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Crystallization and preliminary X-ray studies of mouse centrin1

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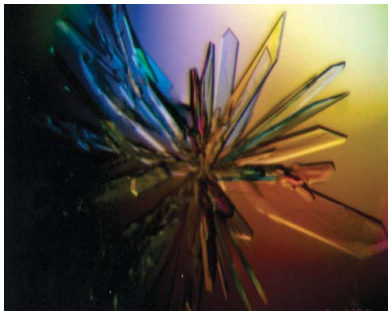

Centrins belong to a family of Ca^{2+}-binding EF-hand proteins that play a fundamental role in centrosome duplication and the function of cilia. To shed light on the structure–function relationship of these proteins, mouse centrin1 has been crystallized. The mouse centrin1 has been expressed in *Escherichia coli* as a GST-centrin fusion protein containing a thrombin protease cleavage site between the fusion partners. Two constructs with different linking-sequence lengths were expressed and purified. Thrombin cleavage yielded functional centrin1 and N-terminally extended centrin1 containing 25 additional residues upstream of its N-terminus. Only N-terminally extended centrin1 (MW $\simeq$ 22 240 Da) could be crystallized at room temperature, using 20–25%(*w*/*v*) PEG 1500, 5–10%(*v*/*v*) ethylene glycol and 1–2%(*v*/*v*) dioxane. Crystals were suitable for X-ray analysis, diffracting to 2.9 Å at 295 K using a rotating-anode X-ray source. They belong to space group *C*2, with unit-cell parameters a = 60.7, b = 59.6, c = 58.3 Å, β = 109.4°. Assuming the asymmetric cell to be occupied by one centrin1 molecule of 22.2 kDa, the unit cell contains 45% solvent with a crystal volume per protein weight, V_M, of 2.2 Å^3 Da^{-1}.

1. Introduction

Centrins, also termed 'caltractins', are eukaryotic low-molecular-weight (~20 kDa) Ca^{2+}-binding proteins which are highly conserved from yeast and protists to humans (Salisbury, 1995; Middendorp *et al.*, 1997). In lower eukaryotes one centrin gene has been found, whereas in mammalian genomes at least four centrin isogenes (*CETN1*, *CETN2*, *CETN3* and *CETN4*) have been identified (Giessl, Trojan *et al.*, 2004). In general, centrins are acidic proteins that contain four helix–loop–helix subdomains, the so-called EF-hands, which represent potential Ca^{2+}-binding sites. These conserved sites allow them to be classified as members of a closely related subfamily within the large superfamily of Ca^{2+}-binding EF-hand proteins that includes calmodulin, troponin C and parvalbumin (reviewed in Wolfrum *et al.*, 2002). An N-terminal extension, which is not found in other related small EF-hand proteins, is the most distinct feature of the centrins (Salisbury, 1995).

The functions of centrins in diverse species and tissues are still unknown and several different roles have been proposed for these proteins. Centrins were first identified in green algae as the major component of contractile rootlets, where they form polymers which contract in response to an increase of the intracellular Ca^{2+} concentration (Salisbury, 1995; Schiebel & Bornens, 1995). Cdc31p is the centrin analogue in the yeast *Saccharomyces cerevisae*, where it is required for the normal duplication and separation of the spindle pole body, the microtubule-organizing centre (MTOC; Baum *et al.*, 1986). Also in higher eukaryotes, centrins are generally associated with MTOCs, the centrioles of centrosomes and basal bodies of cilia and flagella as well as the spindle poles of mitotic cells (Schiebel & Bornens, 1995; Salisbury, 1995; Wolfrum *et al.*, 2002; Giessl, Pulvermüller *et al.*, 2004). During the cell cycle, centrins play a fundamental role in centrosome duplication (Salisbury *et al.*, 2002). In vertebrate photoreceptor cells, centrin isoforms are additionally localized in the connecting cilium linking the photoreceptor inner segment and outer segment compartments (Pulvermüller *et al.*, 2002; Giessl, Pulvermüller *et al.*, 2004). In these specialized neurons, centrins interact with

the $\beta\gamma$-dimer of the heterotrimeric G-protein transducin (Gt) of visual signal transduction, which may regulate light-dependent movements of Gt in a strict Ca^{2+}-dependent manner (Pulvermüller *et al.*, 2002; Wolfrum *et al.*, 2002; Giessl, Pulvermüller *et al.*, 2004). As calmodulin (CaM), these centrin functions are supposed to be regulated by intramolecular structural changes induced by Ca^{2+} binding.

Whereas the molecular structure of Ca^{2+}-dependent calmodulin (and its homologues) have been well studied (Crivici & Ikura, 1995), attempts to crystallize centrin have not yet been successful. Only two NMR structural studies of C-terminal domains of human centrin2 and the centrin (caltractin) of the green algae *Chlamydomonas reinhardtii* have been reported (Hu & Chazin, 2003; Matei *et al.*, 2003). Here, we describe the first crystallization and preliminary X-ray analysis of the full-length mouse centrin1 with its N-terminus extended by 25 residues (~22.2 kDa, 197 amino-acid residues).

2. Materials and methods

2.1. The mouse centrin1 gene in pGEX expression vectors

Mouse centrin1 cDNA was cloned by standard PCR methods into the pGEX-4T3 expression vector (Amersham Biosciences) using *Bam*HI and *Eco*RI restriction sites, leading to an in-frame fusion with the glutathione S-transferase (GST) gene (Pulvermüller *et al.*, 2002). In this gene product, centrin1 and GST are connected by a short linker sequence containing a thrombin cleavage site, which after cleavage yields centrin1 (19.8 kDa, 174 amino-acid residues) with two additional residues (Gly-Ser) at its N-terminus. In a second construct, an oligonucleotide sequence coding for the amino-acid sequence PGISGGGGGIRLRAPLRSQLLWR was inserted into the plasmid at the junction of the thrombin cleavage site and the N-terminus of centrin. Cleavage of the corresponding fusion protein with the protease thrombin yielded N-terminally extended centrin1 (22.2 kDa, 197 amino-acid residues) containing the additional GSPGISGGG-GGIRLRAPLRSQLLWR peptide sequence.

2.2. Expression and purification of centrins

Escherichia coli harbouring the respective expression plasmid was grown in 200 ml LB medium containing 100 µl ml^{-1} ampicillin. The culture was diluted 1:20 with the same medium and grown to an approximate A_{600} of 0.6–0.8. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 m*M* with further incubation at 301 K for 3 h. Cells were harvested, resuspended in 30 ml buffer *A* (20 m*M* BTP, 100 m*M* NaCl pH 7.5) and ultrasonically disrupted on ice (Bandelin Sonopuls GM 70, 10 × 30 s, 50 W output). The extract was centrifuged (30 min at 15 000*g*, 277 K) and the supernatant was loaded onto a glutathione Sepharose 4B column (1.6 × 10 cm; Amersham Biosciences). The column was washed with buffer *B* (20 m*M* BTP, 1 *M* NaCl pH 7.5) and the fusion protein was eluted with 100 ml buffer *A* containing 10 m*M* reduced glutathione. All fractions containing protein were concentrated with a Centricon concentrator (Amicon, 30 kDa molecular-weight cutoff membrane) and incubated at 277 K overnight with 50 units of thrombin (Amersham Biosciences). Completeness of digestion was analyzed by SDS–PAGE electrophoresis. After cleavage, the protein solution was dialyzed against buffer *A* to remove reduced glutathione. The dialyzed protein solution was passed over benzamidine Sepharose 6B (1.5 × 5 cm) and glutathione Sepharose 4B (1.5 × 5 cm, Amersham Biosciences) to remove thrombin and GST protein. The eluted protein was dialyzed against buffer *C* (20 m*M* BTP pH 7.5). Centrin was purified further by anion-exchange chromatography (Q-Sepharose, 1.0 × 6 cm, equilibrated and eluted with a linear gradient of 0–1 *M* NaCl in buffer *C*). Purified centrin was dialyzed against buffer *C* and concentrated with a Centricon concentrator (Amicon, 10 kDa molecular-weight cutoff membrane) to 10–20 mg ml^{-1}. No additional Ca^{2+} was added to any of the buffers; it was assumed that the high-affinity Ca^{2+}-binding sites of centrin were already occupied. The purity of centrin was analyzed by 15% SDS–PAGE.

2.3. Functional assay of centrin

Kinetic near-infrared light-scattering measurements were performed as described previously to test the functionality of purified recombinant centrins (Pulvermüller *et al.*, 2002). The binding of the Gt–centrin complex to light-activated rhodopsin embedded in rod outer segment disk membranes was triggered by activation of rhodopsin with a flash of green light (500 ± 20 nm), attenuated by appropriate neutral density filters. The flash intensity was quantified photometrically by the amount of rhodopsin bleached and expressed as a mole fraction of the light-activated rhodopsin ($R^*/R = 32\%$). The scattering signal was interpreted as a light-induced gain of protein

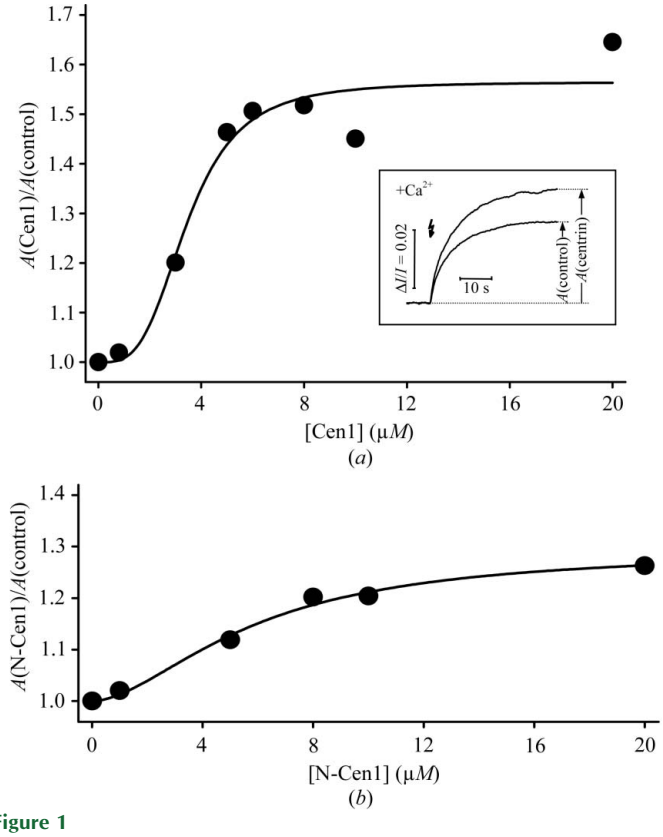

Figure 1
Effect of centrin1 on the Gt-binding signal analyzed by kinetic light scattering (KLS). The dependence of the amplitude of flash-induced KLS Gt-binding signals on centrin1 (*a*) and N-terminally extended centrin1 (*b*) observed in the presence of Ca^{2+} is shown. In both traces the centrin-dependent enhancement of the Gt-binding signals is normalized to the amplitude of the Gt-binding signal without added centrin (control). Measurements were performed in the presence of 100 µ*M* $CaCl_2$. (*a*) Data points were fitted using the Hill equation: $f = (A[\text{Cen1}]^n)/([\text{Cen1}]^n + EC_{50}^n) + 1$, with the following parameters: $A = 0.56 \pm 0.05$, $n = 3.3 \pm 1.4$ and $EC_{50} = 3.5 \pm 0.4$ µ*M*. (*b*) As in (*a*), but with N-terminal extended centrin1 (N-Cen1) instead of centrin1. Data points were fitted as in (*a*) with the following parameters: $A = 0.29 \pm 0.03$, $n = 1.7 \pm 0.4$ and $EC_{50} = 5.8 \pm 0.8$ µ*M*, where *A* is the maximal centrin-dependent enhancement of the Gt-binding signal, *n* is the Hill coefficient and EC_{50} is the effective concentration of half-maximal binding in micromoles. The inset in (*a*) shows an example of kinetic light-scattering binding signals (2 µ*M* rhodopsin; 0.5 µ*M* Gt) with (10 µ*M*) and without centrin1. The flash symbol indicates the time of rhodopsin activation by light. Measuring conditions as described in §2.

mass bound to rhodopsin-containing disk membranes and quantified as described by Heck *et al.* (2000). Light-scattering binding signals were corrected by a reference signal (N-signal) measured on a sample without added Gt (Pulvermüller *et al.*, 1993; Heck *et al.*, 2000).

2.4. Crystallization of centrin

The sparse-matrix method (Jancarik & Kim, 1991) was applied using Crystal Screen from Hampton Research (San Diego, CA, USA). The crystallization was performed at 293 K by the hanging-drop vapour-diffusion method using 24-well Linbro plates. Each hanging drop was prepared on a siliconized cover slip by mixing equal volumes (2 µl each) of purified centrin1 (10–20 mg ml^{-1}) and reservoir solution (1 ml) and sealed with Baysilone vacuum grease (Hampton Research). For preparation of large single crystals, the crystallization method was changed from the hanging-drop to the sitting-drop vapour-diffusion method (droplet size 20 µl).

3. Results and discussion

Centrin1 and N-terminally extended centrin1 were expressed in *E. coli* as GST-centrin fusion proteins and purified to homogeneity by glutathione Sepharose and anion-exchange chromatography. The yield of centrin1 was 3–4 mg per litre of cell culture and the yield of N-terminally extended centrin1 was 6–8 mg per litre of cell culture. The functional activities of recombinant centrins were determined by the kinetic light-scattering assay by measuring binding of the Ca^{2+}-dependent Gt–centrin complex to light-activated rhodopsin (Fig. 1). The additional residues in N-terminally extended centrin1 did not abolish the formation of a complex with Gt and light-activated rhodopsin. The complex formation is Ca^{2+}-dependent (data not shown) as observed earlier for native centrin1 (Pulvermüller *et al.*, 2002). However, the additional residues in N-terminally extended centrin1 led to a twofold increase of the EC_{50} value for formation of the complex between Gt–centrin and light-activated rhodopsin (Fig. 1*b*). The different *A* values and Hill coefficients (*n*) obtained from the fit to the Hill equation may indicate different compositions of the Gt–centrin complexes.

Both centrin1 and N-terminally extended centrin1 aggregated in buffer *C* and no crystals were obtained in the original Hampton Research crystallization screens. However, systematic addition of ethylene glycol and dioxane in PEG 1500 to the protein solution (10–20 mg ml^{-1}) decreased the aggregation of the proteins. Nevertheless, crystals have only been obtained for N-terminally extended centrin1. Introduction of the additional residues at the N-terminus probably reduced the ability of centrin1 to form oligomeric structures, facilitating its crystallization. It has already been shown that algal centrins and human centrin1 and centrin2 isoforms form homodimers (Wiech *et al.*, 1996; Tourbez *et al.*, 2004). Our recent binding studies indicate that the mouse centrin1, centrin2 and centrin4 isoforms also oligomerize (Giessl, Pulvermüller *et al.*, 2004).

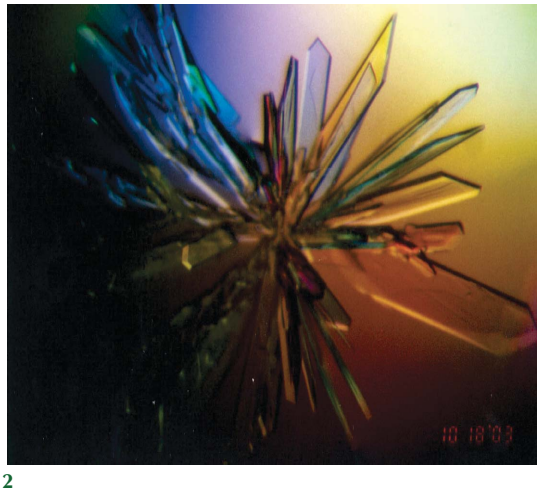

Figure 2
Crystals of N-terminally extended mouse centrin1. The maximum dimensions are 0.2 × 0.6 × 0.1 mm.

Table 1
Crystal data and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 60.70, b = 59.60, c = 58.31, β = 109.41
Content of AU	1 molecule
Matthews coefficient V_M (Å^3 Da^{-1})	2.2
Resolution limits (Å)	25.0–2.9 (3.0–2.9)
No. observations	10227
No. unique reflections	4301 (425)
Completeness (%)	97.2 (97.0)
Mosaicity	0.35
R_{merge}†	0.076 (0.293)
$I/\sigma(I)$	9.2 (2.6)
Mean redundancy	2.4 (2.4)

† $R_{merge} = \sum_{hkl}\sum_j |I_{hkl}(j) - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl}(j)$, where $\langle I_{hkl}\rangle$ is the mean intensity and $I_{hkl}(j)$ are individual intensity measurements of the reflection (*hkl*).

For X-ray studies, single crystals of N-terminally extended centrin1 were mounted in a quartz capillary and sealed with wax after an addition of a small amount of mother liquor. A complete diffraction data set (Table 1) was collected at 295 K using a rotating-anode X-ray generator (MAC Science/Siemens, Cu anode) and a 300 mm imaging-plate detector (MAR Research, Norderstedt, Germany) operating at 45 kV and 60 mA. Data were processed using the *HKL* package (Otwinowski & Minor, 1997). The crystals of N-terminally extended centrin1 diffracted to 2.9 Å. The crystals belong to the monoclinic space group *C*2, with unit-cell parameters a = 60.7, b = 59.6, c = 58.3 Å, β = 109.4°. The asymmetric unit contains one centrin1 molecule with a solvent content of 45% by volume and a crystal volume per protein weight, V_M, of 2.2 Å^3 Da^{-1} (Matthews, 1968). Interestingly, attempts to determine the structure by molecular replacement using different models of calmodulin-type proteins did not succeed. This is likely to indicate a substantial structural difference between centrin and the structurally known calmodulin-type proteins. Hence, we are attempting to solve the phase problem by MIR or MAD/SAD methods.

References

Baum, P., Furlong, C. & Byers, B. E. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 5512–5516.
Crivici, A. & Ikura, M. (1995). *Annu. Rev. Biophys. Biomol. Struct.* **24**, 85–116.
Giessl, A., Pulvermüller, A., Trojan, P., Park, J. H., Choe, H.-W., Ernst, O. P., Hofmann, K. P. & Wolfrum, U. (2004). *J. Biol. Chem.* **279**, 51472–51481.

Giessl, A., Trojan, P., Pulvermüller, A. & Wolfrum, U. (2004). *Cell Biology and Related Disease of the Outer Retina*, edited by D. S. Williams, pp. 195–222. Singapore: World Scientific Publishing Company.
Heck, M., Pulvermüller, A. & Hofmann, K. P. (2000). *Methods Enzymol.* **315**, 329–347.
Hu, H. & Chazin, W. J. (2003). *J. Mol. Biol.* **330**, 473–484.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Matei, E., Miron, S., Blouquit, Y., Duchambon, P., Durussel, I., Cox, J. A. & Craescu, C. T. (2003). *Biochemistry*, **42**, 1439–1450.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Middendorp, S., Paoletti, A., Schiebel, E. & Bornens, M. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 9141–9146.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pulvermüller, A., Giessl, A., Heck, M., Wottrich, R., Schmitt, A., Ernst, O. P., Choe, H.-W., Hofmann, K. P. & Wolfrum, U. (2002). *Mol. Cell Biol.* **22**, 2194–2203.
Pulvermüller, A., Palczewski, K. & Hofmann, K. P. (1993). *Biochemistry*, **32**, 14082–14088.
Salisbury, J. L. (1995). *Curr. Opin. Cell Biol.* **7**, 39–45.
Salisbury, J. L., Suino, K. M., Busby, R. & Springett, M. (2002). *Curr. Biol.* **12**, 1287–1292.
Schiebel, E. & Bornens, M. (1995). *Trends Cell Biol.* **5**, 197–201.
Tourbez, M., Firanescu, C., Yang, A., Unipan, L., Duchambon, P., Blouquit, Y. & Craescu, C. T. (2004). *J. Biol. Chem.* **279**, 47672–47680.
Wiech, H., Geier, B. M., Paschke, T., Spang, A., Grein, K., Steinkotter, J., Melkonian, M. & Schiebel, E. (1996). *J. Biol. Chem.* **271**, 22453–22461.
Wolfrum, U., Giessl, A. & Pulvermüller, A. (2002). *Adv. Exp. Med. Biol.* **514**, 155–178.