Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta xx (2008) xxx–xxx
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamcr

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

Philipp Trojan [a,1], Sebastian Rausch [b,1], Andreas Gießl [a], Clementine Klemm [c], Eberhard Krause [c], Alexander Pulvermüller [b,*,2], Uwe Wolfrum [a,*,2]

[a] *Zell-und Matrixbiologie, Institut für Zoologie, Johannes Gutenberg-Universität Mainz, Müllerweg 6, D-55099 Mainz, Germany*
[b] *Institut für Medizinische Physik und Biophysik, Charité – Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany*
[c] *Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany*

Received 14 November 2007; received in revised form 21 December 2007; accepted 3 January 2008

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

* Corresponding authors. U. Wolfrum is to be contacted at Tel.: +49 6131 39 25148; fax: +49 6131 39 23815. A. Pulvermüller, Tel.: +49 30 450 524176; fax: +49 30 450 524952.
E-mail addresses: alexander.pulvermueller@charite.de (A. Pulvermüller), wolfrum@mail.uni-mainz.de (U. Wolfrum).
[1] These authors contributed equally to this work.
[2] Both authors share senior authorship.

0167-4889/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2008.01.006

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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

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

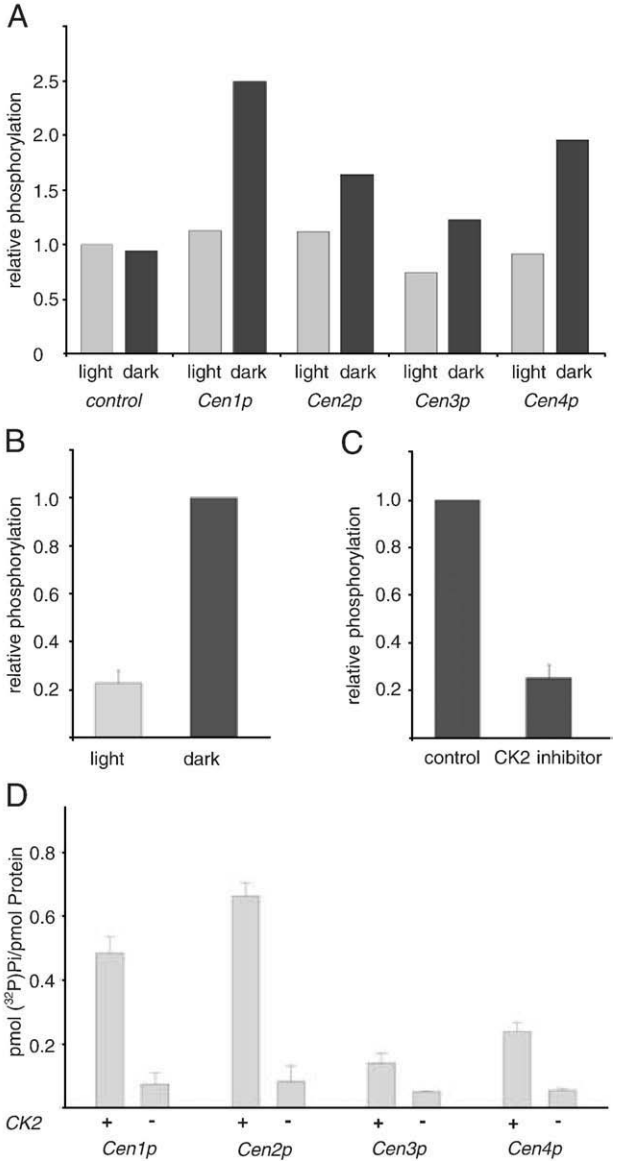

Fig. 1. Light-dependent phosphorylation of murine centrin isoforms. (A), *In vitro* phosphorylation of recombinant murine Cen1p–4p. Light adapted (grey bars) or dark adapted (black bars) bovine retina lysates were incubated with Cen1p–4p respectively in the presence of [γ-^{32}P]ATP. After immunoprecipitation 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.

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

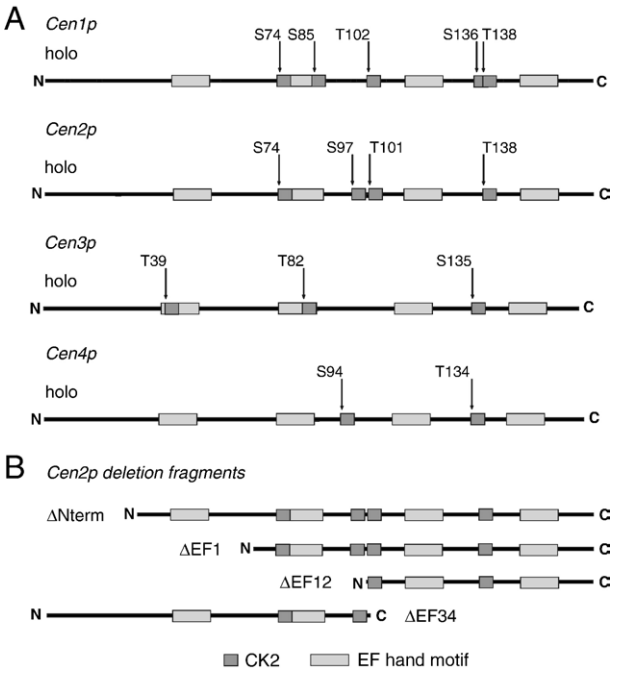

Fig. 2. Identifications of putative target amino acids for CK2 in murine Cen1p–4p. (A), Schematic representation of murine Cen1p–4p proteins. Amino acid sequences have been screened for typical CK2 target sequences (S/T-X-X-D/E). Murine Cen1p contains five such motifs at serines 74 (S^{74}), 85 (S^{85}), 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.

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

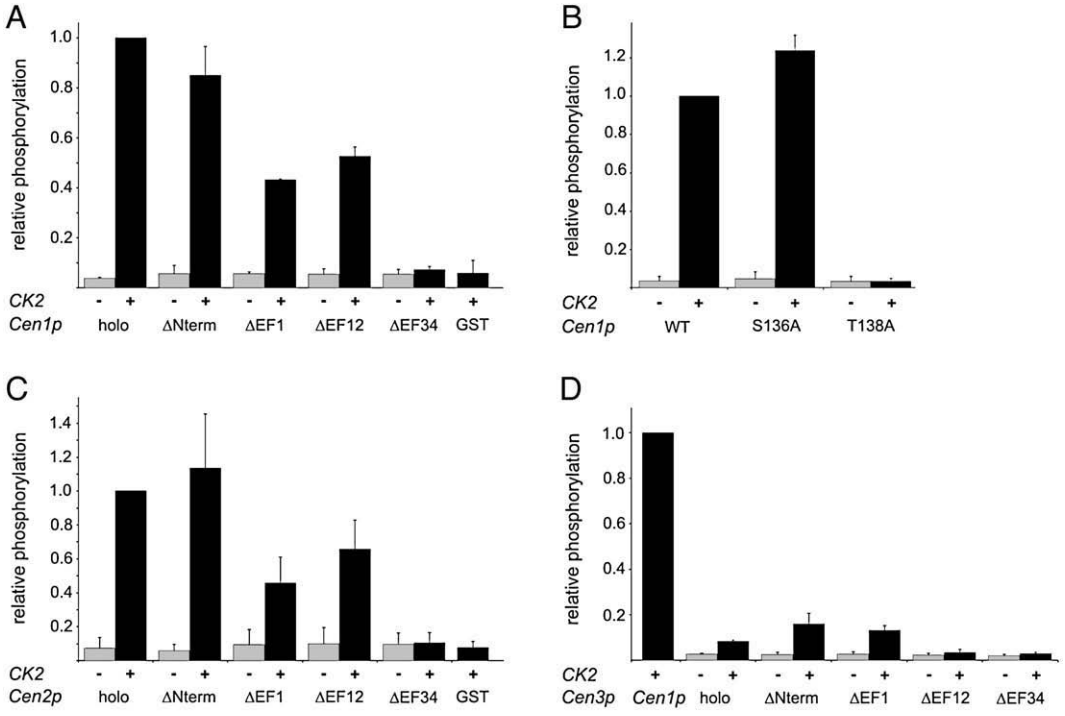

Fig. 3. Identification of functional phosphorylation sites for CK2-mediated phosphorylation in the murine ciliary Cen1p–3p. Recombinant murine Cen1p–3p holo proteins and deletion fragments were incubated with [γ^{32}P]ATP in the presence (dark bars) or absence (light bars) of CK2. Incorporated radioactivity 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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

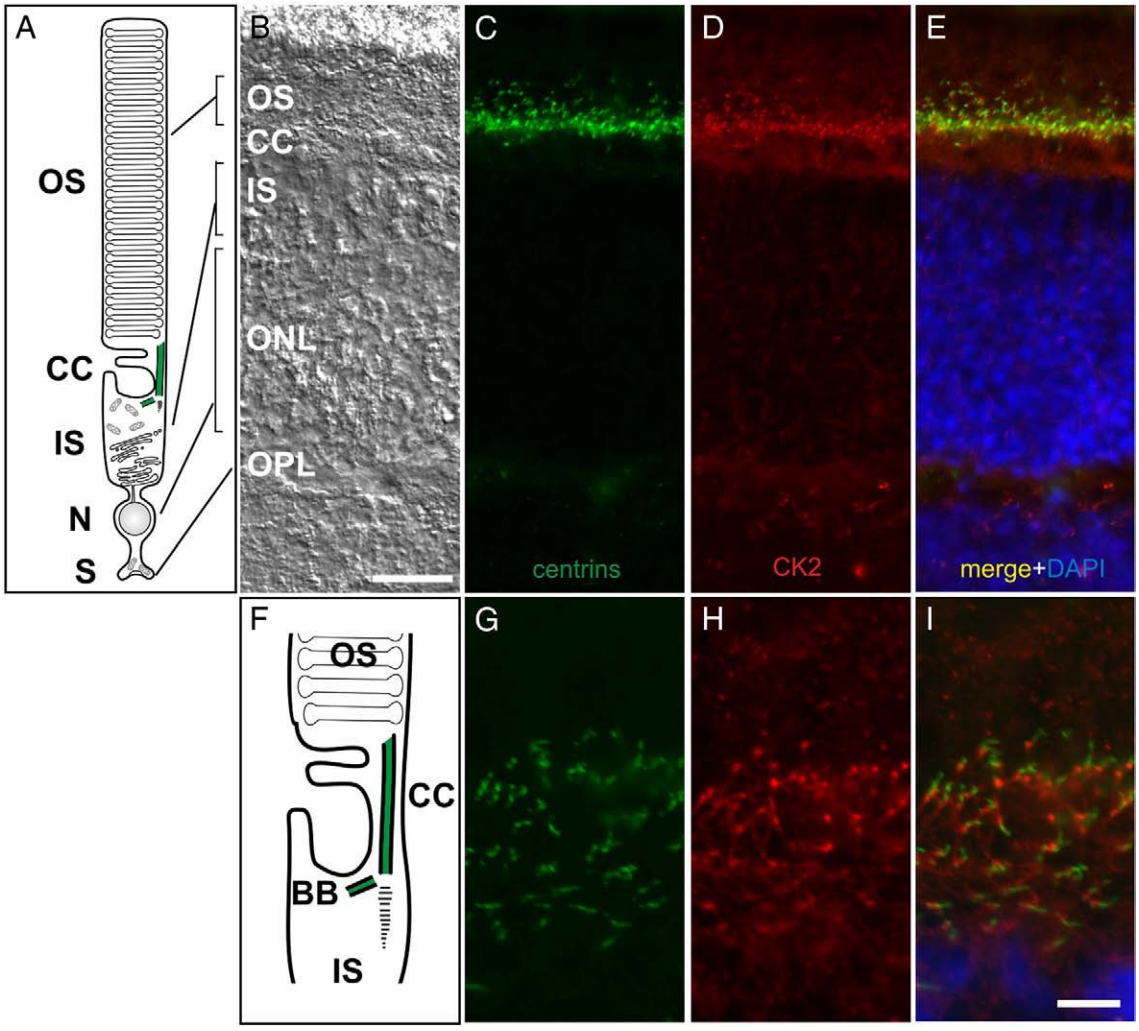

Fig. 4. Subcellular localizations of centrins and CK2 in murine retinas. (A), Schematic representation of a vertebrate photoreceptor cell. The photoreceptors are composed of the light sensitive outer segment (OS) which is linked via the connecting cilium (CC, green) to the inner segment (IS). The nuclei (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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

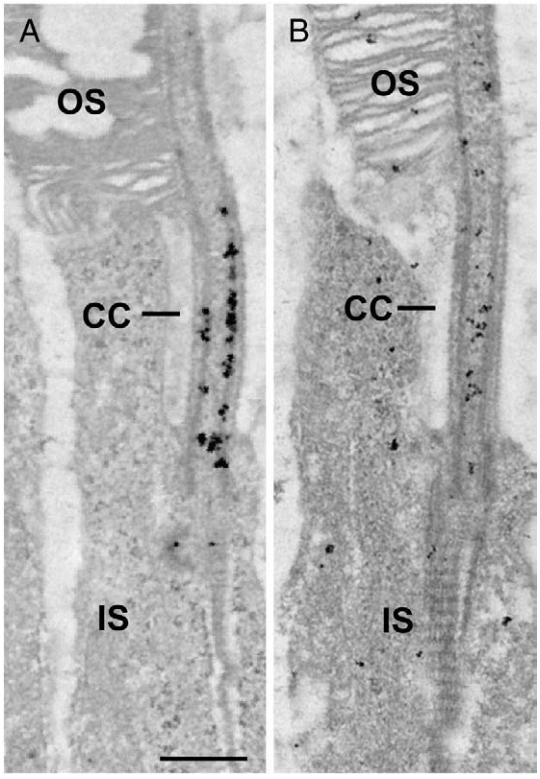

Fig. 5. Immunoelectron microscopic localization of centrins and protein kinase CK2 in a mouse photoreceptor cell. (A and B), longitudinal ultrathin sections through part of mouse rod photoreceptor cells. (A), Silver-enhanced immunogold labeling by pan-centrin antibody. Centrins are localized at the inner 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).

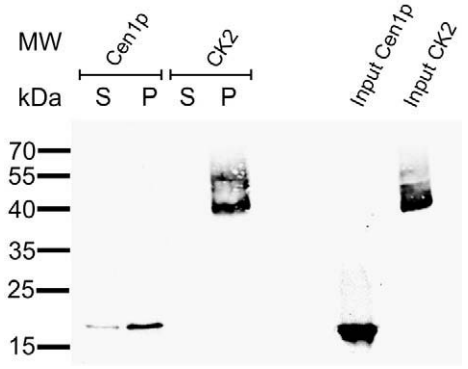

Fig. 6. Direct bindings of Cen1p and CK2 to microtubules. *In vitro* spin down assays were performed to demonstrate binding of centrins and CK2 to microtubules and analyzed by specific antibodies to Cen1p and CK2 in Western blots. This analysis of the supernatant (S) and the pellet (P) reveal direct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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

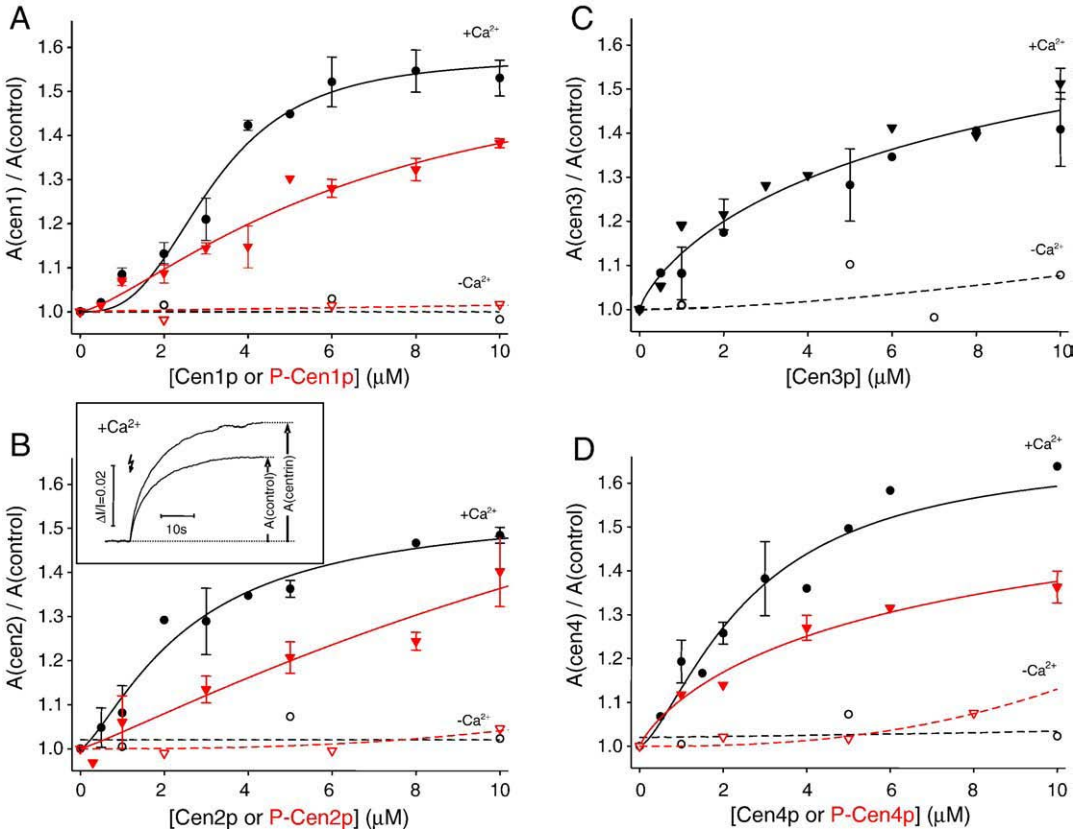

Fig. 7. Effect of centrin isoform phosphorylation on the G_t-binding signal analyzed by kinetic light-scattering. Shown is the dependence of the amplitude of flash-induced kinetic light-scattering G_t-binding signals on the concentration of Cen1p (A), Cen2p (B), Cen3p (C) and Cen4p (D), respectively. 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.

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

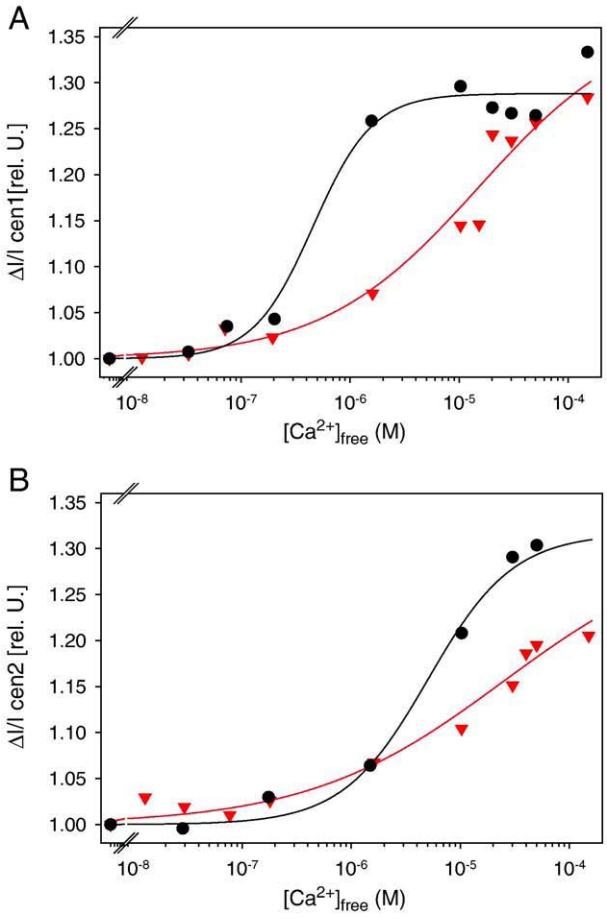

Fig. 8. Calcium titration of the centrin enhanced KLS G_t-binding signal. Amplitudes of KLS G_t-binding signals for (A) p-Cen1p and Cen1p and (B) p-Cen2p and Cen2p are shown as a function of free Ca^{2+} concentration in solution (grey triangles with dotted lines represent phosphorylated and black circles 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.

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

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.

Acknowledgments

The authors are most grateful to E. Sehn, G. Stern-Schneider, D. Wünschig (University of Mainz, Germany) and I. Semjonow and J. Engelmann (Charité Berlin, Germany) for their skillful assistance. We also thank Drs. S. Klumpp and H.-W. Choe for their helpful comments on the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG) to A.P. (Pu186/3), U.W. (Wo548/6), and the FAUN-Stiftung, Nürnberg, Germany to U.W.

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.

References

[1] D.W. Litchfield, Protein kinase CK2: structure, regulation and role in cellular decisions of life and death, Biochem. J. 369 (2003) 1–15.
[2] H. Kuhn, J.H. Cook, W.J. Dreyer, Phosphorylation of rhodopsin in bovine photoreceptor membranes. A dark reaction after illumination, Biochemistry 12 (1973) 2495–2502.
[3] O. Hisatomi, S. Matsuda, T. Satoh, S. Kotaka, Y. Imanishi, F. Tokunaga, A novel subtype of G-protein-coupled receptor kinase, GRK7, in teleost cone photoreceptors, FEBS Lett. 424 (1998) 159–164.
[4] D.J. Kelleher, G.L. Johnson, Purification of protein kinase C from bovine rod outer segments, J. Cyclic Nucleotide Protein Phosphor. Res. 10 (1985) 579–591.
[5] U. Walter, Cyclic-GMP-regulated enzymes and their possible physiological functions, J. Cyclic Nucleotide Protein Phosphor. Res. 17 (1984) 249–258.
[6] B.A. Hollander, M.Y. Liang, J.C. Besharse, Linkage of a nucleolin-related protein and casein kinase II with the detergent-stable photoreceptor cytoskeleton, Cell Motil. Cytoskelet. 43 (2) (1999) 114–127.
[7] Q. Liu, G. Tan, N. Levenkova, T. Li, E.N. Pugh Jr., J. Rux, D.W. Speicher, E.A. Pierce, The proteome of the mouse photoreceptor sensory cilium complex, Mol. Cell Proteomics 6 (2007) 1299–1317.
[8] E.J. Helmreich, K.P. Hofmann, Structure and function of proteins in G-protein-coupled signal transfer, Biochim. Biophys. Acta 1286 (1996) 285–322.
[9] V.Y. Arshavsky, T.D. Lamb, E.N. Pugh Jr., G proteins and phototransduction, Annu. Rev. Physiol. 64 (2002) 153–187.
[10] M.E. Burns, V.Y. Arshavsky, Beyond counting photons: trials and trends in vertebrate visual transduction, Neuron 48 (2005) 387–401.
[11] K. Palczewski, A.S. Polans, W. Baehr, J.B. Ames, Ca(2+)-binding proteins in the retina: structure, function, and the etiology of human visual diseases, BioEssays 22 (2000) 337–350.
[12] P.D. Calvert, K.J. Strissel, W.E. Schiesser, E.N. Pugh Jr., V.Y. Arshavsky, Light-driven translocation of signaling proteins in vertebrate photoreceptors, Trends Cell Biol. 16 (2006) 560–568.
[13] M. Sokolov, A.L. Lyubarsky, K.J. Strissel, A.B. Savchenko, V.I. Govardovskii, E.N. Pugh Jr., V.Y. Arshavsky, Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation, Neuron 33 (2002) 95–106.
[14] A. Pulvermuller, A. Giessl, M. Heck, R. Wottrich, A. Schmitt, O.P. Ernst, H.W. Choe, K.P. Hofmann, U. Wolfrum, Calcium-dependent assembly of centrin–G-protein complex in photoreceptor cells, Mol. Cell. Biol. 22 (2002) 2194–2203.
[15] R. Roepman, U. Wolfrum, Protein networks and complexes in photoreceptor cilia, in: E. Bertrand, M. Faupel (Eds.), Subcellular Proteomics From Cell Deconstruction to System Reconstruction, vol. 43, 2007, pp. 209–235.
[16] A. Giessl, A. Pulvermuller, P. Trojan, J.H. Park, H.W. Choe, O.P. Ernst, K. P. Hofmann, U. Wolfrum, Differential expression and interaction with the visual G-protein transducin of centrin isoforms in mammalian photoreceptor cells, J. Biol. Chem. 279 (2004) 51472–51481.
[17] A. Giessl, P. Trojan, A. Pulvermüller, U. Wolfrum, Centrins, potential regulators of transducin translocation in photoreceptor cells, in: D.S. Williams (Ed.), Cell Biology and Related Disease of the Outer Retina. World Scientific Publishing Company Pte. Ltd., Singapore, 2004, pp. 122–195.
[18] A. Giessl, P. Trojan, S. Rausch, A. Pulvermuller, U. Wolfrum, Centrins, gatekeepers for the light-dependent translocation of transducin through the photoreceptor cell connecting cilium, Vision Res. 46 (2006) 4502–4509.
[19] U. Wolfrum, A. Gieβl, A. Pulvermüller, (2002). Centrins, a novel group of Ca2+-binding proteins in vertebrate photoreceptor cells. Adv. Exp. Med. Biol. 514:155–78., 155–178.
[20] J.L. Salisbury, A. Baron, B. Surek, M. Melkonian, Striated flagellar roots: isolation and characterization of a calcium-modulated contractile organelle, J. Cell Biol. 99 (1984) 962–970.
[21] V.E. Martindale, J.L. Salisbury, Phosphorylation of algal centrin is rapidly responsive to changes in the external milieu, J. Cell Sci. 96 (1990) 395–402.
[22] W. Lutz, W.L. Lingle, D. McCormick, T.M. Greenwood, J.L. Salisbury, Phosphorylation of centrin during the cell cycle and its role in centriole separation preceding centrosome duplication, J. Biol. Chem. 276 (23) (2001) 20774–20780.
[23] J.L. Salisbury, Centrin, centrosomes, and mitotic spindle poles, Curr. Opin. Cell Biol. 7 (1995) 39–45.
[24] E. Schiebel, M. Bornens, In search of a function for centrins, Trends Cell Biol. 5 (1995) 197–201.
[25] S.M. Meyn, C. Seda, M. Campbell, K.L. Weiss, H. Hu, B. Pastrana-Rios, W.J. Chazin, The biochemical effect of Ser167 phosphorylation on *Chlamydomonas reinhardtii* centrin, Biochem. Biophys. Res. Commun. 342 (2006) 342–348.
[26] G. Wald, Molecular basis of visual excitation, Science 162 (1968) 230–239.
[27] M. Heck, K.P. Hofmann, Maximal rate and nucleotide dependence of rhodopsin-catalyzed transducin activation: initial rate analysis based on a double displacement mechanism, J. Biol. Chem. 276 (2001) 10000–10009.
[28] M. Heck, A. Pulvermuller, K.P. Hofmann, Light scattering methods to monitor interactions between rhodopsin-containing membranes and soluble proteins, Methods Enzymol. 315 (2000) 329–347.
[29] A. Pulvermuller, K. Palczewski, K.P. Hofmann, Interaction between photoactivated rhodopsin and its kinase: stability and kinetics of complex formation, Biochemistry 32 (1993) 14082–14088.

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006

[30] M.W. Pinkse, P.M. Uitto, M.J. Hilhorst, B. Ooms, A.J. Heck, Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns, Anal. Chem. 76 (2004) 3935–3943.
[31] U. Wolfrum, A. Schmitt, Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells, Cell Motil. Cytoskelet. 46 (2000) 95–107.
[32] G. Danscher, J. Obel, O. Thorlacius-Ussing, Electron microscopic demonstration of metals in rat mast cells. A cytochemical study based on an improved sulphide silver method, Histochemistry 66 (1980) 293–300.
[33] L.A. Pinna, Casein kinase 2: an 'eminence grise' in cellular regulation? Biochim. Biophys. Acta 1054 (1990) 267–284.
[34] J.A. Ubersax, J.E. Ferrell Jr., Mechanisms of specificity in protein phosphorylation, Nat. Rev., Mol. Cell Biol. 8 (2007) 530–541.
[35] P. Monzo, N.C. Gauthier, F. Keslair, A. Loubat, C.M. Field, Y. Marchand-Brustel, M. Cormont, Clues to CD2-associated protein involvement in cytokinesis, Mol. Biol. Cell 16 (2005) 2891–2902.
[36] A.C. Lim, S.Y. Tiu, Q. Li, R.Z. Qi, Direct regulation of microtubule dynamics by protein kinase CK2, J. Biol. Chem. 279 (2004) 4433–4439.
[37] D.A. Canton, D.W. Litchfield, The shape of things to come: an emerging role for protein kinase CK2 in the regulation of cell morphology and the cytoskeleton, Cell Signal. 18 (2006) 267–275.
[38] H. Kuhn, N. Bennett, M. Michel-Villaz, M. Chabre, Interactions between photoexcited rhodopsin and GTP-binding protein: kinetic and stoichiometric analyses from light-scattering changes, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 6873–6877.
[39] A. Schleicher, K.P. Hofmann, Kinetic study on the equilibrium between membrane-bound and free photoreceptor G-protein, J. Membr. Biol. 95 (1987) 271–281.
[40] J.E. Allende, C.C. Allende, Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation, FASEB J. 9 (1995) 313–323.
[41] S. Nakajo, Y. Masuda, K. Nakaya, Y. Nakamura, Determination of the phosphorylation sites of calmodulin catalyzed by casein kinase 2, J. Biochem. (Tokyo) 104 (1988) 946–951.
[42] J.L. Salisbury, A mechanistic view on the evolutionary origin for centrin-based control of centriole duplication, J. Cell. Physiol. 213 (2007) 420–428.
[43] S. Klumpp, D. Selke, J. Hermesmeier, Protein phosphatase type 2C active at physiological Mg2+: stimulation by unsaturated fatty acids, FEBS Lett. 437 (1998) 229–232.
[44] D. Selke, H. Anton, S. Klumpp, Serine/threonine protein phosphatases type 1, 2A and 2C in vertebrate retinae, Acta Anat.(Basel) 162 (1998) 151–156.
[45] C. Chen, K. Nakatani, Y. Koutalos, Free magnesium concentration in salamander photoreceptor outer segments, J. Physiol. 553 (2003) 125–135.
[46] J.S. Penn, R.E. Anderson, Effect of light history on rod outer-segment membrane composition in the rat, Exp. Eye Res. 44 (1987) 767–778.
[47] R.E. Anderson, J.S. Penn, Environmental light and heredity are associated with adaptive changes in retinal DHA levels that affect retinal function, Lipids 39 (2004) 1121–1124.
[48] Y. Gao, H.Y. Wang, Casein kinase 2 is activated and essential for wnt/beta-catenin signaling, J. Biol. Chem. 281 (2006) 18394–18400.
[49] M.I. Simon, M.P. Strathmann, N. Gautam, Diversity of G proteins in signal transduction, Science 252 (1991) 802–808.
[50] A.I. den Hollander, R.K. Koenekoop, M.D. Mohamed, H.H. Arts, K. Boldt, K.V. Towns, T. Sedmak, M. Beer, K. Nagel-Wolfrum, M. McKibbin, S. Dharmaraj, I. Lopez, L. Ivings, G.A. Williams, K. Springell, C.G. Woods, H. Jafri, Y. Rashid, T.M. Strom, Z.B. van der, I. Gosens, F.F. Kersten, E. van Wijk, J.A. Veltman, M.N. Zonneveld, S.E. van Beersum, I.H. Maumenee, U. Wolfrum, M.E. Cheetham, M. Ueffing, F.P. Cremers, C.F. Inglehearn, R. Roepman, Mutations in LCA5, encoding the ciliary protein lebercilin, cause Leber congenital amaurosis, Nat. Genet. 39 (2007) 889–895.
[51] J.C. Besharse, C.J. Horst, The photoreceptor connecting cilium — a model for the transition tone, in: R.A. Bloodgood (Ed.), Ciliary and Flagellar Membranes, Plenum, New York, 1990.
[52] B. Schermer, K. Hopker, H. Omran, C. Ghenoiu, M. Fliegauf, A. Fekete, J. Horvath, M. Kottgen, M. Hackl, S. Zschiedrich, T.B. Huber, A. Kramer-Zucker, H. Zentgraf, A. Blaukat, G. Walz, T. Benzing, Phosphorylation by casein kinase 2 induces PACS-1 binding of nephrocystin and targeting to cilia, EMBO J. 24 (2005) 4415–4424.
[53] M. Fliegauf, J. Horvath, C. von Schnakenburg, H. Olbrich, D. Muller, J. Thumfart, B. Schermer, G.J. Pazour, H.P. Neumann, H. Zentgraf, T. Benzing, H. Omran, Nephrocystin specifically localizes to the transition zone of renal and respiratory cilia and photoreceptor connecting cilia, J. Am. Soc. Nephrol. 17 (2006) 2424–2433.
[54] J. Hu, Y.K. Bae, K.M. Knobel, M.M. Barr, Casein kinase II and calcineurin modulate TRPP function and ciliary localization, Mol. Biol. Cell 17 (2006) 2200–2211.
[55] J.H. Park, N. Krauss, A. Pulvermuller, P. Scheerer, W. Hohne, A. Giessl, U. Wolfrum, K.P. Hofmann, O.P. Ernst, H.W. Choe, Crystallization and preliminary X-ray studies of mouse centrin1, Acta Crystallogr., Sect. F. Struct. Biol. Cryst. Commun. 61 (2005) 510–513.
[56] J.H. Park, A. Pulvermuller, P. Scheerer, S. Rausch, A. Giessl, W. Hohne, U. Wolfrum, K.P. Hofmann, O.P. Ernst, H.W. Choe, N. Krauss, Insights into functional aspects of centrins from the structure of N-terminally extended mouse centrin 1, Vision Res. 46 (2006) 4568–4574.
[57] W. Bildl, T. Strassmaier, H. Thurm, J. Andersen, S. Eble, D. Oliver, M. Knipper, M. Mann, U. Schulte, J.P. Adelman, B. Fakler, Protein kinase CK2 is coassembled with small conductance Ca(2+)-activated K+ channels and regulates channel gating, Neuron 43 (2004) 847–858.

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

Please cite this article as: P. Trojan, et al., Light-dependent CK2-mediated phosphorylation of centrins regulates complex formation with visual G-protein, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamcr.2008.01.006