Actin Organization, Bristle Morphology, and Viability Are Affected by Actin Capping Protein Mutations in *Drosophila*

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Abstract. Regulation of actin filament length and orientation is important in many actin-based cellular processes. This regulation is postulated to occur through the action of actin-binding proteins. Many actin-binding proteins that modify actin in vitro have been identified, but in many cases, it is not known if this activity is physiologically relevant. Capping protein (CP) is an actin-binding protein that has been demonstrated to control filament length in vitro by binding to the barbed ends and preventing the addition or loss of actin monomers. To examine the in vivo role of CP, we have performed a molecular and genetic characterization of the β subunit of capping protein from *Dro-sophila melanogaster*. We have identified mutations in the *Drosophila* β subunit—these are the first CP mutations in a multicellular organism, and unlike CP mutations in yeast, they are lethal, causing death during the early larval stage. Adult flies that are heterozygous for a pair of weak alleles have a defect in bristle morphology that is correlated to disorganized actin bundles in developing bristles. Our data demonstrate that CP has an essential function during development, and further suggest that CP is required to regulate actin assembly during the development of specialized structures that depend on actin for their morphology.

T HE actin cytoskeleton is important for many cellular processes. The organization of the actin cytoskeleton is thought to be under the spatial and temporal control of actin-binding proteins that mediate the polymerization/depolymerization of actin filaments and their assembly into diverse supramolecular structures. Most of the support for this hypothesis comes from numerous biochemical studies that have described how different actin-binding proteins modify the state of actin filaments in vitro. In many cases, however, the role of a particular protein within the cell remains obscure. Genetic studies are being used to determine if the in vivo function of a given actin-binding protein is related to its defined in vitro properties, and if so, if this function is essential to the organism. For example, in the mouse, gene knockouts have highlighted the relative importance of profilin, a monomer-binding protein, vs. gelsolin, a severing and barbed-end capping protein: the profilin knockout is lethal (Witke, W., A.H. Sharpe, and D.J. Kwiatkowski. 1993. *Mol. Biol. Cell.* 4S:1499), whereas the gelsolin knockout survives and has a very mild phenotype (Witke et al., 1995).

Capping protein (CP,[1] also CapZ in muscle; Casella et al., 1987; Schafer et al., 1995) is an example of such a protein that was initially identified and characterized by biochemical methods. CP is an αβ heterodimer that binds to and "caps" the fast-growing (barbed) end of the actin filament. This capping activity stabilizes the length of the filament by inhibiting the addition and loss of actin monomers (reviewed in Schafer and Cooper, 1995). In vivo studies have shown that CP contributes to the assembly and organization of actin in yeast (Amatruda et al., 1992), the assembly of thin filaments in chicken skeletal muscle (Schafer et al., 1993, 1995), and the control of actin assembly and cell motility in *Dictyostelium* (Hug et al., 1995). While these studies suggest that CP is a key regulator of actin polymerization in both muscle and nonmuscle cells, each of these experimental systems has limitations. In particular, CP function in vivo has yet to be studied in a multicellular organism containing many differentiated tissues with specialized actin structures. *Drosophila* provides an attractive model system for such studies. A number of recent reports have established the importance of actin-binding proteins in the development of a variety of tissues, including bristles (Cant et al., 1994; Petersen et al., 1994; Verheyen and Cooley, 1994; Tilney et al., 1995) and oocytes (for a review, see Cooley and Theurkauf, 1994). Such studies are providing significant new insights into the in vivo function of actin-binding proteins.

In this report, we describe our molecular and genetic characterization of the β subunit of CP in *Drosophila melanogaster*. We demonstrate that *Drosophila* CP is the

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1. *Abbreviations used in this paper:* AP; alkaline phosphatase; CP, capping protein; EMS, ethylmethanesulfonate; UTR, untranslated region.

functional homologue of other CPs by virtue of its ability to substitute for yeast CP in vivo, and by its localization to the Z discs of skeletal muscle. We have identified mutations in the β subunit gene; these are the only CP mutations in a multicellular organism, and they cause death during the early larval period. This lethality is the first demonstration that a barbed-end capping protein has an essential function. Phenotypic characterization of these mutants has revealed that CP is important in actin-mediated processes, including the organization of the actin bundles present in the developing bristle. Thus, our genetic studies have demonstrated that CP modifies actin structures in vivo, and is essential to the organism.

Materials and Methods

Cloning and Sequencing

Standard protocols for cloning, library screens, and Southern and Northern blots were derived from Sambrook et al. (1989). A genomic library made from the Canton-S wild-type strain (Maniatis, et al., 1978) was probed with CP β subunit cDNA clones from nematode (Waddle et al., 1993, kindly provided by J. Waddle, Department of Genetics, Washington University School of Medicine, St. Louis, MO) and chicken (Caldwell et al., 1989) at low stringency (3× SSC, 65°). Several genomic clones were obtained. Partial DNA sequencing revealed that one of these clones contained the CP β subunit gene, and a fragment of this clone was used to screen a Drosophila cDNA library made from adult heads (Itoh et al., 1986). Two cDNA clones were isolated. One of these appears to be near or at full length; it is 1.47 kb in length and includes a 107-nucleotide 5' untranslated region (UTR), an open reading frame of 276 amino acids, a 519-nucleotide 3' UTR, and a 17-nucleotide poly A tail. The other clone appears to be identical in its 5' start site and nucleotide sequence, but is truncated in the 3' UTR. Sequencing of the CP β subunit gene was carried out with both double- and single-stranded templates using the chain termination method (Sanger et al., 1977) with Sequenase (United States Biochemical Inc., Cleveland, OH). In places with strong secondary structure, cycle sequencing was performed with the fmol kit (Promega Biological Research Products, Madison, WI). Both strands of the coding region were sequenced; complete coverage was obtained by primer walking. The region between nucleotides 870 and 890 proved extremely difficult to sequence because of the presence of strong "stops"—we report the best consensus from multiple sequencing reactions. The nucleotide sequence is available from GenBank under accession number U35420.

Yeast Induction and Phalloidin Staining

To express Drosophila CP in yeast, we used the shuttle vector pBJ319. pBJ319 is a yeast expression vector that produces a glutathione S-transferase (GST) fusion protein under control of the GAL1 promoter. In addition, a second protein may be coexpressed from the GAL10 promoter. pBJ319 carries the 2-μm replication origin and the selectable marker LEU2. This vector and related vectors are available from American Type Culture Collection (Rockville, MD) and were constructed by Christopher Hug (Dept. of Cell Biology, Washington University, St. Louis, MO) using the pRS420 series of vectors (Christianson et al., 1992), the GAL1/10 promoter (Johnston and Davis, 1984), and the GST cassette from pGEX-3X (Smith and Johnson, 1988). An EcoRI fragment containing the full-length CP β cDNA was ligated to pBJ319 DNA that was partially cleaved with EcoRI and gel purified to obtain the full-length linear population. A recombinant plasmid containing an insert at the correct EcoR1 site (downstream of the GAL10 promoter) and in the correct orientation was identified by restriction digest, and this clone was designated pBJ319-Dmβ. Three partial cDNA clones encoding the Drosophila CP α subunit were obtained from a male larval cDNA library (Elledge, S.J., personal communication) using the yeast two-hybrid system (Durfee et al., 1993). The longest cDNA contains the complete α-coding region, except for ~30 residues at the amino terminus (Muhua, L., R. Hopmann, J.A. Cooper, and K.G. Miller, unpublished results). This cDNA was excised from the two-hybrid vector by a double digest with BamHI and BglII, gel purified, ligated to pBJ319-Dmβ that was partially cleaved with BamHI and gel puri-

fied to isolate the linear population. A recombinant with the α gene in the correct BamHI site and in the correct orientation was identified by restriction digest, and the final product designated pBJ319-Dmαβ.

The CP double-null strain YJC170(MATα cap1-Δ1::TRP1 cap2-Δ1:: HIS3 ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 rho⁺) was transformed with pBJ319-Dmαβ using a standard lithium acetate protocol (Ito et al., 1983), and transformants were selected for leucine prototrophy.

To test for the ability of Drosophila CP to rescue the yeast CP null phenotype, overexpression of the Drosophila heterodimer was induced. Parallel cultures of the transformed strain were established by diluting an overnight culture 1/100 into glucose-free, Leu⁻ medium and grown 6–8 h. One of the cultures was induced by the addition of 2% galactose (Amatruda et al., 1992), and growth was continued until the cultures reached an OD_{600} of 0.3–0.6 (~12 h). Cells were fixed with formaldehyde and stained with rhodamine-phalloidin (Sigma Chemical Co., St. Louis, MO) to visualize filamentous actin (Amatruda et al., 1990).

Production of Anti-β Subunit Antibodies

Rabbit and rat polyclonal antibodies recognizing the CP β subunit were generated by immunization with an insoluble GST-β subunit fusion protein. The crude serum was affinity purified against a maltose-binding protein-CP β subunit fusion protein (New England Biolabs Inc., Beverly, MA; Guan et al., 1987; Maina et al., 1988) that was coupled to Affi-gel 10 (Bio Rad Laboratories, Hercules, CA). Bound antibodies were eluted with 0.2 M glycine, pH 2.4, and immediately neutralized with 2 M Tris pH 8.8.

Immunoblots

Immunoblots were performed essentially as described in Kellerman and Miller (1992) with the following modifications. Protein samples were fractionated on 12.5% SDS-polyacrylamide gels and electro-transferred onto 0.1-μm nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) in 50 mM Tris/380 mM glycine/0.1% SDS/20% methanol. Filters were blocked in TBS/0.1% Tween 20/5% dry milk for 30–60 min and then incubated with the affinity-purified rat primary antibody at ~2 μg/ml in blocking solution. In one experiment, α-tubulin was used for a loading control, and was detected with hybridoma supernatant from a mouse monoclonal line recognizing α-tubulin (gift from Doug Kellogg and Bruce Alberts, University of California, San Francisco) at a dilution of 1:10. Immunopositive bands were detected with an alkaline-phosphatase (AP)-conjugated goat anti-rat secondary antibody (Cappel Research Products, Durham, NC) at 1:1,000 dilution or AP-conjugated goat anti-mouse (Boehringer Mannheim, Indianapolis, IN) at 1:5,000 dilution, as well as with standard color development reagents (BioRad Laboratories). To measure the relative amounts of CP β present in wild-type, Df(2) ast2/SM1, and $cpb^{6.15}/cpb^{F19}$ adults, extracts from adult males were immunoblotted and doubly probed for CP β and α-tubulin as described above. Quantitation was done by capturing an image of the blot with a CCD camera and quantifying the bands with Bitimage 1.5.5 software (NIH Image shareware). The integrated density of each band (within a defined area) was measured, then the value for the CP β band was normalized to the value for α-tubulin in the same lane. These normalized values for CP were then expressed as a percentage of wild type. The background signal on the blot was negligible and so was not subtracted.

In Situ Hybridization to Polytene Chromosomes

The full-length CP β cDNA clone was used as a hybridization probe to salivary gland chromosomes from wild-type (the isoA derivative of Canton-S, provided by Ian Duncan, Department of Biology, Washington University, St. Louis) third instar larvae using procedures developed by the Ashburner laboratory (Department of Genetics, University of Cambridge, Cambridge, England) and described in Wallrath and Elgin (1995).

Screen for Mutations in the β Subunit Gene

Flies were raised at 25° on standard corn meal medium (Lewis, 1960). Information on genetic markers and balancer chromosomes may be found in Lindsley and Zimm (1992). The deficiency chromosomes Df(2) ast2 and Df(2) dp⁷⁹ᵇ, obtained from the Drosophila Stock Center (Bloomington, IN) were demonstrated to remove the CP β subunit gene by Southern blot as follows. Genomic DNA prepared from heterozygous deficiency flies was simultaneously hybridized with a probe for the CP β gene and a probe for ftz, a known single-copy gene. The intensities of the CP β–specific band in the deficiency lanes were compared to those in the wild-type lane;

when normalized for loading to the *ftz* band, the CP β–specific band was half as intense in the deficiency lanes, demonstrating that the CP β gene was indeed missing from these deficiency chromosomes (data not shown).

The isogenic *shv b cn bw* stock used in the mutagenesis was provided by Clarissa Cheney (Department of Genetics, Washington University School of Medicine). *shv b cn bw* males were mutagenized with ethylmethanesulfonate (EMS; Sigma Chemical Co.) according to the procedure of Lewis and Bacher (1968) and mated to SM6, *Cy/Sco* virgin females. F1 male progeny of the genotype *shv b cn bw*/*SM6, *Cy* were mated individually in vials to Df(2) *ast2/*SM1, *Cy* virgin females, and the progeny were examined for the presence or absence of straight-winged flies, which correspond to the genotype *shv b cn bw*/*Df (2) *ast2*. The absence of this class of progeny in a particular vial suggested that a lethal mutation was generated on the mutagenized chromosome within the defined interval containing the CP β subunit gene. Candidate chromosomes were retested for lethality over Df(2) *dp*[79b], and those that were lethal over both deficiencies were used in complementation tests as described in the text.

Generation of β Subunit Transgenic Line

A 4.5 kb EcoRI genomic fragment containing the entire CP β subunit gene (Fig. 6) was inserted into the EcoRI site of the YES P-element transformation vector, which is marked with y^+ (Patton et al., 1992). DNA preparation and injection were performed essentially as described by Spradling (1986). YES-CP β DNA was coprecipitated with "helper" DNA (the transposase source pπ25.7) and resuspended in injection buffer at a concentration of 300 ng/μl for the P element construct and 50 ng/μl for the helper, and injected into yw^{67C23} host embryos. One y^+ survivor that transmitted the transgene to its progeny was obtained. This line, designated yw^{67C23}, P[YES-β], has the transgene inserted into the X chromosome and is homozygous viable.

Indirect Immunofluorescence Microscopy

Myofibrils were prepared from adult thoraces and incubated with antibodies as described by Saide et al. (1990). The primary antibody, affinity-purified rabbit polyclonal antibodies recognizing the CP β subunit, was used at a concentration of ~10 μg/ml, and the secondary antibody, rhodamine-conjugated donkey anti–rabbit IgG (Chemicon International, Inc., Temecula, CA) was used at a dilution of 1:200. The tissue was counterstained with FITC-phalloidin (Sigma Chemical Co.) at a concentration of 0.1 μg/ml.

Developing bristles were examined in mutant and control pupae derived from mating *shv cpb*[6.15] *b cn bw/cpb*[F19] *cn bw sp* males to *cpb*[F19] *cn bw sp/Gla Bc* females. *shv cpb*[6.15] *b cn bw/cpb*[F19] *cn bw sp* mutant progeny were selected just after puparium formation based on the absence of the *Bc* marker (see Lindsley and Zimm, 1992, for description of *Bc* phenotype). Sibling pupae (*Gla Bc/cpb*) were used as controls. The dorsal epithelial tissue from 44–50-h pupae was dissected and fixed as described in Cant et al. (1994), except that tissue was fixed in 3.7% formalin in PBS instead of paraformaldehyde. To visualize actin alone, the pelts were fixed for 30 min at room temperature and then stained with rhodamine-phalloidin (Sigma Chemical Co.) at a concentration of 0.5 μg/ml. To double label for CP and actin, pelts were fixed as above, but for 10 min instead of 30 min, since the longer fixation interfered with CP staining. To visualize CP, the tissue was incubated with the affinity-purified rabbit polyclonal antibody described above at ~10 μg/ml overnight at 4°C, washed 3 × 10 min, and then incubated with the rhodamine secondary antibody described above overnight at 4°C. After washing, the tissue was counterstained with 1 μg/ml FITC-phalloidin for 2 h at room temperature.

Tissues were mounted in 90% glycerol/PBS + 1 mg/ml paraphenylenediamine and imaged on a Diaphot inverted microscope (Nikon Inc., Melville, NY) with epifluorescence illumination. Images were photographed with hypersensitized Tech-Pan film (Eastman Kodak Co., Rochester, NY).

Phenotypic Analysis of Bristles

The bristle phenotype of the viable *cpb*[6.15]/*cpb*[F19] mutant flies was quantitated in two ways. First, 50 mutant and 31 control (*shv b cn bw*) flies were scored for the number of bristles that expressed a bent or split phenotype. Second, the length of the posterior sternopleural bristle was measured in mutant (*n* = 20) and control (*n* = 15) flies. Measurements of live anesthetized flies were made under the dissecting microscope to the nearest 0.025 mm, using a device ruled in 0.1-mm divisions.

To compare numbers of actin bundles in the developing bristles of mutant and control pupae, animals were dissected, fixed, and stained with rhodamine-phalloidin as described above. The ocellar bristles were selected for analysis because they are large and easily identifiable in pupae. Estimates of the number of bundles were obtained by focusing through the specimen and summing the number of bundles visible in each focal plane near the base of the bristle.

Scanning EM

Adult flies were anesthetized with CO_2 and then transferred to preservative (25% glycerol in 95% ethanol). The wings were removed and the abdomens were punctured, and the flies were left in this preservative for at least 2 d. The flies were then completely dehydrated by passing through an ethanol series: 70% ethanol for 15 min; 95% ethanol for 15 min, and three changes of 100% ethanol for 15 min each (Rose, 1984). The ethanol was then exchanged with four changes of hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, PA) to prevent distortion of the specimens as they dried. The specimens were allowed to dry overnight in a fume hood, mounted on stubs with white glue, sputter-coated with gold to a thickness of ~500 Å in a coating unit (E5000; Polaron Equipment Ltd., Watford, Hertfordshire, England), and imaged in a scanning EM (S-450; Hitachi Instruments, Inc., San Jose, CA) operated at a 20-kV accelerating voltage.

Results

The Drosophila CP β Subunit Is Encoded by a Single-copy Gene and Shows a High Degree of Similarity to the β Subunits of Other Organisms

As the first step in generating mutations in CP, we cloned the CP β gene from *Drosophila* libraries. A genomic clone containing the CP β gene was isolated by screening a genomic library with heterologous probes. This genomic clone was used to obtain two cDNA clones, one of which appears to be full length. Genomic blots probed with the full-length cDNA and washed at reduced stringency did not show any additional bands that would suggest multiple β genes (data not shown).

Sequence analysis indicates that this gene encodes the CP β homologue of *D. melanogaster*. The open reading frame predicts a protein of 276 amino acids with a molecular mass of 31.3 kD (Fig. 1 *A*). This is consistent with the mobility of the protein in SDS-PAGE gels (see below).

The *Drosophila* CP β subunit is similar to β subunits from other species (Table I). Particularly striking is the degree of conservation between the *Drosophila* and vertebrate β subunits. The high degree of similarity is distributed throughout the protein, with the exception of the carboxy terminus. In vertebrates, two isoforms of the β subunit are generated by alternative splicing, producing a muscle (β1) and a nonmuscle (β2) isoform that diverge at amino acid 246 (Schafer et al., 1994). The *Drosophila* sequence is 86% identical to the vertebrate before amino acid 246, but then diverges at the same place as the β1 and β2 isoforms and is similar to neither after that point; nor is it very similar to any of the other invertebrate β subunits (Fig. 1 *B*).

We see no evidence of multiple β isoforms in *Drosophila*. Two-dimensional Western blots of adult or late embryo extract detect only one isoform of CP β, and PCR amplification of the cDNA and genomic clones with primers that flank the point of isoform divergence in other species yields products of exactly the same size (data not

A

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CCCGAAATCCTAGCTAGAGTTTAAGATCGGCCCTGCCCAGCCAGCGAACGCAGGCCCCTAACATAAACAG  70
TGTAAAACTATCGAAACAAGGGAAAGCAGAAAGTACCAAAATGTCGGAAATGCAGATGGATTGTGCTTTG  140
                                        M  S  E  M  Q  M  D  C  A  L
GATCTGATGCGGAGGCTGCCGCCCCAGCAGATCGAGAAGAACCTCATTGATCTGATAGACTTGGCACCGG  210
 D  L  M  R  R  L  P  P  Q  Q  I  E  K  N  L  I  D  L  I  D  L  A  P
ATCTCTGCGAGGACTTGCTCTCCTCCGTGGACCAGCCGCTGAAGATCGCCAAGGACAAGGAGCACGGCAA  280
 D  L  C  E  D  L  L  S  S  V  D  Q  P  L  K  I  A  K  D  K  E  H  G  K
GGACTATCTGCTGTGCGACTATAACCGGGATGGGGACTCCTACAGATCGCCCTGGTCGAACTCCTACTAT  350
 D  Y  L  L  C  D  Y  N  R  D  G  D  S  Y  R  S  P  W  S  N  S  Y  Y
CCGCCGCTGGAGGATGGCCAAATGCCCTCGGAACGACTGCGCAAACTGGAAATCGAGGCGAACTATGCCT  420
 P  P  L  E  D  G  Q  M  P  S  E  R  L  R  K  L  E  I  E  A  N  Y  A
TCGATCAGTACAGGGAGATGTACTACGAGGGAGGCGTCTCCTCCGTCTACCTATGGGATCTGGATCACGG  490
 F  D  Q  Y  R  E  M  Y  Y  E  G  G  V  S  S  V  Y  L  W  D  L  D  H  G
GTTTGCCGCCGTTATACTGATCAAAAAGGCGGGAGATGGCAGCAAGATGATCCGCGGCTGCTGGGATTCC  560
 F  A  A  V  I  L  I  K  K  A  G  D  G  S  K  M  I  R  G  C  W  D  S
ATCCATGTGGTCGAGGTACAGGAGAAGACCACCGGCAGGACGGCCCACTACAAGCTCACCTCCACGGCAA  630
 I  H  V  V  E  V  Q  E  K  T  T  G  R  T  A  H  Y  K  L  T  S  T  A
TGCTCTGGCTGCAGACCAACAAACAGGGTTCGGGAACCATGAATCTGGGCGGATCCCTCACCCGGCAGCA  700
 M  L  W  L  Q  T  N  K  Q  G  S  G  T  M  N  L  G  G  S  L  T  R  Q  Q
GGAGCAGGACGCCAACGTCAGCGAGTCGTCGCCGCACATCGCCAACATTGGCAAGATGGTCGAGGAGATG  770
 E  Q  D  A  N  V  S  E  S  S  P  H  I  A  N  I  G  K  M  V  E  E  M
GAGAACAAGATCAGGAACACCCTGAACGAGATATACTTTGGCAAGACCAAGGACATCGTGAACGGACTAA  840
 E  N  K  I  R  N  T  L  N  E  I  Y  F  G  K  T  K  D  I  V  N  G  L
GGAGCACACAATCGCTGGCCGATCAGCGCCAGCAGGCGGCCATGAAGCAGGACCTCGCAGCGGCAATCCT  910
 R  S  T  Q  S  L  A  D  Q  R  Q  Q  A  A  M  K  Q  D  L  A  A  A  I  L
GCGACGCAATGTCAAGCCCGAATCGAACTGAGTACTGAGCGGCTGAAGGCTGAATTTCCACATAAACCAA  980
 R  R  N  V  K  P  E  S  N  .
TCCAGTCCAGGGGCGTCCCACAAGTAGGCTGCTCCCAAGAAGAGCGCAGGAATATGCGAATTGCAAGGCA  1050
CTGTCGTCGCCACTAAATATTGTCATAAATAGAAATAATTTTCATAATATTTCGGCTGCACCCACCCAAT  1120
TCGCTGTTACTCGCCCAATTGTGTTGAGCAGCTCCGTGGAGGACTCCGCTTGGACTGGACTAAAGAGCAG  1190
ACTAAAATATTAATACGCATTCTAGGCAGTAATTGTTACATGAGAATGTACACAGAGAGCACATATGTA  1260
ATTTGCCCGCCGTTCACTGTCCTCCACATGTTGCCGTTGAGTCGCGCTCATCCAGAGTTCAGCCGAAATG  1330
CCAACTTCGCGCCCTCAAGACGAGTATCCATAAGTATTTGTATCTTATTTGTAATAATGCATATTTGCAC  1400
CGAAGTCGAGCACTTGCGTTTCGTTCTTAATCCTTCCGCGAATAAATATTGTGTTTATTAATTGTAAAAA  1470
AAAAAAAAAAAGG  1484
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B

Figure 1. Sequence of the *Drosophila* CP β subunit and comparison of COOH-terminal peptide sequences. (*A*) Nucleotide and amino acid sequence of the CP β cDNA clone are displayed using the MapDraw program (DNA-Star). These sequence data are available from GenBank/EMBL/DDBJ under accession number U35420. (*B*) The MegAlign program (DNAStar), using the Clustal method with the PAM250 residue weight table, was used to align the *Drosophila* carboxy-terminal amino acid sequence with all the other known CP β subunits. The β1 isoforms are the muscle-specific isoforms, and the β2 are the nonmuscle isoforms. Identities to the *Drosophila* sequence are shaded with solid black, and residues conserved to within 2 distance units are stippled. The carboxy-terminal sequence of *Drosophila* does not strongly resemble either vertebrate isoform, nor does it resemble any of the invertebrate proteins.

shown). Thus, we believe that the *Drosophila* CP β gene encodes only one isoform that functions in muscle as well as in nonmuscle tissue, in contrast to the situation in vertebrates.

Table I. Comparison of CP β Subunit Sequence Identity between Drosophila and Other Species

	D. melanogaster
Saccharomyces cerevisiae	46
Dictyostelium discoideum	56
Caenorhabditis elegans	65
Chick β1	79
Mouse β1	79
Chick β2	82
Mouse β2	82
Human β2	82

Sequence comparisons of the entire coding regions were made using the Align program (DNAStar). References and GenBank accession numbers for sequences are as follows: *S. cerevisiae* (Amatruda et al., 1990), M31720; *D. discoideum* (Hartmann et al., 1989), J04958; *C. elegans* (Waddle et al., 1993), Z18854; chick β1 (Caldwell et al., 1989), J04959; chick β2, mouse β1, and mouse β2 (Schafer et al., 1994), U07826, U10406, and U10407; human β2 (Barron-Casella, E.A., et al., 1995).

Evidence that Drosophila CP Is the Functional Homologue of Other CPs

CP has been extensively characterized biochemically in several model systems, including yeast and chicken (reviewed in Schafer and Cooper, 1995). So that we might interpret our genetic results in the context of the available biochemical data, we wished to determine whether *Drosophila* CP was homologous in a functional sense to CP in these organisms.

Rescue of the Yeast CP Mutant. To determine if *Drosophila* CP could substitute for yeast CP in vivo, we expressed *Drosophila* CP in a CP null yeast strain. In a wild-type budding yeast cell, actin is organized into cables in the mother cell and cortical patches in the bud. In CP null cells, the mother cell loses cables but gains cortical patches, since these are no longer polarized to the bud. A yeast strain carrying disruptions in both the α and β genes was transformed with a plasmid expressing both the α and β subunits of *Drosophila* CP from an inducible promoter. In uninduced cells (Fig. 2 *A*), the CP null phenotype is evident: actin cables are absent in the mother cells, and cortical actin patches are present in both the mother and the bud. In induced cells (Fig. 2 *B*), partial rescue of the phe-
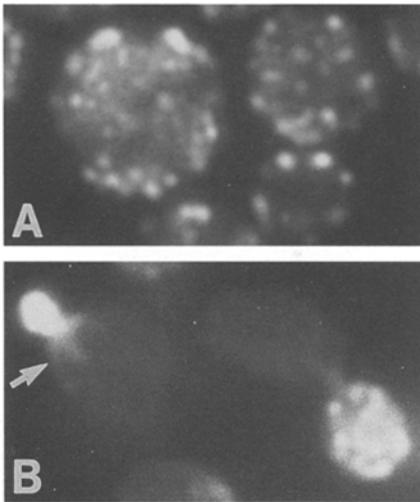
Figure 2. Rescue of yeast CP mutant phenotype with *Drosophila* CP. A yeast strain lacking endogenous CP was transformed with a plasmid expressing *Drosophila* CP from a galactose-inducible promoter. Cells were grown in the absence of galactose, then a portion of the culture was induced by the addition of galactose. Cells were fixed and stained with rhodamine-phalloidin to visualize filamentous actin. (*A*) Uninduced, (*B*) induced. Arrow in *B* indicates mother cell with actin cables restored.

notype is observed. In many cells, the polarized pattern of actin patches is restored, and in some cases, cables can be seen in the mother cells (*arrow*).

Drosophila CP Is Present in the Z Discs of Skeletal Muscle. To look for evidence that *Drosophila* CP is functionally equivalent to chicken CP, we examined its distribution in muscle. Anti-CP antibodies stain the Z discs of thoracic muscle from adult flies (Fig. 3), a distribution similar to chicken striated muscle (Casella et al., 1987).

CP β Is Expressed Throughout Development

To learn at what times during development CP may be important, we examined the expression profile of both the transcript and the protein. A Northern blot shows a doublet of 1.9/1.8 kb at all stages of development (Fig. 4). The two transcripts are likely to be the result of splicing in the 5′ untranslated region (Hopmann, R., and K.G. Miller, unpublished observations). The difference between the size of the transcripts observed on the Northern blot and the length of the cDNA clone (1.5 kb) could result from aberrant migration of the transcript on the gel, possibly due to exclusion by ribosomal RNA, or the presence of a long poly A tail on the transcript. The amount of CP β message remains relatively constant during development, when normalized for loading using a control probe (rp49).

An immunoblot probed with affinity-purified anti–β subunit antibodies (Fig. 5 *A*) exhibited a band of 32 kD at all stages of development. Similar to the transcript, the protein product also appears to be expressed at a fairly constant level, suggesting that CP may function throughout the fly's life cycle.

Mutations in the CP β Gene Are Lethal in Drosophila

To generate mutations in the β subunit gene, first we de-
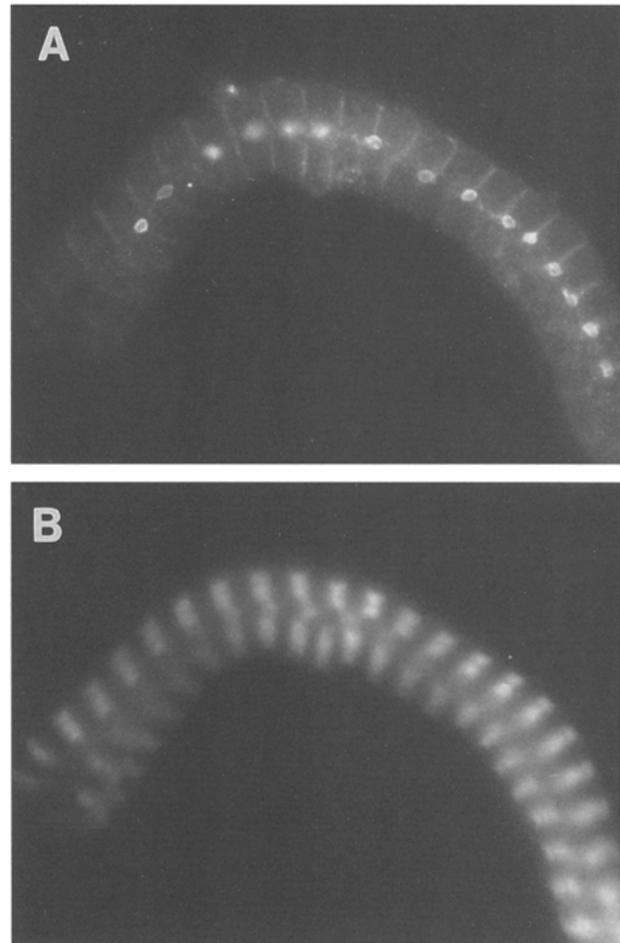
Figure 3. Muscle immunofluorescence. Unfixed skeletal muscle from adult wild-type flies was incubated with the affinity-purified rabbit antibody against the CP β subunit and a rhodamine-conjugated secondary antibody, and then counterstained with FITC-phalloidin, which binds to actin in the I band. The figure shows a tubular muscle, probably a jump muscle. (*A*) Capping protein. (*B*) Actin. CP signal corresponds to the center of the I band, which is the position of the Z disc. The nuclei in the lumen also stain for CP. The presence of CP in nuclei has been reported previously (Ankenbauer et al., 1989; Schafer et al., 1992, 1993), although its function in the nucleus is unknown.

termined the position of the gene by in situ hybridization to polytene chromosomes. A single site of hybridization was detected on polytene band 22A4, on the left arm of the second chromosome. The deficiencies Df(2) *ast2* and Df(2) *dp*[79b] delete the region between 22A2,3 and 22B2,3, based on the reported cytology (Lindsley and Zimm, 1993). We confirmed that these deficiencies delete the β gene by Southern blots (see Materials and Methods).

To determine if loss of CP function was lethal to the fly, a screen for lethal mutations uncovered by the two deficiencies was initiated using the chemical mutagen EMS (see Materials and Methods). We tested 3,300 mutagenized chromosomes and identified 14 mutations that were lethal when heterozygous with either deficiency. We then learned of an existing collection of EMS-induced lethals within the same region (Wieschaus, E., unpublished results). These
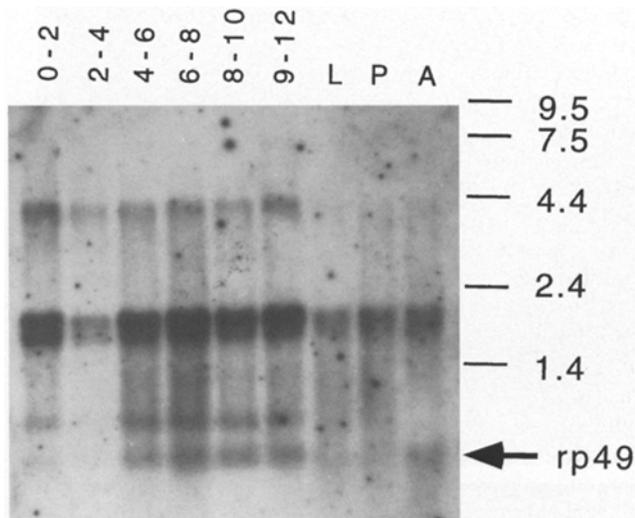
Figure 4. Developmental Northern. Poly A selected RNA from the indicated time periods was electrophoresed on a formalde-hyde-agarose gel, blotted, and probed with a β subunit antisense riboprobe. The positions of the RNA molecular weight standards are indicated to the right, as well as the position of the rp49 loading control. A prominent doublet appears at 1.8/1.9 kb, and fainter bands appear at 4.4 and 1.0 kb. The first five lanes are from embryos of the indicated age, the last three lanes show RNA from larvae (*L*), pupae (*P*), and adults (*A*).

Figure 5. Western blot of wild-type and mutant extracts. (*A*) A developmental immunoblot probed with the affinity-purified rat antibodies recognizing the CP β subunit. 250 μg of total protein was loaded in each lane. The level of CP β remains relatively constant throughout development. Occasionally, a slightly smaller product was observed that most likely represents a breakdown product, since the amount varied between different preparations of the same time point. Lane *1*, 0–2-h embryos; lane *2*, 2–4-h embryos; lane *3*, 0–6-h embryos; lane *4*, 6–12-h embryos; lane *5*, 12–24-h embryos; lane *6*, 3rd instar larvae; lane *7*, late pupae; lane *8*, adult females; lane *9*, adult males. (*B*) An immunoblot of protein extracts from adult males, comparing CP β levels between wild type (lane *1*), hemizygous Df(2) *ast2*/SM1 (lane *2*), and mutant transheterozygote $cpb^{6.15}/cpb^{F19}$ (lane *3*). The equivalent of one male fly was loaded in each lane. The blot was probed with the affinity-purified rat polyclonal as described in *A*, as well as an mAb against α-tubulin as an additional control for loading. The positions of CP β and α-tubulin (*αT*) are indicated to the right of the figure. The filter was imaged with a CCD camera, and the bands were quantified with Bitimage software. The CP β values were normalized to α-tubulin, and then compared to wild type. The hemizygote Df(2) *ast2*/SM1 has 72% of the wild-type level of CP β, and the mutant transheterozygote $cpb^{6.15}/cpb^{F19}$ has 48%.

investigators screened more than 10,000 chromosomes and defined 12 lethal complementation groups that roughly corresponded to our defined interval. All of our alleles were assigned to these complementation groups, and the average number of alleles per group (≥5) suggested that the region had been saturated for lethal genes.

To identify the CP β complementation group, we constructed a β subunit transgene and tested whether it could rescue the lethality of representative alleles from each complementation group when hemizygous with Df(2) *ast2*. The transgene consisted of a 4.5 kb genomic fragment containing the entire CP β transcription unit (Fig. 6) inserted into the YES P element transformation vector (Patton et al., 1992). Of the 12 complementation groups, only alleles from one group were rescued by the β subunit transgene; furthermore, each of the nine alleles in this group could be fully rescued to produce viable, fertile adults. Since these 12 complementation groups probably represent all of the lethal genes in the region, and only one of these can be rescued by the transgene, it follows that the transgene contains only one essential gene. Given the small size of the transforming fragment, it is likely that CP β is the only gene present, and therefore corresponds to the rescued locus.

Additional evidence that these alleles are mutations in the CP β gene came from intragenic complementation tests. One pair of alleles ($cpb^{6.15}/cpb^{F19}$) complemented to produce viable transheterozygous adults that showed reduced levels of CP β compared to wild type (Fig. 5 *B*; see below). From these data, we conclude that we have identified mutant alleles of the CP β subunit. We have designated the gene *cpb*, for capping protein beta.

Analysis of the Mutant Phenotype

Animals that carry a mutant *cpb* allele heterozygous with a deletion of the CP β gene (i.e., hemizygotes) are able to complete embryogenesis and hatch into first instar larvae. The mutant larvae appear morphologically normal, but they are sluggish and die before the first molt.

We examined the transheterozygous adults described above for the phenotypic effects of reduced CP levels. Quantitation of the immunoblot in Fig. 5 *B* showed that the transheterozygous flies have 48% of wild type levels of CP. This contrasts to 72% of wild type for the deficiency heterozygote Df(2) *ast2*/ SM1, which carries one normal copy of the CP β gene. The $cpb^{6.15}/cpb^{F19}$ flies have reduced viability, but display no gross morphological defects, and appear to walk, jump, and fly normally. Males are fertile, but females are semi-sterile and lay small eggs (Hopmann, R., and K.G. Miller, unpublished results); this is a characteristic of the "dumpless" phenotype, where the late stage of cytoplasmic transport to the oocyte is impaired (Spradling, 1993, and references therein).

The most obvious external phenotype of $cpb^{6.15}/cpb^{F19}$ adults affects the bristles. All of the macrochaetae (large bristles) on the thorax and head appear consistently shorter and blunter than the wild type and have a rough contour. To quantitate the altered morphology, we measured the length of one specific bristle, the posterior sternopleural bristle, in mutant and control flies. The posterior sterno-pleural bristle is a relatively large macrochaete present on
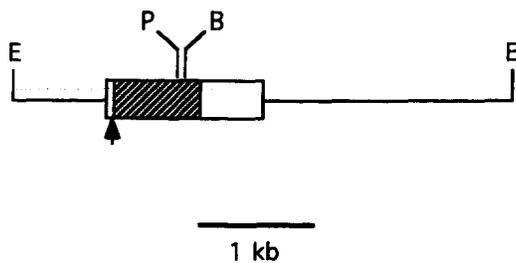
Figure 6. Restriction map of the rescuing genomic fragment of CP β. A 4.5-kb genomic fragment containing the CP β transcription unit was sufficient to rescue the lethality of all the *cpb* alleles. The box represents the transcribed region, and the hatched portion of this box corresponds to the coding region. The arrowhead indicates the position of a ~100-bp intron in the 5′ UTR. *E*, EcoRI; *P*, PstI; *B*, BamHI.

the lateral thorax between the base of the wing and the second leg. The average length of this bristle was 0.24 mm ± 0.05 (n = 20) in mutant flies and 0.30 mm ± 0.01 (n = 15) in control flies. All classes of macrochaetae in mutant flies show similar reductions in length.

In addition to the global changes in morphology noted above, a subset of the macrochaetae express more extreme phenotypes. Examples of these severe phenotypes are shown in Fig. 7. Some macrochaetae bend sharply (*C*), split (*D*), or have a combination of these defects (*B*). Acute bends were most often observed in the scutellar bristles, whereas splitting was often seen in the anterior verticals of the head. Swellings in the midportion of the bristle, termed "knobs" (*H*), were occasionally observed, most often in the posterior sternopleurals. Although most macrochaetae did not express these severe phenotypes, >90% of mutant flies had at least one bent or split bristle, and 50% had two or more (n = 50). This contrasts to the *shv b cn bw* control strain, in which these phenotypes were never observed (n = 31).

The most severely affected bristles also tend to display a striking abnormality in the pattern of surface grooves. The grooves in the mature bristle reflect the position of actin bundles that are present during bristle morphogenesis, but they disappear after bristle elongation has been completed (Overton, 1967; Appel et al., 1993). In wild type bristles, these grooves are straight and run longitudinally (Fig. 7 *E*), consistent with the longitudinal arrangement of the actin bundles. In the most strongly affected bristles of the $cpb^{6.15}/cpb^{F19}$ transheterozygote, these grooves are highly irregular (Fig. 7, *F–H*). They appear more numerous and truncated, they branch or merge with other grooves, and occasionally deviate greatly from the longitudinal axis; in knobs (*H*), the grooves are perpendicular to the long axis. It is often the case that the grooves are relatively normal at the base of the bristle, but become more disorganized toward the tip (Fig. 7, *D* and *F*).

To test the hypothesis that the abnormalities reflect aberrant actin organization, we examined the actin bundles in developing bristles of transheterozygous mutant pupae and compared them to controls. Dorsal epithelial pelts from pupae aged 44–50 hours were dissected, fixed, and stained with rhodamine-phalloidin to visualize the actin bundles (Fig. 8). Panels *A–I* show bristles or segments of bristles

from mutant pupae, and were selected to illustrate the range of phenotypes observed. Panels *J* and *K* are from control pupae. Several different types of abnormalities were observed. First, macrochaetae on all portions of the head and thorax showed an increase in the number of actin bundles (Fig. 8, *A–C* vs. *J* and *K*). To quantify this phenotype, we counted the number of actin bundles in one easily identified class of bristle, the ocellar bristle (located on the dorsal surface of the head between the eyes). Ocellar bristles from mutant pupae had an average of 18.0 bundles (n = 8), compared to 11.0 (n = 11) in controls. Second, it was also evident that some actin bundles in the mutant bristles were displaced from the periphery, in contrast to the control bristles, in which all the bundles were tightly apposed to the cell membrane. Finally, the bundles in mutant bristles were variable in thickness. Some bundles appeared thicker, and others were thinner than the bundles in control bristles, which are of uniform thickness. These types of defects, seen in all macrochaetae, probably do not cause the severe adult phenotypes illustrated in Fig. 7; they are more likely to lead to the more common short, blunt, and rough-textured phenotype.

In addition to the differences noted above, a subset of the mutant macrochaetae had actin bundles that were extremely disorganized (Fig. 8, *D–G*), with bundles forming spiral patterns or "knots." In control bristles, the bundles always run parallel to the long axis of the bristle. We would predict that bristles with this type of actin misorganization would give rise to mature bristles with abnormal groove patterns like those shown in Fig. 7. In addition, some branched bristles were observed in the mutant pupae (Fig. 8, *H* and *I*), which are likely to be the precursors of the branched bristles seen in adults. Thus, the abnormal morphology of the mutant bristles can be correlated with the abnormalities of the actin cytoskeleton during bristle morphogenesis.

CP Immunolocalization in Bristles

To examine whether the localization of CP in developing bristles was consistent with the phenotype of the *cpb* mutant bristle, mutant and control pupae aged 44–50 h were double labeled for CP and actin (Fig. 9). Panels *A/A′* and *B/B′* show two different portions of a bristle in a control pupa. The arrowheads delimit regions where the surface of the bristle is in focus and the peripheral actin bundles can be seen (*A′* and *B′*). In these regions, patches of CP are present along the length of the actin bundles (*A* and *B*). Controls labeled with secondary antibody alone did not show this pattern (not shown). The remainder of the image in panels *A/A′* is mostly in cross-section, and emphasizes that these patches of CP are only seen at the periphery, and not in the interior of the bristle. The association of CP with the actin bundles in the developing bristle is consistent with the observance of a bristle phenotype when CP levels are reduced. The distribution of CP patches suggests that capped barbed ends of actin filaments are present all along the length of the bundles, and therefore, continuous actin filaments do not run the entire length of the bristles.

$cpb^{6.15}/cpb^{F19}$ mutant pupae were also examined to determine the pattern of CP staining in developing bristles.
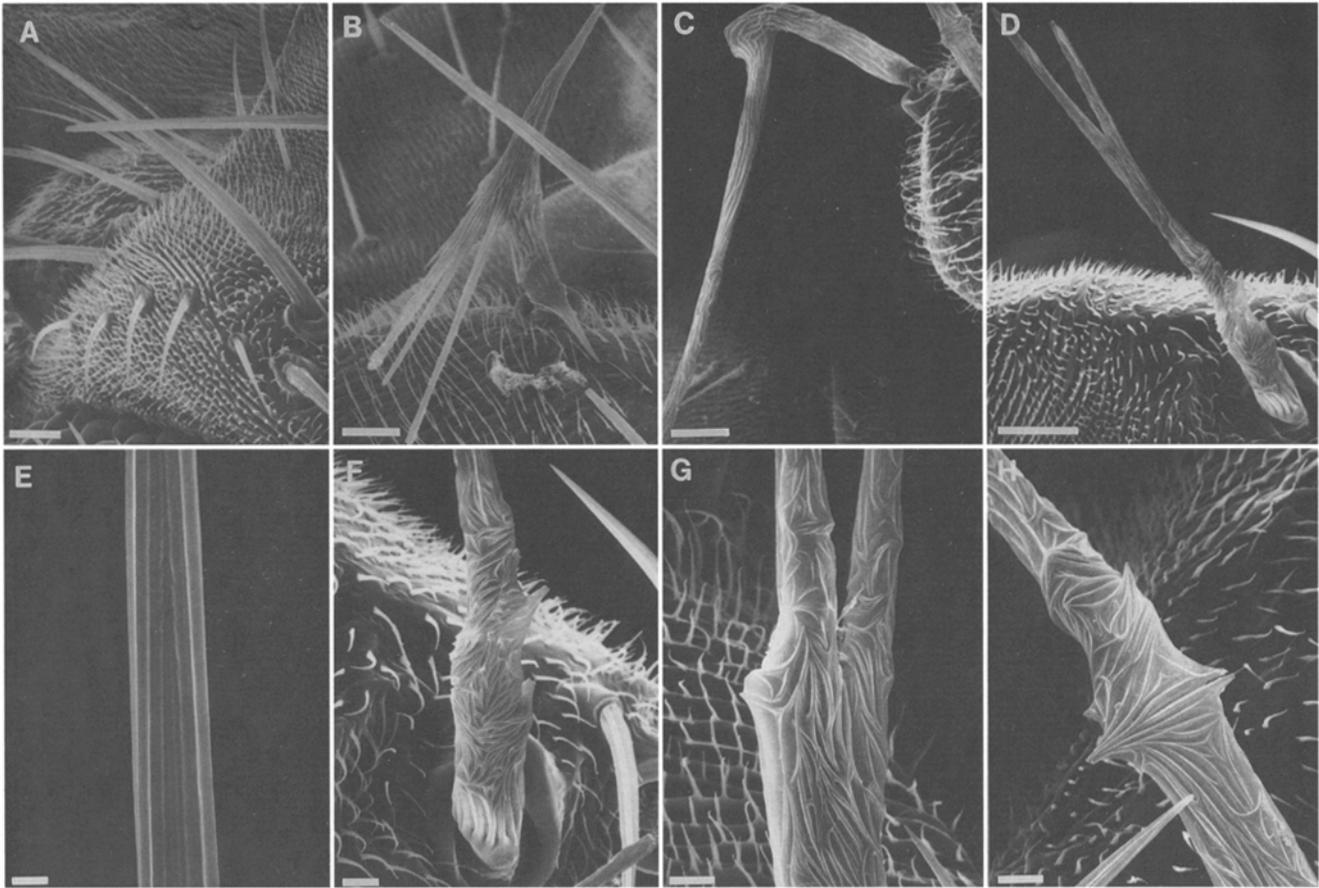
Figure 7. Examples of the more severe bristle phenotypes observed in $cpb^{6.15}/cpb^{F19}$ transheterozygotes. Scanning electron micrographs of macrochaetae from Oregon R (*A* and *E*) and mutant (*B–D* and *F–H*) adults. *B–D* illustrate the extreme bending and branching observed in a subset of macrochaetae. A relatively normal macrochaete is also visible in *B*. *E–H* emphasize the highly disorganized pattern of surface ridges observed in severely affected bristles; note bristle in *H* which has several grooves that are perpendicular to the long axis of the bristle. *F* is a higher magnification of the bristle in *D*, and it shows a short segment of normal-appearing grooves at the base that become disorganized distally. Bar, 20 μm in *A–D* and 5 μm in *E–H*.

Fig. 9, *C/C'* and *D/D'*, show two different focal planes of a double-labeled mutant bristle. CP patches are observed and show an association with the actin bundles as seen in control animals (*arrowheads*). Although the immunoblot of extract from $cpb^{6.15}/cpb^{F19}$ adults shows a 52% reduction in CP β levels (Fig. 5 *B*), we would not expect to easily detect this decrease by immunofluorescence. Since the CP present in mutant bristles is organized normally, it is likely that the reduction in the amount of CP is what causes the abnormalities in the actin cytoskeleton.

Discussion

All eukaryotic cells require the correct organization and function of cytoskeletal elements to maintain their shape, to undergo division, to transport cytoplasmic components, to associate and communicate with other cells, to change shape, and to move. To provide insight into the actin cytoskeleton's contribution to cellular physiology, we are studying the structure, function, and regulation of the actin cytoskeleton during *Drosophila* development. In particular, we have focused on actin-binding proteins that play critical roles in actin structure and function. Capping

protein/CapZ, a barbed-end actin capping protein, is of particular interest because it has been implicated as an important regulator of actin organization in many different types of cells. Our studies demonstrate that this is indeed the case. CP function is essential for viability in *Drosophila*, and is required for the morphogenesis of specialized actin structures.

CP Is an Essential Actin-binding Protein

To determine if CP is essential in *Drosophila*, we undertook a screen for lethal mutations in the β subunit. We identified nine alleles of *cpb* that are lethal when hemizygous with a deficiency removing the gene. The lethality of *cpb* mutations in *Drosophila* is in itself informative—it is the first demonstration of an essential function for any barbed-end capping protein, and argues that the barbed-end capping activity of CP measured in vitro is physiologically relevant in vivo. This is in contrast to yeast, in which CP mutations are not lethal, and cause only a mild impairment in growth (Amatruda et al., 1992). This result also contrasts with experiments on gelsolin, a severing/barbed–end capping protein. Null mutations of gelsolin in mice
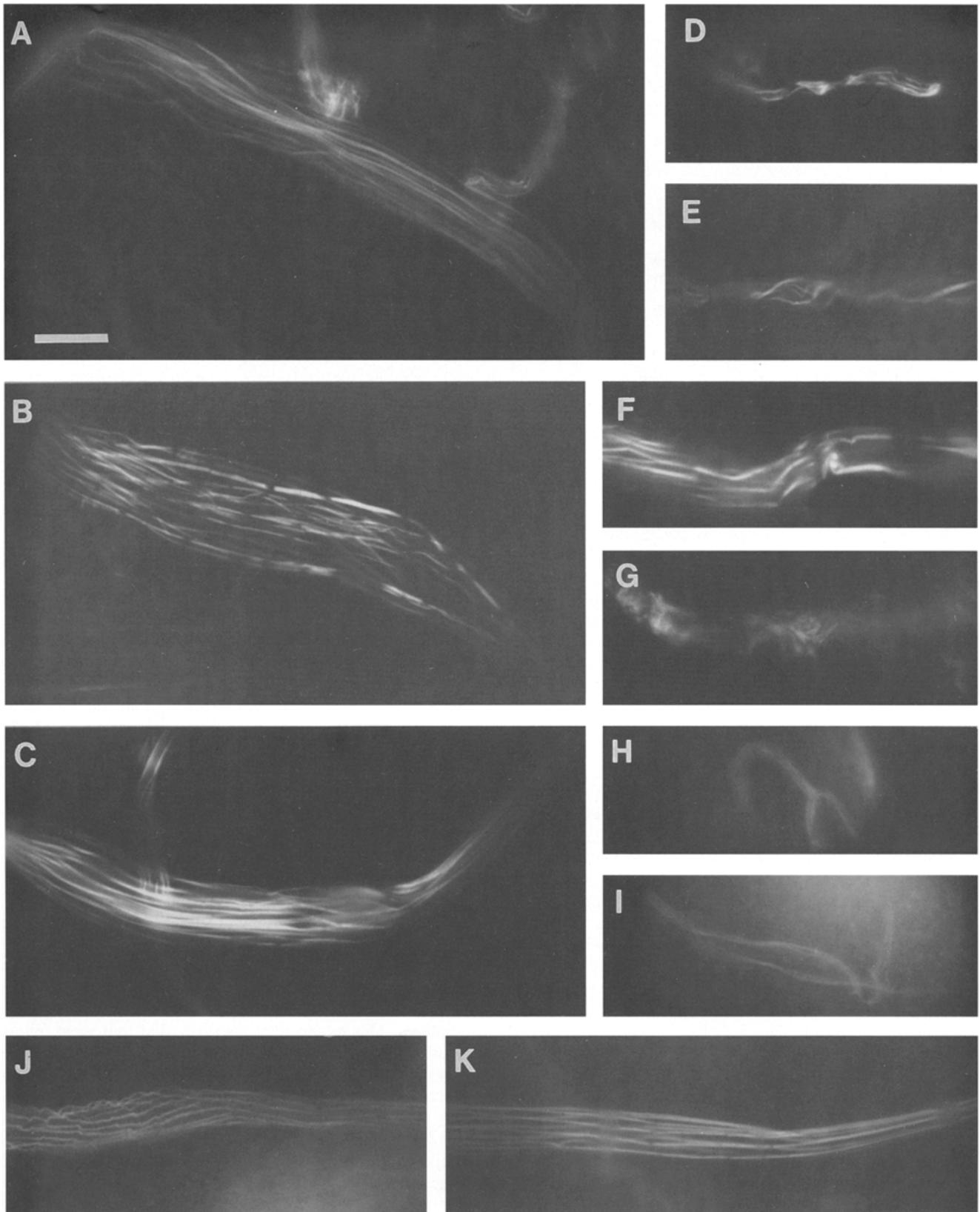
Figure 8. Phalloidin staining of actin bundles in pupal bristles. This figure shows the range of phenotypes observed in bristles from $cpb^{6.15}/cpb^{F19}$ pupae (*A–I*). *J* and *K* are of control pupae, $cpb^{6.15}/Gla\ Bc$ or $cpb^{F19}/Gla\ Bc$. *A–C* illustrate the increase in the number of actin bundles as well as variability in bundle thickness. *D–I* were selected to show examples of the more extreme disorganization of the actin bundles seen in a subset of macrochaetae; pupal bristles of this type are hypothesized to develop into adult bristles with phenotypes such as those shown in Fig. 7. *D–G* highlight the disorganization of the bundles. *H* and *I* show examples of branching. Bar, 10 μm.
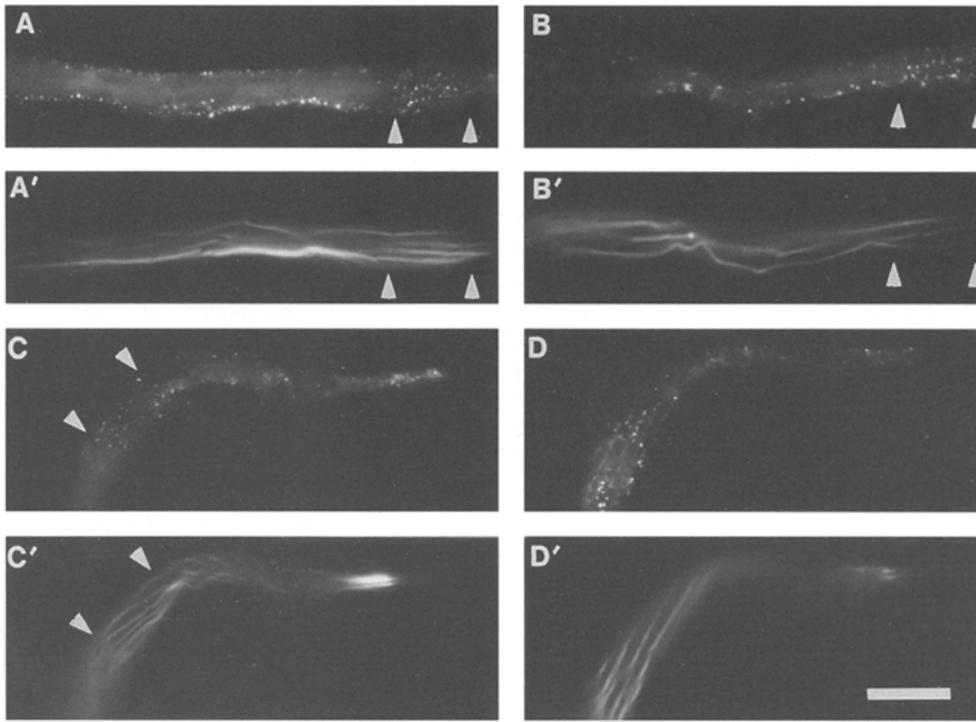
Figure 9. Colocalization of CP and actin. Control pupae (*cpb*$^{6.15}$*/Gla Bc* or *cpb*^{F19}*/Gla Bc*, *A/A'* and *B/B'*) and mutant pupae (*cpb*$^{6.15}$*/cpb*^{F19}, *C/C'* and *D/D'*) aged ~44–50 h were labeled with anti–CP β antibodies to visualize CP (*A–D*) and FITC-phalloidin to visualize the actin bundles (*A'–D'*). Patches of CP staining are associated with actin bundles (*arrowheads*) in both mutant and control bristles. Similar staining is seen in *shv b cn bw* pupae, and is not observed in secondary antibody controls (not shown). The wild-type bristle has some actin bundles that are bent, but this damage occurred in handling and is distinct from the actin abnormalities seen in panel *C'* and Fig. 8. Bar, 10 μm.

(Witke et al., 1995) and severin in *Dictyostelium* (Andre et al., 1989) have a mild or undetectable phenotype. Thus, the examination of CP mutations in *Drosophila* shows that the requirement for CP function in a multicellular organism is different than in a single-celled eukaryote, and that CP may have a more important role than other barbed-end capping proteins in the regulation of actin assembly.

Hemizygous *cpb* flies die during the early larval stage. Early larval lethality is seen in flies with null or near null alleles of other actin-binding proteins such as α-spectrin (Sliter et al., 1988; Lee et al., 1993), α-actinin (Fyrberg et al., 1990), and profilin (Verheyen and Cooley, 1994). In each case, it is likely that the ability to complete embryogenesis reflects the maternal contribution of protein. However, the quantity of maternally contributed protein eventually becomes insufficient to support development, and a mutant phenotype is expressed. By analogy, we expect that at least some of our alleles are null or near null, and that embryonic development is supported by maternally contributed CP. Consistent with this hypothesis, CP is present in ovaries and early embryos before the onset of zygotic transcription (Fig. 5 *A*, lane *1*, and Hopmann, R., and K.G. Miller, unpublished data).

Reduced CP Leads to Abnormal Bristle Morphology by Affecting the Structure of the Actin Bundles

Fortuitously, one combination of mutant alleles results in a mild phenotype with survival to adulthood. The viable transheterozygous adults were examined to determine if the decreased levels of CP affected external morphology. The only visibly affected structures were the macrochaetae, which were uniformly shorter and rougher than the wild type. A subset of macrochaetae also exhibited dramatic bending and/or splitting, and an extremely disorganized pattern of surface grooves.

Actin appears to have an important function during bristle morphogenesis (Appel et al., 1993; Tilney et al., 1995). Bristles are derived from cytoplasmic extensions of the trichogen cell within the pupal epithelium (Lees and Waddington, 1942). These processes contain actin bundles that run longitudinally just underneath the cell membrane, as well as microtubules scattered throughout the core of the process, also oriented longitudinally (Overton, 1967; Appel et al., 1993). The cell protrudes between the actin bundles, forming ridges; later in development, the cuticular layer is deposited, the actin bundles are broken down, and the cell regresses, leaving a hollow tube with longitudinal grooves in the surface (Overton, 1967).

To determine if the adult bristle phenotype reflected abnormalities in the actin cytoskeleton, we examined the actin bundles in bristles of mutant and wild-type pupae. Although in most mutant macrochaetae the actin bundles had a fairly normal organization, a subset of macrochaetae had extremely disorganized bundles, suggesting that the abnormal groove patterns occasionally observed in mature bristles resulted from the aberrant organization of actin bundles. This experiment also revealed that most mutant bristles had an increased number of actin bundles, resulting in an apparent overall increase in the amount of F-actin. Increased F-actin suggests that CP regulates actin assembly in the bristle by preventing the polymerization of actin, similar to the case in *Dictyostelium* (Hug et al., 1995), but opposite that of yeast (Karpova et al., 1995). This type of regulation is likely to depend on CP's barbed-end capping activity, as has been shown in vitro. The finding that CP is associated with the actin bundles in the developing bristle is consistent with such a role. Since CP is present in a punctate pattern along the full length of the bundles, it is likely that the bundle is composed of many short filaments, and barbed-end assembly is regulated throughout the entire bristle, not just at the base or the tip. The fact that

there are patches of CP implies that there are groups of actin filaments with their barbed ends at these positions. The patches do not have a regular periodicity, suggesting that these groups of filaments have variable lengths and/or may interdigitate with actin filaments with barbed ends in different patches.

Aberrant Bundle Organization May Result Directly from Unregulated Actin Polymerization at the Barbed End

How might reduced levels of CP lead to the observed effects on the actin bundles, i.e., bundles that are more numerous and sometimes thicker or thinner than normal, branched, or aberrantly positioned? One possible mechanism is that unrestricted growth at the barbed ends allows individual filaments to grow longer than normal. Since the bundle is composed of groups of shorter filaments, if these groups of filaments are longer but the overall length of the bundle is not increased, there would be increased overlap of filaments. This would lead to a greater number of filaments in a given cross-sectional plane and a thicker bundle. Abnormally long filaments might also branch off the main bundle because of steric hindrances, leading to more numerous but thinner bundles. Such branching and excess polymerization could also cause the abnormal positioning of actin bundles, such that they deviate from the normal longitudinal orientation and/or become displaced from the membrane.

Another explanation for the abnormal arrangement of bundles is that CP has an additional role in the positioning of actin filaments. Antibody inhibition and dominant negative experiments designed to interfere with CP function during myofibrillogenesis indicate that CP may be important for specifying actin filament position in the sarcomere (Schafer et al., 1995). No other evidence suggesting such a role for CP in nonmuscle cells currently exists; if future experiments support this hypothesis, it would be the first example of a structural role for CP in nonmuscle cells.

How does the phenotype of *cpb* mutant bristles compare to that of other actin-binding protein mutants? To our knowledge, there are only four loci affecting bristle development that have been shown to correspond to actin-binding proteins: *singed, forked, chickadee*, and *cpb. singed* encodes the *Drosophila* homologue of fascin, an actin-bundling protein (Cant et al., 1994). *singed* mutations cause the macrochaetae to be short and gnarled, with mild disorganization of the grooves. Microchaetae are also affected in more severe alleles. *forked* encodes a novel protein that is also likely to be a bundling protein, although the evidence for this is indirect (Peterson et al., 1994; Tilney et al., 1995). Both proteins colocalize with the actin bundles. *forked* mutations produce bristles that are likewise short and gnarled, but are also sometimes sharply bent or split at the end. The weakest alleles affect only macrochaetae, the strongest affect all bristles as well as trichomes (hairs). Strong hypomorphic alleles of *chickadee*, which codes for profilin, cause an increase in the number of actin bundles (Verheyen and Cooley, 1994), similar to the *cpb* mutant bristles. This increase is consistent with in vitro evidence suggesting that profilin regulates the polymerization of new actin filaments by sequestering mono-

mers (Tilney et al., 1973). *chickadee* mutant bristles often bend sharply and/or branch in a manner similar to the bristles of the *cpb* mutant. Unlike the punctate, bundle localization of CP, however, antibodies against profilin show general cytoplasmic staining in the developing bristle (Cooley, L., personal communication). Furthermore, the extreme groove phenotype seen in some *cpb* mutant bristles appears to be unique; severe alleles of *singed, forked*, or *chickadee* cause only minor deviations in the groove pattern.

Our initial analysis of external morphology in the mutant flies has revealed the macrochaetae to be the only structures affected by the reduction of CP. We do not interpret this as a lack of a role for CP in other tissues. Rather, the macrochaetae may be more sensitive than other tissues to lower levels of CP during elongation because of their large actin content.

Actin Polymerization May Not Drive Bristle Growth

Clearly, the proper regulation and organization of the actin cytoskeleton is crucial for normal bristle development, but the precise role of actin in this process is unclear. Fristrom and Fristrom (1993) hypothesize that the polymerization of actin at the tip of the growing bristle is the driving force for elongation, with the internal microtubules providing tracks for transport of materials, analogous to the growth of neurites. We have observed bristles in the *cpb* transheterozygote in which the grooves are oriented perpendicularly to the long axis, and observed a similar arrangement of actin bundles during bristle development. If actin polymerization drives elongation, then the direction of growth should always follow the direction of actin polymerization. Thus, our observation of mutant bristles with ridges perpendicular to the axis of outgrowth seems inconsistent with such a role for actin.

If actin is not providing the force for bristle elongation, then what is its role in bristle development? Lees and Picken (1944) proposed a model for bristle morphogenesis based on careful measurements of bristle growth; they posited that the force for bristle elongation is derived from positive hydrostatic pressure existing in the greatly enlarged trichogen cell, and that the shape of the bristle is dictated by the properties of the cell membrane. We further suggest that the peripheral actin bundles may act as a framework that limits the increase of bristle diameter while allowing growth in length, thus specifying the shape of the bristle.

How do actin-binding proteins contribute to this role of actin as a framework? Tilney et al. (1995) have shown that the actin bundles in *singed* and *forked* mutant microchaetae (small bristles) have reduced numbers of actin filaments. It seems reasonable to assume that the bent and twisted phenotype of the mutant bristles stems from the loss of structural integrity associated with the smaller actin bundles. The fact that the phenotype is more severe in macrochaetae than microchaetae is consistent with this; a larger structure would be expected to require a stronger frame.

chickadee (profilin) and *cpb* mutations produce bristles with increased numbers of actin bundles. In the case of *chickadee*, these bundles are uniformly thinner than wild

type. For *cpb*, the bundles are more heterogeneous in thickness, with some thicker than wild type and others thinner, as well as extremely disorganized in some cases. Again, the presence of thinner actin bundles could result in a loss of structural integrity and allow bending. This has also been suggested by Verheyen and Cooley (1994). Furthermore, the irregular organization of these bundles may cause unreinforced points at the cell membrane that allow the "ballooning" of the membrane, forming supernumerary processes. Actin would then polymerize to reinforce the new process, leading to splitting and branching of the bristles.

The relationship between the actin cytoskeleton and bristle development, while intrinsically interesting, may also have broader implications. A favorite paradigm of actin function is that the unidirectional polymerization of actin can generate force that causes, for example, the extension of filopodia and lamelopodia and the propulsion of *Listeria* cells through the cytoplasm. Our findings are inconsistent with such a mechanism as the driving force for bristle growth, and suggest the possibility that peripheral actin bundles can act as a framework that limits growth to a specific direction and dictates shape. Perhaps actin has a similar role in other cell types.

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