Capping Protein and the Arp2/3 Complex Regulate Nonbundle Actin Filament Assembly to Indirectly Control Actin Bundle Positioning during *Drosophila melanogaster* Bristle Development^D

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Drosophila melanogaster bristle development is dependent on actin assembly, and prominent actin bundles form against the elongating cell membrane, giving the adult bristle its characteristic grooved pattern. Previous work has demonstrated that several actin-regulating proteins are required to generate normal actin bundles. Here we have addressed how two actin regulators, capping protein, a barbed end binding protein, and the Arp2/3 complex, a potent actin assembly nucleator, function to generate properly organized bundles. As predicted from studies in motile cells, we find that capping protein and the Arp2/3 complex act antagonistically to one another during bristle development. However, these proteins do not primarily act directly on bundles, but rather on a dynamic population of actin filaments that are not part of the bundles. These nonbundle filaments, termed snarls, play an important role in determining the number and spacing of the actin bundles. Reduction of capping protein leads to an increase in snarls, which prevents actin bundles from properly attaching to the membrane. Conversely, loss of an Arp2/3 complex component leads to a loss of snarls and accumulation of excess membrane-attached bundles. These results indicate that in nonmotile cells dynamic actin filaments can function to regulate the positioning of stable actin structures. In addition, our results suggest that the Arpc1 subunit may have an additional function, independent of the rest of the Arp2/3 complex.

INTRODUCTION

The actin cytoskeleton is of vital importance in many cellular processes: it is an integral part of the contractile ring during cell division, its nucleation at the leading edge drives cell locomotion, its polymerization is important for endocytosis, and it provides a structural framework for specialized cell shapes. The last few years have seen an explosion in knowledge about how actin assembly is both initiated and limited. Two important players are the Arp2/3 complex and capping protein. The Arp2/3 complex consists of actin related proteins 2 and 3 along with five other subunits, Arpc1-Arpc5 (Machesky *et al.*, 1994). On activation by a member of the Wiscott Aldrich Syndrome protein (WASP) family, this complex can nucleate new actin filament assembly as well as create branched networks of filaments through side binding to existing filaments (Egile *et al.*, 1999; Machesky *et al.*, 1999;

Rohatgi *et al.*, 1999; Winter *et al.*, 1999a; Yasar *et al.*, 1999). In addition to its actin nucleation activity, the Arp2/3 complex can cap the slow growing, or pointed, ends of actin filaments (Mullins *et al.*, 1998). Capping protein, a heterodimer of alpha and beta subunits, binds the fast growing, or barbed, ends of actin filaments and prevents both depolymerization and the addition of new monomers (Isenberg *et al.*, 1980). The importance of capping protein and the Arp2/3 complex is evidenced by the fact that they are two of the five components absolutely required for formation of actin comet tails that drive intracellular movement of the pathogenic bacteria *Listeria monocytogenes* (Loisel *et al.*, 1999).

Although much has been learned in the last several years about the proteins and mechanisms regulating actin filament assembly during motility and in vitro (for review see Pollard and Borisy, 2003), much less is understood about the processes governing the assembly of more stable actin structures in cells that do not move. Many questions remain unanswered about such structures. For example, how are stable actin structures positioned within the cell? Do the same proteins control filamentous actin assembly in stationary cells as in the leading edge of moving cells? Do these proteins function in the same way in these very different contexts?

To investigate these questions, we are using the large bristles, or macrochaetes, of the fruit fly *Drosophila melanogaster*. The long bristle shaft (up to 400 μ m in length) is generated from a single cell that undergoes extension during pupal development (Lees and Picken, 1944; Lees

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Abbreviations used: APF, after puparium formation; Arp2/3, actin-related protein 2/3; cpb, capping protein beta; SEM, scanning electron microscopy; WASP, Wiscott Aldrich Syndrome protein.

and Waddington, 1944). Bristle cell elongation is dependent on actin assembly (Tilney *et al.*, 2000), and bundles of actin filaments lie against the membrane, parallel to the long axis of the bristle (Overton, 1967; Appel *et al.*, 1993). These cortical bundles give the adult bristle its grooved appearance; the bundles lie at the positions of the valleys between the tall ridges of cuticle. Although it is apparent that the barbed ends of the actin filaments in bundles are oriented toward the bristle tips (Tilney *et al.*, 1996), it is not clear that extension of actin filaments in the bundles provides the pushing force of membrane protrusion. Rather, the bundles might serve as structural girders that maintain the cellular extension. Between the bundles are very dynamic masses of actin filaments termed snarls (Tilney *et al.*, 1996, 2003). Snarls were proposed to be transient structures that are not stabilized by cross-linking to other filaments or attachment to the membrane (Tilney *et al.*, 2003), perhaps byproducts or aberrantly formed structures that are eliminated. Whether snarls have any function is unknown.

Mutations in genes encoding many actin-regulating proteins, such as the cross-linkers *forked* and *singed* (encoding fascin; Tilney *et al.*, 1995), *chickadee* (encoding profilin, an actin monomer-binding protein that promotes filament assembly; Verheyen and Cooley, 1994), the actin monomer sequesterer *twinfilin* (Wahlstrom *et al.*, 2001), and capping protein (Hopmann *et al.*, 1996), result in deformed bristles. Close examination of bristle development reveals aberrant actin bundle morphologies in these mutants. For example, *capping protein beta* mutant pupal bristles have irregularly sized actin bundles that are displaced from the membrane. We hypothesized that this may be due to unregulated assembly of the filaments within the actin bundles, though the precise mechanism remained unclear (Hopmann *et al.*, 1996). In previous work we have used the bristle system to elucidate the antagonistic interaction between capping protein and profilin: we found that a *chickadee* mutation was able to suppress the bristle phenotype of a transheterozygous combination of *capping protein beta* mutant alleles (Hopmann and Miller, 2003). Reduction of capping protein leads to an increase in actin filament assembly that is abrogated by loss of profilin. Thus these proteins appear to act in a very similar manner to their function at the leading edge of motile cells (for review see Pollard and Borisy, 2003).

In this article we report that, as predicted from studies in motile cells, capping protein and the Arp2/3 complex act antagonistically to one another during bristle development. However, we show that the role of capping protein in actin bundle organization is quite different from previously thought. Instead of primarily acting directly on actin filaments within bundles, our results indicate that capping protein and the Arp2/3 complex regulate actin snarls. Actin bundle positioning is affected by the quantity and persistence of these dynamic actin filaments since their overabundance displaces bundles from the membrane, whereas their absence results in excess actin bundles attached to the membrane.

MATERIALS AND METHODS

Oregon R was used as the wild-type strain throughout this work. Flies were raised on standard cornmeal agar medium at 25°C.

Scanning Electron Microscopy

Adult flies were processed for scanning electron microscopy (SEM) as described previously (Hopmann *et al.*, 1996; Hopmann and Miller, 2003) with the following exceptions: after removal of the wings and legs under CO₂ anesthesia, flies were stored in 75% ethanol at 4°C for several days to weeks before processing. In some cases flies were rehydrated in 50% ethanol for 1 h

and then in distilled water for 1 h and were fixed using the Osmium-Thiocarbohydrazide-Osmium method (Kelley *et al.*, 1973). The samples were dehydrated in an ethanol series and were critical point-dried from CO₂. Mounted samples were sputter-coated with 30 nm of gold and examined in a Hitachi S-450 SEM (Hitachi, Tokyo, Japan) operated at 20 kV accelerating voltage. For quantitative analysis, the four scutellar bristles were scored without knowledge of the genotype using the scale described in the legend for Figure 1. *p* values were determined using a chi square test.

Phenotypic Analyses at the Dissecting Microscope Level

To determine the frequency of the abnormal bristle phenotype, flies were scored under CO₂ anesthesia for the number of major defects, defined as sharp bends, branches, or spikes visible under the dissecting microscope. In most cases flies were scored without knowledge of the genotype. For comparisons between *cpb*^{F19/cpb}^{6.15}; +/+ and *cpb*^{F19/cpb}^{6.15}; *arp3*^{EP3640/+}, the visible markers used to select the appropriate flies made blind scoring impossible. In this case two different observers, who did not know how the relevant genotypes were marked, blindly scored a subset of the same flies as used for the quantitation in two independent experiments. The results obtained were comparable to those reported. *p* values were determined using a chi square test.

Developmental Staging of Pupae

In most cases animals were picked as white prepupae and allowed to develop in a humid chamber at 25°C for the indicated length of time after puparium formation (APF). We have previously noted that *cpb* mutants are developmentally delayed relative to wild type (Hopmann and Miller, 2003), but in this staging we found that wild-type and mutant bristles reached approximately the same length at the same time APF. Thus much of the developmental delay likely occurs during the five preceding days of embryogenesis and larval development. *arp3/arp3* pupal development was delayed by several hours compared with wild type, so comparing these two genotypes at the same time APF was not reasonable. Instead, we examined bristles that were newly emerging (indicated "early" in Figure 3) or fully elongated (indicated "late" in Figure 3). In some cases pupae were maintained at 20°C instead of 25°C; at this temperature animals develop more slowly, but there was no temperature effect on bristle development.

Fluorescent Microscopy

Preparation, staining, and confocal imaging of pupal bristles was performed as described previously (Hopmann and Miller, 2003). Primary antibodies used were affinity purified rat anti-capping protein beta (Hopmann *et al.*, 1996), rabbit anti-Arp 3 (Stevenson *et al.*, 2002), and mouse anti-profilin (chickadee; Verheyen and Cooley, 1994). Secondary antibodies were Alexa-488 anti-rat and Alexa-568 anti-rabbit (both from Molecular Probes, Eugene, OR). Actin was visualized either with GFP-actin or Alexa-568 phalloidin (Molecular Probes).

Transmission Electron Microscopy

The scutellar region of the cuticle was dissected from pharate adults in 0.1 M sodium phosphate, pH 6.8, and fixed for 1 h in 1% glutaraldehyde in the above buffer. Specimens were rinsed extensively and washed overnight in this buffer and then transferred to 0.1 M sodium phosphate, pH 6.0. They were then treated with 1% osmium tetroxide in this buffer for 1 h followed by extensive washing. This was followed with a dehydration series from 10 to 100% ethanol with 10% increases at each 10-min step. Samples were then soaked overnight at 4°C in a 1:1 solution of 100% ethanol and LR White Resin (London Resin Company, Berkshire, England), followed by overnight in 3:1 resin:ethanol and then 3 d at 4°C in 100% resin with one change after the first day. Finally, samples were placed in gelatin capsules filled with 100% resin, left at room temperature for 1 d, and then incubated at 60°C for 48 h to polymerize the resin. All steps were done with mixing, and in some cases a vacuum was applied to the samples for 10 min at various steps in an attempt to increase the penetration of the resin into the bristles. Eighty- to 90-nm sections were cut using a Reichert-Jung Super Nova ultratome (Leica Microsystems, Nussloch, Germany) equipped with a diamond knife and analyzed on a Hitachi H-600 transmission electron microscope. Resin penetration was a constant problem throughout, and only samples that were well embedded were used for analysis. Cuticle areas were measured using ImageJ (NIH, Bethesda, MD).

RESULTS

Capping Protein Function Is Opposed by the Arp2/3 Complex, But the Arpc1 Subunit Has a Distinct Function

To examine the interplay between proteins known to control actin assembly in motile cells in the context of stable actin structures in nonmotile cells, we studied the effects of combinations of mutations of actin assembly regulators in *Drosophila* bristle cells. Because *capping protein β* (hereafter

indicate the statistical significance of the differences between the indicated genotypes and $cpb^{F19}/cpb^{6.15}; +/+$. The numbers of flies scored for each genotype was as follows: $cpb^{F19}/cpb^{6.15}; +/+$, 82; $cpb^{F19}/cpb^{6.15}; arp3/+$, 44; $cpb^{F19}/cpb^{6.15}; wsp1/+$, 37; $cpb^{F19} \text{ } arp1^{Q25st}/cpb^{6.15}; +/+$, 82.

referred to as *cpb*) mutations result in excess F-actin (Hopmann and Miller, 2003) and loss of the Arp2/3 complex, a potent actin nucleator (Welch *et al.*, 1997b; Mullins *et al.*, 1998), is predicted to reduce actin assembly, we hypothesized that mutations in the genes encoding Arp2/3 complex subunits would suppress the phenotypes observed in *cpb* mutants. *cpb* mutants have defects in macrochaete morphology (Hopmann *et al.*, 1996). Among other defects, bristles have abnormally organized surface grooves and regions that lack grooves entirely. Additionally, protruding spikes, split ends, and sharp bends are occasionally apparent. Figure 1A, a and b, shows two representative *cpb* mutant bristles. This phenotype was suppressed by reducing the amount of the Arp 2/3 complex subunit, *arp3*. Macrochaetes from flies of the genotype $cpb^{F19}/cpb^{6.15}; \text{ } arp3^{EP3640}/+$ were often nearly wild type in appearance, with normal groove patterns (Figure 1A, compare c–d with a–b). The extent of phenotypic suppression was quantitated in two ways. In the first assay we scored scutellar bristles for defects by SEM. Our second assay was to count the number of severely defective thoracic macrochaetes per fly visible at the dissecting microscope level. In both assays, scoring was performed

without knowledge of genotype. Both assays showed that the suppression of the *cpb* phenotype by *arp3* was strong and statistically significant (Figure 1, B and C), supporting the hypothesis that capping protein and the Arp2/3 complex act in opposition to one another in the developing bristle.

To confirm that the suppression by *arp3* was due to its role in the Arp2/3 complex, we examined the effect of mutations in *arp1* and *wasp* (an activator of the Arp2/3 complex) and deficiencies that remove *arp4* and *arp5*. As expected, a mutation in *wasp* and the deficiencies that remove *arp4* and *arp5* suppressed the *cpb* phenotype to a similar extent as did the mutation in *arp3* (Figure 1 and Supplementary Figure 1). Surprisingly however, mutations in *arp1* enhanced the *cpb* phenotype (Figure 1 and Supplementary Figure 1). In flies of the genotype $cpb^{F19} \text{ } arp1^{Q25st}/cpb^{6.15}; +/+$, normal-looking bristles were completely absent, and cuticle morphology defects appeared more severe. In addition to very irregular groove patterns, numerous sharp bends, spikes, splits, and fusions of neighboring bristles were observed (Figure 1A, g and h). We interpret the bristle fusions to indicate that during pupal development, the cellular protrusions that form the bristles lack rigidity and thus sometimes lie across one another.

Figure 1. The capping protein mutant bristle phenotype is suppressed by a reduction in function of *arp3* and *wasp*, but enhanced by mutations in *arp1*. (A) Adult bristle phenotypes of capping protein mutants alone and in combination with mutations in genes encoding Arp2/3 complex components or its activator, WASp. Two scanning electron micrographs, one with a weak and one with a strong phenotype, of representative bristles from flies of the indicated genotypes are shown. All scale bars, 5 μ m. (B) Blind scoring of scutellar bristles by SEM. Bristles were scored as follows: I, completely normal or having a slightly rounded or slightly split tip; II, normal groove pattern proximally, but randomly oriented grooves distally, may also have protruding spikes or a split tip; III, same as II, but has randomly oriented grooves proximally; and IV, same as III, but has a sharp bend in the bristle shaft. Data from three independent experiments were combined. p values indicate the statistical significance of the differences between $cpb^{F19}/cpb^{6.15}; +/+$ and $+/+; +/+$ or the indicated genotypes and $cpb^{F19}/cpb^{6.15}; +/+$. The number of bristles scored for each genotype was as follows: $+/+; +/+$, 17; $cpb^{F19}/cpb^{6.15}; +/+$, 81; $cpb^{F19}/cpb^{6.15}; \text{ } arp3/+$, 32; $cpb^{F19}/cpb^{6.15}; \text{ } wsp1/+$, 29; $cpb^{F19} \text{ } arp1^{Q25st}/cpb^{6.15}; +/+$, 22. (C) Frequency of major bristle defects. The number of severely defective thoracic macrochaetes (severe defects were defined as bends, splits, and obvious bulges) per fly was counted, and data from two independent experiments were combined. p values

When chitin secretion occurs, they become “glued” together. This enhancement of the *cpb* bristle phenotype was observed in two different *arp1* alleles (Supplementary Figure 1) isolated in independent screens in different labs (Hudson and Cooley, 2002). The strength of the suppression seen (Supplementary Figure 1) correlates with allele strength (Hudson and Cooley, 2002). Because mutations in *arp3* and *wasp* and deficiencies that remove *arp4* and *arp5* all suppress the *cpb* phenotype, we conclude that the Arp2/3 complex acts in opposition to capping protein in developing bristles as it does in motile cells. However, our observation that mutations in *arp1* enhance the *cpb* phenotype suggests that Arp1 might have an additional role, independent of the rest of the Arp2/3 complex.

Capping Protein and the Arp2/3 Complex Act on Actin Snarls to Indirectly Affect Actin Bundle Positioning

To uncover the mechanisms by which capping protein and the Arp2/3 complex act in opposition to one another, we sought to determine the structure(s) on which these assembly regulators act by examining their localization. We used anti-capping protein serum (Hopmann *et al.*, 1996) and an Arp3-specific antibody (Stevenson *et al.*, 2002) to stain pelts from pupae in which bristles were just starting to emerge. We found that capping protein did not localize to actin bundles in early bristles, but instead localized to snarls, clumps of filamentous actin visible between the actin bundles at the membrane (Figure 2, a–e). In previous work, we found that capping protein was located in small puncta distributed along the length of the actin bundles late in bristle development (Hopmann *et al.*, 1996). Staining of pupal bristles at various times through development revealed that capping protein first localized to the bundles at 40–42 h APF (unpublished data).

Arp3 distribution was determined at these early time points, and its localization was similar to capping protein (Figure 2, f–j). It was absent from actin bundles but present on snarls. Examination of pelts doubly labeled for capping protein and Arp3 showed that these proteins had partially overlapping distributions (Figure 2, k–o). The colocalization of capping protein and Arp3 on snarls and absence from bundles suggests that these two actin regulators do not act on the bundles during early bristle development, but rather on the snarls.

Because both capping protein and Arp3 localized to snarls, we wondered if other actin regulators are on snarls as well. We stained newly emerging bristles with an antibody specific for profilin (Verheyen and Cooley, 1994) and found that it was distributed fairly uniformly throughout the bristles, both on and off of snarls. Like capping protein and Arp3, it was completely excluded from the actin bundles (Figure 2, p–t). The difference between profilin's localization and that of capping protein and Arp3 was especially evident when we compared computed cross-sections through the bristles; profilin was abundant in the center of the bristles (Figure 2t), whereas capping protein (Figure 2e) and Arp3 (Figure 2j) were restricted to the membrane. This staining pattern appears consistent with profilin's role as an actin monomer binding protein that functions to promote actin filament assembly.

If the Arp2/3 complex and capping protein do regulate snarls, our expectation is that loss of Arp3 would lead to a loss of snarls, whereas loss of capping protein would result in excess snarls. A further expectation is that reduction of Arp3 should abrogate the excess snarl phenotype of *cpb* mutants, because of the opposing effects of these regulators

Figure 2. Capping protein and Arp3 are on actin snarls between bundles. (a–c) Emerging bristle in a wild-type pupa at 32–33 h APF stained for actin (red) and capping protein beta (green). Note that capping protein is absent from the long actin bundles (arrow) and instead localizes to the snarls between bundles (arrowhead). (d) Magnified view of the snarl indicated by the arrowhead in a–c. (e) Cross-section of a similarly stained bristle from a slightly older animal. (f–h) Emerging bristle in a wild-type pupa at 32–33 h APF expressing GFP-actin and stained for Arp3 (red). Arp3 is coincident with the actin snarls between bundles. (i) Magnified view of the snarl indicated by the arrowhead in f–h. (j) Cross-section of a similarly stained bristle from a slightly older animal. (k–m) Emerging bristle in a wild-type pupa at 32–33 h APF stained for Arp3 (red) and capping protein beta (green). The locations of Arp3 and capping protein beta partially overlap. (n) Magnified view of the snarl indicated by the arrowhead in k–m. (o) Cross-section of a similarly stained bristle from a slightly older animal. (p–r) Emerging bristle in a wild-type pupa at 32–33 h APF expressing GFP-actin and stained for profilin (red). Profilin is excluded from the actin bundles and fairly uniform on the membrane between them. (s) Magnified view of the snarl indicated by the arrowhead in p–r. (t) Cross-section of the bristle shown in p–r. All scale bars are 4 μ m and do not apply to the magnified views or cross-sections.

Figure 3. Loss of Arp3 results in a loss of actin snarls and formation of excess actin bundles. Newly emerged bristles are indicated as early and those near the end of bristle elongation are designated late. Grazing images are single confocal planes and projections are of all confocal planes through the bristle. Cross-sections were computationally derived from stacks of images through the bristles.

on actin assembly. To test these hypotheses, we first examined the effect of loss of Arp3 on developing bristles in comparison to wild-type bristles. Snarls were clearly evident between the bundles in wild-type bristles just after emergence (Figure 3, a and b). Once the bristles were fully elongated, actin snarls were no longer visible, leaving only 10–15 regularly spaced actin bundles against the membrane (Figure 3, j–l). A strikingly different pattern was observed when we examined developing bristles of pupae homozygous for a strong loss of function allele of *arp3* (Hudson and Cooley, 2002). In early *arp3*^{EP3640} mutant bristles, we were unable to detect snarls and an excess number of actin bundles were juxtaposed to the membrane (Figure 3, d and e). This was especially clear in cross-sectional images of bristles. In wild type, space was visible between the actin bundles (Figure 3c), whereas in *arp3* mutants bundles were present nearly continuously along the membrane (Figure 3f). These excess bundles persisted throughout bristle development, such that when the bristles were fully elongated, there was still a substantial excess of actin bundles on the membrane in *arp3* mutants compared with wild type (Figure 3, compare m–o to j–l). These excess membrane-associated actin bundles likely are the reason that bristles in homozygous *arp3* mutant adults have more, smaller grooves than wild-type bristles (Hudson and Cooley, 2002).

Because loss of *arp3* resulted in a large reduction of snarls, we hypothesized that the snarls are Arp 2/3 complex-nucleated structures. In the absence of functional Arp2/3 complex, there should be virtually no snarl actin filaments to be

capped by capping protein. Therefore, if capping protein acts via snarls, concomitant reduction of capping protein should have little effect on the Arp 2/3 complex loss of function phenotype. To test this, we examined developing bristles in pupae of the genotype *cpb*^{F19}/*cpb*^{6.15}; *arp3*^{EP3640}/*arp3*^{EP3640}. Bristles of this genotype were difficult to examine because most of these animals died before or during the pupal stage. However, observations of six newly emerged bristles showed they were identical to bristles of *arp3* pupae. We were unable to detect snarls but saw an overabundance of actin bundles juxtaposed to the membrane (Figure 3, g–i). These observations support the idea that Arp 2/3 complex and capping protein affect bundles indirectly via snarls.

If capping protein exerts its effect on bundle positioning by controlling snarls, the snarls should be affected in capping protein mutants. Because reduction of capping protein amount increases actin assembly, excess snarls should form when capping protein amount is reduced. To determine if this is the case, we compared developmental time courses in wild-type and *cpb* mutants. In wild-type pupae, actin snarls were clearly evident at 32–33 h (Figure 4Aa), began diminishing around 35–36 h (unpublished data), and were nearly undetectable at 38–39 h (Figure 4Ac). By contrast, in *cpb* mutants snarls persisted much longer; they were still clearly evident at 38–39 h (Figure 4Ag). Furthermore, examination of confocal images of planes through the middle of the bristles showed that although the center of wild-type bristles was devoid of actin bundles, this region was full of bundles in *cpb* mutants (Figure 4A; compare h with d). This suggests that excess persisting snarls interfere with the ability of bundles to localize adjacent to the membrane.

To further test the idea that snarls regulate bundle positioning, we used a mutation in a different actin assembly promoter that affects bundles, profilin. Mutations in the *Drosophila* profilin homolog, *chickadee*, suppress the bundle displacement from the membrane seen in *cpb* mutants (Hopmann and Miller, 2003). Reducing the amount of profilin balances the reduction of capping protein so that actin assembly is more normal in this mutant combination. If the effect of profilin is also via snarls, the amount and persistence of snarls should resemble wild type in capping protein/profilin mutant combinations. Indeed we found that snarls in *cpb*^{F19} *chic*²²¹/*cpb*^{6.15} + bristles looked similar to wild type: they were obvious at 32–33 h (Figure 4A, i), reduced at 35–36 h (unpublished data), and were not visible at 38–39 h (Figure 4Ak). Additionally, in contrast to *cpb* mutants, the actin bundles in *cpb*^{F19} *chic*²²¹/*cpb*^{6.15} + bristles were properly localized against the membrane (Figure 4A, compare l with h). Overexpression of profilin in the bristle resulted in excess snarls and actin bundles displaced into the center of the bristle (Figure 4A, m–p). Thus, like capping protein and the Arp2/3 complex, profilin regulates actin assembly in snarls. In addition, bundle positioning correlates with the amount and persistence of snarls in this genotype as well.

Because capping protein and the Arp2/3 complex both localized to snarls and had opposing effects on snarl abundance, we predicted that loss of one copy of *arp3* should suppress the excess snarl phenotype seen in *cpb* mutants. Although no suppression was observed at early time points—bristles on pupae of the genotype *cpb*^{F19}/*cpb*^{6.15}; *arp3*^{EP3640}/+ looked nearly identical to *cpb* bristles from 32 to 39 h APF (Figure 4A, compare q–t to e–h)—we did see suppression beginning at 41–42 h APF. At this time point, we found bristles of two different types in both genotypes:

Figure 4. Time course of actin in developing bristles in wild type and mutants. (A) Actin in developing bristles at the indicated times APF was visualized with fluorescently labeled phalloidin. Arrow indicates an actin bundle, and arrowhead indicates an actin snarl. "Grazing" is a single optical section of the surface, and "Middle" is through the center, of the bristle. All scale bars, 4 μ m. (B) Bristle actin at 41–42 h APF. For each genotype, a single confocal section through the center of a bristle with snarls and one without snarls is shown. Actin is visualized with fluorescent phalloidin. The numbers in boxes in the upper left of each image indicate how many bristles of that type (with or without snarls) were observed. All scale bars, 8 μ m.

those lacking snarls and having membrane-localized bundles and those with snarls and interior bundles. However, bristles with snarls and interior bundles were far more prevalent in $cpb^{F19}/cpb^{6.15}; +/+$ pupae than in $cpb^{F19}/cpb^{6.15}; arp3^{EP3640}/+$ pupae (Figure 4B). At 46 h APF, no snarls were seen in either genotype, but a range of bundle position phenotypes were observed. We categorized them as all membrane associated, mostly membrane associated, mostly internal, or all internal. Representative examples of each are shown in Figure 5A. Although both $cpb^{F19}/cpb^{6.15}; +/+$ and $cpb^{F19}/cpb^{6.15}; arp3^{EP3640}/+$ pupae had bristles in all of the categories, more of the $cpb^{F19}/cpb^{6.15}; arp3^{EP3640}/+$ bristles had all or mostly membrane attached bundles ($p < 0.01$;

Figure 5B). These results indicate that capping protein and the Arp2/3 complex act in opposition to one another and likely act primarily through effects on snarls.

Arpc1 Plays a Distinct Role in Bundle Positioning Late in Bristle Development

Our observation that *arpc1* mutations enhanced rather than suppressed the *cpb* phenotype (Figure 1 and Supplementary Figure 1) was very puzzling. To attempt to understand this unexpected result, we examined actin in bristles during a developmental time course. As with $cpb^{F19}/cpb^{6.15}; arp3^{EP3640}/+$, we saw no difference between $cpb^{F19}/cpb^{6.15}$

Figure 5. Actin bundle distribution at the end of bristle elongation. (A) Confocal projections of representative fully elongated (45–46 h APF) *cpb^{F19}/cpb^{6.15}* bristles. Actin is in red (Alexa-568 phalloidin), and membrane is in green (either mCD8-GFP or fluorescein-conjugated tomato lectin [Vector Laboratories, Burlingame, CA]). Insets, computed cross-sections through the bristles. All scale bars are 8 μ m and do not apply to the insets. (B) Quantitation of actin bundle distribution in wild type and mutants. *p* values indicate the statistical significance of the differences between *cpb^{F19}/cpb^{6.15}; +/+* and *+/+; +/+* or the indicated genotypes and *cpb^{F19}/cpb^{6.15}; +/+*. The number of bristles scored for each genotype was as follows: *+/+; +/+*, 11; *cpb^{F19}/cpb^{6.15}; +/+*, 25; *cpb^{F19}/cpb^{6.15}; arp3/+*, 19; *cpb^{F19} arp1^{Q25st}/cpb^{6.15}; +/+*, 21.

and *cpb^{F19}arp1^{Q25st}/cpb^{6.15} +* bristles at 32–42 h APF (Figure 4A, compare u–x with e–h, and 4B). However, we did observe a difference at 45–46 h APF. At this time point, a much greater number of *cpb^{F19}arp1^{Q25st}/cpb^{6.15} +* bristles fell into the “mostly internal” or “all internal” bundles categories ($p < 0.025$), and none were categorized as “all membrane associated” (Figure 5B). This suggests that Arpc1 becomes important late in bristle development for attachment of actin bundles to the membrane.

Proper Bundle Positioning Is Important For Organized Cuticle Secretion

We wondered why having the correct number of properly spaced bundles is important for bristle development. We postulated that this might be important for proper cuticle secretion at the end of bristle elongation. To address this idea, we analyzed cross-sections through the tips of wild-type and homozygous *arp3* pharate adult macrochaetes by transmission electron microscopy. To determine if *arp3* mutants were deficient in their ability to secrete the proper amount of cuticle material, we measured the cuticle area and compared this to the area that had previously been occupied by the bristle cell (the hole in the center of each bristle). We found no significant difference between wild-type and *arp3* mutants (for wild type, the average ratio of cuticle area/cell area was 3.2 ± 0.7 ; for *arp3* the ratio was 2.5 ± 1.0 , $p = 0.1$). Although there was no difference in total amount of cuticle secretion, we did observe striking defects in the organization of the cuticle. Although wild-type bristles were quite uniform and had an average of 8.1 large ridges (range 7–10, $n = 8$) at the tips (Figure 6, a and b), *arp3* bristles had an average of 11.2 ridges (range 7–16, $n = 10$) at the tips (Figure 6, c and d) and this difference was significant ($p < 0.005$). Additionally, 5 of the 10 *arp3* bristles we analyzed had an irregular shape or a dramatic protrusion of the cuticle (Figure 6d). This was never seen in wild type. We conclude that loss of functional Arp2/3

complex leads to a reduction of actin snarls, resulting in an overabundance of actin bundles associated with the membrane that interfere with orderly cuticle secretion.

Figure 6. Cuticle secretion is disorganized in *arp3* mutants. Transmission electron micrographs of cross-sections of the tips of wild-type (a and b) and *arp3/arp3* (c and d) bristles are shown. Note that *arp3* mutant bristles have more ridges than wild type and that there are occasional irregular protrusions (arrow in d). All scale bars, 0.5 μ m.

DISCUSSION

Capping Protein and the Arp 2/3 Complex Act Antagonistically to Regulate Actin Assembly in Snarls

Our results establish that the Arp2/3 complex and capping protein have opposing actions on dynamic actin structures, called snarls, in *Drosophila* bristle cells. In the absence of the Arp2/3 complex, no snarls are apparent, whereas when capping protein is reduced, snarls persist longer during development. Combinations of mutations that affect both proteins are more similar to wild type in number and persistence of snarls, as would be expected if the two activities need to be balanced for proper regulation of actin assembly. Because most other studies of capping protein and the Arp2/3 complex have utilized in vitro assays or motile cells in culture, the observation that these regulators function similarly in nonmotile cells in vivo is important, albeit not surprising. What is surprising, however, is that the main structural features important for the morphology of these cells, the large cortical actin bundles, are not the direct target of these activities, despite the fact that their organization and positioning is strongly affected by altered amount of these regulators.

Nonbundle Actin Snarls Contribute to the Proper Size and Distribution of Cortical Actin Bundles

Here we present evidence that early in bristle development capping protein and the Arp2/3 complex control actin assembly not in actin bundles, but rather in the actin snarls that are present between these bundles. We hypothesize that snarls influence the proper positioning of the cortical actin bundles by competing with them for binding sites on the membrane. When the level of snarls is severely reduced (in homozygous *arp3* mutant pupae), excess actin bundles associate with the membrane. This results in adult bristles that are shorter than wild type (unpublished data) and have more, smaller grooves in their cuticle (Hudson and Cooley, 2002). In the presence of too many snarls (in transheterozygous *cpb* mutant pupae), actin bundles are unable to form against the membrane and instead develop internally. Because they are not stabilized by attachment to the membrane, they become irregularly sized and highly variable in number. This results in a cell membrane that is inadequately supported by actin bundles. Adult bristles that have numerous defects, such as irregular groove patterns and smooth regions in the cuticle, as well as bends, splits, and protrusions in the shaft (Hopmann *et al.*, 1996) are produced.

Tilney *et al.* (2003) have previously demonstrated that actin snarls are dynamic and have suggested that their 2-min half-life is due to their failure to be stabilized either by cross-linking to other filaments or by forming stable attachments to the membrane. These authors argued that those filaments that do not become stabilized are turned over, and thus the actin bundles develop in a Darwinian "survival of the fittest" manner. The snarls were proposed to be nonproductive actin structures. We propose here that the snarls have a function in forming properly shaped bristles: they are important for proper positioning of bundles. Snarls may have additional functions as well. They cause protrusions in the membrane between the bundles (Tilney *et al.*, 1996, 2003) and lie beneath the regions that will become the ridges in the cuticle of the adult bristle (the actin bundles lie below the grooves; Overton, 1967). Thus the Arp2/3 complex-directed actin assembly in snarls may push the membrane out, much in the way that this complex causes membrane protrusion at the leading edge of motile cells (Welch *et al.*, 1997a; Mullins *et al.*, 1998; Svitkina and Borisy, 1999). This region of the

bristle membrane is then kept free from actin bundles so that the proper number and size of bundles are formed. We have shown that when too many bundles form, cuticle is secreted in a disorganized manner. Thus it may be important that some regions of the membrane are kept clear of actin bundles to allow for proper secretion of chitin. Alternatively, actin filaments in the snarls could be directly involved in membrane deposition that occurs during cell extension and/or in the secretion of cuticle (patches of cuticulin are evident on developing bristles as early as 36 h APF; Overton, 1967). Snarls may be analogous to actin patches in *Saccharomyces cerevisiae* that are turned over very rapidly (Smith *et al.*, 2001), contain the Arp2/3 complex (Winter *et al.*, 1997) and capping protein (Amatruda and Cooper, 1992), and are involved in endocytosis and cell wall synthesis (Utsugi *et al.*, 2002; Kaksonen *et al.*, 2003).

The interplay we see between actin bundles and snarls in bristles is reminiscent of that seen between actin cables and patches in *S. cerevisiae*. Yeast cells lacking functional capping protein have diminished actin cables and excess patches (Amatruda *et al.*, 1990, 1992). Conversely, long-term lack of Arp3 results in cells that have lost patches and accumulate actin bundles (Winter *et al.*, 1997). The antagonistic relationship between capping protein and the Arp2/3 complex, as well as their opposing effects on actin patches and bundles, might thus be a common theme in the regulation of the actin cytoskeleton.

*Why Do Mutations in *arp1* Enhance the *cpb* Bristle Phenotype?*

We have shown that mutations in *arp3* and *wasp*, as well as deficiencies that remove *arp4* and *arp5*, all suppress the *cpb* bristle phenotype as would be expected from the opposing biochemical functions of the Arp2/3 complex (filament assembly nucleator) and capping protein (prevents further assembly of actin filaments). So it is surprising to find that mutations in *arp1* have the opposite phenotype. This is an especially unexpected result given that previous work has demonstrated that adult bristles completely lacking Arp1 or Arp3 have an identical mild phenotype of more, smaller grooves (Hudson and Cooley, 2002). It is tempting to suggest that there is a genetic background issue at play here. We believe this is unlikely, however, because two different *arp1* alleles isolated in independent labs in different genetic backgrounds gave the same result.

One explanation for the enhancement seen by mutations in *arp1* is that in the absence of this subunit, a complex forms that in some way acts as a neomorph. Arp1 contains the WASp interaction capability of the complex (Pan *et al.*, 2004), so complexes lacking this subunit might be unactivatable, and thus might block the interaction between actin filaments and other important binding proteins. Although we cannot rule this possibility out, we think it is unlikely because of the following: Gournier *et al.* (2001) have shown that the Arp1 (therein referred to as p41) and Arp5 (p16) subunits form an interacting pair. They find that when complexes are formed in the absence of Arp5, Arp1 also fails to assemble into the complex. If this neomorph model were correct (and assuming fly Arp2/3 complex components behave in the same way as their human homologues), we would expect that reduction of Arp5 would lead to loss of Arp1 from the complex and cause enhancement of the *cpb* phenotype. However, this is not what we observed. A deficiency chromosome lacking the *arp5* region in fact suppressed the *cpb* bristle phenotype.

To explain *arp1* enhancement of bundle displacement observed in *cpb* mutants, we note that the difference in phenotype

between *cpb^{6.15}/cpb^{F19}* and *cpb^{6.15} +/cpb^{F19} arpc1^{Q25st}* is only apparent at late developmental times. In both genotypes early in development, bundles are displaced from the membrane. However, in *cpb^{6.15}/cpb^{F19}* at late times, bundles often are associated with the membrane, whereas in *cpb^{6.15} +/cpb^{F19} arpc1^{Q25st}* they rarely are. Interestingly, in *cpb* null epithelial clones, bundles also remain displaced (Hopmann and Miller, 2003 and unpublished data). These similar phenotypes suggest that capping protein and Arpc1 both participate in a late membrane-attachment process. Thus, we suggest that in cases where bundles form not associated with the membrane initially, capping protein and Arpc1 promote bundle attachment late in development. The ability of some bundles to associate with the membrane in *cpb^{6.15}/cpb^{F19}* bristles is likely due to the fact that in this genotype capping protein is not completely eliminated but rather is reduced to 48% of the wild-type level (Hopmann *et al.*, 1996). When capping protein is completely absent or when both capping protein and Arpc1 are reduced in amount, then this late membrane attachment does not occur. Interestingly, capping protein localization on bundles starts at ~42 h APF (Hopmann *et al.*, 1996 and unpublished data), around the time when the effect of capping protein and Arpc1 on bundle membrane association is manifest. Under normal circumstances, this late bundle attachment activity is not essential, because bundles are already associated with the membrane. Similarly, in cases where snarls are reduced and excess bundles form (*arp3*, and presumably, *arpc1* homozygous) this late attachment activity is not needed because the bundles form initially against the membrane. Obviously, other as yet unidentified proteins must be important for membrane association at early times.

Capping protein has previously been observed to be required for attachment of actin filaments to the Z disks during myofibrillogenesis (Schafer *et al.*, 1995); a role in membrane attachment in bristles would be consistent with this function. Welch *et al.* (1997a) have suggested that Arpc1 (therein referred to as p41-Arc) might serve as a link to the membrane through its WD repeats binding to pleckstrin homology domains, which could in turn bind to phosphatidylinositol 4,5-bisphosphate. Additionally, Arpc1 binds to the activating domain of WASp (Pan *et al.*, 2004), which is itself activated at the membrane.

We suggest here that Arpc1 has a function outside the Arp2/3 complex. Although work in yeast (Winter *et al.*, 1999b) demonstrating a unique function for Arpc1 was later shown to be an experimental artifact (Pan *et al.*, 2004), recent studies in the moss *Physcomitrella patens* has revealed that removal of Arpc1 results in different phenotypes than removal of Arpc4 (Harries *et al.*, 2005; Perroud and Quatrano, 2006). Thus, it will be important to explore this phenomenon of potential differential Arp2/3 complex subunit functions further in the future.

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