A Coronin7 Homolog with Functions in Actin-driven Processes*

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Dictyostelium discoideum Coronin7 (DdCRN7) together with human Coronin7 (CRN7) and Pod-1 of Drosophila melanogaster and Caenorhabditis elegans belong to the coronin family of WD-repeat domain-containing proteins. Coronin7 proteins are characterized by two WD-repeat domains that presumably fold into two β-propeller structures. DdCRN7 shares highest homology with human CRN7, a protein with roles in membrane trafficking. DdCRN7 is present in the cytosol and accumulates in cell surface projections during movement and phago- and pinocytosis. Cells lacking CRN7 have altered chemotaxis and phagocytosis. Furthermore, loss of CRN7 affects the infection process by the pathogen Legionella pneumophila and allows a more efficient internalization of bacteria. To provide a mechanism for CRN7 action, we studied actin-related aspects. We propose that the CRN7 role in chemotaxis and phagocytosis is through its effect on the actin cytoskeleton.

Coronins are highly conserved proteins of the WD-repeat domain superfamily with representatives in all eukaryotes with the exception of plants and distant protists. Structurally they are characterized by WD (tryptophan-aspartic acid) repeats that form a β-propeller as in the -subunit of heterotrimeric G-proteins followed by a coiled coil domain that mediates oligomerization. CRN7 also associated with F-actin in vivo. It was present in the Triton X-100-insoluble cytoskeleton, colocalized with F-actin, and its distribution was sensitive to drugs affecting the actin cytoskeleton. We propose that the CRN7 role in chemotaxis and phagocytosis is through its effect on the actin cytoskeleton.

1 The abbreviations used are: CRN7, coronin7; GFP, green fluorescent protein; GST, glutathione S-transferase; m.o.i., multiplicity of infection; PST, proline, serine, and threonine-rich; TRITC, tetramethylrhodamine isothiocyanate; PIPES, 1,4-piperazinediethanesulfonic acid; mAb, monoclonal antibody.

2 The online version of this article (available at http://www.jbc.org) contains supplementary Movies 1 and 2.

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ting to membranes, tyrosine phosphorylation of CRN7 is required, which most likely depends on src kinase, because the broad-range tyrosine kinase inhibitor genistein and the specific Src inhibitor SU6656 led to a redistribution of CRN7 to the cytosol (14–16).

We used *D. discoideum* to study the role of CRN7 in a lower eukaryote and to reveal the primordial function of CRN7 proteins. *D. discoideum* is a single-celled amoeba that lives on the soil feeding on bacteria and yeast. It is a professional phagocyte using chemotaxis to find its prey and phagocytosis to take up its food source. It shares these properties with neutrophils and has been used as a convenient model system to explore the underlying mechanisms (17, 18). Chemotaxis is also used to organize multicellular development. From an evolutionary point of view *Dictyostelium* deviated from the animal fungal lineage after the plant animal split (19). However, despite this early divergence, it shares many more genes with animals than do fungi. Throughout the text we will use the recently proposed nomenclature for coronin proteins (20).

**EXPERIMENTAL PROCEDURES**

**Development, Growth, and Transformation of Dictyostelium**—Strains were either grown on a lawn of Klebsiella aerogenes on SM agar plates or cultivated in a shaking suspension or in a submerged culture at 21 °C in axenic medium (21). Development was initiated by plating 5 × 10^5^ cells/ml on a phosphate agar plate and monitored over 24 h. Every 30 min pictures were taken. Mutants were maintained in the presence of 5 μg/ml blasticidin (MP Biomedicals Inc., Eschwege, Germany) or 10–20 μg/ml of G418 (Roche Applied Science). corB^-^ cells were generated using a replacement vector. Transformants were screened by PCR and confirmed by Southern and Northern blot analysis. Cell lines used were A(2x) (wild type), corB^-^, and A(2x) expressing GFP-CRN7 (residues 1–962), GFP-CRN7N (1–393), GFP-CRN7C (511–962), GFP-CRN7N+PST (1–510), GFP-CRN7C+PST (394–962), and GFP-CRN7PST (394–510). The cDNAs were cloned either into pDDEXH79, conferring G418 resistance (22), or pBsrN2GFP, conferring blasticidin resistance.

**GST Fusion Protein Expression and Purification**—Full-length CRN7 and the N- and C-terminal deletion constructs of CRN7 and the proline-, serine-, and threonine-rich (PST) domain were retrieved from the pGEM-T^-^ easy vector with NotI or BamHI and fused to the C terminus of GST using pGEX-4T expression vectors (GE Healthcare). For protein expression *Escherichia coli* BL21 (DE), DH5α, XL1 blue, or Arctic Express RIL (Stratagene) were used. Induction was done with 0.5 mm Isopropyl β-D-thio-galactoside when an A_600 of 0.6 was reached. Culturing was continued for further 5 h at 37 °C, room temperature, or 10 °C (Arctic Express). The cells were lysed, and the lysates were separated into soluble and insoluble fractions. The fusion proteins were purified from the soluble fraction, and the GST tag was subsequently removed by thrombin cleavage.

**Generation of Coronin Mutants**—To construct the corB replacement vector, the full-length cDNA fragment was cloned into pGem-T easy (Promega). A 250-bp fragment was removed from the N-terminal WD-repeat stretch using BglII and XbaI and replaced by the 1.4-kb blasticidin cassette (23). The vector was transformed into Ax2 cells. Colonies were selected with blasticidin (3.5 μg/ml) and tested by PCR and Southern blot analysis. PCR primers used were Bract15Rp (GATGGGAT-TAATTTGGTATC) and Coro7–18f (GGATCCATGTTTAAAGTATCAAATAGACACACA). As probes in Southern blot analysis, a blasticidin gene sequence of ~300 bp in length was used. A further probe of 540 bp was derived from gene DDB0215560 located 5' to corB. To amplify this probe we used the forward DDB021-f primer (AAGAGTCATTTGCAATAACTTTCTTGG) and the reverse DDB021-r primer (CTCTTGATCTGATCATAATGG). For each cell biological analysis of the mutant cells, each experiment was done at least three times.

**Phagocytosis and Pinocytosis Assays**—Quantitative phagocytosis of TRITC-labeled heat-killed yeast cells was performed as described (5). For quantitative analysis of small particle uptake, yellow fluorescent 1-μm latex beads (Fluoresbrite™ carboxyl NYO 1.0-μm microspheres from Polysciences Europe GmBH) and rhodamine-labeled *L. pneumophila* were used. *Dictyostelium* cells were harvested and adjusted to a density of 2 × 10^6^ cells/ml. Latex beads were added to the cells corresponding to a 200:1 ratio relative to *Dictyostelium* cells, yeast particles in a ratio of 6:1, and bacteria in a ratio of ~500:1. Relative fluorescence was determined for different time points (24). Fluid phase pinocytosis was carried out as described (5, 25).

For infection with *L. pneumophila* Ax2, wild type and mutant cells were cultured in HL5 medium (26). For infection, 25 ml of HL5 medium were inoculated with 1.5 ml of the pre-cultured cells. The cells were grown for 3 days at 24.5 °C to a density of about 5 × 10^9^ to 1 × 10^6^ cells/ml. Cells were harvested (300 × g, 7 min, room temperature) and washed with Sorensen phosphate buffer. The cells were adjusted to a density of 1 × 10^6^ cells/ml in infection medium (1:1 mixture of HL5 and phosphate buffer). 300 μl of cells were transferred per well of a 24-well plate and allowed to settle down for 30 min at room temperature.

*L. pneumophila* Corby (27) was cultured on BCYE plates (buffered charcoal yeast extract agar: 10 g/liter yeast extract (BD Difco), 5 g/liter N-(2-acetamido)-2-aminoethanesulfonic acid (GERBU), 2 g/liter activated charcoal, 1.5% agar (BD Difco), pH 6.9, with KOH (Roth) and was completed by adding 0.25 g/liter Fe(III)NO3_9H2O and 0.4 g/liter cysteine) for 3 days at 37 °C and a CO2 concentration of 5%. The bacteria were harvested in 1 ml of H2O and adjusted to a density of 1 × 10^7^ colony-forming units/ml.

The medium was removed from the cells and replaced by 300 μl of infection medium with a bacteria multiplicity of infection (m.o.i.) 10. After 3 h at 25.5 °C the supernatant was removed, and extracellular bacteria were washed out with phosphate buffer and infection medium containing 100 μg/ml gentamicin (stock solution, 10 mg/ml in H2O, O). Afterward, the cells were allowed to recover for 45 min in freshly added infection medium containing 100 μg/ml gentamicin at 25.5 °C to get rid of all extracellular bacteria. The dead bacteria and gentamicin were removed by washing with phosphate buffer. Cells were covered with 1 ml of infection medium for the rest of infection. At the indicated time points (3 h = colony-forming units
immediately after gentamicin treatment) cells were harvested and transferred to 1.5-ml tubes. Solubilization of the cells was ensured with centrifugation steps of 12,000 × g for 6 min at room temperature and through strong mechanical force (vortex). Dilutions of these homogenates (in H2O) were prepared, and aliquots were plated on BCYE plates for 4 days at 37 °C and 5% CO2 to determine the colony-forming unit values.

Adhesion Assay—For cell substrate adhesion 1 × 10⁶ cells in Ax2 growth medium were added per well (Nunclon 142475, lot 101491). Incubation was for 4 h without shaking. Then plates were shaken on a rotary shaker for 1 h at 65 rpm followed by 1 h at 120 rpm and one more hour at 200 rpm. The number of cells detached was determined in a hemocytometer. Significant detachment was observed after shaking at 200 rpm. At the end of the experiment the total number of cells was determined.

Analysis of Cell Shape and Cell Migration during Chemotaxis—Analysis of cell shape and cell migration was done as described (28). Chemotaxing GFP-CRN7-expressing cells were observed under a cAMP gradient in a chemotaxis chamber (μ-slide chemotaxis hydrophobic, uncoated, and sterile; ibidi, Martinsried, Germany).

Determination of the cAMP-induced Actin Polymerization Response—F-actin levels upon stimulation were determined using TRITC-phalloidin (29). Aggregation-competent cells were resuspended at 2 × 10⁶ cells/ml and stimulated with 1 μM cAMP, and 50-μl samples were taken at various time points. Cells were immediately lysed in lysis buffer (3.7% formaldehyde, 0.1% Triton X-100, 0.25 μM TRITC-phalloidin (Sigma), 10 mM PIPES, pH 6.8, 5 mM EGTA, 2 mM MgCl₂) and incubated for 1 h in the dark and then centrifuged at 10,000 × g for 5 min. Pellets were extracted with methanol overnight, and fluorescence (540-nm excitation/565-nm emission) was measured with a fluorimeter. To test for association of CRN7 with the F-actin, extracts were precleared by centrifugation at 120,000 × g for 15 min. At the end of the experiment, the total number of cells was determined.

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Western Blotting and Immunofluorescence Analysis—For generation of CRN7-specific monoclonal antibodies, a stretch of DNA encoding the PST domain (amino acids 394–510) was amplified and cloned into a pGEX-4T GST expression vector. Thrombin-cleaved PST domain was used for immunization of mice (31). mAbs K67-146-1 and K67-31-5 have been used in this study. GFP, CRN7, severin, α-actin, pspA, actin, and contact site A (csA) were detected with monoclonal antibodies K3-184-2 (32), K67-146-1 (this work), 102-200-1, 47-16-1 (33, 34), mud1 (35), act1–7 (36), and 33-294-17 (37), respectively. Secondary antibodies used were anti-mouse IgG peroxidase or anti-mouse IgG cy3 conjugate (Sigma).

Fixation of cells was either with cold methanol (−20 °C for 10 min) or picric acid paraformaldehyde (15 min with 4% paraformaldehyde at room temperature) for TRITC- or fluorescent isothiocyanate-phalloidin staining. Live cell imaging was performed with cells expressing GFP-fused proteins. Cells were monitored using a confocal microscope (Leica, Wetzlar, Germany). To investigate the effect of cytochalasin A, cells on coverslips were incubated for 30 min in the presence of 20 μM cytochalasin A (Sigma) before fixation. Control cells were treated with corresponding DMSO concentrations (solvent control).

Cell Fractionation and Analytical Gel Filtration Analysis—GFP-CRN7 cells were collected by centrifugation and resuspended at a density of 2 × 10⁶/ml. Subcellular fractionation was performed according to standard protocols (38). The cells were washed 3 times with cold homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 250 mM sucrose, pH 7.4) and homogenized with a syringe and a 22G/1 needle. Alternatively, lysis was achieved by passage of the cells through nucleopore filters (GE Healthcare). Cytosolic and membrane fractions were separated by ultracentrifugation (10,000 × g and 100,000 × g for 15 min) and analyzed by SDS-PAGE.

Isolation of the cytoskeleton containing detergent-insoluble fraction was performed by resuspending the cells in 10 volumes of ice-cold cytoskeletal isolation buffer (1% Triton X-100 in 80 mM PIPES, pH 6.8, 5 mM EGTA, 1 mM MgCl₂) supplemented with a protease inhibitor mixture (Roche Applied Science). Lysis was controlled by light microscopy. Cells were incubated for 20 min on ice followed by 10 min at room temperature. The lysate was separated into a Triton X-100-insoluble pellet and supernatant by centrifugation at 10,000 rpm for 15 min. For analysis, cell equivalents were separated by SDS-PAGE, blotted, and immunostained for different antigens.

For analytical gel filtration analysis, cell-free extracts from Ax2 cells and purified CRN7NPST protein were used. Analytical gel filtration was done using a Superdex-75 and Superdex-200 column. Probes were separated by SDS-PAGE and stained with Coomassie Blue. Endogenous CRN7 from Ax2 cell-free extracts was blotted and labeled with K67-146-1.

F-actin Interaction Assays—All CRN7 proteins used in this study were preclerased by centrifugation at 120,000 × g for 1 h at 4 °C. Dictyostelium actin isoform and actin sedimentation assays were performed as described (39–42). CRN7 proteins and actin were incubated for 30 min at room temperature in 1× polymerization buffer (10×: 100 mM imidazole, pH 7.6, 20 mM MgCl₂, 2 mM CaCl₂). To evaluate the binding ratio between CRN7NPST and actin in more detail, we performed a Scatchard plot analysis. To mimic physiological conditions, we added potassium glutamate (100 mM) to the standard polymerization buffer. The concentrations of protein and actin were determined photometrically at 290 nm and by gel analysis with standards of bovine serum albumin. Actin was polymerized in 50-μl samples with a final concentration of 5 μM and increasing concentrations of CRN7NPST up to 10 μM. Binding to F-actin was determined by high-speed centrifugation at 100,000 × g for 1 h. The pellets were solubilized in the original volume, equal amounts of pellet and supernatant were resolved by SDS-page (10% acrylamide), and proteins were visualized by Coomassie Brilliant Blue R-250 staining. To quantify the co-sedimentation of CRN7NPST with actin, the protein bands were scanned and calculated relative to co-sedimented F-actin (ImageJ). Sedimentation of F-actin was tested also in low-speed centrifugations (15,000 × g, 30 min) to test for F-actin bundling activity of the polypeptides (39). In vitro actin binding and depolymerization assays were carried out as described (41, 42). Microscopic examination of actin filaments was done according to Korneeva and Jockusch (43).
RESULTS

**CRN7 Characterization and Mutant Generation**—CRN7 is encoded by the *corB* gene of *D. discoideum* located on chromosome 1. It is a 962-amino acid protein with a molecular mass of 105,000 harboring two WD40-repeat domains (position 21–332 and 551–840) made from seven antiparallel four-stranded β-sheets each that presumably fold into a β-propeller structure. The two domains are separated by a proline-, serine-, and threonine-rich stretch (PST domain, residues 394–510) (Fig. 1A). The second WD repeat is followed by a unique domain of 122 amino acids that contains a C-terminal acidic domain and ends with EWD as do all vertebrate CRN7 proteins. DdCRN7 is most closely related to human CRN7 (29% identity) with which it also shares the PST domain followed by *C. elegans* POD-1 (24% identity) and the less well conserved *Drosophila* Pod-1. Also, the *C. elegans* and *Drosophila* proteins do not harbor a PST domain. Instead, in both proteins the two WD40-repeat domains are separated by unrelated sequences.

The *corB* gene was expressed as an ~3-kb mRNA present throughout development (Fig. 1B). At the protein level, the 105-kDa CRN7 was detected at all stages of the *D. discoideum* life cycle as revealed with monoclonal antibodies (Fig. 1C). Immunofluorescence analysis revealed that the protein is present in the cytosol and at the cell cortex where it co-localized with F-actin (Fig. 1D). Full-length GFP-tagged CRN7 (GFP-CRN7), which is overexpressed with about six-fold higher levels than the endogenous protein (Fig. 1G) exhibited a similar distribution. In live cell imaging it accumulated strongly at the leading edge of the cells (Fig. 1E), and during chemotactic migration in a cAMP gradient it redistributed always to the front (supplemental Movie 1 shows GFP-CRN7-expressing cells moving in a cAMP gradient; GFP-CRN7 is always seen in the leading front). The role of individual domains for subcellular distribution was identified by expressing distinct CRN7 domains as GFP fusion proteins (Fig. 1A). We found that the information for the localization resides within the first 393 amino acids of the protein as this protein showed the CRN7-specific distribution, whereas the C-terminal WD40-repeat

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**FIGURE 1.** *Expression and distribution of CRN7.* A, shown is a diagram depicting the domain organization of CRN7 (dictyBase gene number DDB0190225) and GFP-tagged truncated CRN7 proteins. The corresponding amino acid positions are given. B and C, shown is CRN7 transcript and protein accumulation during development (in hours). RNA was analyzed by reverse transcription-PCR. The loading control in B is Ig7, a constitutively expressed mitochondrial rRNA. For Western blot analysis whole cell homogenates (2 × 10^5 cells per lane) were probed with mAb K67-146-1. Actin detected with mAb act1–7 is shown for control. D, CRN7 colocalizes with F-actin. CRN7 was detected with mAb K67-31-5 and F-actin with fluorescein isothiocyanate (FITC) phalloidin. Fixation was done using paraformaldehyde. Bar, 3 μm. E, shown is localization of GFP-tagged full-length CRN7 and GFP-tagged CRN7 domains analyzed by live-cell imaging. Arrowheads indicate accumulation of the proteins at macro- and pinocytic cups and in the leading front. F, shown is subcellular fractionation. CRN7 was detected with mAb K67-146-1; α-actinin was used for control. L, lysate; P1, and S1, supernatant and pellet fraction, respectively, after 10,000 × g spin; P2, and S2, supernatant and pellet fraction, respectively, after 100,000 × g spin. G, a Western blot analysis shows the levels of endogenous and GFP-tagged CRN7 using monoclonal antibody K67-146-1.
domain (residues 394–962) was uniformly dispersed throughout the cytosol (Fig. 1E).

Conventional coronins harbor an oligomerization domain at their C termini. Such motifs are missing in Coronin7 proteins. Because they have two WD-repeat domains, dimerization may not be needed. When we tested the oligomerization state of CRN7 by carrying out analytical gel filtration analysis, we found that CRN7 eluted as a monomer (data not shown).

An association of CRN7 with membranes could not be demonstrated. In differential centrifugation experiments of Ax2 cell lysates that had been generated by passage through a nucleopore filter, the majority of CRN7 was present in the supernatant fraction after a low speed spin (10,000 × g) and remained in the supernatant after centrifugation at 100,000 × g. α-Ac-tinin, a cytosolic F-actin cross-linking protein, showed a similar distribution (Fig. 1F). We conclude that CRN7 is primarily a soluble protein.

**Generation and Characterization of corB Cells**—To address the in vivo function of CRN7, we generated corB mutant cells by introducing a gene replacement vector into Ax2 wild type cells. Genomic DNA extracted from transformed cells was analyzed by Southern blotting. Almost all clones tested were positive for substitution of the endogenous corB gene by the replacement vector. In Western blot analysis of total protein extracts no CRN7 protein was detected, and Northern blot analysis revealed the absence of mRNA (Fig. 2, A–E).

Mutant cells were slightly smaller than wild type and were mostly mononucleated as were Ax2 cells (Fig. 2F). We analyzed the growth behavior under different conditions, uptake of fluid nutrient and of particles as well as chemotaxis, which are essential for Dictyostelium growth and development. We found that in axenic medium corB cells grew more slowly and did not reach the same final densities at the stationary phase as Ax2 (8 × 10⁶ cells).
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CRN7 Role in Phagocytosis—Phagocytic pathways in Dictyostelium are similar to the ones in mammalian cells (44–47). We analyzed the distribution of CRN7 during phagocytosis by performing a live cell analysis of GFP-CRN7-expressing cells and carried out quantitative measurements of phagocytosis of corB− cells. GFP-CRN7 accumulates at the phagocytic cup during uptake of TRITC-labeled yeast particles and E. coli. During engulfment of yeast particles GFP-CRN7 accumulates at the nascent phagocytic cup with enrichment at the tips, suggesting that CRN7 may be involved in the final closure of the cup, whereas bacteria are surrounded by a homogenous coat of GFP-CRN7 (Fig. 4, A and B, arrowheads; supplemental Movie 2 shows GFP-CRN7 accumulating at the phagocytic cup upon attachment of a yeast particle and then released after complete engulfment). Once yeast and E. coli cells were completely ingested, they were no longer surrounded by a coat of CRN7 (Fig. 4B, arrow). Quantitative analysis revealed that corB− cells internalized yeast particles at a 2-fold higher rate than control Ax2 and GFP-CRN7-expressing cells (Fig. 4C). The phagocytosis defect was completely reverted to normal when GFP-tagged full-length CRN7 was expressed (data not shown).

To see if this phenotype is either due to an increased uptake of yeast particles by single cells or an increase in the number of phagocytosing cells, the number of intracellular yeast particles after 15 min of phagocytosis was determined. Analysis of more than 400 cells per cell line showed that in the case of corB− more cells (75%) contained one or two yeast particles as compared with Ax2 (55%). We also assayed the uptake of latex beads, which have different surface properties and were smaller in size (1 μm). Uptake was reduced in the mutant to ~40% of wild type (Fig. 4D), whereas phagocytosis of dead rhodamine-labeled L. pneumophila was enhanced in the mutant and was nearly twice as high as in Ax2 (Fig. 4E).

As uptake of particles also correlates with adhesion, we tested the substrate adhesion of corB− and Ax2 wild type cells. We found that corB− cells adhered more strongly to a plastic surface than wild type. Whereas ~68% of wild type cells were detached after shaking at 200 rpm for 1 h, only ~31% of mutant cells were detached.

CRN7 Is Involved in the Uptake of L. pneumophila—In recent years D. discoideum has been used as surrogate host to study infection of cells by pathogenic bacteria like Legionella or Mycobacterium (7, 8). GFP-CRN7-expressing cells infected with L. pneumophila showed an association of the GFP fusion protein at the sites of pathogen uptake, implying a role for CRN7 in this process (Fig. 5A). Next we tested the corB− strain in standard infection assays using L. pneumophila Corby. D. discoideum cells seeded into 24-well plates were infected with L. pneumophila at an m.o.i. of 10. After 3 h, extracellular bacteria were killed by gentamicin and removed by washing. The number of intracellular bacteria and bacterial replication over 4 days was monitored.

The most remarkable difference noted between mutant and wild type was in the number of intracellular bacteria recovered after gentamicin treatment. After 3 h, the corB− mutant had internalized about 8-fold more bacteria than Ax2 (Fig. 5B). Similar results were obtained in quantitative phagocytosis experiments of rhodamine-labeled dead L. pneumophila (Fig. 4E). In

FIGURE 3. Growth behavior of corB− cells. A, growth in axenic medium is altered. 2 × 10^5 cells/ml were used for inoculation. Over a period of 8–10 days, the cells were counted. The mutant cell line did not reach the same final density at the stationary phase as Ax2. B, the mutant cell line grown with E. coli B/r in shaking suspension also did not reach the same final density as Ax2. C, mutant cells plated with Klebsiella on SM plates displayed colonies smaller in diameter than wild type. The experiments were carried out more than six times. The independent experimental data were averaged and are depicted in the diagram. The error bars are very small and can hardly be seen.
this experiment the increase of phagocytosed bacteria was \( \approx \) 2-fold higher than in Ax2. Despite the higher uptake rate of corB\(^{-}\), the ensuing course of infection was comparable, indicating that the intracellular replication of the bacteria is not further influenced by the mutation (data not shown). To test whether the increased number of intracellular bacteria observed in the corB\(^{-}\) mutant after 3 h of infection was due to higher phagocytosis rates or due to less killing of internalized bacteria in the initial hours of infection, the infection assay was slightly modified. Fifteen minutes after infecting the cells with an m.o.i. of 10, gentamicin was added, and the extracellular bacteria were killed as described. Then the bacterial colony-forming units were assessed 3, 24, 48, and 98 h after infection, and the change in intracellular bacteria was normalized for each strain to the t3 value, which is the value observed 3 h post-infection. The difference between the wild type and the corB\(^{-}\) mutant remained, indicating that CRN7 mainly interferes with uptake of \( L. \) pneumophila (Fig. 5C). GFP-CRN7 overexpression supports this notion as the cells have a phenotype converse to corB\(^{-}\) (Fig. 5, B and C).

**corB\(^{-}\) Cells Show Directional Change Defects in Chemotaxis**—Chemotaxis is essential for \( D. \) discoideum biology. We, therefore, tested the chemoattractant-induced cell migration behavior of aggregation-competent corB\(^{-}\) and Ax2 cells to examine whether CRN7 had any effect on migration. During migration toward an exogenous cAMP source, Ax2 cells and Ax2 cells expressing GFP-CRN7 are well polarized, form pseudopodia almost exclusively at the front, and migrate in a highly directed fashion toward the cAMP source (supplemental Movie 1). corB\(^{-}\) cells were less polarized, and pseudopod extension initially occurred in a more random fashion. Speed, persistence, and roundness differed slightly but not significantly from Ax2, whereas the value for the direction change, which represents the average change of angle between frames in the direction of movement, was significantly higher in the mutant (Table 1, Fig. 6, A and B). This was confirmed by counting the lateral pseudopods of a representative number of cells. corB\(^{-}\) cells produced more lateral pseudopods with respect to wild type and GFP-CRN7 overexpressor, indicating this to be the possible reason for speed reduction and directional change increase (Fig. 6C).

Underlying these motile events are changes in the actin cytoskeleton. One of the first responses to a hormone stimulus is a peak of actin polymerization where the amount of F-actin

![Figure 4](image)
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**TABLE 1**

Analysis of chemotaxis

Images were taken at 40× magnification every 30 s. In all cases cells were analyzed over a period of 30 min. Cells were recorded, and after tracing of the cells, the centroid of the cells was determined by computer-assisted analysis (DIAS). This allows calculations of speed, roundness (ratio of the long and short axis of the cell), and direction change. Persistence is an estimation of movement in the direction of the path; direction change represents the average change of angle between frames in the direction of movement. The data shown here are derived from five independent experiments. The data from ~30 cells were used for statistic evaluation. The statistical significance with respect to wild type Ax2 cells was calculated using a t test. NS, not significant at \( p > 0.05 \).

|                      | Ax2        | GFP-CRN7   | corB<sup>a</sup> |
|----------------------|------------|------------|------------------|
| Speed (\( \mu \text{m/min} \)) | 8.94 ± 0.88 (NS) | 8.94 ± 1.88 (NS) | 7.58 ± 1.71 (NS) |
| Persistence (\( \mu \text{m/min-degree} \)) | 3.08 ± 0.97 (NS) | 3.82 ± 1.14 (NS) | 2.7 ± 1.27 (NS) |
| Roundness (%)         | 45.19 ± 6.37 (NS) | 54.5 ± 7.15 (NS) | 61.79 ± 6.71 (NS) |
| Direction change (degree) | 15.46 ± 6.07 (NS) | 13.89 ± 6.29 (NS) | 25.69 ± 10.56<sup>a</sup> |

<sup>a</sup> \( p < 0.01 \).

CRN7 Associates with the Actin Cytoskeleton—Phagocytosis, phagocytosis, and chemotaxis depend on the actin cytoskeleton. We, therefore, tested whether CRN7 contributes directly to the regulation of the actin cytoskeleton. This might then provide a mechanism for its action in these events. We tested a possible cytoskeletal association by preparing Triton-insoluble cytoskeletons from Ax2 cells. Immunoblot analysis of pellet and supernatant fractions revealed an enrichment of CRN7 in the detergent-insoluble fraction, suggesting its association with the actin cytoskeleton. For control, we used α-actinin that largely stays in the supernatant (Fig. 8A). To analyze the cytoskeletal association of CRN7 further, the actin cytoskeleton was disrupted in GFP-CRN7-expressing cells with cytochalasin A. After incubation, cells were fixed, immunolabeled with anti-actin mAb act1−7, and analyzed by confocal microscopy. In control cells treated with solvent only, GFP-CRN7 co-localized with actin and was enriched in the cell cortex. Drug-treated cells showed an altered actin distribution with an accumulation inside the cell. CRN7 still colocalized with the actin structures, suggesting that CRN7 associates with the actin cytoskeleton (Fig. 8B).

We next tested whether CRN7 not only associates with the F-actin cytoskeleton but does so in a regulated manner after a cAMP stimulus. We carried out the experiments with Ax2 cells that had been starved for 6 h and stimulated temporally with 1 \( \mu \text{M} \) cAMP. Samples were taken before and between 5 and 120 s after the stimulation, and Triton X-100-insoluble cytoskeletons were prepared. The proteins were separated by gel electrophoresis and analyzed by Western blotting using mAb K67-146-1. We found that CRN7 was present in the Triton X-100-insoluble actin cytoskeleton and that its accumulation paralleled the one of F-actin (Fig. 7, A and B).

CRN7 Binds Directly to F-actin and Protects from Depolymerization—Based on the F-actin association observed in the above studies, we directly assayed an F-actin interaction of CRN7 in co-sedimentation assays using recombinant CRN7 polypeptides and Dictyostelium actin. In these assays we employed full-length CRN7 (GST–CRN7), the N-terminal WD repeat including the PST domain (CRN7NPST, residues 394–146), and the C-terminal WD repeat (GST–CRN7CPST, residues 394–962). The polypeptides were expressed as GST fusions and used nearly doubles. This is followed by a rapid depolymerization and another longer-lasting but lower F-actin peak that coincides with pseudopod extension and is thought to be due to the regulated action of F-actin cross-linking proteins such as filamin (29, 30, 48). When we assayed the F-actin response to a cAMP stimulus, Ax2 and corB<sup>−</sup> cells showed the typical response with a first sharp peak within the first 10 s and a second smaller peak of F-actin polymerization. In corB<sup>−</sup> cells depolymerization might be slightly altered. GFP-CRN7 overexpression did not have an effect on the actin polymerization response (Fig. 7A).
FIGURE 6. Chemotactic movement of corB<sup>−</sup> cells. A, shown are computer-generated cell tracks and stack images of corB<sup>−</sup> and Ax2-expressing GFP-CRN7 in comparison to Ax2. Aggregation-competent cells were deposited on coverslips and after settling down challenged with cAMP. The star indicates the location of the pipette filled with cAMP. The cell perimeters were outlined and are shown for two representative cells each. Bar, 8 μm. B, cell shape changes of aggregation-competent cells were analyzed by DIAS software. Images were taken every 10 s. The outlines were traced manually, and the changes of direction (arrows) were calculated using the DIAS image analysis software. The green areas indicate new membrane protrusions, and the red areas indicate retractions. C, shown is lateral pseudopod formation of wild type and mutant cell line. Arising lateral pseudopods were counted from more than 60 single cells of each cell line monitored during the first 10 min while migrating in a cAMP gradient.
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FIGURE 7. CRN7 effect on actin polymerization. A, F-actin polymerization response upon cAMP stimulation is shown. Samples were taken at the indicated time points after stimulation with 1 μM cAMP. The amount of F-actin was normalized relative to the F-actin level of unstimulated cells. The relative F-actin content was determined by fluorimetric measurements of TRITC-phalloidin. The data are the average of six independent experiments for each cell line. B, shown is association of CRN7 with the actin cytoskeleton upon a CAMP stimulus. A maximum increase in protein levels of endogenous CRN7 was observed after 5 s of stimulation corresponding to the F-actin changes. The data represent the average of seven independent experiments.

after cleavage with thrombin from the GST part except for full-length CRN7 and CRN7CPST, which could not be cleaved with thrombin and were used as GST fusion proteins in the assays. In control experiments G-actin was polymerized and sedimented at 100,000 × g, whereas the CRN7 polypeptides with the exception of CRN7CPST under the same conditions mainly stayed in the supernatant. In co-sedimentation experiments GST-CRN7 and CRN7NPST co-sedimented with F-actin, whereas the PST domain and the C-terminal WD domain did not co-sediment (Fig. 8C). These results indicate that the N-terminal WD domain of CRN7 harbors an F-actin binding site. We then tested whether CRN7NPST has F-actin bundling activity and performed the sedimentation at low g forces (15,000 × g) (39). CRN7NPST stayed in the supernatant together with F-actin, indicating that the protein does not bundle actin filaments (Fig. 8D). GST-CRN7 also did not have a bundling activity (data not shown).

To determine the stoichiometry of the interaction of CRN7NPST with F-actin, we used increasing concentrations of the recombinant protein keeping the actin concentration constant. The amount of protein in the supernatants and pellets was determined by densitometric analysis of Coomassie Blue-stained SDS-polyacrylamide gels and then analyzed using the method of Scatchard (Fig. 8E). The quantity of CRN7NPST in the pellet increased up to a ratio of 1:1 (CRN7NPST to actin). Thereafter, the amount in the pellet stayed constant. The data suggest that one CRN7NPST molecule binds to one actin molecule. A second lower affinity binding site for actin cannot be excluded. The observed binding behavior might lead to a stabilization of the filament and protection against depolymerization. We tested this directly by carrying out dilution-induced depolymerization assays using pyrene-labeled F-actin. The decrease of fluorescence was clearly inhibited at increasing concentrations of CRN7NPST. Disintegration of actin filaments started to be delayed at roughly equimolar concentrations of actin and CRN7NPST and was completely inhibited at a 5-fold molar excess of the actin binding domain (Fig. 8F). This behavior parallels the one of mammalian Coronin-1B (CRN1), a small coronin, which also decorated actin filaments and protected in vitro the actin filaments from dilution-induced depolymerization. In contrast, yeast coronin (CRN11) did not have a protective role, although it binds to the sides of actin filaments and shows a 1:1 saturation stoichiometry of coronin binding to actin (2, 49).

In addition, we investigated the interaction between CRN7NPST and F-actin by visualizing the filaments by immunofluorescence microscopy. In control experiments, the filament formation after 30 min of polymerization was not influenced by the addition of 10 μM bovine serum albumin (1:1 molar ratio) to the solution. However, the addition of 10 μM CRN7NPST (1:1 molar ratio) resulted in a strong increase of long actin filaments, suggesting that the polypeptide stabilizes filaments (Fig. 8G). This type of analysis as well as dilution-induced depolymerization assays were not successfully carried out in the case of full-length CRN7 and CRN7CPST as the proteins could only be isolated in low amounts from E. coli and could not be cleaved from the GST part.

DISCUSSION

Coronin7 proteins seem to have acquired different roles during evolution. For the C. elegans and D. melanogaster proteins, a function in cell polarity has been identified presumably mediated through its role in organizing specific aspects of the actin cytoskeleton, whereas human CRN7 mainly functions in trafficking processes in the Golgi apparatus (14). Our studies in D. discoideum for the first time also address an F-actin interaction of a Coronin7 protein in vitro in detail.

Cell fractionation studies of Dictyostelium lysates supported this interaction. They revealed that the majority of CRN7 is present in the cytosolic fraction as a monomer (data not shown). When we separated cytoskeletal from soluble fractions, CRN7 distributed between both fractions, and subcellular localization studies of endogenous and GFP-tagged CRN7 indicated a co-localization with the actin cytoskeleton and in particular with F-actin-rich structures that are formed around pino- and phagosomes. We identified the first WD-repeat domain as being responsible for this distribution using GFP-tagged fusion proteins. The results suggested the presence of an F-actin binding region that was confirmed by in vitro studies. The second WD-repeat domain did not participate in the F-actin association. For human CRN1 an actin binding site has been
mapped to the first WD repeat, where a surface-exposed arginine residue at position 30 appears to be crucial as the F-actin binding capability in vitro is strongly reduced upon mutation of this arginine residue to an aspartate (50). This residue and its surroundings are well conserved in mammalian and invertebrate small coronins. In DdCRN7 the arginine residue is replaced by an asparagine at position 26 with the surrounding sequence from amino acids 17 to 40 being well conserved. In Drosophila, C. elegans, and mammalian CRN7 this sequence is not conserved. The second WD-repeat domain of DdCRN7 does not harbor a region of homology. However, the suggestion that this amino acid stretch represents the F-actin binding site has to be taken with caution as recent studies indicate that a more extended region of CRN1 is in contact with actin molecules in the filament (49).

Disruption of the corB gene resulted in several phenotypic alterations. Although the corB/H11002 cells displayed no significant alterations in motility and polarization when we analyzed their behavior in a cAMP gradient, pseudopod extension initially occurred in a more random fashion. Instable pseudopods may result from a less stable actin network. Our findings that CRN7 associates with the Triton-insoluble cytoskeleton after a cAMP stimulus support such a notion. We propose that CRN7 is involved in actin dynamics during formation and extension of pseudopods and temporally associates with filaments that are formed and stabilizes them.

When we followed GFP-CRN7 in vivo we noticed a highly dynamic behavior during cell migration where the protein constantly relocated to the leading pseudopod. A similar dynamic behavior was observed during uptake of yeast particles and Legionella. In both instances CRN7 accumulated at the forming phagocytic cup and then quickly dispersed into the cytosol. An involvement of CRN7 in these processes was confirmed when we carried out quantitative analysis of uptake in corB− cells. Loss of CRN7 led to an increased uptake of yeast cells and Legionella, whereas uptake of latex beads was reduced. Growth rates on E. coli B/r and Klebsiella were not affected, pointing to an unimpaired uptake of these bacterial species.

**FIGURE 8.** Association of CRN7 with F-actin. A, Triton X-100-insoluble cytoskeletons of Ax2 cells are shown. CRN7 was detected with mAb K67-146-1. α-actinin and actin were used as a control. Detection was with mAb 47-16-1 and act1–7, respectively. L, lysate; P, Triton-insoluble pellet; S, supernatant. B, GFP-CRN7 distribution is sensitive to drugs that affect the actin cytoskeleton. Incubation with cytochalasin A (Cyt A, 20 μM) was for 30 min. DMSO represents the solvent control. GFP-CRN7-expressing cells were fixed and stained with mAb act 1–7. Bar, 8 μM. Co-sedimentation of CRN7 polypeptides with actin is shown. C, full-length CRN7 (GST-CRN7) and different CRN7 polypeptides (CRN7NPST, GST-CRN7CPST, CRN7PST) and actin were copolymerized and centrifuged at 100,000 g, and the pellets were solubilized in the original volume. The first two lanes show the sedimentation of the recombinant proteins in the absence of actin, and the second two lanes show the experiment with actin added. The experiments were carried out in parallel; the actin sedimentation is shown only once. D, low speed sedimentation of F-actin polymerized in the presence of CRN7NPST and centrifuged at 15,000 g for 30 min. All supernatants (S) and pellets (P) were subjected to and analyzed by SDS-PAGE. E, a Scatchard plot evaluates the molar ratio of binding of CRN7NPST to actin. Ratios from 0.2:1 to 2:1 (CRN7NPST to actin) were used in the experiment. Coomassie Blue-stained gels were scanned and quantified with ImageJ. F, shown is a dilution-induced F-actin depolymerization assay. Increasing concentrations of CRN7NPST were used. Pyrene-labeled F-actin was diluted below the critical concentration of the minus end, and the fluorescence decrease was monitored. G, shown is polymerization of actin filaments on coverslips stained with TRITC-phalloidin and incubated alone (A’), with bovine serum albumin (B’), and CRN7NPST in a molar ratio of 1:1 (C’). Bar, 1 μM.
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Data from previous work show that different mechanisms are employed during uptake of particles that differ in size and surface properties and that functionally independent binding sites for bacteria and other particles like yeast or latex beads exist on the cell surface of D. discoideum (51, 52). For example, E. coli cells lacking glucose residues on their surface were not recognized by a presumably lectin-type receptor and were not taken up (52). Mutants in Phg1p, a nine-transmembrane protein, caused a defect in phagocytosis of latex beads and E. coli but not of K. aerogenes (53), and a SadA mutant, which lacks another nine-transmembrane cell adhesion protein, did not take up latex beads (54). These reports show that differential adhesion will affect uptake of particles.

In these mutants the phagocytosis defect is frequently associated with reduced cell substrate adhesion, whereas in the CRN7-deficient cells we observed an increased adhesion to plastic surfaces and a decreased uptake of latex beads, which also have a hydrophilic surface. It is difficult to reconcile these results; however, the CRN7 effects might be due to its regulation of the actin cytoskeleton. Mutants with increased surface adhesion have not been tested for phagocytosis (55).

Several mutants with defects in cytoskeletal proteins also have altered adhesion properties (56). We can envision that alterations in the cortical actin cytoskeleton brought about by a factor. Several mutants with defects in cytoskeletal proteins also have altered adhesion properties (56). We can envision that alterations in the cortical actin cytoskeleton brought about by a factor.

Mechanisms that regulate the phagocytosis process play an important role in the host defense against invading microorganisms, and several studies have addressed the relationship between coronins and the intracellular survival of pathogens (57). TACO, the CRN4 homolog in mice, was found to be important for the lysosomal delivery of phagosomes containing Mycobacterium bovis. Here TACO remained associated with phagosomes containing live M. bovis, preventing phagosomes from fusing with lysosomes and allowing the mycobacteria to survive (58, 59). Also D. discoideum coronin (GFP-CRN12) was observed at the phagocytic cup upon internalization of L. pneumophila but was lost 60 s after internalization (8).

Up to now no long coronin has been implicated in innate immunity against natural pathogens as all previous studies involved exclusively small coronin proteins. We show that GFP-CRN7-overexpressing cells and corB− cells display a strong converse phenotype upon infection with L. pneumophila. Whereas GFP-CRN7-expressing cells display a reduced internalization and an increased replication rate of L pneumophila, corB− is more susceptible to internalization but restrains the replication rate to the one of Ax2. Additionally, internalization of L pneumophila in corB− is comparable with the increase of the phagocytosis rate in experiments with TRITC-labeled yeast cells. We propose that CRN7 plays a role in regulating internalization of these particles in a repressive manner. Our experiments reveal that the loss of CRN7 renders Dictyostelium more susceptible to L. pneumophila infection, making this protein an important component of the innate immunity system.

The mechanism for the CRN7 role in uptake processes and in chemotaxis is most likely provided by its impact on the dynamics of the actin cytoskeleton. CRN7 normally will prevent polymerization of filaments and might allow formation and stabilization of distinct F-actin structures that are built, for example, at a phagosome or in an extending pseudopod.

Comparing the activities of the long coronins, we conclude that CRN7 proteins from lower eukaryotes function in actin-regulated processes. In the case of Drosophila Pod-1, an additional function by cross-linking actin filaments and microtubules has been acquired, and for C. elegans CRN7 (POD-1), a role in regulation of trafficking processes by a so far unknown mechanism has been shown. The mammalian CRN7 has diverged farthest in terms of function. It is both a cytosolic and a membrane-associated protein and has roles in membrane trafficking. It will be interesting to see whether it also acts in innate immunity and whether its huge cytosolic pool might interact with F-actin as well.

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