Effect of Fgd1 on Cortactin in Arp2/3 Complex-Mediated Actin Assembly†

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ABSTRACT: Mutations in faciogenital dysplasia protein (Fgd1) result in the human disease faciogenital dysplasia (FGDY). Fgd1 contains a RhoGEF domain specific for Cdc42. Fgd1 also contains a Src homology (SH3) binding domain (SH3-BD) that binds directly to the SH3 domain of cortactin, which promotes actin assembly by actin-related protein (Arp)2/3 complex. Here, we report the effect of ligation of cortactin's SH3 domain by the Fgd1 SH3-BD on actin polymerization in vitro. Glutathione *S*-transferase (GST)-fused Fgd1 SH3-BD enhanced the ability of cortactin to stimulate Arp2/3-mediated actin polymerization. However, a synthetic peptide containing only the SH3-BD sequence had no effect. The SH3-BD peptide bound to cortactin and inhibited the effect of GST-Fgd1 SH3-BD, suggesting that GST dimerization was responsible for the stimulating effect of GST-Fgd1 SH3-BD. When GST-Fgd1 SH3-BD was prepared as a heterodimer with a control GST fusion protein (GST-Pac1), no stimulatory effect on actin polymerization was observed. In addition, when cortactin was dimerized via its N-terminus, away from the C-terminal SH3 domain, actin polymerization with Arp2/3 complex increased markedly, compared to free cortactin. Thus, cortactin ligated by Fgd1 is fully active, indicating that the cell can use Fgd1 to target actin assembly. Moreover, if Fgd1 is multimerized, then cortactin's activity should be enhanced. Fgd1 and cortactin may participate as scaffolds and signal transducers in a positive feedback cycle to promote actin assembly at the cell cortex.

The actin cytoskeleton is organized into bundles and networks of actin filaments that serve as frameworks for a variety of cellular structures. The actin cytoskeleton demonstrates dynamic changes based on polymerization or depolymerization in response to various internal and external stimuli. Actin dynamics plays an important role in a variety of cellular processes including endocytosis, phagocytosis, membrane trafficking, and cell motility. Actin polymerization can drive cell protrusion and intracellular motility.

Cortactin is a *c-src* substrate associated with sites of dynamic actin assembly at the leading edge of migrating cells (*1*, *2*). The N-terminal acidic domain (NTA)[1] of cortactin binds directly to actin-related protein (Arp)2/3 complex, a nucleator of actin assembly (*1*). The NTA domain is followed by 6.5 tandem repeats, containing 37 amino acids each. The tandem repeat region binds directly to F-actin (*1*). Alone, Arp2/3 complex is not able to nucleate actin assembly. Cortactin activates Arp2/3 complex and stabilizes branched filament networks induced by Arp2/3 complex. Both the NTA domain and the tandem repeat domains are required for Arp2/3 complex activation (*3*).

Cortactin also contains a C-terminal Src homology (SH3) domain (*4*, *5*). The SH3 domain of cortactin binds specifically to certain proline-rich proteins such as dynamin, Wiskott-Aldrich syndrome (WASp)-interacting protein (WIP), CortBP-1/Shank2, Shank-3, and ZO-1 (*6*−*9*). Dynamin-2 binds cortactin and modulates Arp2/3 complex-mediated actin assembly in vitro and in vivo (*10*). The interaction of WIP with cortactin increases the efficiency of Arp2/3 complex-mediated actin polymerization in vitro and cell protrusive activity in vivo (*11*).

Faciogenital dysplasia protein (Fgd1) protein has been identified as binding to the SH3 domain of cortactin, via a proline-rich region near the N-terminus of Fgd1 (*12*). Mutations in *FGD1* result in the human disease faciogenital dysplasia (FGDY, Aarskog syndrome), which includes skeletal dysplasia and multiple congenital anomalies (*13*). During embryogenesis in the mouse, Fgd1 is expressed in regions of ossification including skeletal elements involved in the FGDY phenotype (*14*). Fgd1 contains a DH/PH domain with guanine-nucleotide exchange activity specific for Cdc42 (*15*), as well as a FYVE-finger domain and a second PH domain. Microinjection of Fgd1 into fibroblasts induces actin polymerization and activates Cdc42 (*16*). Abnormal targeting of Fgd1 causes abnormal assembly of actin (*12*). Activation of Cdc42 appears to account for other

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[1] Abbreviations: Arp, actin-related protein; DH/PH, Dbl homology and pleckstrin homology domain; FGDY, faciogenital dysplasia syndrome; Fgd1, faciogenital dysplasia protein; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; NTA, *N*-terminal acidic domain; PRD, proline-rich domain; SH3, Src homology; SH3-BD, SH3 binding domain; WIP, WASp-interacting protein; WASp, Wiskott-Aldrich syndrome.

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cellular activities of Fgd1 related to signaling and the cell cycle (*17*, *18*).

In this study, we asked whether Fgd1 binding to the SH3 domain of cortactin would alter cortactin's ability to activate actin assembly by Arp2/3 complex. Cortactin was fully active when Fgd1 was bound. Moreover, dimerization of the Fgd1 SH3 binding domain increased cortactin's activity. Direct dimerization of cortactin alone via its N-terminus also increased its activity. Therefore, increased local concentrations of Fgd1 or other ligands for the SH3 domain of cortactin may provide a cellular mechanism to target or enhance Arp2/3-mediated actin assembly.

MATERIALS AND METHODS

Protein Purification. Arp2/3 complex was purified from bovine calf thymus (*19*). Actin was purified from chicken muscle and gel filtered (*20*, *21*). Cortactin was prepared as described (*22*), with the following modifications: After cleavage with TEV protease, the sample was dialyzed against buffer A (20 mM Tris, pH 8.3, 40 mM NaCl, 1 mM EDTA). The dialysate was clarified, applied to a MonoQ column (Pharmacia) (1.6 × 10 cm; $V_t = 20$ mL) preequilibrated with buffer A and eluted with a 20−1000 mM NaCl gradient. Fractions containing cortactin were identified by SDS−PAGE. A wild-type glutathione *S*-transferase (GST)-Fgd1 SH3-BD fusion protein, corresponded to amino acid residues 147−169 and a mutant GST-Fgd1 SH3-BD (P159 and P162 were replaced by alanine) were prepared as described (*12*).

Actin Polymerization Assays. Actin polymerization assays were performed as described (*23*). Cortactin (0.03−0.4 μM), 0.1 μM GST-Fgd1 SH3-BD, and/or Fgd1 SH3-BD peptide (0.1−1 μM) were incubated with 100 nM Arp2/3 complex in 10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, and 1 mM EGTA at 25 °C. Actin polymerization was initiated by the addition of 2 μM monomeric actin containing pyrene-labeled actin (5%). Polymerization was followed by continuous measurement of pyrene fluorescence on a PTI Quantmaster TM spectrofluorometer (Photon Technology International, Santa Clara, CA) with excitation at 368 nm and emission at 386 nm (*3*, *24*). Calculation of the concentration of barbed ends created was performed as described (*25*), using the slope of the pyrene actin polymerization curves at the point of 50% polymerization.

Formation of GST-Heterodimer. GST-Fgd1 SH3-BD (0.1 μM) and GST-Pac1 were mixed in molar ratios of 1:1, 1:5, and 1:10 and then dialyzed 24 h at 4 °C against 10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, and 1 mM EGTA, 0.5 mM DTT. GST-Pac1was prepared by Dr. Wei-Lih Lee of our laboratory, as described (*23*).

Miscellaneous. Chemicals and reagents were from Fisher Scientific and Sigma Chemical Co., unless stated otherwise. The synthetic peptide Fgd1 SH3-BD, containing 23 amino acids ($_{147}$RPSPLKRAPGPKPQVPPKPSYLQ$_{169}$) of the N-terminal proline-rich domain, was synthesized in the lab of Dr. Robert Mecham (Department of Cell Biology, Washington University). The sequence was verified by mass spectrometry (M_r of 2539 Da).

RESULTS

Cortactin provides Arp2/3 complex with the ability to nucleate actin assembly. To do this, cortactin must bind both Arp2/3 complex and F-actin (*3*). Fgd1 is composed of several domains: an N-terminal proline-rich domain (PRD), an adjacent DH and pleckstrin homology (PH) domain, a FYVE domain, and a second C-terminal PH domain (Figure 1A). The PRD domain contains at least two potential SH3 binding sequences. However, only one of them, corresponding to amino acid residues 158−165 (KPQVPPKP), binds directly to the SH3 domain of cortactin (Figure 1A) (*12*).

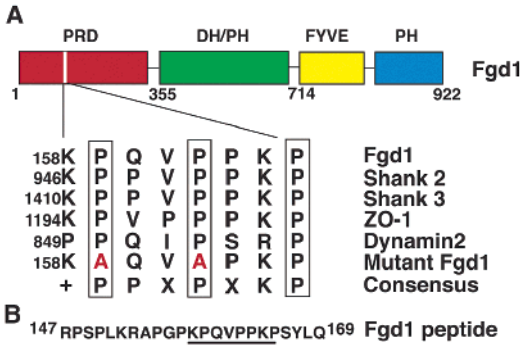

FIGURE 1: Fgd1 domain structure and cortactin SH3 binding domain sequence. (A) The diagram illustrates the structural domains of Fgd1 including the proline-rich domain (PRD), Dbl homology and pleckstrin homology domain (DH/PH), FYVE domain, and a second PH domain. Also shown is a sequence alignment of SH3 binding motifs within PRD domains in various cortactin-binding proteins and a consensus sequence. (B) Sequence for the Fgd1 SH3-BD synthetic peptide used here. The cortactin binding sequence is underlined.

We asked whether interaction with Fgd1 altered cortactin's ability to activate actin nucleation by Arp2/3 complex, using pure proteins in vitro. Pyrene actin polymerization assays were performed with purified Arp2/3 complex and cortactin. As previously shown (*3*), cortactin at 30 and 400 nM enhanced the rate of actin polymerization in the presence of 100 nM Arp2/3 complex (Figure 2A,B, curves b and d).

First, the Fgd1 SH3-BD was added in the form of a GST fusion protein (GST-Fgd1 SH3-BD; residues 147−169). 100 nM GST-Fgd1 SH3-BD enhanced the rate of actin polymerization (Figure 2A,B, compare curve c with b, and e with d). In contrast, the addition of a mutant form of GST-Fgd1 SH3-BD (both P159 and P162 replaced by alanine) (Figure 1A) that does not bind cortactin had no effect (Figure 2C, curve c). These data indicated that, by binding to cortactin, GST-Fgd1 SH3-BD enhanced Arp2/3 actin polymerization.

We investigated the mechanism of this effect. Cortactin is a monomer (*26*), and GST is known to dimerize with itself with high affinity (*27*). Thus, we asked whether the effect of GST-Fgd1 SH3-BD was due to dimerization via GST. A synthetic peptide containing only the Fgd1 SH3-BD sequence (residues 147−169) (Figure 1B) was added to the actin polymerization assays. This peptide had the same sequence (23 amino acids) as the GST-Fgd1 SH3-BD, except without GST. The peptide alone had no effect on cortactin, even at high concentrations up to 500 nM (Figure 3A).

The result indicates that the SH3-BD peptide binds to cortactin but does not stimulate its activity. If so, then the peptide should compete with GST-Fgd1 SH3-BD. When increasing amounts of SH3-BD peptide (100, 500, and 1000 nM) were added to reactions with 100 nM GST-Fgd1 SH3-BD, the rate of actin polymerization decreased to near the control level as predicted (Figure 3B,C). We conclude that

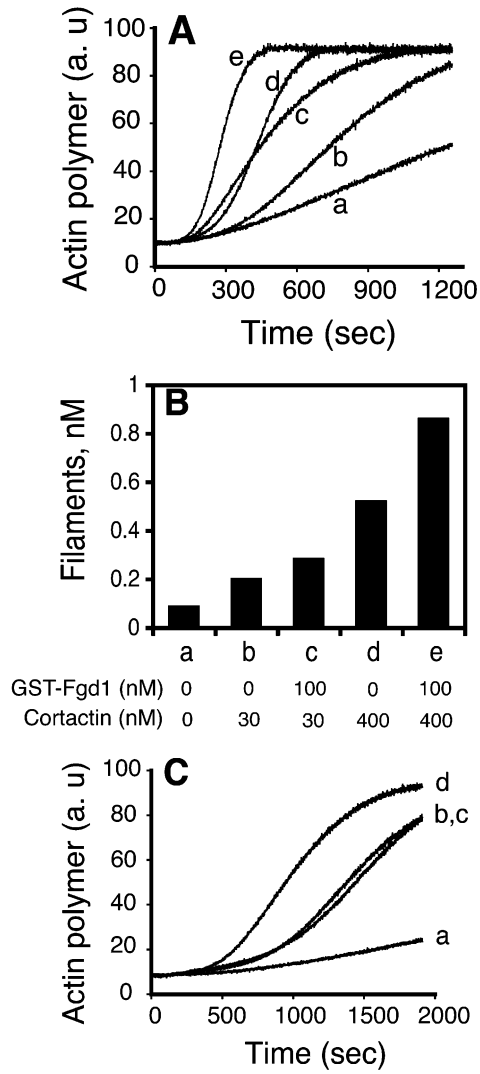

FIGURE 2: Effect of GST-Fgd1 SH3-BD on cortactin in actin polymerization assays. Fluorescence of pyrene-labeled actin is plotted vs time. All reactions contained 100 nM Arp2/3 complex and 2 μM actin, with 5% pyrene labeled. (A) Wild-type GST-Fgd1 SH3-BD. (a) control with only actin and Arp2/3 complex; (b) 30 nM cortactin; (c) 30 nM cortactin + 100 nM GST-Fgd1 SH3-BD; (d) 400 nM cortactin; (e) 400 nM cortactin +100 nM GST-Fgd1 SH3-BD. (B) Number of filaments nucleated. The concentration of free barbed ends was calculated from the curves in panel A. (C) GST-Fgd1 SH3-BD mutant. (a) control with only actin and Arp2/3 complex; (b) 100 nM cortactin; (c) 100 nM cortactin + 100 nM mutant GST-Fgd1 SH3-BD; (d) 100 nM cortactin + 100 nM GST-Fgd1 SH3-BD.

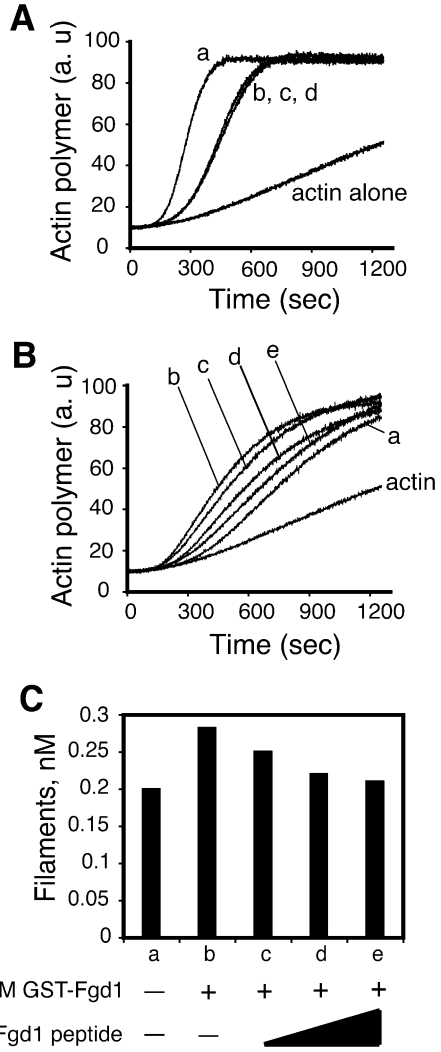

FIGURE 3: Effect of Fgd1 SH3-BD synthetic peptide on cortactin in actin polymerization assays. All reactions contained 100 nM Arp2/3 complex and 2 μM actin, 5% pyrene labeled. (A) Fgd1 SH3-BD synthetic peptide has no effect. Curves: (a) 400 nM cortactin + 100 nM GST-Fgd1 SH3-BD; (b) 400 nM cortactin; (c) 400 nM cortactin + 100 nM peptide; (d) 400 nM cortactin + 500 nM peptide. (B) Fgd1 SH3-BD synthetic peptide competes with GST-Fgd1 SH3-BD. 30 nM of cortactin was present in all reactions. Curves: (a) control with no addition; (b) 100 nM GST-Fgd1 SH3-BD; (c) 100 nM GST-Fgd1 SH3-BD + 100 nM peptide; (d) 100 nM GST-Fgd1 SH3-BD + 500 nM peptide; (e) 100 nM GST-Fgd1 SH3-BD + 1000 nM peptide. (C) Number of filaments nucleated. The concentration of free barbed ends was calculated from the curves in panel B.

the SH3-BD peptide competes with GST-Fgd1 SH3-BD for binding to the cortactin SH3 domain, and that the bound synthetic peptide does not enhance or inhibit the ability of cortactin to stimulate Arp2/3 complex-mediated actin polymerization.

These results suggested that GST-Fgd1 SH3-BD stimulated actin polymerization by dimerizing cortactin. Alternatively, the GST-Fgd1 SH3-BD protein might have bound to cortactin more avidly than did the SH3-BD peptide, or the GST portion of the fusion protein itself might have had a stimulatory affect on cortactin. To distinguish among these possibilities, we created heterodimers of GST-Fgd1 SH3-BD with a nonspecific GST fusion protein available in our lab, GST-Pac1. Pac1p is a yeast protein that regulates dynein and is not involved in actin polymerization (*28*). Addition of increasing molar ratios of GST-Pac1 to GST-Fgd1 SH3-BD completely inhibited the ability of GST-Fgd1 SH3-BD to stimulate cortactin (Figure 4). Thus, the ability of GST-Fgd1 SH3-BD to activate cortactin does not depend on the GST portion of the fusion protein interacting with cortactin. Instead, dimerization appears to be the cause.

To test this hypothesis further, we asked whether we could activate cortactin by dimerization in a different manner. Since the SH3 domain of cortactin is located near its C-terminus, we dimerized cortactin at its N-terminus. GST was fused directly to the N-terminus of cortactin. GST-cortactin stimulated actin polymerization with Arp2/3 complex markedly better than did cortactin (Figure 5 A,B).

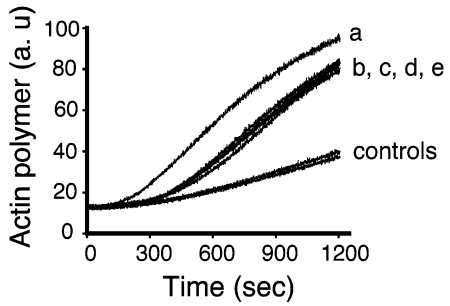

FIGURE 4: Effect of heterodimer formation on GST-Fgd1 SH3-BD. All reactions had 100 nM Arp2/3 complex and 2 μM actin, 5% pyrene labeled. The two control curves contained no other addition or only GST-Pac1. Curves: (a) 30 nM cortactin and 100 nM GST-Fgd1 SH3-BD; (b) 30 nM cortactin; (c) 100 nM GST-Fgd1 SH3-BD + 100 nM GST-Pac1; (d) 100 nM GST-Fgd1 SH3-BD + 500 nM GST-Pac1; (e) 100 nM GST-Fgd1 SH3-BD + 1000 nM GST-Pac1.

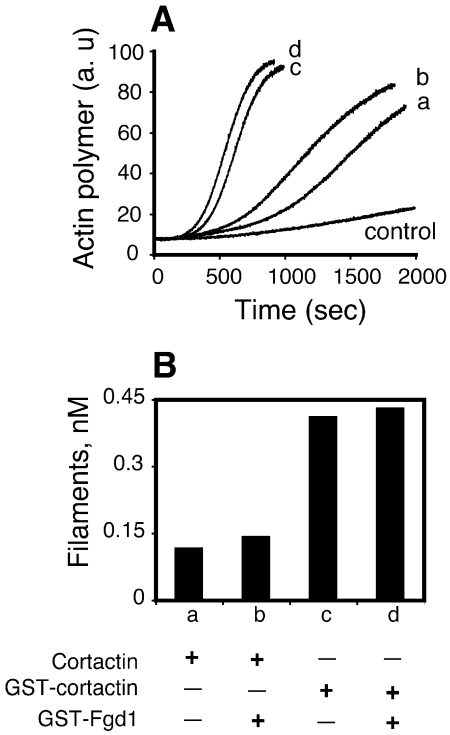

FIGURE 5: Effect of dimerization of cortactin at its N-terminus. (A) Pyrene actin polymerization vs time. All reactions contained 100 nM Arp2/3 complex and 2 μM actin, 5% pyrene-labeled. The control curve had no other addition. Curves: (a) 100 nM cortactin; (b) 100 nM cortactin + 100 nM GST-Fgd1 SH3-BD; (c) 100 nM GST-cortactin; (d) 100 nM GST-cortactin + 100 nM GST-Fgd1 SH3-BD. (B) Number of filaments nucleated. The concentration of free barbed ends was calculated from the curves in panel A.

DISCUSSION

To determine how Fgd1 protein might influence actin assembly via cortactin, we examined the effect of the Fgd1-SH3 binding domain on actin assembly nucleated by Arp2/3 complex and cortactin. One important result was that cortactin is fully active when its SH3 domain is bound to a monomeric ligand. Cortactin's ability to nucleate and stabilize actin filament assembly depends on its N-terminal acidic and central tandem repeat regions, which bind Arp2/3 complex and F-actin, respectively. Full-length cortactin has the same activity in this regard as the active fragment of cortactin containing only the NTA and tandem repeat regions. We found here that occupation of the SH3 domain of cortactin by a monomeric ligand, the SH3 binding peptide of Fgd1, allows cortactin to retain its full activity for actin assembly. Thus, SH3 ligands may target cortactin and thereby actin assembly to locations in the cell.

Another important result came from the observation that GST fusions of the SH3 binding domain of Fgd1 enhanced the activity of cortactin in Arp2/3 complex-mediated actin assembly. We determined that this increase was due to dimerization of cortactin caused by dimerization of GST. The effect was not specific for GST, as heterodimers of GST-Fgd1−SH3-BD with GST-Pac1 (a control protein) did not enhance cortactin activity. Furthermore, increased activity was seen with cortactin dimerized via its N-terminus, as well as via its C-terminal SH3 domain.

One potential implication of this observation for actin assembly in vivo is that signaling scaffold complexes might act to multimerize cortactin at specific locations in the cell. Indeed, Fgd1 and cortactin both show punctate, nonrandom distributions in the cytoplasm (*12*). At these locations, the ability of cortactin to promote and stabilize actin filament assembly may be increased relative to its activity free in the cytoplasm.

In general, complexes composed of signaling and scaffold proteins that bind to actin in multiple different ways seem to display enhanced ability to promote actin assembly. In these cases, simple bringing the various reactants close to each other in space may be sufficient to increase the rate of the reaction for actin polymerization. Alternatively, the reactants may be held in specific positions that promote the polymerization reaction. The former hypothesis is supported here by the fact that dimerization of cortactin via its N- or C-terminal regions increases actin assembly. In a previous study, a GST fusion of the WA fragment of WASp was far more potent in stimulating Arp2/3 complex than was cleaved WA alone (*19*). The W (WH2) domain binds an actin monomer, while cortactin binds an actin filament, so the molecular mechanisms may differ.

Fgd1 in a Positive Feedback Cycle with Membrane-Based Actin Assembly. Fgd1 has multiple domains, each capable of specific interactions with components of the plasma membrane and the machinery of actin polymerization. These domains include an N-terminal PRD followed by a Dbl-homology (DH) and pleckstrin homology (PH) domain (DH/PH), a FYVE domain, and a C-terminal PH domain (*12*). The DH/PH domain functions as a guanidine nucleotide exchange factor (GEF) specific for Cdc42. Cdc42 at the plasma membrane can activate WASp family proteins, which in turn activate Arp2/3 complex (*16*, *29*). Fgd1 binds cortactin via one of at least two SH3 domain-binding motifs in its PRD (*12*). An obvious role for the FYVE and PH domains of Fgd1 would be to assist in targeting Fgd1 to the plasma membrane via interactions with phosphoinositides (*30*). On the other hand, the N-terminal PRD of Fgd1 is necessary and sufficient for targeting of Fgd1 to the cortical region of the cell (*31*). Truncation of the cortactin SH3 domain decreases localization of Fgd1 at the cortical region. These observations suggest that cortactin, via its SH3 domain, may target Fgd1 to the subcortical actin cytoskeleton, and that abnormal Fgd1 localization results in actin cytoskeleton abnormality and significant change in cell shape. To combine these observations into one model, one might envision a role

for Fgd1 as a scaffolding and signaling molecule in a positive feedback cycle for actin assembly. Initially, Fgd1 may be targeted to the membrane and cortex via interactions with phosphoinositides and cortactin. Once recruited, Fgd1 may then promote actin assembly via its effects on Cdc42 and cortactin. Additional actin assembly mediated by Arp2/3 complex would then recruit additional cortactin and thus additional Fgd1 in a positive feedback cycle. These results support the view that Fgd1 activates Cdc42 and targets actin assembly in osteoblasts as its essential function in skeletal development (*12*).

Other Protein Ligands for the SH3 Domain of Cortactin. WIP has a PRD that binds directly to the SH3 domain of cortactin. WIP colocalizes with cortactin at the cell periphery. In vitro, WIP enhances cortactin-mediated activation of Arp2/3 complex (*11*). A point mutation in the cortactin SH3 domain (W525K) that inhibits proline-rich ligand binding prevents WIP from activating Arp2/3-mediated actin polymerization. WIP's effect in this system may involve its WH2 (WASp homology2) domain, which binds to actin monomers and may deliver them to Arp2/3 complex to enhance actin nucleation and polymerization (*11*).

Dynamin2 also has a PRD that binds to the SH3 domain of cortactin. Dynamin2 modulates Arp2/3 complex-mediated actin assembly (*10*). Mutating the W252 residue of the cortactin SH3 domain to K abolished the effect of dynamin2. Dynamin2 appears to oligomerize with itself. Here, we found that dimerization of cortactin increased its activity, so the effect of dynamin2 on cortactin may be caused by multimerization as well (*10*, *32*). Other ligands for the SH3 domain of cortactin include ZO-1 and Shank proteins (*1*), which have not been tested for effects on actin assembly via cortactin.

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