Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation

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Cortactin is a *c-src* substrate associated with sites of dynamic actin assembly at the leading edge of migrating cells. We previously showed that cortactin binds to Arp2/3 complex, the essential molecular machine for nucleating actin filament assembly. In this study, we demonstrate that cortactin activates Arp2/3 complex based on direct visualization of filament networks and pyrene actin assays. Strikingly, cortactin potently inhibited the debranching of filament networks. When cortactin was added in combination with the active VCA fragment of N-WASp, they synergistically enhanced Arp2/3-induced actin filament branching. The N-terminal acidic and F-actin binding domains of cortactin were both necessary to activate Arp2/3 complex. These results support a model in which cortactin modulates actin filament dendritic nucleation by two mechanisms, (1) direct activation of Arp2/3 complex and (2) stabilization of newly generated filament branch points. By these mechanisms, cortactin may promote the formation and stabilization of the actin network that drives protrusion at the leading edge of migrating cells.

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Results and discussion

Cortactin binds filamentous actin (F-actin) and localizes to sites of dynamic actin assembly [1–3]. Although the function of cortactin is largely unknown, it is overexpressed in human tumors [4]. We recently reported that cortactin binds directly to Arp2/3 complex [1], a seven-protein complex that plays a central role in various cellular processes that depend on dynamic actin assembly [5, 6]. Arp2/3 complex nucleates actin filaments in vitro and generates branched filaments with a characteristic 70° angle between branches [7, 8]. The branched filaments that form in vitro closely resemble the meshwork of actin filaments that forms at the leading edge of migrating cells. Arp2/3 complex has been localized in vivo to filament branch points in lamellipodia [9].

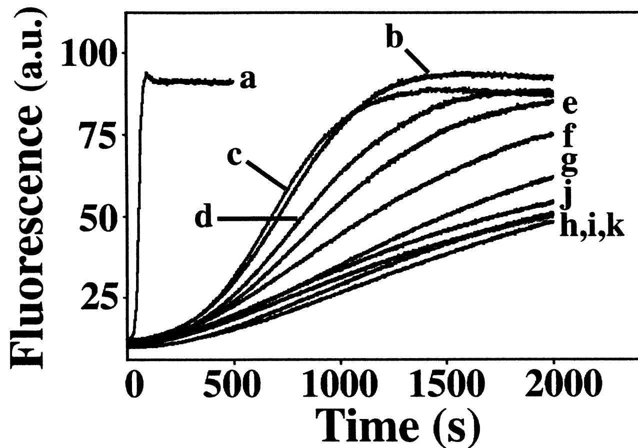
Disassembly of the actin filament network is essential for actin dynamics and cell movement. The process of disassembly includes debranching, which is the process of breaking the network at branch points. Debranching is promoted by dissociation of the phosphate from ADP-Pi-actin filaments, which result from ATP hydrolysis as actin filaments age [8, 10]. ADF/cofilin also promotes disassembly [10, 11].

In cells, Arp2/3 complex is inactive until cellular signals result in its activation [12]. In higher eukaryotes, the only known activators of Arp2/3 complex are the Wiskott-Aldrich syndrome family of proteins (WASps) [12]. WASp family members contain a C-terminal acidic domain that binds Arp2/3 complex. The C-terminal region also contains a verprolin homology and connecting (VC) domain, which binds monomeric actin (G-actin) [12].

Using purified proteins, we tested cortactin for the ability to activate Arp2/3 complex. Recombinant cortactin and the bovine Arp2/3 complex were mixed with pyrene-labeled monomeric actin, and we monitored the actin polymerization as a function of time by measuring the increase in fluorescence. Cortactin activated Arp2/3 complex (Figure 1). The level of activation increased with the cortactin concentration and was saturable, with half-maximal activation of 100 nM Arp2/3 complex occurring with 360 nM cortactin. Compared with the constitutively active VCA fragment of N-WASp (VCA) (curve a), cortactin is a relatively weak activator of Arp2/3 complex.

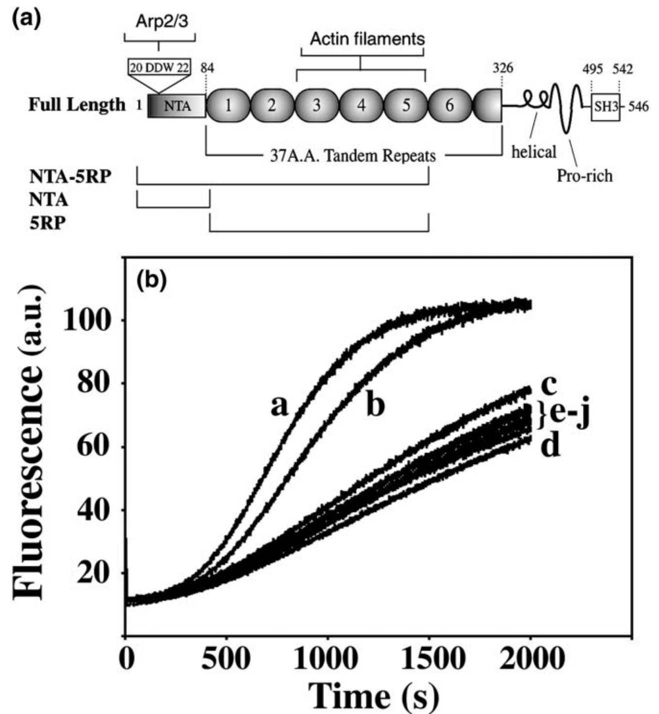
The N-terminal acidic domain of cortactin is necessary and sufficient for binding Arp2/3 complex [1]. Cortactin's central portion, consisting of five or six 37 amino acid tandem repeats, binds F-actin [1, 13]. The C-terminal portion, comprised of proline-rich and SH3 domains, mediates protein-protein interactions with a number of signaling proteins [14–16]. To determine which domains of cortactin were necessary for Arp2/3 activation, we tested truncated cortactin proteins in the pyrene actin assay (Fig-

Figure 1



Cortactin stimulates the actin nucleation activity of Arp2/3 complex. Cortactins or the GST-VCA fragment of human N-WASp were incubated with Arp2/3 complex in 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM imidazole (MKEI) (pH 7.0) at 25°C. Actin polymerization was initiated by the addition of monomeric actin (7.5% pyrene-labeled) and monitored by the continuous measurement of fluorescence at 386 nm [22]. 100 nM Arp2/3 was tested with increasing concentrations of cortactin. Curves (a–i) had 100 nM Arp2/3 complex. Other additions were as follows: curve a, 100 nM GST-VCA; curve b, 2 μM cortactin; curve c, 5 μM cortactin; curve d, 500 nM cortactin; curve e, 200 nM cortactin; curve f, 50 nM cortactin; curve g, 30 nM cortactin; curve h, 10 nM cortactin; and curve i, 0 nM cortactin. Controls without Arp2/3 included curve j, 2 μM cortactin alone; and curve k, buffer alone.

Figure 2



Domains required for activation of Arp2/3 complex. **(a)** Diagram of cortactin structure and tested mutants. **(b)** Structural requirements for cortactin activation of the Arp2/3 complex. The Arp2/3 (25 nM) was tested with 500 nM of cortactin proteins in the pyrene actin assay. Curves (a–e) had 25 nM Arp2/3. Other additions were as follows: curve a, full-length cortactin; curve b, NTA-5RP; curve c, 5RP; curve d, NTA; and curve e, alone. Controls without Arp2/3 included curve f, full-length; curve g, NTA-5RP; curve h, 5RP; curve i, NTA cortactins; and curve j, buffer only.

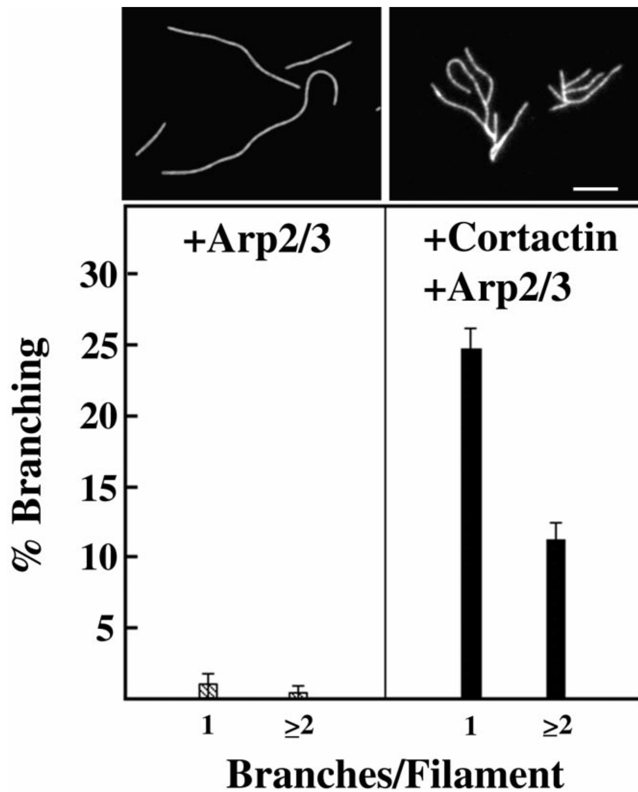
ure 2). The N-terminal fragment of cortactin ("NTA-5RP"), which contains both Arp2/3 and F-actin binding domains, was almost as potent as full-length cortactin in activating Arp2/3 complex. However, when expressed as separate domains, neither the N-terminal acidic domain ("NTA") nor the "5 repeats" region ("5RP") independently activated Arp2/3 complex. These results indicate that cortactin plays a role in stabilizing the binding of Arp2/3 to F-actin. Furthermore, the fact that the proline-rich and SH3 domains of cortactin are not necessary for the activation of Arp2/3 complex indicates that the activity is not due to an SH3 binding cofactor present as a contaminant in Arp2/3 preparation.

We determined the ability of cortactin to promote branched-filament formation by Arp2/3 complex by imaging filament networks directly with fluorescence microscopy (Figure 3) [8]. Cortactin, Arp2/3, and monomeric actin were incubated under the conditions used in the pyrene actin assays. Rhodamine-phalloidin was present during the reaction to stabilize and visualize the actin filaments. Neither cortactin nor Arp2/3 alone promoted branched-filament formation (0% and 1.6% branching, respectively). Cortactin alone also did not bundle or cross-link filaments. However, when added together, cortactin (500 nM) and Arp2/3 complex (200 nM) promoted actin filament branching: 36% of the filaments were branched, and approximately one third of the branched filaments had two or more branches (Figure 3). For comparison, VCA (200 nM) and Arp2/3 (200 nM) induced 52% filament branching, of which slightly more than half had two or more branches (data not shown).

We next asked whether cortactin and VCA act synergistically to promote actin branching. For these experiments, we reduced the concentration of Arp2/3 complex to 50 nM to minimize branching induced with cortactin alone. When cortactin and VCA were added in combination, the level of branching was greatly increased compared to branching with VCA or cortactin alone (representative experiment shown in Figure 4a, n = 3).

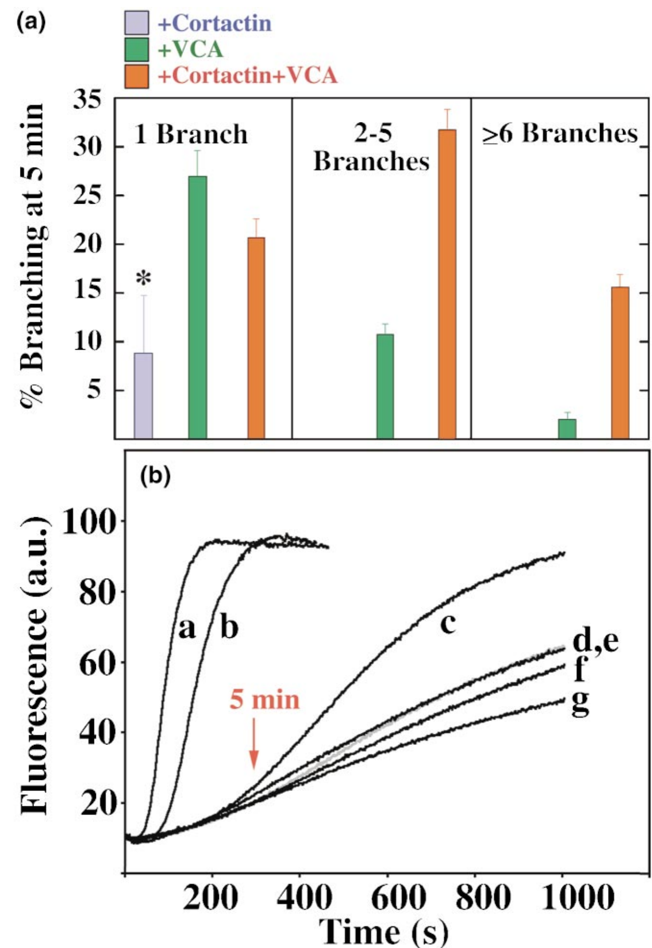
We also performed pyrene actin assays of polymerization rates to assess new barbed-end formation in response to the combination of cortactin and VCA. In the representative experiment shown in Figure 4b, the concentration of barbed ends was 0.15 nM in the presence of Arp2/3 or buf-

Figure 3



Cortactin induces Arp2/3 complex to form branched actin filaments. The upper panels show representative images of filaments. The lower panels show quantitative analysis of branching in the filament networks. Arp2/3 ("Arp2/3") (200 nM) or 500 nM Cortactin + 200 nM Arp2/3 ("Cortactin + Arp2/3") were incubated with 2.5 μM actin and 2.5 μM rhodamine-phalloidin for 35 min at 22°C. Polymerization reactions were diluted into fluorescence buffer (1× MKEI, 100 mM dithiothreitol, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 3 mg/ml glucose, and 0.5% methylcellulose), applied to glass coverslips coated with 0.1% nitrocellulose, and then visualized by fluorescence microscopy [8]. The scale bar represents 3 μm. Branching was quantified as follows: % Branching = (number of branched filaments/number of total filaments) × 100. Percent branching was subcategorized into the percent of total filaments with one branch and the percent of total filaments with two or more branches. Error bars represent standard error of the mean (SEM; n = 2).

Figure 4

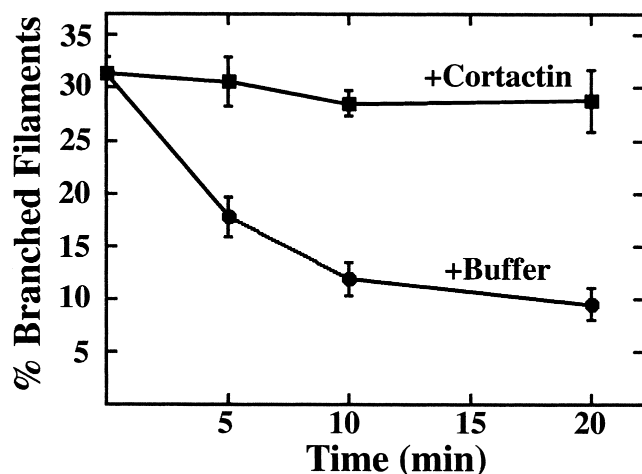


Cortactin synergizes with the active fragment of N-WASp to induce branched filament formation. **(a)** Quantitative analysis of filament branching at 5 min after the initiation of polymerization. Arp2/3 (50 nM) was incubated with G-actin (3 μM) and rhodamine-phalloidin (3 μM) and either 500 nM cortactin (+cortactin), 400 nM GST-VCA (+VCA), or both cortactin and GST-VCA (+cortactin, +VCA). The percent of total actin filaments with one branch, between two and five branches, or six branches or more was quantified. *Cortactin + Arp2/3 samples had very few filaments at 5 min (see pyrene assay, panel b for comparison), so the sample size is relatively small and the error bar is relatively large. Error bars represent SEM. **(b)** Pyrene actin assay. Arp2/3 (50 nM) was incubated with pyrene-labeled G-actin (3 μM) and either cortactin (500 nM), GST-VCA (400 nM), or both cortactin and GST-VCA. Curves (a–d) had 50 nM Arp2/3. Other additions were as follows: curve a, 500 nM cortactin + 400 nM GST-VCA; curve b, 400 nM GST-VCA; curve c, 500 nM cortactin; and curve d, no additions. Controls without Arp2/3 included curve e, cortactin; curve f, buffer; and curve g, GST-VCA. The arrow indicates the time point corresponding to the branching assay analyzed in panel (a).

fer alone, 0.30 nM in the presence of cortactin + Arp2/3, 1.75 nM in the presence of VCA + Arp2/3, and 3.05 nM in the presence of VCA + cortactin + Arp2/3. The increase in barbed-end concentration induced by cortactin + VCA is significantly greater than the sum of the increases induced by cortactin and VCA alone. In four independent experiments, an equivalent level of synergy between cortactin and VCA was consistently observed (see Supplementary material available with this article on the internet for a table of all results).

Actin filament networks created by Arp2/3 complex debranch over time in vitro [8, 10] and model the disassembly that occurs in vivo. We asked whether cortactin stabilizes branched networks by inhibiting debranching. Actin filaments were prepolymerized in the presence of VCA (600 nM) and Arp2/3 (100 nM) and in the absence of cortactin. At 8 min, the actin network was fully polymerized and

Figure 5



Cortactin inhibits debranching of VCA/Arp2/3-nucleated actin filaments. G-actin (3 μM) was polymerized in the presence of Arp2/3 (100 nM) + GST-VCA (600 nM) for 8 min. At that time, rhodamine-phalloidin (0 min sample), 500 nM cortactin (+Cortactin), or buffer (+Buffer) was added to individual parallel reactions. The cortactin and buffer samples were incubated for 5, 10, or 20 additional min before the debranching reactions were stopped with equimolar rhodamine-phalloidin and visualized. Error bars represent SEM (n = 3).

had begun to debranch. Cortactin (500 nM) or buffer was added, and we removed samples and added them to rhodamine-phalloidin to arrest debranching and visualize the filament network. This experiment was designed to measure debranching. One cannot rule out the possibility that some new filaments were formed during the course of the reaction; however, only minimal G-actin was available for de novo filament formation, because the majority of the actin was in the polymerized form. As shown in Figure 5, cortactin stabilized the branched-filament networks, whereas control networks disassembled over the course of the experiment. These results indicate that cortactin plays a role in stabilizing branch points in actin filament networks.

In higher eukaryotes, cortactin is the only known activator of Arp2/3 complex that is not a member of the WASp family [12]. Homology between cortactin and WASps is restricted to the acidic Arp2/3 binding domain [1, 12]. Notably, cortactin does not contain the VC G-actin binding domain found in WASps and, accordingly, does not bind monomeric actin [13]. In WASps, the VC domain is necessary for the activation of Arp2/3 complex [17, 18], and this finding suggests that cortactin activates Arp2/3 by a different mechanism. Cortactin binds to filamentous actin [13] and to Arp2/3 [1]. Furthermore, the association of Arp2/3 with F-actin may be necessary for the optimal activation of Arp2/3 [18–20]. Thus, the major function of cortactin may be to stabilize the connection between Arp2/3 complex and actin filaments. This mechanism of action is consistent with the requirement for both the F-actin and Arp2/3 binding domains of cortactin in the activation of Arp2/3 complex and may also explain the synergy of cortactin with VCA.

Cortactin localizes to sites of dynamic actin assembly in vivo, including the actin tail of rocketing endosomes [2, 3]. Of note, N-WASp is present only at the rim of the endosome [2, 21], whereas Arp2/3 complex and cortactin are also present throughout the F-actin tail [2, 3]. These results are consistent with our proposed mechanism in which cortactin binds to F-actin and Arp2/3 complex to stabilize sites of actin filament branching.

The finding that cortactin inhibits debranching of Arp2/3-induced filament networks represents a novel mechanism for promoting actin assembly. In contrast to WASp family members, cortactin stabilizes branched filaments once they are formed. The mechanism for this stabilization of branch points may involve the ability of cortactin to bind F-actin and Arp2/3 complex simultaneously, perhaps by increasing the affinity of Arp2/3 complex for either the mother filament or the daughter branch. The latter could involve increasing the affinity of Arp2/3 complex for the pointed end of actin filaments, which has been proposed to be important in regulating branch stability [10]. Whereas cortactin inhibits debranching, ADF/cofilin accelerates debranching and promotes filament disassembly [10, 11]. Thus, the relative activities of cortactin and ADF/cofilin may be important determinants of the extent and speed of dynamic actin assembly in cells.

In conclusion, we have discovered that cortactin promotes branched actin filament network formation by Arp2/3 complex. The mechanism has two parts. First, cortactin activates Arp2/3, both separately and in combination with the VCA domain of N-WASp; second, cortactin inhibits disassembly of preformed Arp2/3-nucleated filament networks.

Supplementary material

Supplementary data and additional details describing the experimental methods are available at http://current-biology.com/supmat/supatin.htm.

Note added in proof

A recently published paper (Urono T, Liu J, Zhang P, Fan YX, Egile C, Li R, *et al.*: **Activation of Arp 2/3 complex-mediated actin polymerization by cortactin.** *Nat Cell Biol 2000*, **3**:259-266.) presents findings similar to those described herein and in our previous publication (Weed et al., 2000). This topic and these papers were discussed in a minireview (Zettle M, Way M: **New tricks for an old dog?** *Nat Cell Biol 2001*, 3:E74-E75).

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