Functional Surfaces on the Actin-binding Protein Coronin Revealed by Systematic Mutagenesis\textsuperscript{*S}

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Coronin is a conserved actin-binding protein that co-functions with ADF/cofilin and Arp2/3 complex to govern cellular actin dynamics. Despite emerging roles for coronin in a range of physiological processes and disease states, a detailed understanding of the molecular interactions of coronin with actin and other binding partners has been lacking. Here, we performed a systematic mutational analysis of surfaces on the yeast coronin $\beta$-propeller domain, which binds to F-actin and is conserved in all coronin family members. We generated 21 mutant alleles and analyzed their biochemical effects on actin binding and ADF/cofilin activity. Conserved actin-binding residues mapped to a discrete ridge stretching across one side of the $\beta$-propeller. Mutants defective in actin binding showed loss of synergy with ADF/cofilin in severing filaments, diminished localization to actin structures \textit{in vivo}, and loss of coronin overexpression growth defects. In addition, one allele showed normal actin binding but impaired functional interactions with ADF/cofilin. Another allele showed normal actin binding but failed to cause coronin overexpression defects. Together, these results indicate that actin binding is critical for many of the biochemical and cellular functions of coronin and that the $\beta$-propeller domain mediates additional functional interactions with ADF/cofilin and possibly other ligands. Conservation of the actin-binding surfaces across distant species and in all three major classes of coronin isoforms suggests that the nature of the coronin-actin association may be similar in other family members.

Many processes including cell motility, intracellular transport, and endocytosis rely on dynamic reorganization of the actin cytoskeleton. As rapidly as actin networks are assembled \textit{in vivo} they must be disassembled and turned over, which requires a high degree of spatial and temporal control and the coordinated activities of numerous actin-associated proteins (1, 2). Central among these factors is ADF/cofilin, which binds to actin filaments and induces their severing into short fragments (3). However, ADF/cofilin does not function alone in regulating the disassembly of actin networks but rather in close harmony with several other actin-binding proteins. One such protein is coronin, which is highly conserved from yeast to mammals. Coronin was first identified in \textit{Dictyostelium}, where it co-localizes with F-actin at the corona (or crown-like cell cortex) and at sites of phagocytosis (4, 5). Genetic disruption studies in a variety of model systems have demonstrated that coronin has important functional roles in endocytosis, phagocytosis, and cell motility (6–8). Biochemically, coronins universally bind to and bundle actin filaments, suggesting that their mechanistic effects on actin dynamics may be conserved. Coronin binds with high affinity to the sides of actin filaments independently of ADF/cofilin, bundles filaments, and synergizes with ADF/cofilin in severing actin filaments (9–12). Recent studies further suggest that coronin may function with ADF/cofilin and another actin-binding protein, Aip1, to induce abrupt bursts of actin disassembly, resulting in a loss of discrete lengths of polymer (10, 13, 14). Coronin also directly associates with the Arp2/3 complex and inhibits its actin nucleation activity, which may locally contribute to actin disassembly (7, 15, 16). Thus, coronin is multi-functional in promoting the disassembly and turnover of filamentous actin networks, and we may have yet to learn the full range of its effects.

The domain structure of coronin is conserved in diverse species and consists of an N-terminal folded $\beta$-propeller domain, followed by a variable (or "unique") region, and a C-terminal coiled-coil domain. The $\beta$-propeller domain is a common fold found in proteins of diverse biological functions, and although it typically has no catalytic activity, it often serves as a structural platform for mediating protein-protein interactions (17). The crystal structure of the mouse coronin-1A $\beta$-propeller domain shows that it is comprised of seven blades, assembled from five canonical WD repeats and two noncanonical repeats (18). Additional sequences C-terminal to the seven blades are disordered and associate with the underside of the propeller domain, possibly to provide added structural integrity. F-actin is the only binding partner of the coronin $\beta$-propeller domain that has been identified in any species (9, 11, 19). In contrast, the much smaller C-terminal coiled-coil domain mediates multiple functions and interactions, including coronin homo-oligomerization and actin cross-linking (9, 20, 21), direct inhibition of Arp2/3 complex (7, 15, 16, 22), and binding to ATP-rich F-actin, which blocks ADF/cofilin severing (12).

To date, none of the activities of either the $\beta$-propeller or the coiled-coil domain have been mapped at higher resolution, which has left the precise molecular nature of the interactions between coronin and its binding partners poorly understood. Earlier efforts to map the actin binding activity of coronin relied on truncation mutants of the $\beta$-propeller domain, which led...
to issues of instability and/or insolvency of the incomplete domain fragments (20, 23, 24). One later study identified a conserved residue (Arg30) in the β-propeller domain of coronin-1B that mediates F-actin binding and is important for its localization and function in vivo (11). However, this site likely represents only a part of the actin-binding footprint on coronin, because it is predicted to be relatively extensive based on three-dimensional reconstructions of coronin-1A decorated actin filaments (19) and on modeling of conserved electrostatic surfaces on the β-propeller domain (18).

Here, we used a systematic mutagenesis approach to identify surfaces on the β-propeller domain of Saccharomyces cerevisiae coronin (Crn1) required for actin binding and/or functional synergy with ADF/cofilin in severing actin filaments. Our analysis identified five conserved surfaces important for actin binding, which form a ridge or a belt-shaped footprint extending across the β-propeller structure. Actin binding was required for coronin synergy with ADF/cofilin in severing actin filaments and for normal localization and function of coronin in vivo. Further, we identified two surfaces that did not appear to be involved in actin binding but that made important contributions to coronin function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions and Yeast Strains**—Standard molecular biology techniques and yeast growth and transformation methods were used. To generate plasmids for expressing mutant GST-Crn1 polypeptides in *Escherichia coli*, PCR-based site-directed mutagenesis was performed on pBG203 (pGAT2-CC) (9). The mutations introduced in each allele either generated a unique silent restriction site or eliminated a unique restriction site. All of the mutant plasmids were verified by restriction digestion and then DNA sequencing. Wild type GST-Crn1ΔCC polypeptide was expressed from plasmid pBG206 (9). To generate a corresponding set of plasmids expressing mutant GST-Crn1ΔCC polypeptides, the DNA sequences encoding residues 1–600 were PCR-amplified from the first set of mutant plasmids, digested with BamHI/NsiI and cloned into pGAT2. For overexpression of *crn1* alleles in yeast, a DNA fragment encoding residues 1–651 of Crn1 was PCR-amplified from the first set of mutant plasmids, digested with BamHI/NotI, and cloned into pRS426 (2μ, URA3) with a GAL promoter insertion. pBG222 (9) was used to overexpress wild type CRN1.

A CRN1-GFP plasmid (pBG1114) was constructed in two steps. First, a 2.4-kb fragment (including 500 bp upstream and the CRN1 ORF lacking its stop codon) was amplified from *S. cerevisiae* genomic DNA, digested with BamHI/Spel, and cloned into the GFP-containing plasmid pBG999 (12). The resulting construct was then digested with NotI/SacI and used as a vector for insertion of a 303-bp NotI/SacI fragment (containing CRN1 stop codon and 300-bp downstream sequences) amplified from yeast genomic DNA. The resulting CRN1-GFP plasmid was confirmed by DNA sequencing. For the plasmids expressing GFP fusions of *crn1* alleles, each mutant *crn1* DNA fragment (including 500 bp upstream and *crn1* ORF lacking stop codon) was amplified from the corresponding mutant plasmid (CEN, TRP1, expressing mutant *crn1* allele under endogenous promoter), digested with BamHI/Spel, and cloned into BamHI/Spel-digested pBG1114.

**Protein Purification**—The wild type and mutant GST-Crn1ΔCC polypeptides used in F-actin binding and Cof1 synergy assays were expressed in *E. coli* strain BL21 (DE3) and purified as described (9), followed by removal of the GST tag by thrombin digestion. The full-length Crn1 polypeptides (wild type and mutant) used in F-actin bundling assays were purified similarly, but the GST tag was not removed. *S. cerevisiae* Cof1 was purified from *E. coli* as described (25). Yeast actin was purified as described (26). Rabbit skeletal muscle actin (RMA)2 was purified (27) and gel-filtered. RMA was labeled on Cys375 with pyridyliodoacetamide (28).

**F-actin Co-sedimentation Assays**—Wild type Crn1ΔCC showed similar binding to RMA and yeast actin (supplemental Fig. S2), and therefore RMA was used for further analysis. Wild type and mutant Crn1ΔCC polypeptides (0.75 μM) were incubated with different concentrations (0, 2, 4, 8, 16, and 24 μM) of preassembled F-actin (RMA) for 10 min and then centrifuged for 20 min at 80,000 rpm in a TLA100 rotor (Beckman Instruments, Fullerton, CA). Pellets and supernatants were separated and analyzed on Coomassie-stained gels followed by densitometry of Crn1 bands using ImageJ software (National Institutes of Health) to calculate the percentage of Crn1 bound to F-actin. Sigma plot software (Systat Software Inc., San Jose, CA) was used for curve fitting, and from these curves the concentration of F-actin required for 50% Crn1 binding was determined. For the analysis of Crn1-3ΔCC and Crn1-21ΔCC, the highest concentration of F-actin used was 16 μM, which was necessary to accommodate larger volumes of these mutant Crn1 polypeptides in the reactions, as a result of lower stock concentrations.

For comparing F-actin bundling activities of full-length wild type and mutant Crn1 proteins, a low speed pelleting assay was used. Crn1 (0.5 μM) was incubated for 10 min with 2 μM preassembled F-actin (RMA) and then centrifuged for 3 min at 14,000 rpm. Pellets and supernatants were separated and analyzed on Coomassie-stained gels followed by densitometry of actin bands using ImageJ software to calculate the percentage of actin in the pellet.

**Actin Assembly Kinetics**—For Cof1 synergy assays, gel-filtered monomeric RMA (5% pyrene labeled) in G buffer (10 mM Tris, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.2 mM DTT) was converted to Mg-ATP-actin and then mixed with 15 μl of proteins or control buffer (20 mM Tris, pH 7.5, 50 mM KCl) and 3 μl of 20× initiation mix (40 mM MgCl₂, 10 mM ATP, 1 mM KCl) in 60-μl reactions. The pyrene signal was monitored at 25 °C in a fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ) with an excitation of 365 nm and an emission of 407 nm. The rates of assembly were calculated from the slopes of the curves at 25–50% polymerization.

**Overexpression Analysis in Yeast**—Plasmids expressing wild type CRN1 and mutant *crn1* alleles under control of the GAL promoter were transformed into *crn1*Δ cells (BGY11) (9). The cell cultures were serially diluted and plated on selective medium containing glucose or galactose. Crn1 protein levels in

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2 The abbreviation used is: RMA, rabbit skeletal muscle actin.
overexpressing cells were determined by immunoblotting whole cell extracts with anti-Crn1 antibodies and anti-tubulin antibodies as a loading control (9).

*In Vivo Localization Analysis*—Wild type CRN1-GFP and mutant crn1-GFP plasmids were transformed into a crn1Δ ABP1-RFP strain (BGY3017). Cells were grown to early log phase in selective medium and imaged on a Zeiss Axioskop-2 mot plus microscope (Carl Zeiss, Thornwood, NY) using a Hamamatsu IEEE1394 digital CCD camera (Hamamatsu Photonics, Bridgewater, NJ) running OpenLab software (Improvision, Lexington, MA). All of the images were acquired using the same exposure times and settings and were processed identically.

**RESULTS**

**Scanning Mutagenesis of the Coronin β-Propeller Domain**—The primary goal of our analysis was to identify actin-binding surfaces on Crn1 and test their importance for its biochemical and cellular functions. When we initiated the study, the structure of the β-propeller domain of coronin had not been reported, and therefore we used a “charge-to-alanine” strategy to design mutations (29, 30). From an alignment of coronin sequences of distant species (Fig. 1A and supplemental Fig. S1), we first identified clusters of charged residues, which were likely to reside on the protein surface. Alanine substitutions were introduced at these sites to generate many of the alleles. Once the structure of the β-propeller domain was reported (18), we introduced additional alleles, targeting conserved solvent-exposed residues missed by our charge-to-alanine strategy. In total, we generated 21 alleles, each containing two to four alanine substitutions (Table 1). Eighteen of these alleles carried exclusively charge-to-alanine substitutions, whereas the remaining three alleles included alanine substitutions at noncharged residues: Thr199 (crn1-7), Leu206 (crn1-8), and Gln367 (crn1-21). Most of our alleles mutated surface residues that are highly conserved in the coronin family, but a few alleles targeted residues in Crn1 that are charged but less well conserved (Fig. 1A and supplemental Fig. S1). Modelling of the mutated residues on the β-propeller domain structure of coronin-1A showed that they are distributed across all seven blades (Fig. 1, B and C).

**Mutant Analysis for Defects in F-actin Binding**—The β-propeller domain of Crn1 binds to F-actin (9) and similarly is implicated in actin binding in a number of other coronin family members (11, 31). More recently, we showed that Crn1 harbors a second F-actin-binding site in its coiled-coil domain (12). This suggests that the observed high affinity (Kd = 5–10 nm) actin binding interaction of full-length Crn1 (9) stems from the combined contributions of these two actin-binding sites. Consistent with this view, we found that a coronin polypeptide lacking its coiled-coil domain (Crn1ΔCC) bound to F-actin with much lower affinity (micromolar range; Fig. 2, A and B, see below) than intact Crn1. A similar observation has been reported for coronin-1B lacking its coiled-coil domain (11), suggesting that the presence of two separate actin-binding sites may be a conserved feature in coronins.

Because we were focused on mapping the actin binding activity of the β-propeller domain, we isolated our mutants as Crn1ΔCC polypeptides. Each Crn1ΔCC polypeptide was expressed in *E. coli* as a GST fusion, and then the GST tag was removed. Thirteen of the mutants (Crn1-2, Crn1-4, Crn1-6, Crn1-10, Crn1-13, Crn1-15, Crn1-16, Crn1-17, Crn1-18, Crn1-19, Crn1-20, Crn1-21, and Crn1-22) were readily expressed and isolated as soluble polypeptides. Expression of five others (Crn1-5, Crn1-8, Crn1-9, Crn1-12, and Crn1-14) was not detected, suggesting that the mutations might have interfered with protein folding and/or stability. Indeed, four of those alleles carried mutations in β-sheets of the propeller blades, suggesting that they may have destabilized this fold. The remaining mutant (Crn1-12) carried a mutation in a loop of blade 6 that is exposed at the bottom of the propeller structure and that may contact the C-terminal stabilizing sequence. Two other alleles (Crn1-7 and Crn1-11) were expressed in *E. coli* but were found to be sensitive to degradation during purification and thus were excluded from the biochemical analysis. One other allele (Crn1-3) was prone to degradation to a lesser degree, which enabled us to purify enough for inclusion in the analysis.

We first compared the abilities of wild type and mutant Crn1ΔCC polypeptides (0.75 μM) to bind a range of concentrations of F-actin (0–24 μM) in co-sedimentation assays. Pellets and supernatants were analyzed on Coomassie-stained gels, and the concentration of F-actin required to pellet 50% of the Crn1ΔCC was determined. Wild type Crn1ΔCC exhibited concentration-dependent binding, reaching 50% bound at ~5 μM F-actin (Fig. 2, A and B). Four mutants (Crn1-3, Crn1-4, Crn1-15, and Crn1-16) exhibited actin-binding profiles similar to wild type Crn1ΔCC (Fig. 2B) and were therefore designated as pseudo-wild type alleles. Five other mutants (Crn1-10, Crn1-18, Crn1-20, Crn1-21, and Crn1-22) required 2–3-fold higher concentrations of F-actin to reach 50% binding compared with wild type Crn1ΔCC (Fig. 2B) and were designated as “moderately defective” in actin binding. The remaining five mutants (Crn1-2, Crn1-6, Crn1-13, Crn1-17, and Crn1-19) showed little detectable actin binding, even at the highest F-actin concentrations tested (Fig. 2A), and were designated as “severely impaired” in actin binding. Among the five severely impaired alleles, Crn1-2 and Crn1-17 showed some actin binding at the highest F-actin concentrations tested, suggesting that they may be impaired to a lesser degree than the other three severe alleles.

As an independent test for loss of actin binding activity, we purified as full-length Crn1 proteins the five severe mutants (Crn1-2, Crn1-6, Crn1-13, Crn1-17, and Crn1-19) along with wild type and two moderately impaired mutants (Crn1-10 and Crn1-22) and compared their abilities to bundle F-actin. Bundling by Crn1 requires both the β-propeller and coiled-coil domains (9), and therefore impairments in actin binding by the β-propeller domain should disrupt bundling. Wild type and moderately impaired mutant Crn1 proteins bundled F-actin efficiently, suggesting that mild defects in actin binding do not substantially impair bundling activity (Fig. 2C). On the other hand, the severe mutants showed losses of bundling activity to different degrees. Crn1-6 and Crn1-19 had the most striking losses of activity, followed by Crn1-2 and Crn1-13, which retained some activity. Crn1-17 exhibited the weakest defects in bundling, suggesting that this mutant may have the least severe defects in actin binding among these five alleles. This
Structure and Function Analysis of Coronin

A

M. musculus Coronin IA  
D. discoideum Coronin  
S. cerevisiae Coronin

B

C

(Top)  
(Bottom)
Structure and Function Analysis of Coronin

| Allele    | Mutations       | Protein expression in E. coli/yeast | F-actin binding in vitro | Synergy with Cof1 in vitro | Overexpression phenotype |
|-----------|-----------------|------------------------------------|--------------------------|---------------------------|-------------------------|
| crn1-1    | Wild type       | Yes/Yes                            | ++                       | +                         | ++                      |
| crn1-2    | K10A, R12A      | Yes/Yes                            | ++                       | +/–                       | +/–                     |
| crn1-3    | D102A, D103A, K105A | Yes/Yes                          | +/–                       | 15.9 ± 2.7                | +/–                     |
| crn1-4    | D119A, H120A    | Yes/Yes                            | ++                       | 81.9 ± 2.1                | –                       |
| crn1-5    | K131A, K134A    | No/No                              | ++                       | 85 ± 3.5                  | +/–                     |
| crn1-6    | R141A, K142A    | Yes/Yes                            | +/–                       | 20.5 ± 2.5                | –                       |
| crn1-7    | T199A, R202A, D203A | No/Yes                      |                           |                           |                         |
| crn1-8    | K204A, K205A, L206A, R207A | No/No             |                           |                           |                         |
| crn1-9    | D203A, R207A    | No/No                              |                           |                           |                         |
| crn1-10   | R212A, E213A, E214A, K215A | Yes/Yes          | ++                       | 82.8 ± 5.4                | +/–                     |
| crn1-11   | E262A, K263A    | No/Yes                             |                           |                           |                         |
| crn1-12   | E285A, K288A    | No/No                              |                           |                           |                         |
| crn1-13   | K295A, D297A    | Yes/Yes                            | ++                       | 17.5 ± 2.3                | –/–                     |
| crn1-14   | R301A, E304A    | No/No                              |                           |                           |                         |
| crn1-15   | D308A, E309A    | Yes/Yes                            | +/–                       | 72.8 ± 1.1                | +/–                     |
| crn1-16   | E312A, E315A    | Yes/Yes                            | ++                       | 10.2 ± 4.7                | +/–                     |
| crn1-17   | E320A, R323A    | Yes/Yes                            | +/–                       | 52.1 ± 3.8                | +/–                     |
| crn1-18   | K336A, E337A    | Yes/Yes                            | +/–                       | 93.7 ± 4.8                | +/–                     |
| crn1-19   | R361A, R362A, E364A, E365A | Yes/Yes          |                           | 10.9 ± 2.1                | –                       |
| crn1-20   | E368A, D369A    | Yes/Yes                            | ++                       | 84.2 ± 3.7                | +/–                     |
| crn1-21   | Q367A, D369A    | Yes/Yes                            | ++                       | 88.2 ± 0.8                | +/–                     |
| crn1-22   | E384A, E385A    | Yes/Yes                            | ++                       | 76.9 ± 5.9                | +/–                     |

* From the data in Fig. 2 (A and B). ++, wild type; +/–, moderately defective; –, severely impaired.

* From the data in Fig. 3C.

* From three independent experiments as in Fig. 4D. The symbols (+, ++, +/–, and –) represent a range of phenotypes, from severe growth defects to no growth defects.

* The polypeptide was highly sensitive to degradation and could not be purified.

Mutant Analysis for Defects in Synergizing with ADF/Cofilin to Sever Actin Filaments—Another important activity of the coronin β-propeller domain is its synergy with ADF/cofilin in severing actin filaments (12). Crn1∆CC strongly enhances the effects of Cof1 in severing filaments and increasing the number of free barbed ends, which leads to an increase in the rate of pyrene-actin assembly. Therefore, we used this assay to screen each of our mutants (as Crn1∆CC polypeptides) for functional defects (Fig. 3, A–C, and supplemental Fig. S3). With the exception of Crn1-16 (see below), all pseudo-wild type alleles and alleles designated as moderately defective in F-actin binding showed normal functional synergy with Cof1, comparable with wild type Crn1∆CC. In contrast, the five alleles that were severely impaired in F-actin binding were also defective in synergizing with Cof1. Further, as observed above in actin bundling assays, Crn1-17 was less defective compared with the other four severe alleles. These data suggest a close correlation between the loss of F-actin binding and the loss of the ability to synergize with Cof1 in severing actin filaments.

Interestingly, one mutant (Crn1-16) that was pseudo-wild type for F-actin binding exhibited a dramatic loss of synergy with Cof1, comparable with the loss of activity observed for the most severely impaired actin-binding alleles (e.g. Crn1-13) (Fig. 3, B and C). This result was observed using Crn1-16 isolated from multiple independent preparations and suggests that the surface mutated in Crn1-16 (which does not appear to be directly involved in actin binding) is required for functional synergy with Cof1. For convenience, we refer to the surface mutated in this allele as the “Cof1 regulatory surface” from here on.

Next, we investigated the importance of the Cof1 regulatory surface and an actin-binding surface for coronin function in vivo. Previously, we have shown that wild type Crn1 and Crn1∆CC constructs expressed from the endogenous promoter on low copy plasmids rescue the growth defects of a crn1Δ cof1-22 strain at 34 °C (12). Here, we used this assay to test whether crn1-19∆CC and/or crn1-16∆CC alleles could rescue the growth defects. Both wild type CRN1 and crn1∆CC complemented growth as expected, but crn1-19∆CC and crn1-16∆CC both failed to complement (Fig. 3D). This suggests that the genetic functions that Crn1 shares with Cof1 in vivo require its actin binding and the Cof1 regulatory surfaces.

Overexpression Phenotypes of Coronin Mutants—As an additional test for in vivo function, we compared wild type CRN1 and mutant crn1 alleles for defects in cell growth after overexpression (Fig. 4). Previously, we have shown that galactose-induced overexpression of wild type full-length CRN1 causes severe defects in cell growth and that this phenotype depends on the coiled-coil domain (15). Here, we asked whether this phenotype also depends on the β-propeller domain of Crn1 by comparing the overexpression phenotypes of full-length Crn1 (1–651) and Crn1 (400–651) in a crn1Δ strain. Immunoblotting of whole cell extracts verified that both Crn1 polypeptides were overexpressed (Fig. 4A). As expected, full-
length Crn1(1–651) caused strong defects in cell growth. In contrast, Crn1(400–651) did not (Fig. 4).

Next, we screened our crn1 alleles in this assay. Immunoblotting verified that 16 of 21 alleles were overexpressed. Expression was not detected for the remaining five alleles (crn1-2, crn1-6, crn1-13, crn1-17, and crn1-19) (Fig. 4C). As mentioned earlier, these five alleles were poorly expressed in E. coli and thus may have inherent defects in folding and/or stability. For the 16 alleles that were verifiably overexpressed, we directly compared cell growth (one experiment shown in Fig. 4B; effects observed in multiple experiments listed in Table 1). Nine alleles (crn1-4, crn1-10, crn1-11, crn1-15, crn1-16, crn1-18, crn1-20, crn1-21, and crn1-22) caused clear defects in cell growth, similar to wild type CRN1. All of these alleles were classified above as pseudo-wild type or only moderately defective in F-actin binding in vitro, with the exception of crn1-11, which could not be purified from E. coli. The remaining seven alleles failed to cause strong cell growth defects upon overexpression. Five of them (crn1-2, crn1-6, crn1-13, crn1-17, and crn1-19) were designated as severely impaired in F-actin binding in vitro. These results suggest a close correlation between the loss of F-actin-binding and the loss of the overexpression phenotype (Table 1).

The remaining two alleles, crn1-3 and crn1-7, also failed to cause strong growth defects after overexpression. For Crn1-7, the result was not surprising given that we observed degradation to be a problem during its purification, which precluded us from examining its biochemical activities in vitro. On the other hand, immunoblotting showed that Crn1-7 was successfully overexpressed in vivo, which means that we cannot rule out the possibility that this surface mutated in Crn1-7 contributes to F-actin binding interactions and to protein folding. For Crn1-3, the result was more surprising, given that this mutant showed normal binding to F-actin (Fig. 2B) and normal synergy with Cof1 (Fig. 3C and supplemental Fig. S3B), and in vivo this mutant was overexpressed (Fig. 4C). One possible explanation is that the surface mutated in Crn1-3 mediates a distinct (as yet undefined) in vivo function and/or interaction of the β-propeller domain required for the overexpression phenotype. A second possibility is that this allele might alter post-translational modifications to affect Crn1 function. This mutant could therefore serve as a useful tool for future dissections of coronin in vivo function and regulation.

Interestingly, crn1-16, which was specifically impaired in synergizing with Cof1 but had normal actin binding, showed a similar overexpression phenotype to wild type CRN1. The simplest interpretation of this result is that the CRN1 overexpression phenotype does not depend on this specific functional interaction between Crn1 and Cof1 mediated by this surface.

In Vivo Localization Analysis of crn1 Alleles—Finally, we examined how specific crn1 alleles influence its localization in vivo. Crn1 localization to cortical endocytic actin patches is sensitive to latrunculin A, suggesting a requirement for fila-
mentous actin that is presumed to depend on direct binding of Crn1 to F-actin (9, 32). However, in the absence of specific alleles that disrupt F-actin binding, this model has never been tested directly for Crn1. Our identification of F-actin-binding mutants enabled us to perform this test.

Previously, we have shown that Crn1 localization depends on both the β-propeller and coiled-coil domains (15) and that each domain binds to F-actin (9, 12). Therefore, a prediction of the

model above is that actin-binding mutations in the β-propeller domain should impair Crn1 localization to cortical actin patches. To address this, we constructed a low copy plasmid expressing full-length wild type Crn1 with a C-terminal GFP tag under the control of the CRN1 promoter. This CRN1-GFP plasmid complemented the growth defects of a crn1Δ cof1-22 strain similar to untagged CRN1 (Fig. 5A), suggesting that the GFP tag does not interfere with function in vivo. Crn1-GFP was localized in a strain lacking endogenous CRN1 and carrying an integrated Abp1-RFP to mark actin patches. Crn1-GFP localized to cortical puncta that overlapped with Abp1-RFP (Fig. 5B). The overlap was incomplete, which is expected because Crn1 arrives to patches later than Abp1 (14, 33). We examined localization for three of the severe actin-binding mutants (Crn1-2-GFP, Crn1-13-GFP, and Crn1-19-GFP) and found that each had reduced GFP intensity at cortical patches and increased cytoplasmic fluorescence compared with wild type Crn1-GFP (Fig. 5C). By comparison, a moderately defective actin-binding mutant (Crn1-20-GFP) showed normal localization similar to wild type Crn1-GFP, as did Crn1-16-GFP (Fig. 5C). These observations support the model that Crn1 localization depends on its direct binding interactions with F-actin but that minor defects in actin binding are not sufficient to disrupt localization.

**DISCUSSION**

Our main objective in this study was to dissect the structure and function of the β-propeller domain of coronin, which is conserved in all coronin family members. Specifically, we sought to map the actin-binding footprint on this domain and to test the importance of actin binding for coronin biochemical and cellular functions. F-actin binding is the defining property of the coronin family, yet our understanding of the coronin-actin interaction has been limited. Until now, there has been little information available about the specific residues on the surfaces of coronin and actin that mediate their direct interaction. To address this, we generated 21 mutant alleles of the yeast coronin β-propeller domain, collectively introducing alanine substitutions at 46 surface residues. Mutants were analyzed for their ability to bind F-actin *in vitro*, which revealed five evolutionarily conserved actin-binding surfaces (Fig. 6). We then used these alleles to assess the importance of actin binding by Crn1 for its biochemical and cellular functions and for its localization *in vivo*.

The Actin-binding Footprint on Coronin—Previous studies predicted that the actin-binding footprint on the β-propeller domain of coronin might be extensive based on electrostatic mapping and modeling (18, 19), but until now only a single actin-binding residue on coronin has been identified, Arg30 in coronin-1B (11). Our scanning mutagenesis approach has now identified five evolutionarily conserved surfaces on coronin that are important for actin binding. These surfaces are distributed among four of the seven blades that form the β-propeller domain (blades 2, 3, 6, and 7), as well as a portion of the C-terminal extension that associates with the bottom face of the structure. Four of the actin-binding surfaces are located on the top face of the β-propeller, and the fifth (Crn1-19) is located closer to the bottom face (Fig. 1). Introducing a right-handed
tilt to the rendered view of the structure puts all five actin-binding surfaces (and Arg30, identified as an actin-binding residue in Ref. 11) on a “ridge” or belt stretching across one side of the /H9252-propeller domain structure (Fig. 6, transparent pink band).

Our finding that the top of the /H9252-propeller is heavily involved in actin binding is consistent with the top of the structure being more solvent-exposed and therefore available for protein-protein interactions compared with the bottom, which packs against the C-terminal extension (18). In addition, our data agree with predictions made from an electrostatic potential analysis of the /H9252-propeller domain, which suggested that very large basic surfaces might be involved in actin binding (Fig. 5 in Ref. 18). Indeed, all five of our actin-binding sites lie within those broad patches. On the other hand, our data are only in partial agreement with the modeling predictions made from three-dimensional reconstructions of coronin-1A decorated actin filaments (19). In that study, only one of the five actin-binding surfaces that we have identified (Crn1-19) was suggested to bind actin. Thus, our scanning mutagenesis approach has both defined the positions of multiple actin-binding surfaces on coronin at high resolution and provided the critical functional validation of these sites.

One other point made by our data is that the actin-binding surface on coronin spans almost half of the circumference of the /H9252-propeller domain. It is difficult to imagine how all of these actin-binding surfaces could be interacting with a single actin subunit in the filament. It seems more likely that each coronin molecule may therefore interact with two or more actin subunits in the filament. Indeed, a similar suggestion was made in the above-mentioned EM study (19).

**Importance of Actin Binding for Coronin Localization and Function**—Using the specific alleles defective in actin binding, we tested how weakened actin binding affinity affects Crn1 biochemical activities, localization, and cellular functions. We observed a close correlation between reduced actin binding affinity (Crn1-2, Crn1-6, Crn1-13, Crn1-17, and Crn1-19) and the following: (a) loss of Crn1 synergy with Cofl in severing actin filaments in vitro, (b) loss of the Crn1 overexpression phenotype, and (c) diminished Crn1 localization to actin patches. Collectively, these observations suggest that yeast coronin localization and function are highly dependent on its ability to directly associate with F-actin. This agrees well with a previous study on mouse coronin-1B showing that the Arg30 mutation, which reduces actin binding affinity, causes defects in cell motility and lamellipodial dynamics and diminished co-localization with cortical actin networks (11). Together, these studies show that...
F-actin binding plays a central role in coronin function across distant species and reinforces the view that its F-actin binding interactions are likely to be highly conserved.

As mentioned above, our data and those of Cai et al. (11) indicate that coronin localization depends on direct interactions with F-actin. However, these observations do not rule out the possibility that coronin localization has additional requirements. Indeed, one recent study reported that Crn1 localization depends on specific surfaces of Cof1 (14). We have been unable to find any evidence for a direct physical association between Crn1 and Cof1 in solution using a variety of assays (12). Therefore, we favor the possibility that Crn1 and Cof1 interact specifically on F-actin (similar to Aip1 and Cof1) or alternatively that Cof1 alters the conformation of F-actin in a manner that promotes Crn1 association with F-actin in vitro. Perhaps specific surfaces on Cof1 and Crn1 (such as Crn1-16) are required for their functional interactions when they are bound to F-actin. What remains perplexing, however, is that Crn1 localization to F-actin structures depends on Cof1, but Crn1 binds to F-actin with higher affinity than Cof1 in vitro. This suggests that we have yet to fully define the rules governing Crn1 localization in vivo, which may be complex, and depend on the relative expression levels of many different actin-binding proteins and the competition or cooperation among them in binding F-actin.

Our analysis has also provided new insights into the mechanism underlying the CRN1 overexpression phenotype. Previously, we showed that the coiled-coil domain of Crn1 is required for the overexpression growth defects (15). The coiled-coil domain of Crn1 directly inhibits Arp2/3 complex, and the overexpression phenotype is suppressed in strains expressing specific alleles of the subunit of Arp2/3 complex that binds Crn1 (p35/APRC2) (15). This suggested that the Crn1 overexpression phenotype arises at least in part from elevated Crn1 levels misregulating Arp2/3 complex. Here we have shown that the overexpression phenotype was strong in Crn1-16, which is mutated at the Cof1 regulatory surface. Taken together, these results show that the arrest in cell growth arises from a mechanism that involves direct interactions of Crn1 with both F-actin and Arp2/3 complex but not the observed synergy between Crn1 and Cof1 in severing actin filaments.

**Additional Functional Surfaces on the β-Propeller Domain—** We also identified two surfaces on coronin (Crn1-16 and Crn1-3) that are important for its biochemical and/or cellular functions but that are not obviously involved in binding actin. Crn1-16 showed normal binding to F-actin and localized normally in vivo but failed to synergize with ADF/cofilin in vitro and failed to complement crn1/H9004 cof1-22 growth defects in vivo. As mentioned above, coronin and cofilin may interact directly when bound to F-actin, which could lead to their observed synergy in severing actin filaments (10, 12). The surface mutated in Crn1-16 could mediate such interactions with Cof1 when both proteins are bound to F-actin and/or F-actin is in a specific conformational state. Alternatively, the Crn1-16 surface might bind directly to F-actin in a specific Cof1-induced conformation. Indeed, this is consistent with the Crn1-16 surface lying within the actin-binding belt, in close proximity to the Crn1-2 F-actin-binding surface (Fig. 6). For these reasons, Crn1-16 may be a valuable mutant tool for future mechanistic studies aimed at dissecting coronin-ADF/cofilin synergy in promoting actin disassembly.
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The other functional surface we identified (Crn1-3) shows normal binding to actin and normal synergy with Cof1 in vitro but does not exhibit the Crn1 overexpression phenotype. This suggests that the Crn1-3 surface could mediate interactions with another cellular binding partner of coronin besides actin or ADF/cofilin. Because this surface contains residues that are highly conserved in the coronin family, its molecular interactions and cellular function are likely conserved in other species, making it another important tool for dissecting coronin biology in diverse systems.

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FIGURE 6. Functional surfaces on the β-propeller domain of coronin. Surface residues identified in this study as important for Crn1 biochemical and/or cellular functions modeled on the structure of the β-propeller domain of murine coronin-1A (18). A, rendered structure showing that all of the functional surfaces lie along a single ridge or belt stretching across the domain (transparent pink band). Color coding for residues: red, F-actin binding; green, Cof1 regulatory surface; purple, unexplained requirement for coronin overexpression phenotype; orange, an actin-binding residue (R30) identified in mouse coronin-1B (11). B, cartoon structure of the same view as in A with the same color coding. CT, C terminus.