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Dynamin2 and Cortactin Regulate Actin Assembly and Filament Organization

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Summary

The GTPase dynamin is required for endocytic vesicle formation. Dynamin has also been implicated in regulating the actin cytoskeleton, but the mechanism by which it does so is unclear [1–4]. Through interactions via its proline-rich domain (PRD), dynamin binds several proteins, including cortactin [1], profilin [5], syndapin [6], and murine Abp1 [7], that regulate the actin cytoskeleton. We investigated the interaction of dynamin2 and cortactin in regulating actin assembly in vivo and in vitro. When expressed in cultured cells, a dynamin2 mutant with decreased affinity for GTP decreased actin dynamics within the cortical actin network. Expressed mutants of cortactin that have decreased binding of Arp2/3 complex or dynamin2 also decreased actin dynamics. Dynamin2 influenced actin nucleation by purified Arp2/3 complex and cortactin in vitro in a biphasic manner. Low concentrations of dynamin2 enhanced actin nucleation by Arp2/3 complex and cortactin, and high concentrations were in hibitory. Dynamin2 promoted the association of actin filaments nucleated by Arp2/3 complex and cortactin with phosphatidylinositol 4,5-bisphosphate (PIP2)-containing lipid vesicles. GTP hydrolysis altered the organization of the filaments and the lipid vesicles. We conclude that dynamin2, through an interaction with cortactin, regulates actin assembly and actin filament organization at membranes.

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Supplementary Material

Supplementary Material including complete details of the Experimental Procedures and materials used in this work and four figures, as cited in the text, is available at http://images.cellpress.com/supmat/supmatin.htm.

Results and Discussion

Dynamin2 and Cortactin Influence Actin Dynamics In Vivo

To investigate how dynamin influences actin dynamics in vivo, we examined the effects of dynamin2 on actin assembly by using cultured cells expressing GFP-capping protein (CP) as a probe for dynamic actin. Dynamin2 was found in PtK1 cells at the cell periphery and in a punctate distribution throughout the lamella; dynamin2 was largely, but not completely, colocalized with GFP-CP (Figure 1A). GFP-CP reveals sites of dynamic actin assembly at foci within lamellae and at the periphery of PtK1 cells [8] (see Movie 1 in the Supplementary Material available with this article online), locations that are also enriched in F-actin, Arp2/3 complex, and cortactin [8, 9]. Thus, dynamin2 is localized at sites where actin assembly occurs.

To determine if the GTPase activity of dynamin2 influences actin assembly in vivo, we observed actin dynamics in cells expressing either wild-type dynamin2 or a mutant form (dynamin2 K44A) defective in binding and hydrolysis of GTP [10–12]. Dynamics at the cell periphery and at foci appeared qualitatively normal in cells expressing wild-type dynamin2 (see Movie 2 in the Supplementary Material). Actin dynamics at foci were quantified from a time-based Fourier analysis of the fluctuation of GFP-CP fluorescence in well-spread regions of the lamella (see the Experimental Procedures). The fluctuation of GFP-CP in cells expressing dynamin2 was reduced slightly to 85% of that in control, mock-transfected cells (Figure 1B). Expression of dynamin2 K44A altered the dynamic distribution of GFP-CP in two ways (see Movie 3 in the Supplementary Material). First, the morphology and dynamics of foci were different. Foci were longer (1.5 ± 0.05 μm) than foci in control cells (0.90 ± 0.03 μm) and those in cells expressing wild-type dynamin2 (0.86 ± 0.02 μm). Foci in cells expressing dynamin2 K44A moved slightly faster than foci in cells expressing dynamin2 (0.14 ± 0.02 μm/s compared with0.09 ± 0.001 μm/s for dynamin2-expressing cells and 0.09 ± 0.007 μm/s for control cells), traveled greater distances (3.8 ± 0.06 μm compared with 1.6 ± 0.2 μm for cells expressing dynamin2 and 1.5 ± 0.2 μm for control cells), and persisted for longer times (31 ± 4.6 s compared with 20 ± 4.6 s for cells expressing dynamin2 and 20 ± 3.5 s for control cells). Second, new punctate structures containing GFP-CP appeared. The punctate structures were dynamic, appearing as transient, local bursts of actin assembly. These structures did not move laterally, in contrast to the behavior of foci that moved several microns. The fluctuation in GFP-CP fluorescence in well-spread lamellae was decreased to 46% of that in control, mock-transfected cells (Figure 1B), and this reduction indicates that overall actin dynamics were decreased. Decreased actin dynamics in cells expressing dynamin2 K44A is consistent with the longer foci, in which the actin filaments may disassemble slowly, and local, transient bursts of actin assembly at punctate structures that are spatially restricted.

The decreased actin dynamics observed in cells expressing dynamin2 K44A could result from a direct effect of dynamin2 on actin polymerization or secondarily from of a block in clathrin-mediated endocytosis. To elucidate the relationship between actin assembly at foci and endocytosis, we measured the fluorescence fluctuation of GFP-CP in cells expressing the SH3 domain of amphiphysin1, which inhibits clathrin-mediated endocytosis

[13, 14]. Expression of the amphiphysin1 SH3 domain had little effect on the fluctuation in GFP-CP at foci; the fluctuation of GFP-CP was decreased to ~80% of that in control, mock-transfected cells (Figure 1B). The morphology of foci was unchanged in cells expressing the amphiphysin1 SH3 domain. A mutant form of the amphiphysin1 SH3 domain, which does not bind dynamin or inhibit endocytosis, also had little effect on dynamics of foci. Thus, the effects of expressed dynamin2 K44A on actin dynamics do not appear to be secondary to inhibition of clathrin-meditated endocytosis.

We examined the participation of cortactin in actin assembly by using cells expressing mutant forms of cortactin. Dynamin2 binds cortactin via interactions of two SH3 domain binding motifs in its PRD with the cortactin SH3 domain [1]. A point mutation in the cortactin SH3 (cortactin W525K) domain greatly reduced binding of dynamin2 to GST fusions of the cortactin SH3 domain in a pull-down assay (see Figure S1 in the Supplementary Material available with this article online). Expression of full-length cortactin W525K in PtK1 cells decreased actin dynamics, as measured from the fluorescence fluctuation of GFP-CP (Figure 1C). Expression of wild-type, full-length cortactin or an N-terminal fragment of cortactin, both of which bind and activate Arp2/3 complex in vitro [15], did not affect actin dynamics (Figure 1C). These control experiments show that cortactin W525K did not decrease actin dynamics by inhibiting assembly due to binding and sequestering Arp2/3 complex. Actin dynamics at foci also were decreased in cells expressing cortactin W22A, which does not bind Arp2/3 complex and does not activate actin assembly in vitro[16]. We conclude that the interactions of cortactin with dynamin and Arp2/3 complex contribute to actin assembly in vivo.

Dynamin2 Regulates Actin Assembly by Arp2/3 Complex and Cortactin In Vitro

To determine how dynamin2 influences actin assembly, we examined the effect of dynamin2 on actin assembly nucleated by Arp2/3 complex and cortactin by using purified proteins in vitro. The effects of dynamin2 on actin assembly by Arp2/3 complex and cortactin were biphasic (Figure 2A). Low concentrations of dynamin2 enhanced actin assembly by decreasing the lag period for filament formation. Enhanced actin assembly was achieved at molar ratios of dynamin and cortactin of less than 1:1. Addition of GTP to the reactions with Arp2/3 complex, cortactin, and dynamin2 did not affect the rate of actin assembly (Figure S2). Substitution of cortactin W525K for wild-type cortactin eliminated the effects of dynamin2, suggesting that the effects of dynamin2 on actin assembly were mediated through cortactin (Figure 2B). The mechanism whereby dynamin2 enhanced actin nucleation is unclear but may involve formation of cortactin dimers. In other studies, dimerization of cortactin, either by association with a dimeric ligand via its SH3 domain, or as a fusion with GST (GST-cortactin), enhanced cortactin’s ability to activate nucleation by Arp2/3 complex (Hou et al., personal communication). Dynamin2 at low concentrations forms dimers or tetramers [17] that could support the dimerization of cortactin.

High concentrations of dynamin2 (molar ratios of dynamin and cortactin of greater than 1:1) inhibited actin assembly (Figure 2). In the absence of cortactin, dynamin2 did not alter the time course of polymerization of actin monomers or the level of F-actin formed at steady state, indicating that dynamin2 does not sequester actin monomers. The inhibitory

effect of dynamin2 might result from the association of cortactin with oligomeric dynamin, preventing cortactin's interaction with Arp2/3 complex or F-actin. In support of this idea, cortactin, but not Arp2/3 complex, sedimented with dynamin2 under conditions that promote dynamin oligomerization (Figure S3).

Dynamin2 GTPase Activity Influences Actin Filament Organization

Actin filaments nucleated by Arp2/3 complex and cortactin form branched networks [15]. To determine if dynamin2 influences this organization of actin filaments, we examined filaments directly by using fluorescence microscopy [18]. Because dynamin also binds phospholipids, we included phospholipids to examine if dynamin recruits actin filaments to membranes. Actin filaments and lipid vesicles were visualized with rhodamine-phalloidin and fluorescein-phosphatidylethanolamine (PE), respectively.

Actin filaments, some of which were branched, formed in reactions containing Arp2/3 complex and cortactin (Figure 3A). PIP_2-containing lipid vesicles (PC:PIP_2, 90:10 mol:mol) appeared as small green punctae distributed uniformly on the coverslip surface and did not affect the organization of actin filaments formed in reactions of Arp2/3 complex and cortactin. Addition of dynamin2 induced aggregation of the lipid vesicles (Figures 3B and 3D), consistent with the binding of dynamin2 to PIP_2-containing lipid [19]. Actin filaments were associated with the lipid aggregates and appeared as loose bundles (Figures 3B and 3D). Similar F-actin/lipid structures formed in reactions containing phosphatidylserine vesicles (data not shown). In reactions containing Arp2/3 complex, cortactin, dynamin2, and lipid vesicles comprised of phosphatidylcholine (PC), to which dynamin2 does not associate, bundles of actin filaments formed that were not associated with PC vesicles (Figure 3C). Actin filament bundles may form if cortactin, which also binds F-actin [20], associates with assembled dynamin, creating a multivalent template for binding filaments. Different ionic conditions for optimal actin polymerization by Arp2/3 and cortactin (low salt) and for dynamin disassembly (high salt) preclude testing if dynamin2 oligomerization promotes bundle formation.

The association of actin filaments with PIP_2-con taining lipid aggregates was most favorable in reactions containing dynamin and cortactin (Table 1). In the absence of cortactin, or in the presence of cortactin W525K, fewer lipid aggregates contained associated actin filaments (Table 1). GST-VCA, a potent activator of Arp2/3 complex [21], was less effective than cortactin in recruiting actin filaments to lipid aggregates (Table 1; also compare Figure 3D and Figure 3E). We conclude that dynamin2, through its interactions with anionic phospholipids and with cortactin, can promote the association of actin filaments with PIP_2-containing membranes.

To investigate the role of dynamin's GTPase activity in actin filament organization, we added GTP to actin assembly reactions containing Arp2/3 complex, cortactin, dynamin2, and PIP2-containing lipid vesicles. The bundles of actin filaments associated with lipid were thinner and appeared as single cable-like structures with lipid aggregates dispersed along the bundles (Figure 3F). This change in filament organization was not observed in reactions containing GTPγS (Figure 3G). If GTP was added to reactions after the formation of loosely bundled filaments associated with lipid aggregates (such as those shown in Figure 3B),

actin filaments were reorganized as thin, cable-like bundles (Figure S4). Therefore, GTP hydrolysis by dynamin can influence the organization of actin filaments associated with PIP2-containing membranes.

We found that dynamin2 influences actin dynamics in vivo and affects the organization of actin filaments in vitro. Biochemical studies of actin assembly by Arp2/3 complex and cortactin reveal a modest stimulation of actin nucleation at low dynamin2 concentrations and inhibition at high concentrations, effects that are mediated by the interaction of dynamin2 and cortactin. GTP hydrolysis did not influence these effects of dynamin2 on actin assembly, but GTP hydrolysis did induce changes in the organization of actin filaments formed in these reactions. Quantifying actin dynamics in vivo revealed that the GTPase activity of dynamin2 promoted actin dynamics, suggesting that actin filament organization may be linked to actin dynamics in vivo. The mechanism whereby dynamin2 GTPase activity exerts this effect remains unclear. One possible mechanism by which this occurs is by enhancing F-actin turnover. Previous studies of the effects of dynamin2 K44A on F-actin turn over in podosomes also support this notion [2]. An alternate mechanism whereby dynamin2 GTPase activity may promote actin dynamics is through orientation of filaments associated with membranes. Dynamin2, which is associated with F-actin tails of motile Listeria and of rocketing PIP5K-induced organelles [3, 4], may organize filaments in the F-actin tails to provide for optimal propulsion of Listeria or of the organelles. The altered F-actin tail morphology and reduced speed of such organelles in cells expressing mutants of dynamin2 with defective GTPase activity [3, 4] is consistent with a role for dynamin2 GTPase activity in forming effective F-actin tails. We conclude that, through its interactions with cortactin, dynamin2 contributes to actin dynamics by regulating actin assembly and actin filament organization at membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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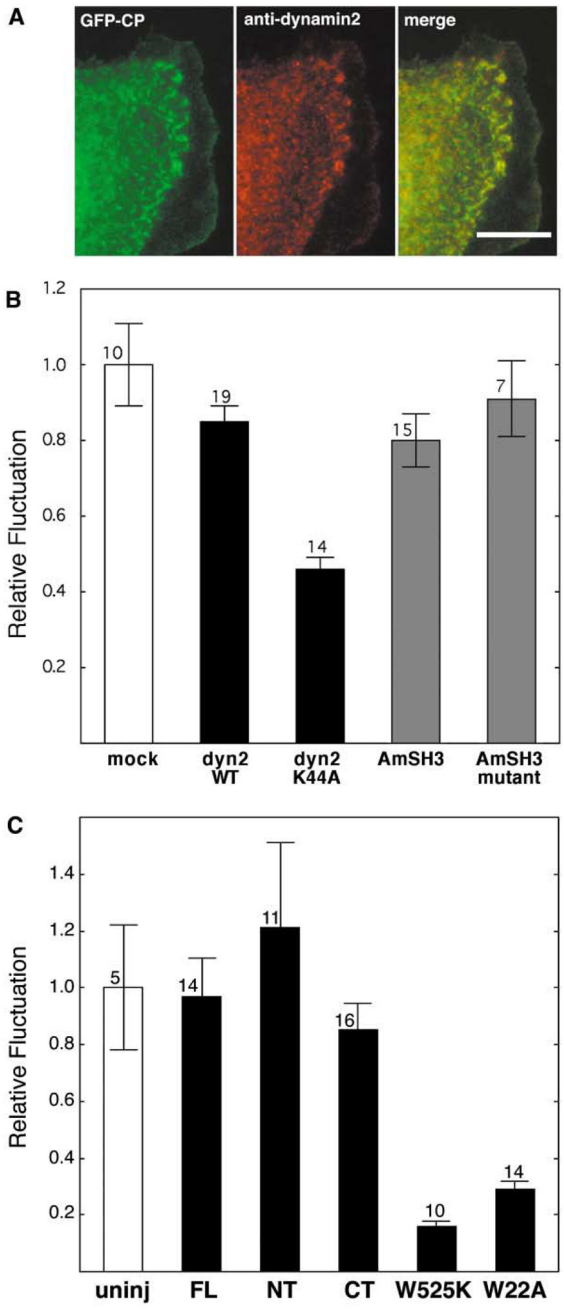

Figure 1.

Dynamin2 Regulates Actin Dynamics in PtK1 Cells

(A) Dynamin2 is localized at sites of actin assembly in PtK1 cells. Dynamin2 was localized with rabbit anti-dynamin2 PRD antibodies (red) in cells expressing GFP-CP (green). Dynamin2 and GFP-CP overlap at punctate foci within the lamella and at the cell periphery (yellow in merged view). The scale bar represents 10 μm.

(B and C) Actin dynamics in living PtK1 cells were quantified from the fluctuation in GFP-CP fluorescence. Values were normalized to the fluctuation measured in control, mock-transfected or uninjected cells (white bars). Error bars represent the standard error

of the mean, and the number beside each error bar indicates the number of cells analyzed in each group. (B) Actin dynamics in cells expressing dynamin2, dynamin2 K44A, the SH3 domain of amphiphysin1, or a mutant form of the amphiphysin1 SH3 domain. Movies that illustrate the effects of expressed dynamin2 (Movie 2) and dynamin2 K44A (Movie 3) on the distribution and dynamics of GFP-CP are available online as Supplementary Material. (C) Actin dynamics in cells expressing mutant forms of cortactin that do not bind Arp2/3 complex (cortactin W22A) or dynamin2 (cortactin W525K). Cells were injected with plasmids for expression of full-length and mutant cortactins, as indicated: full length cortactin (FL), the N-terminal fragment 1–330 (NT), the C-terminal fragment 350–546 (CT), cortactin W525K (W525K), and cortactin W22A (W22A).

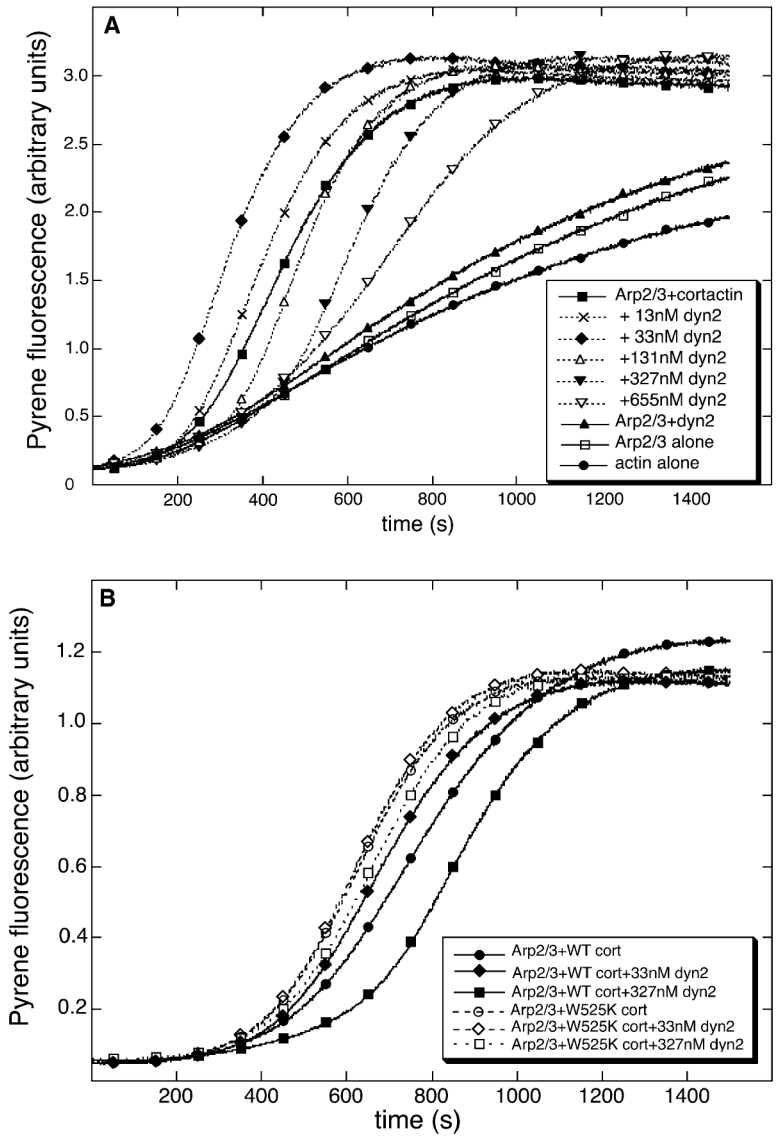

Figure 2.

Dynamin2 Acts through Cortactin to Influence Arp2/3 Complex-Mediated Actin Assembly In Vitro

(A) Actin polymerization over time in reactions containing 2 μM actin (10% pyrene-labeled), 50 nM Arp2/3 complex, 90 nM cortactin, and varying concentrations of dynamin2 as indicated. The fluorescence of pyrene-actin over time is plotted.

(B) Dynamin2 at low (33 nM) or high (327 nM) concentrations had no effect on actin assembly in reactions containing cortactin W525K (open symbols) compared with reactions containing wild-type cortactin (closed symbols). The fluorescence of pyrene-actin over time is plotted.

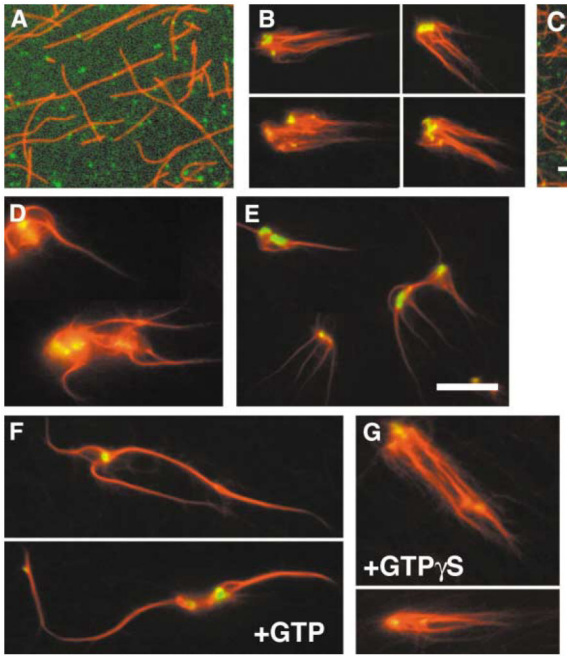

Figure 3.

Dynamin2 Alters the Organization of Actin Filaments Associated with PIP_2-Containing Membranes

(A–G) Actin filaments and lipid vesicles were visualized with rhodamine-phalloidin (red) and fluorescein-PE (green), respectively. Reactions contained (A, B, and D–G) 50 μM PC:PIP_2 or (C) 50 μM PC vesicles and the following proteins, which are described in the legends of the individual panels. (A) 62 nM Arp2/3 complex and 450 nM cortactin. Long actin filaments formed, some of which were branched; actin filaments were not bundled or associated with PC:PIP_2 vesicles. (Because rhodamine-phalloidin was not present during the actin assembly reaction, the extent of filament branching is low [18]). (B and D) 62 nM Arp2/3 complex, 450 nM cortactin, and 450 nM dynamin. Actin filament bundles associated with aggregates of PC:PIP_2 vesicles; single actin filaments distributed on the coverslip surface are not detected in these images because the exposure time for collecting images of the actin filament bundles was short. (C) 62 nM Arp2/3 complex, 450 nM cortactin, 450 nM dynamin in the presence of PC vesicles. Actin filament bundles not associated with lipid formed. (E) 62 nM Arp2/3 complex, 1.5 nM GST-VCA, and 450 nM dynamin. GST-VCA was substituted for cortactin with an amount providing a similar rate of actin assembly as 450 nM cortactin in pyrene-actin assembly assays; lipid aggregates formed but less F-actin was associated with the aggregates (the exposure time used to obtain images in [D] and [E] was identical). (F) 62 nM Arp2/3 complex, 450 nM cortactin, 450 nM dynamin2, and 200 μM GTP. GTP induced the formation of thin actin filament bundles that resembled cables with lipid aggregates dispersed along the cables.(G) Arp2/3 complex, 450 nM cortactin, 450 nM dynamin, and 200 μM GTPγS. Actin cables were not formed, and lipid-F-actin structures similar to those observed in the absence of guanine nucleotide were observed. The scale bars represent 10 μm.

Table 1.

Quantitation of F-Actin/Lipid Structures Formed in Reactions of Actin Polymerization with Arp2/3 Complex, Cortaxtin, Dynamin2, and GST-VCA

Condition	**Number of Actin/Lipid Structures/50 Fields**
Reactions Containing PC:PIP_2 Vesicles:	
Arp2/3 + cortactin	0; n = 2
Arp2/3 + cortactin + dynamin	169 ± 34; n = 4
Arp2/3 + cortactin W525K + dynamin2	36 ± 4; n = 3
Arp2/3 + dynamin2	35; n = 1
Arp2/3 + GST-VCA	0; n = 1
Arp2/3 + GST-VCA + dynamin2	66 ± 14; n = 3

Reactions contained 100 nM Arp2/3 complex; either 400 nM cortactin, 400 nM cortactin (W525K), or 1.5 nM GST-VCA; 400 nM dynamin2; and 50–100 μM PC:PIP_2 (90:10, mol:mol), as indicated. The mean (±SE) number of F-actin/lipid structures in 50 microscope fields observed with a 40× objective lens is tabulated; the number of reactions performed and counted for each condition is indicated(n). F-actin/lipid structures are defined as lipid aggregates having associated actin filaments.