F Actin Bundles in *Drosophila* Bristles I. Two Filament Cross-links Are Involved in Bundling

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Abstract. Transverse sections though *Drosophila* bristles reveal 7–11 nearly round, plasma membrane-associated bundles of actin filaments. These filaments are hexagonally packed and in a longitudinal section they show a 12-nm periodicity in both the 1,1 and 1,0 views. From earlier studies this periodicity is attributable to cross-links and indicates that the filaments are maximally cross-linked. *singed* mutants also have 7–11 bundles, but the bundles are smaller, flattened, and the filaments within the bundles are randomly packed (not hexagonal); no periodicity can be detected in longitudinal sections. Another mutant, *forked* (f^{36a}), also has 7–11 bundles but even though the bundles are very small, the filaments within them are hexagonally packed and display a 12-nm periodicity in longitudinal section. The *singed–forked* double mutant lacks filament bundles. Thus there are at least two species of cross-links between adjacent actin filaments. Hints of why two species of cross-links are necessary can be gleaned by studying bristle formation. Bristles sprout with only microtubules within them. A little later in development actin filaments appear. At early stages the filaments in the bundles are randomly packed. Later the filaments in the bundles become hexagonally packed and maximally cross-linked. We consider that the forked proteins may be necessary early in development to tie the filaments together in a bundle so that they can be subsequently zippered together by fascin (the *singed* gene product).

Cross-linked bundles of actin filaments are common features of eukaryotic cells. Examples include the acrosomal processes of sperm, the brush border of intestinal epithelial cells, the stereocilia of the ear, stress fiber bundles in cultured cells, and the contractile ring of dividing cells. In these and other systems some of the proteins involved have been identified, isolated, characterized, cloned, and sequenced and in certain cases their structures solved by x-ray diffraction; conserved and/or key domains have been identified, in vitro assays performed to determine function, and some successful attempts have been made to reconstruct "life-like" bundles in vitro. Less is known about how these bundles, along with the myriad of actin binding proteins, assemble, are positioned, and how their size and shape are regulated in vivo. We addressed this by examining stages in the differentiation of stereocilia in the ear (for review see Tilney et al., 1992) and others have carried out comparable studies on the formation of the brush border in intestinal epithelial cells (see Heintzelman and Mooseker, 1992). What is required is a way in vivo of selectively eliminating or reducing the amount of specific actin bundling proteins at known stages in the formation of a bundle. In short, to forward our understanding of how bundles are assembled and positioned requires a system in which a combination of molecular biological and molecular genetic approaches can be applied, yet at the same time a system in which changes in the assembly of the bundle can readily be monitored. Furthermore, it would be useful if such a system had mutants available whose actin filaments are compromised and accordingly display a recognizable phenotype. We believe a system of choice is the *Drosophila* bristle.

In the older literature two extraordinary papers on *Drosophila* bristles are available. The first, published in 1944 by Lees and Picken, describes in detail the growth of the bristle in the wild type and in a number of mutants and demonstrates that each bristle contains 8–12 parallel ridges, at least in the adult wild type, with variations in this pattern in many of the mutants. In the second paper, published in 1967 by Jane Overton, thin sections of pupal wild type and mutant bristles were carefully examined. She described within the wild-type bristle a large population of microtubules and 8–12 bundles of filaments associated with the plasma membrane. These bundles are located between the ridges described by Lees and Picken (1944). In longitudinal section each bundle displays a 12-nm period. *singed* mutants exhibit distinctly smaller and more flattened bundles that Overton (1967) correlated with the bristle phenotype.

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Although the morphology of the bundles, e.g., their filamentous nature and their periodicity, provided clues to their identity, it was not until 1993, 26 years later, that Appel et al. (1993) identified these bundles as being composed of actin filaments using fluorescent phalloidin. Thus, not only do *Drosophila* bristles have beautiful actin bundles, but numerous mutations that affect bristle morphology are currently available. We describe three informative mutations in this paper, *forked*, *singed,* and a *singed–forked* double mutant.

To our knowledge no detailed fine structural studies have been carried out on bristles since 1967. Accordingly, we have begun an examination of the actin filament bundles in *Drosophila* bristles in thin sections. This paper, which we hope will be the first in a series devoted to actin filaments in *Drosophila* bristles, is designed to provide an overview of this fantastic system as a background for further in-depth papers. Besides describing how the actin filaments are cross-linked into bundles in the wild type, we will describe the actin bundles in *singed* and *forked* single and double mutants. We chose to focus on these genes because antibodies to their gene products localize to the actin bundles (Cant et al., 1994; Petersen et al., 1994) and when either product is missing, the bundles are abnormal. Furthermore, both genes have been cloned and sequenced (Paterson and O'Hare, 1991, *singed*; Hoover et al., 1993, *forked*). The singed protein product is homologous to the actin cross-linking protein, fascin (Bryan et al., 1993), a protein first isolated by Kane (1976). The *forked* gene produces six transcripts of differing lengths. There are ankyrin-like repeats in some of the encoded proteins, but otherwise they do not resemble any actin associated proteins. With this information in hand, we will relate our observations to earlier studies on the formation of actin bundles in vivo during microvillar and stereocilia extension and suggest why two cross-links may be necessary for the formation of a bundle.

Materials and Methods

Drosophila Stocks

Wild type (Canton-S strain) and the sn^3 and sn^{X2} mutants were obtained from L. Cooley (see Cant et al., 1994 for details). The f^{36a} mutant was obtained from Nancy Petersen (see Petersen et al., 1994). The *y w* sn^3 f^{36a} stock was obtained from the Mid-Atlantic Drosophila Stock Center (Bowling Green, OH) and is referred to as the *singed–forked* double mutant in this report. The *In(1)dl49* sn^{X2} chromosome was maintained over the *In(1)Mud* chromosome. Male third instar larvae hemizygous for the *In(1)dl49* sn^{X2} chromosome were selected under a dissecting microscope (where the developing male gonad can be identified) and allowed to pupate. All other mutations were maintained as viable homozygotes. Complete descriptions of genes and symbols can be found in Lindsley and Zimm (1992) and on FlyBase (1994).

Developmental Staging of Pupae

White prepupae (0 time) were collected and placed in a petri dish containing a moist piece of filter paper and incubated at 25°C. From the earlier work of Lees and Picken (1944) wild-type fly bristles begin to appear 31 h after puparium formation. By 35 h the bristles are approximately one-fifth their adult length and by 41 h they are two-thirds of the adult length reaching their mature length by 44–48 h. By 54 h the cuticle forms and the actin bundles disappear (see Overton, 1967). Unfortunately, we cannot examine the bristles at 54 h by scanning microscopy as they are obscured by an inner cuticular layer that up to now we have been unable to remove as it adheres tightly to the pupa. Thus the ridges along the bristles can only be visualized in adult flies.

Dissection of Pupae

For examination of the mature filament bundles in wild type and mutants, the animals were killed 42–44 h after puparium formation. The pupae were placed on their backs in a dissecting dish with their ventral sides up, pinned through their abdomen to the dish, and covered with mammalian ringers. Using a pair of fine scissors and forceps the outer pupal case was removed, the posterior end of the abdomen and the anterior end of the head (the developing mouth parts and labium) snipped off to allow the fixative to penetrate, and the rest of the animal, along with the insect pin, dropped into fixative. This procedure, leaving the animal still impaled on the insect pin, proved useful as it eliminated problems of air bubbles attached to the carcass inducing the specimen to float in the fixative and at the same time facilitated subsequent identification of the dorsal and ventral surfaces. The latter is a problem at this stage as the animals consist primarily of epidermis and imaginal discs and are so soft that distortion (e.g., by folding or crunching) by handling is hard to avoid. Furthermore the eyes are not yet fully formed and are not pigmented.

Fixation and Methods for Transmission Electron Microscopy

Two types of fixatives were employed. The most successful was immersion in 2% glutaraldehyde (from an 8% stock purchased from Electron Microscope Sciences, Fort Washington, PA) in 0.05 M phosphate buffer at pH 6.8. Fixation for 4 h was begun at room temperature and then the sample put at 4°C. After 4 h the tissue was then placed in 1% OsO_4 in 0.05 phosphate buffer at pH 6.2 at 4°C for 1 h. In the second procedure, fixation by immersion was carried out for 45 min at 4°C. The fixative, which was made just before use, consisted of 1% glutaraldehyde, 1% OsO_4 in 0.05 phosphate buffer at pH 6.2. This procedure was designed for fixing actin filaments in situ. A rationale for this fixation is outlined in Tilney and Tilney (1994). After fixation by either method the specimens were washed three times in water at 4°C to remove the phosphate and en bloc stained with 0.5% uranyl acetate overnight in the dark at 4°C. The specimen was then dehydrated in acetone and flat embedded in Epon or Spurr. The pupae were oriented before embedding. After polymerization, individual pupae were oriented in the microtome so that transverse sections through the thorax could be obtained. Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with an electron microscope (Philips 200; Philips Sci., Mahwah, NJ). As it is essential to have perfect transverse and longitudinal sections through the bristles, an appendage that curves in space, it was necessary to constantly reorient the block, an extremely tedious undertaking. Photographs were taken of sections on uncoated grids.

Scanning Microscopy

Adult *Drosophila* were fixed for several hours by immersion in 70% alcohol. They were then dehydrated completely, air dried, placed upon stubs, coated with tungsten-platinum, and examined with a scanning microscope (AMR 1000; Amray Inc., Bedford, MA). This method of fixation proved ideal as it induces the bending of the back with a displacement of the wings so that the dorsal surface of the thorax and its bristles are clearly exposed. Since these are the bristles we are examining in the pupae, this method is ideal.

To determine the microchaete length we enlarge the scanning micrographs photographically and measure with a ruler. At the magnifications used each microchaete is at least 10 cm long so errors in measurements are minimal. All measurements are then translated into microns.

Results

Bristle Types

Adult *Drosophila melanogaster* have ~5,000 bristles that function as mechanoreceptors and chemoreceptors. The large bristles, macrochaetes (Fig. 1 *a*, *arrows*), develop in specific locations that have been evolutionarily conserved for 50 million years (see Sturtevant, 1970). The smaller bristles, microchaetes, are arranged in rows with remark-

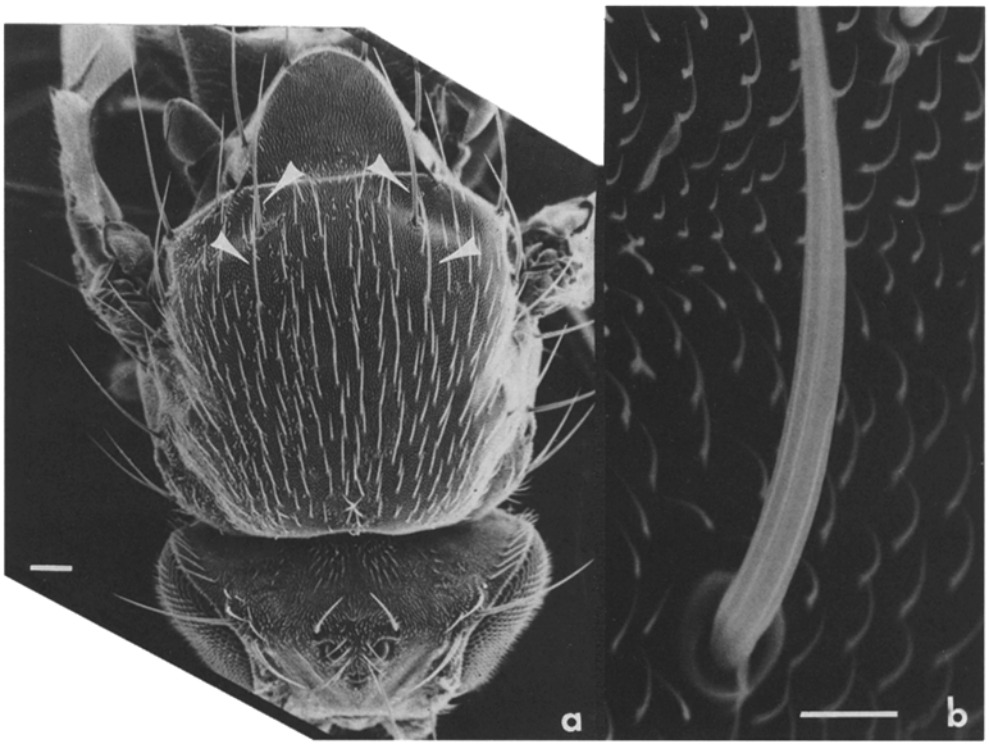

Figure 1. (*a*) Scanning electron micrograph of the thorax of an adult (wild type) *Drosophila melanogaster*. There are four macrochaete bristles (*arrows*) extending from the posterior margin of the thorax and seven at the lateral margins, most of which are difficult to trace. Covering the rest of the thorax are microchaete bristles. If thin sections are cut across the mid-anterior half of the thorax only sections of microchaetes will be found. (*b*) A microchaete bristle at higher magnification. Each microchaete extends from a socket; the axis of the microchaete is fluted containing ridges and valleys. Between microchaetes are tiny hairs, apical extensions of epidermal cells. Bars: (*a*) 100 μm; (*b*) 10 μm.

ably regular spacing (Fig. 1 *a*). Besides these bristles, the surface of *Drosophila* contains numerous trichomes or "hairs" (Fig. 1 *b*).

Although light microscopic observations on bristles are usually carried out on the large bristles or macrochaetes, for fine structural analysis these are inappropriate as they are sparse. Thus we have investigated the microchaetes. For future work it is important to stay with one bristle type because the macrochaetes and microchaetes have different numbers and sizes of actin filament bundles. This point will become important in subsequent publications where we compare wild-type and mutant bristles. Accordingly, we have concentrated on the dorsal thorax of pupae and adults because this surface contains only microchaetes and trichomes with the exception of the lateral and posterior margins; there are four macrochaetes that extend from the posterior surface (see Fig. 1 *a*, *arrows*) and seven that extend from each lateral surface. Since these macrochaetes extend directly posteriorly from their attachments on the thorax, sections are cut across the mid-anterior two-thirds of the thorax.

The microchaete extends from a circular socket (Fig. 1 *b*). The shaft of the bristle, although tapering to a point, is longitudinally grooved, and although only one side of the bristle is visible at any one time by scanning microscopy, we estimate that there must be 8–10 ridges around the circumference running from just above the socket to the tip. Invariably the shaft immediately within and just outside the socket is smooth. For completeness we should add that the macrochaetes also have sockets and grooved shafts but the number of ridges is at least double that of the microchaetes, i.e., 15–25. In contrast to the microchaetes, the hairs do not have a socket; sometimes grooves are present on their surfaces.

The Bristle Complex

Thin sections through the surface ectoderm at stages in the elongation of the bristle, e.g., 31–44 h after puparium formation, reveal that each bristle is a complex of four cells (see Lees and Waddington, 1942; Hartenstein and Posakony, 1989) (Figs. 2 and 3). These are the polyploid bristle cell or tricogen, the socket cell or tormogen that surrounds the bristle at its base, a neuron, and a glial sheath cell or thecogen (see Figs. 2 and 3). At early stages in development, e.g., 35 h, dendrites of the neuron grow upwards with an electron dense cap secreted by the glial sheath cell and become surrounded by the bristle cell like a mesaxon lying within a Schwann cell. The nuclei of the four cells lie near each other (Fig. 3).

The Bristle

The shafts of microchaetes 42–44 h after puparium formation contain mitochondria, elements of the rough ER, ribosomes, a central core of microtubules oriented parallel to the long axis, and 7–11 round bundles of filaments associated with the plasma membrane. Immediately outside the plasma membrane that undulates slightly is a clear space and peripheral to this space is the outer layer of cuticle called the cuticulin (Fig. 4).

The fiber bundles, although round in transverse section, vary in diameter in a predictable way. On one side of the bristle the bundles are distinctly larger than those on the other side giving the bristle a bilateral asymmetry (Fig. 4). The side of the bristle with the larger bundles is invariably located nearest the epidermis. The spacing between adjacent bundles around the circumference of the bristle within a single section is not uniform.

The bundles are composed of hexagonally packed fila-

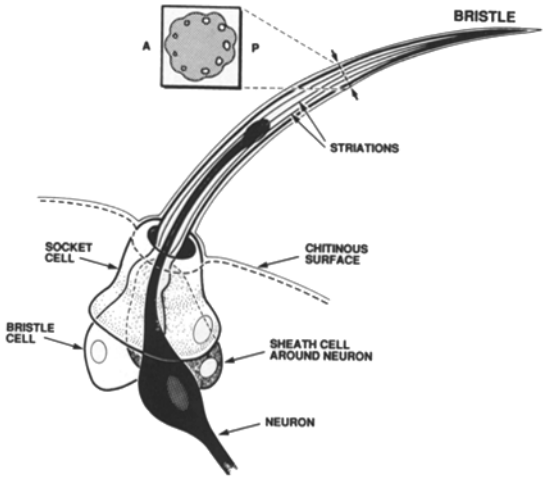

Figure 2. Drawing illustrating the four cells that make up each microchaete bristle. Most important to this report is the bristle cell. Surrounding its base is a socket cell. The dendrites of a neuron extend up into the bristle cell. The neuron is surrounded by a sheath or glial cell.

ments. This lattice is remarkably precise (Fig. 5, *c* and *d*). Only occasionally are there filaments missing in this lattice. In longitudinal section the filaments are strictly parallel to each other and in favorable sections a transverse periodicity of 12 nm is detectable (Fig. 5, *a* and *b*). This periodicity was noticed earlier by Overton (1967) and Lawrence (1966). Similar periodicities have been detected in stereocilia (Tilney and DeRosier, 1986), in microvilli of

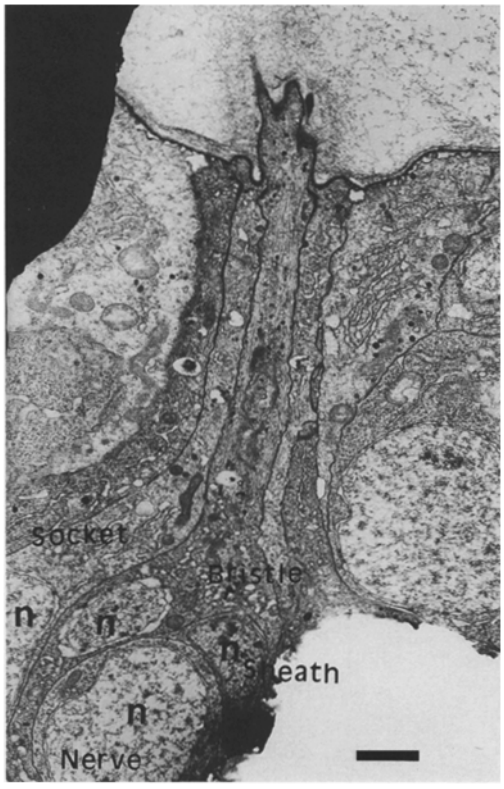

Figure 3. Thin section through the basal end of a microchaete. The nuclei of each of the four cells lie close to each other (*n*). Bar, 1 μm.

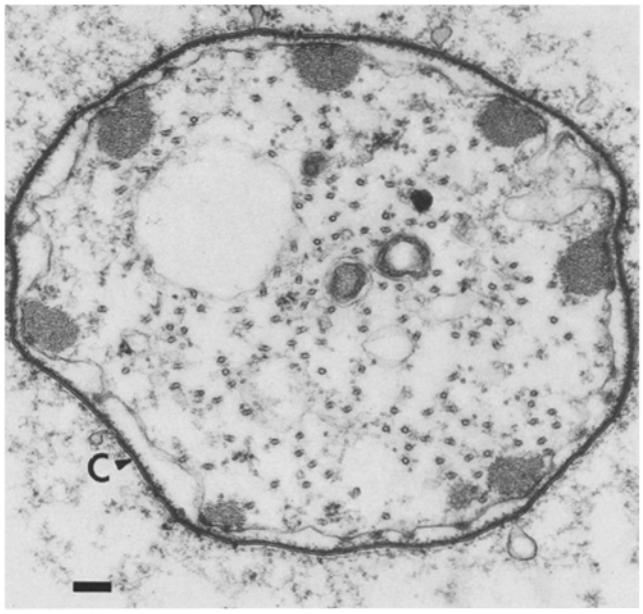

Figure 4. Transverse thin section through a bristle cell of a pupa 42 h after puparium formation. Surrounding the plasma membrane is the cuticulin layer (*C*). Attached to the plasma membrane are nine nearly spherical bundles of actin filaments (*arrows*). Of particular interest is that the bristle is bilaterally asymmetric with large actin filament bundles along one margin and smaller ones along the other. The larger bundles are always positioned nearest the surface of the thorax. Within the center of each bristle cell is a large population of microtubules. Bar, 0.1 μm.

sea urchin eggs (Spudich and Spudich, 1979), in filopodia (Edds, 1980), and in the acrosomal processes of sperm (Tilney et al., 1987) and their nature has been analyzed in detail (see DeRosier and Tilney, 1982). In short, the periodicity arises from the pattern of cross-links that bundle actin filaments together into hexagonal arrays. It is noteworthy that we see two distinct patterns in Fig. 5, *a* and *b*. These patterns form the so-called 1,0 and 1,1 views of a bundle (see Tilney et al., 1987). Since these patterns are important in understanding what happens to the actin filament bundles in the *Drosophila* mutants (described below) that are missing one or both cross-links, we have elected to include a brief summary of how these patterns arise.

Each actin filament is composed of a series of identical subunits arranged in a double helical pattern. Because of the helical nature of actin filaments, cross-linking by a given type of bivalent cross-linking protein cannot occur at random positions because the filament twists in space. To achieve maximal cross-bridging the filaments must be in a hexagonal bundle. In such the position of the cross-link is dictated by the helical symmetry of the actin. The maximum ratio of cross-links is one for every 4.3 actin subunits. Fig. 6 illustrates how maximally cross-linked filaments appear in thin sections. In Fig. 6 *a* we are looking down a hexagonally packed, maximally cross-linked bundle; in Fig. 6 *b* the same bundle is rotated by 30°. If one takes each bundle and flips it on its back, one will see the two patterns illustrated in Fig. 6, *c* and *d*. In Fig. 6 *c*, the so-called 1,1 view, one sees that the bridges (heavy lines) repeat at approximately equal intervals corresponding to the

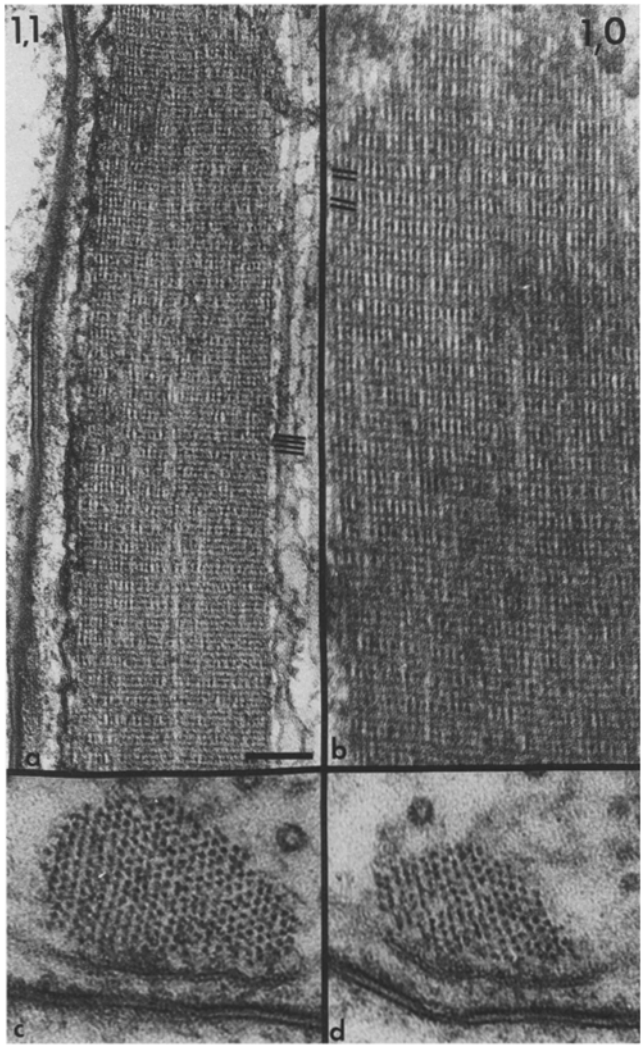

Figure 5. Thin sections through the actin filament bundles in bristle cells of thoracic microchaetes. All are at the same magnification. (*a*) Longitudinal section of a 1,1 view of an actin filament bundle. The 12-nm periodicity can easily be seen by turning the micrograph 90°. We have indicated the 12-nm periodicity with fine lines. (*b*) Longitudinal section of the 1,0 view of an actin filament bundle. One sees a pair of bridges, then a space, then a pair of bridges and a space, etc. (*fine lines*). (*c* and *d*) Cross sections through two actin filament bundles from the same bristle cell. The actin filaments are hexagonally packed. Bar, 0.1 μm.

12-nm repeat in a real biological preparation. In Fig. 6 *d*, the 1,0 view, one sees a pair of bridges and a space that repeats. In the space between the pair of bridges another bridge connecting the filaments normal to the plane of the paper also must exist. This is indicated by a black dot. If one now removes the perspective, such as one sees in a thin section through a bundle, one gets the two patterns that are illustrated in Fig. 6, *e* and *f* even though the only difference between these is that the bundle is rotated by 30°. Both views are illustrated in longitudinal sections through the actin bundles of a *Drosophila* bristle (Fig. 5) where we see the 1,1 and 1,0 views with the 12-nm periodicities predicted by this diagram. Thus the microchaete bundle is dominated by a single cross-link that is present in a stoichiometry of ~1/4.3.

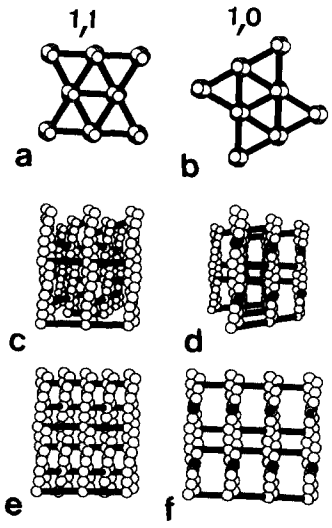

Figure 6. Drawing illustrating the two patterns of a hexagonally packed bundle of actin filaments that are maximally cross-linked by a single species of cross-link that will be encountered in longitudinal sections of actin filament bundles, the so-called 1,0 and 1,0 views (from Tilney et al., 1987).

Where the filament bundles approach the overlying plasma membrane the plasma membrane appears somewhat denser (Fig. 4). Even so, the filaments are separated from the bilayer proper by ~150–200Å (Fig. 5, *c* and *d*). This separation is constant from the tip of the bristle to immediately above the socket. At the socket the filaments remain in bundles but the bundles separate from the plasma membrane as they gradually taper at their bases. No traces of filament bundles are present in the nuclear region.

As seen most easily in scanning micrographs, the bristles taper along their lengths. Since transverse sections through bristles show varying diameters and the sizes of the bundles are proportional to the bristle diameter, we presume that the fiber bundles also taper as one moves from their greatest diameter just outside the socket to their tips. Thus fewer filaments would be present near the tip of the bundle than at its base.

The singed Bristle

We examined two alleles of *singed*, sn^3, and sn^{X2}. Both present similar phenotypes. Antibodies against the *singed* gene product or fascin show no reactivity in the bristles of either mutant (Cant et al., 1994).

The most noticeable changes by scanning microscopy are in the macrochaetes that are bent, gnarled, and twisted and considerably shorter than the wild type (Fig. 7 *a*, *arrows*). The microchaetes are also affected although the phenotype is more subtle. They often appear irregularly bent and in some cases take the form of a question mark. Furthermore, they are shorter (see Table I) and tend to lie down on the surface of the thorax as if not very stiff. At higher magnification the ridge distribution and pattern is slightly different from the wild type (Fig. 7 *b*). Although usually parallel, adjacent ridges often come together and merge or the reverse. The ridges tend to be more prominent and the valleys deeper and broader than in the wild type. Near the base of the microchaete one sees a very complex pattern of ridges and valleys.

What is immediately apparent in all our thin sections through the *singed* microchaetes is that the actin filament bundles are thinner and generally ribbon-like (Fig. 8). These bundles are still associated with the plasma mem-

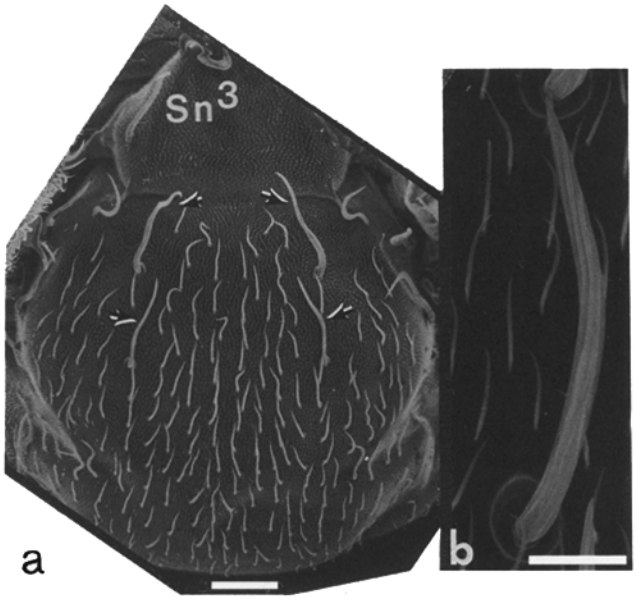

Figure 7. (*a*) Scanning electron micrograph of a thorax of a *singed* (*sn3*) mutant. (*b*) At high magnification is a microchaete. These bristle cells appear less rigid than the wild type and lie over the surface of the thorax. It is fluted with ridges and valleys but often this fluting is distorted. Bars: (*a*) 100 μm; (*b*) 10 μm.

brane. The number of bundles in cross sections through microchaetes ranges from 7 to 11, but we have found some sections in which there are only five. The bilateral asymmetry is also maintained with the larger bundles on the side of the bristle nearest the epidermis. The spacing of the bundles around the circumference of the bristle is less uniform than in the wild type so that there are often large gaps where the circumference of the bristle is devoid of bundles. Furthermore, we often see elongated bundles, albeit still associated with the plasma membrane, as if several bundles had fused together.

At higher resolution we see that adjacent filaments, although aligned parallel to each other, are randomly spaced relative to each other. In general the filaments are not hexagonally packed but are loosely associated and in longitudinal section no periodicities that could be interpreted as bridges are present. The total number of actin filaments per bundle is reduced approximately sixfold relative to the wild type.

The forked Bristle

As with *singed*, the most noticeable change in the bristles of *forked* mutants is in the macrochaetes that are very short and grotesquely malformed (Fig. 9 *a*). The microchaetes are dramatically shorter (Table I) yet, like the wild type, extend posteriorly over the thorax. In some cases their bases just outside the socket are enlarged. We have

Table I. Length of Microchaetes

	Length of microchaete	No. measured
Wild type	63.1 μm ± 1.99	40
singed mutant	56.9 μm ± 1.68	37
forked mutant	35.5 μm ± 0.63	28
singed–forked mutant	42.9 μm ± 0.71	47

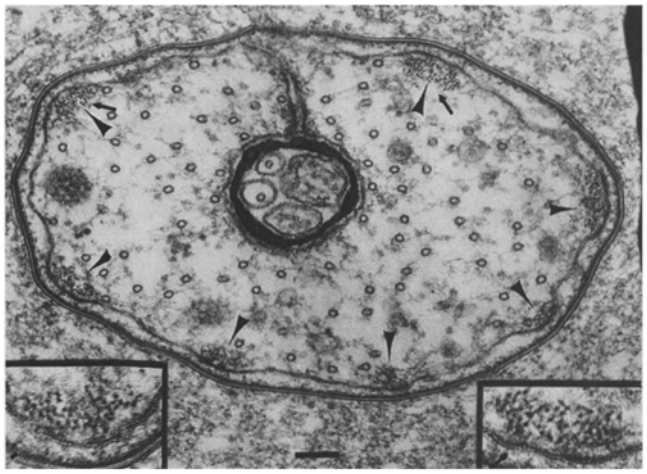

Figure 8. Thin, transverse section through a *singed* (sn^3) mutant bristle cell. Associated with the plasma membrane are bundles of actin filaments that give the bristle bilateral asymmetry with larger ones on one side. Unlike the wild type, these bundles are smaller, flatter and the filaments within them are disordered, not hexagonally packed (see insets). Within the center of the bristle are microtubules and the dendrites of the neuron that lie within the bristle cell proper like a mesaxon. Bar, 0.1 μm.

not found any that are forked. The trichomes on the other hand often appear forked, unlike the wild type or the situation in *singed*. Higher magnification of the microchaetes reveals that the surface is not perfectly smooth but, like the wild type, consists of ridges and valleys. Adjacent ridges merge and separate again as one looks towards the tip (Fig. 9 *b*).

In thin sections the filament bundles are very small containing ~1/10–1/50 the number of filaments of the wild type (Fig. 10 *a*). Often, if one does not look carefully or if the material is not adequately fixed, these bundles are not seen. Presumably this is why Petersen et al. (1994) did not resolve them in their electron micrographs or by phalloidin immunofluorescence as they would be close to the limit of detection. Nevertheless bundles are present in all our sections of microchaetes and they are located near the

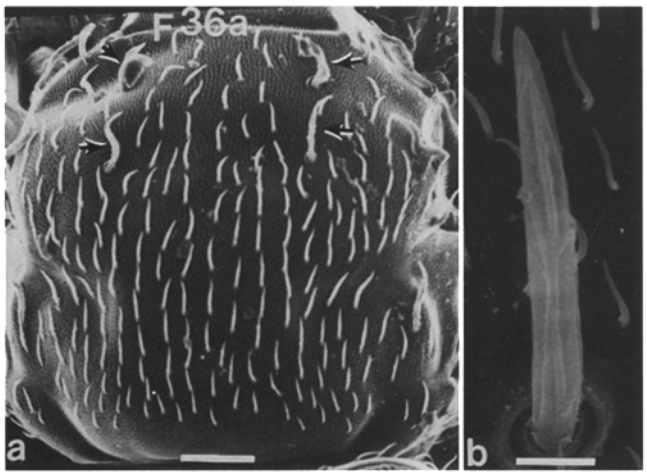

Figure 9. (*a*) Scanning electron micrograph of the thorax of a *forked* (f^{36a}) mutant. The microchaetes are shorter than the wild type and often distorted. (*b*) At higher magnification the bristle cell can be seen to be fluted. Bars: (*a*) 100 μm; (*b*) 10 μm.

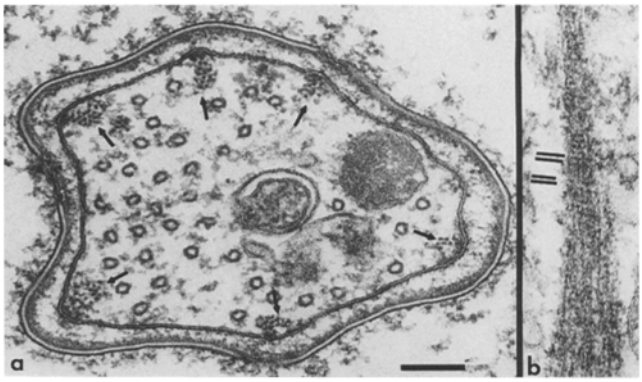

Figure 10. (*a*) Transverse section through a bristle cell of a *forked* (f^{36a}) mutant. Associated with the plasma membrane are tiny bundles of actin filaments; larger bundles are present on one side of the bristle giving it bilateral asymmetry. Even though the bundles are much smaller than normal, the filaments making up the bundles are hexagonally packed. (*b*) Longitudinal section of these tiny bundles reveals the 12-nm periodicity in the 1,1 view depicted here. Bar, 0.1 μm.

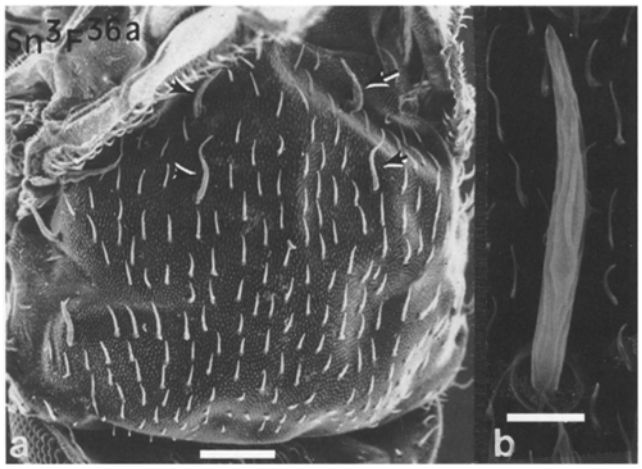

Figure 11. (*a*) Scanning electron micrograph of the thorax of a *singed* (sn^3)–*forked* (f^{36a}) double mutant. The microchaetes are shorter than those of the wild type and more distorted. (*b*) At higher magnification ridges and valleys on the surface of the microchaetes can be seen, but the pattern is irregular. Bars: (*a*) 100 μm; (*b*) 0.1 μm.

plasma membrane of the bristle. Approximately the same number of bundles (6–11) are present as is found in the wild type and they exhibit the same bilateral asymmetry in the bristles with the larger bundles nearest the epidermis. At higher resolution we see that the filaments in the bundle are hexagonally packed (Fig. 10 *a*) although sometimes, because of a flattening of the bundle, they form a rectangular shape. In longitudinal sections through the bristles we see the 12-nm period of the 1,1 and 1,0 views (Fig. 10 *b*) in several micrographs, indicating the presence of a cross-link. This is difficult as the bundles are very small. We have reproduced one here (Fig. 10 *b*); the quality is mediocre, but the 12-nm periodicity is detectable. We have tried to get better images, but because the bundles are so small, it requires an exorbitant amount of sectioning and/or luck.

The singed–forked Bristle

As with the single mutants, the double mutant macrochaetes are very short, sometimes forked, and usually badly deformed (Fig. 11 *a*). The microchaetes are shorter than the wild type (see Table I), but appear straight and stiff. They are not forked. Irregularly spaced ridges and valleys are present. As with the *singed* and *forked* mutants, these ridges merge or split (Fig. 11 *b*).

No actin filaments at all are visible in thin sections through some bristles although there are numerous microtubules. Other bristles have a linear row or monolayer of actin filaments in discrete clusters located immediately beneath the plasma membrane (Fig. 12). The number of these clusters is difficult to determine accurately as they are very small, but we estimate that there may be 5–10. The total number of actin filaments per cluster is ~10. On one occasion we found a cluster that was ~1 1/2–2 rows thick. No bundles of actin filaments such as are seen in wild type, or in *forked*, or *singed* mutants were found.

Stages in the Formation of Bristles

We have chosen to include here a brief description of the actin filament bundles of microchaetes in the early stages in bristle formation as this information relates directly to our understanding of the cross-links. More details will be presented in a future publication.

We find variation in bristle development not only on the same organism but among organisms. In one animal fixed 35 h after puparium formation we find tiny microchaete bristles. In thin sections of these we see numerous microtubules, but surprisingly no actin filament bundles or for that matter any actin filaments at all. Thus tiny bristles can elongate from the epidermal surface without actin fila-

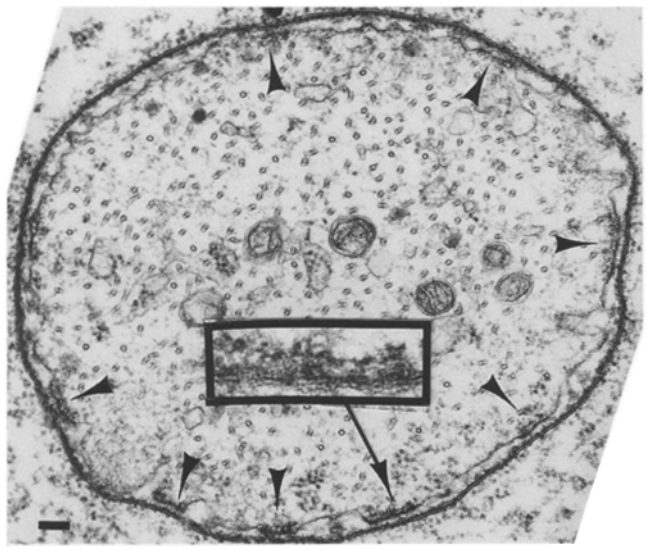

Figure 12. Transverse section of a bristle cell of a *singed* (sn^3)–*forked* (f^{36a}) double mutant. At first glance one does not see any actin filament bundles associated with the plasma membrane, but careful examination shows that there are tiny patches of actin filaments associated with the limiting membrane (see *arrows*). At higher magnification these patches appear to form a monolayer of actin filaments tightly associated with the plasma membrane. Bar, 0.1 μm.

ments, presumably by using the microtubules. In the same organism we also find tiny microchaetes that contain bundles of actin filaments generally located near the limiting membrane and in clusters. There is no pattern to the clusters, e.g., they do not appear in discrete bundles as described for older pupae with long bristles. In other animals fixed at the same time we find bristles that appear a little older. These have 7–11 discrete bundles of actin filaments. The actin filaments have small hexagonally packed regions but most of the filaments are randomly oriented relative to each other (Fig. 13).

Discussion

Two Cross-links Are Present in the Bundle

The *singed* gene product is homologous in sequence to the actin filament cross-link, fascin (Bryan et al., 1993). Furthermore, recombinant *Drosophila* fascin will cross-link actin filaments in vitro and produce bundles that show the 12-nm periodicity (Cant et al., 1994), and finally, antibodies to *Drosophila* fascin (Cant et al., 1994) stain the wild-type bundles. Since the actin filaments in the *singed* mutant appear as bundles, a situation where no fascin is produced (Cant et al., 1994), it means that there must be at least one more actin cross-link. A likely candidate is the forked product because antibody staining shows that the forked protein is present in wild-type bundles (Petersen et al., 1994) and that bundles do not form in the *forked* and *singed* double mutant. Unfortunately experiments have not yet been carried out to see if actin filaments are cross-linked in vitro by recombinant forked protein. Without this key experiment we have only mutant analysis (presented here) and, at the light microscope level, immunological localization (Petersen et al., 1994) to suggest that the second cross-link is a forked protein. By morphological, transcriptional, and immunological criteria (Hoover et al., 1993; Petersen et al., 1994) the f^{36a} allele examined here exhibits a null phenotype. Although several forked isoforms encode a cluster of five ankyrin-like repeats, we suspect that forked protein may not function to localize actin bundles to the bristle cell plasma membrane. First, in the absence of forked protein small actin bundles comparable in number and position to those in the wild type are seen in cross sections of microchaetes. Second, complete rescue of the forked phenotype in f^{36a} mutants can be achieved by transgenic expression of forked proteins that do not contain the ankyrin-like repeats (Petersen et al., 1994).

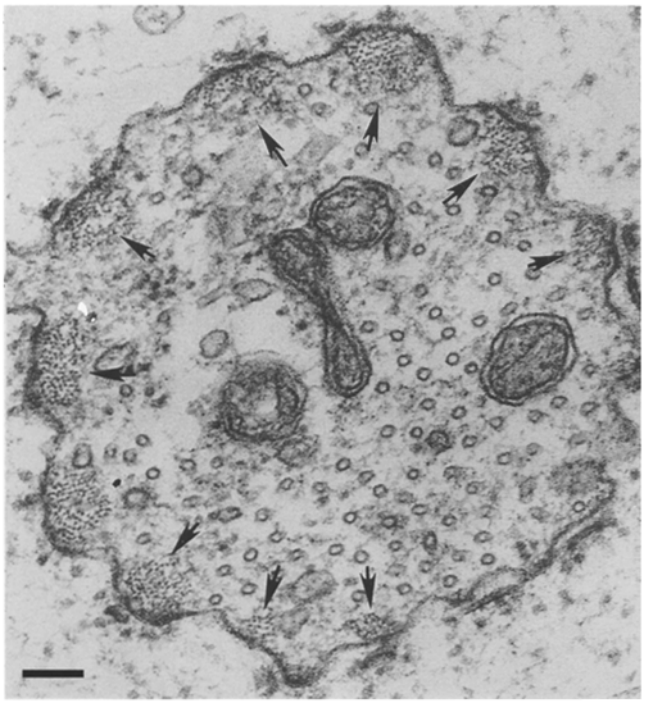

Figure 13. Thin transverse section through a developing bristle in the wild-type pupa 36 h after puparium formation. Associated with the plasma membrane are bundles of actin filaments. Of interest is that the filaments within the bundles are not well ordered. There are, however, patches of hexagonally packed filaments in these bundles. Bar, 0.1 μm.

The Phenotype of singed, forked, and the singed–forked Double Mutant

Since we have such distinctive changes in the actin filament bundles in the *singed*, *forked,* and double mutants, we expected to find the bristle morphology to be severely affected. Surprisingly, this is not the case. The microchaetes of the mutants are shorter than the wild type and often wavy in profile, but neither effect is extreme. Even the ridges are still present on these mutant bristles although they are often poorly spaced and join and separate from one another. This is puzzling as we assumed that the actin filament bundles would play a major cytoskeletal role in bristle formation. This assumption is based on the studies by Lees and Picken (1944) and Overton (1967) who described, in the *singed* and *forked* mutants, severely distorted macrochaetes that have a similar ultrastructure to the microchaetes even though they are 4–6 times longer and contain twice as many actin filament bundles. Thus to produce an obvious phenotype as assayed by light microscopy, the bristle must have to be very long and contain an abundant number of actin bundles.

The lack of a pronounced microchaete phenotype in the mutants makes us suspect that other cytoskeletal elements, most likely microtubules, must be primarily responsible for the microchaete cell shape, although both play an essential role in the larger macrochaetes. Thus to collect more mutants for further studies on genes that affect actin filaments bundles and their attachment to the plasma membrane will require the analysis of macrochaetes. What is particularly encouraging is that bristle phenotype is also sensitive to the overproduction of the forked proteins (Petersen et al., 1994). This fact may prove extremely useful in identifying new gain-of-function mutations in genes that affect not only actin bundling, actin-membrane associations, and pattern determination, but also the relation of actin bundles to other cytoskeletal elements that contribute to the shape of the bristle.

What Can Be Learned from the singed–forked Double Mutant?

This is a particularly revealing mutant for three reasons. First, it shows that the gene products of *forked* and *singed*

are both necessary to form a bundle of actin filaments. We have no idea whether an additional species of cross-link or other molecules such as tropomyosin may be required. Second, although actin filament bundles do not form, there is a monolayer or patch of actin filaments associated with specific sites on the plasma membrane. Since the number of these sites is in the normal range of 6–10, there seem to be specific regions on the plasma membrane containing proteins that interact directly or via intermediate molecules with actin filaments. Since we now know some of the cross-linking macromolecules and, in the case of fascin (the singed protein), precisely where it is located in the bundle, we can begin to explore with other mutants how the actin bundle is connected to the plasma membrane. The double mutant also shows that only the outer row of the actin bundle is connected to the plasma membrane and this pattern exists in the absence of cross-links between filaments. Furthermore, our scanning micrographs show that the pleated nature of the bristle is maintained. If the ridges and valleys are indeed related to an underlying actin cytoskeleton, then only those filaments attached to the plasma membrane should contribute to this grooved pattern. Now we can ask the question: if all the actin filaments are eliminated, would we still see a linear pattern of ridges and valleys by scanning microscopy? If not, what gives order to the limiting membrane and the cuticle of the bristle cell? Third, and this point is reinforced by looking at bristle initiation in pupae, it is not the actin bundles that account for the elongated shape of the bristle cell. Instead it is likely that the microtubules specify cell shape as the bristle sprouts without actin filaments and bristle elongation occurs with only tiny ribbons of actin filaments as in this double mutant.

How, by Elimination of a Cross-link, Is the Number of Actin Filaments Reduced?

In both the *singed* and the *forked* mutants the number of filaments per bundle is 10–50 times less than in the wild type. Why should a cross-link mutant affect filament number? This constitutes a fascinating puzzle that should be addressed in the future. At the same time it makes us consider the more general question of how the appropriate stoichiometry of cross-links to F-actin is regulated so that essentially no excess of any of these components is found in the adult bristle. Are there transcriptional controls, translational controls, or controls regulated by turnover? Is this regulation related to the disappearance of the actin bundles in pupae older than 54 h (Overton, 1967) when their bristles become reinforced extracellularly by chitin? Clearly this is an area that should be examined.

Which Cross-link Induces Hexagonal Packing of the Filaments?

In the *singed* mutants where fascin is not expressed (Cant et al., 1994) the filaments, although appearing in clusters, are not precisely packed but instead are separated by varying distances. Furthermore, longitudinal sections show no 12-nm periodicity. In contrast, in *forked* mutants where fascin is expressed but the forked protein is not (Petersen et al., 1994), the packing of adjacent actin filaments in the bundles is hexagonal and in longitudinal sections a 12-nm periodicity is observed. Thus fascin is responsible for the hexagonal packing and the 12-nm cross-link pattern. Since in the wild type we find hexagonal packing and the 12-nm periodicity attributable to the fascin cross-link, we would like to determine the function of the forked product and its stoichiometry relative to fascin.

At Least Two Types of Cross-linking Macromolecules Are Commonly Present in Actin Bundles

Three cases come to mind. In microvilli of intestinal epithelial cells two actin cross-bridging molecules have been described that bundle actin: villin (Matsudaira and Burgess, 1982) and fimbrin (Bretscher and Weber, 1980; Bretscher, 1981; Glenney et al., 1981). In stereocilia of the ear two cross-bridging proteins must also be present. One of these is fimbrin. The other is unknown but it must exist because when detergent extracted stereocilia are incubated in high salt, all the fimbrin is removed but the filaments remain attached together (Tilney et al., 1989). The third case is the one described here, the *Drosophila* bristle. Although there are other actin filament bundles found in nature, they have not been characterized in enough detail to determine if there is only one cross-link present or, if there are at least two as in stereocilia, whether the second is in low copy number relative to the first.

Why Are There Two Species of Cross-links in Actin Bundles?

There are three possibilities: (*a*) redundancy with overlap of function; (*b*) different functions for each cross-link; and (*c*) the necessity for two different kinds of cross-link in the de novo formation of the bundle. A number of examples for apparent redundancy of actin binding proteins have been proposed because deletion of one or more actin binding protein genes fails to present a recognizable phenotype (Wessels et al., 1991). The lack of a recognizable phenotype might be dependent upon an appropriate assay that might be very difficult to determine. Thus in times of stress or at different stages in the cell cycle or developmentally one might see a phenotype that is invisible under routine observation. An example to illustrate this point was recently described by Doberstein et al. (1993) for *Acanthamoeba*. Cells lacking a specific myosin I showed no phenotype unless the *Acanthamoeba* was placed, not in culture medium, but in distilled water at which time the cells lysed. This is, in fact, similar to the natural environment for this amoeba and it shows that those cells that lack myosin I have a malfunctioning contractile vacuole.

For the second possibility there is evidence in vitro of slightly different functions for different cross-links. For example, in microvilli, villin is a calcium-induced actin filament depolymerizing protein (Matsudaira and Burgess, 1982) as well as a cross-link. Fimbrin also cross-links actin and is calcium sensitive but it does not lead to the rapid depolymerization of actin filaments. Thus in the brush border both cross-links bundle actin, but they appear to react differently to calcium. In stereocilia we know that acoustic trauma (Tilney et al., 1982) induces a loss in the hexagonal packing and the 12-nm periodicity, yet the remaining actin filaments stay bundled, reminiscent to the situation when fimbrin is removed in detergent-high salt-

extracted hair cells (Tilney et al., 1989). This indicates that the cross-links are differentially labile and accordingly may play different roles.

The third alternative seems the most likely. A stage-specific requirement for formation of bundles with different actin cross-linking proteins has been described in all three cases. In intestinal epithelial cells in chick embryos the microvilli first sprout from the surface with an extremely disordered core filament bundle (Chambers and Grey, 1979). Then the filaments are zippered together to form a compact bundle. A similar sequence occurs in the formation of stereociliary bundles in the chick ear. Stereocilia sprout on embryonic day 8 with disordered actin filament bundles in which no 12-nm periodicities are seen. Several days later the actin filaments within each stereocilium become hexagonally packed and reveal a 12-nm periodicity in longitudinal section (Tilney and DeRosier, 1986). This two-step actin bundle assembly also occurs in developing *Drosophila* bristles where actin filaments first form at random and then become hexagonally packed and the 12-nm period appears. Our idea is that one cross-link is required early to bring filaments together. The second cross-link then zippers these filaments together into a hexagonally packed bundle with a 12-nm period. There is evidence for the sequential appearance of cross-links during the formation of microvilli. Villin, one of the cross-links, appears at day 8, but fimbrin, the other cross-link that in vitro forms well-ordered bundles, is not present until a day or so later (Ezzell et al., 1989; Shibajama et al., 1987). In stereocilia, fimbrin appears after sprouting (unpublished observations) as if this is the second cross-link to appear. In bristle formation there is evidence that the forked protein is most abundant during the earliest stages in actin bundle formation, thereafter its amount relative to actin and fascin decreases (Petersen, N. S. 1994. *J. Mol. Biol. Cell.* 5:273a). Thus a general protocol for the formation of a well-ordered, maximally packed bundle involves the sequential use of two actin cross-links; one to hold adjacent filaments together during the initial stages of elongation, and the second to zipper together the filaments into a hexagonal bundle with a 12-nm period.

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