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Long continuous actin bundles in *Drosophila* bristles are constructed by overlapping short filaments

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The actin bundles essential for *Drosophila* bristle elongation are hundreds of microns long and composed of cross-linked unipolar filaments. These long bundles are built from much shorter modules that graft together. Using both confocal and electron microscopy, we demonstrate that newly synthesized modules are short (1–2 μm in length); modules elongate to ~3 μm by growing over the surface of longitudinally adjacent modules to form a graft; the grafted regions are initially secured by the forked protein cross-bridge and later by the fascin cross-bridge; actin bundles are smoothed by filament addition and appear continuous and without swellings; and in the absence of grafting, dramatic alterations in cell shape occur that substitutes cell width expansion for elongation. Thus, bundle morphogenesis has several components: module formation, elongation, grafting, and bundle smoothing. These actin bundles are much like a rope or cable, made by overlapping elements that run a small fraction of the overall length, and stiffened by cross-linking.

Introduction

Looks can be deceiving. Cellular extensions are often supported by bundles of actin filaments; these bundles can be more complex than they appear. Two questions about these bundles have puzzled us for some time: What controls the length of actin bundles (because this must control the length of a cell extension)? and what controls the number of actin filaments in these bundles (because this must influence the diameter of the cell extension)? We recognize that in certain cells the length and diameter of actin bundles are rigorously controlled, such as: in ear stereocilia, in intestinal microvilli, and to a less rigorous extent, in the acrosomal process of certain invertebrate sperm, in actin filament cables in *Drosophila* nurse cells, in *Drosophila* bristles, and in the ectoplasmic specializations in Sertoli cells (for reviews see Bartles, 2000; DeRosier and Tilney, 2000). We initially thought that bundle length simply reflects actin filament length, a situation that seems justified for intestinal microvilli or even stereocilia of birds and mammals where the maximum length is 5.5 μm. This is not the case for *Drosophila* bristles, nurse cell cables, the acrosomal process of *Thyone* sperm, and the stereocilia of lizard ears (Tilney et al., 1996; Guild et al., 1997; unpublished data), where these bundles are composed of actin filaments that do not run the full length of the bundle. Thus, what controls bundle length and diameter and how individual filaments are integrated into a long continuous cable becomes a fascinating problem.

The actin bundles in *Drosophila* bristle cells represent one of the best places to study this process. Bristle cells represent the prominent visible component of the peripheral nervous system, cover much of the adult epidermis, and can be extremely long: up to 400 μm in the case of macrochaetes. These cells sprout during metamorphosis and elongate over the course of 16 h. Growth takes place at the bristle tip (Lees and Picken, 1944) and is driven by actin filament polymerization (Tilney et al., 2000b). The polarized filaments are cross-bridged into modular bundles 1–5 μm in length by at least two cross-linking proteins, forked and fascin (Tilney et al., 1998, 2000a). These modules are then assembled by the end to end joining into 7–11 stiff bundles, which run longitudinally along the bristle shaft and are attached to the plasma membrane to support the growing cell (Tilney et al., 1996). After elongation is complete, the actin bundles begin to breakdown (Overton, 1967; Guild et al., 2002) after the formation of a thickened cuticular layer, which hardens and ultimately supports the cell from the outside (Tilney et al., 1996).

Here, we used confocal and electron microscopy to examine bundle formation at the growing bristle tip and find that linear arrays of modules morph into continuous actin bundles by overlap and smoothing resulting in a stiff and continuous bundle.

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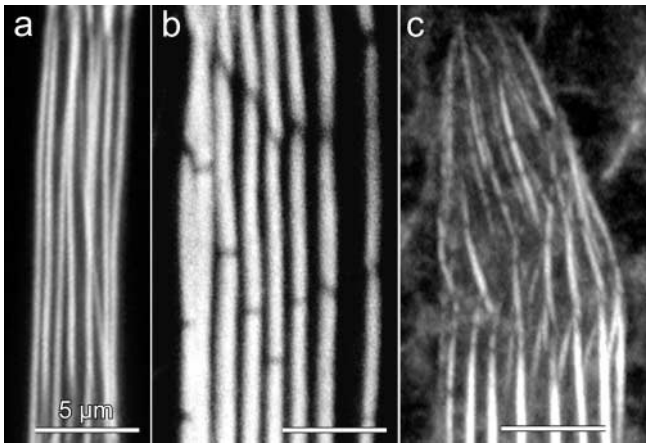

Figure 1. **Actin bundle morphogenesis during bristle development illustrated by confocal images of phalloidin-stained actin bundles.** A portion of a bristle is shown in each panel. Bristle tips are up. (a) Microchaete from of a 44-h pupa showing that the bundles are continuous with no gaps. (b) Macrochaete from a 48-h pupa bristle during an early stage in bundle disassembly. Note the gaps between modules in the bundles. (c) Macrochaete tip from a 33-h pupa showing very small modules that eventually morph into smooth bundles (bottom). Bars, 5 μm.

Results

Continuous actin bundles are assembled from and broken down into modules

If the actin bundles in bristle cells are examined after staining with fluorescently labeled phalloidin (Appel et al., 1993; Petersen et al., 1994; Tilney et al., 1996; Wulfkuhle et al., 1998) or if they are examined in living cells using GFP decoration (Guild et al., 2002), we see bundles that extend from the socket region at the base of the bristle shaft to the tip of the bristle. At higher magnification, these bundles are continuous (Fig. 1 a). However, once the bristles have fully elongated and a chitinous exoskeleton is laid down (Tilney et al., 1996), the actin bundles breakdown. During this breakdown, the bundles appear to have been "sawed" into shorter segments (Fig. 1 b). Further disassembly of the bundles occurs by the removal of subunits from the apical end of each module so that module length becomes shorter and shorter (Guild et al., 2002).

Close examination of the elongating bristle tips in young pupae showed that tiny bundles derived from microvilli (unpublished data) cluster together to form larger bundles (Tilney et al., 2000b), which when examined by confocal microscopy appeared as linear strings of short modules (Fig. 1 c, top). These newly assembled modules are considerably shorter and thinner than the modules seen in older bundles (Fig. 1 b). The gaps between the newly assembled short modules must be subsequently filled in with actin filaments because these gaps disappear as the bundle matures toward the base of the bristle (Fig. 1 c, bottom).

Adjacent modules join by overlap to form a continuous bundle

To examine bristles prepared with a minimum of manipulation, we labeled the bundles with GFP actin and examined live pupae after careful removal from the pupal case. Nevertheless, we occasionally bend a bristle. Close examination of these bends (Fig. 2) showed that the actin bundles were fractured revealing two tapered ends that before breaking had overlapped, much like horticulture grafts in which a branch of one twig overlaps another at the graft site. We presume that such a graft is the result of the joining of adjacent modules, a conclusion that is consistent with the observation that during bundle breakdown the gaps between the modules are also frequently in transverse register (Tilney et al., 1996).

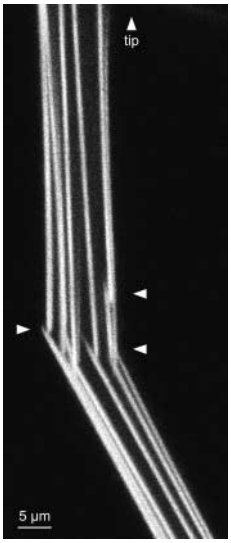

Figure 2. **Smooth actin bundles are built from modules grafted together.** Confocal image through a portion of an unfixed 46-h pupal bristle in which the bundles have been labeled with GFP actin. The bristle was bent during preparation and the actin bundles fractured (arrowheads). Of interest here is that individual bundle fracture points occur at the same place along the bristle longitudinal axis and that module overlap is disrupted at the breaks. The tip (upward arrowhead) indicates the orientation of the *Drosophila* bristle. Bar, 5 μm.

Newly synthesized modules are short and in transverse register

Scans of the fluorescence intensity along newly formed bundles (Fig. 3, a–c) quantified the longitudinal variation in actin filament numbers, with modules 1.82 ± 0.43 (SD) μm in length (Fig. 3 d) separated by short gaps. These are considerably shorter than the 2.8 ± 2.2 μm modules that appear later during bundle breakdown (Tilney et al., 1996; Guild et al., 2002). By comparing module positions in adjacent bundles (Fig. 3, b and c) we note that modules were in transverse register soon after their synthesis. The length of newly synthesized modules can vary widely in length (Fig. 3 d). Another example can be seen in Fig. 1 c, where 22 single modules measured 1.00 ± 0.29 μm.

Module elongation and grafting results in transient bundle discontinuities

Modules are elongated, grafted, and smoothed into bundles relatively quickly. We can appreciate this speed by recalling that 400-μm macrochaetes elongate at an average rate of 25 μm/hr (Tilney et al., 1996) and that the transition from modules to smooth bundles occurs within 15 μm of the bristle tip (Fig. 1 c). Thus, module formation, grafting, and bundle smoothing must take place within an ~30-min window.

We looked for evidence for module–module overlap before bundle smoothing by examining bristle tips stained with fluorescent phalloidin. By acquiring thin confocal sections we could image modules in profile that had elongated over one another before grafting (Fig. 4, a and b).

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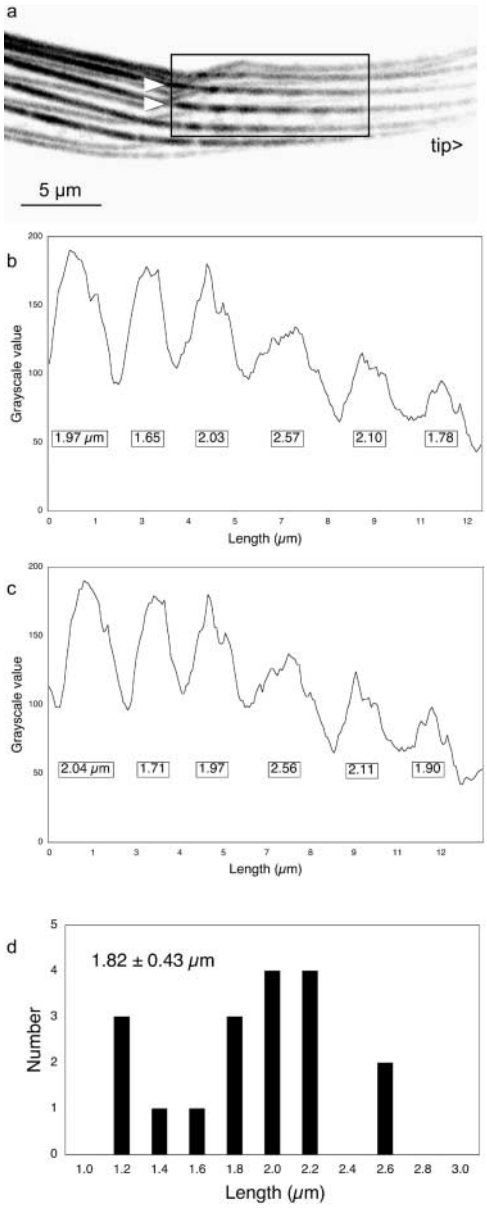

Figure 3. **Grafting modules are short and in transverse register.** (a) Confocal image of a portion of a macrochaete from a 37-h pupa labeled in vivo with GFP actin showing short modules near the tip. Four bundles forming from modules are boxed. Bar, 5 μm. (b and c) Histograms showing actin concentration along forming bundles. Two of the bundles shown in a (arrowheads) were scanned using the ImageJ application and the resulting grayscale values plotted as a function of the bundle length. In each histogram, the distance between minimum grayscale values was taken to represent the maximum length of an individual module (boxed values). Note that module lengths in a single bundle were different and that the linear arrangement of module lengths in adjacent bundles was similar indicating that the modules in the two adjacent bundles were in transverse register. (d) Length distribution histogram of all of the modules within the boxed region shown in a.

Sprouting bristles form many thin actin bundles which coalesce into thicker bundles by the action of the forked cross-linking proteins (Tilney et al., 1998, 2000a). We suspected that forked proteins might also be involved in grafting so we stained bristles with antiforked antibody to highlight potential grafting regions along the forming bundles.

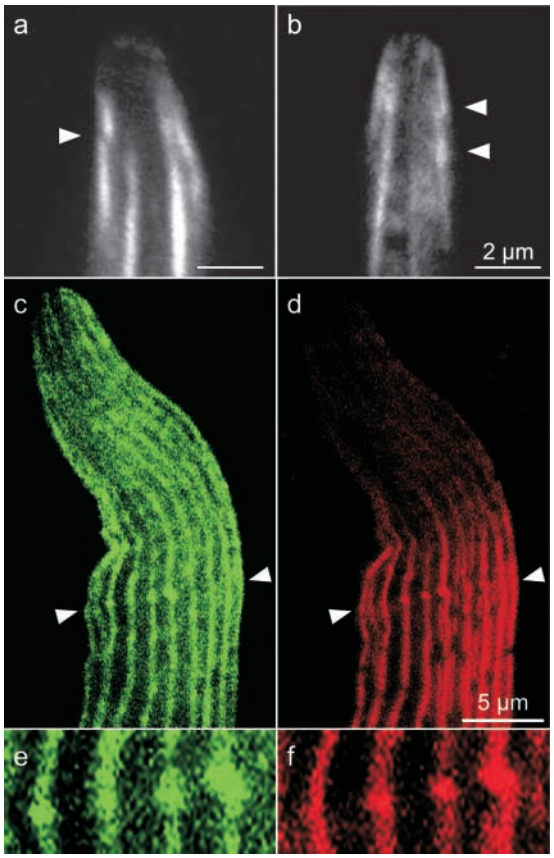

Figure 4. **Actin modules overlap during grafting.** (a and b) Confocal images of growing bristle tips from 44-h wild-type macrochaetes stained with rhodamine phalloidin showing overlapping modules (arrowheads). (c–f) Confocal micrographs of a wild-type 36-h pupal bristle stained with antiforked antibody (green) and phalloidin (red). A macrochaete showing thin actin bundles (d) containing the forked cross-bridge (c). Periodic "knuckles" representing module overlap can be seen along the bundles using the antiforked antibody. Many of these knuckles are in transverse register (e.g., arrowheads). (e and f) A higher magnification view of four actin-forked knuckles shown in c and d between the arrowheads. As expected, sprouting bristles that lack forked protein (*forked*^{36a} mutant) were not stained with the forked antibody but were stained with phalloidin (not depicted). Bars: (a and b) 2 μm; (c and d) 5 μm.

In thin optical sections, we could identify regions along single bundles that exhibited overlapping modules—regions we call knuckles—by virtue of increased forked (Fig. 4, c and e) and phalloidin (Fig. 4, d and f) staining in the overlapping regions. These knuckles were often seen in transverse register. We conclude that modules overlap after synthesis and before bundle smoothing and that this overlap, the knuckle, contains forked cross-linkers.

Fine structure analysis of the grafted region

We also examined longitudinal sections through bundles that had just completed the first few modules (Fig. 5). We believe these three serial sections illustrate a graft in which the ends of the modules are being "glued" or cross-linked together. This can be appreciated by noting that the space between bundles (Fig. 5 a, arrow) in one section is filled in the other sections (Fig. 5, b and c). The grafted region also exhibits a 12-nm striping typical of the transverse period across

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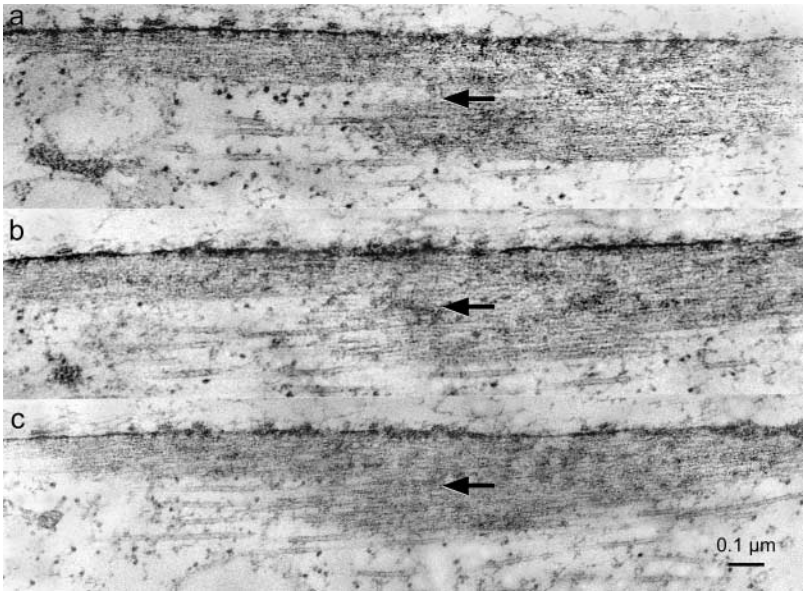

Figure 5. **Actin bundles are made smooth by the addition of actin filaments to grafting modules.** Three serial longitudinal sections through a bristle from a 35-h pupa. At this age, the bristles are very short so stages in grafting of modules should be present. In these sections, a graft can be seen and the overlap visualized (arrows). We believe that cross-linking of the filaments in the grafted region is beginning. Bar, 0.1 μm.

a bundle in longitudinal section due to fascin cross-bridging (Tilney et al., 1995) indicating that the fascin cross-bridge is ultimately present in the graft.

Additional actin polymerization smooths grafting modules

We examined actively elongating macrochaetes labeled with GFP actin by time-lapse confocal microscopy to catch smoothing in action. By concentrating on bristles with bent tips we could use module–module junctions as fiduciary marks. We found that tipward module ends show an increase in fluorescence indicating the position of module–module grafting (Fig. 6, a and b, asterisks). These fluorescent regions elongate in a baseward direction within 15 min (Fig. 6, b and c, arrows). Thus, new filament formation, or at least accumulation, seems to begin at the module knuckles and "paint" the module from the apical end toward the module below merging modules into a smooth bundle.

Module grafting results in smooth and continuous bundles

Using confocal microscopy, we carefully examined individual bundles in bristles that had achieved 50% of their mature length to look for overlapping regions where grafting occurred in unbent bristles. We consistently failed to locate such regions, rather the bundles appeared continuous with no apparent thickenings that one might expect for a graft. An example of this is shown in Fig. 7 a, showing several smooth bundles over a 50-μm length. Ultimately these bundles will reveal approximately sixteen 3-μm modules like those seen in Fig. 1 b as they undergo disassembly.

We also examined longitudinal sections of bundles by electron microscopy to identify graft sites at higher resolution (Fig. 7 b). We could not find any position along the longitudinal sections where a graft might exist. Taking these two serial sections together we saw that the bundle was the same width all along its length and gaps were not apparent. Because the average mature module length is ~3 μm (Tilney et al., 1996), this 9-μm long segment must have included at least one graft region, but we could find no evidence of this. In hindsight, this is not surprising because our thin sections are 500–700 Å in thickness, yet each filament is only 50 Å thick and adjacent cross-linked filaments are separated by 50–70 Å. Thus, when we examine a thin section through a bundle we are seeing the superposition of 3–5 filaments so that we could only recognize a graft point if there is considerable space between filaments in the overlapped zone.

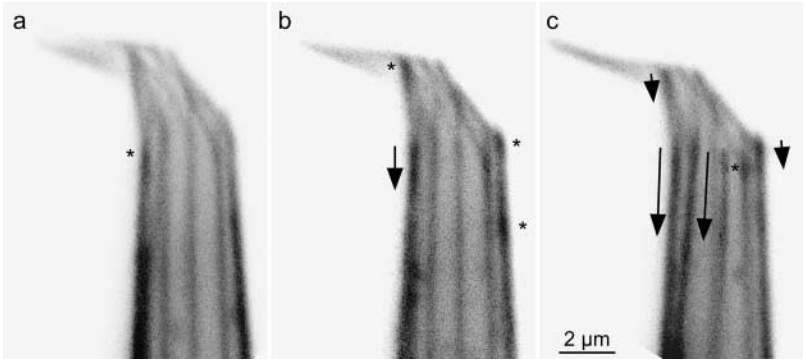

Figure 6. **Actin bundles become smooth by actin filament addition shortly after module grafting.** A bristle tip expressing GFP actin was visualized by time-lapse confocal microscopy at 15-min intervals (displayed left to right). Each image represents a 3.5-μm optical section containing five actin bundles. Images were aligned using fiduciary marks provided by several module–module junctions that were bent in this preparation. Grafting areas characterized by increased actin fluorescence are indicated by asterisks. These grafts are subsequently the site of baseward actin addition (arrows) that result in a smoother and thicker bundle. Bar, 2 μm.

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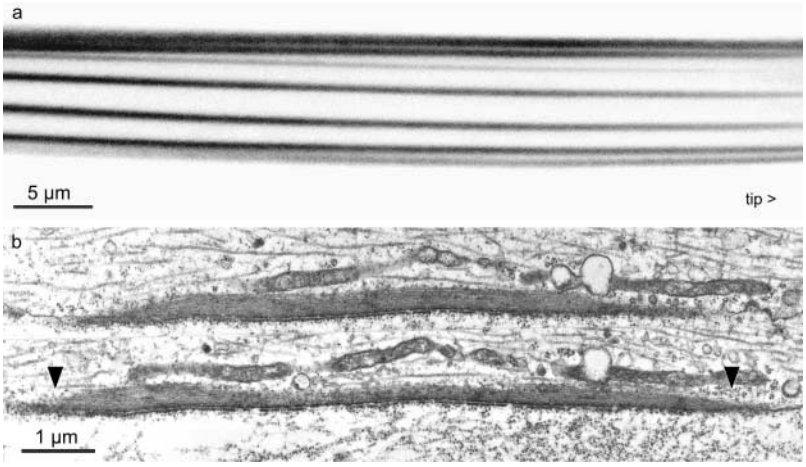

Figure 7. **Actin bundles are smooth and continuous after grafting.** (a) Confocal image (1-μm optical section) of a macrochaete from a 33-h pupa labeled in vivo with GFP actin showing smooth bundles. (b) Two serial longitudinal sections through an actin bundle from a bristle of a 41-h pupa. Of interest is that these sections, together, illustrate a 9-μm segment (arrowheads) within a single bundle, a region that should contain one or two modules. No evidence can be found of the grafted regions. Bars: (a) 5 μm; (b) 1 μm.

Module grafting occurs at any position around the circumference of a module

Because the actin bundles are attached to the plasma membrane, we tried to determine if module overlap, as revealed by fracture, was always on the same side of the bundle relative to the plasma membrane. In other words, can grafting take place at any position around the circumference of the module. Here we examined thin optical sections from fixed bristles stained with fluorescent phalloidin. We selected "center cut" sections of the bristle to be sure that there was a lateral association with the plasma membrane in the image. We examined 38 module–module fractures and found that the point of overlap is not fixed, but occurred with similar frequency on either the cytoplasmic (47%) or the membrane surface (53%) of the upper module (Fig. 8). In most cases, the overlap zone appeared as a graft of two tapered ends, not as an end to end abutment.

What is the "glue" that attaches the overlapping modules at the graft region?

We addressed this question by examining bristles lacking one or both of the cross-bridges, forked or fascin. Scanning electron micrographs of adult wild-type bristles reveal that each is fluted like Greek or Roman columns (Fig. 9 a). Previous work demonstrated that this fluted appearance is related to the distribution of the actin bundles formed earlier during pupal development and that run the length of the bristle (Overton, 1967; Appel et al., 1993; Tilney et al., 1995, 1996). Thus, examination of the surface of the adult bristle informs us of where the actin bundles were located during bristle elongation before the exoskeleton was deposited. In particular, continuous ridges are a measure of grafting over extended longitudinal distances.

In the absence of fascin (*singed* mutant), adult bristles of normal length formed but they were frequently bent or twisted (Fig. 9 b). This phenotype, although present in microchaetes was more pronounced in macrochaetes where the rate of bristle elongation is greater (both elongate over an ~16-h period but macrochaetes are typically fourfold longer than microchaetes). Unlike the wild-type, where the flutes parallel the long axis and are equally spaced around the bristle circumference, in the fascin-less mutant the fluting twists and turns. The flutes are not equally spaced nor are they equal in size (Fig. 9 b, vertical arrowheads). Besides this twisted appearance we also find, particularly in the lower half of the bristle, a honeycomb of short ridges 5–10 μm in length, most of which are connected to major longitudinal ridges (Fig. 9 b, horizontal arrowheads). Interestingly, the short grooves that interconnect to more major grooves are usually tapered at their tip where a connection is made. Overall, the ridges and valleys have a collective longitudinal orientation and continuous ridges (a measure of grafting) of over 50 μm in length were common on most bristles, especially near the tip. If we stain wild-type and *singed* pupal bristles with fluorescent phalloidin (Fig. 9, e and f) it is apparent that the fluted pattern seen in our SEM images reflects the distribution and size of the actin bundles during bristle growth. By looking directly at the actin cytoskeleton in this way it is readily apparent that significant module

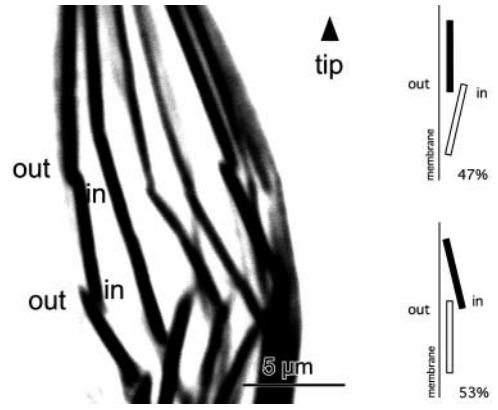

Figure 8. **There is no preferred surface for module grafting.** Confocal image through a portion of a 48-h pupal bristle stained with fluorescent phalloidin. The left-most bundle (along the membrane) exhibits two breaks. The orientation of module ends relative to one another is indicated. Bristle tip is up (arrowhead). 38 examples of module–module juxtaposition along the plasma membrane in 12 bristles were analyzed. In about half the cases (47%) a module was grafted to the inside (cytoplasmic side) of the adjacent tip-wise module (top example). Approximately half the grafting (53%) occurred on the outside (nearest the plasma membrane) of a module above it (lower example). Bar, 5 μm.

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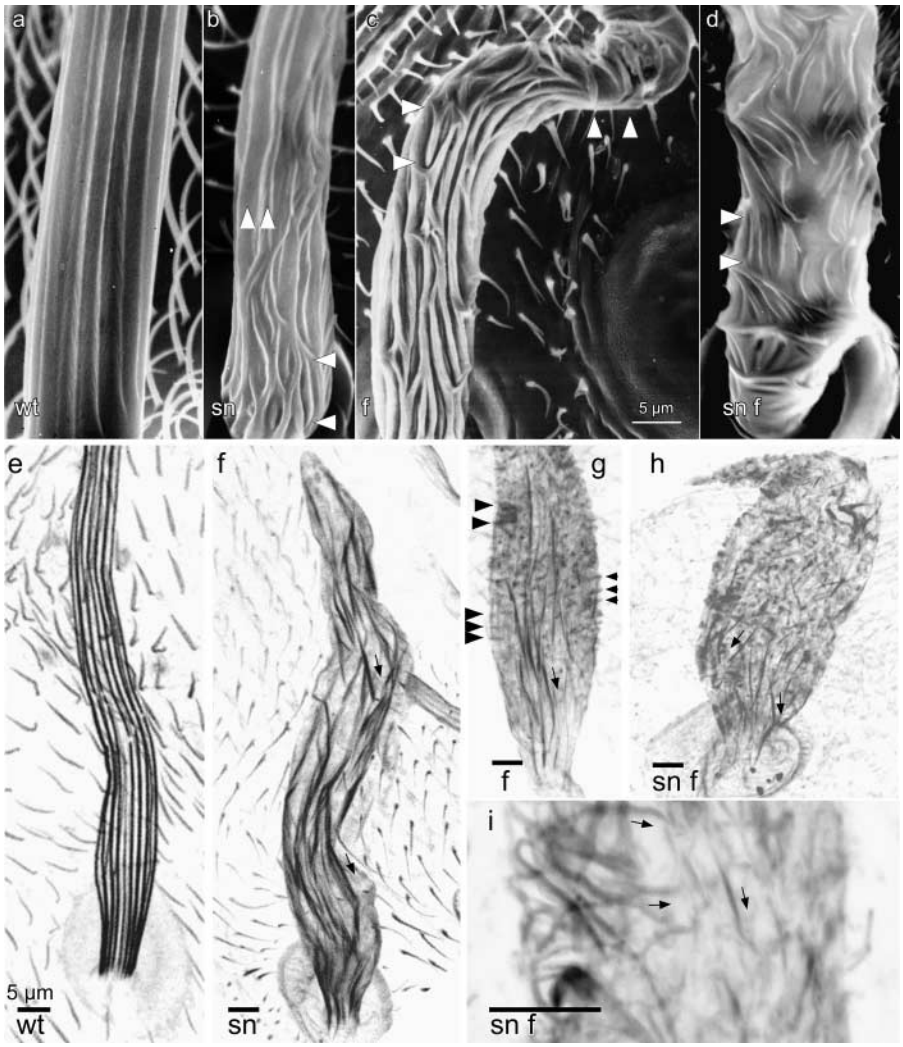

Figure 9. **Actin filament cross-linkers are required for module grafting.** (a–d) Scanning electron micrographs of wild-type and mutant adult bristles showing the cuticular morphology of ridges and valleys. Bristle bases are at the bottom of each panel. (a) Wild-type bristle showing long parallel ridges and valleys. (b) Bristle from a *singed* mutant (lacking fascin) showing twisted morphology accompanied by both very short (horizontal arrowheads) and much longer (vertical arrowheads) ridges and valleys. Note that the ends of the ridges are tapered and interconnect. (c) Bristle from a *forked* mutant (lacking forked proteins). All ridges and valleys are short (e.g., horizontal arrowheads), whereas others run perpendicular to the bristle longitudinal axis (e.g., vertical arrowheads) and the cuticular valleys rarely fuse. (d) Bristle from a *singed-forked* double mutant exhibiting only very short ridges and valleys (arrowheads) that are largely oblique to the bristle longitudinal axis. The ridges rarely connect. (e–i) Confocal micrographs of wild-type mutant pupal bristles stained with fluorescent phalloidin showing internal actin bundles. (e) Wild-type bristle showing long, parallel, and continuous actin bundles. (f) Bristle from a *singed* mutant showing a twisted morphology. Although the bundles are shorter than the wild type, they are considerably longer than single modules (3 μm) and many interconnect (arrows). Thus, much of the F actin is cross-linked into longitudinal bundles. (g) Bristle from a *forked* mutant that exhibits very limited longitudinal bundling (arrow) and far more short bundles, some running perpendicular to the bristle longitudinal axis (arrowheads). *forked* bristles are shorter and wider than either wild-type or *singed* bristles. (h) Bristle from a *singed-forked* double mutant exhibiting vast numbers of short bundles and very few organized bundles (arrows). (i) Higher magnification of another *singed-forked* bristle highlighting the very short modules that are arranged in a variety of orientations (arrows). These bundles exhibit weak fluorescence and rarely fuse. Bars, 5 μm.

grafting is taking place in the absence of fascin demonstrated by long bundles oriented parallel to the longitudinal axis of the bristle shaft (Fig. 9 f, arrows). From both the SEM and confocal images we conclude that grafting of bundles together certainly takes place in the *singed* mutant and, thus, fascin cannot be the major grafting cross-linker.

In the absence of the forked proteins (*forked* mutant), the adult bristles are on average only 50% as long as the wild type (Tilney et al., 2000b; Fei et al., 2002). Most are twisted or deformed and in some cases they exhibit forked tips. These bristles tend to be larger in diameter than the wild-type or the *singed* mutant. Some show an irregular longitudinal fluting along a portion of their length but the ridges are not equally spaced. In other regions, we see large numbers of short ridges (2–10 μm in length) that appear at all angles relative to the longitudinal axis (Fig. 9 c, arrowheads). Some of these short ridges connect to other ridges at the tapered tips and, thus, we conclude that the actin bundles can be grafted together. In most instances, we could not follow any ridges for more than 20 μm anywhere on the bristle and we saw short ridges terminate without abutting other ridges. In confocal images we saw many very short ungrafted modules that were oriented perpendicular to the longitudinal axis of the bristle shaft (Fig. 9 g, arrowheads) plus a small number of thin grafted modules (typically less than 20 μm in length) running parallel to the longitudinal axis of the bristle shaft (Fig. 9 g, arrow). We conclude that grafting in the absence of forked protein is minimal but does occur.

In the double mutant lacking both the forked proteins and fascin, we generally failed to find ridges that ran more that 10 μm anywhere on the bristle (Fig. 9 d), which is again 45% of the wild-type length. Instead we see a variety of short ridges 3–10 μm in length. Some of these were parallel to the longitudinal axis of the bristle, whereas others were perpendicular or oblique (Fig. 9 d). The ridges were seldom connected to one another but terminated freely on the surface. By confocal microscopy, the vast majority of phalloidin-stained material was in the form of short and weakly fluorescent units (Fig. 9 h). These were organized in many orientations and seemed to represent ungrafted modules

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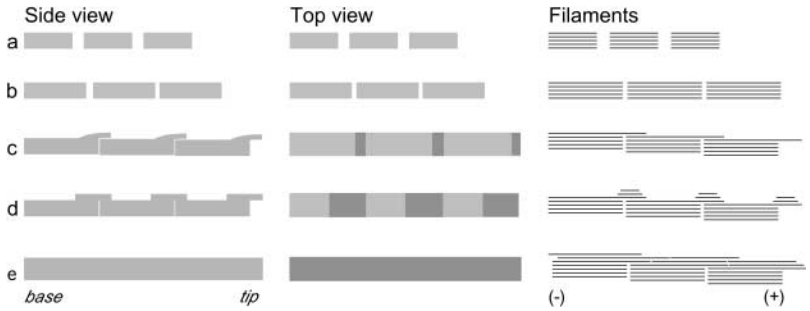

Figure 10. **Model for stages in bundle morphogenesis.** Three views of a developing bundle. The side view (left) and top view (center) represent a time-lapse view of a forming bundle. A schematic representation of actin filaments (right) shows a possible mechanism by which actin filaments accomplish bundle morphogenesis. For simplicity, consider a tandem array of three modules arranged head to tail. The filament polarity in all modules is identical with the barbed ends (+) oriented toward the bristle tip. Short modules are assembled as linear arrays (a) at the tip of a growing bristle and lengthen toward their neighbors by barbed end elongation (b). This elongation results in module overlap and subsequent cross-bridge–mediated grafting giving rise to periodic actin knuckles along the bundle (c). The modules in c–e are staggered to show this process more clearly. The regions of module overlap are extended by filament addition and elongation (d) to fill the gaps between grafts and to smooth the actin filament density along the length of the bundle (e). These events take place during a ~30-min time window.

(Fig. 9 i, arrows). There were very few thin bundles and these were located mainly at the base (Fig. 9 h, arrows). We conclude that the major cross-linking glue for grafting adjacent modules is the forked proteins. Fascin also assists to this process, albeit poorly.

Discussion

Successive modules elongate and graft into smooth bundles in a series of discrete steps

First, modules form in head to tail linear arrays at the growing tip of the bristle (Fig. 10 a). These modules are short but not fixed in length. Second, modules elongate to fill in the gaps between them (Fig. 10 b). Third, modules overlap by one extending over another (Fig. 10 c). This can be seen as a local increase in actin staining intensity (a knuckle) along a bundle from a top-down perspective or as overlapping modules seen in profile. Fourth, module overlaps seem to elongate (Fig. 10 d) resulting in a thickening of the bundle and filling in between the overlaps. Finally, bundle diameter increases with the subsequent loss of module–module distinctions (Fig. 10 e). This maturation process results in a smooth bundle with no detectable discontinuities. However, the modular components of mature bundles are still detectable in these bundles.

Small actin filaments can grow in length by end to end annealing in vitro (Murphy et al., 1988; Andrianantoandro et al., 2001). We do not think filament annealing is responsible for joining the filaments of one module with those of an adjacent one for four reasons. First, bundles fracture when bent. This occurs at discrete points along their length (Fig. 8) and the broken ends taper like the ends of twigs in a horticultural graft (Figs. 2 and 8). Second, when the actin bundles breakdown after exoskeleton formation gaps appear in the bundles separated by ~3 μm, the module length. The ends of adjacent modules are tapered where they overlap each other (Fig. 1 b). Third, cytochalasin treatment results in complete disruption of bundles into short (~3 μm) subbundles (Guild et al., 2002). These three separate sets of observations are inconsistent with filament annealing from sequential modules but consistent with overlapping and cross-bridging of filaments of sequential modules. And finally, the speckled but more-or-less uniform distribution of capβ protein, a barbed end capper, along mature bristle bundles (Hopmann et al., 1996) is inconsistent with annealing.

The forked cross-linkers are the major glue that attaches sequential modules together

A conceptual problem occurs when two modules overlap. How does the irregular surface of one bundle become adequately cross-bridged to a different irregular surface of a second, because annealing does not seem to occur? Earlier studies showed that at least two cross-bridges connect adjacent actin filaments together into bundles (Cant et al., 1994; Petersen et al., 1994; Tilney et al., 1995; Wulfkuhle et al., 1998; Grieshaber and Petersen, 1999). The forked proteins cross-link the filaments into liquid ordered bundles (Tilney et al., 1995, 1996) and facilitate the entry of fascin which results in the formation of a hexagonally packed and maximally cross-linked bundle.

We presented evidence that the forked proteins will play a major role in cross-linking adjacent modules together, albeit imperfectly, being not only present in the grafting knuckles, but from mutant analysis essential for grafting. Still there should be gaps within bundles such as is seen in our serial sections. We have no information on how cross-bridging between the two modules in a graft can be increased, but because introduction of bends in a bristle causes bundle fracture at grafted regions, we conclude that the cross-bridging between modules is no where near perfect or as good as that within the center of a module.

Module grafting may contribute to nontip bristle growth

Lees and Picken (1944) showed that bristles elongate at their tips and it is clear that module birth drives this (Tilney et al., 1996, 2000a; Turner and Adler, 1998). However, module grafting could also contribute to bundle extension and could account for the elongation of the shaft detected recently by the Adler group (Fei et al., 2002). Accordingly, there are two components of cell elongation: module birth (tip growth) and module grafting (shaft elongation). We envision that bundle elongation would begin with two newly synthesized modules and proceed by barbed end filament lengthening of the basal module followed by some filament sliding and some traction over the tipward module. Traction provided by cross-bridging would push the upper module toward the bristle tip, thus, lengthening the bundle.

We have described this combination of sliding and traction between modules before in *Drosophila* nurse cell cables (Guild et al., 1997). These actin cables are assembled from overlapping modules that extend from the plasma mem-

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brane to the nuclear envelope to form a cage around the nucleus. When the cell volume of the nurse cells is reduced during dumping, nurse cell cytoplasm flows into the oocyte. To allow this, the modules of the actin cables slide past one another like elements of an extension ladder.

Loss of grafting leads to dramatic cell shape changes

In the absence of the forked proteins, bristles elongate to only half their normal length and have an abnormally large shaft diameter exhibiting what Lees and Picken (1944) termed "inharmonious" growth. These *forked* mutant bristles exhibit very poor module grafting and contain ungrafted modules oriented almost at random relative to the long axis of the bristle. Both problems are even more pronounced when the fascin cross-linker is also removed in the *singed-forked* double mutant. This has two consequences. First, modules fail to form long continuous actin bundles. We suspect that this causes bristle shortness because newly synthesized modules fail to stand on the shoulders of older modules compromising their ability to effectively "push" growth in a tipward direction. Second, modules may still try to graft by elongating their filaments but their misorientation causes misdirected growth that expands bristle diameter.

Materials and methods

Drosophila stocks, developmental staging, and dissection

The Oregon-R strain of *Drosophila melanogaster* was used as the wild-type in these studies. The *singed* stock (*sn* ^{3}), *forked* stock (*f* ^{36a}) and *singed-forked* (*sn* ^{3} *f* ^{36a}) double mutant stock were obtained from the *Drosophila* Stock Center and maintained as viable homozygotes. A *Drosophila* line (B11-98) containing the GAL4-driver expressed in bristles and socket cells was isolated and generously supplied by John Merriam (UCLA). The B11–98 third chromosome was maintained over a TM6B, Tb balancer. A *Drosophila* line with the second chromosome containing a GFP-actin fusion under the control of a yeast UAS element (Verkhusha et al., 1999) was supplied by V. Verkhusha (Japan Science and Technology Corporation, Kyoto, Japan) and balanced over CyO. Animals expressing GFP actin in their bristles were generated by crossing these two stocks and identifying GFP-expressing non-Tb white prepupae. These animals developed normally and were viable. All observations were confirmed using GFP-actin expression under the direction of scabrous-GAL4 and/or neuralized-GAL4 drivers, both of which express significantly less GFP actin in bristles when compared with the B11–98 GAL4 driver. Flies were maintained on standard cornmeal-molasses-yeast food at 25°C, 60–70% relative humidity, and with a 12 h/12 h day/night cycle. Complete descriptions of genes and symbols can be found in Lindsley and Zimm (1992) and on FlyBase (Flybase Consortium, 2003).

Developmental staging and thoracic dissections were as described in Tilney et al. (2003).

Confocal and electron microscopy

The procedures for fixation, staining with rhodamine- or Texas red–conjugated phalloidin, and confocal microscopy were described previously (Guild et al., 2002).

Rabbit polyclonal antibody directed against the forked proteins was prepared using the 22G-*forked* cDNA clone representing the abundant 2.5-kb mRNA (Hoover et al., 1993; Petersen et al., 1994) cloned into the pET-28b expression plasmid (Novagen, Inc.). The His-tagged protein representing amino acids 12–604 was recovered from inclusion bodies, purified by Ni-NTA chromatography and SDS-PAGE, and used to immunize rabbits (Cocalico Biologicals, Inc.). The resulting antiserum was used at a dilution of 1:1,000. As expected, no forked protein was detected in elongating thoracic bristle cells in the *forked*^{36a} mutant.

Time-lapse confocal microscopy of GFP-actin–expressing cells was performed as described previously (Guild et al., 2002) except that images were collected at 15-min intervals. The confocal images of phalloidin-stained actin bundles presented here are single optical sections (~0.5 μm) or Z-projections of several optical sections that collectively cover half of the bristle diameter (4–5 μm). Pupae expressing GFP actin were dissected from their pupal cases at the indicated times and placed in a well on a microscope slide defined by a silicone rubber isolator (Molecular Probes, Inc.), covered with a drop of Halocarbon-700 oil (Halocarbon Products Corp.) and a coverslip. Animals were imaged immediately using an confocal microscope (model BX50; Olympus Fluoview) equipped with a 60× oil immersion 1.4 NA objective. GFP fluorescence was easily detected using the 488-nm line of the argon laser set at 6%, its minimum intensity. Virtually no sample bleaching was observed under these conditions. All confocal images were processed using Adobe Photoshop (Adobe Systems, Inc.) using the guidelines outlined by Murphy (2001). All images obtained from phalloidin-stained material or from GFP-labeled animals are presented as grayscale images. Some of these images (Figs. 3, 6, 8, and 9) were printed as dark signals on a white background that allows for easier visualization of small actin bundles. Pixel intensity along actin bundles was quantified using the ImageJ application (http://rsb.info.nih.gov/ij/) and plotted using Microsoft Excel. Procedures used for thin section transmission EM and scanning EM have been described previously (Tilney et al., 1998).

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