cDNA Cloning and Life-Cycle Stage-Specific Expression of Coronin from Physarum polycephalum

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Coronin cDNA was cloned from the plasmodia of Physarum polycephalum. The amino acid sequence deduced from the cDNA was comprised of 449 residues and showed 60% identity to that of Dictyostelium discoideum coronin. Southern blot analysis suggested that the coronin gene present in the P. polycephalum genome might be a single copy. Coronin was expressed in diploid plasmodia, while it was not detected in haploid amoebae or spores.

Key words: coronin; Physarum polycephalum; cloning; life-cycle

Coronin, an actin-binding protein, was first identified in an actin-myosin complex from Dictyostelium discoideum, a cellular slime mold. Since the protein was concentrated on the crown-like extensions of amoeba, it was named coronin. The amino acid sequence of D. discoideum coronin is similar to that of β subunit of G proteins. Further, coronin has the TSSGD sequence and WD repeat sequence, which are conserved in the G-β subunits. Hence it might be associated with G protein-mediated signal transduction. It has been found in various eukaryotes, and its functions in various cytoskeleton-based processes such as cell migration, cell division, and membrane trafficking, have been examined.

Physarum polycephalum, a true slime mold, is taxonomically different from D. discoideum. However, similarly to D. discoideum, P. polycephalum shows a unique morphological change during its life cycle. In D. discoideum, the amoebae aggregate with each other to form a haploid migrating cell, following which the migrating cells differentiate into a haploid fruiting body. On the other hand, P. polycephalum amoeba mates with different sexual cells, forming a diploid zygote. The zygote repeats nuclear division without cell division and grows into a plasmodium, a multinuclear cell. The plasmodium can differentiate into fruiting bodies under appropriate conditions. In P. polycephalum, Ishikawa et al. have reported that a partial amino acid sequence of the actin-binding protein from the plasmodia is similar to that of coronin from D. discoideum.

We have analyzed the biochemical features of three β-glucosidases from P. polycephalum (Pp II (+/−) ATCC #24466 and 24467) plasmodia, and have developed an antibody against the secretory β-glucosidase. Although we performed an immuno-screening with the antibody using the cDNA library of plasmodia, several clones unrelated to β-glucosidase were obtained. The nucleotide sequence of one of these clones was homologous to the D. discoideum coronin. To elucidate the function of P. polycephalum coronin, we characterized the isolated coronin cDNA. A cDNA library of P. polycephalum plasmodium was constructed from mRNA with a double stranded Uni-ZAP XR vector (Stratagene, La Jolla, CA) and immuno-screened with β-glucosidase antibody, as described previously. By the screening, 22 clones were obtained from 1.5 × 10⁶ pfu plaques. The nucleotide sequences of the clones were analyzed using universal T7 and T3 promoter primers. We obtained a clone with a nucleotide sequence homologous to that of D. discoideum coronin. The cDNA isolated consisted of 1,259 bp with a poly (A) tail of 19 nucleotides, but the nucleotide sequence did not contain the ATG start codon. To obtain the full-length sequence of coronin, 5’-rapid amplification of cDNA ends (RACE) PCR was carried out using the cDNA pool, following the instructions provided in the GeneRacer Kit (Invitrogen, Carlsbad, CA). The PCR primers for RACE were designed from the known sequence: 5’-CTTGAAGTACGGATGTTG-3’. The full length cDNA consisted of a 5' noncoding region of 63 bp, an open reading frame of 1,347 bp encoding 449 amino acids, and a 3' noncoding region of 78 bp, which included the putative polyadenylation signal (AAATAA). The complete nucleotide sequence has been submitted to the DDBJ, EMBL, and Gen-Bank international nucleotide sequence databases under accession no. AB179828. The molecular weight of P. polycephalum coronin encoded by the open reading frame was calculated to be 50,159, similar to that of D. discoideum coronin. As described above, the amino acid sequences of two peptides of P. polycephalum coronin protein have been reported. These two sequences correspond to positions 4–10 (VVRSSKY) and 185–194 (GSQLATTCHDKKLRIDPQR) of P. polycephalum coronin. The amino acid sequence of P. polycephalum coronin includes five WD repeats and a coiled-coil motif in the C-terminal region (Fig. 1). Like D. discoideum coronin, it is predicted that P. polycephalum

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coronin also forms a β-propeller structure with its WD repeats. Coronin is generally classified into a shorter protein, such as mammalian type I and II coronin, and a longer protein, such as mammalian type III coronin. *P. polycephalum* coronin is of shorter type and shows 60% sequence identity to *D. discoideum* coronin (Fig. 1). In addition, it shows approximately 45% identity with coronins from *Drosophila melanogaster* (accession no. AF467271) and 46%, 39%, and 30% identity to coronin 1A (type I; accession no. BC110374), coronin 2A (type II; accession no. AK292788) and coronin 7 (type III; accession number, BC117289) from humans.

Genomic DNA was extracted from isolated nuclei of *P. polycephalum* microplasmodia by the method of Mohberg and Rusch,7) and was digested using five restriction enzymes (*Xho* I, *Pst* I, *Nde* I, *Nco* I, and *Bam* HI), for which there were no recognition sites in *P. polycephalum* coronin cDNA. The resulting fragments were analyzed by Southern blot using the probe (corresponding to positions 451–1,080 bp of the nucleotide sequence). Except for the fragments digested with *Pst* I and *Nde* I, a single fragment was detected by Southern analysis for the digests (Fig. 2). One or two recognition sites for *Nde* I and *Pst* I might be present in the introns of the coronin gene. Thus *P. polycephalum* genome might have a single coronin gene. There are at least seven coronin genes in the mammalian genome, and they show distinct patterns of expression across different cell and tissue types.2) It is known that only one type of coronin is present in non-mammalian organisms, except for *Caenorhabditis elegans*, which has two different types of coronins.8)

To express the *P. polycephalum* coronin in *E. coli*, the full-length DNA fragment of coronin was amplified by PCR using the following forward and reverse primers: 5′-GGATCCAGGTGTGAAGGTCTCT-3′ and 5′-AAGCTTAAAGCCGAAAGTCTTGTGATTAT−3′. After the amplified fragment was digested with *Bam* HI and *Hind* III, it was ligated into pQE-30 vector (Qiagen, Valencia, CA). Expression of the recombinant coronin in transformed *E. coli* harboring the pQE-30/ coronin plasmid was induced with isopropyl β-D-thiogalactoside. After the cells were disrupted by sonication, the precipitate obtained was dissolved in 20 mM sodium phosphate (pH 7.4) containing 0.5 M NaCl, 40 mM imidazole, and 8 M urea. The suspension was then applied to a His Trap HP column (GE Healthcare). The signals from the labeled probe were visualized by treating the membrane with CDP-star chemiluminescent detection reagent (GE Healthcare) and exposing them to X-ray film.

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**Fig. 1.** Deduced Amino Acid Sequence from the cDNA of *P. polycephalum* Coronin Aligned with That of *D. discoideum.*

White characters against a black background show identical amino acids. Underlining indicates the five predicted WD repeats. Asterisks (*) below sequence indicate the coiled-coil motif.

**Fig. 2.** Southern Analysis of *Physarum* Genomic DNA.

Genomic DNA was digested with five restriction enzymes. Approximately 10 μg of gDNA per lane was subjected to electrophoresis on a 0.8% agarose gel and was transferred to Hybond N+ membrane (GE Healthcare). The probe for analysis was labeled with alkaline phosphatase using an Alkphos Direct Kit (GE Healthcare). The results were then applied to a His Trap HP column (GE Healthcare, Buckinghamshire, UK). The purified coronin was injected into a rabbit to prepare a coronin-specific polyclonal antibody. After the antibody was purified by Protein G column chromatography (GE Healthcare), it was used to examine the life-cycle stage-specific expression of coronin by Western analysis. The reactivity of coronin-specific antibody was examined by Western blot (Fig. 3A). The antibody also reacted with an extra band, of about 20 kDa, except for 50 kDa coronin. Because the band was detected in the
crude extract of control *E. coli* (Fig. 3A, lane 1), it was thought that the band resulted from an unspecific reaction. To analyze coronin expression, the microplasmodium, plasmodium, and amoebae were cultured. Sclerotia and spores formed, as reported previously. As shown in Fig. 3, coronin was found in diploid microplasmodia, plasmodium, and sclerotia. Similarly to *D. discoideum* haploid amoebae, we expected that *P. polycephalum* amoebae would express coronin protein, but coronin expression was not detected in the haploid spores or amoebae. It is possible that cytoskeletal dynamics, like cell migration and phagocytosis in *P. polycephalum* amoebae, are carried out by other proteins, such as tectonin, which is present in *P. polycephalum* and contains six tandem repeats similar to WD repeats. Because we have confirmed that *P. polycephalum* amoebae express tectonin (Y. Minami, unpublished data), it might act as a substitute for coronin. On the other hand, the microplasmodia and plasmodia expressed coronin, and they have a coronin-actin system. Humphries et al. have reported that overexpression of coronin induced growth repression in *Saccharomyces cerevisiae*. Furthermore, de Hostos et al. have reported that *D. discoideum* mutants lacking coronin grow and migrate more slowly than wild-type cells. To elucidate its function, *P. polycephalum* coronin cDNA was incorporated into a vector and then expressed in *Saccharomyces cerevisiae* in this study. However, expression of *P. polycephalum* coronin in the yeast cells did not bring about a clear change in their growth or morphological forms (data not shown). Although it is not clear how coronin functions in diploid cells, it is likely that *P. polycephalum* coronin functions differently than *Saccharomyces* coronin. The difference in coronin expression between *P. polycephalum* and *D. discoideum* amoebae provides information regarding the function of coronin.

References