Crystallographic characterization of the C-terminal coiled-coil region of mouse Bicaudal-D1 (BICD1)

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Bicaudal-D1 (BICD1) is an α -helical coiled-coil protein which is evolutionarily conserved from *Drosophila* to mammals and facilitates the attachment of specific cargo factors to the dynein motor complex. The C-terminal coiled-coil region (CC3) of BICD1 plays an important role in sorting cargo, linking proteins such as the small GTPase Rab6 and the nuclear pore complex component Ran-binding protein 2 (RanBP2) to the dynein motor complex. This report describes the crystallization and X-ray data collection of the BICD1 CC3 region, as well as the preparation of the complex of BICD1 CC3 with a constitutively active mutant of Rab6. The crystals of the BICD1 CC3 region belonged to space group C2, with unit-cell parameters $a = 59.0$, $b = 36.8$, $c = 104.3$ Å, $\alpha = \gamma = 90$, $\beta = 99.8^\circ$. The X-ray diffraction data set was collected to 1.50 Å resolution.

1. Introduction

Bicaudal-D (BicD) was first identified in *Drosophila melanogaster* by genetic screens that were aimed at isolating genes involved in embryogenesis and pattern formation (Mohler & Wieschaus, 1986; Steward & Nüsslein-Volhard, 1986). It is highly conserved across several species from *Caenorhabditis elegans* to mammals (Suter *et al.*, 1989; Wharton & Struhl, 1989). The loss-of-function mutations in *BicD* induce mis-localization of factors important for oocyte differentiation and affect the polarization and organization of the microtubule network during oogenesis (Theurkauf *et al.*, 1993; Swan & Suter, 1996). Based on the genetic data and the localization of the BicD protein, it has been suggested that BicD plays an essential role in oogenesis and embryogenesis by controlling the mRNA localization mediated by the dynein–dynactin motor complex (Swan *et al.*, 1999; Bullock & Ish-Horowicz, 2001; Delanoue & Davis, 2005). The overexpression of BicD in *Drosophila* embryos promotes the minus-end-directed transport of individual mRNA transport particles along microtubules (Bullock *et al.*, 2006).

In mammals, two homologues, BICD1 and BICD2, have been identified (Baens & Marynen, 1997). These proteins mainly localize to the Golgi apparatus in cultured cells and co-localize with dynactin at microtubule plus ends (Hoogenraad *et al.*, 2001). The BICD proteins are cytoplasmic α -helical coiled-coil proteins that consist of about 800 amino-acid residues and possess three functional coiled-coil (CC) regions. The N-terminal half (containing the coiled-coil region CC1 and a part of CC2) of BICD proteins interacts with the dynein–dynactin motor complex, whereas the C-terminal coiled-coil region (CC3) directly binds cargo-specific factors such as the small GTPase Rab6, which is involved in the regulation of membrane traffic from the Golgi apparatus to the endoplasmic reticulum (ER) in a coat complex coatamer protein I (COPI)-independent pathway, and Ran-binding protein 2 (RanBP2), which is a component of the nuclear pore complex (Hoogenraad *et al.*, 2001, 2003; Matanis *et al.*, 2002; Navarro *et al.*, 2004; Fumoto *et al.*, 2006; Wanschers *et al.*, 2007; Dienstbier & Li, 2009; Splinter *et al.*, 2012). The cargo-binding ability

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Table 1

Recombinant protein-production information.

Protein	BICD1
Source organism	Mouse
DNA source	cDNA
Forward primer	5'-AAACCCGGGAACAAATATGAAATGAAAG-3'
Reverse primer	5'-ACGCGTGCAGCTATTGCTGCTCGGGACTGCTC-3'
Expression vector	pMAL-c2x
Expression host	BL21 Star (DE3)
Protein	Rab6
Source organism	Mouse
DNA source	cDNA
Forward primer	5'-AAACCCGGGGGAATCCGCTGAGGAAATCAAG-3'
Reverse primer	5'-AAACTCGAGTTATGTTGCTCTGAGGCTTTCCAG-3'
Expression vector	pET-49b
Expression host	BL21 Star (DE3)

of the BICD CC3 region could promote association with the dynein-dynactin motor complex by releasing the autoinhibitory interaction with the N-terminal CC1 region (Hooenraad *et al.*, 2001, 2003; Splinter *et al.*, 2012). These findings suggest that BICD protein acts as a linker between the dynein-dynactin complex and cargoes for Golgi-ER trafficking, nuclear positioning and lipid droplet trafficking (Larsen *et al.*, 2008; Splinter *et al.*, 2010).

Intracellular cargo transport driven by the dynein-dynactin motor complex plays critical roles in neuronal development, morphology, survival and function (Hirokawa *et al.*, 2010). Various mutations in the *BICD2* gene have been found in patients suffering from dominant congenital spinal muscular atrophy (DCSMA) with lower extremity predominance (LED) (Oates *et al.*, 2013). The mutations Ser107Leu and Arg501Pro in CC1 of BICD2 increase binding affinity to the dynein-dynactin motor complex (Neveling *et al.*, 2013). The Glu772Gly mutation of BICD2, which is located in the CC3 region, decreases Rab6 binding (Peeters *et al.*, 2013). These reports suggest that the cargo-sorting mediated by BICD proteins and the dynein motor complex is linked to neurological diseases. However, the precise mechanism by which the BICD CC3 region binds to cargo factors and the N-terminal CC1 region is unknown. Here, we report the expression, purification, crystallization and preliminary X-ray crystallographic characterization of the BICD1 CC3 region.

2. Materials and methods

2.1. Protein expression

The nucleotide fragments encoding CC3 (residues 711–808) of mouse BICD1 were amplified by PCR using cDNAs (Gene ID 12121). Using *Xma*I and *Sal*I restriction-enzyme sites, the amplified nucleotide fragment encoding BICD1 CC3 was subcloned into a pMAL-c2x plasmid (New England Biolabs) in which the nucleotides encoding an HRV3C protease-recognition site and an *Xma*I restriction-enzyme site were inserted using *Eco*RI and *Sal*I restriction-enzyme sites. The nucleotide fragment encoding mouse Rab6 (residues 8–200) was amplified by PCR using cDNAs (Gene ID 19346). The amplified nucleotide fragment was digested by the restriction enzymes *Xma*I and *Xho*I, and was subcloned into a pET-49b plasmid (Novagen). The constitutively active mutant (Rab6Q72L) of Rab6, in which Gln72 was replaced by Leu, was generated using the Quik-Change mutagenesis method (Qiagen). The plasmids overexpress BICD1 CC3 and Rab6Q72L with an N-terminal hexahistidine-tagged maltose-binding protein (MBP) and glutathione *S*-transferase (GST) linked to the HRV3C protease site, respectively. The constructs had an additional three residues (GPG) at the N-terminus after protease

cleavage of the MBP or GST tag. Table 1 summarizes the detailed information for the recombinant protein production.

The BICD1 CC3 contains five methionine residues. For multi-wavelength anomalous dispersion (MAD) experiments, the MBP-fused BICD1 CC3 was expressed in *Escherichia coli* BL21 Star (DE3) cells (Life Technologies) by using a technique based on the inhibition of methionine biosynthesis for the substitution of methionine by selenomethionine (Van Duyne *et al.*, 1993; Doublé, 1997). The cells transformed with pMAL-c2x-BICD1 CC3 were cultured with 1 l M9 medium until the OD_{600 nm} reached 0.4. Induction of recombinant protein expression using 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was initiated 30 min following the addition of the amino acids required for the inhibition of methionine synthesis. The cultures were grown at 298 K overnight. The GST-fused Rab6Q72L was expressed in *E. coli* BL21 Star (DE3) cells. 20 ml of an overnight culture of cells transformed with pET-49b-Rab6Q72L were inoculated into 2.5 l Luria-Bertani (LB) medium containing 50 μ g ml⁻¹ kanamycin. The expression of recombinant protein was induced by the addition of IPTG to a final concentration of 0.1 mM. The recombinant protein was expressed at 298 K overnight. The cells were harvested by centrifugation at 5000g for 10 min at 277 K.

2.2. Purification of the BICD1–Rab6Q72L complex

Cells that expressed the MBP-fused BICD1 CC3 or the GST-fused Rab6Q72L were disrupted by sonication at 277 K. Insoluble cellular debris was removed by centrifugation at 39 150g using a Model 7000 centrifuge (Kubota) and the supernatants were applied onto an amylose resin column (New England Biolabs) or a glutathione Sepharose 4B column (GE Healthcare), respectively. The columns were washed with 50 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM dithiothreitol (DTT). The fusion proteins were eluted with 50 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT, 20 mM maltose or 10 mM glutathione. The fractions, to which ammonium sulfate was added to a final concentration of 1.0 M, were applied onto a HiPrep Butyl Sepharose column (GE Healthcare) and washed with 20 mM Na HEPES buffer pH 7.5 containing 1 M ammonium sulfate, 1 mM DTT. The fusion proteins were eluted using a linear ammonium sulfate concentration gradient (1.0–0 M). Fractions containing the fusion proteins were pooled and exchanged with 20 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT using a HiPrep desalting column (GE Healthcare). All purification processes were performed at 277 K.

The solutions of purified MBP-BICD1 CC3 and GST-Rab6Q72L were mixed and incubated overnight at 277 K in 20 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 1 mM GTP. To remove unbound MBP-BICD1 CC3, the complex solution was loaded onto a glutathione Sepharose column and washed with 20 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT. The MBP and GST tags were removed with 1 μ M GST-fused HRV3C protease on resin at 277 K overnight. The BICD1–Rab6Q72L complex was eluted with 20 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT. The complex was purified on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 10 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT.

2.3. Crystallization and X-ray diffraction

For crystallization, purified BICD1 CC3–Rab6Q72L complex was concentrated to 27.5 mg ml⁻¹ in a solution containing 10 mM Na HEPES buffer pH 7.5, 150 mM NaCl, 1 mM DTT using an Amicon 20 (10K MWCO PES, Vivascience). The protein concentration of the

Table 2

X-ray diffraction data of the BICD1 CC3 region crystal.

Values in parentheses are for the outer shell.

Data-set name	Peak	Edge	Remote
Wavelength (Å)	0.97924	0.97973	0.96448
Space group	C2	C2	C2
Unit-cell parameters			
<i>a</i> (Å)	59.0	59.1	59.0
<i>b</i> (Å)	36.8	36.9	36.9
<i>c</i> (Å)	104.3	104.6	104.7
$\alpha = \gamma$ (°)	90.0	90.0	90.0
β (°)	99.8	99.9	99.9
Resolution range (Å)	50.0–1.50 (1.53–1.50)	50.0–1.55 (1.58–1.55)	50.0–1.60 (1.63–1.60)
Total No. of reflections	255485	231256	208751
No. of unique reflections	35669	32364	29185
Completeness (%)	99.6 (96.9)	99.3 (92.3)	99.2 (89.9)
Multiplicity	7.2 (5.7)	7.2 (5.5)	7.2 (5.5)
Mean <i>I</i> / σ (<i>I</i>)	44.6 (5.9)	42.4 (3.1)	36.9 (2.1)
<i>R</i> _{merge} (%)	9.0 (27.6)	7.3 (42.2)	7.9 (62.4)

crystallization sample was determined from the absorbance at 280 nm. Initial crystallization screening was performed by the sitting-drop vapour-diffusion method with a commercial crystallization screening kit at 277 and 293 K. Crystallization drops were prepared by mixing 1 μ l protein solution and 1 μ l reservoir solution and were equilibrated against 50 μ l reservoir solution. The conditions for crystallization screening were optimized by changing the precipitant concentration and the buffer pH. The composition of the crystals was analyzed using 17.5% polyacrylamide gel electrophoresis, and staining was carried out with Simply Stain Blue (Invitrogen).

The crystals were flash-cooled in liquid nitrogen. X-ray diffraction data were collected on the BL-17A beamline at the Photon Factory, Tsukuba, Japan using an ADSC Q270 CCD detector. Optimal peak, edge and remote wavelengths were determined by X-ray fluorescence. Data collection was performed using an angular range of 360°, a step size of 0.5° and an exposure time of 0.5 s. The camera was fixed at a distance of 159.2 mm. The diffraction data were processed using *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

The CC3 region of BICD1 was insoluble, while its complex with Rab6Q72L was highly soluble in 50 mM Na HEPES buffer pH 7.5 containing 150 mM NaCl, 1 mM DTT. A BICD1 CC3 region suitable for crystallization trials was not obtained from the current construct. In gel-filtration chromatography, the BICD1–Rab6Q72L complex eluted with an apparent molecular mass of 71.8 kDa, corresponding to a complex with 2:2 stoichiometry (the calculated molecular weight

was 34 166 Da for the 1:1 complex). The final yield of the purified BICD1–Rab6Q72L complex was 4.5 mg per litre of cell culture.

In the initial screening using the purified BICD1–Rab6Q72L complex, crystallization was achieved with Wizard III solution No. 7 consisting of 100 mM Tris–HCl pH 8.5, 50% 2-methyl-2,4-pentanediol (MPD), 200 mM ammonium phosphate. However, analysis of the composition of crystals by SDS–PAGE revealed that the crystal contained the BICD1 CC3 region only but not Rab6Q72L (Fig. 1). Despite further crystallization trials using screening kits from Hampton Research and Jena Bioscience, crystals of the BICD1 CC3–Rab6Q72L complex could not be obtained using the current construct. Although the BICD1 CC3 region forms a stable complex with Rab6 in solution, the reagents used in these crystallization conditions may affect the interaction of the BICD1 CC3 region with Rab6 as well as the solubility of the BICD1 CC3 region alone. Therefore, we focused only on the crystals of the BICD1 CC3 region alone for structural determination of the unique CC region.

The best crystals of the BICD1 CC3 region were obtained using a solution consisting of 13.8 mg ml^{−1} protein, 50 mM Tris–HCl pH 8.2, 24% MPD, 100 mM ammonium phosphate, 5 mM TCEP–HCl, 75 mM NaCl, 0.5 mM DTT equilibrated against 48% MPD, 200 mM ammonium phosphate, 100 mM Tris–HCl pH 8.2, 10 mM TCEP–HCl at 277 K (Fig. 2). The crystals grew to maximum dimensions of 0.4 × 0.2 × 0.1 mm after a week. The crystals diffracted to a resolution of 1.50 Å and belonged to space group C2, with unit-cell parameters *a* = 59.0, *b* = 36.8, *c* = 104.3 Å, $\alpha = \gamma = 90$, $\beta = 99.8^\circ$. A Matthews coefficient of 2.36 Å³ Da^{−1} was calculated under the assumption that two BICD1 CC3 regions were present in the asymmetric unit, which corresponded to a solvent content of 47.8% (Matthews, 1968). To facilitate structural determination of the BICD1 CC3 domain using the MAD method, X-ray fluorescence was used to detect the Se atoms in these crystals in addition to determining the peak points of the Se *K* edge. Table 2 summarizes the detailed statistics of the diffraction data.

Automated phasing of MAD X-ray data performed by the *auto-SHARP* program identified the positions of nine Se atoms in the crystal (Vonnrhein *et al.*, 2007). The positions of these Se atoms were used to calculate the initial phase with an average figure of merit (FOM) of 0.46 at 1.50 Å resolution. Further density modification using the *SOLOMON* program (Abrahams & Leslie, 1996) improved the average FOM to 0.89 at 1.50 Å resolution. Structural analysis of the BICD1 CC3 region is now in progress.

Figure 1

The analysis of the crystal composition by SDS–PAGE. 17.5% polyacrylamide gel was used in the experiment. SDS–PAGE of molecular-weight markers (lane 1; labelled in kDa), the purified sample (lane 2) and the crystals (lane 3).

Figure 2

The crystals of the BICD1 CC3 region.

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References

- Abrahams, J. P. & Leslie, A. G. W. (1996). *Acta Cryst.* **D52**, 30–42.
- Baens, M. & Marynen, P. (1997). *Genomics*, **45**, 601–606.
- Bullock, S. L. & Ish-Horowicz, D. (2001). *Nature (London)*, **414**, 611–616.
- Bullock, S. L., Nicol, A., Gross, S. P. & Zicha, D. (2006). *Curr. Biol.* **16**, 1447–1452.
- Delanoue, R. & Davis, I. (2005). *Cell*, **122**, 97–106.
- Dienstbier, M. & Li, X. (2009). *Biochem. Soc. Trans.* **37**, 1066–1071.
- Doublié, S. (1997). *Methods Enzymol.* **276**, 523–530.
- Fumoto, K., Hoogenraad, C. C. & Kikuchi, A. (2006). *EMBO J.* **25**, 5670–5682.
- Hirokawa, N., Niwa, S. & Tanaka, Y. (2010). *Neuron*, **68**, 610–638.
- Hoogenraad, C. C., Akhmanova, A., Howell, S. A., Dortland, B. R., De Zeeuw, C. I., Willemsen, R., Visser, P., Grosveld, F. & Galjart, N. (2001). *EMBO J.* **20**, 4041–4054.
- Hoogenraad, C. C., Wulf, P., Schiefermeier, N., Stepanova, T., Galjart, N., Small, J. V., Grosveld, F., de Zeeuw, C. I. & Akhmanova, A. (2003). *EMBO J.* **22**, 6004–6015.
- Larsen, K. S., Xu, J., Cermelli, S., Shu, Z. & Gross, S. P. (2008). *PLoS One*, **3**, e3763.
- Matanis, T., Akhmanova, A., Wulf, P., Del Nery, E., Weide, T., Stepanova, T., Galjart, N., Grosveld, F., Goud, B., De Zeeuw, C. I., Barnekow, A. & Hoogenraad, C. C. (2002). *Nature Cell Biol.* **4**, 986–992.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Mohler, J. & Wieschaus, E. F. (1986). *Genetics*, **112**, 803–822.
- Navarro, C., Puthalakath, H., Adams, J. M., Strasser, A. & Lehmann, R. (2004). *Nature Cell Biol.* **6**, 427–435.
- Neveling, K. *et al.* (2013). *Am. J. Hum. Genet.* **92**, 946–954.
- Oates, E. C. *et al.* (2013). *Am. J. Hum. Genet.* **92**, 965–973.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Peeters, K., Litvinenko, I., Asselbergh, B., Almeida-Souza, L., Chamova, T., Geuens, T., Ydens, E., Zimoń, M., Irobi, J., De Vriendt, E., De Winter, V., Ooms, T., Timmerman, V., Tournev, I. & Jordanova, A. (2013). *Am. J. Hum. Genet.* **92**, 955–964.
- Splinter, D., Razafsky, D. S., Schlager, M. A., Serra-Marques, A., Grigoriev, I., Demmers, J., Keijzer, N., Jiang, K., Poser, I., Hyman, A. A., Hoogenraad, C. C., King, S. J. & Akhmanova, A. (2012). *Mol. Biol. Cell*, **23**, 4226–4241.
- Splinter, D. *et al.* (2010). *PLoS Biol.* **8**, e1000350.
- Steward, R. & Nüsslein-Volhard, C. (1986). *Genetics*, **113**, 665–678.
- Suter, B., Romberg, L. M. & Steward, R. (1989). *Genes Dev.* **3**, 1957–1968.
- Swan, A., Nguyen, T. & Suter, B. (1999). *Nature Cell Biol.* **1**, 444–449.
- Swan, A. & Suter, B. (1996). *Development*, **122**, 3577–3586.
- Theurkauf, W. E., Alberts, B. M., Jan, Y. N. & Jongens, T. A. (1993). *Development*, **118**, 1169–1180.
- Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.
- Vonrhein, C., Blanc, E., Roversi, P. & Bricogne, G. (2007). *Methods Mol. Biol.* **364**, 215–230.
- Wanschers, B. F. J., van de Vorstenbosch, R., Schlager, M. A., Splinter, D., Akhmanova, A., Hoogenraad, C. C., Wieringa, B. & Fransen, J. A. M. (2007). *Exp. Cell Res.* **313**, 3408–3420.
- Wharton, R. P. & Struhl, G. (1989). *Cell*, **59**, 881–892.