A Balance of Capping Protein and Profilin Functions Is Required to Regulate Actin Polymerization in *Drosophila* Bristle

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Profilin is a well-characterized protein known to be important for regulating actin filament assembly. Relatively few studies have addressed how profilin interacts with other actin-binding proteins in vivo to regulate assembly of complex actin structures. To investigate the function of profilin in the context of a differentiating cell, we have studied an instructive genetic interaction between mutations in profilin (*chickadee*) and capping protein (*cpb*). Capping protein is the principal protein in cells that caps actin filament barbed ends. When its function is reduced in the *Drosophila* bristle, F-actin levels increase and the actin cytoskeleton becomes disorganized, causing abnormal bristle morphology. *chickadee* mutations suppress the abnormal bristle phenotype and associated abnormalities of the actin cytoskeleton seen in *cpb* mutants. Furthermore, overexpression of profilin in the bristle mimics many features of the *cpb* loss-of-function phenotype. The interaction between *cpb* and *chickadee* suggests that profilin promotes actin assembly in the bristle and that a balance between capping protein and profilin activities is important for the proper regulation of F-actin levels. Furthermore, this balance of activities affects the association of actin structures with the membrane, suggesting a link between actin filament dynamics and localization of actin structures within the cell.

INTRODUCTION

Regulation of actin filament dynamics is essential for many cellular processes, including cell motility, cytokinesis, cellular differentiation, and endocytosis. Many actin-binding proteins participate in regulating actin filament elongation or disassembly, and their functions have been analyzed by various in vitro assays. Yet, it is often unclear how well the biochemical properties of these proteins, as defined in vitro, correspond to their functions in vivo. Furthermore, biochemical analyses can be incomplete, not accurately reproducing the complexities of actin dynamics in the cell. Genetic studies in whole animals complement in vitro studies by providing functional information in a cellular context.

We are using the fly bristle as a model system to study actin assembly in vivo. During pupal development, the bristle cell elaborates a long process that contains prominent longitudinal bundles of actin filaments associated with the plasma membrane (Overton, 1967; Appel *et al.*, 1993). Cuticle is deposited on the surface of this process, and then as development proceeds, the actin cytoskeleton disassembles,

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the cell regresses, and the remaining hollow tube of cuticle becomes the adult bristle. Mutations in actin regulatory proteins such as capping protein (*cpb*; Hopmann *et al.*, 1996), profilin (*chickadee*; Verheyen and Cooley, 1994), ADF/cofilin (*twinstar*; Gunsalus *et al.*, 1995; Chen *et al.*, 2001), ADF/cofilin phosphatase (*slingshot*; Niwa *et al.*, 2002), and twinfilin (*twf*; Wahlstrom *et al.*, 2001) perturb bristle morphogenesis through effects on the actin cytoskeleton. The bristle seems to be particularly sensitive to reductions in the level of actin-binding proteins, most likely because the cells are very large and require massive amounts of actin polymerization during development.

Bristle growth is dependent on actin filament polymerization (Tilney *et al.*, 2000a). Actin polymerization requires a free end and actin monomers competent to polymerize (Pollard *et al.*, 2000). Capping protein and profilin are two actin-binding proteins that regulate filament elongation by modulating these two parameters. Capping protein binds to actin filament barbed ends with high affinity and prevents the addition or loss of subunits (Isenberg *et al.*, 1980; Casella *et al.*, 1986). Profilin is an enigmatic protein that seems to stimulate actin polymerization in some contexts and repress it in others. Profilin binds to actin monomers and was originally thought to limit actin polymerization through monomer sequestration (Carlsson *et al.*, 1977). Subsequent data suggest profilin promotes actin polymerization at free

barbed ends, but sequesters when barbed ends are capped (Pantaloni and Carlier, 1993). A few studies have shown that polymerization enhancement predominates in specific situations in vivo (Finkel *et al.*, 1994; Rothkegel *et al.*, 1996; Benlali *et al.*, 2000; Boquet *et al.*, 2000; Wolven *et al.*, 2000; Lu and Pollard, 2001), but monomer sequestration has been invoked in others (Verheyen and Cooley, 1994). Thus, the function of profilin in a cellular context, particularly in differentiating cells of multicellular organisms, remains somewhat unclear.

In this article, we focus on an informative genetic interaction between *cpb*, which encodes the β subunit of capping protein, and *chickadee* (*chic*; encodes profilin). Null mutations in either gene are lethal, but partial loss-of-function alleles are viable and affect bristle development (Verheyen and Cooley, 1994; Hopmann *et al.*, 1996). Both *cpb* and *chic* mutant flies have shortened bristles that exhibit bending, branching, and disrupted surface grooves. Previous studies have shown that in both cases, the phenotypes result from an abnormal actin cytoskeleton. Herein, we show that *cpb* mutations lead to dramatic increases in F-actin levels in the bristle and this is likely to be the underlying cause of the bristle phenotype. We also show that loss-of-function *chic* mutations suppress the *cpb* bristle phenotype. To our knowledge, this is the first demonstration of a genetic interaction between these two genes. This interaction is evident at the level of the actin cytoskeleton and suggests that profilin stimulates actin assembly in this context. Our results are consistent with the idea that a balance between profilin and capping protein activity contributes to the regulated assembly of actin that is required for normal bristle elongation and morphogenesis. We discuss possible mechanisms by which profilin might stimulate actin polymerization in this cell, and how the disruption of actin filament dynamics leads to disorganization of the actin bundles and abnormal bristle morphology. This work contributes to the emerging picture of how actin-binding proteins coordinate to correctly organize the actin cytoskeleton in eukaryotic cells.

MATERIALS AND METHODS

Fly Culture and Mutant Stocks

Flies were raised on standard cornmeal medium (Lewis, 1960) at 25°C. The generation of $cpb^{6.15}$ and cpb^{F19} was described previously (Hopmann *et al.*, 1996). The $chic^{37}$ allele was provided by L. Cooley (Yale University, New Haven, CT) and the $chic^{221}$ allele and UAS-profilin lines were provided by L. Jones (Yale University, New Haven, CT) and L. Cooley. The P [GAL-4] B-11 driver line was provided by J. Merriam (University of California, Los Angles, CA). The green balancer used in this study was CyO, P {GAL4-Kr.C} DC3, P {UAS-GFP.S65T} DC7, hereafter called CyO, green fluorescent protein (GFP). It is available from the Bloomington stock center (Bloomington, IN). All lines used to generate GFP marked clones were also obtained from the Bloomington stock center.

Fly Crosses and Viability Determination

Because *cpb* and *chic* reside on chromosome arm 2L, recombinant chromosomes were constructed to link *chic* alleles to cpb^{F19}. Transheterozygotes were then made with the $cpb^{6.15}$ allele to generate flies of the experimental genotypes as follows. $cpb^{6.15 \ shv \ pr}$/CyO, GFP females were mated to males from three different lines: cpb^{F19} *cn bw sp*/CyO, GFP; cpb^{F19} $chic^{37}$ *cn*/CyO, GFP; or cpb^{F19} $chic^{221}$ *cn*/CyO, GFP. Seven virgin females were premated to three males in

a vial for 24 h, then transferred to a bottle and incubated at 25°C. The relative viability of the different genotypes was determined by counting balancer (CyO) and nonbalancer adult progeny between days 11 and 17. If fully viable, the number of experimental (nonbalancer) progeny should be equal to one-half the number of balancer progeny. This expectation is based on the observation that balancer homozygotes never survive to adulthood, and the assumption that balancer heterozygotes are fully viable. The relative adult viability of each experimental genotype was calculated by dividing the number observed by the number expected and converting to percentage.

To overexpress profilin in the developing bristle, *w*; P [*w+* GAL4] B-11/TM3, *Sb* females were mated to *w*; l (2)/CyO; P [*w+* UAS-*chic*]/P [*w+* UAS-*chic*] males. The B-11 driver line is an enhancer trap that expresses GAL4 in developing bristles (J. Merriam, unpublished data). Non-*Sb* progeny were examined for bristle phenotypes and subjected to scanning electron microscopy.

Phenotypic Analysis of Bristles

All analyses were performed on adult flies preserved in a solution of 95% ethanol/glycerol (3:1).

To evaluate the frequency of the abnormal bristle phenotype, flies were scored for the number of bristles on the dorsal head and thorax with major defects, which were defined as sharp bends, branches, or split ends. Flies were classified as having zero, one, or two or more defective bristles.

The length of the posterior sternopleural bristle was measured under the dissecting microscope to the nearest 25 μm as described previously (Hopmann *et al.*, 1996). Both left and right bristles were measured and treated as individual data points. Bristles that were obviously broken were not included. Average length was calculated, and error expressed as ± 1 SD.

Scanning Electron Microscopy

Preserved adult flies were processed for scanning electron microscopy as described previously (Hopmann *et al.*, 1996). After processing, flies were mounted on stubs with carbon-adhesive tabs and carefully grounded with colloidal silver liquid (all materials available from Electron Microscopy Sciences, Fort Washington, PA). Specimens were coated and imaged as described previously (Hopmann *et al.*, 1996).

Preparation and Staining of Pupal Pelts

Pupae of the experimental genotypes were selected after examination under an SZX-12 dissecting microscope equipped with fluorescence (Olympus, Tokyo, Japan). Balancer pupae fluoresce green because of the GFP-expressing transgene present on the CyO, GFP balancer, whereas the nonbalancer, experimental pupae are nonfluorescent. Pupae were raised at 25°C then dissected at 45–47 h after pupariation (AP). This time is near the end of bristle elongation when actin bundles are very prominent. Dissections of the dorsal epithelium were performed essentially as described by Tilney *et al.* (1998) with minor modifications. Pelts were fixed in 1 ml of 3.7% formaldehyde in PBSTx (1× PBS + 0.01% Triton X-100) for 30 min at room temperature, followed by 3 × 30-min washes with PBSTx.

Pelts were blocked in lectin block (1× PBSTx, 2 mM CaCl₂, 5% bovine serum albumin) at least 1 h at room temperature, and then stained overnight at 4°C in 0.5 ml of fluorescein-conjugated *Lycopersicon esculentum* (tomato) lectin (Fluor-LE; Vector Laboratories, Burlingame, CA) at 20 μg/ml in lectin block. Pelts were washed 3 × 20 min in 1 ml of lectin block, and stained with 0.5 ml of Alexa-568-phalloidin (Molecular Probes, Eugene, OR) at 1.6 U/ml in lectin block. Pelts were washed 3 × 20 min in lectin block, and mounted in Shandon Immumount (Shandon Southern Instruments, Sewickley, PA). Indirect flight muscles still attached to the pelt were carefully removed during the mounting procedure. Mounted pelts

were examined with a laser scanning spectral confocal microscope model TCS SP2 (Leica, Heidelberg, Germany).

### Generation of Marked Null Clones in Pupae

The mosaic analysis with a repressible cell marker method for induction of GFP-marked mitotic clones was used to generate $cpb^{4.18}$ homozygous clones in pupae (Luo et al., 1999; Lee and Luo, 2001). $cpb^{4.18}$ is a null allele and will be more fully described elsewhere (Hopmann and Miller, unpublished data). In the mosaic analysis with a repressible cell marker system, mutant clones are marked by expression of the mCD8-GFP fusion protein driven by GAL4. Because this GFP fusion is targeted to the plasma membrane, the mutant cells are nicely outlined. Expression of the GFP marker in all other cells is repressed by the presence of GAL80.

The following cross was done to generate animals of the correct genotype for clone induction: y w P [w+ UAS-mCD8-GFP]/Y; cpb4.18 shv P [ry+ FRT] 40A/+; P [w+ tubP-GAL4]/+ males X y w P [ry+ hs70-FLP] 122; P[w+ tubP-GAL80 FRT] 40A/CyO females. Eggs were collected in a food vial for 2–3 d and then adults removed. Developing embryos/larvae in the vial were heat shocked in a 38°C water bath for 2 h, and then allowed to continue development at 25°C. Pupae ~48 h AP were directly examined under a fluorescence-equipped dissecting microscope for the presence of GFP-expressing clones. Animals with clones were dissected and fixed as described above, and stained with rhodamine-phalloidin as described previously (Hopmann et al., 1996).

RESULTS

Capping protein is αn αβ heterodimer that binds tightly to the barbed ends of actin filaments. Previous screens for mutations in the cpb gene, which encodes the β subunit, yielded two hypomorphic alleles, $cpb^{6.15}$ and cpb^{F19} (Hopmann et al., 1996). These alleles partly complement, such that transheterozygotes survive to adulthood, although at reduced frequency. $cpb^{6.15}$ and cpb^{F19} alleles seem to express reduced amounts of normal capping protein because 1) transheterozygous ($cpb^{6.15}/cpb^{F19}$) adults express ~48% of normal levels of β protein with typical electrophoretic mobility (Hopmann et al., 1996), and 2) sequencing reveals no changes in the coding region of either allele (Hopmann and Miller, unpublished data).

Transheterozygous adults display abnormal bristle morphology that is caused by the disorganization of actin bundles in the developing bristle (Hopmann et al., 1996; see below). Based on the in vitro biochemical properties of capping protein from other species, we predicted that the disruption of actin bundle organization in $cpb^{6.15}/cpb^{F19}$ bristles resulted from increases in the amount of F-actin caused by a reduction in barbed-end capping activity. To demonstrate that loss of capping protein function does indeed cause increased levels of F-actin, we generated mitotic clones in the pupal epidermis that were homozygous for the null allele $cpb^{4.18}$. The null mutant cells were marked by the expression of mCD8-GFP, which is targeted to the plasma membrane. Pupae with clones were dissected near the end of bristle elongation, at ~46 h AP, and then fixed and stained with rhodamine-phalloidin to label F-actin. Three clones were observed that contained elongating macrochaetae. In all three cases, the clonal macrochaetae stained much more intensely with rhodamine-phalloidin than the nonclonal macrochaetae within the same pelt. A representative example is shown in Figure 1, a–c. In this image, a clonal bristle ($cpb^{4.18}/cpb^{4.18}$), expressing mCD8-GFP (arrow in c), was
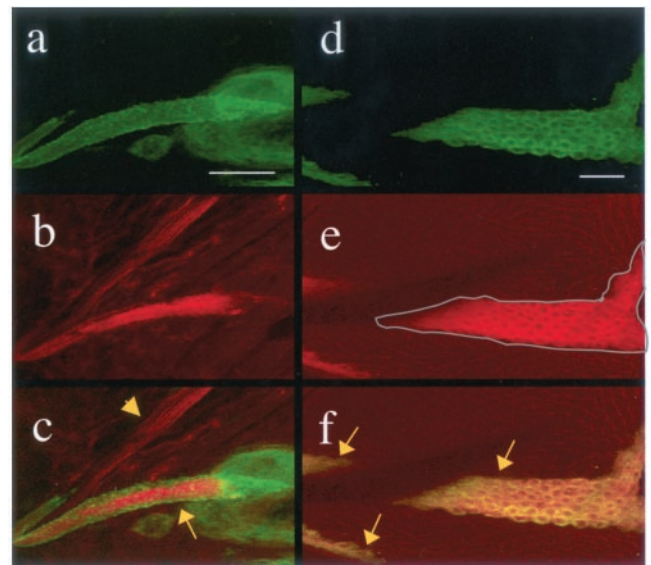
Figure 1. Loss of capping protein function leads to increased F-actin in pupal epithelial cells. Two elongating bristles from a single pupal pelt are shown (a–c). The bristle expressing the GFP marker is within a mitotic clone homozygous for $cpb^{4.18}$ (arrow in c), and the unlabeled bristle is $cpb^{4.18}/+$ (arrowhead in c). Note increased actin labeling in the clonal bristle, which lacks capping protein. F-actin is also elevated in a $cpb^{4.18}$ pupal wing clone, similarly marked with GFP (d–f). Null clones are marked with arrows, and the largest clone is outlined in e. The rest of the field is filled with nonclonal wing epithelial cells. Clonal cells have elevated F-actin. mCD8-GFP (a and d), actin (b and e), and merge (c and f). Bars, 20 μm.

located near a nonclonal bristle ($cpb^{4.18}/+$; arrowhead in c) of similar size. Comparison of phalloidin fluorescence intensity between the two bristles showed more F-actin in the cpb null bristle. This difference in actin levels is also very evident in wing clones (Figure 1, d–f). In this series of images, the entire field consists of wing epithelial cells. As in the bristle clones, null cells are marked with mCD8-GFP (d). For clarity, the boundary of the largest clone is outlined in gray in e. Within in the boundary, the cells stain brightly with rhodamine-phalloidin; outside the boundary, the nonclonal cells exhibit much fainter fluorescence. This result indicates that loss of capping protein in wing epithelial cells leads to significant increases in the concentration of F-actin, as it does in bristle cells.

To study how profilin and capping protein work together to regulate the assembly and organization of bristle actin bundles, we looked for a genetic interaction between mutations in chickadee and cpb. chickadee (chic) is the single gene encoding profilin in flies. Several alleles of chic affect bristle morphology (Verheyen and Cooley, 1994). Some aspects of the chic bristle phenotype are reminiscent of cpb: bristles are shorter and thicker than wild type and are often bent, split, or branched. This phenotype was originally interpreted to result from increased F-actin levels, similar to cpb mutants. However, chic bristles do not display groove patterns that are as aberrant as cpb bristles. This difference corresponds to subtle differences in the actin bundle phenotypes of chic and cpb. Although chic mutant bristles have more numerous and

Table 1. Reduced profilin levels suppress *cpb* semilethality

	No. CyO observed	No. non-CyO expected	No. non-CyO observed	Relative adult viability, %[a]
$cpb^{6.15}$ +/CyO × cpb^{F19} + /CyO	208	104	45	43 (n = 253)
$cpb^{6.15}$ +/CyO × cpb^{F19} $chic^{37}$/CyO	542	271	206	76 (n = 748)
$cpb^{6.15}$ +/CyO × cpb^{F19} $chic^{221}$/CyO	604	302	228	75 (n = 832)

[a] No. observed/no. expected × 100.

thinner actin bundles, somewhat like *cpb*, the actin bundles as not as disorganized as those in *cpb* bristles (Verheyen and Cooley, 1994).

Given that *cpb* and *chic* mutations affect actin bundle morphology in the bristle, and the encoded proteins are known to regulate actin assembly, it seemed likely that the two genes would have a genetic interaction. We used enhancement or suppression of the *cpb* bristle phenotype as an assay to test the following hypotheses: if profilin's primary role in the bristle is to sequester monomer and inhibit actin polymerization, then reduction of profilin would lead to increased amounts of F-actin, and *chic* mutations would enhance the *cpb* bristle phenotype. Conversely, if profilin's main function is to stimulate actin polymerization, we would expect *chic* mutations to suppress the *cpb* bristle phenotype. Although it could be argued that this rationale is based on an oversimplified view of these proteins' functions, the results of this analysis were indeed informative.

Because null alleles of capping protein are homozygous lethal, we based our genetic interaction studies on the bristle phenotype of transheterozygous $cpb^{6.15}/cpb^{F19}$ animals. We reduced profilin levels by introducing *chic* alleles into this background. Two alleles of *chic* were used. $chic^{37}$ is a hypomorphic allele that is caused by a small deletion in the 5′ untranslated region. When homozygous, $chic^{37}$ reduces (but probably does not eliminate) profilin expression in the bristle and is associated with a strong bristle phenotype. $chic^{221}$ is a null, homozygous lethal allele caused by a larger deletion removing part of the 5′ untranslated region as well as a large portion of the coding region (Verheyen and Cooley, 1994).

Reduction of profilin strongly suppressed multiple aspects of the $cpb^{6.15/cpbF19}$ phenotype. First, heterozygosity for either *chic* allele improved the viability of $cpb^{6.15/cpbF19}$ transheterozygotes (Table 1). $cpb^{6.15/cpbF19}$ adults were observed at 43% of the expected frequency at 17 d from mating. Heterozygosity for either *chic* allele improved survival significantly (76% for $chic^{37}$, p < 0.005; 75% for $chic^{221}$, p < 0.005). Second, a developmental delay associated with loss of capping protein function was suppressed. At 13 d after mating, $cpb^{6.15}/cpb^{F19}$ adults were observed at 20% of the expected frequency, compared with 43% at day 17. Heterozygosity for *chic* caused the developmental delay to be less pronounced: 60% for $chic^{37}$, 67% for $chic^{221}$ at day 13 vs. 76 or 75% at day 17. The suppression of *cpb* effects by *chic* suggests that capping protein and profilin have opposing functions in *Drosophila* development.

Even more striking were the effects of *chic* heterozygosity on the *cpb* bristle phenotype. $cpb^{6.15}/cpb^{F19}$ adults have a moderate bristle phenotype characterized by bending, branching, or splitting of some of the large bristles (macrochaetae). Furthermore, the cuticular surfaces of many macrochaetae seem knobby or rough. The roughening is caused by the disruption of longitudinal grooves seen in normal adult bristles (Hopmann *et al.*, 1996) and Figure 2A). Rare $cpb^{6.15}$ homozygous adult escapers have a similar bristle phenotype (Hopmann, unpublished data). In the adult, the bending, splitting and abnormal groove patterns seen in $cpb^{6.15/cpbF19}$ macrochaetae were dramatically suppressed by both *chic* alleles. Figure 2A, a–d, shows a typical range of phenotypes of $cpb^{6.15/cpbF19}$ bristles, demonstrating that although some macrochaetae were strongly affected and others mildly, all were affected to some degree. For $cpb^{6.15}$ + /cpb^{F19} $chic^{37}$, a similar phenotypic series (Figure 2, e–h) shows that weakly affected macrochaetae (Figure 2, e) were nearly indistinguishable from wild type (Figure 2, m), and that the most strongly affected macrochaetae (Figure 2, g and h) were comparable with the least affected $cpb^{6.15/cpbF19}$ macrochaetae. The $chic^{221}$ allele exhibited an even greater degree of phenotypic suppression (Figure 2, i–l). The majority of macrochaetae in this genotype seemed completely normal (compare with wild-type, Figure 2, m).

We quantified these differences in bristle phenotype in two ways. First, for each genotype we counted the number of bristles on the dorsal thorax and head that exhibited major defects (Figure 3A). Major defects were defined as sharp bends, branches, or split ends that could be easily seen under the dissecting microscope. Each fly was categorized as having zero, one, or two or more defective bristles. Although 78% of $cpb^{6.15/cpbF19}$ flies had at least one major bristle defect, the presence of either *chic* allele greatly reduced the frequency of bristle defects. 26% of $cpb^{6.15}$ + /cpb^{F19} $chic^{37}$ flies exhibited major bristle defects, and for $cpb^{6.15}$ + /cpb^{F19} $chic^{221}$ no major defects were observed. The difference in effect of the two *chic* alleles correlated with the relative strength of the alleles (hypomorph vs. null) and could also be discriminated in more subtle aspects of the bristle phenotype. When viewed at low magnification, both $cpb^{6.15/cpbF19}$ and $cpb^{6.15}$ + /cpb^{F19} $chic^{37}$ flies exhibited minor defects such as rough, knobby bristles, although this was less pronounced in the latter genotype. In contrast, $cpb^{6.15}$ + /cpb^{F19} $chic^{221}$ bristles seemed completely normal.

We also quantified the differences in the bristle phenotype by measuring the length of one particularly long bristle, the posterior sternopleural bristle, in the various genotypes. We have shown previously that reduction of capping protein concentration in $cpb^{6.15/cpbF19}$ flies resulted in a significant shortening of this particular bristle compared with wild type (Hopmann *et al.*, 1996). In the current experiment (Figure 3B), the average length of the sternopleural bristle in Oregon
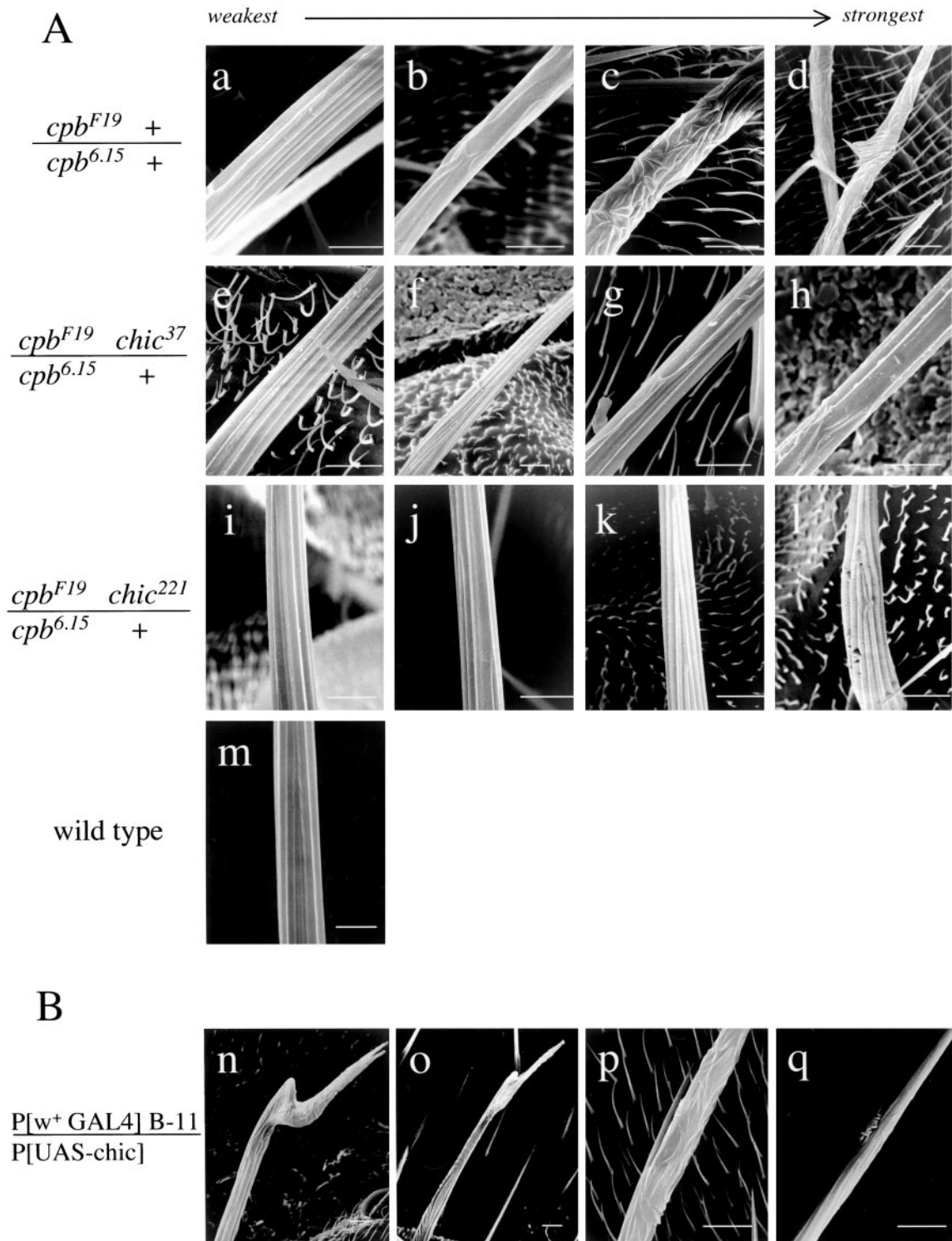
Figure 2. Adult bristle phenotypes of *cpb* and *chic* mutants are consistent with opposite effects on actin polymerization. (A) *cpb* bristle phenotype is suppressed by *chic* mutations. Four scanning electron micrographs of macrochaetae from each genotype are shown to illustrate the range of phenotypic severity, from weakest (a, e, and i) to strongest (d, h, and l). Genotypes are as follows: $cpb^{F19}/cpb^{6.15}$ (a–d), cpb^{F19} $chic^{37}/cpb^{6.15}$ (e–h), cpb^{F19} $chic^{221}/cpb^{6.15}$ (i–l), and wild type (m). Bars, 10 μm. (B) Overexpression of profilin in the bristle causes a *cpb*-like phenotype. Four examples of affected macrochaetae from B-11 UAS-chic adults are shown (n–q), ordered from weakest to strongest phenotype with respect to the groove pattern. Bars, 10 μm.
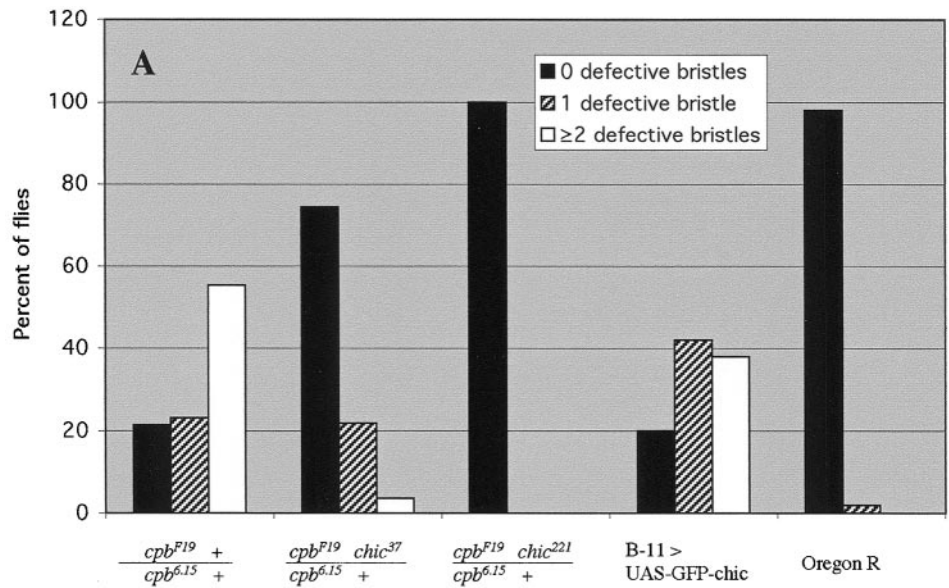
Figure 3. *cpb* bristle phenotype is suppressed by *chic*. (A) Frequency of major bristle defects. Flies were categorized as having zero defective bristles (black bars), one defective bristle (striped bars), or two or more defective bristles (white bars). The raw data was converted to percentage of the total number of flies of each genotype. Complete genotypes are as in Figure 1. Sample sizes: $cpb^{6.15}/cpb^{F19}$ (n = 56), $cpb^{6.15}/cpb^{F19}$ $chic^{37}$ (n = 55), $cpb^{6.15}/cpb^{F19}$ $chic^{221}$ (n = 15), B-11 UAS-chic (n = 50), and Oregon R (wild type, n = 50). (B) Length of sternopleural bristle. Sample sizes: $cpb^{6.15}/cpb^{F19}$ (n = 30), $chic^{37}/chic^{37}$ (n = 28), $cpb^{6.15}/cpb^{F19}$ $chic^{37}$ (n = 28), $cpb^{6.15}/cpb^{F19}$ $chic^{221}$ (n = 28); B-11 UAS-chic (n = 43); and Oregon R (wild type, n = 22).
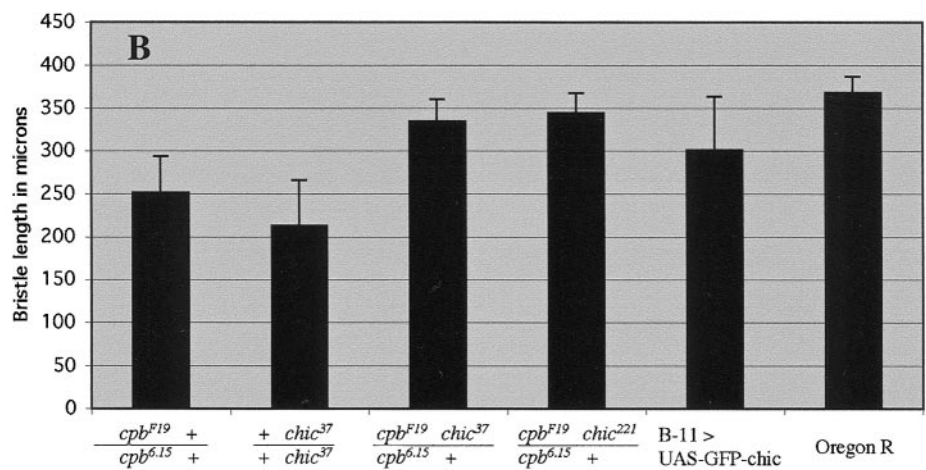
R flies was 368 vs. 252 μm in $cpb^{6.15/cpbF19}$, an ~30% reduction. The sternopleural bristle was even shorter in $chic^{37}$ homozygotes, averaging 213 μm. However, in the background of $cpb^{6.15/cpbF19}$, heterozygosity for either *chic* allele restored the sternopleural bristle to nearly wild-type length.

The suppression of *cpb* mutant phenotypes by *chic* suggests that profilin functions antagonistically to capping protein. Therefore, we predicted that overexpression of profilin in the bristle is likely to cause a phenotype similar to *cpb* loss-of-function. We tested this by combining a UAS-chic transgene to a GAL4 enhancer trap line (designated B-11) that is expressed in the elongating bristle shaft. B-11 UAS-chic pupae overexpress profilin in the bristle shaft but not in most other tissues. Adults of this genotype exhibited a strong bristle phenotype that seemed very similar to the *cpb*

loss-of-function phenotype (Figure 2B, n–q). Macrochaetae were often bent or split at the ends, or had slender barbs branching off. This bristle phenotype was quantified in the same manner as the cpb loss of function genotypes. The frequency of major bristle defects was very similar to $cpb^{6.15/cpbF19}$ (Figure 3A). Eighty percent of the B-11 UAS-chic adults had at least one abnormal bristle, compared with 78% for $cpb^{6.15}/cpb^{F19}$. Likewise, bristle length was also decreased in B-11 UAS-chic adults, although this phenotype was variable (Figure 3B). The mean length of the posterior sternopleural bristle was 301 μm, but individual bristle length ranged widely. Most strikingly, groove patterns were highly disorganized in the profilin over expressing bristles, implying the actin cytoskeleton is disorganized as it is in *cpb*

mutants. This suggests that an increase of profilin has similar consequences as a reduction of capping protein.

It is worth noting that profilin loss of function also leads to severe bristle phenotypes that at a superficial level seem similar to the profilin overexpression phenotype (Verheyen and Cooley, 1994, Figure 7, for bristle phenotype of chic[37] homozygotes). Specifically, both conditions cause bristles to be shorter and have abnormal morphology. However, analysis of scanning electron micrographs points out clear differences (compare Verheyen and Cooley, 1994, Figure 7, to Figure 2B, m–p). chic[37] bristles are extremely short and highly branched, and the surface grooves are very visible and oriented longitudinally. Conversely, B-11 UAS-chic bristles are only slightly shorter than wild type, and never display the high degree in branching seen in chic[37]. However, the surface grooves are often shallow and very disorganized, especially in the distal half of the bristle. In all respects, the effects of profilin overexpression in the bristle are much more similar to capping protein loss of function than they are to profilin loss of function.

Previously, we noted that in $cpb^{6.15}/cpb^{F19}$ bristle cells the association of actin bundles with the plasma membrane seen in normal bristles is partially disrupted (Hopmann *et al.*, 1996). To look at this more carefully we stained pupal pelts with a fluorescein-conjugated lectin from tomato (Fluor-LE) to label the plasma membrane in addition to Alexa-Fluor-568-phalloidin to label the actin bundles. In grazing longitudinal sections of wild-type bristles, actin bundles were visible as longitudinal stripes (Figure 4A, a–c). In medial longitudinal sections (Figure 4A, d–f) and z-planes extracted from a confocal series (cross sections perpendicular to longitudinal axis; Figure 4A, g), actin bundles were present at the perimeter of the cell and not in the interior. In addition, the membrane glycoproteins(s) bound by the lectin probe were excluded from the membrane domains overlying the actin bundles. In favorable images (Figure 4A, h–j), this caused the lectin labeling also to seem striped in grazing sections, and in the merged image the actin and lectin stripes were interdigitated. This alternating pattern was also evident in z-planes (Figure 4A, g) in which actin spots alternated with higher concentrations of lectin staining. In $cpb^{6.15}/cpb^{F19}$ bristle cells, the association between the actin bundles and the plasma membrane was partly disrupted such that actin bundles were visible in the interior of the bristle shaft. Although in grazing section some actin bundles were still associated with the membrane (Figure 4A, k–m), a medial section of the same bristle cell revealed many actin bundles in the center of the bristle shaft (Figure 4A, n–p). The displacement of actin bundles in the cpb mutant is also apparent in z-planes (Figure 4A, q). The disorganization of the actin cytoskeleton was reflected in the lectin staining. Although there was some striping visible in grazing sections (Figure 4A, k and m) it was not as striking as in wild type nor as well organized relative to actin. In contrast, chic[37] homozygotes do not demonstrate displacement of the bristle actin bundles (our unpublished data).

Consistent with the adult bristle phenotypes, the disorganization of actin bundles seen in $cpb^{6.15}/cpb^{F19}$ pupal macrochaetae was suppressed by *chic* mutations (Figure 4B). Actin bundles in $cpb^{6.15} + /cpb^{F19}$ chic[37] (Figure 4B, a–f) and $cpb^{6.15} + /cpb^{F19}$ chic[221] (Figure 4B, h–m) bristles were more uniform in thickness and better organized than in $cpb^{6.15}/cpb^{F19}$. In

fact, in most macrochaetae the actin staining was very similar to wild type. In grazing sections (Figure 4B, a–c, h–j), actin bundles were well organized and in medial sections (Figure 4B, d–f, k–m) actin bundles were mostly absent from the center of the bristle shaft and located at the cell periphery. Lectin staining was striped and alternated with actin bundles. Z-planes (Figure 4B, g and n) emphasize that both *chic* alleles restored association of the actin bundles with the membrane although the degree of suppression seemed greater for the chic[221] allele.

If the adult phenotype of bristles overexpressing profilin truly reflects a similar mechanism as the *cpb* loss of function, one would expect the phenotype of actin bundles in the pupal bristle to resemble *cpb*. To test this, P[GAL4] B-11/P [UAS-chic] pupae were stained for actin and membrane as described above (Figure 5). The actin bundle phenotype seen in these pupae was strikingly similar to the *cpb* transheterozygote. Actin bundles were more numerous and heterogeneous and were partially displaced from the plasma membrane. Although the top grazing section (a–c) showed bundles still opposed to the membrane and striped lectin labeling, the medial section (d–f) revealed bundles in the center and the bottom grazing section (g–i) showed less organized membrane labeling and no actin bundles. The z-plane (j) further illustrates how bundles from one side of the bristle seemed to be mislocalized in the center of the bristle shaft. This displacement of actin bundles from the membrane is very similar to that seen in *cpb* loss of function mutants, and very different from defects seen in *chic* loss of function mutants.

DISCUSSION

Capping protein loss of function leads to dramatic increases in F-actin in the fly bristle, resulting in aberrant organization of the actin cytoskeleton. Reduction of profilin suppresses the disorganized actin phenotype caused by reduction of capping protein function, suggesting that profilin promotes actin assembly in the elongating bristle. These results emphasize the idea that the balance of activities of actin-binding proteins is critical for assembling actin structures that are organized and positioned properly.

Numerous studies have demonstrated the importance of the actin cytoskeleton for the normal elongation and morphogenesis of the fly bristle. Tilney *et al.* (2000a) showed that inhibitors of actin polymerization significantly decreased the elongation rates of bristles whereas inhibitors of microtubule polymerization had little effect. The morphology of bristle actin bundles is affected by changes in the amount of cross-linking proteins (Tilney *et al.*, 2000b) as well as mutations in genes that encode regulators of actin dynamics, including ADF/cofilin (*twinstar*; Gunsalus *et al.*, 1995; Chen *et al.*, 2001), *twinfilin* (Wahlstrom *et al.*, 2001), and ADF/cofilin phosphatase (*slingshot*; Niwa *et al.*, 2002). Yet many of these alterations do not cause severely displaced and disoriented actin bundles. In contrast, mutations in capping protein strongly affect not only the amount of F-actin but also the position and orientation of actin structures. In this regard, the phenotype of *twinfilin (twf)* mutants is particularly noteworthy. Twinfilin is a monomer-sequestering protein that is structurally related to ADF/cofilin (Palmgren *et al.*, 2002). In *twf* mutant bristles, F-actin levels are increased and
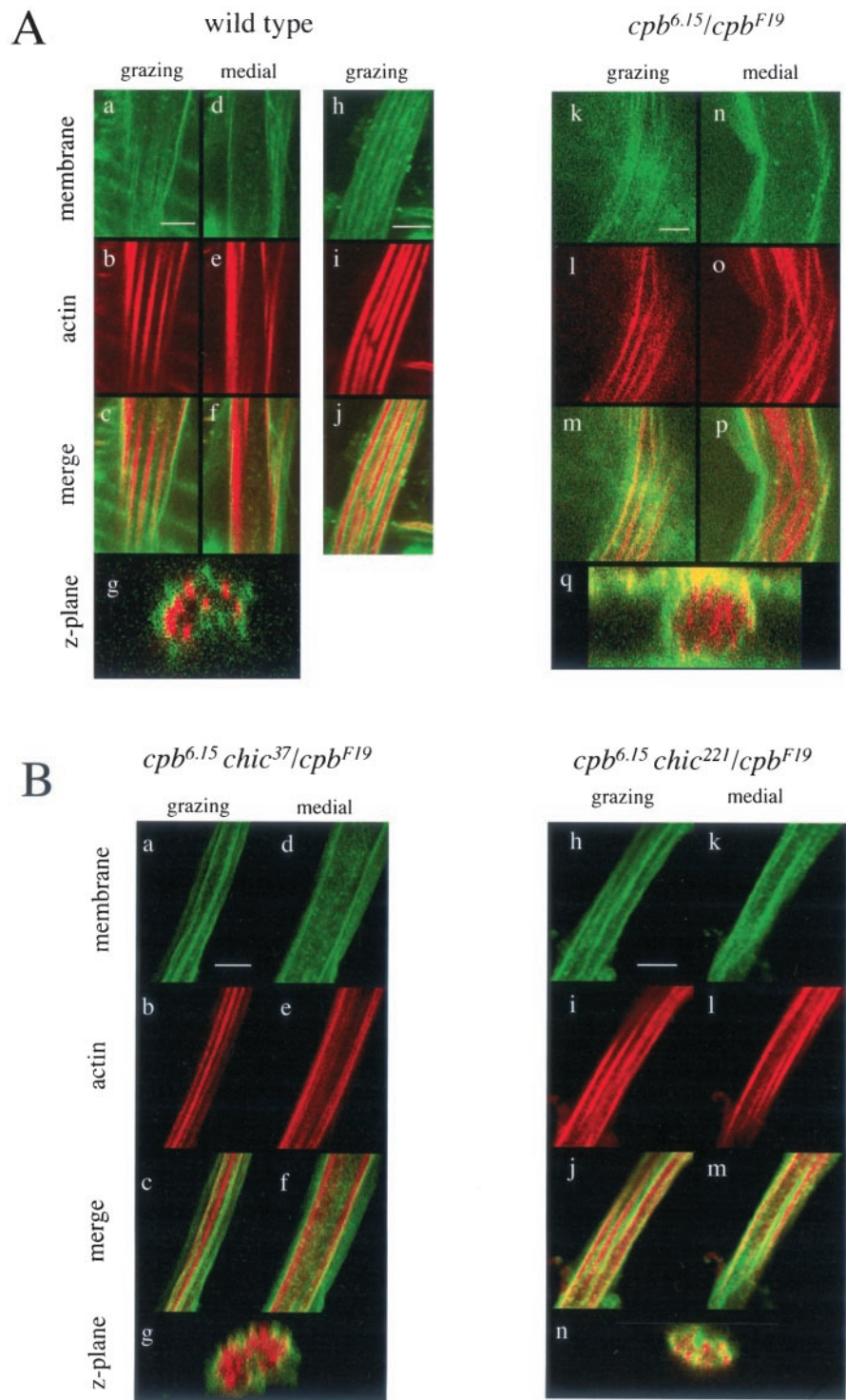
Figure 4. chic suppresses actin bundle disorganization and displacement in *cpb* pupal bristles. (A) *cpb* loss-of-function causes bristle actin bundles to become disorganized and displaced from the plasma membrane. Confocal sections of pupal bristles are from Oregon R (wild type, a–j); *cpb^F19^ cn bw sp/cpb^6.15^ shv pr* (k–q). Membrane was labeled with fluorescein-conjugated tomato lectin (Fluor-LE; a, d, h, k, and n) and actin was labeled with Alexa-568-phalloidin (b, e, i, l, and o). Merged images are also shown (c, f, j, m, and p). Grazing (a–c, h–j, k–m) and medial (d–f, n–p) sections are single confocal xy-planes. Z-plane cross sections (g and q) are extracted from series of xy-planes spaced 0.15 μm. For Oregon R, the z-plane is derived from a different bristle than that shown in a–f. Bars, 4 μm. (B) *chic* suppresses the actin bundle abnormalities of *cpb* pupal bristles. *cpb^6.15^ shv pr/cpb^F19^ chic^37^ cn* (a–g); *cpb^6.15^ shv pr/cpb^F19^ chic^221^ cn* (h–n). Membrane (a, d, h, and k), actin (b, e, i, and l), merge (c, f, j, and m), and z-plane merge (g and n). Bars, 4 μm.

the actin bundles are very disorganized, like they are in *cpb* mutants. Furthermore, the actin bundles show the same dramatic displacement from the membrane in *twf* as they do in *cpb*. This contrasts with the phenotype of *chic* bristles, which do not show displacement of bundles, and under-

scores the fact that although twinfilin and profilin both have sequestering activity in vitro, they clearly have different roles in vivo.

What the analysis of individual mutant phenotypes does not tell us is how the different actin regulatory proteins work
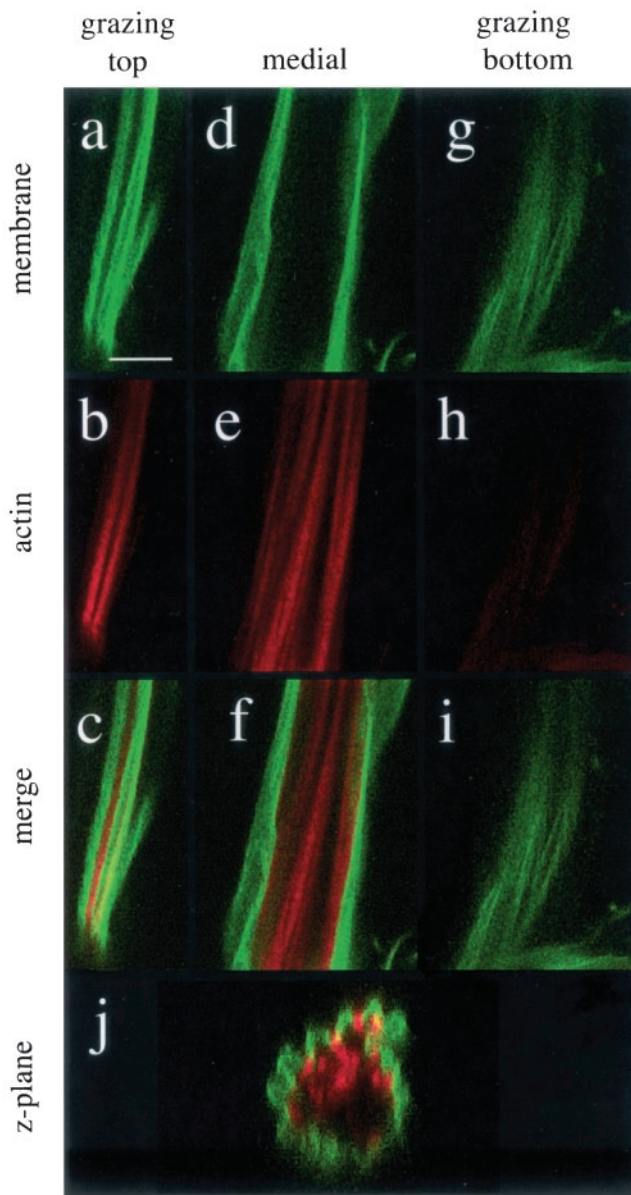
Figure 5. Profilin overexpression mimics capping protein loss of function effects on bristle actin bundles. Confocal sections from a P[GAL4] B-11/P [UAS-chic] pupa. Membrane was labeled with Fluor-LE (a, d, and g) and actin with Alex-568-phalloidin (b, e, and h), merge (c, f, and i), and z-plane (j). Bars, 4 μm.

together to generate normal actin bundles. Our analysis of *cpb chic* double mutants demonstrates this clearly. Because the original phenotypic characterization of *cpb* and *chic* single mutants suggested that they both led to increased levels of F-actin, our original expectation was that *chic* loss of function would enhance *cpb* loss of function. Instead, we observed the opposite effect. This approach has yielded valuable insights regarding the importance of the balance of capping protein and profilin activities in normal cells. In other cases, mutant combinations do exhibit predictable phe-

notypes. For example, double heterozygous combinations of *twf* and *tsr*, which encodes ADF/cofilin, exhibit a moderate bristle phenotype even though the single mutant heterozygotes show little or no bristle phenotype (Wahlstrom *et al.*, 2001). This is consistent with the proposed function of both proteins: reduction of twinfilin leads to increases in F-actin assembly due to reduced sequestering activity, and reduction of ADF/cofilin leads to a decreased rate of actin depolymerization. Thus, it is expected that the two mutations behave synergistically and cause an increase in F-actin. We anticipate that additional mutant combinations will be equally informative about the complex interplay of activities required to construct normal actin bundles, at present, formulating a model that incorporates the many different actin regulators is difficult because there is limited data of this type available.

Our results support the idea that profilin has polymerization-promoting activity, as demonstrated by previous work. Expression of vertebrate or plant profilins in mammalian tissue culture cells led to increases in F-actin (Finkel *et al.*, 1994; Rothkegel *et al.*, 1996) and profilin null clones in the developing *Drosophila* eye exhibited greatly reduced levels of F-actin (Benlali *et al.*, 2000).

However, the observation that profilin acts in an opposite manner to capping protein, seeming to stimulate actin polymerization in the fly bristle seems at first difficult to reconcile with the original characterization of the *chic* bristle phenotype. In *chic* mutants, the elongating bristle seemed to have an increased number of actin bundles that were thinner than wild-type bundles (Verheyen and Cooley, 1994). This phenotype was thought to reflect an overall increase in the amount of F-actin, which is consistent with a monomer-sequestering role for profilin. We suggest two possible explanations for this seeming paradox. First, biochemical data on profilin activity have shown that its activity is dependent on the state of the barbed ends. Profilin-actin can add to free barbed ends but not to capped ones (Pollard and Cooper, 1984; Pantaloni and Carlier, 1993; Kang *et al.*, 1999). Thus, in wild-type bristles, barbed ends may be maximally capped (except at the growing tip) and profilin's primary function would be to sequester monomer. In a *chic* mutant bristle, reduction in profilin-mediated sequestering activity might lead to the observed increase in F-actin. We would then predict that when capping protein is reduced, barbed ends are not maximally capped and thus, profilin's polymerization-promoting activity would predominate, which is consistent with our observations.

Another interpretation of the *chic* bristle phenotype is suggested by the results of inhibitor studies performed on cultured *Drosophila* pupae (Tilney *et al.*, 2000a; Guild *et al.*, 2002). Exposure of cultured pupae to cytochalasin D, an inhibitor of actin polymerization, causes the actin bundles in elongating bristles to fall apart by splitting into thinner subbundles, reminiscent of *chic* mutant bristles that exhibit an increased number of thinner bundles. The similarity of these two phenotypes suggests that continued actin polymerization is required to maintain the integrity of actin bundles, and reductions in actin polymerization cause the actin bundles to "unravel." Although it is clear that profilin can promote actin polymerization, the mechanism by which it does this is less well understood. Studies in yeast have demonstrated that profilin's nucleotide exchange activity is required for its function (Wolven *et al.*, 2000; Lu and Pollard, 2001). Because ATP-actin is more readily incorporated onto

barbed ends of filaments (Pollard, 1986), this activity can explain profilin's effects on actin assembly (Blanchoin and Pollard, 1998; Didry *et al.*, 1998). However, there is reason to believe *Drosophila* profilin may not work this way. Plant profilins do not catalyze nucleotide exchange (Perelroizen *et al.*, 1996; Eads *et al.*, 1998), and some even seem to repress it (Kovar *et al.*, 2001). A comprehensive mutational analysis of profilin in fission yeast (*Schizosaccharomyces pombe*) by Lu and Pollard (2001) has identified tyrosine79 as critical to its ability to stimulate nucleotide exchange. When tyrosine79 is mutated to arginine, *S. pombe* profilin loses its exchange activity. Notably, the majority of plant profilins naturally contain arginine at the comparable position, whereas all characterized vertebrate profilins, which tend to have very high exchange activity, contain aspartate. Thus, there is a correlation between arginine at position 79 and low activity, tyrosine and moderate activity, and aspartate and high activity. Interestingly, *Drosophila* has arginine: it is the only nonplant profilin, besides that of shrimp, known to have arginine at this position (Lu and Pollard, 2001). The exchange activity of *Drosophila* profilin is unknown, but it seems reasonable to predict that *Drosophila* profilin has low activity.

Although plant profilins do not enhance nucleotide exchange by actin monomers, some stimulate actin polymerization in vitro in thymosin-β4/actin solutions (Perelroizen *et al.*, 1996). Thymosin-β4 is a true monomer sequestering protein in that T-β4-actin cannot add to a growing filament, whereas profilin-actin adds readily to the barbed ends of actin filaments. Profilin is thought to shuttle monomer out of the T-β4 pool (Pantaloni and Carlier, 1993), and this may be the relevant mechanism in other cell types. Studies in *Drosophila* may prove useful in elucidating the details as well as the physiological relevance of alternate mechanisms of profilin activity.

In this article, as well as previous work we have demonstrated that a reduction of capping protein function leads to increased F-actin and abnormal actin organization. It is likely that the aberrant actin cytoskeleton underlies all of the defects observed in the adult bristle such as decreased length, bending, branching, and abnormal groove patterns. Although some of the correlations between the actin abnormalities and adult phenotypes are fairly obvious, it may seem counterintuitive that increases in F-actin levels would lead to shorter bristles. One might expect increased F-actin polymerization to give rise to longer bristles. Indeed, Tilney *et al.* (2000a,b) have shown that treatment of cultured pupae with jasplakinolide, a drug that stabilizes F-actin, increases the growth rate of the bristle shaft. However, their experiments were done for 6–7 h, whereas bristle elongation takes ~16 h at 25°C. Perhaps the increased growth rate would not be maintained were it possible to expose the growing bristle to drug for the entire elongation period. We hypothesize that in *cpb* mutants, actin is overpolymerized at the beginning of bristle elongation. Some component required for actin bundle assembly may be limiting in the bristle (Tilney *et al.*, 2000b); therefore, in a *cpb* mutant bristle, the limiting component would be prematurely depleted due to the increase in F-actin. Comparing the growth rates of wild-type and mutant bristles can test this idea.

Although our data demonstrate that reduction of capping protein function leads to increases in F-actin, we have not quantified these changes. It would have been desirable to measure the concentrations of F-actin in the various mutant genotypes directly, but technical limitations prevented us from doing so in a controlled manner. Phalloidin staining often varies greatly between experiments, so the subtle differences we might expect to see in different genotypes could be obscured. We are currently developing more quantitative methods for measuring actin in situ.

One of the most puzzling features of the *cpb* mutant phenotype is the displacement of actin bundles from the membrane. An increase in the amount of F-actin in the bristle does not, by itself, seem to explain this phenotype. In bristles where the cross-linking protein fascin is overexpressed, F-actin amounts are increased and bundles are considerably larger, but they do not show significant displacement from the membrane (Tilney *et al.*, 2000b). A structural function of capping protein in physically linking the bundles to the plasma membrane would explain this phenotype. Previous studies in chicken myoblasts have uncovered a structural requirement for capping protein in organizing actin filaments within the sarcomere (Schafer *et al.*, 1995). However, a structural role seems unlikely given that the displacement of bundles is suppressed when profilin dosage is reduced. Instead, the proper regulation of actin assembly may be important for the positioning of actin bundles. As noted above, *twf* mutant bristles also exhibit this displacement phenotype. Because capping protein and twinfilin are known to associate in yeast (Palmgren *et al.*, 2001), this raises the interesting possibility that these two proteins work together in regulating actin assembly such that the association of bundles with the membrane is established and/or maintained. Intriguingly, treatment of cultured pupae with okadaic acid, an inhibitor of protein phosphatases, causes a similar displacement of actin bundles (Tilney *et al.*, 2000b), suggesting the phosphorylation status of one or more proteins may be relevant.

In this article, we have shown that the balanced activities of capping protein and profilin are essential in the regulation of actin dynamics and organization in the elongating *Drosophila* bristle. Our data are consistent with the emerging idea that the activity of profilin is context dependent, and that in many cells, profilin promotes actin assembly. Our data also suggest that perturbations of actin dynamics in the bristle lead to a striking displacement of actin bundles from the membrane. In the future, we hope to clarify the role of capping protein in the bristle and better understand how it is integrated with the many other actin regulators functioning in the bristle such that actin bundles are correctly assembled and positioned.

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