Drosophila Singed, a Fascin Homolog, Is Required for Actin Bundle Formation during Oogenesis and Bristle Extension

Kelly Cant, Brenda A. Knowles, Mark S. Mooseker,* and Lynn Cooley

Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510; and *Departments of Biology, Cell Biology and Pathology, Yale University, New Haven, Connecticut 06511

Abstract. *Drosophila singed* mutants were named for their gnarled bristle phenotype but severe alleles are also female sterile. Recently, singed protein was shown to have 35% peptide identity with echinoderm fascin. Fascin is found in actin filament bundles in microvilli of sea urchin eggs and in filopodial extensions in coelomocytes. We show that *Drosophila* singed is required for actin filament bundle formation in the cytoplasm of nurse cells during oogenesis; in severe mutants, the absence of cytoplasmic actin filament bundles allows nurse cell nuclei to lodge in ring canals and block nurse cell cytoplasm transport. Singed is also required for organized actin filament bundle formation in the cellular extension that forms a bristle; in severe mutants, the small disorganized actin filament bundles lack structural integrity and allow bristles to bend and branch during extension. Singed protein is also expressed in migratory cells of the developing egg chamber and in the socket cell of the developing bristle, but no defect is observed in these cells in *singed* mutants. Purified, bacterially expressed singed protein bundles actin filaments in vitro with the same stoichiometry reported for purified sea urchin fascin. Singed-saturated actin bundles have a molar ratio of singed/actin of approximately 1:4.3 and a transverse cross-banding pattern of 12 nm seen using electron microscopy. Our results suggest that singed protein is required for actin filament bundle formation and is a *Drosophila* homolog of echinoderm fascin.

THE gnarled, kinky bristle phenotype of X-linked *singed* mutants was first described by Mohr (1922), and *singed* mutants have been used extensively since then in *Drosophila* research to provide visible genetic markers. Depending on the allele, *singed* bristles vary from short and gnarled to wavy and bent. In severe mutants, large bristles (macrochaetes), small bristles (microchaetes), and hairs on the head, thorax, legs, and wings are all affected to varying degrees (Fig. 1, *A* and *B*). Bristles are formed during pupation when the trichogen cell sends out a shaft of cytoplasm with a cytoskeletal core comprised of central microtubules and 8–12 fibrous bundles dispersed peripherally at the plasma membrane (Overton, 1967). The fibrous bundles consist of actin filaments (Appel et al., 1993). The morphology of the bristle appears to reflect the organization and integrity of the cytoskeletal core present at the time of cuticle deposition during bristle development. Aberrant bristle morphology has been correlated with defects in the organization and composition of actin filament bundles in *singed* bristles (Overton, 1967). Electron microscopy analysis of gnarled *singed*^{3} bristles showed that the microtubule component of the developing shaft appeared normal; however, the actin filament bundles appeared very small, flat and ribbon-like rather than round and columnar. This decrease in size of the actin filament bundles suggested that *singed* mutants may have a defect in actin organization, such as in actin bundle formation.

The severity of the bristle defect generally correlates with the *singed* female sterile phenotype such that *singed* mutants with gnarled bristles are also female sterile. Severe *singed* females are sterile due to a defect in oogenesis (Bender, 1960; Gutzeit and Strauß, 1989). In *Drosophila*, oogenesis is divided into 14 stages and begins with a single germline stem cell daughter that undergoes four mitotic divisions to yield a 16 cell cluster (for oogenesis review see King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993). This 16 cell germline-derived cluster is surrounded by somatic follicle cells to form an egg chamber. Since cytokinesis in the germline divisions is incomplete, the 16 cells are interconnected by a series of cytoplasmic bridges called ring canals. One of the 16 cells becomes the oocyte while the 15 remaining cells differentiate into nurse cells. Nurse cells become highly polyploid and function to supply cytoplasm to the oocyte through the ring canals throughout oogenesis. Nurse cell cytoplasm transport into the oocyte occurs in two phases. During the early stages of oogenesis, lasting 2–3 d, nurse cell cytoplasm flows slowly into the oocyte. In late stage 10, the rapid phase of cytoplasm transport begins. During stage 11, final nurse cell cytoplasm transport takes place

Address all correspondence to L. Cooley, Department of Genetics, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.

resulting in a doubling of the oocyte volume in about 30 min and in the regression of the nurse cell cluster. Drug studies have shown that the rapid phase of cytoplasm transport is actin dependent (Gutzeit, 1986).

In sterile *singed* mutants, oogenesis becomes defective at the onset of rapid cytoplasm transport (Gutzeit and Strauß, 1989). Egg chambers of sterile *singed* mutants appear normal prior to rapid cytoplasm transport but subsequently, the nurse cells fail to regress leading to the formation of small eggs (Fig. 1, *C* and *D*). The follicle cells, however, continue their developmental program and synthesize all the components of the egg shell (Bender, 1960; Gutzeit and Strauß, 1989). The follicle cell-derived structures appear affected in sterile *singed* mutants; respiratory appendages are often flattened and fused and the operculum forms at almost a right angle to the long axis of the egg. These defects are likely to be secondary consequences of the failure of nurse cell regression (Gutzeit and Strauß, 1989).

We became interested in *singed* mutants because the *singed* oogenesis phenotype is very similar to the phenotype of *chickadee* mutants (Cooley et al., 1992). *chickadee* encodes *Drosophila* profilin and is required for the formation of nurse cell cytoplasmic actin filament bundles. In the absence of cytoplasmic actin filament bundles, nurse cell nuclei become lodged in ring canals and thus block the flow of nurse cell cytoplasm into the oocyte. This implies a structural role for cytoplasmic actin filament bundles in maintaining the position of the nurse cell nuclei as the nurse cells presumably contract (Cooley et al., 1992). Subcortical actin-containing filaments, which are normal in *chickadee* mutants, probably provide the force for nurse cell contraction that squeezes cytoplasm into the oocyte (Gutzeit, 1986). In addition to similarities in oogenesis phenotype, severe *chickadee* mutants also have a bristle defect (Verheyen and Cooley, 1994).

The reduction in actin filament bundles in *singed* bristles and the failure of nurse cell cytoplasm transport in egg chambers of sterile *singed* females both suggest that the singed protein may interact with the actin-based cytoskeleton. The cloning of the *singed* gene revealed an open reading frame of 1,536 nucleotides encoding a 57-kD protein, but the protein function remained elusive (Paterson and O'Hare, 1991). Recently, the gene encoding echinoderm fascin was cloned and found to have 35% peptide identity and 67% peptide similarity with the *singed* gene (Bryan et al., 1993). Sea urchin fascin has been extensively characterized as a 58-kD protein that bundles actin filaments into hexagonally packed, linear arrays with a characteristic transverse cross-banding

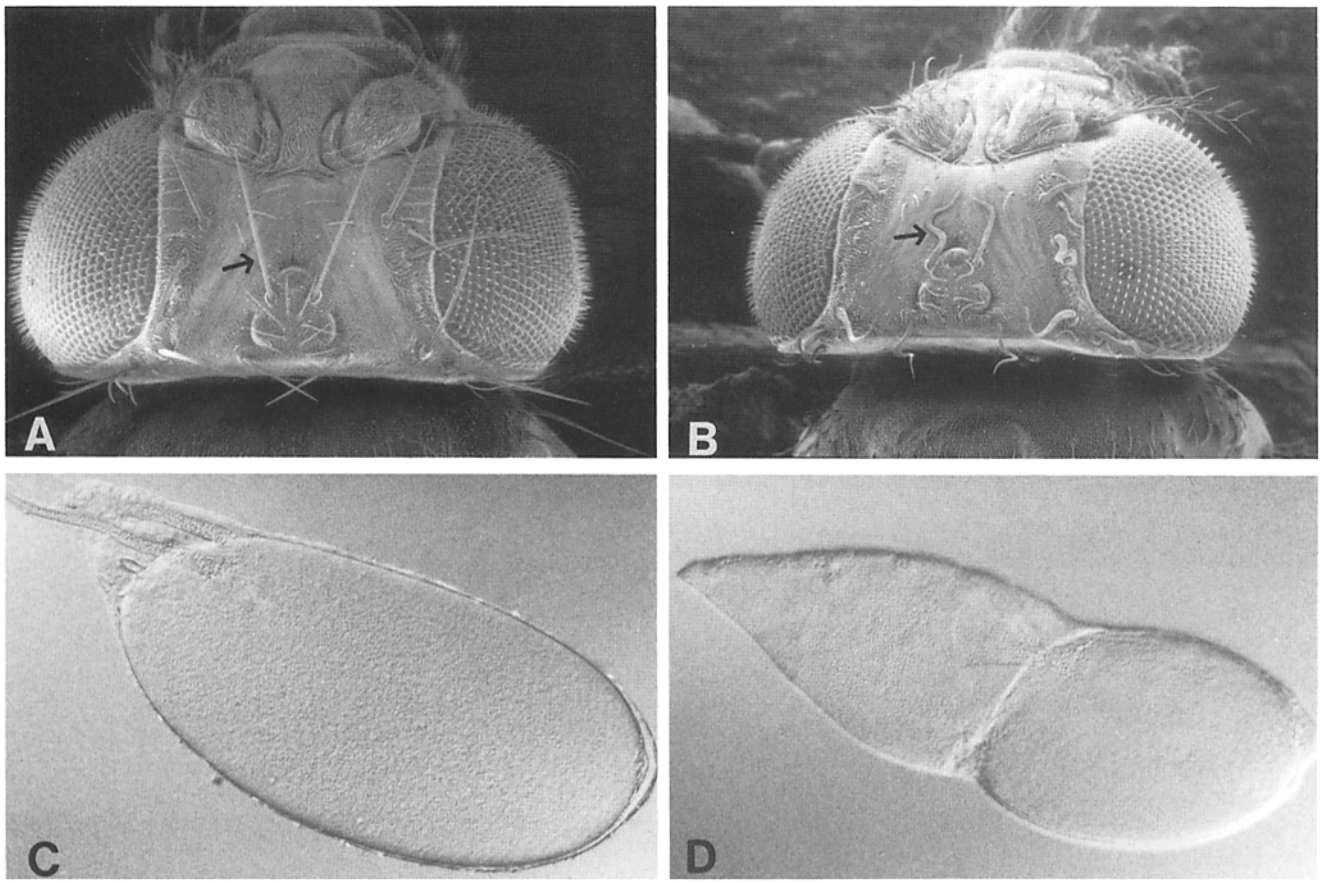

Figure 1. Severe *singed*^{X2} mutants have defective bristle development and oogenesis. Scanning electron micrographs are shown of heads from wild type (*A*) and *singed*^{X2} flies (*B*). Wild type bristles are long and straight (A, *arrow*) while *singed* bristles are bent and curved with a spiral-like or branched appearance (B, *arrow*). Mature eggs from wild type (*C*) and sterile *singed*^{X2} females (*D*) differ in size. Dorsal appendages and egg shell chorion have formed on both eggs yet the *singed*^{X2} egg appears half the size of the wild type egg and the nurse cells fail to regress.

pattern of about 12-nm periodicity (Bryan and Kane, 1978; DeRosier and Censullo, 1981; DeRosier et al., 1977; Kane, 1976; Spudich and Amos, 1979). Fascin has been implicated in the organization of actin filaments in numerous structures. In sea urchin, fascin is found in actin filament bundles in egg microvilli upon fertilization and in filopodial extensions formed on coelomocytes in a hypotonic environment (Otto and Bryan, 1980; Otto et al., 1979, 1980). Fascin has also been localized to the actin bundles of starfish oocyte spikes and sperm acrosomes (Maekawa et al., 1982; Otto and Schroeder, 1984). Given the sequence homology between the *singed* and the *fascin* genes, we have sought to characterize the *singed* phenotypes and to determine whether the biochemical characteristics of singed resemble those of fascin. In this paper, we show that singed protein is required for the formation of actin filament bundles in nurse cells and developing bristles, and we describe the actin bundling properties of bacterially expressed singed protein. Our results suggest that *Drosophila* singed protein is functionally homologous to echinoderm fascin.

Materials and Methods

Drosophila Stocks

All fly stocks were maintained under standard culturing conditions. Four spontaneous alleles were studied (Table I): $singed^{2}$, $singed^{3}$, $singed^{4}$, and $singed^{36a}$. $singed^{X2}$ was induced by x-ray mutagenesis. Some of the sites of mutation for these alleles have been described (Paterson and O'Hare, 1991; Roiha et al., 1988). Transcription of *singed* in $singed^{3}$ uses a cryptic promoter and starts 200-bp closer to the *olfE* promoter; the developmental profile of the cryptic promoter allows adequate expression during oogenesis but not bristle formation (Paterson and O'Hare, 1991). $cn;ry^{506}$ flies were used as the wild type control.

Antibody Production

The *singed* open reading frame of 1,536 bp was cloned in-frame adjacent to the carboxy-terminal segment of *Schistosoma japonicum* glutathione S-transferase (GST)[1] in the expression vector pGEX-2T (Amrad Corp., Melbourne, Victoria, Australia; Smith and Johnson, 1988). This construct allows isopropyl-1-thio-β-galactopyranoside (IPTG) induction of a GST-singed fusion protein in *Escherichia coli*. Fusion protein expression was induced with 1 mM IPTG for 4 h at 37°C. Due to the highly insoluble nature of this fusion protein, it was isolated from inclusion bodies, electrophoresed on preparative 8% SDS-polyacrylamide gels, and identified by 0.3 M $CuSO_4$ stain. The fusion protein band was excised and the protein extracted by incubating the gel slice overnight in elution buffer (50 mM Tris, pH 8, 0.1 mM EDTA, 0.2 mM NaCl, 0.1% SDS, 5 mM DTT). Purified protein was used to immunize mice. Antisera were screened for reactivity with the fusion protein by Western immunoblot and for immunoreactivity to *Drosophila* ovaries by western immunoblot and immunofluorescence.

An immunoreactive mouse was used for hybridoma cell line production. Monoclonal cell lines were screened by ELISA, Western immunoblot, and immunofluorescence. Purification of IgG was carried out using HiTrap Protein G (Pharmacia Fine Chemicals, Piscataway, NJ).

Western Immunoblotting

Drosophila ovary extracts were obtained by grinding ovaries in Laemmli sample buffer and boiling for 5 min. Protein concentration was determined using Bio-Rad's (Hercules, CA) modified Bradford assay. Extracts were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Towbin et al., 1979). Following the transfer, the nitrocellulose membrane was blocked with Blotto (5% powdered milk and 0.1% Tween 20 in PBS) for 2 h at room temperature. The membrane was then incubated overnight with monoclonal antibody supernatant diluted 1:10 in Blotto. After washing the membrane for 1 h in PBS-Tween 20 (0.1%), the membrane was incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (31434; Pierce, Rockford, IL) at a 1/10,000 dilution in Blotto for 1 h at room temperature. After four 15-min washes with PBS-Tween (0.1%), the signals were detected using ECL Western immunoblot detection reagents (Amersham Corp., Arlington Heights, IL) following the instructions of the manufacturer. Scanning densitometry was used to quantitate the signal intensity.

1. *Abbreviation used in this paper*: GST, glutathione S-transferase.

Table I. Alleles of singed Used in This Study

Allele	Bristle morphology	Fertility	Mutation site
$singed^{2}$	bent	fertile	polymorphism in 5′ untranslated region
$singed^{4}$	bent	fertile	unknown
$singed^{3}$	gnarled	fertile	promoter deletion, cryptic promoter used
$singed^{36a}$	gnarled	sterile	insertion, 5′ untranslated region
$singed^{X2}$	gnarled	sterile	unknown

Egg Chamber Staining Procedure

Ovaries were dissected and separated into individual egg chambers in ice-cold *Drosophila* EBR saline solution (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, pH 6.9). Egg chambers were fixed in 100 μl of devitellinizing buffer (6% formaldehyde, 16.7 mM KH_2PO_4/K_2HPO_4, pH 6.9, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$) and 600 μl of heptane for 10 min with gentle agitation. Egg chambers were then washed extensively with PBT (1× PBS, 0.3% Triton X-100, 0.5% BSA).

For actin staining, 5 μl rhodamine-conjugated phalloidin (R415; Molecular Probes, Eugene, OR) was vacuum dried and resuspended in 100 μl of PBT. Fixed egg chambers were incubated in rhodamine-conjugated phalloidin solution for 20 min in the dark and then were rinsed extensively in PBT. If nuclear staining was also desired, phalloidin-stained egg chambers were incubated in 1 μg/ml DAPI for 5 min. After washing egg chambers thoroughly in PBS, they were equilibrated in 50% PBS/glycerol and then mounted onto slides.

For antibody staining, fixed egg chambers were blocked for 10 min in PBT. Egg chambers were incubated overnight at 4°C in 12 μg/ml purified anti-singed IgG. Egg chambers were washed extensively in PBT for 1 h and then were incubated for 2 h at room temperature in FITC-conjugated goat anti-mouse IgG secondary antibody diluted 1/200 in PBT. Egg chambers were washed extensively in PBT for 1 h, rinsed in PBS, and then mounted in 50% PBS/glycerol.

Bristle Staining

White prepupae were collected and dissected at 40–44 h of pupal development according to Schweisguth and Posakony (1992). The dorsal epithelial tissue was fixed in 4% paraformaldehyde for 30 min and then rinsed extensively in PBT for 2 h. Tissue was then incubated for 20 min in either rhodamine-conjugated phalloidin (5 μl/100 μl PBT), 50 μg/ml unconjugated phalloidin to stabilize actin filaments without fluorescent labeling, or no phalloidin as control. After rinsing the tissue for 30 min in PBT, it was incubated overnight at 4°C in 12 μg/ml purified anti-singed IgG. The tissue was rinsed extensively for 1 h in PBT and incubated for 3 h at room temperature in FITC-conjugated goat anti-mouse IgG secondary antibody diluted 1/200 in PBT. Tissue was washed extensively in PBT for 1 h, rinsed in PBS, and mounted in 50% PBS/glycerol. Antibody staining was not affected by the presence or absence of phalloidin.

Singed Protein Production and Purification

The *singed* open reading frame was cloned into the pET 14b expression vector (Novagen, Inc., Madison, WI). This construct allows bacterial expression of the entire singed peptide sequence with an additional six histidine residues followed by a thrombin cleavage site at the amino terminus. To optimize solubility of the fusion protein, transformed bacteria were induced at 25°C with 0.05 mM IPTG for 3 h. Soluble protein extracts in 0.1% Triton X-100, 5 mM imidazole, 0.5 M NaCl, 160 mM Tris-HCl, pH 7.9, were incubated with Ni^{2+} charged beads for 1 h to bind the singed protein. These

beads were then used to make a column that was extensively washed with a 60 mM imidazole buffer containing 0.5 M NaCl, 20 mM Tris, pH 7.9. Purified singed protein with the NH_2-terminal histidine tag was eluted in 150 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9. DTT (0.1 mM) was immediately added to eluted protein to prevent precipitation. Singed protein was dialyzed overnight into thrombin cleavage buffer, 20 mM Tris, pH 8.4, 150 mM KCl, 1 mM DTT at room temperature. $CaCl_2$ was added to dialyzed protein to bring the solution to 2.5 mM $CaCl_2$. To remove the NH_2-terminal histidine tag, thrombin (Novagen) was added at 1 U/μg protein and incubated at room temperature for 2 h. Following thrombin cleavage, the purified singed protein was incubated again with Ni^{2+} charged beads for 30 min. The flow through containing purified singed protein was collected and any uncleaved His-tagged singed protein should remain bound to the column. Purified singed protein was dialyzed into storage buffer (20 mM Tris, pH 8.4, 90 mM KCl, 0.1 mM EDTA, 0.1 mM DTT) and stored on ice. Approximately 1–3 mg of protein was obtained from a 200 ml bacterial culture. Purified protein remained soluble at 4°C for approximately 10 d. Singed solubility appeared to be sensitive to oxidizing conditions, therefore DTT was added every 3 d to prevent precipitation of protein.

Low-speed Cosedimentation Assay with Singed and Actin

G-actin was purified from chicken skeletal muscle acetone powder according to Spudich and Watt (1971). G-actin was polymerized in polymerization buffer (75 mM KCl, 2.5 mM $MgSO_4$, 10 mM imidazole, 10 mM EGTA, 0.1% NaN_3, pH 7.3). The approximate singed/actin ratio required to achieve apparent saturation of binding was determined by adding a range of purified singed (0.87–14 μM based on 57 kD) to a fixed concentration of F-actin (7 μM, based on 43 kD, stabilized as filaments with a 1:1 molar ratio of phalloidin) in a final solution of 85 mM KCl, 10 mM imidazole, 1 mM EDTA, 2.5 mM $MgSO_4$ pH 8.0. Samples were incubated at room temperature for 1 h and then centrifuged 15 min at 16,000 *g* in an Eppendorf microfuge (Beckman Instruments, Palo Alto, CA). The relative distribution of actin and singed in the pellet and supernatant fractions was assessed by SDS-PAGE (8–15% gradient gels) and protein bands were visualized with Coomassie stain. Density of protein bands was quantitated using scanning densitometry (transmittance scanning densitometer; Bio-Rad). Saturation was determined as the point at which singed/actin protein ratio in the pellet remained constant. The molar ratio of singed to actin in the pellet fraction was graphed as a function of total singed protein concentration.

We tested the supernatant and pellet fractions of singed protein for actin bundling activity. A mixture containing 1:3 molar ratio of singed to actin was sedimented as above. More than 98% of the actin sedimented whereas the majority of singed remained soluble. F-actin (14 μM) was added to this soluble fraction of singed, incubated for 1 h at room temperature, and then centrifuged. The pellet and supernatant fractions from both the original sedimentation and the sedimentation with the soluble singed fraction were analyzed by SDS-PAGE.

To test bundling ability of singed recovered from a singed-actin pellet, singed/actin bundles were sedimented by centrifugation of a mixture containing 1:2 molar ratio singed/actin. The pellet fraction of singed-actin bundles was dissolved in 0.5 M KCl to dissociate singed. The F-actin was removed by high speed centrifugation (23 psi in an airfuge for 1 h; Beckman Instruments) while the singed remained soluble. The recovered singed was then dialyzed into singed storage buffer and the concentration of singed was determined. Both the singed recovered from bundles and the original singed preparation were used to make mixtures of 1:2 molar ratio of singed:actin. These mixtures were sedimented and the supernatant and pellet fractions were analyzed by SDS-PAGE.

Microscopy

Fluorescent microscopy was carried out on a Zeiss Axiophot equipped with differential interference contrast and epifluorescence optics.

Confocal imaging was performed using a Bio-Rad MRC 600 scanning laser confocal microscope. Optical sections were presented individually or were combined using the Comos software package (Bio-Rad).

Scanning electron microscopy was performed on a ISI model SS-40 scanning electron microscope. Flies were dehydrated in isoamyl alcohol and mounted on stubs.

Mixtures of F-actin and singed in a 1:2 ratio were visualized by darkfield light microscopy and electron microscopy. Light microscopy was performed on a Zeiss light microscope with a darkfield condenser. Samples for electron microscopy were stained with 0.2% aqueous uranyl acetate on parlodion and carbon-coated grids and examined on a Zeiss 10CA electron microscope at an accelerating voltage of 80 kV.

Results

Nurse Cell Cytoplasmic Actin Filament Bundles Are Absent in Sterile Singed Mutants

Most *singed* mutants with severe bristle defects are female sterile and produce egg chambers in which the final rapid phase of cytoplasm transport to the oocyte is incomplete. Since this process is actin dependent (Gutzeit, 1986), we investigated actin distribution in *singed* egg chambers. Actin filaments are normally present subcortically in nurse cells, including the ring canals connecting adjacent cells. Subcortical actin-containing filaments likely support nurse cell contraction to push nurse cell cytoplasm through the ring canals into the oocyte (Cooley et al., 1992). In late stage 10, just before the rapid phase of cytoplasm transport, actin filament bundles form in the nurse cell cytoplasm (Fig. 2 *A*); these bundles probably have a structural role in anchoring the nurse cell nuclei in a central position away from ring canals (Cooley et al., 1992). We analyzed actin-based structures using rhodamine-conjugated phalloidin to stain F-actin in egg chambers from sterile *singed* mutants. *singed* mutant egg chambers were indistinguishable from wild type before the rapid phase of nurse cell cytoplasm transport. However, in egg chambers from sterile *singed* mutants, the cytoplasmic actin filament bundles rarely formed (Fig. 2 *C*) and nurse cell nuclei became dramatically rearranged (Fig. 2, *B* and *D*). The nuclei in the four nurse cells adjacent to the oocyte appeared to be pushed into the ring canals, to extend into the oocyte (Fig. 2 *D*), and to block the flow of cytoplasm into the oocyte. Some nuclei also appeared pushed into ring canals between adjacent nurse cells (not shown).

Monoclonal Antibodies Recognize a 57-kD Protein That Is Reduced in Singed Mutants

To analyze singed protein expression in wild type and *singed* mutants, we made monoclonal antibodies from a mouse immunized with a GST-singed fusion protein. These monoclonal antibodies recognized a 57-kD protein by Western immunoblot in whole fly extracts of males and females (not shown) and in ovary extracts (Fig. 3). *singed* mutants also contained a 57-kD protein; however, the level of singed protein was reduced. To quantitate levels of singed protein in wild type and *singed* ovaries, 25 μg of protein extract was loaded in each lane and kelch antibody was used as a loading control (Xue and Cooley, 1993). Fertile *singed* alleles, $singed^2$, $singed^3$, and $singed^4$, contained markedly reduced levels of the 57-kD protein relative to wild type levels as determined by densitometric scanning of autoradiograms like the one shown in Fig. 3. In sterile *singed* mutants, $singed^{X2}$ and $singed^{36a}$, the 57-kD protein was absent or nearly absent. Three transcripts are generated from the *singed* locus that differ only in the 3′-untranslated region and encode the same 57-kD protein (Paterson and O'Hare, 1991). The absence of protein in severe, sterile *singed* alleles suggested that all transcripts are affected. The increased severity of the mutant phenotype appeared to correlate with the decreased amount of singed protein detected.

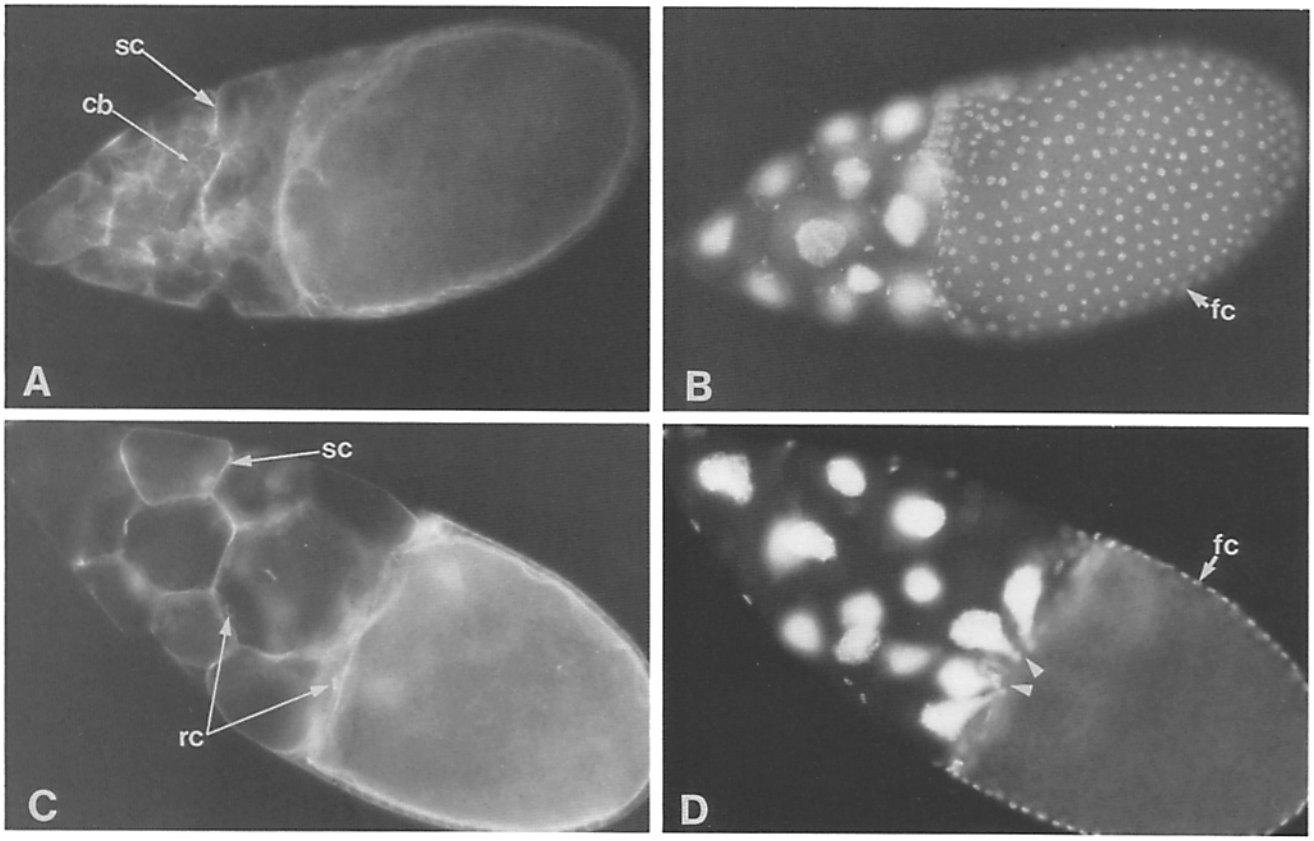

Figure 2. Absence of nurse cell cytoplasmic actin filament bundles and subsequent nuclear rearrangement in egg chambers from sterile *singed*^{X2} mutants. Stage 10B egg chambers from wild type (*A* and *B*) and *singed*^{X2} egg chambers (*C* and *D*) were double stained with rhodamine-conjugated phalloidin (*A* and *C*) and DAPI (*B* and *D*). In wild type, cytoplasmic actin bundles (*cb*) form during late stage 10 (*A*) and the nurse cell nuclei are located centrally in the nurse cell (*B*). In *singed*^{X2} mutants, cytoplasmic actin filament bundles are rare (*C*) and nurse cell nuclei become rearranged and appear to be pushed into ring canals (D, *arrowheads*). Subcortical actin filaments (*sc*) and ring canals (*rc*) appear unaffected in mutants. In the DAPI stained wild type egg chamber (*B*) the small nuclei of the follicle cells (*fc*) are seen in the surface focal plane.

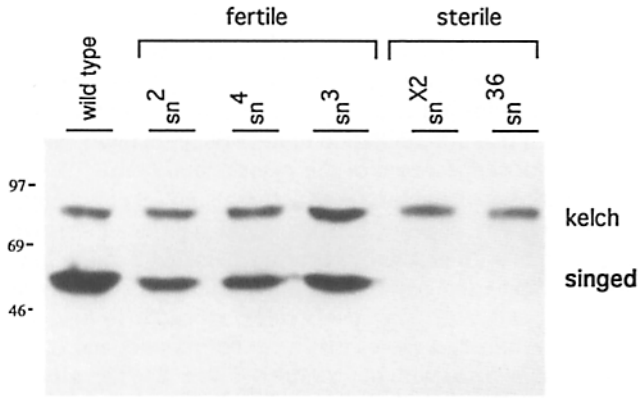

Figure 3. Singed monoclonal antibody recognizes a 57-kD protein that is reduced in *singed* mutants. Western immunoblot analysis of singed protein expression in ovary extracts from wild type and fertile and sterile *singed* mutants was performed. 25 μg of total protein was loaded per lane. The blot was probed with singed monoclonal antibody 7C and kelch monoclonal antibody 1B (Xue and Cooley, 1993). The singed monoclonal antibody recognized a single 57-kD protein that is reduced in fertile singed mutants and absent or nearly absent in female sterile *singed* mutants. Kelch monoclonal antibody recognized an 80-kD protein and was used as a loading control. Protein molecular weight markers are indicated in kD on the left edge of the gel.

Nurse Cells and Specific Somatic Follicle Cells Contain Cytoplasmic Singed Protein

We stained egg chambers from wild type and both fertile *singed*^{3} and sterile *singed*^{X2} mutants with singed monoclonal antibody purified IgG. In the germarium and subsequent early stages of oogenesis, singed was detected at low levels in nurse cell cytoplasm. Several migratory populations of follicle cells expressed very high levels of singed. At stage 9, abundant staining was present in border cells and posterior follicle cells (Fig. 4 *A*). The border cells formed spike-like projections visible by anti-singed immunofluorescence as they migrated between nurse cells from their anterior position in the egg chamber to the anterior margin of the oocyte (Fig. 4, *A* and *B*). As border cells migrate between nurse cells, the majority of follicle cells surrounding the egg chamber migrate posteriorly along the outside of the egg chamber to form a columnar epithelium around the oocyte. A small number of follicle cells remain, surrounding the nurse cells in a squamous epithelium. The follicle cells migrating around the outside of the egg chamber do not express abundant singed protein (Fig. 4 *A*). In early stage 10, singed was expressed abundantly in the centripetal follicle cells as they migrated along the nurse cell–oocyte interface (Fig. 4 *B*). Singed expression in these cells appeared localized near the

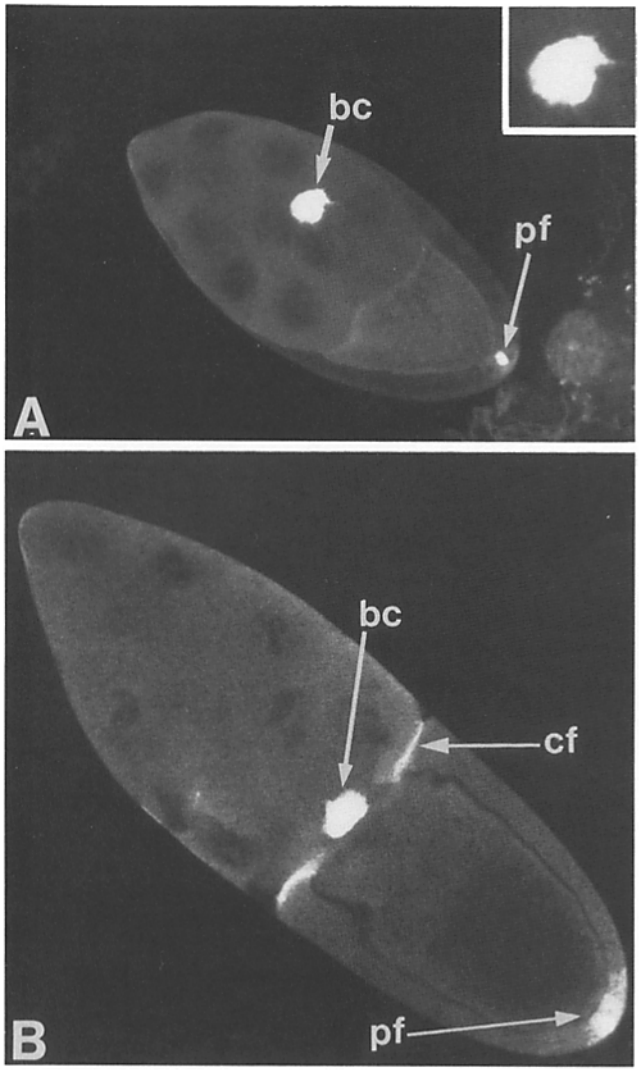

Figure 4. Localization of singed protein in migratory cells in stage 9 egg chambers. Wild-type egg chambers were stained with purified singed monoclonal antibody IgG (12 μg/ml) and examined using confocal microscopy. In stage 9 egg chambers, singed is abundantly expressed in the cytoplasm of border cells (*bc*) and posterior follicle cells (*pf*) (*A*). As these cells migrate between nurse cells toward the anterior margin of the oocyte, singed protein is also detected in several spike-like cellular extensions (*inset*). In early stage 10 egg chambers (*B*), the border cells in the anterior margin of the oocyte, the posterior follicle cells, and centripetal follicle cells (*cf*) all contain abundant singed protein. As centripetal follicle cells migrate along the nurse cell oocyte interface, singed protein appears to have a subcellular localization near the follicle cell–nurse cell interface. These image projections represent the combination of serial confocal optical sections.

follicle cell–nurse cell interface. These data suggest that only the follicle cells that migrate to the interior of the egg chamber express abundant singed protein.

During stage 10 there was a dramatic increase in singed expression in the nurse cells. Nurse cell cytoplasm showed a low concentration of singed protein in early stage 10 egg chambers (Fig. 4 *B*) and a high concentration at the end of stage 10 when actin filament bundles form. In stage 11, during rapid nurse cell cytoplasm transport, singed protein staining continued to increase throughout the nurse cell cytoplasm and also intensified in subcortical regions (Fig. 5 *A*). Polyclonal sera and 10 different monoclonal supernatants all demonstrated this staining pattern. Gentle extraction of egg chambers with 0.5% Triton X-100 or saponin decreased the nurse cell cytoplasm staining intensity but did not alter the diffuse character of the staining pattern (not shown). In fertile *singed*^{3} mutants, singed protein staining was slightly reduced, but the localization and character of the staining was not significantly affected (Fig. 5 *C*). The presence of singed protein in both wild type and fertile *singed*^{3} mutants correlated with the presence of normal cytoplasmic actin filament bundles (Fig. 5, *B* and *D*). Singed protein was not detected in nurse cells, border cells, posterior follicle cells, or centripetal follicle cells in egg chambers from sterile *singed*^{X2} mutants (Fig. 5 *E*). The absence of protein in nurse cells in sterile *singed*^{X2} mutant egg chambers (Fig. 5 *E*) correlated with the near absence of cytoplasmic actin filament bundles (Fig. 5 *F*).

Absence of Singed Protein Correlates with Disrupted Actin Filament Bundles in Bristle Development

In *Drosophila*, each bristle organ is comprised of four clonally derived cells: the trichogen, tormogen, nerve cell, and thecogen. The bristle shaft forms from a single cytoplasmic extension of the trichogen cell. The extension is supported by 8–12 submembranous actin filament bundles and a central region of microtubules (Appel et al., 1993; Overton, 1967). Bristle elongation occurs between 30–45 h of pupal development. External ridges and microfilament bundles are apparent at 35 h, and the bristle has extended to 75% of its final length by 41 h (Lees and Picken, 1945). As the bristle is extending, the plasma membrane protrudes between adjacent actin filament bundles resulting in a ridged appearance during bristle elongation and in the adult after cuticle deposition. In the adult cuticle, ridges represent the outpatching of the cell membrane while grooves represent the position of submembranous actin filament bundles that were present during bristle development (Fig. 6 *A*). The tormogen cell forms the socket that surrounds the bristle shaft at its base. Final differentiation of the bristle includes innervation and disappearance of the cytoskeletal core.

We analyzed *singed* bristle structure using scanning electron microscopy of adult bristles. Wild type adult bristles were straight with gradually tapering ends and parallel longitudinal ridges and grooves running the length of the bristle (Figs. 1 *A* and 6 *A*). *singed*^{X2} bristles appeared to have an increased number of ridges that were not parallel and often intersected or fused with other ridges (Fig. 6 *B*). The scutellar bristles on *singed*^{X2} mutants were the most severely deformed; some regions of the shaft appeared collapsed and the bristles appeared spiraled, bent, and branched (Figs. 1 *B* and 6 *C*).

By examining the organization of actin filaments and the localization of singed protein in developing bristles, we found that singed appeared to be required to form organized actin filament bundles. In wild type pupae, singed protein was present in the cytoplasm of the extending bristle and in the tormogen cell (Fig. 7, *A* and *C*). In about one-third of the bristles examined we also observed an increased singed protein staining in a bundle-like pattern coincident with actin

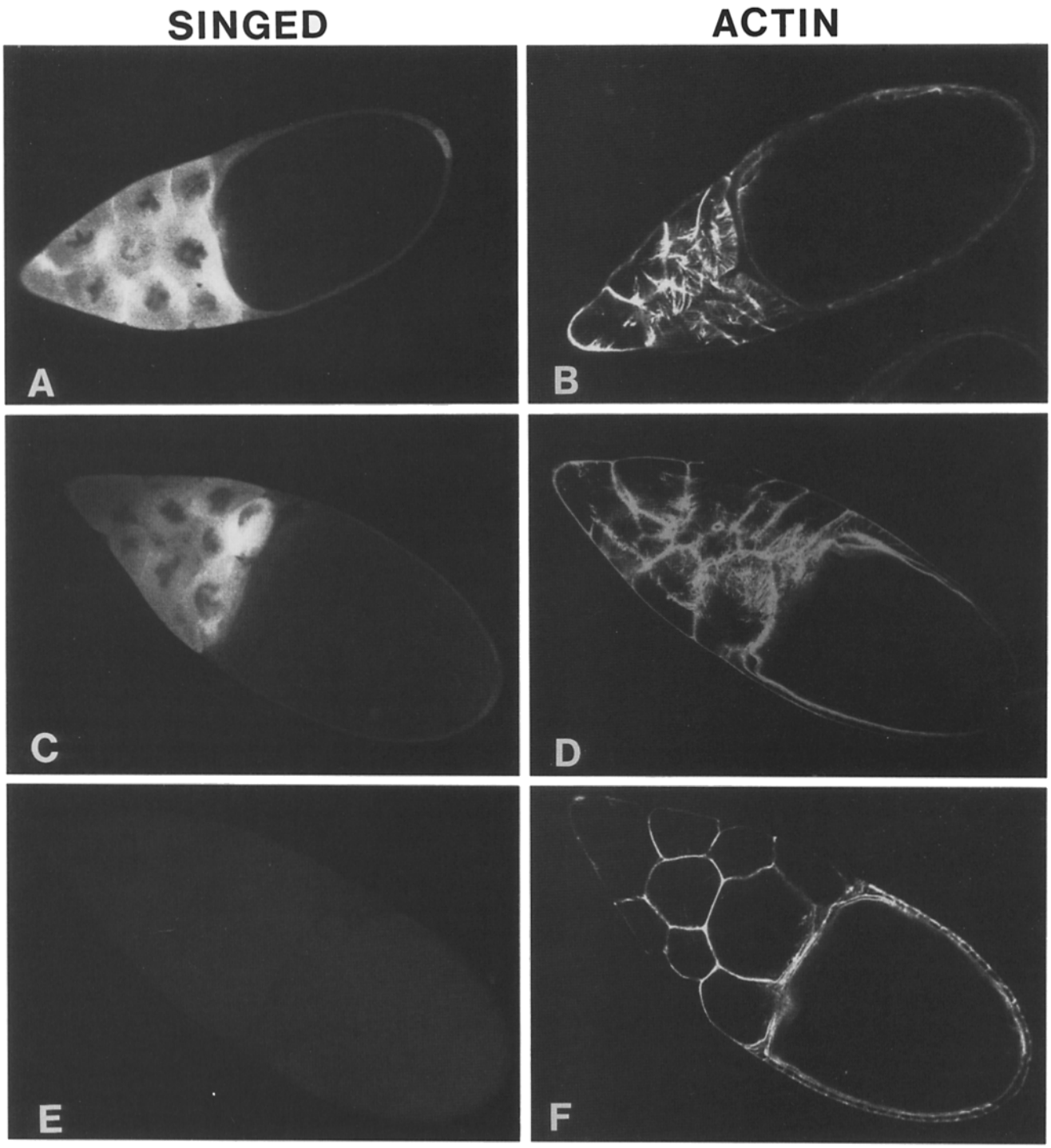

Figure 5. Singed protein expression is necessary for cytoplasmic actin filament bundle formation. Stage 11 egg chambers from wild type and fertile and sterile alleles of *singed* mutants were stained with either purified singed monoclonal antibody IgG (*singed*) or rhodamine-conjugated phalloidin (*actin*) during the rapid phase of nurse cell cytoplasm transport. In wild type, cytoplasmic actin filament bundles are present (*B*), and singed protein is expressed abundantly in the nurse cell cytoplasm (*A*). In egg chambers from fertile *singed*^{3} females, singed protein expression is slightly reduced (*C*) but the actin filament structures appear normal (*D*). In egg chambers from sterile *singed*^{X2} females, singed protein is absent (*E*), and cytoplasmic actin filament bundles are nearly absent (*F*) while subcortical actin filaments appear normal.

filament bundles (Fig. 7 *C*). In wild type bristles, rhodamine-conjugated phalloidin staining showed discrete and parallel actin bundles extending the length of the bristle (Fig. 7, *B* and *D*). Actin filaments were also present in the tormogen cell (not shown). Actin filaments and singed protein expression were then analyzed in bristles from *singed*^{3} and *singed*^{X2} pupae. *singed*^{3} mutants have moderately gnarled bristles, whereas *singed*^{X2}, a putative null allele, have the most severely gnarled bristles. In *singed*^{3} bristles, singed protein was nearly absent and the actin filament bundles ap-

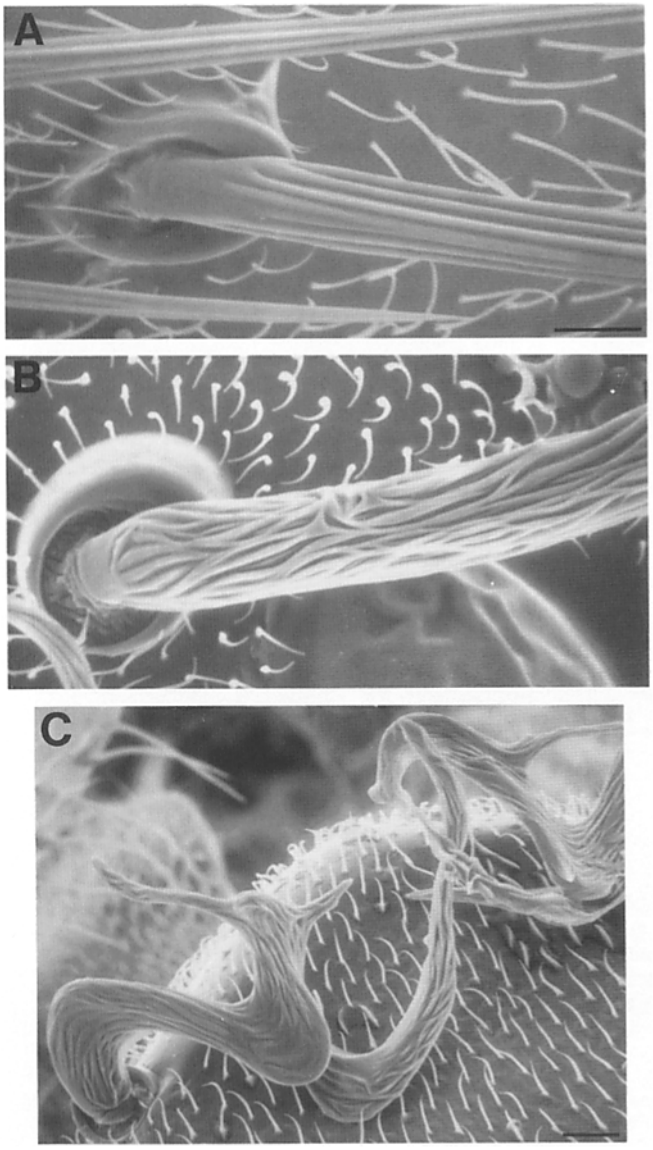

Figure 6. Aberrant bristle morphology in *singed*^{X2} mutants. Scanning electron microscopy images of adult bristles from wild type (*A*) and *singed*^{X2} mutants (*B* and *C*) are shown. Wild type bristles are straight with parallel longitudinal ridges and grooves in the cuticle. In *singed*^{X2} bristles, the ridges and grooves are highly irregular and often intersect or fuse (*B*). The scutellar *singed*^{X2} bristles are most deformed and appear curved, bent, branched and sometimes collapsed (*C*). *A* and *B* are the same magnification. Bars, 10 μm.

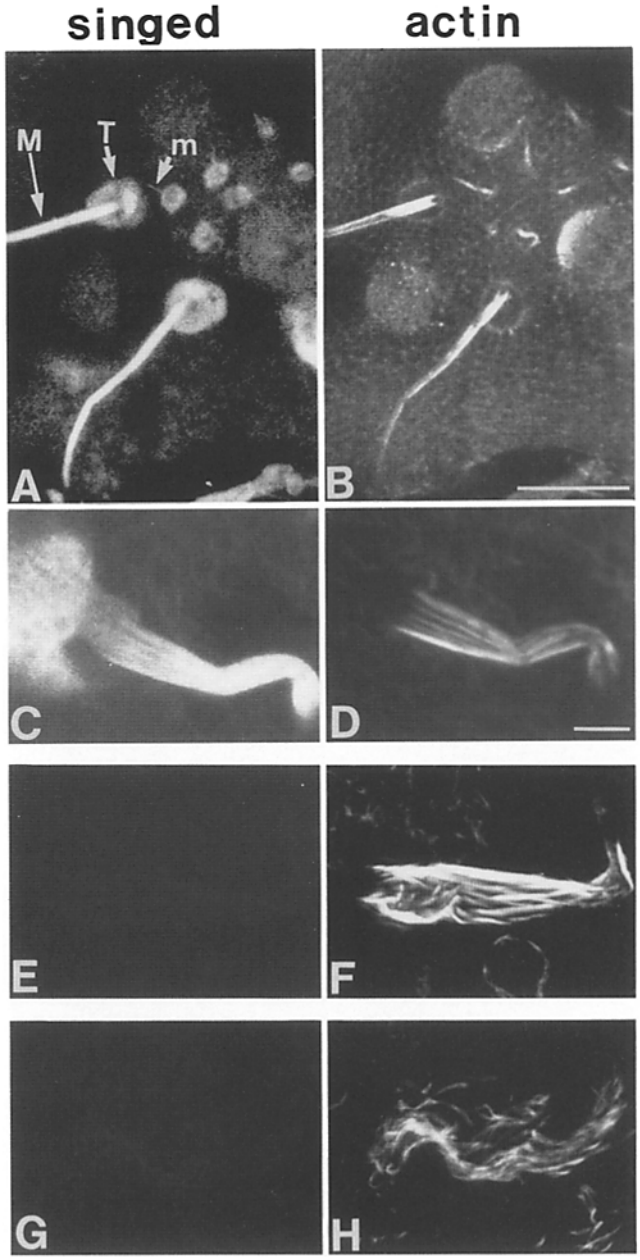

Figure 7. The absence of singed protein during bristle extension correlates with defective actin filament bundles. The dorsal epithelia of 40–44-h-old pupae were double stained with purified singed monoclonal antibody IgG (*A*, *C*, *E*, and *G*) and rhodamine-conjugated phalloidin (*B*, *D*, *F*, and *H*). In wild type pupae (*A–D*), singed protein is expressed in the cytoplasm of the bristle extensions in both macrochaetes (*M*) and microchaetes (*m*) and in the tormogen socket cell (*T*). Wild type bristle extensions contain parallel actin filament bundles (*B* and *D*). In addition to the diffuse cytoplasmic localization of singed protein in the bristle extension, singed often appears more concentrated in a bundle-like pattern (*C*) coincident with submembranous actin filament bundles (*D*). In *singed*^{3} bristles (*E* and *F*) singed protein is nearly absent and actin filament bundles are disorganized. In *singed*^{X2} bristles singed protein is also nearly absent (*G*) and the actin filament bundles appear diffuse and disorganized (*H*). The overall shape of the *singed*^{X2} bristle is curved and branched (*H*). Note that the rhodamine-conjugated phalloidin staining signal does not bleed into the FITC channel (*E* and *G*). *C–H* are the same magnification. Bars, 10 μm.

peared disorganized (Fig. 7, *E* and *F*). In *singed*^{X2} bristles, singed protein was also absent, although the rhodamine–phalloidin staining of actin filament bundles appeared more diffuse, faint, and extremely disorganized (Fig. 7, *G* and *H*). Immunofluorescence may not be sensitive enough to detect differences in protein expression in *singed*^{3} versus *singed*^{X2} bristles.

Bacterially Expressed Singed Protein Bundles F-Actin

The phenotypic characterization of singed mutants suggested that singed protein is required for actin filament bundle formation in egg chambers and bristles. We used purified, bac-

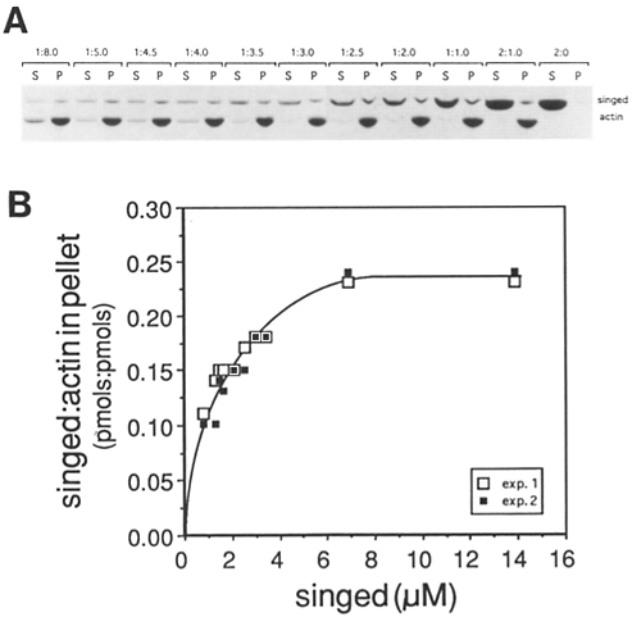

Figure 8. Co-sedimentation of singed with actin. (*A*) Mixtures of singed and F-actin at molar ratios of singed:actin varying from 1:8 to 2:1 were centrifuged at low speed. The distribution of actin and singed in the supernatant (*S*) and pellet (*P*) fractions were visualized by coomassie staining of SDS-PAGE gels. The concentration of F-actin remained constant at 7 μM, while the singed concentration increased from 0.87 to 14 μM. (*B*) Quantitation of two low speed co-sedimentation experiments was done using scanning densitometry. Exp 1 refers to quantitation of the protein bands shown in *A*; exp 2 refers to the same experiment repeated. The molar ratio of singed/actin in the pellet fraction is plotted against the total concentration of singed prior to centrifugation. The molar ratio of singed/actin in the pellet becomes saturated at a ratio of about 0.23, or 1:4.3.

terially expressed singed protein to explore whether singed has functional homology with the actin filament bundling protein fascin. The singed protein used in our in vitro experiments was made as a recombinant protein in the pET expression system and contained three extra amino acids, glycine, serine, and histidine, at the amino terminus. The protein was approximately 57 kD and was recognized by singed monoclonal antibodies on Western immunoblots (not shown).

In preliminary studies, purified singed protein (3.5 μM) was mixed with F-actin (7 μM) in a 1:2 molar ratio and bundles of F-actin were visible by dark field microscopy (not shown). To quantitate the interaction between singed and actin, low speed cosedimentation assays were conducted on samples containing 7 μM phalloidin-stabilized F-actin and increasing concentrations of purified singed (0.87–14 μM) such that the singed/actin molar ratios ranged from 1:8 to 2:1 (Fig. 8 *A*). Samples were centrifuged at low speed to pellet actin filament bundles. The amount of protein in the soluble and pellet fractions was determined using scanning densitometry. Less than 2% of either F-actin or singed protein alone sedimented upon low speed centrifugation (Fig. 8 *A*, singed to actin ratio 2:0). To quantitate the stoichiometry of singed binding to actin, we measured the ratio of singed to actin in the pellet fractions (Fig. 8 *B*). The saturation of actin bundles with singed was reached when the amount of singed in the pellet no longer increased despite increasing singed protein concentration (compare 7 to 14 μM singed in Fig. 8 *B*). The molar ratio of singed to actin in saturated singed–actin bundles was calculated as approximately 0.23 from densitometry data. This can also be seen by extrapolating the saturation curve to the ordinate in Fig. 8 *B*. The ratio of 0.23 converts to a singed/actin molar ratio of 1:4.3 and this is consistent with the stoichiometry of fascin–actin bundles of 1:4.6.

While both singed–actin and fascin–actin bundles contained a molar ratio of approximately 1:4.5, the conditions for saturation varied dramatically. Saturation of singed–actin bundles required a molar ratio of 1:1 singed/actin because most singed remained unbound. Saturation of fascin–actin bundles required a molar ratio of about 1:4 fascin/actin, in which most fascin was bound (Bryan and Kane, 1978). The high concentration of singed required to saturate actin suggested that singed bound actin with low affinity. To eliminate the alternative possibility that the singed protein preparation contained a mixture of active and inactive protein, we tested the bundling activity of both singed recovered from bundles and singed remaining soluble. Both these fractions retained qualitatively similar bundling activities compared to the original singed protein preparation (see Materials and Methods). The soluble fraction of singed retained bundling activity. Singed recovered from bundles maintained its ability to bundle F-actin, yet, as with the original singed preparation, most of the singed remained soluble. These data suggested that the singed protein preparation contained homogeneously active protein with a low affinity for actin. Bacterially expressed sea urchin fascin is not soluble (Bryan et al., 1993) and therefore no direct comparison can be made with bacterially expressed singed.

Negatively Stained Singed–Actin Bundles Have a Transverse Cross-banding Pattern

Electron microscopy of negatively stained actin bundles typically reveals a transverse cross-banding pattern characteristic of the bundling protein cross-linking actin filaments. Incubation of singed and phalloidin-stabilized F-actin at a 1:2 singed/actin ratio resulted in F-actin bundles with a transverse cross-banding pattern (Fig. 9). We saw two characteristic views of negatively stained singed–actin bundles. The bundle in Fig. 9 had several regions of cross-band doublets. The interval between pairs of cross-bands within a doublet was 12 nm ± 1.1 (average of 15 intervals such as the bracket labeled *a* in bundle shown in Fig. 9), whereas the interval between doublets was 24.9 nm ± 1.7 (average of 12 intervals such as the bracket labeled *b* in Fig. 9). The larger space occurred at sites of actin filament crossover and thus the cross-band at this point is difficult to see. While the doublet view was more common, a region of continuous 12-nm cross-banding repeats and less discernible actin filaments is suggested near the right end of the bundle in Fig. 9, and was clearly visible on other bundles. We measured the intervals between eight continuous cross-bands on a different bundle and we found a 12-nm transverse periodicity. Negatively stained fascin–actin bundles also have two similar characteristic patterns on electron micrographs depending on the orientation of the bundle in the microscope. The 12-nm transverse periodicity and the stoichiometry of singed/actin

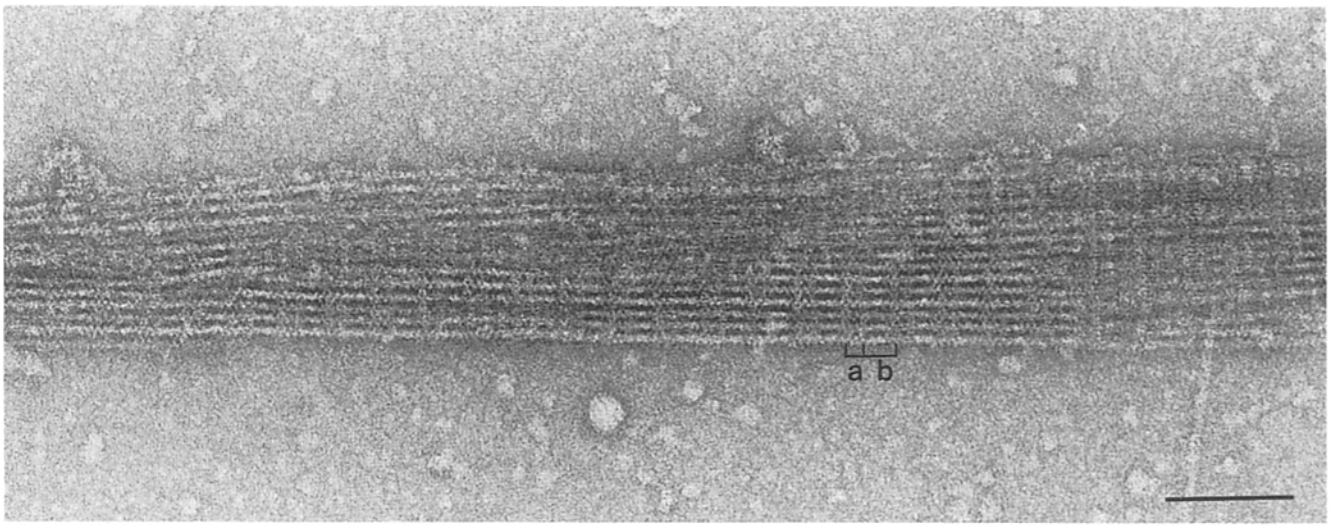

Figure 9. Electron micrograph of a negatively stained singed–actin filament bundle. Singed–actin filament bundles were formed by mixing purified bacterially expressed singed protein and phalloidin-stabilized F-actin. Negative staining reveals transverse cross-bands appearing as doublets that represent singed protein cross-linking actin filaments. The small interval (bracket *a*) within a single doublet is 12 nm ± 1.2, while the large interval between doublets (bracket *b*) is 24.9 nm ± 1.7. A distinct characteristic view showing a continuous 12 nm transverse periodicity is suggested near the right end of this bundle. Magnification is 168,000. Bar, 100 nm.

of 1:4.3 determined by cosedimentation assay closely resemble fascin/actin interactions. We could not determine whether singed–actin bundles contained uniformly polarized actin filaments.

Discussion

Singed Is Functionally Homologous to Fascin

Several lines of evidence indicate that the *Drosophila singed* gene encodes a homolog of echinoderm fascin. *Drosophila singed* and sea urchin *fascin* encode proteins of similar molecular weight with 35% peptide identity and 67% overall similarity (Bryan et al., 1993). We have shown that actin filaments bundled with bacterially expressed singed protein resemble fascin-bundled actin filaments. Electron micrographs of negatively stained singed-bundled actin filaments and fascin-bundled actin filaments (Kane, 1976; Byran and Kane, 1978; Spudich and Amos, 1979) both show parallel actin filaments with a transverse cross-banding pattern of about 12 nm. Analysis of fascin–actin bundles using optical diffraction and image reconstruction showed that the bundles consist of actin filaments arranged in a hexagonal lattice with nine fascin cross-band links per 41 actin monomers (DeRosier and Censullo, 1981; DeRosier et al., 1977). Therefore, this model places restriction on the molar ratio of fascin to actin. The transverse periodicity and the stoichiometry of both fascin–actin bundles (12 nm and 1:4.6) and singed–actin bundles (12 nm and 1:4.3) agrees with the stoichiometry fixed by the geometry of hexagonally packed bundles described for fascin–actin bundles.

The phenotypes of *singed* mutants suggest that singed protein, like fascin, organizes actin filaments into bundles in vivo. Defective actin filament bundles in *singed* mutants can account for both the nurse cell cytoplasm transport arrest and the bristle deformity in *singed* mutants. Sterile $singed^{x2}$ egg chambers contain no singed protein and nurse cell cytoplasmic actin filament bundles are nearly absent. The absence of cytoplasmic actin filament bundles allows the nurse cell nuclei to lodge into ring canals and block nurse cell cytoplasm transport. In $singed^{x2}$ bristles, the absence of singed protein correlated with both the faint, disorganized actin staining in developing bristles and the subsequent aberrant adult cuticle pattern. In wild type, dense actin filament bundle columns appear to promote bristle extension in a single direction possibly by providing structural support and by directing cytoplasmic forces toward the tip through symmetric and circumferential force opposition. In electron micrographs of wild type developing bristles, actin filament bundles have an approximately 12-nm cross-banding periodicity (see Fig. 13 in Overton, 1967) similar to in vitro singed–actin bundles. In *singed* mutants, a decrease in actin bundling protein may result in bundles that contain fewer actin filaments and lack of the usual rigid parallel organization ensured by saturated cross-linking binding sites. We suggest that the thin, disorganized filament bundles in gnarled *singed* bristles cannot provide the structural support for continuous, straight extension. This decrease in structural integrity could allow actin filament bundles to bend or divide causing bristles to curve and branch. Lateral bending of actin filament bundles could explain the cross-weave cuticle ridge pattern seen with scanning electron microscopy and result in the spiral curvature of some *singed* bristles. Singed may affect the assembly and stabilization of actin filament bundles, the structural integrity of actin filament bundles, or actin monomer/polymer dynamics.

In the bristle extension, singed protein appears predominantly cytoplasmic, with an occasional diffuse, bundle-like pattern coincident with filamentous actin. In the nurse cells of egg chambers, singed protein appears diffusely cytoplasmic with an increased concentration along the nurse cell oocyte interface and subcortically during the rapid phase of nurse cell cytoplasm transport. We propose that singed protein bound to actin filament bundles is difficult to observe

in the presence of a large excess of unbound protein. A potentially analogous situation has been found in sea urchin eggs. In eggs, most fascin is present in the cytoplasm as determined by fractionation assays (Otto et al., 1980). Upon fertilization, 30–35% of the fascin shifts to the pelletable fraction suggesting that only a portion of fascin is recruited to the newly formed microvillar cytoskeleton. Fascin associated with actin bundles is best visualized in isolated sea urchin egg cortex microvilli (Otto et al., 1980) and permeabilized coelomocyte filopodia extensions (Otto and Bryan, 1980; Otto et al., 1979). In these preparations, unbound, cytoplasmic fascin would not be present.

Our phenotypic and biochemical data argue strongly that mutations in the fascin-encoding *singed* gene are responsible for all of the *singed* phenotypes observed. It is unlikely but formally possible that mutations affecting the *singed* gene also affect neighboring genes and that they contribute to the phenotypes. Transgenic experiments with copies of the cloned *singed* gene would test the ability of the fascin homolog to rescue *singed* mutants; however, such experiments have not yet been carried out.

Singed Expression in Cells Undergoing Dynamic Cytoskeletal Rearrangements

Singed appears to be abundantly expressed in specific subsets of migratory somatic follicle cells in egg chambers. The border cells migrate between the nurse cells to take up position at the nurse cell–oocyte interface. During their migration, cell extensions are clearly visible with singed immunofluorescence. Posterior follicle cells also express abundant singed. These cells have migratory potential although normally the oocyte blocks their path (Montell et al., 1992). As centripetal follicle cells migrate inward along the nurse cell–oocyte interface, they express high levels of singed. Therefore, specific migratory follicle cells of the egg chamber express singed abundantly.

The presence of singed in cells undergoing dynamic actin reorganization associated with migration is not surprising. Fascin has been localized to filopodial extensions that form rapidly on sea urchin coelomocytes. HeLa cell 55-kD protein (Yamashiro-Matsumura and Matsumura, 1986, 1985), which was also recently shown to have homology with fascin peptide sequence (Bryan et al., 1993), immunolocalizes to highly motile microspikes and also stress fibers. However, border cell and follicle cell migrations appear unaffected in sterile *singed* mutants, even though no singed protein is detected in these cells. Additional actin bundling proteins could be present that can compensate for the absence of singed during cytoskeletal rearrangements associated with migration. A requirement for *singed* in egg chamber somatic cells remains unclear. Mosaic analysis has shown that females with *singed* mutant germline-derived cells and wild type somatic follicle cells exhibit a *singed* phenotype suggesting that singed is required in the germline (Perrimon and Gans, 1983). However, mosaic females with *singed* somatic cells and wild type germline-derived cells have not been described, and a somatic cell defect can not be excluded.

Aberrant Actin Filament Bundle Formation in Drosophila Mutants with Bristle Defects

The highly organized, actin-based cytoskeletal framework in developing bristles requires multiple proteins. In addition to *singed*, several fly bristle morphology mutants, including *forked* (Petersen et al., 1994), *chickadee* (Verheyen and Cooley, 1994), and *Stubble-stubbloid* (Appel et al., 1993), also have defective actin filament bundle structures. The *forked* bristle phenotype most closely resembles the *singed* phenotype. The *forked* locus contains many transcripts, none of which encode proteins with homology to any known actin binding protein (Hoover et al., 1993). However, forked protein is localized on actin filament bundles in developing bristles and in severe null *forked* alleles, actin filament bundles are absent (Petersen et al., 1994). In addition, overexpression of small *forked* transcripts disrupts the structural integrity of developing bristles (Petersen et al., 1994). High concentrations of bundling protein during the initial phase of bundle formation in vitro can result in rapid aberrant cross-linking of actin filaments without regard to order (Stokes and DeRosier, 1991). Forked may indeed be a novel actin binding protein that directly affects actin filament bundle organization and acts together with singed to provide highly ordered, rigid actin filament bundles.

In addition to *singed* and *forked*, *chickadee*, and *Stubble-stubbloid* mutations have recently been shown to affect the actin filament bundles in bristles (Verheyen and Cooley, 1994; Appel, 1993). Developing *stubbloid* mutant bristles contain filament bundles that become disorganized at the tip accounting for the frayed appearance of the ends of adult *stubbloid* bristles (Appel et al., 1993). In both *Stubble* and *chickadee* mutants, the bristles are thicker and there is an increased number of actin filament bundles that are smaller and irregularly arranged (Appel et al., 1993; Verheyen and Cooley, 1994). *Stubble* bristles end abruptly while *chickadee* bristles typically appear branched and bent. *chickadee*, *Stubble*, and *stubbloid* mutants differ from *singed* and *forked* mutants in that the bundles remain dense and columnar, although thinner. The *Stubble-stubbloid* gene encodes a protein that resembles a type II transmembrane protein with an extracellular serine protease domain (Appel et al., 1993) but the protein localization in the bristle shaft is unknown. Stubble-stubbloid protein may act in a complex to tether actin filament bundles to the membrane. *chickadee* encodes profilin (Cooley et al., 1992) and the protein is present in the cytoplasm of the bristle extension (Verheyen and Cooley, 1994). Profilin may be functioning to regulate nucleation of new filaments and/or the rate of actin filament polymerization. The structural integrity of the developing bristle extension appears to require a wide array of proteins for the formation of organized, dense actin filament bundles.

Conclusions

We have found that singed protein is expressed in a variety of cells and that a deficiency of singed protein affects actin integrity in distinct cell types differently, ranging from absence of actin bundles in nurse cells to disorganized actin bundles in bristles. In contrast, migratory cells in egg chambers, which have intense singed staining in wild type, appear unaffected in severe *singed* mutants. Cell types that appear less affected may contain additional cytoskeletal proteins that act redundantly with singed or can substitute for singed in severe *singed* mutants. In addition, the developmental time frame constraining actin rearrangement in a given cell

probably influences the severity of the *singed* phenotype. The most stringent requirement for singed protein appears to be in nurse cells, which have only minutes to complete a massive actin assembly that may require immediate bundling to prevent actin filament depolymerization. In the developing bristle there may be adequate time for other actin binding proteins to stabilize filaments and allow minimal organization and elongation.

Both nurse cell cytoplasm transport and bristle extension provide excellent in vivo models for the functional analysis of actin binding proteins. Future mutagenesis screens for female sterile mutants with disrupted cytoplasm transport and aberrant bristle morphology could uncover additional actin binding proteins. The regulation of actin binding proteins can also be explored by studying genes that interact genetically with mutants such as *singed*.

We thank Kevin O'Hare for *singed* cDNA and helpful discussion; Joe Bryan for sharing fascin sequence homology; Joe Wolenski for enthusiastic instruction and assistance with cosedimentation experiments; Barry Pickos for help with SEM; Esther Verheyen for instruction in pupal dissection. We especially thank Lew Tilney and David DeRosier for valuable comments on the manuscript and members of the Cooley and Mooseker Labs for many helpful discussions and encouragement. We are grateful to the Bloomington Stock Center for *singed* alleles; Yale University School of Medicine, Section of Immunology Monoclonal Antibody Facility; Yale University School of Medicine, Molecular and Developmental Neurobiology Program for use of the confocal microscope.

This work was supported by Public Health Service grant GM43301 (L. Cooley), the Pew Charitable Trusts (L. Cooley), National Institutes of Health grant DK25387 (M. S. Mooseker), NIH Postdoctoral grant (B. A. Knowles), and NIH Medical Scientist Training Program grant, GM07205 (K. Cant).

Received for publication 24 November 1993, and in revised form 13 January 1994.

References

Appel, L., M. Prout, R. Abu-Shumays, A. Hammonds, J. Garbe, D. Fristrom, and J. Fristrom. 1993. The *Drosophila Stubble* gene encodes an apparent transmembrane serine protease required for epithelial morphogenesis. *Proc. Natl. Acad. Sci. USA.* 90:4937–4941.

Bender, H. 1960. Studies on the expression of various *singed* alleles in *Drosophila melanogaster*. *Genetics.* 45:867–883.

Bryan, J., R. Edwards, P. Matsudaira, J. Otto, and J. Wulfkuhle. 1993. Fascin, an echinoid actin-bundling protein, is a homolog of the *Drosophila singed* gene product. *Proc. Natl. Acad. Sci. USA.* 90:9115–9119.

Bryan, J., and R. Kane. 1978. Separation and interaction of the major components of sea urchin actin gel. *J. Mol. Biol.* 125:207–224.

Cooley, L., E. Verheyen, and K. Ayers. 1992. *chickadee* encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell.* 69:173–184.

DeRosier, D., and R. Censullo. 1981. Structure of F-actin needles from extracts of sea urchin oocytes. *J. Mol. Biol.* 146:77–99.

DeRosier, D., E. Mandelkow, A. Stillman, L. Tilney, and R. Kane. 1977. Structure of actin-containing filaments from two types of non-muscle cells. *J. Mol. Biol.* 113:679–695.

Gutzeit, H., and A. Strauß. 1989. Follicle cell development is partly independent of germ-line cell differentiation in *Drosophila* oogenesis. *Roux Arch. Dev. Biol.* 198:185–190.

Gutzeit, H. O. 1986. The role of microfilaments in cytoplasmic streaming in *Drosophila* follicles. *J. Cell Sci.* 80:159–169.

Hoover, K., A. Chien, and V. Corces. 1993. Effects of tranposable elements on the expression of the *forked* gene of *Drosophila melanogaster*. *Genetics.* 135:507–526.

Kane, R. 1976. Actin polymerization and interaction with other proteins in temperature-induced gelation of sea urchin egg extracts. *J. Cell Biol.* 71:704–714.

King, R. C. 1970. Ovarian development in *Drosophila melanogaster*. Academic Press, New York. 227 pp.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lees, A. D., and L. E. R. Picken. 1945. Shape in relation to fine structure in the bristles of *Drosophila melanogaster*. *Proc. Roy. Soc. Lond. Ser. B.* 132:396–423.

Lees, A. D., and C. H. Waddington. 1943. The development of the bristles in normal and some mutant types of *Drosophila melanogaster*. *Proc. Roy. Soc. Lond. Ser. B.* 131:87–110.

Maekawa, S., S. Endo, and H. Sakai. 1982. A protein in starfish sperm head which bundles actin filaments *in vitro*: purification and characterization. *J. Biochem.* 92:1959–1972.

Mahowald, A. P., and M. P. Kambysellis. 1980. Oogenesis. *In* The Genetics and Biology of *Drosophila*, Vol. 2D. M. Ashburner and T. R. F. Wright, editors. Academic Press, New York. 141–224.

Mohr, O. 1922. Cases of mimic mutations and secondary mutations in the X chromosome of *Drosophila melanogaster*. *Z. Ind. Abst. Vererb.* 28:1–22.

Montell, D. J., P. Rorth, and A. C. Spradling. 1992. *slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes Drosophila C/EBP. *Cell.* 71:51–62.

Otto, J., and J. Bryan. 1980. The incorporation of actin and fascin into the cytoskeleton of filopodial sea-urchin coelomocytes. *Cell Motility.* 1:179–192.

Otto, J. R. Kane, and J. Bryan. 1979. Formation of filopodia in coelomocytes: localization of fascin in 58,000 dalton actin cross-linking protein. *Cell.* 17:285–293.

Otto, J., R. Kane, and J. Bryan. 1980. Redistribution of actin and fascin in sea urchin eggs after fertilization. *Cell Motility.* 1:31–40.

Otto, J., and T. Schroeder. 1984. Assembly-disassembly of actin bundles in starfish oocytes: an analysis of actin-associated proteins in the isolated cortex. *Dev. Biol.* 101:263–273.

Overton, J. 1967. The fine structure of developing bristles in wild type and mutant *Drosophila melanogaster*. *J. Morph.* 122:367–380.

Paterson, J., and K. O'Hare. 1991. Structure and transcription of the *singed* locus of *Drosophila melanogaster*. *Genetics.* 129:1073–1084.

Perrimon, N., and M. Gans. 1983. Clonal analysis of the tissue specificity of recessive female-sterile mutations of *Drosophila melanogaster* using a dominant female-sterile mutation *Fs(1)K1237*. *Dev. Biol.* 100:365–373.

Petersen, N., D. H. Lankenau, H. Mitchell, P. Young, and V. Corces. 1994. Forked proteins are components of fiber bundles present in developing bristles of *Drosophila melanogaster*. *Genetics.* 136:173–182.

Roiha, H., G. Rubin, and K. O'Hare. 1988. P Element insertions and rearrangements at the *singed* locus of *Drosophila melanogaster*. *Genetics.* 119:75–83.

Schweisguth, F., and J. Posakony. 1992. *Suppressor of hairless*, the *Drosophila* homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell.* 69:1199–1212.

Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene.* 67:31–40.

Spradling, A. 1994. Developmental genetics of oogenesis. *In* The Development of *Drosophila melanogaster*. Vol. 1. M. Bate and A. Martinez-Arias, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1–70.

Spudich, J., and L. Amos. 1979. Structure of actin filament bundles from microvilli of sea urchin eggs. *J. Mol. Biol.* 129:319–331.

Spudich, J., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246:4866–4871.

Stokes, D., and D. DeRosier. 1991. Growth conditions control the size and order of actin bundles *in vitro*. *Biophys. J.* 59:456–465.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedures and applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Verheyen, E., and L. Cooley. 1994. Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. *Development.* In press.

Xue, F., and L. Cooley. 1993. *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell.* 72:681–693.

Yamashiro-Matsumura, S., and F. Matsumura. 1985. Purification and characterization of an F-actin-bundling 55-kilodalton protein from HeLa cells. *J. Biol Chem.* 260:5087–5097.

Yamashiro-Matsumura, S., and F. Matsumura. 1986. Intracellular localization of the 55-kD actin bundling protein in cultured cells: spatial relationship with actin, alpha-actinin, tropomyosin, and fimbrin. *J. Cell Biol.* 103:631–640.