Actin filaments and microtubules play different roles during bristle elongation in *Drosophila*

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Accepted 30 January; published on WWW 7 March 2000

SUMMARY

Developing bristles in *Drosophila* pupae contain 7-11 bundles of crosslinked actin filaments and a large population of microtubules. During bristle growth the rate of cell elongation increases with bristle length. Thin section EM shows that bundle size is correlated with the amount of cytoplasm at all points along the bristle. Thus, as the bristle elongates and tapers, fewer actin filaments are used. To ensure penetration of inhibitors we isolated thoraces and cultured them in vitro; bristles elongate at rates identical to bristles growing in situ. Interestingly, inhibitors of actin filament assembly (cytochalasin D and latrunculin A) dramatically curtailed bristle elongation while a filament stabilizer (jasplakinolide) accelerated elongation. In contrast, inhibitors of microtubule dynamics (nocodazole, vinblastine, colchicine and taxol) did not affect bristle elongation. Surprisingly, the bristle microtubules are stable and do not turn over. Furthermore, the density of microtubules decreases as the bristle elongates. These two facts coupled with calculations and kinetics of elongation and the fact that the microtubules are short indicate that the microtubules are assembled early in development and then transported distally as the bristle grows. We conclude that actin assembly is crucial for bristle cell elongation and that microtubules must furnish other functions such as to provide bulk to the bristle cytoplasm as well as playing a role in vesicle transport.

Key words: Actin, *Drosophila*, Bristle, Bundle assembly, Regulation

INTRODUCTION

We are interested in determining how bundles of actin filaments are arranged in animal cells to give the cells their characteristic shapes. Although over the years we have studied many different systems (e.g. the microvilli of intestinal epithelial cells, the acrosomal processes of invertebrate sperm, the stereocilia of the vertebrate ear, and the cytoplasmic bundles in nurse cells in ovarian follicles) we have recently concentrated our attention on the sensory bristles of *Drosophila* as a model system. This is a particularly advantageous system because there currently exists a large number of mutations that affect the actin cytoskeleton and result in malformed bristles that are either too short or too long, bent or twisted, forked or lumpy, short and stiff or javelin-like, or absent altogether. Since the shaft of each bristle is composed of the cytoplasm of a single cell, genetic mutations which result in changes in bristle shape should logically involve components or modifiers of the cytoskeleton.

Thin sections through the shaft of the most abundant bristles, the microchaetes, reveal a large population of microtubules and 7-11 peripheral bundles of crosslinked actin filaments (Overton, 1967; Appel et al., 1993; Tilney et al., 1995). Not surprisingly, mutations that affect the actin filament crosslinking proteins markedly affect the morphology of the bristle. Thus, we can directly link changes in the cytoskeleton to the overall shape of the cell (Tilney et al., 1995, 1996).

Much interest has been generated in studying the effects of GTPase-mediated signal transduction pathways on the actin cytoskeleton and on remodeling the actin cytoskeleton (for recent reviews see Zigmond, 1996; Tapon and Hall, 1997; Hall, 1998). As might be expected, alterations in cdc42 (Eaton et al., 1996), Ras (Harden et al., 1995; Eaton et al., 1996), and Rho (Hacker and Perrimon, 1998; Strutt et al., 1997) affect the morphology and/or appearance of hairs and bristles in *Drosophila*. It is tempting to relate the action of these GTPases and/or the cascades they participate in to the generation and/or maintenance of the actin cytoskeleton of these epidermal extensions. However, since both microtubules and actin filaments are present in bristle cells and hairs, either or both of these components may be targets of these cascades. Further complicating this issue is the fact that there are two distinct steps in the formation of hairs and bristles. The first step is the specification of where on the cell surface the cell extension will occur (Wong and Adler, 1993; Eaton et al., 1996; Turner and Adler, 1998). The second step is cell elongation, the topic of this paper. These two steps may or may not involve the same cytoskeletal elements and/or regulating machinery.

Here we report our studies using compounds that affect actin filament and microtubule dynamics to see what each polymer contributes to bristle elongation. Our results show that actin filament assembly is essential for elongation. In contrast, microtubules are not directly involved in elongation. Surprisingly, the microtubules are highly stable and are formed...
at the start of elongation and are then moved out along the shaft as the cell elongates. We conclude that they contribute bulk to the bristle and appear to be the fly’s equivalent of the family of intermediate filaments in vertebrates. These conclusions and the culture methods we employ provide the groundwork for studying how crossovers between actin filaments are regulated and what regulates the rate of bristle elongation, which is the topic of other manuscripts.

**MATERIALS AND METHODS**

*Drosophila stocks and developmental staging*

The Oregon-R strain of *Drosophila melanogaster* was used as the wild-type in these studies. Flies were maintained on standard cornmeal-molasses-yeast food at 25°C, 60-70% RH, with a 12 hour/12 hour day/night cycle.

All animals were staged from the point of puparium formation, an easily recognizable and brief stage lasting 30 minutes at the beginning of metamorphosis (Bainbridge and Bownes, 1981). White prepupae were collected and placed on double sided scotch tape in a Petri dish that was put back into a 25°C incubator. At the appropriate time of incubation the Petri dish was removed and the pupae dissected.

**Dissection of pupae and culturing the dorsal thoracic epithelium**

After removing the pupal case we filleted the pupae as outlined in detail in Tilney et al. (1998). We carried this out under Grace’s insect cell culture medium (Gibco-BRL) equilibrated to 25°C rather than under phosphate buffered saline. The fillet which consists of the dorsal surface of the thorax was placed on its back and very delicately the large tracheoles and fat bodies were removed with fine forceps. It is important not to clean thoroughly, e.g. do not remove the muscles and all the fat bodies as it is easy to damage the underlying epidermis containing the bristles. These isolated thoracic fillets were then placed in 60 x 15 mm Petri plates with 5 ml of Grace’s medium containing inhibitor. The inhibitors were diluted into the medium from concentrated stocks made in dimethyl sulfoxide (DMSO) or methanol. Control incubations using DMSO or methanol alone (typically 0.1-0.2%) were performed in parallel. After swirling the medium in the Petri dishes to dilute the compounds, the plates were returned to the 25°C incubator for 5-7 hours. At the end of this period thoraces were removed and fixed for light or electron microscopy. The volume of medium used was just sufficient to cover the tissue allowing for good oxygen exchange.

**Sources and the concentration of the inhibitors used**

Jasplakinolide and latrunculin A were obtained from Molecular Probes Inc. (Eugene, OR) and the remaining inhibitors from Sigma Chemical Co. (St Louis, MO). Stock solutions of the reagents were made up as follows: Cytochalasin D (2 mM) was dissolved in methanol, while latrunculin A (1 mM), jasplakinolide (1 mM), taxol (1 mM), nocodazole (10 mM), vinblastine (1 mM), and demicicolchicine (10 mM) were all prepared in DMSO. Each reagent was stored as aliquots at −20°C and diluted to final concentration in culture medium just before use.

**Light and electron microscopy**

All these methods are described by Tilney et al. (1998).

**Microfilament and microtubule number and density measurements**

To measure density of microtubules or the percentage of cytoplasm occupied by actin filaments or bundles of actin filaments, we cut transverse thin sections of microchaetes. Since the bristle tapers, the diameter of the cross section through each bristle tells us approximately where the section through the bristle was cut, e.g. near the tip the diameter must be very small, at the base it must be maximal. We then traced each transverse section on mylar film and cut out the tracing and weighted it. We then cut out the region occupied by the 7-11 actin bundles in that bristle and weighed them. The ratio of area or weight occupied by the bundles relative to the cytoplasmic area or weight of the bristle in that section was calculated for transverse sections of bristle with different diameters. This was done for mature length bristles, e.g. 48 hours or bristles that were fixed at stages in their elongation phase. Since we calculated the area in each section we can then go back and by counting the total number of microtubules in that section we can determine microtubule density.

**RESULTS**

**Kinetics of elongation of microchaetes**

To measure the lengths of microchaete bristles what we did was to sacrifice pupae at known times after the initiation of pupariation, then isolate the thorax and stain the actin bundles with fluorescent phalloidin. The stained actin bundles in the bristle allowed us to measure bristle length by confocal microscopy. We had to employ this method rather than videomicroscopy as the elongating bristles at early stages cannot be detected by either phase contrast or Nomarski interference microscopy. By measuring the length of bristles on thoraces of different ages we determined the kinetics of bristle elongation (Fig. 1). For each time point we measured a minimum of 40 bristles and determined the standard error of the mean. For these measurements a single batch of prepupae collected at the same time was used as variation exists between batches of flies. Overall the rate of bristle elongation can be fitted best by an exponential curve. What is apparent is that the rate of elongation is slow at first and increases over time as the bristles increase in length and taper.

**Bristles on cultured thoraces show the same growth kinetics as bristles elongating in situ**

*Drosophila* pupae are covered by the inner pupal case, a
covering that we have been unable to remove during the period of bristle elongation. To allow the penetration of drugs, it is necessary to isolate the pupal thorax free from the rest of the pupa so that the underside of the epithelium is exposed. To do this we isolate the thorax by ‘filleting’ the pupa.

Accordingly, we isolated thoraces from the pupae of 36 hours of age and incubated them in culture medium at 25°C in Petri plates for 5 hours. During this period the rate of elongation of bristles from isolated thoraces and from bristles elongating in situ was compared. They are indistinguishable (Fig. 2).

Transverse thin section of microchaetes from animals 38 hours and 40 hours after puparium formation were used to evaluate cytoplasmic and actin bundle area. It is clear from the largest diameter sections that these represent cuts through the bristle base and that these diameters are similar to those seen in mature bristles (Table 1). What is important is the percentage of cytoplasm occupied by actin bundles is similar to that seen in mature bristles.

### Actin bundles at the bristle base form fully at early stages of elongation

Lees and Picken (1944) demonstrated not only that elongation of macrochaete bristles occurs by tip growth, but also that immediately following bristle sprouting at 33 hours the diameter of the macrochaete base increases to the adult diameter and remains constant throughout elongation. We have confirmed their observations using electron microscopy of thin transverse sections cut through the base of microchaetes. Thus, the largest bristle diameter in mature bristles (Table 1) if anything is smaller than the largest diameter seen in bristles that are in early stages of elongation, e.g. a 40 hour old pupae (Table 2). In all our studies we have concentrated on microchaetes not only because they are more numerous (200/thorax) than macrochaetes (22/thorax), but they are centrally located and thus there is a good probability of cutting thin transverse sections through them. On the other hand, the larger size of macrochaetes is useful for light microscopy so much of our information also applies to macrochaetes, although our measurements are exclusively on microchaetes.

But what is key to understanding the kinetics of bristle elongation is understanding whether the bundles at the bristle base increase in size throughout elongation or if their size reaches a maximum before elongation. Another way of phrasing this question is does a tapered bristle form like building an Egyptian pyramid where the base is laid out first and support all the subsequent layers, or do the supporting elements in the base increase in size as the pyramid grows upward? To address this we measured the area occupied by the 7-11 actin bundles in cross sections of bristle bases from mature bristles (Table 1) and from elongating bristles (Table 2). As has been shown previously (see Tilney et al., 1996) maximally crosslinked actin bundles form in three stages: (1) tiny bundles appear, (2) they aggregate into larger bundles and finally (3) the actin filaments in the bundles become maximally crosslinked and then by adding filaments increase in size to the mature size. What we need to compare then is the stage-3 bundles at the base of a tiny bristle with the bundles of a fully elongated bristle. Since the filaments within each bundle are

### Table 1. Actin bundles in 48 hour pupae

<table>
<thead>
<tr>
<th>Bristle diameter (µm)</th>
<th>Total area (µm²)</th>
<th>Area occupied by actin bundles (µm²)</th>
<th>% Bristle area occupied by actin bundles</th>
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<tr>
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<td>9.06</td>
<td>0.82</td>
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Mean (± sem) = 7.7 ± 0.6%
hexagonally packed by fascin (see Tilney et al., 1995), an arrangement that packs the maximum number of filaments in the smallest area, a reasonably accurate way to determine filament number is by measuring the space (area) occupied by the bundles in each cross section. We found that the area occupied by the bundles, and thus the total number of actin filaments, is the same at both stages (compare Tables 1 and 2). It is in essence like building a pyramid. This conclusion is depicted visually by the tracing in Fig. 3 which compares the base of a 38 hour bristle (a) with that of a mature base of 48 hours (b) at the same magnification. In both cases, a similar fraction of each cross-section is occupied by the actin bundles.

**Actin bundles occupy a constant percentage of cytoplasmic volume along the length of the bristle**

In thin sections cut perfectly normal to the bristle axis, it is possible to determine precisely how many filaments are present in each bundle by simply counting the filaments which appear as dots. One must repeat this for each of the 7-11 bundles in that section. Unfortunately the bristle extends posteriorly in an arc with continuous curvature. Thus even if the bundles on the underside of the bristle are cut perfectly transversely those on the upper surface will be slightly oblique, making an accurate count of the total number of filaments in a bristle cross section impossible. Accordingly we measured the areas occupied by the filaments relative to the total area of cytoplasm in the transverse sections, a measurement that represents the number of filaments per bundle.

In Table 1 we present our measurements of the cross-sectioned areas of 12 thin sections cut through 48 hour bristles, i.e. bristles that have reached their mature length. We measured the total cross-sectional area occupied by all the bundles in a section, and expressed this as a percentage of the total cross-sectional area of the bristle. Small cross-sectional areas (e.g. 0.51 µm²) are from sections cut near the tip of the bristle while large areas (e.g. 9.06 µm²) are from sections cut near the base. Even though there is some variation in the percentage of area occupied by bundles in different sections, this variation is not due to the location of the section near the tip or near the base. Instead sections near the tip or near the base have similar areas occupied by bundles, e.g. about 7.7% (Table 1). This means that the total number of filaments is roughly proportional to the total amount of cytoplasm at any position along the length of the bristle.

We should mention that many of our values are taken from sections cut near the bristle tip. This is because the bristle curves over the thorax. To obtain cross sections of the bristles is very labor intensive as the angle of the block relative to the knife must be continually changed. For full length bristles, e.g. from 48 hour pupae it is easiest to orient the thorax so that nearly transverse sections are cut across it. This will usually give us cross sections of the bristles near their tips as the inner pupal case overlies the surface of the pupae and compresses the bristles beneath it. Thus, most of our measurements for Table 1 are near the bristle tip. To obtain transverse sections of bristles near their base one must orient the thorax so the sections are cut nearly parallel to the surface of the thorax because the bristles at their base extend nearly perpendicular to the surface of the thorax.

Using this technique we also made measurements and calculated the space occupied by the bundles where the bristle has achieved only half its mature length, e.g. 38-40 hours. The mean area occupied by bundles relative to the cross sectional area of the bristle is 7.1±1.1% (Table 2) a value not significantly different from what we found for the fully elongated bristle (7.7±0.6%, see Table 1). To determine the relative area occupied by the bundles in earlier stages of elongation (e.g. in 34 or 36 hour pupae) is not possible as in these early stages large bundles have not yet formed, except at the base, and we cannot accurately determine this value from the 25 or so tiny bundles located at the tip of the bristle (Stage 1 in elongation).

**What is the total length of polymerized actin in a fully elongated bundle?**

We estimated the total length of actin present in a microchaete in order to compare the amount of the cytoskeleton devoted to actin vs. microtubules (see below). This is a difficult calculation to make because the bristle tapers. From actual filament counts there are a total of 560 filaments in the bundles near the tip, 1760 in bundles from the middle region (from actual counts of 4 of the 11 bundles and an extrapolation of the rest) and in excess of 4000 in the bundles at the base. By making the simplifying assumption that the mature bristle represents a right circular cone that tapers equally along its length with a base radius (r) of 2 µm and a height (h) of 70 µm (Tilney et al., 1995), we can use simple geometry to estimate its volume \( V=\frac{1}{3}\pi r^2 h \) as 293 µm³. From our cross-sectional measurements (Table 1) we also know that the actin bundles comprise ~7.7% of this volume, or 22.6 µm³. Finally, we know that maximally cross-linked filaments are packed to a density of \( 2.8\times10^4 \) filaments/µm² (e.g. there are 2042 filaments contained in 4 bundles that occupy a 7.35×10⁻² µm² area in the bristle shown in Fig. 13 of Tilney et al., 1996). Thus, we estimate that a mature microchaete with an actin bundle volume of 22.6 µm³ containing cross-linked filaments at a density of 2.8×10⁴ filaments/µm² possesses approximately 0.63 meters of F-actin. Amazingly, a 250 µm long macrochaete with an 8.8 µm base diameter
Actin drives Drosophila bristle elongation

Actin filament assembly drives bristle elongation

Cytochalasin D in low concentrations caps the barbed end of actin filaments and accordingly inhibits further elongation from the barbed end (Cooper, 1987). In the case of the bristle the barbed ends are oriented toward the bristle tip (Tilney et al., 1996). At the concentrations used here (10-20 μM) cytochalasin D also sequesters monomers. Thus if there is turnover of the filaments they will be unable to reassemble and will gradually disappear. Crosslinking of filaments as occurs in the bristle bundles may stabilize the filaments thereby slowing turnover, but even so the existing bundles should not be able to elongate because of capping.

We isolated the thoraces of 36 hour old pupae, a time when the bristles have emerged and started to elongate, and cultured them for 5 hours in the presence of either 10 or 20 μM cytochalasin D. In the presence of cytochalasin bristle elongation was dramatically slowed (Fig. 4) while the controls elongated normally.

The sponge toxin latrunculin A binds monomeric actin and thereby inhibits filament elongation (Bubb et al., 1994). Thus if filaments turn over, newly freed monomers will be bound by latrunculin and thus be incapable of reassembly and newly synthesized monomers will be incapable of assembly. When we incubated thoraces isolated from 35 hour pupae in 1 μM latrunculin A for 6 hours, bristle elongation was also dramatically slowed (Fig. 4).

Since the bristles do not elongate and no new actin filaments can assemble while in cytochalasin or latrunculin any bundles that are visible after drug treatment were presumably formed prior to treatment. In fact if we examine phalloidin stained bristles after drug treatment we see bundles. What is interesting is that the microchaetes often appear twisted and in some bristles the bundles appeared wavy (Fig. 5A). The bundles extend right to the tip of the bristle as if the bundles formed prior to treatment just abruptly stop elongating (Fig. 5A). It is also true that following cytochalasin treatment the bristles usually contain more bundles than the untreated controls as if the existing bundles have split lengthwise into subbundles. Why this occurs will be treated in a subsequent publication.

This fragmentation into subbundles was much less pronounced in latrunculin-treated bristles. We also find that cytochalasin-treated bristles, the base of the bristle in microchaetes and to a lesser extent in microchaetes seem to be composed of a jumble of short fluorescent units (Fig. 5B). These units appear to be modules that have separated from the basal end of the bundles above them. These units measured are on average 3 μm in length and sometimes appear pointed. They are randomly oriented with respect to each other. After latrunculin treatment we did not find randomly oriented actin bundles in the basal cytoplasm.

In contrast to the effects of cytochalasin or latrunculin, when

Fig. 4. Compounds that affect actin polymerization profoundly affect bristle cell elongation. For each drug, a group of same age thoraces (dissected from single cohorts of pupae 33-36 hours after puparium formation) were divided into two groups and cultured in the presence or in the absence (control) of drug for 6 or 7 hours. Bristle length was determined from confocal images of 40 phalloidin-stained microchaetes for each group. The length of the bar represents the bristle growth as a percentage of the control.

containing a similar fraction of bundled actin contains over 10 meters of F-actin!

Actin filament assembly drives bristle elongation

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Fig. 5. Cytochalasin D stops actin filament growth and promotes actin bundle breakdown. Thoraces from pupae 35 hours after puparium formation were cultured for 7 hours in the presence of 20 μM cytochalasin D, fixed, and stained with rhodamine conjugated phalloidin. Bristle cells were visualized by confocal microscopy. (A) The bundles at the macrochaete tip end abruptly in what looks like a square top (arrow). In contrast, bundles in wild-type bristles taper to a point (C). At the base of the bristles modules separate from their bristle shaft and are now present in the cytoplasm of the cell body (arrowhead). (B) A higher magnification view of the base of a macrochaete showing a large number of modules that have separated from the bundles in the bristle shaft. (C) A control macrochaete cultured in the absence of inhibitor is shown for comparison. Note that the tip of the bristle tapers as it curls under the shaft. Bars, 10 μm.

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we incubated isolated thoraces in jasplakinolide, a membrane permeant sponge toxin that, like phalloidin, stabilize any filaments that form (see Bubb et al., 1994), the bristles elongate 50-75% faster than untreated controls (see Figs 5 and 6 of Tilney et al., 2000). Of interest here is that examination of thin cross sections through bristles treated with jasplakinolide for 5 hours (36-41 hour pupae) or 2 hours (41-43 hour pupae) bundles of actin filaments are found throughout the bristle cytoplasm. More specifically, and this is documented by Tilney et al. (2000) at the tip of the bristle where elongation is occurring during incubation with jasplakinolide we see 7-11 cortical bundles, but they are much smaller than normal. Besides these bundles there are internal bundles composed of ~25 filaments. Since the rate of elongation is faster than the untreated controls the smaller bundles are sufficient to allow extension of the bristle shaft.

To summarize since cytochalasin and latrunculin, compounds that inhibit actin filament growth, slowed bristle growth and jasplakinolide, a compound that stimulates filament assembly, accelerated bristle elongation, we conclude that actin polymerization plays a major role in bristle elongation. It would be interesting to know the mature length of cytochalasin or jasplakinolide treated bristles. This is impossible as it would mean culturing thoraces in these drugs for more than 12 hours, a time when cultured thoraces without drugs are dying.

**Microtubule density in mature bristles is constant along the bristle shaft**

Apart from the 7-11 peripheral bundles of maximally crosslinked actin filaments the bristles also contain a large population of microtubules. In order to evaluate the contribution of these microtubules to bristle elongation we started by determining the total number of microtubules in transverse sections of fully elongated bristles (48 hour pupae) and at the same time measured the area occupied by the cytoplasm (Table 3). These sections were cut through the tip, mid regions and through the base of the bristle. While the total numbers of microtubules varied, we found that the overall density of microtubules was similar (54.5±5 microtubules/µm² of cytoplasm) throughout the length of the bristle except at the base where the density was slightly lower. Thus the total number of microtubules, just like the total number of actin filaments, is proportional to the amount of cytoplasm present or to the taper of the bristle.

**What is the total length of microtubules in a fully elongated bundle?**

We can estimate the total length of microtubules in a microchaete by simplifying its geometry to that of a right circular cone with a base radius of 2 µm and a height of 70 µm. Using our assessment of microtubule density we estimate that a mature microchaete with a volume of ~293 µm³ and a microtubule density of 54 microtubules/µm² of cytoplasm contains approximately 16 mm of microtubule.

**The density of microtubules changes as the bristles elongate**

We next compared the density of microtubules from comparable regions of bristles at different times during bristle elongation (34-48 hours after puparium formation). In 34 hour old pupae or in bristles that have just sprouted from the surface the microtubule density was 107±10 µm², in 36 hour old pupae the density was 160±13, in 38 hour old pupae the density was 125±23, and in 40 and 48 hour old pupae the density was 54±9 and 54±5, respectively (Table 4). Thus during the early stages of bristle elongation microtubule density increases reaching a maximum in 36 hour pupae and declines thereafter to reach the density of fully elongated bristles at 48 hours. From Fig. 1 we know that the bristles in a 40 hour pupa are half the length of fully extended bristles. Since the bristles taper and, as already shown, the diameter of the bristle base is established prior to 40 hours, this means that relatively little new tubulin is required to form microtubules after 40 hours. We calculate that there need only be 25% more polymer (microtubules) in a 48 hour vs a 40 hour pupa (see Discussion).

**Microtubules do not drive bristle elongation**

We tested four compounds that affect microtuble dynamics. First, elongating bristles were cultured in the presence of nocodozole, demicolchicine, or vinblastine. Much to our surprise as microtubules are abundant in the cytoplasm of elongating bristles, none of these agents had any effect on bristle elongation when compared to untreated controls run in parallel (Fig. 6). We found the same results using nocodozole in four separate experiments, and in two separate experiments with colchicine. One might argue that bristle cells and/or Drosophila are resistant to these drugs or are impermeable to them. This seems unlikely since these same agents block cytoplasmic streaming and transport of organelles from the

<table>
<thead>
<tr>
<th>Bristle diameter (µm)</th>
<th>Area (µm²)</th>
<th>Number</th>
<th>Number/µm²</th>
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<tbody>
<tr>
<td>0.80</td>
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<tr>
<td>1.61</td>
<td>2.07</td>
<td>138</td>
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<tr>
<td>1.70</td>
<td>2.28</td>
<td>138</td>
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<tr>
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<td>3.29</td>
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<tr>
<td>2.10</td>
<td>3.49</td>
<td>138</td>
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<tr>
<td>2.91</td>
<td>6.66</td>
<td>309</td>
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<tr>
<td>3.30</td>
<td>8.6</td>
<td>119</td>
<td>14</td>
</tr>
<tr>
<td>3.39</td>
<td>9.06</td>
<td>508</td>
<td>56</td>
</tr>
</tbody>
</table>

Mean = 54±16

Table 3. Microtubule density in mature bristles

Table 4. Microtubule density in elongating bristle cell shafts

<table>
<thead>
<tr>
<th>Pupal age (hours)</th>
<th>Number of microtubules/µm² cross section</th>
<th>Number observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>107±10</td>
<td>7</td>
</tr>
<tr>
<td>36</td>
<td>160±13</td>
<td>12</td>
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<tr>
<td>38</td>
<td>125±23</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>54±9</td>
<td>7</td>
</tr>
<tr>
<td>48</td>
<td>54±5</td>
<td>10</td>
</tr>
</tbody>
</table>

Transverse thin sections of microchaetes from animals 48 hours after puparium were used to determine the cytoplasmic area and the number (± sd) of microtubules along the length of the bristle.

Transverse thin sections of microchaetes from animals of increasing age were used to determine the number (± sd) of microtubules along the length of the bristle.
nurse cells to the oocytes during oogenesis in Drosophila (see Gutzeit, 1986; Cooley and Theurkauf, 1994). Furthermore, Turner and Adler (1998) showed that all three inhibitors, at the same concentrations used here, inhibited the initiation of hair and bristle growth in cultured Drosophila pupal wing primordia. Note that these results and our results are not in conflict because Adler and Turner were studying bristle initiation while we are studying bristle elongation.

We also studied the effect of taxol, an agent that stabilizes microtubules and inhibits disassembly. If microtubule assembly is important in regulating bristle elongation we would expect taxol to inhibit elongation by tying up all the existing tubulin in microtubules. As shown in Fig. 6 taxol does not affect bristle elongation.

**The microtubules in elongating bristles are stable**

Since bristle elongation occurs in the presence of microtubule inhibitors we asked whether the bristle microtubules break down when exposed to these drugs. Accordingly we cut thin sections of bristles treated with nocodozole, vinblastine, and colchicine. The isolated thoraces of 36 hour pupae were cultured for 5 hours in drugs and then fixed at 41 hours. In all cases (Fig. 7) the microtubules look and remain normal. What is particularly significant is that after 5 hours in nocodozole and vinblastine there was no significant reduction in the number of microtubules per µm² of a cross section as compared to the controls (compare Tables 4 and 5). For demicolchicine the average number was reduced about 40%. Since demicolchicine is the most toxic and irreversible of these microtubule inhibitors we assume that this drop in microtubule number in the presence of demicolchicine may denote toxicity. Treatment with the microtubule-stabilizing drug taxol did not cause any significant change in the number of bristle microtubules (data not shown). Overall then these inhibitors neither affect growth nor significantly influence the numbers of microtubules. Since these inhibitors have no effect on the microtubules we conclude that bristle microtubules like ciliary or flagellar microtubules are not...
turning over and thus constitute a stable rather than a dynamic population.

The microtubules in the bristles do not extend the full length of elongating bristles

From our observations that the number of microtubules present in sections cut at any point along the bristle length is proportional to the cytoplasmic area (Table 3) and that the bristle tapers, it is obvious that few microtubules could extend the entire length of the bristle. Thus most must be considerably less than 70 \( \mu \text{m} \) in length, the length of a mature microchaete. Perhaps even more revealing is data we have analysed from serial cross sections of the same bristle. These sections allow us to follow individual microtubules from one section to another. One sequence is illustrated in Fig. 8. If we examine regions at high magnification one can find individual microtubules that are present in sections 1 or 2 but absent from section 3. Bristle taper would account for these. More interesting are cases where sections cut further and further out along the bristle show an occasional microtubule appearing for the first time in one of the middle serial sections and the same tubule is present as one progresses toward the bristle tip, but absent towards the base. These images mean that there are microtubules that start midway in the bristle, not just in the basal region. The implication is that the microtubules are short.

DISCUSSION

Actin filament assembly, but not microtubule assembly, is required for bristle elongation

We show that inhibitors of actin assembly (cytochalasin and latrunculin) rapidly slow the elongation of bristles while the...
addition of jasplakinolide, which stabilizes actin filaments, promotes faster bristle growth. In contrast, agents that inhibit microtubule assembly (nocodazole, vinblastine, and demicolchicine) or stabilize microtubules (taxol) have no effect on elongation. Thus although microtubules are present in large numbers, their assembly does not contribute to bristle elongation.

In retrospect these conclusions are not surprising as in other well studied systems such as the elongation of growth cones in nerves, the expansion of lamellodopida such as occurs in cells crawling over the substrate, the elongation of the acrosomal process in invertebrate sperm and the extension of the tail of *Listeria* all require the assembly of actin filaments, but not microtubules.

**Actin filament bundles occupy a constant cytoplasmic volume along the length of the bristle**

Because the bristle smoothly tapers from its base which is 4-5 μm in diameter to its tip which is less than 0.5 μm in diameter, we wondered how closely the filament number corresponds to the taper. From our immunofluorescence studies it is clear that each bundle decreases in fluorescence from the base to the tip, but quantitatively does this decrease correlate with the amount of cytoplasm in the bristle at each point or in some other way? The most direct way to obtain such quantitative information is to count the number of filaments in a cross section at known distances from the bristle base. It turns out that this is not feasible for two reasons. First, one cannot tell how far out on a particular bristle a section is cut and second, because each bristle is curved, sections cut perfectly normal to the bundles on the upper surface of a bristle will be slightly but significantly oblique to the lower surface. Accordingly, we accumulated a number of random transverse sections through bristles. In these we measured the diameter of the bristle, the total area included within the plasma membrane of the bristle, and the area occupied by the actin bundles in that cross section. Since the actin filaments in the bundles are hexagonally packed along their length, a packing which is the closest that can be achieved and is thus a constant, we can ask whether the fraction of cytoplasm occupied by the filaments is similar in bristle cross-sections of varying diameters. This value seems constant for any bristle diameter, and thus constant along the length of a smoothly tapered bristle. What this in turn indicates is that whatever regulates filament number and thus bundle size is somehow proportional to the amount of cytoplasm present.

**Bristle elongation is independent of bundle size**

Even though actin assembly is essential for bristle elongation what is interesting is that there seems to be no minimum bundle size necessary for elongation, or to put this another way, there is no minimum number of actin filaments that must be present in individual bundles. Perhaps the most remarkable case is the *singed/forked* double-mutant in which the actin bundles consist of a monolayer of actin filaments attached to the plasma membrane yet bristles still form and in the case of the microchaetes they elongate to 40% of the wild-type length (Tilney et al., 1995). A second example comes from experiments where bristles were grown in the presence of jasplakinolide, a sponge toxin that enhances filament polymerization and stabilizes existing actin filaments. Thus once monomers of actin assemble into filaments, they remain because the filament once formed cannot depolymerize. Of significance here is that the bristle elongates faster than the wild-type even though only tiny actin bundles are formed during jasplakinolide treatment in the new elongating portion of the bristle. Thus, in the absence of crosslinkers (*singed/forked* double-mutant) or in the presence of jasplakinolide small numbers of actin filaments, perhaps even individual filaments, are fully capable of eliciting bristle elongation. Large, well-packed and fully crosslinked filaments are not essential.

Why then is it necessary to form large bundles in the first place? We believe this is to make sure that the bristles are straight, stiff, and rigid, not twisted or bent and floppy. Thus if the bundles are tiny such as in the *forked* mutant or the *singed/forked* double-mutant, the bristles are twisted or bent or irregularly curved (see Tilney et al., 1995) and we assume not as stiff as the wild type.

**The rate of bristle elongation increases as the bristle grows longer**

We learned that bristles elongate with increasing speed as they grow longer. From earlier studies (Tilney et al., 1996) we know that the actin bundles are made up of repeating units or modules, on average 3 μm in length, that are attached end to end. Since modules are the same length throughout the bristle this means that the modules must be formed faster and faster. However, the total amount of actin polymers must decrease as the bristles elongate because the number of filaments per module decreases in proportion to the bristle taper.

Further complicating the situation is that the rates of elongation of macrochaetes and microchaetes on the same area of the thorax differ. Both types of bristles emerge at the same time during development (32 hour pupae) and reach their full length at about the same time (48 hour pupae) yet the macrochaetes measure ~250 μm in length compared to the ~70 μm long microchaetes. We should also mention that each macrochaete has 16-25 actin bundles attached to the plasma membrane while each microchaete has 7-11. Furthermore, the size of the actin bundles in macrochaetes (which relates to the number of filaments per bundle) is proportionally larger than those in the microchaetes. Since the total amount of actin is greater in macrochaetes than in microchaetes it means that actin assembly is considerably faster in macrochaetes.

Perhaps increased elongation rates can be explained by thinking about module construction being accomplished by factories dumping F-actin into bundles much like concrete is poured into cylindrical molds during building construction. The increasing elongation rates observed in single cells could be accomplished by dumping concrete (F-actin) at a constant rate into smaller and smaller molds (bundles). In this case, elongation rates will be controlled by bundle size, i.e. by crosslinkers. In the same vein, macrochaetes may elongate faster than microchaetes for the simple reason that they have more F-actin factories to fill their molds. Here, elongation rates could be set by the number of actin polymerizing factories.

The bundling idea may also help to explain how short thick bristles form in some mutants, e.g. mutants like *Stubble*. In these cases, mutants producing bristles shorter than the wild-type could be pouring the normal amount of concrete into too many molds or into molds of larger than normal diameter. Thus
all the concrete is used up before the bristle elongates to a normal length. We see evidence for both of these themes (internal bundles and large bundles) in such bristles (unpublished results).

**What is the function of the microtubules during bristle elongation?**

In many systems we know that cytoplasmic streaming involving particle movements along microtubules is driven by motors such as kinesin and dynein. Although we have no information on cytoplasmic movements in bristles, it seems likely that they occur because as the bristles elongate a chitinous exoskeleton is laid down just outside the plasma membrane. Although chitin is a polysaccharide, what is frequently overlooked is that the exoskeleton is 50% protein. Within the bristle cytoplasm are numerous small vesicles approximately 0.2 μm in diameter whose interior is filled with a dense material morphologically similar to the material that makes up the developing exoskeleton. Furthermore, we have morphological evidence that suggests that these dense vesicles fuse with the plasma membrane. Presumably these vesicles are formed from the bulk of the ER and the Golgi present in the cell body and are transported out along the shaft of the bristles for exocytosis. Since these distances are large 70 or 250 μm (microchaetes and macrochaetes, respectively), something other than diffusion probably accounts for their transport.

Even so, one wonders why there is such a large population of microtubules in these bristles and why their density in the cytoplasm decreases later in development when most of the exoskeleton is being deposited. Before answering these questions what we have discovered here is that the microtubules in the bristle are not dynamic but unusually stable.

It is well recognized that the speed of conduction of the action potentials down axons is related to the diameter of the neuron as well as whether or not the neuron is myelinated. Accordingly, to achieve the greater conduction speeds in axons like the squid giant axon, the diameter of the axon is huge. Fibers such as neurofilaments, actin filaments and microtubules fill up the cytoplasm and maintain the large diameter of these neurons. This filling function can be demonstrated by applying pressure on a cut axon: out comes the cytoplasm like toothpaste out of a tube. The neurofilaments have traditionally been regarded as the major fibers used to provide bulk to this cytoplasm; the other fibrous components being responsible for cytoplasmic transport. Other members of the neurofilament/intermediate filament family also provide bulk to cells such as cells that resist abrasion like skin cells, epithelial and endothelial cells and fibroblasts.

Interestingly insect axons, unlike mollusks (squid) and vertebrate axons, do not seem to contain intermediate filaments or neurofilaments. So we began to wonder how axons and bristles maintain their large diameters. What is prominent in these cells are microtubules and, in the case of the bristle, microtubules that are not dynamic and do not turn over but are extremely stable. Accordingly, it is our contention that a major function of this large population of microtubules is to give bulk to the cytoplasm, acting as a kind of stuffing. In essence our idea is that stabilized microtubules in insects can provide the function of intermediate filaments found in other organisms.

**How do microtubules fill an elongating bristle?**

Over fifty years ago Lees and Picken (1944) compared the diameter of the basal portion of a mature macrochaete to the diameter of the bristle just after it emerged from the thorax in a 32 hour pupa. The diameters are the same. These investigators also determined using mutants that the bristles elongate by growth at the tip. From these two bits of data they showed that the taper of the bristle from the basal portion of the shaft to its tip occurs as the bristle elongates.

From our transverse sections we determined that the maximum density of microtubules (160 microtubules/μm²) are present in bristles of 36 hour pupae. Simplifying the shape of this bristle to a right circular cone with a base diameter of 4 μm and a height of 20 μm (in a 36 hour pupa) we estimate that the microchaete contains approximately 12 mm of microtubules. The microtubule density gradually decreases to only ~55 microtubules/μm² as the bristle elongates and matures in 48 hour pupae. At this stage the microchaete contains approximately 16 mm of microtubules. Thus, 36 hour microchaetes have already polymerized 75% (12 mm/16 mm) of its mature microtubule population. Furthermore, the bristle microtubules seem to be very stable. Even when cultured in the presence of compounds that prevent microtubule assembly or...
turnover, bristle cells still elongate and their microtubules and microtubule density is still reduced to the same extent we see in wild-type bristles. The easiest way to reconcile these observations with those shown by Lees and Picken (1944) is to postulate that microtubules assemble prior to 36 hours and as the bristle elongates existing microtubules, not tubulin, are translocated out the length of the bristle (Fig. 9). This idea is not novel as in the axon it is known that assembly of microtubules nucleated by γ-tubulin occurs in the cell body. A large body of evidence has now accumulated showing that during slow axonal transport microtubules are translocated from the cell body and down the axon (see a recent review by Baas, 1999, for references). Thus, outward translocation of polymerized and stable microtubules may be a cytoskeletal hallmark of polarized cells with cytoplasmic extensions.

This work was supported by a grant from the National Institutes of Health GM-52857 to L. G. Tilney and from the University of Pennsylvania Research Foundation to G. M. Guild.

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