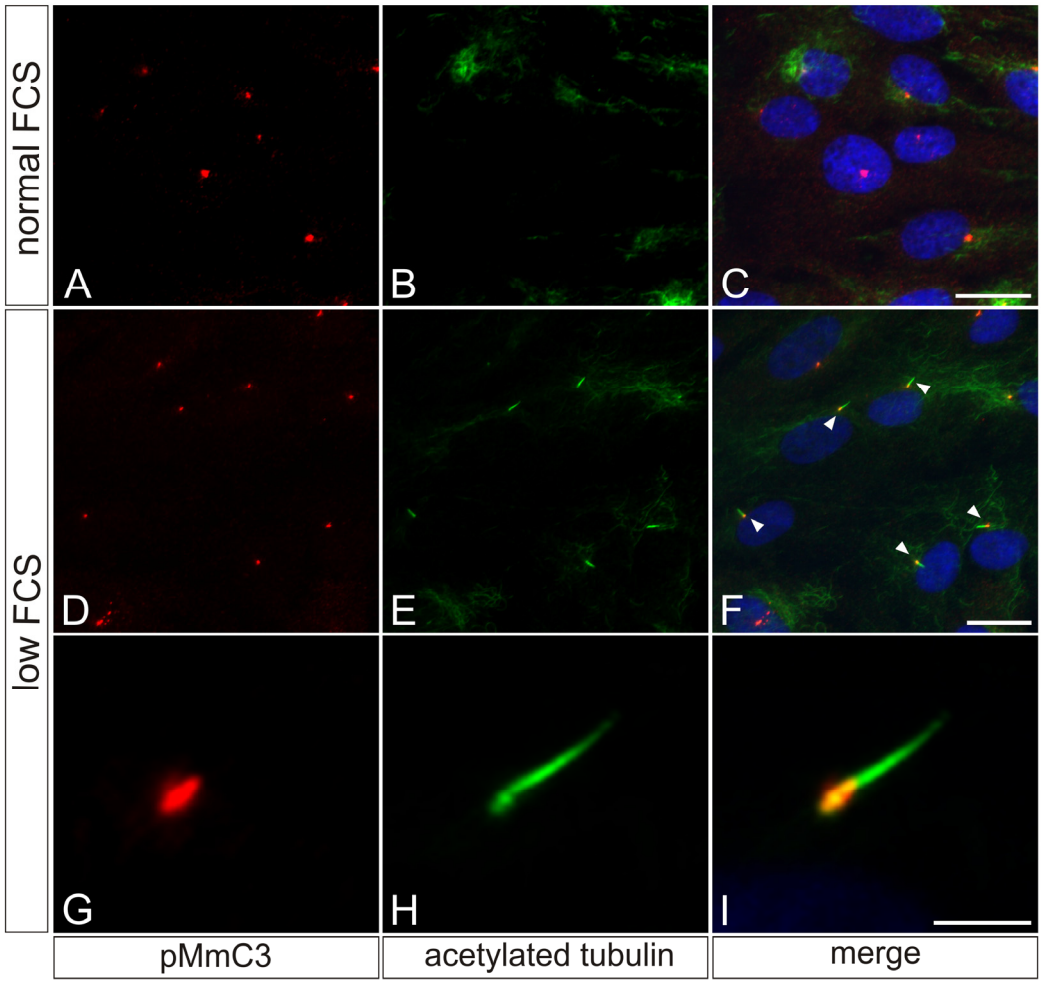

Abb. 7: Lokalisation von Centrinen und acetyliertem Tubulin während der Ciliogenese in Kulturzellen. **(A-I)** Indirekte Immunfluoreszenzanalyse der subzelluären Lokalisation von Centrinen und acetyliertem Tubulin in hTERT-RPE1 Zellen. Die DNA der Zellkerne ist zusätzlich mittels DAPI markiert. **(A-C)** Doppelmarkierung gegen Centrin **(A)** und acetyliertes Tubulin **(B)** in hTERT-RPE1 Zellen während der Interphase des Zellzyklus unter idealen Kulturbedingungen (normal FCS). Centrine sind an den Centrosomen der Zellen lokalisiert, wohingegen acetyliertes Tubulin nur als leichte Markierung in der Nähe der Centrosomen zu erkennen ist. **(D-I)** Zellen wurden mittels geringer Serumzugabe (low FCS) in G0 arretiert und dadurch die Bildung primärer Cilien induziert. Centrine **(D)** sind als punktförmige Markierungen zu erkennen. Antikörper gegen acetyliertes Tubulin **(E)** markieren die primären Cilien. **(F)** Übereinanderlagerung von **(D)** und **(E)** zeigt deutlich, dass die Centrine mit acetyliertem Tubulin partiell colokalisiert sind (Pfeilspitzen). **(G-I)** Hochauflösende Aufnahmen eines einzelnen primären Ciliums zeigen, dass Centrine **(G)** eine punktförmige Lokalisation aufweisen. Acetyliertes Tubulin **(H)** hingegen dekoriert das gesamte Cilium. **(I)** Die Übereinanderlagerung von **(G)** und **(H)** zeigt, dass Centrine nur am Basalkörper und der Übergangszone des Cilium lokalisiert sind. Größenbalken: A-D: 13 µm; H-I: 0.5 µm.

Eine weitere mögliche Rolle könnten die Centrine als Ca^{2+}-Sensoren an der Übergangszone von Cilien spielen. Primäre Cilien besitzen eine Reihe unterschiedlicher Ca^{2+}-Kanäle in ihrer Plasmamembran. So führt die Aktivierung des Ca^{2+}-Kanals Polycystin-2 zu einer Erhöhung der intrazellulären Ca^{2+}-Konzentration (Satir und Christensen, 2007). Die Funktion von

Polycystin-2 ist an die Wnt-Signaltransduktionkaskade gekoppelt (Habas und Dawid, 2005). Wie bereits diskutiert, können G-Proteine in diesen Signalweg involviert sein (Gao und Wang, 2006). Diese G-Proteine können direkt mit Centrinen an der Übergangszone von Cilien interagieren oder regulieren möglicherweise die Aktivität der Protein Kinase CK2 (Pulvermüller *et al.*, 2002; Gießl *et al.*, 2004a*; Gao und Wang, 2006). Die Phosphorylierung durch die CK2 verringert die Ca^{2+}-Affinität der Centrine und führt zur Freisetzung gebundener regulatorischer G-Proteine von der Übergangszone der Cilien.

Bereits mehrfach konnte gezeigt werden, dass Centrine für den korrekten Verlauf des Zellzyklus notwendig sind (Schiebel und Bornens, 1995; Salisbury *et al.*, 2002). Bei Defekten der Centrine kommt es in Kulturzellen zu Defekten bei der Duplikation und Separation der Centrosomen (Salisbury *et al.*, 2002). Primäre Cilien sind ebenfalls in die korrekte Abfolge der Zellteilung involviert (Satir und Christensen, 2007). Durch die Bildung des primären Ciliums unterbrechen die Zellen ihre normale Zellteilung und arretieren in der G0 Phase des Zellzyklus (Wheatley, 1971). Erst nach der Degradation des Ciliums beginnen die Zellen wieder mit ihrem normalen Zellzyklus (Satir und Christensen, 2007). Der Abbau des Ciliums wird durch Veränderungen der intrazellulären Ca^{2+}-Konzentration ausgelöst (Satir und Christensen, 2007). Die Centrin-Isoformen Cen2p und Cen3p sind sowohl an der Übergangszone primärer Cilien als auch an den Centrosomen jeder somatischen Zelle lokalisiert (Dissertation Publikation III) (Wolfrum und Salisbury, 1998). Möglicherweise stellt die Funktion der Centrine als intrazelluläre Ca^{2+}-Sensoren die Verbindung zwischen der Arretierung des Zellzyklus und seiner normalen Fortsetzung her.

Der im Rahmen dieser Arbeit beschriebene Mikrotubuli-assoziierte Reaktionskomplex aus Centrinen, Protein Kinase CK2 und Protein Phosphatasen der 2C Familie stellt einen neuartigen Regulationsmechanismus für signalinduzierte Translokationsprozesse dar. Möglicherweise spielen diese Reaktionskomplexe nicht nur bei der lichtabhängigen Translokation des visuellen G-Proteins in den Photorezeptorzellen von Vertebraten eine wichtige Rolle, sondern repräsentieren einen generellen Regulationsmechanismus an allen centrosomalen Strukturen in jeder somatischen Zelle.

5. Zusammenfassung

Centrine sind kleine Ca^{2+}-bindende Proteine aus der Familie der EF-Hand Proteine. Erstmals wurden Centrine als Hauptbestandteil der kontraktilen Flagellenwurzeln von Grünalgen beschrieben. Mittlerweile sind Centrine in nahezu allen eukaryotischen Organismen nachgewiesen worden und gelten als eukaryotische Markerproteine. In Säugetieren wurden bis zu vier Gene nachgewiesen, die an Centrosomen oder davon abgeleiteten Strukturen, wie Spindelpolkörpern und Basalkörper, aber auch in Übergangszonen von Cilien exprimiert werden.

Bislang ist nur wenig über die zelluläre Funktion der Centrine bekannt. In eukaryotischen Kulturzellen sind Centrine zur korrekten Duplikation und Separation der Centrosomen während des Zellzyklus essentiell. In den Photorezeptorzellen der Vertebraten werden alle vier Centrin-Isoformen exprimiert und sind differenziell am Verbindungscilium und dessen Basalkörperkomplex lokalisiert.

In der vorliegenden Arbeit konnte gezeigt werden, dass die Centrine im zellulären Kontext der Photorezeptorzellen nicht nur durch die Bindung von Ca^{2+} reguliert werden, sondern auch durch reversible Phosphorylierungen. Die Phosphorylierung der Centrin-Isoformen findet in der Retina von Vertebraten lichtabhängig während der Dunkeladaption statt. Die Protein Kinase CK2 (CK2) ist für die beschriebenen lichtabhängigen Phosphorylierungen hauptverantwortlich. Obwohl alle Centrin-Isoformen mehrere mögliche Zielsequenzen für die CK2 besitzen, kommt es nur zur Phosphorylierung einer einzigen Aminosäure in Cen1p, Cen2p und Cen4p. Im Gegensatz dazu stellt die Isoform Cen3p kein Substrat für die CK2 dar.

Zudem wurden hier erstmals Phosphatasen identifiziert, die in der Lage sind Centrine zu dephosphorylieren. Die Dephosphorylierung durch die PP2Cα und PP2Cβ ist sehr spezifisch, da keine andere Phosphatase der Retina die CK2-vermittelte Phosphorylierung der Centrine rückgängig machen kann. Hoch auflösende licht- und elektronenmikroskopische Analysen zeigten erstmals, dass die Centrine sowohl mit der CK2 als auch mit der PP2Cβ im Verbindungscilium der Photorezeptorzellen colokalisiert sind. Cen1p und CK2 sind in der Lage, direkt an Mikrotubuli zu binden, was die notwendige räumliche Nähe zwischen Enzymen und Substrat herstellt.

Bisherige Arbeiten zeigten, dass alle Centrine Ca^{2+}-abhängig mit dem visuellen G-Protein Transducin interagieren. Diese Wechselwirkung dürfte an der Regulation der lichtabhängigen Translokation des visuellen G-Proteins Transducin zwischen dem Außen-

und dem Innensegment der Photorezeptorzelle beteiligt sein. In der vorliegenden Arbeit zeigten Interaktionsstudien, dass die Bindungsaffinitäten der Centrine für Transducin durch die CK2-vermittelte Phosphorylierung drastisch verringert wurden. Dieser beobachtete Effekt beruht auf deutlich verringerten Ca^{2+}-Affinitäten der Centrin-Isoformen nach der CK2-vermittelten Phosphorylierung.

In der vorliegenden Arbeit wurde ein neuartiger Regulationsmechanismus der Centrine in den Photorezeptorzellen der Vertebraten beschrieben. Centrine werden nicht nur durch Ca^{2+}-Bindung zur Bildung von Protein Komplexen stimuliert, sondern durch die Phosphorylierung zum Auflösen dieser Komplexe angeregt. Damit reguliert die CK2-vermittelte, lichtabhängige Phosphorylierung der Centrine möglicherweise ebenfalls die adaptive Translokation des visuellen G-Proteins Transducin zwischen dem Außen- und Innensegment der Photorezeptorzellen.

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7. Anhang

7.1. Zuordnung der geleisteten Beiträge zu den einzelnen Publikationen

An dieser Stelle möchte ich ausführen, welche Beiträge ich zu den einzelnen Publikationen, in denen ich als Koautor auftrete, geleistet habe. Daher möchte ich zunächst auf die drei Hauptpublikationen Trojan *et al*., 2008 (I), Thissen *et al*., eingereicht (II) und Trojan *et al*., 2008a (III) eingehen. Anschließend werde ich meine Beiträge zu weiteren Publikationen der Arbeitsgruppe von Prof. Dr. Uwe Wolfrum darstellen. Diese Veröffentlichungen sind im Text mit einem Stern (*) hervorgehoben.

In Trojan *et al*., 2008 sind Sebastian Rausch und ich gleichberechtigte Erstautoren. Mein Beitrag zu dieser Arbeit bestand in der Entwicklung, Etablierung und Durchführung der beschrieben lichtabhängigen Phosphorylierungsexperimenten (Fig. 1 a, b und c). Die Analysen der Primärstrukturen der Centrin-Isoformen wurden ebenfalls von mir geleistet (Fig. 2). Die quantitative *in vitro* Phosphorylierung in Fig. 1c wurde von unseren Kooperationspartnern in Berlin mittels biophysikalischer Methoden durchgeführt. Des Weiteren wurden von mir die Deletionskonstrukte der Centrin-Isoformen 1-4 amplifiziert und kloniert. Diese Deletionskonstrukte wurden von mir für *in vitro* Phosphorylierungen verwendet. Die Generierung der Punktmutationen in Cen1p wurde von Sebastian Rausch durchgeführt und von mir für *in vitro* Phosphorylierungen eingesetzt. Dies führte zur Identifikation der spezifischen Zielsequenzen für die CK2-vermittelte Phosphorylierung der Centrine (Fig. 3). Weiterführende massenspektrometrische Analysen der Phosphorylierungsstellen wurden von Clementine Klemm durchgeführt (Fig. S1). Durch den Einsatz spezifischer Antikörper gegen Centrine und die Protein Kinase CK2 konnte von mir erstmals die Colokalisation beider Proteine am Verbindungscilium mit Hilfe hoch auflösender Lichtmikroskopie visualisiert werden (Fig. 4). Die Dokumentation der subzellulären Lokalisation der Centrine und der CK2 mittels immunelektronenmikroskopischer Analysen wurde mit technischer Unterstützung von Gabriele Stern-Schneider erreicht (Fig. 5). Zusätzlich führte ich die Mikrotubuli-Bindestudien mittels Zentrifugationsassays durch, welche die direkte Bindung der Centrine und der CK2 an Mikrotubuli beweisen (Fig. 6). Die biophysikalischen Analysen der Bindungsaffinitäten der Centrine in phosphoryliertem Zustand wurden von unseren Kollegen in Berlin mit rekombinanten Proteinen durchgeführt (Fig. 7). Die zur bakteriellen Expression notwendigen Expressionsklone wurden von Dr. Andreas Gießl und mir generiert. Ebenso wurden die Ca^{2+}-Affinitäten der Centrine in Berlin von Sebastian Rausch mit diesen rekombinanten Proteinen gemessen (Fig. 8).

In der zweiten Hauptpublikation, Thissen *et al*. eingereicht (II), wurden von Dr. Marie-Christin Thissen und mir während eines Laboraufenthalts in Münster in der Arbeitsgruppe von Prof. Dr. Susanne Klumpp die Grundlagen für diese Publikation gelegt. Während meines Forschungsaufenthalts wurde zunächst die Phosphorylierung von Cen1p durch CK2 *in vitro* validiert (Fig. 1a und b). Dr. Marie-Christin Thissen führte anschließend die Phosphorylierung von Cen1p mit GTP (Fig. 1c) und die *in vitro* Phosphorylierungen der Cen2p-4p durch (Fig. 1d). Die Identifizierung der verantwortlichen Phosphatase PP2Cβ bei der Dephosphorylierung von Cen1p wurde von uns gemeinsam geleistet (Fig. 2). Während meines Aufenthalts in Münster wurde außerdem größtenteils die Charakteristik der Dephosphorylierung von Cen1p analysiert (Fig. 3a, b und d). Die Dephosphorylierung in Abhängigkeit der Ca^{2+}-Konzentration und die Identifikation der PP2Cα erfolgte durch Dr. Marie-Christin Thissen (Fig. 3c und e). Die subzelluläre Lokalisation der Centrine und der PP2Cβ in der Retina von Mäusen wurde von mir mittels indirekter Immunhistochemie analysiert (Fig. 4). Um die räumliche Verteilung der PP2Cβ am Verbindungscilium genauer zu bestimmen führte ich mit technischer Unterstützung von Elisabeth Sehn zudem immunelektronenmikroskopische Analysen der Retina von Mäusen durch (Fig. 5).

Die dritte Hauptpublikation Trojan *et al.* 2008a (III) stellt einen Übersichtsartikel dar, bei dem ich als Erstautor auftrete. Der konzeptionelle Entwurf dieser Arbeit wurde in erster Linie von mir und Prof. Dr. Uwe Wolfrum durchgeführt. Mit Ausnahme des Kapitels 2.3 wurden alle anderen Teile zunächst von mir vorbereitet und anschließend mit Prof. Dr. Uwe Wolfrum überarbeitet. Das Kapitel 2.3 wurde von den Koautoren Dr. Alexander Pulvermüller, Prof. Dr. Hui-Woog Choe und Prof. Dr. Norbert Krauss geschrieben und von uns überarbeitet. Des Weiteren steuerte ich verschiedene Abbildungen und Tabellen bei (Fig. 1, Fig 2c, Fig. 3, Fig. 4, Fig. 10, Fig. 11, Table 1). Weitere Abbildungen wurden in erster Fassung von Dr. Andreas Gießl zusammengestellt und anschließend von mir überarbeitet (Fig. 2a, b, Fig. 5, Fig. 6, Fig. 7, Fig. 9). Die Abbildung Fig. 8 wurde von Dr. Alexander Pulvermüller zusammengestellt.

Neben den drei Hauptpublikationen Trojan *et al*., 2008 (I), Thissen *et al*., eingereicht (II) und Trojan *et al.*, 2008a (III), konnte ich weitere wichtige Beiträge zu Veröffentlichungen unserer Arbeitsgruppe beisteuern. So war es mir möglich, in der Publikation Gießl *et. al*, 2004*, mit der Durchführung von RT-PCR Analysen erstmals die Expression aller bekannten Centrine in der Retina der Maus nachzuweisen (Fig. 4). Zudem wurden die hoch auflösenden lichtmikroskopischen Analysen mittels spezifischer Antikörper gegen die Centrin-Isoformen von Dr. Andreas Gießl und mir durchgeführt (Fig. 7). Mit der technischen Unterstützung von

Gabriele Stern-Schneider führte ich die immunelektronenmikroskopische Analyse der Lokalisation von Cen3p am Verbindungscilium durch (Fig. 8a). Weitere RT-PCR Analysen fanden Eingang in das Buchkapitel Gießl *et al*. 2004a* und zeigten erstmals die Expression der damals neuen Centrin-Isoform 4 in der Retina der Ratte (Fig. 2). Zusätzlich fanden erste Ergebnisse zur lichtabhängigen Phosphorylierung Eingang in diesen Übersichtsartikel (Fig. 6). Durch den Einsatz spezifischer Antikörper konnten Dr. Andreas Gießl und ich die subzelluläre Lokalisation von Nucleophosmin und dem Retinitis Pigmentosa GTPase Regulator (RPGR) in der Retina von Mäusen dokumentieren und leisteten dadurch einen wesentlichen Beitrag zur Publikation Shu *et al.* 2005* (Fig. 6). Einen weiteren Übersichtsartikel veröffentlichten wir mit unseren Berliner Kooperationspartnern (Gießl *et al*., 2006*). Dabei war ich hauptsächlich mit Dr. Andreas Gießl an der Auswahl und Zusammenstellung der Abbildungen beteiligt (Fig. 1-4).

7.2. Abkürzungen

Im Folgenden sind, im Text häufig verwendete, Abkürzungen nochmals in alphabetischer Reihenfolge zusammengefasst. Zusätzlich sind allgemein gebräuchliche Abkürzungen chemischer und physikalischer Einheiten und Chemikalien aufgelistet, welche im Text nicht näher definiert wurden. Aminosäuren sind im Text stets im Einbuchstabencode angegeben.

5´GMP	Guanosinmonophosphat
ATP	Adenosintriphosphat
BAD	Bcl-Xt/Bcl-2 associated death promotor
BB	Basalkörper (engl.: basal body)
Ca^{2+}	Calcium
CC	Verbindungscilium (engl.: connecting cilium)
CDC31	Centrin-Gen der Bäckerhefe *Saccharomyces cerevisae*
Cdc31p	Centrin-homologes Protein der Bäckerhefe *Saccharomyces cerevisae*
DNA	Desoxyribonukleinsäure (engl.: deoxyribonucleic acid)
cDNA	komplementäre DNA (engl.: complementary DNA)
Cen1p	Centrin-Isoform 1
Cen2p	Centrin-Isoform 2
Cen3p	Centrin-Isoform 3
Cen4p	Centrin-Isoform 4
cGMP	zyklisches Guanosinmonophosphat
CK2	Protein Kinase CK2 (früher Casein Kinase)
DAPI	4,6-Diamidino-2-Phenylindol; blau fluoreszierende Zellkernfärbung
G1/S	Übergang von G1-Phase des Zellzyklus zur S-Phase
G2 Phase	G2 Phase des Zellzyklus
GCL	Ganglienzellschicht (engl.: ganglion cell layer)
GDP	Guanosindiphosphat
G_t	visuelles G-Protein Transducin
$G_t\alpha$	alpha-Untereinheit von Transducin
$G_t\beta\gamma$	nicht-dissoziierbare beta-gamma Untereinheiten von Transducin
GTP	Guanosintriphosphat
$h\nu$	Photon

HsCETN1-3	Centrin-Gene 1-3 des Menschen
INL	innere Körnerschicht (engl.: inner nuclear layer)
IPL	innere plexiforme Schicht (engl.: inner plexiform layer)
IS	Innensegment der Photorezeptorzellen (engl.: inner segment)
MAP	Mikrotubuli-bindendes Protein (engl.: microtubule associated protein)
MmCentn1-4	Centrin-Gene 1-4 der Maus
N	Zellkern (engl.: nucleus)
Na^+	Natrium
ONL	äußere Körnerschicht (engl.: outer nuclear layer)
OPL	äußere plexiforme Schicht (engl.: outer plexiform layer)
OS	Außensegment der Photorezeptorzellen (engl.: outer segment)
PCM	pericentrioläre Matrix
PDE	Phosphodiesterase
RHO	Rhodopsin
RHO*	lichtaktiviertes Rhodopsin
RnCentn1-4	Centrin-Gene 1-3 der Ratte
RPE	retinales Pigmentepithel
RPGR	Retinitis Pigmentosa G-Protein gekoppelter Rezeptor
S	Synapse
VFL2	Centrin-Gen der Grünalge *Chlamydomonas reinhardtii*

7.3. Curriculum vitae

Persönliche Angaben:

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich meine Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Ich habe keinen anderen Promotionsversuch unternommen.

Mainz, den 2008

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Philipp Trojan