Sequential Interaction of Actin-related Proteins 2 and 3 (Arp2/3) Complex with Neural Wiscott-Aldrich Syndrome Protein (N-WASP) and Cortactin during Branched Actin Filament Network Formation

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The WASP and cortactin families constitute two distinct classes of Arp2/3 modulators in mammalian cells. Physical and functional interactions among the Arp2/3 complex, VCA (a functional domain of N-WASP), and cortactin were examined under conditions that were with or without actin polymerization. In the absence of actin, cortactin binds significantly weaker to the Arp2/3 complex than VCA. At concentrations of VCA 20-fold lower than cortactin, the association of cortactin with the Arp2/3 complex was nearly abolished. Analysis of the cells infected with Shigella demonstrated that N-WASP located at the tip of the bacterium, whereas cortactin accumulated in the comet tail. Interestingly, cortactin promotes Arp2/3 complex-mediated actin polymerization and actin branching in the presence of VCA at a saturating concentration, and cortactin acquired 20 nm affinity for the Arp2/3 complex during actin polymerization. The interaction of VCA with the Arp2/3 complex was reduced in the presence of both cortactin and actin. Moreover, VCA reduced its affinity for Arp2/3 complex at branching sites that were stabilized by phalloidin. These data imply a novel mechanism for the de novo assembly of a branched actin network that involves a coordinated sequential interaction of N-WASP and cortactin with the Arp2/3 complex.

Dynamic cortical actin assembly is intimately associated with membrane protrusions, cell crawling, phagocytosis, and intracellular vesicle trafficking. While assembly of actin bundles or cables requires formin-like proteins (1, 2), de novo actin polymerization in the cell cortex occurs primarily as the result of the function of actin-related proteins 2 and 3 (Arp2/3), which form a stable protein complex with five other unique proteins (3–5). The Arp2/3 complex serves as a nucleation site for actin assembly, a major rate-limiting step in actin elongation, a major rate-limiting step in actin assembly, and includes the cortactin family proteins (cortactin, Refs. 21–22 and HS1, Ref. 23) in metazoans, and myosins-I (24–26) and Abp1 (27) in yeast. The ability of these proteins to promote actin assembly, however, appears to be modest compared with the WASP-related proteins, and the significance of such modest activations remains unclear.

Cortactin contains an N-terminal acidic domain that binds to the Arp2/3 complex and a central repeat domain consisting of 6.5 tandem repeats of unique 37 amino acids that binds to F-actin (21, 28, 29). Its C-terminal region contains a Src homology 3 (SH3) domain that associates with a variety of cellular proteins including CortBP1 (30), CBP90 (31), ZO-1 (32), and dynamin-2 (33). The structure of cortactin is well conserved in higher eukaryotes. Cortactin-like proteins have been also found in sea urchin, sponge, fruit fly, frog, chicken (35), mouse (36), and human (37), indicating an established function of cortactin at a very early stage of evolution. In contrast, no myosin I- or Abp1-like proteins that contain the acidic motif required for Arp2/3 binding have been found in mammalian cells (38). The only cortactin-related protein is HS1, a hematopoietic cell-specific protein, which also contains a similar N-terminal region, the repeat, and the C-terminal SH3 domains. The major difference between HS1 and cortactin is that HS1 has only 3.5 tandem repeats (23, 34).

While WASP proteins potentiate strongly the actin nucleation activity of the Arp2/3 complex, the branched filaments formed by WASP and the Arp2/3 complex are unstable and quickly undergo debranching by a mechanism that is still not clear (10, 39, 40). On the other hand, cortactin has a modestly lower affinity for actin polymerization, the branched actin filament network formed is stable and plays a critical role in cell migration and invasion.
activity for actin nucleation, but promotes significantly the formation of stable actin branches (22, 23). Despite these differences, WASP and cortactin bind to the Arp2/3 complex through a similar structural domain consisting of multiple acidic residues and a single tryptophan (7, 21). A recent study has reported that although both N-WASP and cortactin bind to the Arp3 subunit of the Arp2/3 complex (41), N-WASP binds to Arp2 and p41 subunits as well (41, 42). Interestingly, a cortactin fragment containing only the Arp2/3 binding domain competes poorly with N-WASP for the Arp2/3 complex even at a concentration 1000-fold higher than N-WASP (21, 41). Based on this finding, it has been suggested that a ternary complex of N-WASP, cortactin, and Arp2/3 might exist in the presence of excess amounts of cortactin, and the activities of WASP and cortactin might be synergistic for actin assembly under this condition (41). Indeed, both proteins have been implicated in the same actin-dependent cellular processes such as membrane ruffling (33, 43), podosome dynamics (35, 44), vesicle propulsion (45, 46), and actin comet tail formation by infectious agents (16, 47–49). However, there is evidence suggesting that cortactin may interact with the Arp2/3 complex in a mechanism distinct from N-WASP. For example, both cortactin and N-WASP are abundant proteins, and their cellular concentrations are in micromolar ranges (21, 50). It is also known that the majority of cortactin proteins are intimately associated with the Arp2/3 complex within cells (21). Thus, the precise role of cortactin and N-WASP and the nature of their functional relationship in actin assembly remain to be defined.

Here, we report that although the affinity of VCA, a constitutively active N-WASP peptide, for the Arp2/3 complex is much higher than that of cortactin in the absence of actin, such affinity is significantly reduced once actin polymerization is initiated. The release of VCA from the Arp2/3 complex is further promoted by the presence of cortactin, which apparently has increased its affinity for the activated Arp2/3 complex. Our data suggest that while N-WASP interacts primarily with the free form of the Arp2/3 complex, the more likely target for cortactin is the complex of Arp2/3 and F-actin at a branching site. Thus, we propose that the rapid formation of actin filaments requires a sequential event involving an initial activation of the Arp2/3 complex by N-WASP and a subsequent interaction between activated Arp2/3 complex and cortactin at the branching point.

EXPERIMENTAL PROCEDURES

Proteins—Murine cortactin tagged with 6×His at its C terminus was expressed in Escherichia coli and purified as described previously (21). Human N-WASP-derived VCA peptide tagged by GST was expressed and purified from E. coli as described (16) and further purified by Mono Q chromatography. For some experiments, GST-VCA was digested with thrombin at room temperature overnight, and the released tag-free VCA was further purified by Mono Q chromatography. GST-cort-(1–80), comprising the acidic domain and a region before the central repeat domain, and GST-cort-(1–375), containing both the acidic and the repetitive domains, were expressed in E. coli and purified as described (21). Bovine Arp2/3 complex was purified by a three-step procedure involving Q Sepharose ion-exchange, GST-VCA affinity chromatography and additional Q Sepharose chromatography (23). The protein in the flowthrough fraction of the final Q Sepharose was concentrated by a Centricon 30 (Amicon), and the buffer of the sample was changed to 1× Ca2+-free polymerization buffer containing 10 mM MgCl2, 1 mM EDTA, 0.25 mM ATP, 10 mM imidazole, pH 7.3, 3 mM NaN3, and 0.5 mM diethiothreitol). Protein concentration was determined by the Bradford method using BSA as the standard.

GST Pull-down Assay—To measure the effect of VCA on the binding of cortactin to the Arp2/3 complex, 10 μM Arp2/3 complex was mixed with GST-cort-(1–80) or GST-cort-(1–375), and untagged VCA at different concentrations in a total 200-μl volume of 1× polymerization buffer. After a 15-min incubation at 22 °C, the reaction mixture was supplemented with BSA (1 mg/ml) and transferred to a 1.5-ml vial containing 20 μl of glutathione-Sepharose beads, and then incubated for 30 min with gentle rotation. The beads were pelleted by centrifugation at 300 × g for 1 min and subsequently subjected to 10% SDS–PAGE followed by immunoblotting using anti-Arp3 polyclonal antibody (21). The blot was digitalized by film scanning and quantified by Scion Image software. The result was normalized based on five control samples of the Arp2/3 complex with different amounts on the same gel.

To measure the binding of VCA to the Arp2/3 complex during actin assembly, polymerization of 1.5 μM actin was initiated in the presence of 10 nM Arp2/3 complex, 100 nM GST-VCA, and cortactin at concentrations from 0 to 400 nM in 200 μl of 1× polymerization buffer for 30 min. The reaction mixture was then supplemented with BSA (1 mg/ml), and mixed with 20 μl of glutathione-Sepharose beads, and incubated for 30 min with gentle rotation. The beads were sedimented at 300 × g for 1 min, the supernatant was transferred to a new 50-μl tube. 50 μl of the supernatant was mixed with an equal volume of 2× SDS sample buffer and boiled for 5 min. 16 μl of the sample was subjected to SDS-PAGE (12%) followed by transferring to a nitrocellulose membrane. Arp2/3 complex was detected by immunoblot analysis using Arp3 antibody.

Actin Polymerization—Polymerization of G-actin (10% pyrene-labeled, rabbit skeletal muscle actin from Cytoskeleton Inc.) was performed as described previously (21) with a modification. Briefly, Ca2+-ATP-G-actin in G-actin buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, and 0.5 mM DTT) was mixed with a tenfold-volume of 10× exchange buffer (2 mM EGTA, 1 mM MgCl2) for 3 min at 22 °C to convert to pyrene-labeled G-ATP-G-actin. Polymerization was initiated by adding 60 μl of Mg2+-ATP-G-actin (7.5 μM) to 240 μl of 1.25× polymerization buffer (62.5 mM KCl, 2.5 mM MgCl2, 12.5 mM imidazole, pH 7.3, 1.25 mM EGTA, 0.125 mM CaCl2, 0.625 mM DTT, 0.3125 mM ATP, and 3.75 mM NaCl), containing Arp2/3 complex, GST-VCA, and cortactin at concentrations as indicated. The kinetics of actin polymerization was monitored by monitoring the increase in pyrene fluorescence detected by an LS50B spectrophotometer (PerkinElmer Life Sciences) with filters for excitation at 365 nm and emission at 407 nm.

Fluorescence Microscopy Analysis of Branched Actin Filaments—The analysis was carried out essentially as described previously (10, 13). The specific conditions of actin polymerization for each experiment were described in the corresponding legends. The Arp2/3 complex (Molecular Probes) was added at the times indicated to actin polymerization reactions at a molar concentration equivalent to actin. After 5 min of incubation, the mixture was diluted 400-fold in fresh fluorescein buffer (100 mM KCl, 1 mM MgCl2, 100 mM DTT, 10 mM imidazole, pH 7.3, 0.5% methylcellulose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, and 3 mg/ml glucose). The diluted samples (2.4 μl) were applied onto coverslips precoated with 0.1% nitrocellulose in n-amy acetate, and examined under an Olympus IX-70 inverted microscope using a ×100 objective lens with numerical aperture (NA) of 1.35. Images were captured by a charge-coupled device camera, and further processed on Adobe Photoshop to generate monochromatic images. The lengths of filaments and the number of branch points were measured using image analysis software, and the degree of branching was calculated as the number of branches per micrometer of filament.

Immunofluorescence Analysis of Shigella-infected MDA-MB-231 Cells—Breast cancer MDA-MB-231 epithelial cells expressing GFP (enhanced green fluorescent protein) alone, GFP-N-WASP, or cortactin-GFP were prepared by retrovirus-mediated gene transfer according to the protocol described previously (51). Infection with Shigella flexneri M90T strain (a kind gift of C. Egile) was carried out according to the method described (52). Briefly, overnight culture of the bacteria was diluted in trypticase soy broth (BD Biosciences) at 1:100 and grown for 2 h at 37 °C to an OD600 of 0.2–0.3. Bacteria (100 bacteria/cell) were pelleted, rinsed twice in Ca2+- and Mg2+-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and resuspended with the growth medium (2 ml/well). Cells, seeded on fibronectin-coated coverslips in a 4-well plate a day before infection, were washed once with the growth medium, over layered with the bacterial suspension, and centrifuged at 2000 rpm for 10 min. Cells were fixed for 1 h at 37 °C in 4% paraformaldehyde, permeabilized in containing 0.2% Triton X-100 for 10 min, and washed three times with the growth medium, and incubated in the medium containing 50 μg/ml of gentamicin for an additional 1–2 h at 37 °C before inspection.

Cells were fixed for 20 min in PBS containing 3.7% paraformaldehyde, incubated for 10 min in PBS–50 mM NH4Cl twice to quench the fluorescence and permeabilize the cells, and then left in the medium containing 0.2% Triton X-100 for 10 min. The cells were blocked for 1 h with PBS containing 2% BSA, incubated with anti-GFP polyclonal antibody (Molecular Probes, 1:100), and anti-cortactin antibody (4F11; Upstate Biotechnology Inc., 1:100) for 1 h in PBS containing 0.2% BSA. After three washes with PBS, the
VCA concentration. The total amount of Arp2/3 in the reaction was plotted as a function of the percentage of the normalized values for each sample in relation to complex samples of five different concentrations loaded on the same gel. The percentage of the normalized values for each sample in relation to the total amount of Arp2/3 in the reaction was plotted as a function of VCA concentration.

N-WASP–VCA Inhibits the Association of Cortactin with Arp2/3 Complex in the Absence of Actin—In an effort to understand the relationship of N-WASP and cortactin during actin assembly, we examined whether the two types of Arp2/3 activators could bind simultaneously to the Arp2/3 complex. A result, the interaction of cortactin and Arp2/3 complex was examined in the presence of a VCA peptide derived from human N-WASP (16) by pull-down analysis with murine cortactin recombinant proteins GST-cort(1–80) and GST-cort(1–375), both of which have a similar affinity for the Arp2/3 complex with a $K_d$ of about 1 $\mu$M (21). As shown in Fig. 1, GST-cort(1–375) was able to pull-down 41% of 10 $nm$ Arp2/3 complex at 1.5 $\mu$M, and 52% at 5 $\mu$M, in the absence of VCA. However, this ability to pull-down the Arp2/3 complex was dramatically inhibited by VCA in a dose-dependent manner and nearly abolished by VCA at concentrations significantly lower than GST-cort(1–375). (200 $nm$ VCA abolished the association of Arp2/3 complex with 1.5 $\mu$M cortactin protein, and 600 $nm$ VCA abolished that with 5 $\mu$M cortactin.) Binding of GST-cort(1–80) to the Arp2/3 complex was also inhibited by VCA in a similar manner (Fig. 1). With either of the cortactin proteins, VCA inhibited 50% of cortactin binding to the Arp2/3 complex at ~50 $nm$. This result is consistent with the previously reported relative affinities of VCA and cortactin for the Arp2/3 complex: VCA has a $K_d$ value from 0.1 to 0.2 $\mu$m (16, 21, 23), and cortactin has a $K_d$ from 0.7 to 1.3 $\mu$m (21, 23, 29, 41). Our finding also agrees with a recent report showing that a similar cortactin peptide encoding the N-terminal region (1–80) competed poorly with VCA in GST pull-down assays (41).

**Cortactin and N-WASP Localize in Distinct Areas in the Comet Tail Induced by S. flexneri**—The above data suggest that binding of cortactin and VCA to Arp2/3 complex is mutually exclusive, making it less likely that they form a ternary complex with the Arp2/3 complex as previously suggested (41). To further confirm this notion, we examined the distribution of cortactin and N-WASP in the actin comet tail formed by the infectious agent S. flexneri in the cytoplasm of epithelial cells expressing human N-WASP fused with GFP (GFP-N-WASP). It has been shown that Shigella protein IcsA at the bacterial surface is able to interact with and activate N-WASP, which in turn activates Arp2/3 complex-mediated actin assembly to induce comet tails (16). The bacteria were stained with DAPI, a DNA binding dye (blue); and GFP-N-WASP and cortactin were stained with anti-GFP (green) and cortactin (red) antibodies, respectively. Although both GFP-N-WASP and cortactin were found in the comet tails, two proteins displayed distinct localization profiles in relation to bacteria (Fig. 2). N-WASP was predominantly localized at either one or both polar ends of the bacterial surface (Fig. 2, *arrowheads*). This observation is consistent with previous reports (16, 48). In contrast, cortactin was primarily associated with the entire tail (Fig. 2, *arrows*), where Arp2/3 complex is known to be abundant (16). Colocalization of GFP-N-WASP and cortactin was found, if any, only in a narrow region where the bacterial head and comet tail meet. Thus, it appears that N-WASP is closely associated with the initiation site for actin assembly while cortactin is stably associated with Arp2/3 complex and the more established actin network. Similar distribution pattern of the two proteins was also reported for the actin comet tail induced by vaccinia virus (53).

**Cortactin Is Able to Promote Actin Polymerization in the Presence of VCA at Saturating Concentrations**—To further explore the specific role of VCA and cortactin in actin polymerization mediated by the Arp2/3 complex, we examined Arp2/3 complex-mediated actin assembly in the presence of both cortactin and GST-VCA. At low concentrations of the Arp2/3 complex (~10 nM), which showed very little intrinsic nucleation activity, cortactin had no significant effect on the activation of the complex even at 500 nM until GST-VCA was added (data not shown), suggesting a synergistic function between cortactin and VCA (21, 22). To understand better the nature of this synergistic function, we analyzed the ability of cortactin to promote actin polymerization in the presence of 1 $\mu$m GST-VCA. Since GST-VCA has a 100 nM affinity for the Arp2/3 complex, and 1 $\mu$m VCA was able to abolish binding of 10 $\mu$m cortactin to the Arp2/3 complex (Fig. 1), it was assumed that 1 $\mu$m GST-VCA would have saturated the binding sites on 8 nM Arp2/3 complex available for cortactin. Surprisingly, under this condition cortactin was still able to provoke a strong actin assembly even at concentrations as low as 5 nM (Fig. 3A). A dose dependence analysis with various concentrations of GST-VCA further demonstrated that cortactin induces consistently a half-maximal stimulation at ~20 nM (Fig. 3B and Refs. 21 and 23), indicating that this value reflects a biochemical property of cortactin independent of GST-VCA. The similar result was also obtained with untagged VCA (data not shown). Therefore, it appears that cortactin promotes actin polymerization in a unique mechanism that apparently requires neither the formation of a complex with N-WASP nor a competition with N-WASP for Arp2/3 binding. Previous studies have demonstrated that cortactin stimulates actin polymerization in a
Arp2/3 binding-dependent manner (21, 22), indicating that 20 nM concentration for a half-maximum stimulation likely reflects the affinity of cortactin for the Arp2/3 complex during actin assembly.

**Cortactin Induces the Release of VCA from the Arp2/3 Complex through the Formation of Branched Actin Filaments**

The above observations led us to hypothesize that the relative affinities of VCA and cortactin for the Arp2/3 complex might have been changed during the course of actin polymerization. To test this possibility, we first examined the interaction of VCA and the Arp2/3 complex by pull-down analysis under the conditions where actin polymerization was involved. Actin polymerization was initiated in the presence of GST-VCA and the Arp2/3 complex with or without cortactin. After 30 min when actin polymerization was completed, the reaction mixture was incubated with glutathione beads to pull-down GST-VCA and the Arp2/3 complex-mediated actin polymerization by cortactin. Actin polymerization was performed under the same condition as described for A, except a broader range (from 1 to 500 nM) of cortactin concentrations was used. The time required to reach half-maximal polymerization ($t_{1/2}$) was calculated from each polymerization curve and is presented as a function of cortactin concentration.

**FIG. 2.** Distinct localization of N-WASP and cortactin in the comet tails induced by *S. flexneri*. MDA-MB-231 epithelial cells expressing GFP-N-WASP were infected with *S. flexneri* M90T strain, and the localization of GFP-N-WASP (green) and cortactin (red) in the comet tail induced by the bacteria was analyzed by immunofluorescent staining using anti-GFP and anti-cortactin antibodies, respectively. Host and bacterial nuclei (blue) were stained with DAPI. Panels A to C show examples of GFP-N-WASP (arrowheads) associated at both ends of a bacterium with either a double or a single cortactin tail (arrows). Panels D and E show examples of GFP-N-WASP (green, arrowheads) found at the posterior tip of a bacterium with a single cortactin tail (red, arrow).

**FIG. 3.** Cortactin stimulates actin nucleation by the Arp2/3 complex in the presence of GST-VCA at a saturating concentration. A, kinetics of actin polymerization in the presence of 1 μM GST-VCA. Polymerization of 1.5 μM pyrene-labeled actin was recorded over time in the presence of 1 μM GST-VCA with or without 8 nM Arp2/3 complex and cortactin at 0, 5, or 100 nM, respectively. B, dose dependence of the stimulation of VCA-Arp2/3 complex-mediated actin polymerization by cortactin. Actin polymerization was performed under the same condition as described for A, except a broader range (from 1 to 500 nM) of cortactin concentrations was used. The time required to reach half-maximal polymerization ($t_{1/2}$) was calculated from each polymerization curve and is presented as a function of cortactin concentration.
associated Arp2/3 complex. The Arp2/3 complex remaining in the supernatant was analyzed by immunoblotting of the Arp3 subunit (Fig. 4A). In the absence of cortactin GST-VCA was able to pull-down more than 90% of the Arp2/3 complex with or without adding actin (Fig. 4A, lane 3; Fig. 4B, at 0 nM cortactin). In the presence of cortactin and actin, the ability of GST-VCA to pull-down Arp2/3 was altered. Under this condition, the interaction between VCA and the Arp2/3 complex was remarkably reduced in a cortactin dose-dependent manner, and the reduction reached a half-maximum at 25 nM cortactin, the value that is consistent with its affinity for the Arp2/3 complex as estimated from the actin polymerization assay. In a control experiment without actin, cortactin showed no detectable effect on the ability of VCA to pull-down the Arp2/3 complex, consistent with the view that cortactin binds weakly to the actin-free form of the Arp2/3 complex.

VCA Is Unable to Inhibit the Branching Activity of Cortactin—To further confirm the changes in the relative affinities of cortactin and VCA for the Arp2/3 complex during the course of actin polymerization, we examined the effect of VCA on cortactin-induced actin branch formation. Previous studies have demonstrated that VCA itself is unable to stabilize actin branches (10, 54). Therefore, one would expect that branched actin could become less stable should cortactin be replaced by VCA from the Arp2/3 complex. Thus, actin polymerization was assembled first in the presence of cortactin and the Arp2/3 complex and GST-VCA to a steady state, and additional GST-VCA was added afterward to the reaction to compete for Arp2/3 binding. The actin branching was then compared with that formed without adding further GST-VCA. Consistent with previous reports (22, 23), actin filaments assembled in the presence of 50 nM cortactin and 10 nM Arp2/3 complex with (a and c) or without (b and d) 50 nM cortactin in a total 300-μl volume of reaction. After 15 min of polymerization, 45 μl of the reaction was transferred to a new tube containing either 5 μl of 1× polymerization buffer (a and b) or 5 μl of 10 μM GST-VCA (c and d), the final concentration was 1.1 μM, and incubated for additional 45 min. Actin branching was then examined by fluorescence microscopy. Panels e and f are the samples for actin branching at 3 min after polymerization initiation in the presence (e) or absence (f) of 50 nM cortactin. Bar in panel c indicates a 5-μm length. B, actin polymerization was performed as above and GST-VCA at a series of concentrations was added after 15 min of polymerization. The degree of actin branching was calculated by counting the number of branching points from actin filaments of 200 μm or longer in length and expressed as branch per μm length filament. The value of actin branching was then plotted as a function of GST-VCA concentration.

**Fig. 4.** Cortactin induces dissociation of Arp2/3 complex from VCA under the condition for actin polymerization. A, analysis of VCA-associated Arp2/3 complex during actin polymerization. GST-VCA (100 nM) and Arp2/3 complex (10 nM) was preincubated with or without cortactin at the indicated concentrations for 3 min. G-actin (1.5 μM) was added to the mixture to initiate actin polymerization in a final volume of 200 μl of 1× polymerization buffer at 22 °C. In the control experiment (−actin), the G-actin buffer alone was added. After 30 min of polymerization, the reaction mixture was supplemented with BSA (1 mg/ml) and mixed with 20 μl of glutathione beads. The beads were pelleted by centrifugation. The supernatant fractions were subjected to SDS-PAGE followed by immunoblotting using anti-Arp3 antibody. The bands corresponding to Arp3 were digitalized by film scanning. Lane 1, half the amount of the sample on lane 2; lane 2, 0 nM GST-VCA and cortactin; lane 3, 100 nM GST-VCA, 0 nM cortactin; lanes 4–9, 100 nM GST-VCA plus 10, 25, 50, 100, 200, and 400 nM cortactin, respectively. B, the quantification of the release of Arp2/3 complex from VCA. The digitalized image of the blot as shown in A was quantified using Scion Image. The Arp2/3 complex associated with GST-VCA was estimated by subtracting that in the supernatant from the total amount in the reaction and plotted as a function of cortactin concentration.

**Fig. 5.** VCA is unable to inhibit actin branching activity of cortactin. A, actin (1.5 μM) was polymerized in the presence of 100 nM GST-VCA and 10 nM Arp2/3 complex with (a and e) or without (b and d) 50 nM cortactin in a total 300-μl volume of reaction. After 15 min of polymerization, 45 μl of the reaction was transferred to a new tube containing either 5 μl of 1× polymerization buffer (a and b) or 5 μl of 10 μM GST-VCA (c and d), the final concentration was 1.1 μM, and incubated for additional 45 min. Actin branching was then examined by fluorescence microscopy. Panels e and f are the samples for actin branching at 3 min after polymerization initiation in the presence (e) or absence (f) of 50 nM cortactin. Bar in panel c indicates a 5-μm length. B, actin polymerization was performed as above and GST-VCA at a series of concentrations was added after 15 min of polymerization. The degree of actin branching was calculated by counting the number of branching points from actin filaments of 200 μm or longer in length and expressed as branch per μm length filament. The value of actin branching was then plotted as a function of GST-VCA concentration.

**Fig. 6.** Assembly of the Arp2/3 complex with N-WASP and cortactin. A, GST-VCA (100 nM) was polymerized in the presence of 100 nM GST-VCA and 10 nM Arp2/3 complex with (a and e) or without (b and d) 50 nM cortactin in a total 300-μl volume of reaction. After 15 min of polymerization, 45 μl of the reaction was transferred to a new tube containing either 5 μl of 1× polymerization buffer (a and b) or 5 μl of 10 μM GST-VCA (c and d), the final concentration was 1.1 μM, and incubated for additional 45 min. Actin branching was then examined by fluorescence microscopy. Panels e and f are the samples for actin branching at 3 min after polymerization initiation in the presence (e) or absence (f) of 50 nM cortactin. Bar in panel c indicates a 5-μm length. B, actin polymerization was performed as above and GST-VCA at a series of concentrations was added after 15 min of polymerization. The degree of actin branching was calculated by counting the number of branching points from actin filaments of 200 μm or longer in length and expressed as branch per μm length filament. The value of actin branching was then plotted as a function of GST-VCA concentration.
replacing cortactin associated with Arp2/3 complex on the branched actin filaments that have been already established.

VCA Reduces Its Affinity for the Arp2/3 Complex at Branched Actin Filaments—Cortactin inhibited maximally only 50% of the binding of the Arp2/3 complex to GST-VCA (Fig. 4). The remaining bound Arp2/3 complex could be in the F-actin-free form to which cortactin binds weakly compared with GST-VCA. These Arp2/3 complexes in the free form might be either released from F-actin as a result of a debranching process (54) or those that did not participate in actin nucleation. To verify that VCA may only bind to the free form of Arp2/3 complex and may acquire a relatively lower affinity than cortactin for Arp2/3 complex once it is associated with F-actin, we examined the interaction of GST-VCA with the Arp2/3 complex at branched actins in the absence of cortactin. Previous studies have shown that branched actin filaments initiated by the Arp2/3 complex can be also stabilized by fixation with phallolidin (10, 54). Thus, we analyzed the interaction of VCA with the Arp2/3 complex in the presence of phallolidin that was added at either 3 or 60 min after nucleation of actin assembly. Actin filaments formed under each condition displayed different degrees of actin branching. At 3 min, many actin filaments remained branched, indicating that a significant portion of the Arp2/3 complex was still at actin branches (Fig. 5A, panel f). On the other hand, the most branched actin filaments had undergone debranching at 60 min (Fig. 5A, panel b), and the majority of the Arp2/3 complex was assumed to be in the free form. When GST-VCA was precipitated from the actin assembly reaction fixed at 3 min with glutathione beads, only 60% of the Arp2/3 complex was able to be pulled down (Fig. 6, A and B). In contrast, nearly 90% of the Arp2/3 complex was pulled down by GST-VCA at 60 min. The efficiency of pull-down at 60 min was the same as that observed without phalloidin and cortactin (Fig. 4) where most branched actin filaments have been debranched. Thus, the ability of GST-VCA to interact with the Arp2/3 complex is apparently inversely correlated with the degree of actin branching. In addition, the pull-down of the Arp2/3 complex by GST-VCA reached a maximum level at concentrations as low as 25 nM (Fig. 6B) at either 3 or 60 min, suggesting that the remaining Arp2/3 complex, which was likely associated with branched F-actin, was unable to be accessed by GST-VCA even at high concentrations. Indeed, when the similar assay was performed in the presence of cortactin that was added after phalloidin fixation, cortactin was no longer able to inhibit the interaction between GST-VCA and the Arp2/3 complex (Fig. 6C), indicating that the Arp2/3 complex associated with GST-VCA was in the free form for which cortactin had a much lower affinity than VCA (Fig. 1). Taken together, these results suggest that VCA had reduced its affinity for the Arp2/3 complex on the branched F-actin.

DISCUSSION

In this study, we provide evidence for dramatic changes in the relative affinities of N-WASP and cortactin for the Arp2/3 complex during the course of actin assembly. Although N-WASP shows predominance for the free form of the Arp2/3 complex, it appears that cortactin has a much higher affinity for the Arp2/3 complex once it is incorporated into actin filaments. First, cortactin promotes significantly Arp2/3-mediated actin nucleation even in the presence of VCA at a concentration 200-fold higher than cortactin that would have occupied all the binding sites of the Arp2/3 complex for cortactin. Based on the analysis of actin nucleation and actin branching (21, 23) (also in this study), we estimated the affinity of cortactin for the Arp2/3 complex at ~20 nM, which is significantly lower than 0.7–1.3 μM for the free form of Arp2/3 as reported previously (21, 23, 29, 41). Second, the interaction between VCA and the Arp2/3 complex becomes much weaker in the presence of cortactin and actin. In fact, by analysis of binding to the Arp2/3 complex in the presence of phalloidin or cortactin, VCA appears to bind to only those Arp2/3 complexes that are in the free form released from debranching. Finally, VCA is not able to displace cortactin from the Arp2/3 complex at branching sites of actin filaments.

Reduced affinity of WASP for the Arp2/3 complex after actin nucleation is initiated has been postulated because of a presumed requirement for a rapid motility driven by actin polymerization (16, 55). It has been reported that the Arp2/3 complex requires hydrolysis of ATP for actin nucleation (55, 56), and VCA prefers ATP-bound Arp2/3 complex with a 140 nM affinity to the ADP-bound form with a 0.7 μM affinity (55). Thus, the function of VCA may be to place an actin monomer in contact with an Arp2/3 complex and stimulate ATP hydrolysis. Hydrolysis of ATP to an ADP-P state would then cause a conformational change that allows Arp2, Arp3, and an actin monomer to associate and form a nucleus, and subsequent release of VCA from the Arp2/3 complex. In this study, we have directly observed the reduced binding of VCA to the Arp2/3 complex, which was only apparent when actin polymerization took place in the presence of cortactin. Although the measurement was carried out when actin polymerization was completed, fixation of actin polymerization by phallolidin at different times has mimicked the function of cortactin to stabilize actin branching.
Fig. 7. A model for the formation of de novo branched actin filaments mediated by Arp2/3 complex, N-WASP, and cortactin. In de novo actin assembly, N-WASP acquires a high affinity for the free form of Arp2/3 complex upon stimulation by membrane-associated signaling molecules such as Cdc42 and phosphatidylinositol 4,5-bisphosphate. The association with N-WASP leads to activation of the Arp2/3 complex, facilitation of its binding to an existing actin filament, and initiation of new actin assembly. At this stage, the activated Arp2/3 complex becomes less accessible to N-WASP but is more prone to the association with cortactin, which binds to both the Arp2/3 complex and the nascent actin filament with an affinity 50-fold higher than for the free form of Arp2/3 complex. The complex of cortactin/Arp2/3/F-actin at the branching site is stable and serves as a better nucleation site for assembly of branched actin filaments.

and demonstrated that the change in the affinity of VCA for the Arp2/3 complex occurs at an early phase of actin assembly. Since phalloidin is known to stabilize actin-associated ADP-Pi, (57), an intermediate form from the transition from ATP to ADP, our finding supports the view that hydrolysis of ATP hydrolysis contributes to the change in the affinity of VCA for the Arp2/3 complex. However, given a dramatic increase in the affinity of cortactin from 1 μM to 20 nM, which is even stronger than VCA for ATP-bound Arp2/3 complex, cortactin could play an important role in the dissociation of VCA from the Arp2/3 complex in a manner independent of ATP hydrolysis.

The enhanced affinity of cortactin for Arp2/3 complex during actin assembly could be a result of a substantial conformational change in the Arp2/3 complex (58, 59). However, this structural change could not be simply due to VCA binding because it only occurs when actin is present. Indeed, GST-cort-(1–80) fusion protein, which has the same affinity for the free form of the Arp2/3 complex as intact cortactin (21), is not able to induce actin polymerization or branching, nor inhibit the activity of an intact cortactin (data not shown). Thus, the Arp2/3 complex itself, whether it is in the activated form or not, is not sufficient to account for the increase in its affinity for cortactin during actin polymerization. It is more likely that the acquired high affinity of cortactin for Arp2/3 complex reflects its interaction with both Arp2/3 complex and actin filament at the branching site. In fact, cortactin is known as a potent F-actin binding protein with an affinity from 0.2 to 0.4 nM (21, 28). Our recent measurements using freshly polymerized actin fixed with phalloidin estimated a $K_d$ value even below 100 nM (23). The alignment of activated Arp2/3 complex with a daughter filament may further enhance the affinity of cortactin. Additional evidence supporting this possibility is a previous observation that cortactin absolutely requires its binding to F-actin for its ability to promote actin nucleation and actin branching (21, 22).

Because of its strong dependence on F-actin binding, we propose a model for a possible interaction between cortactin and Arp2/3 complex at a nucleation site as shown in Fig. 7. In this model, N-WASP activates the Arp2/3 complex by a transient interaction, which results in changes in the configuration of the Arp2/3 complex and increase in the association of the Arp2/3 complex with an existing actin filament (60). Once the branching point is established, N-WASP may be released from the complex as a result of either ATP hydrolysis or replacement by cortactin, which has acquired a much higher affinity for the branching point. Since both WASP-related proteins and cortactin are abundantly present in most cells (21), a high affinity of the WASP family proteins for the Arp2/3 complex, 100–200 nM with N-WASP (16, 21) and 400 nM with WAVE (61, 62), would secure an instant activation of the free form of the Arp2/3 complex in a de novo actin assembly. Similarly, the high affinity of cortactin for the activated Arp2/3 complex would be necessary to facilitate the release of WASP from the branching site of actin filaments and to increase stabilized nucleation sites for a rapid growth of actin filaments. Consequently, the sequential reaction will result in a robust polymerization of branched actin network upon stimulation. Supporting evidence for this novel mechanism for actin polymerization is the observation that cortactin, which itself may not directly activate the free form of the Arp2/3 complex as N-WASP does, can potently stimulate both actin nucleation and actin branching (Fig. 3).

Our conclusion is significantly different from a recent report (41) that VCA and cortactin may bind simultaneously to the Arp2/3 complex via different subunits and form a ternary complex with Arp2/3 complex. The apparent discrepancy is at least partially due to different conditions used in their experimental procedure, which was performed with a cortactin fragment containing only the Arp2/3 binding domain and lacking the activity for either actin nucleation or branching. Although the report has described a competition between VCA and the cortactin fragment and the authors indicated the presence of a common binding site at the Arp3 subunit for both types of protein, no clear conclusion was drawn because of a further finding that the cortactin N-terminal peptide could not compete with the VCA binding sites at Arp2 and p41 sites of the free form of the Arp2/3 complex (41). While our study using fully functional cortactin proteins does not rule out the possibility that N-WASP might still associate with p41 and Arp2 subunits in the presence of cortactin, this possibility is very unlikely under the condition when actin polymerization takes place, given the fact that their relative affinities have been dramatically changed. Consistent with our conclusion, cortactin and N-WASP do not appear to be co-localized in the Shigella-induced actin comet tail that is composed of newly formed branched actin network driven by constitutively activated N-WASP and Arp2/3 complex (16, 63). However, cortactin could be colocalized more closely with WASP family proteins in other cellular systems. For example, cortactin is a primary substrate of the Src protein tyrosine kinases (35, 64) and binds to several membrane-associated proteins including dynamin-2, ZO-1, and CortBP1 via its SH3 domain (30, 32, 33, 65). Thus, cortactin is
likely to function in proximity to the plasma and other intracellular membranes as well. Indeed, cortactin is abundantly present in lamellipodium, membrane ruffles, and endosomes (21, 35, 46, 66). By interacting with different components on the membrane, cortactin and N-WASP likely play an important role in the organization of actin network by fine-tuning the size and branches of actin filaments to power movements of particular types of cellular organelles.

It is also possible that WASP-like proteins may facilitate the association of cortactin with the Arp2/3 complex during actin assembly. In fact, it has been reported that cortactin could bind to N-WASP directly though the cortactin SH3 domain (44). A recent study has also described an interaction between cortactin and WIP, a WASP interacting protein (67). Thus, WASP could also affect cortactin through indirect interaction. While the actin branching activity of cortactin as analyzed in vitro does not appear to require the function of its SH3 domain (data not shown) and the VCA fragment used in this study does not contain an apparent SH3 binding domain, a transient interaction with WASP proteins could contribute to recruitment of cortactin to the sites for cortical actin assembly in cells. Since many cellular proteins have also been found to bind to the cortactin SH3 domain, distinguishing specific roles of these proteins in the function of cortactin/WASP/Arp2/3-mediated cortical actin assembly may eventually provide a detailed mechanism for regulating and directing actin assembly in cells.

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Sequential Interaction of Actin-related Proteins 2 and 3 (Arp2/3) Complex with Neural Wiscott-Aldrich Syndrome Protein (N-WASP) and Cortactin during Branched Actin Filament Network Formation

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