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Short Stop provides an essential link between F-actin and microtubules during axon extension

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SUMMARY

Coordination of F-actin and microtubule dynamics is important for cellular motility and morphogenesis, but little is known about underlying mechanisms. *short stop* (*shot*) encodes an evolutionarily conserved, neuronally expressed family of rod-like proteins required for sensory and motor axon extension in *Drosophila melanogaster*. We identify Shot isoforms that contain N-terminal F-actin and C-terminal microtubule-binding domains, and that crosslink F-actin and microtubules in cultured cells. The F-actin- and microtubule-binding domains of Shot are required in the same molecule for axon extension, though the length of the connecting rod domain can be dramatically reduced without affecting activity. Shot therefore functions as a cytoskeletal crosslinker in axon extension, rather than mediating independent interactions with F-actin and microtubules. A Ca^{2+}-binding motif located adjacent to the microtubule-binding domain is also required for axon extension, suggesting that intracellular Ca^{2+} release may regulate Shot activity. These results suggest that Shot coordinates regulated interactions between F-actin and microtubules that are crucial for neuronal morphogenesis.

Key words: F-actin, Microtubules, Shot, Plakins, Ca^{2+}, *Drosophila*

INTRODUCTION

During development, neurons make specific connections with other cells via axons and dendrites. Growth cones, the tips of developing axons and dendrites, are morphologically dynamic migratory organelles whose trajectories are regulated by extracellular guidance cues (Tessier-Lavigne and Goodman, 1996). These cues probably act via specific receptors and signaling pathways on the growth cone cytoskeleton.

Filopodia and lamellopodia at the leading edge of the growth cone contain F-actin, and the core of the growth cone contains polarized arrays of microtubules. Cellular morphogenesis in general, as well as growth cone migration in particular, probably requires precise coordination between these dynamic F-actin and microtubule networks. These networks remain largely separate as growth cones or other migratory cellular structures move forwards on substrates, but interact at the interface where extending microtubules invade F-actin rich lamellopodia (Forscher and Smith, 1988; Rochlin et al., 1999). These interactions appear targeted and affect the interacting microtubules and F-actin (Kaverina et al., 1998; Kaverina et al., 1999). Drugs that affect F-actin polymerization affect microtubule dynamics and conversely, altering microtubule dynamics affects F-actin assembly (Lin and Forscher, 1993; Baas, 1997; Rochlin et al., 1999).

Members of the plakin family of rod-like proteins may link F-actin to microtubules into structures important for cell integrity, function and morphogenesis. ACF7 (Actin Crosslinking Family 7) (Leung et al., 1999; Karakesisoglou et al., 2000), BPAG1n (Bullous Pemphigoid Antigen 1, neuronal isoform) (Yang et al., 1999) and plectin isoforms (Svitkina et al., 1996; Andra et al., 1998) all contain binding sites for both F-actin and microtubules. Mutation of plectin affects the maintenance of cytoskeletal structures that allow epithelia to resist contractile stress (Andra et al., 1997) and mutants in BPAG1n affect the stability of microtubules required for axonal transport (Yang et al., 1999). *shot* encodes a family of proteins whose modular structures are most closely related to vertebrate ACF7 (Leung et al., 1999). *shot* is allelic to *kakapo* (Lee et al., 2000), a gene required to stabilize mechanoresistant microtubule arrays in neuronal support and muscle attachment cells (Gregory and Brown, 1998; Prokop et al., 1998). *shot* is required for growth cone extension in selected contexts: motor axon extension into target fields (Van Vactor et al., 1993; Lee et al., 2000), sensory axon extension along neuronal substrates (Kolodziej et al., 1995) and the formation of lateral dendritic branches (Prokop et al., 1998; Gao et al., 1999). Shot also organizes apical accumulations of F-actin and microtubules in tracheal cells that are required for lumen formation during tracheal tube fusion (Lee and Kolodziej, 2002).

These phenotypes are suggestive of plakins functioning as crosslinkers, but could also reflect independent interactions occurring between plakins and F-actin and plakins and microtubules. Plakins are large (200-600 kDa) and rod-like, so interactions with the cytoskeleton can occur at spatially well-separated sites. For example, a ~4000 amino acid central rod

domain separates the N-terminal F-actin and C-terminal microtubule binding domains in ACF7 (Leung et al., 1999). Individual cytoskeletal interaction domains in plakins can have physiological activity (Andra et al., 1998). Interactions between plakins, F-actin and microtubules can even be mutually exclusive. An additional microtubule binding site in ACF7 and other plakins, the M1 domain, appears active only in the absence of an adjacent actin binding site (Yang et al., 1999; Karakesisoglou et al., 2000).

We have addressed the importance of F-actin/microtubule crosslinking activity and its possible regulation during development by identifying the domains in Shot proteins that are required for axon extension. These include key sites of cytoskeletal interaction. The long, 600 kDa Shot isoforms A (Shot L(A)) and B (Shot L(B)) isoforms bind F-actin via their N-terminal domain and microtubules via the C-terminal domain. Axon extension requires F-actin and microtubule binding sites to be present in the same molecule, indicating that axon extension requires Shot to link F-actin to microtubules. An evolutionarily conserved Ca^{2+}-binding site next to the microtubule-binding motif is also required. However, much of the central rod domain can be removed without affecting axon extension, a surprising result considering the conservation of central domain length in evolution. Our structure/function analysis indicates that crosslinker proteins whose activity requires intracellular Ca^{2+} coordinate F-actin and microtubule dynamics during neuronal morphogenesis. Remarkably, this mechanism is specific to developmental context because Shot interacts independently with F-actin and with microtubules during tracheal development (Lee and Kolodziej, 2002).

MATERIALS AND METHODS

Molecular biology

pEGFPN2 (Clontech) was used to express GFP (green fluorescent protein) fusions in mammalian cells. cDNA sequences (Lee et al., 2000) encoding the N-terminal regions of Shot isoforms A (amino acids 1 to 398; Accession Number, CAA09869), B (amino acids 1 to 287; Accession Number, CAA09870) or C (amino acids 1 to 357; Accession Number, AAF24340) were PCR amplified as *Eco*RI-*Acc*65I fragments and subcloned into pEGFPN2. cDNA sequences encoding the C-terminal region of the long isoforms, C-Shot L (amino acids 4698 to 5201; Accession Number, AAF24343) or regions of C-Shot L were PCR amplified as *Bgl*II-*Sal*I DNA fragments and subcloned into pEGFPN2. The amplified C-Shot L-coding sequence was used to make GFP fusion expression vectors containing full-length *Shot L(A)*, *Shot L(B)* and *Shot L(C)* cDNAs. Sequences covering either the two EF-hand motifs (amino acids 4711 to 4849; Accession Number, AAF24343) or most of GAS2 (Growth Arrest-Specific 2 protein homology) motif (amino acids 4859 to 4905; Accession Number, AAF24343) were deleted from a DNA fragment encoding *Shot L(A)* to produce DNA fragments encoding *Shot L(A)-ΔEF-hand* or *Shot L(A)-ΔGAS2*, respectively. A pEGFPN2 expression vector expressing the N-terminal and plakin-like domains from Shot L(C) (amino acids 1 to 1315, Accession Number, AAF24340) was also constructed: *EGFPN2-Shot L(C)-plakin-like-GFP*. For rescue experiments, the *Shot L(A)-GFP* cDNA or derivatives were subcloned into pUASTNEXT (P. A. K., unpublished), a derivative of the GAL4 expression vector pUAST (Brand and Perrimon, 1993).

In vitro *Drosophila* embryo primary culture

Embryos were collected for 5-6 hours, dechorionated in 50% bleach, and placed on a glass coverslips coated with 1% gelatin (Sigma). Embryos were gently dissociated with a needle and cells were grown in 5% CO_2 at 25°C as described (O'Dowd, 1995) for 2 days prior to immunohistochemistry. The neuronal identity of cells was confirmed by anti-HRP labeling or the expression of other neuronal markers. All cells with extensive neurites were neurons.

Immunohistochemistry

NIH3T3 cells transfected with lipofectamine (Gibco BRL) or *Drosophila* embryonic cells were fixed with 4% formaldehyde for 15 minutes at room temperature and then permeabilized with 0.1% Triton X-100 for 5 minutes. Fixed cells were washed three times with phosphate-buffered saline (PBS), preblocked in 1% bovine serum albumin (BSA)/PBS, and treated with primary antibodies (rabbit anti-GFP (Clontech), monoclonal rat anti-tubulin (Harlan Sera-Lab, UK)) for 1 hour. After three washes in PBS, cells were incubated in 1% BSA/PBS with R6G phalloidin (Molecular Probe) and FITC or Cy5-conjugated secondary antibodies (Jackson Laboratories). The cells were washed four times with PBS, and mounted in SlowFade (Molecular Probe). Immunohistochemistry and microscopy of fly embryos with mAb 22C10 (sensory axons) (Fujita et al., 1982), mAb 1D4 (motor axons) (Van Vactor et al., 1993) and anti-Shot (Strumpf and Volk, 1998) has been previously described (Kolodziej et al., 1995).

Yeast two hybrid screen

A C-Shot L-LexA DNA-binding domain fusion was expressed using pMW101 (Watson et al., 1996) in *S. cerevisiae* strain EGY48 (Gyuris et al., 1993) containing the *lacZ* reporter plasmid pMW108 (Watson et al., 1996). Clones (200,000) from a 0-24 hour *Drosophila* embryonic cDNA yeast two hybrid library (Gyuris et al., 1993) were screened for the ability to confer leucine prototrophy and *lacZ* activation in the presence of C-Shot L (Gyuris et al., 1993).

In vitro actin-binding assays

cDNA sequences encoding the two calponin homology repeats in the type A and B N-terminal domains, and the single calponin repeat in the type C N-terminal domain were subcloned in frame into pGEX6P1 (Pharmacia). Bacterially produced GST fusion proteins were purified using glutathione-conjugated Sepharose beads (Pharmacia). F-actin binding assays were performed with the non-muscle actin binding kit (Cytoskeleton). Soluble and pellet protein samples separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were quantitated by silver staining followed by densitometry or transferred to Immobilon P (Millipore) membranes. GST fusion proteins on the membranes were identified with anti-GST (gift of Dr Ron Wisdom, UC Davis) at a dilution of 1:1000 in PBS/0.1% Tween-20 and subsequent incubations with HRP-conjugated anti-rabbit (Jackson) and Supersignal ECL substrate (Pierce).

Rescue experiments

Transgenic flies containing GAL4-dependent promoter (Brand and Perrimon, 1993) driven-*Shot L(A)-GFP*, *Shot L(B)-GFP*, *Shot L(C)-GFP*, *Shot L(A)-ΔGAS2-GFP*, *Shot L(A)-Δrod1-GFP* and *Shot L(A)-ΔEF-hand-GFP* cDNAs were obtained (Robertson et al., 1988). *Shot L(A)-Δrod1-GFP* lacks amino acid residues 1205-4599 (Accession Number, AAF24343), approximately 75% of the central domain, including all of the spectrin repeats (see Fig. 7). The GAL4 enhancer trap line *1407* (Luo et al., 1994) was used to drive pan-neural expression of these GAL4-dependent *Shot* transgenes in wild-type and $shot^3$ mutant embryos. Levels of green fluorescence and labeling of axon tracts were comparable among different transgenic lines. In genetic crosses used to test the activity of *Shot L(A)* and derivatives, wild-type embryos expressed *lacZ* in salivary glands. Mutant embryos were distinguished from wild-type embryos either by the absence of epidermal Shot protein expression or by the absence of *lacZ* staining.

RESULTS

Shot proteins are structurally diverse, conserved in evolution and vary in their actin binding properties

Shot proteins are modularly assembled from different N-terminal, central rod and C-terminal domains to generate multiple isoforms (Gregory and Brown, 1998; Strumpf and Volk, 1998; Lee et al., 2000). The long 600 kDa isoforms (Fig. 1A) are similar to vertebrate ACF7 and contain one of four N-terminal domains (type A, B, C or D), a ~4000 amino acid long central domain likely to form a rod-like structure and a 581 amino acid long C-terminal domain (Lee et al., 2000).

The type A and B Shot N-terminal domains contain two calponin homology (CH) motifs (Fig. 1B) and are therefore predicted to bind F-actin (Gimona and Winder, 1998), whereas the type C (one CH motif) and D (no CH motif) N-terminal domains (Fig. 1B) are not (Gimona and Winder, 1998). Type C and D isoforms are only found in non-neuronal cells (Lee et al., 2000), suggesting that the ability to bind F-actin may be important for axon extension. We verified that GFP fusions with the type A or B N-terminal domains associate with cortical F-actin and F-actin rich stress fibers in transfected cells (Fig. 2A). By contrast, the type C N-terminal domain-GFP fusion does not associate with F-actin (Fig. 2A). The in vitro F-actin-binding properties of the different N-terminal domains were also measured (Fig. 2B). The GST fusion containing the two CH motifs found in the type A and B domains binds to F-actin with a K_d ~ 0.022 μM (Fig. 2C), but the GST fusion containing only the second CH domain found in the type C domain interacted only weakly with F-actin (Fig. 2B). Similar results have been obtained for the actin binding properties of CH-containing domains in BPAG1 (Yang et al., 1996) and ACF7 (Leung et al., 1999; Karakesisoglou et al., 2000).

The C-terminal domain of long Shot isoforms stabilizes microtubules by interactions between the GAS2 homology sequence and α-tubulin

The 581 amino acid C-terminal domain of Shot long isoforms (C-Shot L) and those in related vertebrate proteins contain two EF-hand motifs (Ikura, 1996), a stretch of homology to the Growth Arrest-Specific 2 (GAS2) protein (Brancolini et al., 1992), and additional homology in the remaining 300 amino acids (Strumpf and Volk, 1998; Lee et al., 2000). Overall, C-Shot L shows 40% sequence identity to the C-terminal domain of mouse ACF7 (mACF7) (Fig. 1A).

As plakin-like proteins often link different cytoskeletal structures to each other, we performed a yeast two-hybrid screen (Gyuris et al., 1993) to identify proteins interacting with C-Shot L. Two of the interacting clones encoded different α-tubulin isoforms (Fig. 3A) (Theurkauf et al., 1986). These clones shared the same breakpoint within the α-tubulin sequence and encode the C-terminal two helices. These two helices form a prominent groove on the exterior of the microtubule that is the major structural feature accessible to solvent, and presumably microtubule binding proteins (Nogales et al., 1999). Thus, C-Shot L contains a likely binding site for α-tubulin.

We therefore investigated whether C-Shot L associates with microtubules. When fused to C-Shot L, GFP localizes to microtubules (Fig. 3C-E). We further delineated the microtubule-binding region within C-Shot L (Fig. 3B). A GFP fusion of the C-Shot L sequences that lie C-terminal to the GAS2 region is largely nuclear and only weakly associates with microtubules (Fig. 3F-H). By contrast, a GFP fusion containing the GAS2 motif is sufficient for microtubule association (Fig. 3I-K). C-Shot L appears to contain fewer high-affinity microtubule-binding sites than the C-terminal domain of ACF7, which associates with microtubules via the GAS2 motif (Sun et al., 2001) and also via sequences C-terminal to the GAS2 motif (Sun et al., 2001). As in the case of ACF7 (Sun et al., 2001), the GAS2 motif stabilizes associated microtubules against nocodazole-induced depolymerization (Fig. 3I).

ACF7 has been reported to contain an additional microtubule binding site, the M1 domain, within the plakin-like region (Karakesisoglou et al., 2000). However, we observed no association in transfected cells between a GFP fusion with the

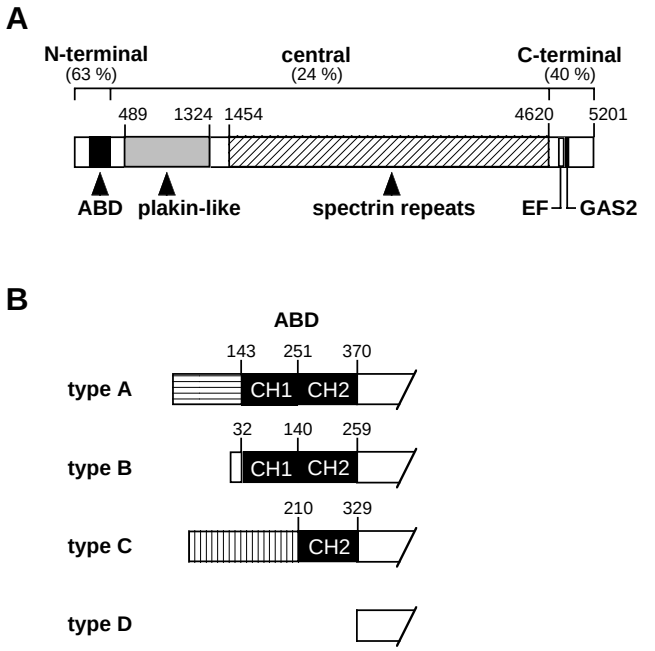

Fig. 1. Structures of Shot isoforms. (A) The F-actin/microtubule crosslinking Shot isoforms required for axon extension. The Shot L(A) isoform contains an N-terminal actin-binding domain, a central domain of ~4000 amino acids and a C-terminal domain that contains EF-hand Ca^{2+}-binding motifs, the GAS2 motif and other conserved sequences. The central domain contains a plakin-like region and 22 spectrin repeats (Strumpf and Volk, 1998). Numbers indicate the start and end positions of the different domains in the amino acid sequence and the percent amino acid identity between *Drosophila* Shot L(A) and mouse ACF7 (Leung et al., 1999) in the N-terminal, central and C-terminal domains. The other Shot isoform that crosslinks F-actin and microtubules, Shot L(B), differs from Shot L(A) only in the N-terminal leader sequence. ABD, actin-binding domain; EF, EF-hand; GAS2, GAS2 homology motif. (B) The structures of the four Shot N-terminal domains (Lee et al., 2000). Numbers indicate the start and end positions of the different features in the amino acid sequence. Type A and type B N-terminal domains differ in their leader sequences (residues 1-143 and 1-32, respectively) and contain two calponin homology motifs (CH1 and CH2). The type C domain contains yet another leader sequence (residues 1-210) and only the CH2 motif. The type D domain starts in the central rod domain.

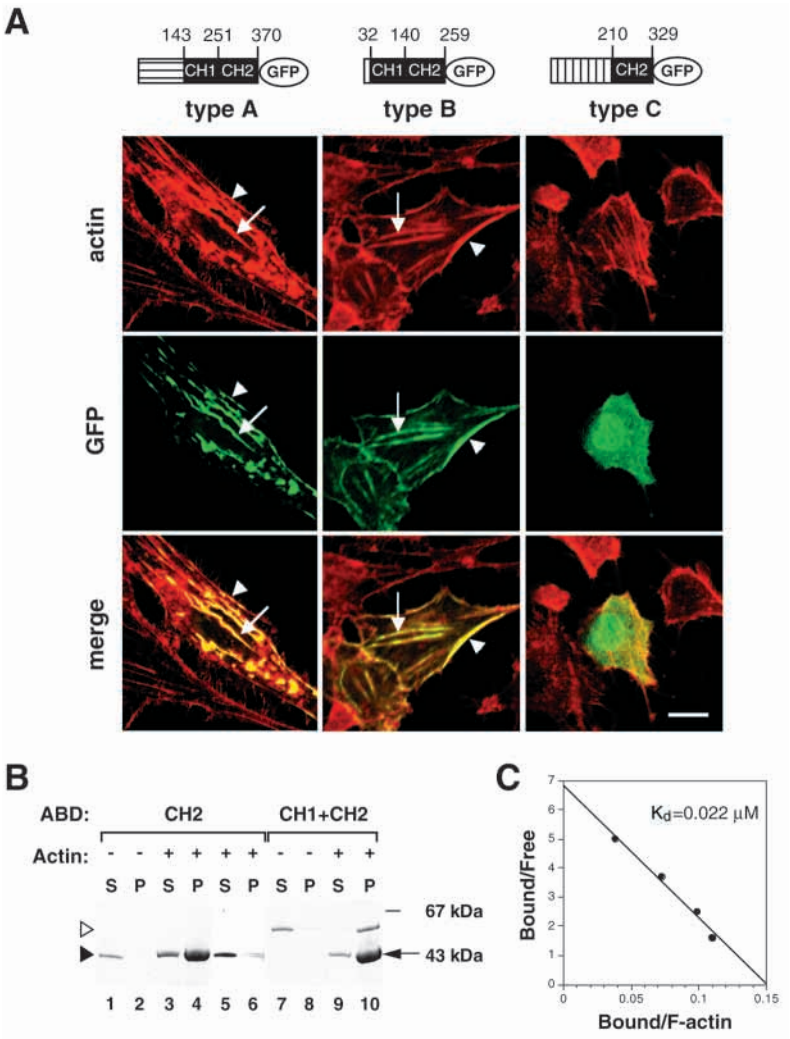

Fig. 2. Shot type A and B N-terminal domains associate with F-actin. (A) NIH3T3 fibroblasts transfected with type A, B, or C Shot N-terminal domains fused to GFP. CH, calponin homology domain. F-actin (red) is visualized using Cy3-conjugated phalloidin. Type A- and B-GFP fusions (green) co-localize with F-actin in the cortical region (arrowhead) and stress fibers (arrows) of transfected cells, but type C-GFP fusions (green) do not. Yellow (see merge panels) indicates the overlap between the F-actin and GFP distributions. Images are 1 μm confocal sections. Scale bar: 10 μm. (B) The in vitro actin-binding properties of Shot N-terminal domains. GST (Glutathione S-Transferase) fusions (2 μM) with either the second calponin homology domain (CH2) or the two calponin homology domains (CH1 + CH2) were incubated with F-actin (19.2 μM) and the samples separated into supernatant (S) or pellet (P) fractions by 1 hour of centrifugation at 150,000 ***g***. The proteins in each fraction were then electrophoretically separated. The positions in the gel of the (CH1 + CH2)-GST fusion (open arrowhead), the CH2-GST-fusion (black arrowhead) and actin (arrow) are indicated. In the absence of F-actin, (lanes 1, 2, 7, 8), little fusion protein pellets. Little CH2-GST fusion is associated with F-actin (lanes 4, 6); most remains in supernatant fractions (lanes 3, 5). As CH2-GST is not well resolved from actin, the samples in lanes 3-4 were transferred electrophoretically to Immobilon P membranes (Millipore) and probed with anti-GST (lanes 5-6) to determine how much CH2 is associated with F-actin. CH2-GST largely remains in the supernatant after F-actin is pelleted (compare lane 5 with lane 6). By contrast, most of the (CH1+CH2)-GST fusion pellets with F-actin under these conditions (compare lane 9 with lane 10). (C) Scatchard plot of the binding reaction between the (CH1+CH2)-GST fusion and F-actin. Binding of the (CH1+CH2)-GST fusion to F-actin (4 μM) was measured at fusion protein concentrations of 100, 200, 300 and 400 nM. The relative amounts of proteins in the supernatant and pellet were determined by quantitative densitometry of silver-stained SDS-polyacrylamide gels. Three data sets were averaged; individual values differed from the average by no more than 9%. Vertical axis, (CH1+CH2)–GST bound/free (CH1+CH2)–GST (μM^{-1}); horizontal axis, bound (CH1+CH2)–GST/F-actin.

corresponding portion of Shot and microtubules (Fig. 3L-N), suggesting that Shot isoforms do not contain an M1 domain.

Shot isoforms that crosslink F-actin and microtubules in cultured cells are present in growth cones

Our identification of N-terminal F-actin and a C-terminal microtubule binding domains in Shot L(A) and Shot L(B) suggests that these isoforms can coordinately bind F-actin and microtubules. We therefore examined the distributions of a full-length Shot L(A)-GFP fusion protein, F-actin and microtubules in transfected NIH3T3 cells. Shot L(A)-GFP is primarily associated with microtubules (Fig. 4A-D), consistent with observations of the distribution of ACF7 in keratinocytes (Karakesisoglou et al., 2000). NIH3T3 cells expressing Shot L(A)-GFP also contain filamentous structures containing F-actin, Shot L(A)-GFP and microtubules (Fig. 4A-D). Forming these structures requires both the F-actin and the microtubule-binding domains in Shot L(A). In transfected cells expressing either the F-actin binding mutant Shot L(C)-GFP (Fig. 4E-H) or the microtubule-binding (Fig. 4I-L) domain mutant Shot L(A)-ΔGAS2-GFP, large filamentous aggregates of Shot, F-actin and microtubules are never observed. These data suggest that the type A and B N-terminal domains mediate the crucial interactions with F-actin and also that the GAS2 motif is the major microtubule-binding site in long Shot isoforms. Aggregates containing F-actin, Shot and microtubules also form in transfected cells expressing Shot L(A)-ΔEF-hand-GFP, which lacks the EF-hand motifs in the C-terminal domain (Fig. 4M-P).

Shot protein is present in axons and dendrites in vivo (Lee et al., 2000), but whether Shot is also present in growth cones has not been determined, owing to the difficulty of imaging *Drosophila* growth cones in embryos. We therefore prepared neuronal cultures from dissociated embryos (O'Dowd, 1995), and examined the distribution of F-actin, Shot and microtubules in neuronal processes (Fig. 4Q-T). Many neuronal processes in culture are complex and can extend for 100-200 μm. Shot is detected along the entire length of these processes, including growth cones (Fig. 4Q-T).

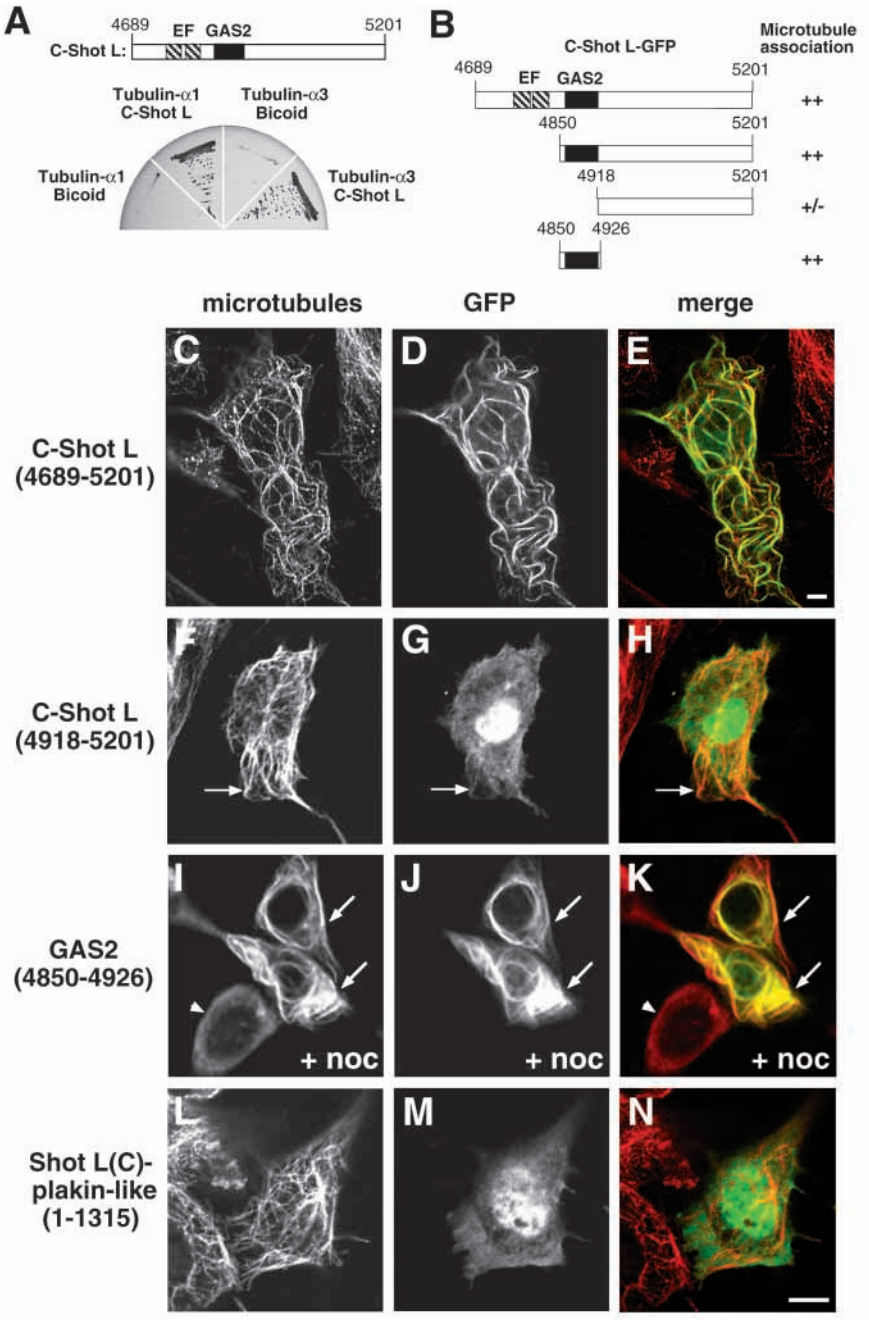

Fig. 3. The C-terminal domain of the long Shot isoforms (C-Shot L) binds to microtubules. (A) C-Shot L contains two EF-hand motifs (hatched) (Ikura, 1996) and homology to GAS2 (black) (Brancolini et al., 1992). EGY48 yeast cells (Gyuris et al., 1993) grow on leucine-depleted selective medium when they express the C-Shot L-lexA fusion and an α-tubulin-activation domain fusion, but do not grow when they express a bicoid-lexA fusion and an α-tubulin-activation domain fusion. The cDNA inserts of the two clones encode the C-terminal regions of tubulin α1 (amino acids 344 to 450, Accession Number, P06603) and tubulin α3 (amino acids 343 to 450, Accession Number P06605) (Theurkauf et al., 1986). EGY48 yeast also do not grow on selective medium when they contain the C-Shot L-lexA fusion and the activation domain alone (data not shown). (B) Summary of the qualitative ability of C-Shot L fragments to associate with microtubules in transfected cells. ++, as in E,K; +/–, as in H. (C-E) An NIH3T3 cell transfected with a C-Shot L-GFP expression vector. (F-H) A cell transfected with a GFP fusion to sequences C-terminal to the GAS2 sequence. (I-K) Cells treated with 1 μM nocodazole for 20 minutes. All cells transfected with the GAS2 motif-GFP expression vector (arrows) still contain microtubules, whereas no control cells (arrowhead) contain detectable microtubules. (L-N) Cells transfected with a GFP fusion to an N-terminal fragment from isoform Shot L(C) that contains the second calponin homology repeat and the plakin-like domain. (C,F,I,L) Microtubule distribution. (D,G,J,M) GFP expression and distribution. (E,H,K,N) Overlap (yellow) between microtubule (red) and GFP (green) distributions. Only GFP fusions with C-Shot L fragments that contain the GAS2 motif associate strongly with microtubules (E,K) and stabilize microtubules in transfected cells against nocodazole treatment (I-K). GFP fusions with a C-terminal fragment lacking the GAS2 motif associate weakly (H, arrow), and GFP fusions with the Shot plakin-like domain do not associate with microtubules (N). Images in C-N are 1 μm confocal sections. Scale bars: 10 μm.

Neuronal expression of actin/microtubule crosslinking isoforms of Shot rescues *shot* axon extension phenotypes

The presence of the long isoforms in growth cones suggests that they could be required for the role of *shot* in motor and sensory axon extension. To investigate this possibility, we introduced the *Shot L(A)-GFP* fusion gene under the control of a GAL4-dependent promoter into the *Drosophila* germline, and expressed it in all neurons in wild-type and *shot* mutant embryos using the neuron-specific GAL4 enhancer trap line 1407 (Brand and Perrimon, 1993; Luo et al., 1994). In these embryos, Shot L(A)-GFP can be detected in neuronal cell bodies, axons and dendrites (Fig. 4U), and motor, sensory and CNS axon extension appear normal (data not shown).

We first examined the ability of a neuronally expressed *Shot L(A)-GFP* transgene to rescue motor axon extension defects in *shot* null mutant embryos. In wild-type embryos, approximately 30 motoneurons innervate an equivalent number of muscles in each abdominal hemisegment (Sink and Whitington, 1991; Landgraf et al., 1997) (Fig. 5A; Table 1). $shot^3$ mutant embryos lack detectable long Shot isoforms and are null mutants (Lee et al., 2000). In $shot^3$ mutant embryos, motor axons extend outward from the CNS, choose the right pathways but then stall short of their muscle targets (Fig. 5B; Table 1) (Lee et al., 2000). Essentially no neuromuscular connections form (Table 1) (Lee et al., 2000). In $shot^3$ mutant embryos that express *Shot L(A)-GFP* in all neurons, wild-type muscle innervation is restored (Fig. 5C; Table 1). Similar

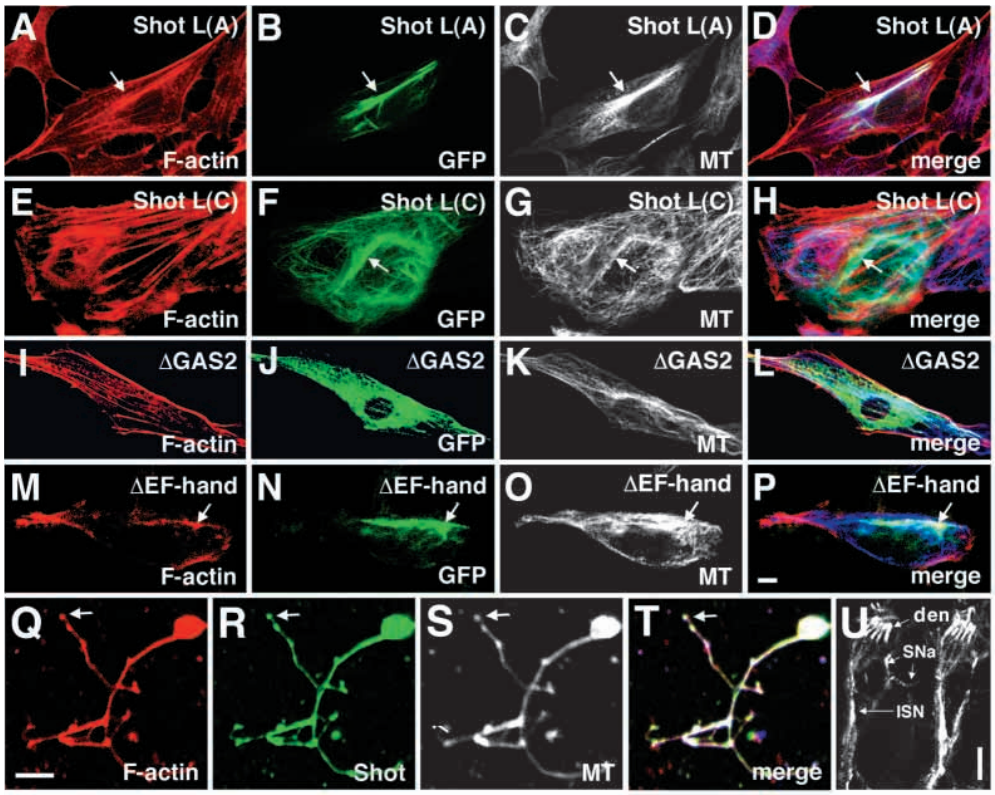

Fig. 4. Long isoforms of Shot crosslink F-actin and microtubules and are found in growth cones and neuronal processes. (A-D) Structures (arrows) containing F-actin, Shot L(A)-GFP and microtubules found in transfected NIH 3T3 cells expressing Shot L(A)-GFP. (E-H) Shot L(C)-GFP is associated with microtubules (arrows). (I-L) Shot L(A)-ΔGAS2-GFP no longer clearly associates with microtubules. Most Shot L(A)-ΔGAS2-GFP is diffusely distributed and some appears to be associated with F-actin (yellow in L). (M-P) Shot L(A)-ΔEF-GFP forms aggregates (arrows) containing F-actin, Shot and microtubules. (Q-T) The distributions of F-actin (Q), Shot long isoforms (R) and microtubules (S) in neurites in culture. Arrow indicates tip of an extending neurite. (T) Merge of Q-S. (A,E,I,M,Q) F-actin (red); (B,F,J,N) Shot GFP fusion protein (green); (C,G,K,O,S) Microtubules (white). In D,H,L,P,T, microtubules are shown in blue. Structures that contain F-actin, Shot-GFP and microtubules appear white, structures that contain only microtubules and Shot-GFP appear blue-green, and structures that contain only F-actin and Shot-GFP appear yellow. (U) Shot L(A)-GFP labels dendrites (den) and axon bundles (ISN, SNa) when expressed in neurons in a *Drosophila* embryo. The dendrites shown are those of the lateral chordotonal cluster. ISN and SNa axons are indicated. Similar levels of expression and localization patterns were observed for other Shot L(A)-GFP derivatives. All panels are 1 μm confocal sections, except U, which is a 5 μm confocal stack. Scale bars: 10 μm in A-P; 25 μm in Q-T.

results were obtained using a *Shot L(B)-GFP* transgene (data not shown). The rescue of the motor axon defects, resolved at the level of individual axons (Table 1), indicates that Shot L(A) or Shot L(B) function is required in neurons for axon extension, rather than in other cells.

We also tested whether the *Shot L(A)-GFP* or *Shot L(B)-GFP* transgenes could rescue sensory axon extension defects in *shot*^{3} mutant embryos. In wild-type embryos, four clusters of sensory neurons are found within each abdominal hemisegment (Fig. 6A). Three of these clusters (dorsal, lateral and one of the two ventral) send their axons ventrally into the intersegmental nerve (ISN). In *shot*^{3} mutant embryos, sensory neurons extend only rudimentary axons (Fig. 6B) (Kolodziej et al., 1995). In *shot*^{3} mutant embryos that express the *Shot L(A)-GFP* or the *Shot L(B)* transgenes in all neurons, sensory axon growth is restored (Fig. 6C and data not shown).

N-terminal F-actin and C-terminal microtubule-binding domains of Shot are required for axon extension

Neuronal expression of Shot isoforms that contain F-actin and microtubule-binding domains can rescue axon extension defects, suggesting that these domains may be critical for the roles of *shot* in neuronal morphogenesis. We therefore examined whether neuronal expression of *Shot L(C)-GFP*, an F-actin binding defective mutant, could rescue sensory and motor axon extension defects in *shot*^{3} mutant embryos. Shot L(C)-GFP expression in neurons did not rescue either the motor (Fig. 5D; Table 1) or sensory axon (Fig. 6D) extension defects observed in *shot*^{3} mutant embryos. Shot L(C)-GFP expression levels and localization in axons were comparable with those observed for Shot L(A)-GFP (data not shown). Shot L(C)-GFP expression in tracheal cells in *shot* mutant embryos rescues tracheal cytoskeletal defects, indicating that this protein retains biological activity in other contexts (Lee and Kolodziej, 2002). However, the F-actin binding domain is required in Shot proteins for axon extension.

To investigate the importance of the microtubule-binding GAS2 domain, we tested whether neuronal expression of Shot L(A)-ΔGAS2-GFP could rescue sensory and motor axon extension defects in *shot*^{3} mutant embryos. This mutant protein also did not rescue motor (Fig. 5F) or sensory (Fig. 6E) axon extension defects in *shot*^{3} mutant embryos. Shot L(A)-ΔGAS2-GFP was expressed at comparable levels with Shot L(A)-GFP and also labeled axons (data not shown). Shot L(A)-ΔGAS2-GFP expression in tracheal cells in *shot* mutant embryos rescues tracheal cytoskeletal defects, indicating that this protein can organize the cytoskeleton in other cells (Lee and Kolodziej, 2002). These results indicate that the microtubule-binding GAS2 domain is also required in Shot proteins for axon extension.

The F-actin/microtubule crosslinking activity of Shot is required for axon extension

The F-actin- and microtubule-binding domains are linked in Shot L(A), Shot L(B) and vertebrate ACF7 proteins by a central

Table 1. Domains in Shot L(A) required for motor axon extension

Genotype*	Wild-type ISN†	One absent‡	One to two absent§	One to three absent¶
Wild type	100 (*n*=65)	0	0	0
shot^3	0 (*n*=82)	0	81	19
Shot L(A)	99 (*n*=101)	1	0	0
Shot L(C)	0 (*n*=102)	0	93	7
Shot L(A)-ΔGAS2	7 (*n*=92)	8	70	16
Shot L(C)/Shot L(A)-ΔGAS2	6 (*n*=100)	13	65	16
Shot L(A)-Δrod1	97 (*n*=72)	3	0	0
Shot L(A)-ΔEF	11 (*n*=116)	11	73	4

Genotype	Wild-type SNa**	Defective SNa††	Wild-type ISNb‡‡	ISNb-missing 1§§	ISNb-missing 2¶¶	ISNb-missing 3***
Wild type	100 (*n*=68)	0	87 (*n*=69)	10	0	3
shot^3	1 (*n*=72)	99	0 (*n*=77)	0	0	100
Shot L(A)	97 (*n*=94)	3	60 (*n*=114)	36	0	4
Shot L(C)	0 (*n*=101)	100	1 (*n*=102)	5	0	94
Shot L(A)-ΔGAS2	11 (*n*=76)	89	28 (*n*=88)	25	3	43
Shot L(C)/Shot L(A)-ΔGAS2	6 (*n*=99)	93	9 (*n*=108)	0	19	72
Shot L(A)-Δrod1	93 (*n*=63)	7	88 (*n*=75)	8	4	0
Shot L(A)-ΔEF	16 (*n*=110)	84	46 (*n*=116)	30	4	20

Late stage 16-17 embryos were scored.

*Genotypes: *shot^3* embryos are also homozygous for the *1407 GAL4* panneural enhancer trap, which does not affect axon extension. Scored embryos contained a second chromosome bearing the indicated transgene recombined with the *shot^3* null mutation and also the *1407 shot^3* second chromosome. The transgenes were all GFP fusions. All transgenes were expressed using *GAL4* dependent (*UAS*) promoters, and most were inserted on the second chromosome. *Shot L(A)* and *Shot L(C)/Shot L(A)-ΔGAS2* embryos contained homozygous *UAS-Shot L(A)-GFP* and the *UAS-Shot L(A)-ΔGAS2-GFP* insertions on the X chromosome and were *shot^3/1407 shot^3* on the second chromosome.

†The wild-type ISN forms three arborizations over the dorsal muscles.

‡One connection missing.

§The two dorsal most connections are missing.

¶All three connections are missing.

**The wild-type SNa bifurcates at the dorsal edge of muscle 12 and innervates lateral muscles.

††One branch or both SNa branches are shorter or missing.

‡‡The wild-type ISNb innervates muscles 12 and 13, and the cleft between muscles 6 and 7.

§§ISNb motor axons terminate near muscle 13 without forming connections.

¶¶ISNb motor axons form two of the three normal connections.

***ISNb motor axons stall over the ventral muscles.

domain predicted to form an approximately 200 nm long rod. To investigate central domain function, we examined the axon extension activity of a Shot L(A)-GFP derivative lacking 75% of the central domain, including all of the spectrin repeats. Surprisingly, neuronally expressed Shot L(A)-Δrod1-GFP appears to rescue fully motor axon extension defects in *shot^3* mutant embryos (Fig. 5E; Table 1). Sensory axon extension is also restored (data not shown). Surprisingly, these data indicate that the length of the central domain is not essential for activity, and that the spectrin repeats, though conserved in evolution, are not essential for axon extension (Table 1).

In these central domain deletion experiments, the F-actin and microtubule binding domains remain physically connected. These rescue results are therefore consistent both with models in which F-actin and microtubule crosslinking is crucial for axon extension, as well as with models in which these interactions are essential yet can occur independently of each other. To distinguish between these possibilities, we tested whether co-expression of the F-actin and microtubule-binding defective Shot L(A) mutants could rescue axon extension. Neuronal co-expression of Shot L(C)-GFP and Shot L(A)-ΔGAS2-GFP did not rescue either motor (Fig. 5G) or sensory axon extension (Fig. 6F) in *shot^3* mutant embryos. Thus, axon extension requires the presence of F-actin and microtubule-binding domains in the same Shot molecule, suggesting that the F-actin/microtubule crosslinking activity of Shot is essential for axon extension.

A conserved Ca^{2+}-binding motif is required for axon extension

Changes in intracellular Ca^{2+} concentration regulate growth cone motility in vivo (Gomez and Spitzer, 1999). Both long Shot isoforms and ACF7 contain two EF-hand Ca^{2+}-binding sequences (Ikura, 1996) immediately preceding the GAS2 microtubule-association motif. To test whether the EF-hand motifs in Shot L are required for Shot activity, we investigated whether Shot L(A)-ΔEF-hand-GFP, which lacks these motifs, is active in axon extension. In cell culture, this derivative can still form aggregates that contain Shot, F-actin and microtubules (Fig. 4M-P), and its expression in tracheal cells in *shot* mutant embryos rescues tracheal defects (S. L. and P. A. K., unpublished). However, the EF-hand motif is required for both motor (Fig. 5H) and sensory axon (Fig. 6G) extension. Sensory axon extension is modestly improved over a null mutant, suggesting that the EF-hand deletion mutant retains residual ability to promote axon extension. These results suggest that intracellular Ca^{2+} concentration may modulate Shot activity in motor and sensory axons.

DISCUSSION

Interactions between F-actin and microtubules may be required for cell motility (Lin and Forscher, 1993; Kaverina et al., 1998; Kaverina et al., 1999; Rochlin et al., 1999), but the underlying

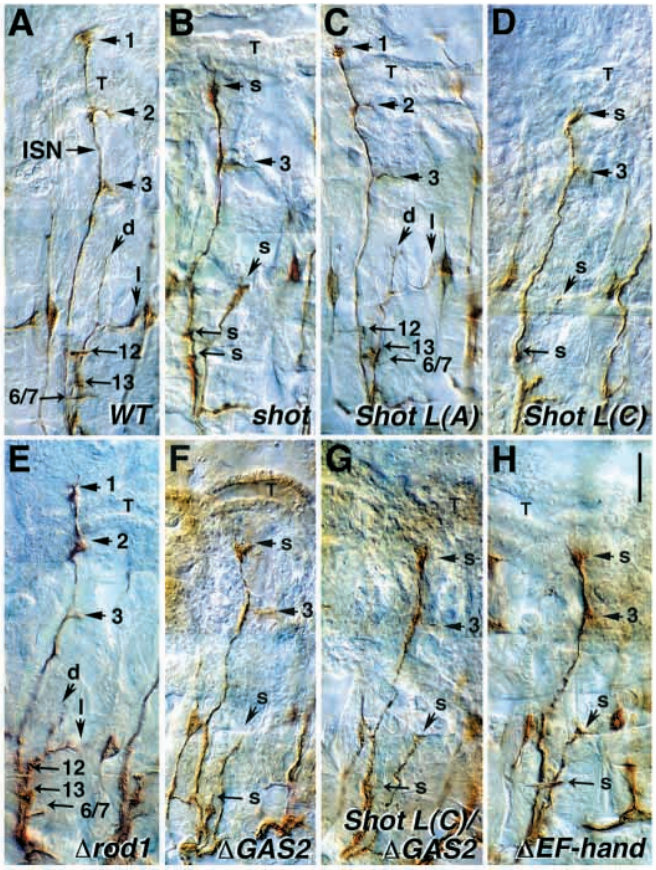

Fig. 5. The F-actin/microtubule crosslinking activity and Ca^{2+} binding domains of Shot L(A) are required in neurons for motor axon extension. (A-H) The pattern of muscle innervation in late stage 16 *Drosophila* embryos. (A) Wild type. In each abdominal hemisegment, the ISN motor axons innervate dorsal muscles, and form three characteristic neuromuscular junctions (1-3) in each abdominal hemisegment (short arrows). The dorsal trunk of the trachea (T) runs approximately between the dorsalmost two connections. The SNa contacts lateral muscles and bifurcates above muscle 12 into a dorsal (d) and a lateral (l) branch (concave arrows). The ISNb (long arrows) innervates ventral muscles 12, 13, and the cleft between muscles 6 and 7. (B) *shot*^{3} null mutant. The ISN stalls (s) at the approximate position where it would normally make the second dorsalmost muscle contact (short arrow). The SNa stalls (s) at the point where it would normally bifurcate (concave arrow). The ISNb stalls (s) in the ventral muscle field (long arrows), and fails to reach muscle 12. (C-H) The indicated transgenes are expressed specifically in all neurons in *shot*^{3} mutant embryos. (C) *Shot L(A)-GFP* expression restores normal muscle innervation. All Shot L(A)-GFP derivatives produced green fluorescence and were present in axons when expressed in Drosophila embryonic neurons (data not shown). (D) *Shot L(C)-GFP* expression does not rescue normal muscle innervation. (E) *Shot L(A)-Δrod1-GFP* (Table 1) expression restores normal muscle innervation. (F) *Shot L(A)-ΔGAS2-GFP* expression does not rescue normal muscle innervation. (G) *Shot L(C)-GFP* and *Shot L(A)-ΔGAS2-GFP* co-expression does not rescue normal muscle innervation. (H) *Shot L(A)-ΔEF-hand-GFP* expression does not rescue normal muscle innervation. Scale bar: 15 μm. Anterior, left; dorsal, top.

mechanisms are not well understood. We show here that the ability of Shot to bring F-actin and microtubules together is crucial for axon extension and that a Ca^{2+}-binding site on Shot may regulate its activity. These results indicate that the F-actin/microtubule interface is essential for neuronal morphogenesis, and suggests a mechanism for its regulation.

shot encodes evolutionarily conserved plakins that link F-actin to microtubules

Shot L(A), Shot L(B), and related ACF7 isoforms (Leung et al., 1999; Sun et al., 1999) contain N-terminal actin-binding domains, a long central rod-like domain, and a C-terminal domain that contains two putative EF-hand motifs and the GAS2 homology region. We show that the GAS2 homology sequence can target GFP to microtubules in cultured cells and stabilizes associated microtubules against depolymerization, most probably by binding to the C-terminal helices of α-tubulin. These findings can therefore explain why *shot* mutant embryos lack microtubule arrays normally found in muscle attachment and neuronal support cells (Prokop et al., 1998).

Shot L(A) and Shot L(B) can coordinately bind F-actin and microtubules. In transfected cells, Shot L(A)-GFP forms filamentous structures with F-actin and microtubules. Assembly of these structures requires the N-terminal calponin

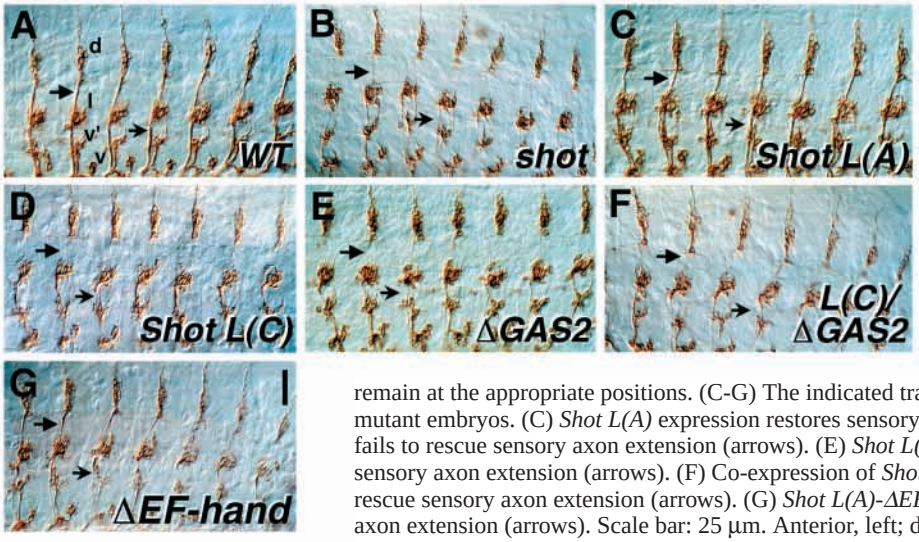

Fig. 6. The F-actin/microtubule crosslinking activity and Ca^{2+}-binding domains of Shot L(A) are required in neurons for sensory axon extension. Sensory neurons and their axons in abdominal hemisegments a1-a7 in filleted stage 16 *Drosophila* embryos. (A) Wild-type. The PNS neurons in each hemisegment are organized into dorsal (d), lateral (l) and two ventral (v,v′) clusters. Neurons in the dorsal and lateral clusters send their axons (arrows) in the intersegmental nerve (ISN) to CNS targets. (B) *shot*^{3} null mutant. Sensory axons are rudimentary or absent (arrows). Cell bodies remain at the appropriate positions. (C-G) The indicated transgenes are expressed in neurons in *shot*^{3} mutant embryos. (C) *Shot L(A)* expression restores sensory axons (arrows). (D) *Shot L(C)-GFP* expression fails to rescue sensory axon extension (arrows). (E) *Shot L(A)-ΔGAS2-GFP* expression fails to rescue sensory axon extension (arrows). (F) Co-expression of *Shot L(C)-GFP* and *Shot L(A)-ΔGAS2-GFP* fails to rescue sensory axon extension (arrows). (G) *Shot L(A)-ΔEF-hand-GFP* expression fails to rescue sensory axon extension (arrows). Scale bar: 25 μm. Anterior, left; dorsal, top.

homology and C-terminal GAS2 motifs, suggesting that these are the primary sites that mediate the interactions of Shot with F-actin and microtubules, respectively.

Interactions of Shot with the cytoskeleton depend on developmental context

We show that axon extension requires the presence of the F-actin and the microtubule binding sites in the same Shot molecule, suggesting that Shot crosslinks F-actin and microtubules, rather than mediating functionally independent interactions with F-actin and with microtubules. Co-expressing F-actin-binding defective Shot with microtubule-binding defective Shot does not rescue axon extension defects in *shot* null mutant embryos. Surprisingly, though the length of the central rod domain is conserved between Shot and related proteins, the precise spacing of F-actin and microtubule-binding sites does not appear to be crucial (Fig. 7).

These experiments directly test the model that plakins crosslink F-actin and microtubules relative to each other to effect morphogenesis. However, whether Shot is a crosslinker or interacts independently with F-actin and microtubules depends on developmental context. In tracheal cells involved in tracheal branch fusion, the N-terminal F-actin binding domain appears functionally redundant with the microtubule-binding GAS2 motif in organizing apical accumulations of F-actin and microtubules (Lee and Kolodziej, 2002). In contrast to neurons, whose growth cones require strong coordination between F-actin and microtubules for motility, the tracheal cells are not motile when Shot is required, and the F-actin and microtubules appear to become apically organized at different times.

The actin/microtubule interface: a crucial target for regulation during motility and morphogenesis

Motility and morphogenesis involve complex changes in the F-actin and microtubule cytoskeletons. Observation and pharmacological manipulation of these networks suggest that coordination between them is required for motility and morphogenesis (Kaverina et al., 1999; Rochlin et al., 1999; Waterman-Storer and Salmon, 1999). Interactions between filopodial F-actin and microtubules are probably involved in filopodial maturation into an axon (Sabry et al., 1991). Shot may be involved in forming F-actin/microtubule complexes essential for this process or others required for growth cone motility.

F-actin/microtubule crosslinkers such as the Shot proteins described here could dynamically affect the cytoskeleton in several ways. First, a change in the interactions between Shot and F-actin or microtubules may change the relative orientation of associated F-actin and microtubules. Second, Shot could also be involved in controlling the polymerization of actin and tubulin, as observed for other F-actin and microtubule interacting proteins, including plakins (Andra et al., 1998; Yang et al., 1999). Both Shot L(A) and ACF7 (Leung et al., 1999) stabilize associated microtubules against depolymerization. Third, bringing F-actin and microtubules into proximity may promote biochemical interactions among proteins associated with these cytoskeletal structures. Regulators of F-actin dynamics appear associated with microtubules (Ren et al., 1998; Glaven et al., 1999), and F-actin and microtubule-based motors can physically interact (Huang et al., 1999). Further biochemical and genetic studies will clarify how Shot binding to F-actin and microtubules affects their dynamic properties.

Our structure/function analysis of Shot indicates that complexes containing F-actin, microtubules and Shot are crucial for growth cone motility in selected contexts. Signaling systems that guide axon extension may therefore use or regulate these complexes. We have shown that Shot proteins contain an essential EF-hand motif. Removing the EF-hand does not appear to prevent Shot proteins from crosslinking F-actin and microtubules in cultured cells, but could affect the kinetics of the interactions of Shot with the cytoskeleton in neurons. Changes in intracellular Ca^{2+} concentration could regulate its subcellular distribution, as observed for ACF7 in keratinocytes (Karakesisoglou et al., 2000). Alternatively, the EF-hand motifs may regulate the association of Shot with other proteins that regulate cytoskeletal dynamics or other cell biological processes important for motility. Through such mechanisms, Shot may link signaling events to growth cone motility.

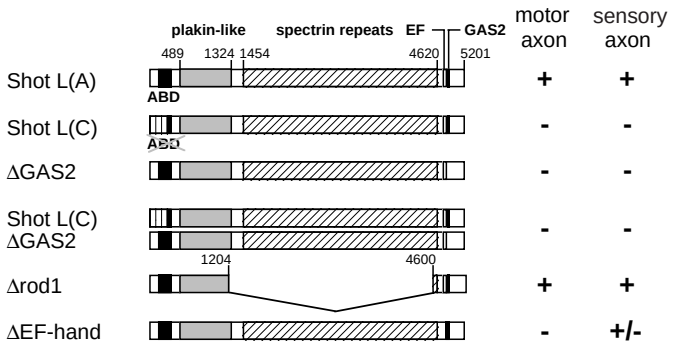

Fig. 7. Domains in Shot L(A) required for axon extension. Neuronal expression of Shot L(A)-GFP rescues (+) motor and sensory axon extension defects in *shot* mutants. Shot L(A) contains an N-terminal actin-binding domain (ABD, black box), a central rod domain consisting of a plakin-like region (gray box), 22 spectrin repeats (hatched box), and a C-terminal domain with Ca^{2+}- (EF-hand) and microtubule- (GAS2) binding motifs. Shot L(C)-GFP contains no ABD and has no rescue activity (–). Derivatives of Shot L(A)-GFP that lack the EF-hand, GAS2 homology or the spectrin repeat sequences were tested for motor and sensory axon extension activity. Shot L(C) and Shot L(A)-ΔGAS2 also have no rescue activity when co-expressed.

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