

## Research Letter

# Dephosphorylation of Centri- nins by Protein Phosphatase 2C $\alpha$ and $\beta$

Marie-Christin Thissen,<sup>1</sup> Josef Krieglstein,<sup>1</sup> Uwe Wolfrum,<sup>2</sup> and Susanne Klumpp<sup>1</sup>

<sup>1</sup>Institut für Pharmazeutische und Medizinische Chemie, Westfälische Wilhelms-Universität Münster,  
Hittorfstr. 58-62, D-48149 Münster, Germany

<sup>2</sup>Institut für Zoologie, Johannes Gutenberg-Universität Mainz, Johannes-von-Müllerweg 6,  
D-55099 Mainz, Germany

Correspondence should be addressed to Susanne Klumpp, klumpp@uni-muenster.de

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In the present study, we identified protein phosphatases dephosphorylating centri-  
nins previously phosphorylated by protein kinase CK2. The following phosphatases known to be present in the retina were tested: PP1, PP2A, PP2B, PP2C, PP5, and alkaline phosphatase. PP2C  $\alpha$  and  $\beta$  were capable of dephosphorylating P-Thr<sup>138</sup>-centrin1 most efficiently. PP2C $\delta$  was inactive and the other retinal phosphatases also had much less or no effect. Similar results were observed for centri-  
nins 2 and 4. Centrin3 was not a substrate for CK2. The results suggest PP2C  $\alpha$  and  $\beta$  to play a significant role in regulating the phosphorylation status of centri-  
nins *in vivo*.

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## 1. Introduction

In the highly specialized vertebrate photoreceptor cells, centri-  
nins are components of the ciliary apparatus localized in the connecting cilium and their basal bodies [1–3]. In fully differentiated photoreceptor cells, CK2 phosphorylates centri-  
nins 1 and 2 during dark adaptation. Since the phosphorylation of the ciliary centri-  
nins drastically reduces the binding to the G-protein 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 centri-  
nins in mammalian photoreceptor cells.

## 2. Materials and Methods

**2.1. Phosphorylation of Centri-  
nins and BAD.** GST-centri-  
nins (0.2  $\mu$ g) or GST-BAD (0.6  $\mu$ g) were incubated in 30 mM Tris-  
HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -glycerophosphate, 0.2  $\mu$ g

CK2, 0.06% 2-mercaptoethanol, 1 mM EGTA, and 100  $\mu$ M ATP including 1  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP in a volume of 10  $\mu$ L for 15 minutes at 37°C. Then unincorporated ATP was removed by centri-  
SEP spin columns.

**2.2. Dephosphorylation of P-Centri-  
nins and P-BAD.** Phosphorylated proteins were incubated with 0.16  $\mu$ g PP1 or 0.05  $\mu$ g PP2A or 1.3  $\mu$ g PP2B or 0.08–0.8  $\mu$ g PP2C $\alpha$  or 0.08–1.5  $\mu$ g PP2C $\beta$  or 0.08–0.8  $\mu$ g PP2C $\delta$  or 0.8  $\mu$ g PP5 or 1.5  $\mu$ g alkaline phosphatase in a total volume of 15  $\mu$ L, respectively. Incubations contained a 10  $\mu$ L aliquot of the completed phosphorylation reaction plus 5  $\mu$ L 50 mM Tris-HCl, pH 7.5, 1% glycerol, 0.1% 2-mercaptoethanol, and an additional 5 mM MnCl<sub>2</sub> for PP1, PP2A, and PP2C $\delta$ ; or 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, and 2  $\mu$ g calmodulin for PP2B; or 1 mM MgCl<sub>2</sub> for PP2C  $\alpha$  and  $\beta$ ; or 100  $\mu$ M oleic acid for PP5. Alkaline phosphatase assays contained 50 mM Tris-HCl, pH 7.9 and 1 mM MgCl<sub>2</sub>. Reactions were stopped after 30 minutes at 37°C by adding 5  $\mu$ L sample buffer (130 mM Tris-HCl, pH 6.8, 10% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.06% bromphenol blue).

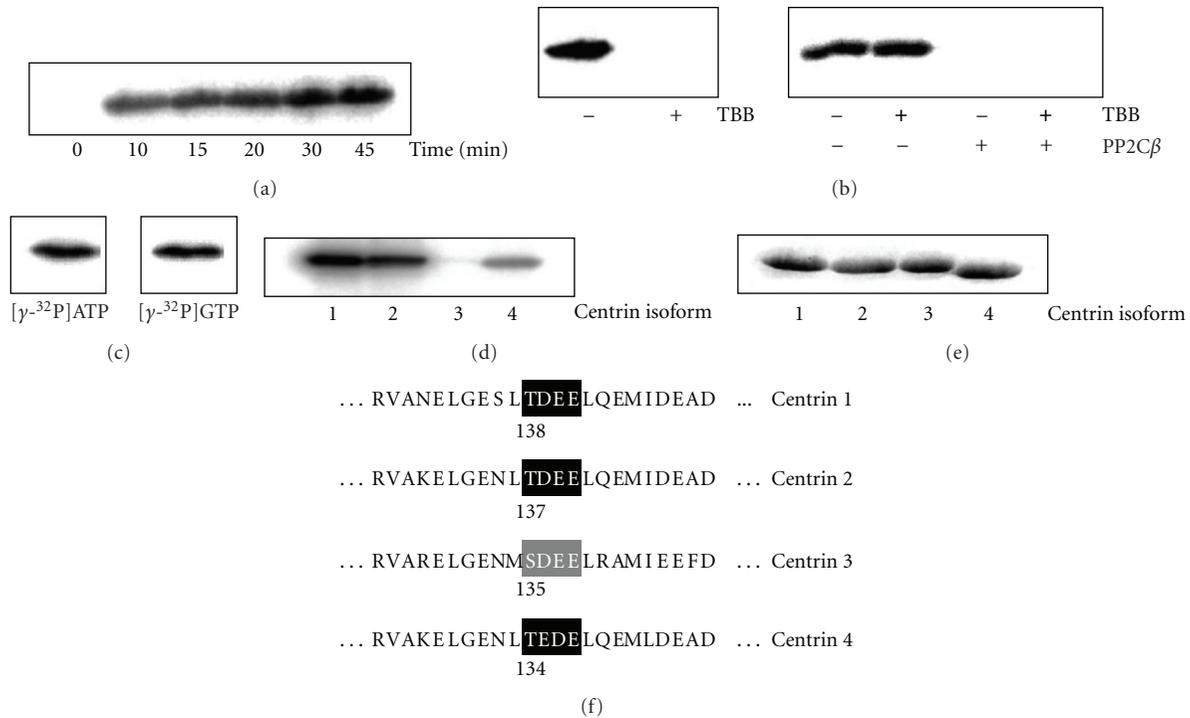


FIGURE 1: Characterization of phosphorylation of centrin1 by CK2. Centrin1 (0.2 μg, resp.) were phosphorylated by CK2 (0.2 μg) using [γ-<sup>32</sup>P]ATP as phosphate source as described in Section 2. (a)–(d) Autoradiograms. (a)–(c) Centrin1 as a substrate for CK2. (a) Time dependence. (b) Effect of the CK2-inhibitor TBB (4,5,6,7-tetrabromobenzimidazole). The inhibitor was present either in the phosphorylation reaction (left) or added after phosphorylation prior to and present upon dephosphorylation by PP2Cβ (right). (c) Phosphorylation with GTP (1 μCi [γ-<sup>32</sup>P]GTP and 100 μM GTP) in comparison to that with ATP. (d) Phosphorylation of centrin isoforms (0.2 μg, resp.) by CK2. (e) Coomassie protein stain of centrin isoforms (0.2 μg, resp.). (f) Sequences of the CK2 phosphorylation site on the centrin1–4.

### 3. Results

**3.1. Phosphorylation of Centrin1 by CK2.** Purified recombinant centrin1 could be phosphorylated in vitro by CK2 using ATP as phosphate source within a few minutes only (Figure 1(a)). Phosphorylation of centrin1 by CK2 was not detectable in the presence of 100 μM of the CK2-inhibitor TBB (Figure 1(b)), left). Guanine nucleotides are playing a uniquely important role in the retina and for vision [3]. Indeed, phosphorylation of centrin1 by CK2 worked equally well using GTP as phosphate source instead of ATP (Figure 1(c)).

Thr<sup>138</sup> of centrin1 is conserved in centrin2 (Thr<sup>137</sup>) and centrin4 (Thr<sup>134</sup>) whereas centrin3 (Ser<sup>135</sup>) carries a serine residue instead (Figure 1(f)). As expected from the amino acid sequence identity, centrin2 and 4 also could be phosphorylated by CK2 (Figure 1(d)). A variety of proteins are phosphorylated by CK2 at serine residues (for review see [4]). Centrin3, however, was not a substrate of CK2 (Figure 1(d)). Coomassie staining was used in parallel to verify equal protein loading (Figure 1(e)).

**3.2. Identification of the Phosphatases Hydrolyzing P-Centrin1.** Phosphatases acting on P-centrin1 included PP1, PP2A, PP2B, PP2Cβ, and PP5. Unspecific alkaline phosphatase was

also tested. 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 (Figure 1(b), right).

Among the 6 phosphatases tested here PP2Cβ was most efficiently dephosphorylating P-centrin1 (Figure 2(a)). All the other phosphatases tested had no or much less effect (Figure 2(a)). This unexpected selectivity prompted us to run the dephosphorylation of P-BAD as an extra control. For that purpose BAD was phosphorylated at Thr<sup>117</sup> by CK2 [5]. Dephosphorylation of P-BAD was run in parallel and identical to the experiments dealing with the putative dephosphorylation of P-centrin1. In analogy to what is known for the majority of phosphorylation sites in any protein, our in vitro studies revealed that P-Thr<sup>117</sup>-BAD more or less could be hydrolyzed by all the phosphatases tested (Figure 2(b)). This was in sharp contrast to the results obtained with phosphatases acting on P-centrin1 (Figure 2(a) versus 2(b)). This unexpected result—strongest dephosphorylation of P-centrin1 by PP2Cβ (Figure 2(a))—was also observed for P-centrin2 and 4 (data not shown).

**3.3. Characterization of Dephosphorylation of P-Centrin1 by PP2Cβ.** An increasing amount of PP2Cβ protein resulted in

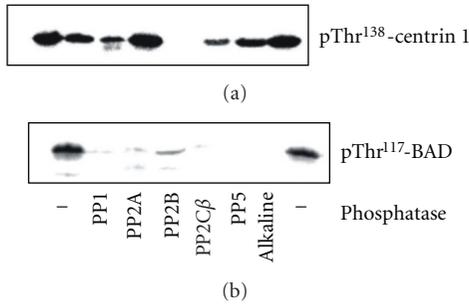


FIGURE 2: Dephosphorylation of P-centrin1 and P-BAD. (a) Incubation of P-Thr<sup>138</sup>-centrin1 (0.2 μg) with phosphatases as indicated. (b) Incubation of P-Thr<sup>17</sup>-BAD (0.6 μg) with phosphatases. The amount of a phosphatase added for the dephosphorylation reactions was the same in (a) and (b) (0.16 μg PP1, 0.05 μg PP2A, 1.3 μg PP2B, 1.5 μg PP2Cβ, 0.8 μg PP5, or 1.5 μg alkaline phosphatase). PP2Cβ is most efficient in dephosphorylating P-centrin1 phosphorylated by CK2. The BAD protein—also phosphorylated by CK2—was run for control to verify activeness of the phosphatases.

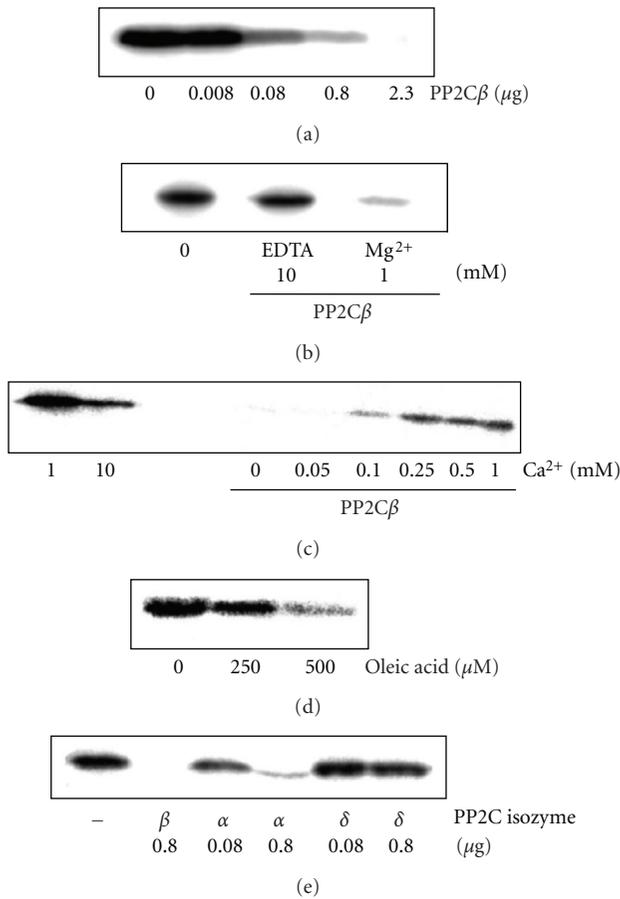


FIGURE 3: Characterization of dephosphorylation of P-centrin1 by PP2C. Centrin1 (0.2 μg) was phosphorylated by CK2 (0.2 μg) and [ $\gamma$ -<sup>32</sup>P]ATP. (a)–(d) Dephosphorylation by 0.08 μg PP2Cβ performed in the presence of 1 mM Mg<sup>2+</sup> unless indicated otherwise. (a) Protein dependence. (b) Requirement for Mg<sup>2+</sup>-ions for activity. (c) Inhibition by Ca<sup>2+</sup>-ions. (d) Stimulation by oleic acid. (e) Effect of PP2C isozymes α, β, and δ on P-Thr<sup>138</sup>-centrin1.

enhanced dephosphorylation (Figure 3(a)). PP2C enzymes are characterized by their requirement for Mg<sup>2+</sup>- or Mn<sup>2+</sup>-cations for activity [6]. In line with that, dephosphorylation of P-centrin1 by PP2Cβ increased upon addition of Mg<sup>2+</sup>-ions (Figure 3(b)). Increasing the Ca<sup>2+</sup>-ion concentration reduced dephosphorylation of P-centrin1 by PP2Cβ (Figure 3(c)). Unsaturated long-chain fatty acids are inhibiting PP2C activity from plants [7] but activate PP2Cα and PP2Cβ in vertebrates [8]. Oleic acid (18 : 1) was capable of stimulating dephosphorylation of P-centrin1 by PP2Cβ (Figure 3(d)).

Dephosphorylation of P-centrin1 was detectable not only with PP2Cβ as shown before but also with PP2Cα (Figure 3(e)). In contrast, P-Thr<sup>138</sup>-centrin1 could not be hydrolyzed by PP2Cδ (Figure 3(e)).

#### 4. Discussion

Phosphorylation of centrin1 by CK2 occurs during dark adaptation in photoreceptor cells of the mammalian retina. It reciprocally regulates the Ca<sup>2+</sup>-mediated binding of centrin1 to the βγ-subunit of the visual heterotrimeric G-protein transducin [1, 9, 10]. 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 centrin1.

Accordingly, in the present study, we addressed the question which phosphatase is capable of dephosphorylating P-Thr<sup>138</sup>-centrin1. All the most abundant retinal phosphatases were tested, that is, PP1, PP2A, PP2B, PP2C α and β, PP5, and alkaline phosphatase [11–14]. Our results were most striking: PP2C α and β most efficiently hydrolyzed P-centrin1; all other phosphatases tested had no or much less effect. This unexpected finding was verified using P-Thr<sup>17</sup>-BAD, phosphorylated by CK2, for control [5]. As expected, P-BAD was dephosphorylated by all those phosphatases which is in sharp contrast to the dephosphorylation of P-centrin1 by PP2C α and β.

Many proteins are phosphorylated at several distinct sites. Knowledge on the reversible phosphorylation of centrin1 currently comprises PKA at Ser<sup>167</sup> [15–17], PKC [15], Cdc2 [15], and CK2 [18]. This report is the first focusing on phosphatases acting on P-centrin1. Because of the unexpected potency of PP2C α and β to dephosphorylate CK2-mediated P-centrin1, we briefly checked whether PP2C α and β might also dephosphorylate P-centrin1 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, PP2C α and β are playing a most decisive role. Overall, dephosphorylation of P-centrin1 by PP2C α and β should increase the affinity of centrin1 to G<sub>t</sub>βγ and finally reduce transport of the G-protein transducin through the connecting cilium.

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