

short stop Is Allelic to kakapo, and Encodes Rod-Like Cytoskeletal-Associated Proteins Required for Axon Extension

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short stop (*shot*) is required for sensory and motor axons to reach their targets in the *Drosophila* embryo. Growth cones in *shot* mutants initiate at the normal times, and they appear normal with respect to overall morphology and their abilities to orient and fasciculate. However, sensory axons are unable to extend beyond a short distance from the cell body, and motor axons are unable to reach target muscles. The *shot* gene encodes novel actin binding proteins that are related to plakins and dystrophin and expressed in axons during development. The longer isoforms identified are predicted to contain an N-terminal actin binding domain, a long central triple helical coiled-coil domain, and a C-terminal domain that contains two

EF-hand Ca²⁺ binding motifs and a short stretch of homology to the growth arrest-specific 2 protein. Other isoforms lack all or part of the actin binding domains or are truncated and contain a different C-terminal domain. Only the isoforms containing full-length actin binding domains are detectably expressed in the nervous system. *shot* is allelic to *kakapo*, a gene that may function in integrin-mediated adhesion in the wing and embryo. We propose that *Shot*'s interactions with the actin cytoskeleton allow sensory and motor axons to extend.

Key words: *Drosophila*; cytoskeleton; *GAS2*; axon; growth cone; short stop; *kakapo*

The tips of developing axons, growth cones, undergo complex morphological changes in response to extracellular guidance cues (Tessier-Lavigne and Goodman, 1996). Rearrangements of the actin cytoskeleton are required for growth cone motility and guidance (Yamada and Wessells, 1973; Letourneau and Marsh, 1984; Bentley and Toroin-Raymond, 1986). Actin is assembled into filaments at the leading edge, transported away from the membrane by a process termed retrograde flow, and then disassembled (Welch et al., 1997). In culture, retrograde flow rates are inversely proportional to the rate of axon extension (Lin and Forscher, 1995). Although the mechanisms are not well understood, actin dynamics in the growth cone are likely coordinated with the movement of other cellular components such as membrane and microtubules.

Mutations that affect axon extension could interfere with different aspects of actin dynamics. For example, mutations in profilin, an actin binding protein (Pollard and Cooper, 1984; Wills et al., 1999), and Rho family GTPases, regulators of actin assembly (Luo et al., 1994; Hall et al., 1998), affect axon extension in the

Drosophila embryo, and mutations in *unc-115*, a novel actin binding protein, alter axon development in *Caenorhabditis elegans* (Lundquist et al., 1998). Although many actin binding proteins are known, relatively few have been shown to affect neuronal morphogenesis *in vivo*, presumably because they are required earlier in development.

We show here that *short stop* (*shot*), a gene required for sensory and motor axon extension, encodes novel proteins that are predicted to contain actin binding domains and are expressed in axons. *shot* mutants were originally described as affecting the entry of a subset of motor axons into target muscle fields in the *Drosophila* embryo (Van Vactor et al., 1993). We demonstrate that strong *shot* mutants, which also affect sensory axon extension (Kolodziej et al., 1995), disrupt the extension of all motor axons. Dye fills of sensory neurons indicate that the timing of outgrowth initiation and the ability of growth cones to form filopodia or lamellopodia appear normal in *shot* mutants. Sensory and motor growth cones can still assess some directional cues in *shot* mutants; they orient and extend properly during early parts of their trajectory but fail to continue advancing.

The *shot* cDNAs match in part those identified recently for *kakapo* (*kak*), which encodes a novel, evolutionarily conserved plakin-like, actin binding protein (Gregory and Brown, 1998; Strumpf and Volk, 1998). *kak* is required for wing epithelium formation (Prout et al., 1997), epidermal muscle attachment cell differentiation (Strumpf and Volk, 1998), and synapse formation (Prokop et al., 1998), but its role in axon extension has not been described. In *shot/kak* mutants defective in neuromuscular synapse formation, motor axons reach their target muscles (Prokop et al., 1998).

Plakins, such as plectin, bullous pemphigoid antigen 1 (BPAG1), and envoplakin, are long rod-like proteins that bind actin filaments via an N-terminal domain and other cytoskeletal

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proteins or membrane proteins via their C-terminal domain or at other sites (Ruhrberg and Watt, 1997). The long isoforms of Shot/Kak (5500 amino acids) and their mammalian homologs contain a C-terminal domain with homology to the mammalian growth arrest-specific 2 (Gas2) protein, a cytoskeletal-associated protein that is induced in fibroblasts during serum starvation (Schneider et al., 1988). We have identified isoforms that likely differ in their actin binding properties, the length of the rod domain, and the C-terminal domain. Shot thus forms a family of rods predicted to vary between 75 and 220 nm in length and could play a key role in organizing the cytoskeleton in the ~500-nm-wide fly axon.

MATERIALS AND METHODS

Drosophila strains and genetics. The diepoxybutane-generated *short stop* alleles used in this study were isolated previously (Kolodziej et al., 1995) and generated on an Oregon R background. The P-element alleles were identified by screening a collection of P-element lethals (Torok et al., 1993) for failure to complement the lethal *shot*³ mutation. Pairwise crosses among all *shot* alleles confirm that they are part of a single complementation group. The *kak*¹ strain and the deficiency *Df(2R)CX1* were obtained from the Bloomington Stock Center, and the deficiency strains *Df(2R)Su(z)2-1.a3* and *Df(2R)Su(z)2-1.a4* were a kind gift of Ting Wu (Harvard Medical School). The *shot* alleles also fail to complement *Df(2R)CX1* and *Df(2R)Su(z)2-1.a4*, but complement *Df(2R)Su(z)2-1.a3*. These deficiencies remove the following chromosomal regions: 49D1;50D13, 49D1-E1;50C10, and 49E1-F2; 50B1-C4. Thus, the complementation behavior of *shot* alleles is consistent with the chromosomal location for *shot*: 50C6–10 on chromosome II. *shot* was physically mapped by hybridizing *shot* cDNA probes to a P1 phage physical array of the fly genome (Genome Systems; Berkeley Drosophila Genome Project) and by identifying the insertion site for *l(2)k06601*, a P-element allele of *shot*, on polytene chromosomes (Berkeley Drosophila Genome Project). We reverted the lethality of *l(2)k05460* and *l(2)k06601* by mobilization of the P-element (Robertson et al., 1988), thus confirming that the lethality of these P-element alleles is attributable to the P-element insertions. To show that the P-elements of *l(2)k05460* (*shot*^{P1}) and *l(2)k06601* (*shot*^{P2}) are inserted in *shot*, the elements were mobilized by crossing in the $\Delta 2-3$ transposase (Robertson et al., 1988). The chromosomes containing excision events were then tested for their ability to complement *shot*³. Viable excisions were recovered for all P-element *shot* alleles. Wild-type dye fills were performed on *yw*^{Harvard} flies; flies heterozygous for *shot* mutations were used as wild type in other experiments.

Cloning of shot. Genomic DNA fragments adjacent to the P-elements of *l(2)k06601* were isolated by plasmid rescue using *EcoRI* or *SacII* and then used as a probe to screen a λ gt10 cDNA library (Clontech, Palo Alto, CA) prepared from 12–24 hr embryonic RNA. Seven cDNA clones were isolated and sequenced on both strands. They contain common 3' but one of four different 5' sequences. Because these partial cDNAs contained long open reading frames without a stop codon, probes derived from the common sequences were used to rescreen both the λ gt10 and a plasmid cDNA library prepared from 12–24 hr embryonic RNA (Brown and Kafatos, 1988). To screen the plasmid library, 50 pools of $\sim 5 \times 10^4$ clones were screened by PCR and then screened by filter hybridization to isolate clones containing the 3' end of *shot*.

Sequencing was performed using the Perkin-Elmer fluorescent sequencing kit (Perkin-Elmer, Norwalk, CT). The samples were analyzed by the Duke University Microchemistry Facility using an ABI automated sequencer, and the results were edited and compiled using EditView (Perkin-Elmer), AssemblyLign (Oxford Biomolecular), and MacVector (Oxford Biomolecular) software. The predicted amino acid sequences were analyzed using MacVector (Oxford Biomolecular) and MacStripe, a coiled-coil prediction program (Lupas, 1997), and compared with nonredundant sequences using Blastp (Altschul et al., 1990).

Immunohistochemistry. Immunohistochemistry with monoclonal antibodies (mAbs) 1D4 (Van Vactor et al., 1993), 22C10 (Fujita et al., 1982), and 49C4 (Kolodziej et al., 1995) has been described. Whole-mount staining of embryos with antibody against Kak/Shot has been described previously (Kolodziej et al., 1995); fixation in B5 fix (3% HgCl₂, 0.12% NaOAc, 5% formaldehyde 1:1 with heptane) for 20 min followed by devitellinization in MeOH and rehydration in PBS/0.1% Triton/0.2%

BSA improved the sensitivity of the antiserum but did not alter observed pattern of expression. Embryos were staged according to previously described morphological criteria (Campos-Ortega and Hartenstein, 1985). Stage 14 and early stage 15 embryos chosen for dye filling were classified according to the degree of dorsal closure and gut morphology of intact embryos. Antibody-stained embryos were filleted and mounted in 90% glycerol for photography; homozygous mutant embryos were identified by the absence of β -galactosidase expression associated with a lacZ-containing CyO second chromosome balancer. *In situ* hybridization experiments were performed with *shot* antisense or sense probes as described (Tautz and Pfeifle, 1989), and the embryos were mounted in Permunt (Fisher Scientific, Pittsburgh, PA). In-focus regions were assembled into a photomontage using Adobe Photoshop. Dye injections into lateral chordotonal neurons were performed as described (Merritt and Whittington, 1995) and mounted whole in 70% glycerol for photography. In-focus regions of digitally recorded optical sections were then photomontaged.

RESULTS

short stop is allelic to *kakapo*

Mutations in *short stop* (*shot*) have variable effects on the outgrowth of sensory axons, with *shot*¹ being the weakest and *shot*³ the strongest allele (Kolodziej et al., 1995). *shot*¹ also affects the outgrowth of a subset of motor axons (Van Vactor et al., 1993); the effects of other *shot* alleles on motor axon outgrowth have not been characterized. To isolate additional *shot* alleles and to clone the gene, we screened a collection of transposable P-element-induced lethals (Torok et al., 1993) for failure to complement *shot*³. We identified four lethal P-element insertion alleles: *l(2)k03010*, *l(2)k03405*, *l(2)k05460*, and *l(2)k06601*. We isolated genomic DNA fragments flanking the P-elements by plasmid rescue (Bier et al., 1989) and found that all of the P-elements are inserted within a 15 kb genomic region based on Southern and sequence analysis (Fig. 1A). We confirmed that these P-elements are insertions in the *shot* gene by mobilizing the P-elements and reverting the lethal phenotypes (Robertson et al., 1988) and physically mapping them to the chromosomal region defined genetically to contain *shot* (see Materials and Methods). Therefore, we renamed *l(2)k05460* and *l(2)k06601* alleles as *shot*^{P1} and *shot*^{P2}, respectively. As described below, embryos homozygous for any of the P-element insertions also lack detectable Shot protein.

Shortly after we completed the *shot* sequence, we found that it matched partial sequences of *kakapo* (*kak*) deposited in the GenBank database (accession numbers AJ011924 and AJ011925). We then tested whether *kak* (Prout et al., 1997) was allelic to *shot* and found that the *kak*¹ allele failed to complement chemically generated and P-element *shot* alleles. The P-element insertions *l(2)k03010* and *l(2)k03405* were also recently identified as alleles of *kak* and renamed as *kak*^{P1} and *kak*^{P2}, respectively. *kakapo*, also identified as *kopopu* (Walsh and Brown, 1998), has been identified as a gene required for adhesion of the wing epithelium (Prout et al., 1997; Walsh and Brown, 1998) and more recently as required for muscle attachment to the epidermis (Gregory and Brown, 1998; Strumpf and Volk, 1998) and neuromuscular synapse formation (Prokop et al., 1998).

The *shot/kakapo* gene encodes novel proteins that differ in their predicted actin binding properties and are related to plakins and dystrophin

We used genomic DNA flanking the *shot*^{P2} insertion to identify *shot* gene transcripts. We screened a λ gt10 cDNA library with adjacent DNA fragments, and 15 different overlapping clones were recovered and partially sequenced. These cDNAs all encode predicted proteins with homology to the plakin and dystrophin

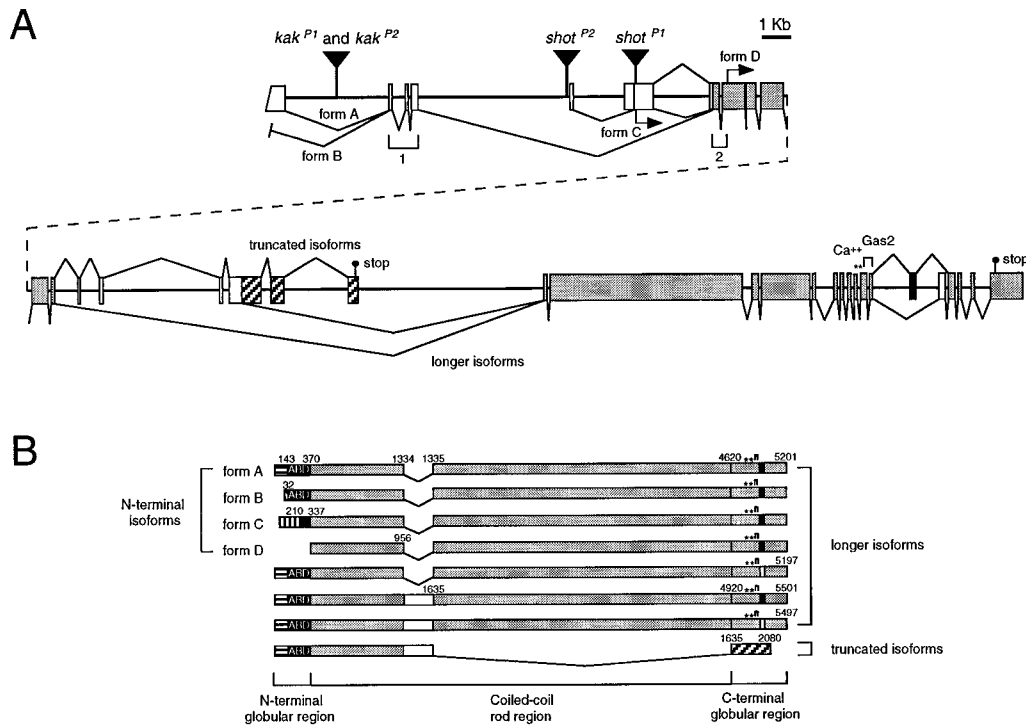


Figure 1. Multiple transcriptional starts and alternative splicing throughout the *shot stop/kakapo* gene generate protein isoforms predicted to differ in their actin binding properties (isoform types A–D), rod domain length, and C-terminal domains. **A**, The genomic structure of the *shot/kak* gene at 50C6–10 on chromosome II and P-element insertion sites. *Inverted triangles* indicate the sites of P-element insertions, and the *arrows* in the 5' end of the gene indicate the likely transcription start points for the mRNAs encoding isoforms C and D, as inferred from the corresponding cDNAs. The *boxes* represent exons (35) mapped in the *shot* gene by comparison of cDNA with genomic DNA sequence (Berkeley Drosophila Genome Project), and the patterns of alternative splicing are indicated. The first exon shown is exon 3. An additional two exons that encode the 5' ends of isoforms A and B, respectively, are located upstream of this genomic region (Gregory and Brown, 1998), and their sizes and positions have not been analyzed in this study. *Gray boxes* indicate exons common to longer isoforms; *white boxes* indicate alternatively spliced exons. *Black box* indicates an alternatively spliced exon in the sequence encoding the long isoform C-terminal domain; *cross-hatched boxes* indicate the exons encoding the C-terminal domain of the shorter isoform. The exons indicated by *brackets 1* and *2* encode the predicted actin binding domain found in isoforms A and B; the exons indicated by *bracket 2* are the only portion of the predicted actin binding sequences retained in the isoform C mRNA. *Asterisks* denote the exons encoding the Ca²⁺-binding EF-hand motifs (Ikura, 1996). The exons encoding the GAS2 domain are indicated. **B**, Some of the predicted proteins encoded by the *shot* gene. Four different N-terminal sequences were identified. Two isoforms (A and B) contain complete predicted actin binding domains (*black box* labeled ABD), following unique sequences of 143 (*horizontal striped box*) or 32 amino acids (*cross-hatched box*), respectively. Isoform C contains a partial ABD (*black box*), following a unique sequence of 210 amino acids (*vertically striped box*), and isoform D contains no globular N-terminal domain. Alternative splicing in the middle of the protein can cause the insertion of a 300 amino acid coiled-coil sequence (*long white box*), and alternative splicing of the C-terminal domain of the long isoform generates additional diversity (*small black* or *white boxes*). The rod domain is shaded in gray. A second C-terminal domain (*long cross-hatched box*) is associated with a shorter rod domain. The *numbers* refer to the amino acid residues at the junction of these different protein domains for each isoform depicted. Some of the combinatorial possibilities are diagrammed for variants of isoform A; the other N-terminal sequences may exist in similar combinations.

families of actin binding proteins (Michalak and Opas, 1997; Ruhrberg and Watt, 1997). These rod-like proteins link actin to other cytoskeletal or membrane proteins. Complete sequencing of seven of these cDNAs revealed four classes (A, B, C, and D) of alternatively initiated or spliced transcripts (Figs. 1, 2A). Interestingly, the predicted proteins encoded by these cDNAs differ in the extent of the actin binding domain that they retain, as well as in their extreme N-terminal sequences (Fig. 2A).

Four cDNAs contained sequences encoding isoforms A and B of Kak (Gregory and Brown, 1998). Isoform A contains a unique 143 amino acid N-terminal sequence; isoform B contains a unique 32 amino acid N-terminal sequence. The N-terminal sequences in isoforms A and B are followed by a 227 amino acid domain that is predicted to bind actin (Fig. 2A). This actin binding domain contains the region of highest homology (60% sequence identity, 88% sequence similarity) to plakins such as plectin, ACF7, and BPAG1 (Fig. 2B). The corresponding domains of BPAG1 (Yang et al., 1996) and plectin (Andra et al., 1998) have been shown to bind actin *in vitro* and *in vivo*.

Two *shot* cDNAs (form C) encode a third unique N-terminal sequence of 210 amino acids followed by half of the actin binding domain (Figs. 1, 2A). This second half of the actin binding domain is less evolutionarily conserved (40% identical with the plakins shown here) than the first half of the actin binding domain (81% identical with these other plakins) (Fig. 2B). The mammalian BPAG1n3 protein also contains a similar half actin binding domain and associates poorly with the actin cytoskeleton in cultured cells (Yang et al., 1999).

Finally, isoform D lacks an identifiable actin binding domain and contains no N-terminal globular domain (Figs. 1, 2A). The likely initiator methionine codon for isoform D is located downstream of sequences encoding the actin binding domain, at the start of sequences encoding the central rod domain (Fig. 1A).

Thus, *shot* encodes various rod-like proteins predicted to differ in their actin binding properties. The genes for other plakins (Ruhrberg and Watt, 1997) also encode similarly spliced actin binding variants (Fig. 2B). Four different 5' isoforms are encoded by the *BPAG1* gene (Brown et al., 1995a,b; Yang et al., 1999).

A

form A	MTSHSYKDRLGFDPNEQQPGSNNMCKRSSSRQTTHHHQSYHHATTSSSSQ	50
	SPARISVSPGGNNGTLEYQQVQREQRDRELYSNNGSLHHHQHHHHHRHS	100
	TGSSASSPLYENSSSPAAPKKAKHSSTQAQPOGGYEDALTQFKDERDAIQ	150
	KKTFTKWNKHLKKANRRVVDLFDLDRDGHNLLSLEVLSGEHLPREKGGK	200
	MRFHMLQNAQMALDFLRYKKIKLVNIRAEDIVDGNPKLTLGLIWTIILHF	250
	QISDIVVGKEDNVSAREALLRWARRSTARYPGVRVNDFTSSWRDGLAFSA	300
	LVHRNRPDLLDWRKARNDRPRERLETAFHIVEKEYGVTRLLDPEDVDTNE	350
	PDEKSLITYISSLYDVFPPEPSIHLPLFDMESQRRVHEYRDLAQQFIYW	398
form B	MMDRESLSEWAKDKPLSILQLDPADRAVLRIADERDAIQKKTFTKWNKH	50
	LKKANRRVVDLFDLDRDGHNLLSLEVLSGEHLPREKGGKMRFHMLQNAQM	100
	ALDFLRYKKIKLVNIRAEDIVDGNPKLTLGLIWTIILHFQISDIVVGKED	150
	NVSAREALLRWARRSTARYPGVRVNDFTSSWRDGLAFSALVHRNRPDLLD	200
	WRKARNDRPRERLETAFHIVEKEYGVTRLLDPEDVDTNEPDEKSLITYIS	250
	SLYDVFPPEPSIHLPLFDMESQRRVHEYRDLAQQFIYW	287
form C	MHSSSQSTLKRTOQLVQRQTNGGRDVLSSGSEVHSSRGPAAITTNIDDSN	50
	LRVRHETKRERTVEAVITDYPGVSPLGSPHERTNFERTVTFQDPTGNSR	100
	RLSETGALVPAGAIPTSSSTHYQQVTRNKRISTEVLGSSVESTKRSQRAPN	150
	GHRVSTHIVRKVTTLSRAEENAQPAEDLLPPAKMIRSRSELEYRRALPPA	200
	IESSSTQRREISDIVVGKEDNVSAREALLRWARRSTARYPGVRVNDFTSS	250
	WRDGLAFSALVHRNRPDLLDWRKARNDRPRERLETAFHIVEKEYGVTRLL	300
	DPEDVDTNEPDEKSLITYISSLYDVFPPEPSIHLPLFDMESQRRVHEYRDL	350
	AQQFIYW	357
form D	MESQRRVHEYRDLAQQFIYW	20

B

dShot/dKak	DERDAIQKKTFTKWNKHLKKANRRVVDLFDLDRDGHNLLSLEVLSGEHL	193
mACF7	DERDRVQKKTFTKWNKHLKVKRKHINDLYEDLDRDGHNLLSLEVLSGIK	126
hPlectin	DERDRVQKKTFTKWNKHLIKAKQRHISDLVEDLDRDGHNLLSLEVLSGDS	224
hBPAG1	DERDKVQKKTFTKWINQHLKVKRKHVNDLYEDLDRDGHNLLSLEVLSGDT	80
dShot/dKak	LPREKGMRFHMLQNAQMALDHLRYKKIKLVNIRAEDIVDGNPKLTLGLI	243
mACF7	LPREKGMRFHMLQNVQIALDHLKQRQVCLVNIRAPDITDGNPKLTLGLI	176
hPlectin	LPREKGMRFHMLQNVQIALDYLKHRQVCLVNIRDDITDGNPKLTLGLI	274
hBPAG1	LPREKGMRFHMLQNVQIALDYLKRRQVCLVNIRNDITDGNPKLTLGLI	130
dShot/dKak	WTIILHFQISDIVVGKE-DNVSAREALLRWARRSTARYPGVRVNDFTSSW	292
mACF7	WTIILHFQISDIYISGDSGDMSTAKEKLLWTKVTFAGYTGKCTNFSSCW	226
hPlectin	WTIILHFQISDIQVSGQSEDMSTAKEKLLWSQRMVEGYQGLRCDNFSSW	324
hBPAG1	WTIILHFQISDIHVTGSEDMSTAKERLLWTKQATEGYAGVRCENFTTCW	180
dShot/dKak	RDGLAFSALVHRNRPDLLDWRKARNDRPRERLETAFHIVEKEYGVTRLLD	342
mACF7	SDGKMFNAITHRYRPPDLVNMERVQVQSNRENLECAFVVAER-LGVTRLLD	275
hPlectin	RDGRMFNAITHRHKPLLDLDMNKVYRQTNLENLDQAFSVAERDLGVTRLLD	374
hBPAG1	RDGKMFNAITHKLRPDLIDMNTVAVQSNLANLEHAFYVAER-LGVTRLLD	229
dShot/dKak	PEVDVTNEPDEKSLITYISSLYDVFPPEP	370
mACF7	AEDVDVPSPEKSVITYVSSLYDAFPRV	303
hPlectin	PEVDVVPQPEKSLITYVSSLYDAMPKV	402
hBPAG1	PEVDVSSPEKSVITYVSSLYDAFPRV	257

Figure 2. The *shot* gene encodes isoforms predicted to vary in their actin binding properties. *A*, The four predicted N-terminal amino acid sequences of Shot/Kak isoforms. The sequence shaded in black encodes the predicted actin binding domain in different isoforms. Only half of this sequence is present in isoform C (accession number AF200422), and isoform D (accession number AF200423) contains no actin binding sequences. The sequences shaded in gray indicate the sequences common to all isoforms. *B*, Comparison of the predicted actin binding domain sequence from *Drosophila* Shot isoform A (dShot; accession number CAA09869) with the corresponding sequences in mouse ACF7 (mACF7; accession number AAC52989), human plectin (hPlectin; accession number CAA91196), and human BPAG1 (hBPAG1; accession number I39160). BPAG1 is also known as dystonin. Sequence identities are indicated by white letters, with identities among all four sequences indicated on a black background. Sequence similarities (+1 or more in a PAM 250 matrix) are indicated by black letters on a gray background. The inverted triangle marks where the second half of the actin binding domain begins, as found in Shot isoform C, hBPAG1n3 (accession number AAD49334) (Yang et al., 1999), and mACF isoform 3 (accession number AAC52990) (Bernier et al., 1996).

A

QLGERMRSCE	TAIGLMKNLOSSVQVEESWVDGTT	ERLSAMPTATSAYELD	1334
KL	LGAAIERKPKIENVNVAGGRLIREAKI	YDSKCLRFDWLVEARPSFSP	1384
PRRDLR	PADSDPGATOYYSORLDNLNTKYDR	LLEQLSQRLKTAIEVNGSD	1434
GLQYAES	LQKPLKTFRVDFBSAGSVPTGDGYA	ARQEDLYTTTTYSTTQISST	1484
KTTKSST	TKSVYSSDGLDAASQEVSTAGLPQS	OIQFNEIRTLKRSQQLGGH	1534
SVLDIAG	IRDPRRTGRVLTIGEAIQLRILDV	RTGEMLVGDRRITLEQAADO	1584
GLIDLQL	AKQLLEPGAGRDASGRELSLLEVI	QREISEAESGYETAKRKIK	1634
VNNTVTV	EQTSGELGSPENPRNIADAITAGS	VDTKTGLYRVKSGOTISLA	1684
EAYERGY	LIRHESVTIKSNALCLSDAIAHGL	VDGAGWIADNRNSGDKFRLD	1734
SAIANQL	IDASVREVVDAKRDTKITLQ	EALQSGVLNAKTGRYVNEVTKEK	1784
LTFAEARN	RQLIVKPYTLKDICDLNLLDKQAQ	ITSPMRREKLSIMQAI EA	1834
GVL	DGNLLK C I T K R K G E L V T L Q E A I A D G I V L P A E C K Y R D F M T G E L I S I P E		1884
AVRQGF	ID E E R Q L F K D P K T G N I Y S V Q Q A L N Y G L L V P D S N Q T V P E P T N R K K		1934
TKSTIT	I V T K Q I I P E A E P I K L N T Q H T K Y V E K S V E I P I S Q E L V K P H R V V S E		1984
FINLEK	S S Y I E Q N V T E R Q I M E L P P G G W R L K D A I E Q R L F N P D T G V F H V Q G T		2034
DRLVN	F E E C I N K H S D H N N S N N Y C I K Y N N G D R K S G A S C V R R C F D H S		2080

B

		EF-hand								
dKak	LKYMNHKKSR	LDLFRKMDKDNGMIP	PRDVFIDGILN	TKFDTSGL	LEMKAV	3765				
dShot	LKYMNHKKSR	LDLFRKMDKDNGMIP	PRDVFIDGILN	TKFDTSGL	LEMKAV	5111				
mACF7	MRYMNHKKSR	MDLFRKMDKDNGMIP	PRDVFIDGILN	TKFDTSGL	LEMKAV	5018				
KIAA0465	MRYMNHKKSR	MDLFRKMDKDNGMIP	PRDVFIDGILN	TKFDTSGL	LEMKAV	1394				
KIAA0728	MRYMNHKKSR	MDLFRKMDKDNGMIP	PRDVFIDGILN	TKFDTSGL	LEMKAV	723				
		EF-hand								
dKak	ADLFDNRNGE	GLIDWQEFIAAL	RPDWQERKPAN	DSDKIHDEVK	KRLVMLCTC	3815				
dShot	ADLFDNRNGE	GLIDWQEFIAAL	RPDWQERKPAN	DSDKIHDEVK	KRLVMLCTC	5161				
mACF7	ADLFDNRNGE	GLIDWQEFIAAL	RPDWQERKPAN	DSDKIHDEVK	KRLVMLCTC	5068				
KIAA0465	ADLFDNRNGE	GLIDWQEFIAAL	RPDWQERKPAN	DSDKIHDEVK	KRLVMLCTC	1444				
KIAA0728	ADLFDNRNGE	GLIDWQEFIAAL	RPDWQERKPAN	DSDKIHDEVK	KRLVMLCTC	773				
		Gas2								
dKak	RQKFRVFQ	VGEK-----Y	FGDSQK	LRLVRI	LRSTVMVR	VGGGWV	ALD	3859		
dShot	RQKFRVFQ	VGEK-----Y	FGDSQK	LRLVRI	LRSTVMVR	VGGGWV	ALD	5205		
mACF7	AKRFQVEQ	GENKYPFLC	NQFGDSQK	LRLVRI	LRSTVMVR	VGGGWV	ALD	5118		
KIAA0465	AKRFQVEQ	GEN-----K	FGDSQK	LRLVRI	LRSTVMVR	VGGGWV	ALD	1488		
KIAA0728	AKRFQVEQ	GENKYPFLC	NQFGDSQK	LRLVRI	LRSTVMVR	VGGGWV	ALD	823		
		Alternative Splice								
dKak	EF	LQKNDPCR	AD-EHLAEL	MPI	FEKIRA	QDVPC	AFP-----I	HMGAGGT	3903	
dShot	EF	LQKNDPCR	AKGATNIEL	AEQF	ILADGVS	QSMAAFT	PRRSTP	NAAATAS	5255	
mACF7	EF	LQKNDPCR	ARGRTNIEL	AEKFI	LPEGAS	QGMPFR	-----	-----	5155	
KIAA0465	EF	LQKNDPCR	ARGRTNIEL	AEKFI	LPEGAS	QGMPFR	-----	-----	1525	
KIAA0728	EF	LQKNDPCR	AKGRTNIEL	AEKFI	LADGAS	QGNAAF	-----	-----	860	
		Alternative Splice								
dKak	VF	VRCNTSR	SVPLSPHVL	HCHPT	THWV	ERSVRS	IPMSR	PSRSLS	SASTP	3963
dShot	SS	PHAHNGG	SSNPYMSG	QGI	IIK	ERSVRS	IPMSR	PSRSLS	SASTP	5304
mACF7	-----	-----	-----	-----	-----	SRGR	RSKPS	SRAASPT	RSSSSA	5177
KIAA0465	-----	-----	-----	-----	-----	SRGR	RSKPS	SRAASPT	RSSSSA	1547
KIAA0728	-----	-----	-----	-----	-----	PRGR	RSRPS	SRGASFN	STSVS	882

Figure 3. Shot proteins contain different C-terminal domains attached to rodlike domains of varying lengths. *A*, Two classes of long isoforms of Shot/Kak exist, one of which (Gregory and Brown, 1998) contains a 300 amino acid sequence insertion (*shaded* residues) predicted to form additional coiled-coil sequence. The Shot/Kak isoforms predicted by most of our cDNAs and reported by Strumpf and Volk (1998) lack this sequence. A novel truncated isoform of Shot/Kak is shown here that contains this 300 amino acid insertion, but its rod region terminates in an alternative sequence of 346 amino acids that may form a globular domain (sequence after *shaded* residues) (accession number AF200424). *B*, The C-terminal domain of the long isoform of Shot contains two EF-hand motifs and a GAS2 homology domain (Strumpf and Volk, 1998), features that are conserved in the C-terminal domain of novel plakin-like vertebrate proteins. These sequences in Shot/Kak are aligned with the corresponding sequences in ACF7 (accession number AAD32244) and available partial sequences of two plakin-like human proteins (KIAA0465, accession number BAA32310; KIAA0728, accession number BAA34448) (Seki et al., 1997). The accession number for the Kak C-terminal sequence is CAA70581, and the accession number for the Shot C-terminal sequence is AF200425. The sequences of the fly and vertebrate proteins are approximately 200 residues longer than shown and contain additional regions of lower homology. Identical residues are indicated in *white*, and similar residues are indicated in *black*. (Figure legend continues)

Two neuronal isoforms, BPAG1n1 (dystonin-1) and BPAG1n2 (dystonin-2), contain an actin binding domain, a third neuronal isoform, BPAG1n3, contains a half-actin binding domain analogous to that found in Shot isoform C, and the epidermal isoform BPAG1e contains no actin binding domain, as in Shot isoform D. Mouse ACF7 is 71% identical to BPAG1 within the predicted actin binding domain and encodes N-terminal isoforms similar to Shot isoforms A, B, and C (Bernier et al., 1996). Both isoforms 1 and 2 of mACF7 are predicted to contain a complete actin binding domain; mACF7 isoform 3 lacks the most conserved portion of the actin binding domain and contains the less conserved portion exactly as in Shot isoform C (Fig. 2B) (Bernier et al., 1996).

Our results, in conjunction with those reported previously (Gregory and Brown, 1998), suggest that the *shot* gene may contain as many as four different promoters (Fig. 1A). We investigated the relationship between the P-element insertions in *shot* and the *shot* promoter and transcription start sites. We used a combination of PCR, Southern analysis, and sequencing to map the *shot*^{P1}, *shot*^{P2}, *kak*^{P1}, and *kak*^{P2} insertion sites and the cDNA sequences onto a 15 kb genomic DNA contig (Fig. 1A). The *kak*^{P1} and *kak*^{P2} P-elements are inserted at the same site, in an intron 1917 bp before the first exon common to mRNAs encoding isoforms A and B (Gregory and Brown, 1998) (Fig. 1A). The *shot*^{P2} insertion is 49 bp upstream of the start of alternative transcript C (Fig. 1A). The *shot*^{P1} insertion is located in an exon common to both isoforms C and D, 131 bp downstream of the alternative splice site that generates isoform C (Fig. 1A). Although they are inserted at different sites with respect to the *shot* transcripts, all of the P-element insertions disrupt the protein expression of the long isoforms of Shot and appear to be similar in their axon growth phenotypes (see below).

***shot/kak* encodes rod-like proteins of varying length that contain one of two alternative C-terminal domains**

The open reading frame in the cDNAs that we initially characterized lacked 3' stop codons, and Northern blot analysis revealed the *shot* mRNAs to be ~17 kb in size [Strumpf and Volk (1998), and data not shown]. Therefore, we rescreened both the λ gt10 (Clontech) and the plasmid cDNA libraries (Brown and Kafatos, 1988) using the most 3' portion of the composite cDNA and isolated overlapping cDNA clones that extended the open reading frame to the 3' end of *shot*.

These clones identify additional isoforms of Shot/Kak, the longest of which encodes a 5501 amino acid protein that is almost completely identical to the previously reported 5497 amino acid protein (Gregory and Brown, 1998; Strumpf and Volk, 1998) (Fig. 1B). The central region of this protein is likely to be rod-like and contains 22 triple helical coiled-coil repeats (Strumpf and Volk, 1998) similar to those found in spectrin (Speicher and Marchesi, 1984) and dystrophin (Dubreuil, 1991). The C-terminal globular domain contains two EF-hand motifs (Ikura, 1996). The C-terminal globular domain also contains a short stretch of sequence homology to the mammalian growth arrest-specific 2 (Gas2) protein (Schneider et al., 1988). Gas2 is a cytoskeletal protein of unknown function that appears to be associated with

microfilaments in cultured cells and is highly induced in cultured fibroblasts during serum starvation (Brancolini et al., 1992).

The cDNA sequences that we isolated for the 3' end of the *shot* gene reveal that the central rod region is also alternatively spliced (Figs. 1, 3A). Seven of eight cDNA clones predict an isoform that shares the same central rod sequence reported previously (Strumpf and Volk, 1998). The other clone encodes a 300 amino acid sequence within the central rod region, as previously reported for Shot/Kak isoforms A and B (Gregory and Brown, 1998).

We also isolated a cDNA in which the sequence for the 300 amino acid region is spliced to a novel sequence that encodes a globular domain of 436 amino acids (Fig. 3A). This 436 amino acid domain shows low homology to the six tandem repeat domains in the C-terminal globular region of plectin (Liu et al., 1996) and is unlikely to form extensive coiled-coil structure. Although it still contains considerable coiled-coil forming sequence (Lupas, 1997), this truncated isoform lacks the 22 triple helical repeats found in the longer isoforms. By comparing the known lengths of plectin (Foisner and Wiche, 1987) and dystrophin (Pons et al., 1990) proteins, and the relative lengths of coiled-coil forming sequence in Shot, plectin and dystrophin (Lupas, 1997), we infer that the truncated isoforms of Shot are ~75 nm long and the long isoforms of Shot are ~200–220 nm long. Thus, *shot* encodes rod-like proteins of varying length with different C-terminal domains, as well as different predicted actin binding properties.

The sequence of the C-terminal domain in the longer Shot isoforms matches the C-terminal sequences of several vertebrate proteins (Seki et al., 1997; Strumpf and Volk, 1998), including the full-length sequence of mouse ACF7 recently reported in GenBank. The sequence of the EF-hand and GAS2 domains are particularly well conserved (Fig. 3B). Sequencing of the *shot* cDNAs also reveals diversity in this C-terminal domain (Fig. 3B). The C-terminal sequence after the Gas2 homology domain is alternatively spliced, with the variant reported here being a closer match to the vertebrate proteins.

Taken together, we have cloned overlapping cDNAs that predict multiple isoforms of Shot/Kak (Fig. 3B) and greatly expand the potential functional diversity of this gene. The gene encodes rod-like proteins of varying lengths, only some of which contain complete N-terminal actin binding domains. These proteins contain two different classes of globular C-terminal domains, which in plakins mediate protein–protein interactions. By analogy, the different Shot proteins may therefore interact with diverse cytoskeletal targets.

The long isoforms of Shot stop are expressed in axons

The previously reported descriptions of *kak* report its expression at high levels in epidermal muscle attachment cells and chordotonal organ support cells and at lower levels in epidermis and in motor axon terminals, but did not analyze its expression in the nervous system during axon development. *shot* mRNAs are expressed also in the CNS and PNS during embryonic stages 13 and 14 when most axon outgrowth begins, and this expression persists

←

Black background indicates sequence identities between *Drosophila* and vertebrate proteins; *gray background* indicates sequence similarities. Gaps to allow a maximal alignment are marked by *dashes*. The amino acid residue numbers for dShot are based on a sequence that includes the sequence of isoform A (Fig. 2A), the long rod domain, and the 300 amino acid sequence found in the middle of some Shot proteins (A). The EF-hand sequence reported previously (Strumpf and Volk, 1998) does not completely match genomic sequence and the EF-hand consensus and has been corrected in this figure. The alternatively spliced region after the GAS2 domain is indicated.

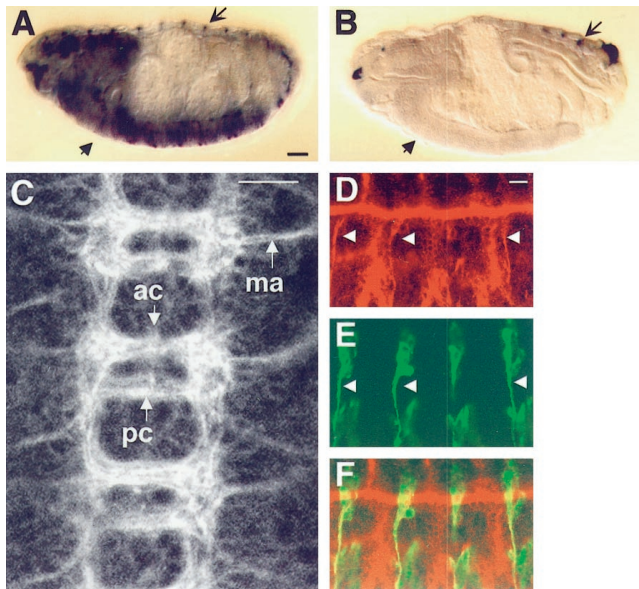


Figure 4. Only Shot isoforms A and B, those that contain complete actin binding domains, are detectably expressed in the nervous system, and the long isoforms of Shot are present in axons. *A*, *In situ* hybridization with a cDNA probe containing 5' sequence derived from the actin binding isoform A and the first half of the actin binding domain coding sequence shared also by isoform B reveals transcripts in the CNS (arrow) and muscle attachment cells (concave arrow) at stage 17 as well as earlier (data not shown). *B*, Whole-mount *in situ* hybridization to a stage 17 embryo with a cDNA probe containing exons specific for isoforms C and D reveals comparable levels of expression in muscle attachment cells (concave arrow), but no detectable expression in the CNS (arrow). Scale bar, 30 μ m. *C*, Confocal (1 μ m) section of a stage 14 wild-type embryo stained with polyclonal anti-Kak (Strumpf and Volk, 1998). This antibody recognizes epitopes in the rod domain present in the long isoforms of Shot/Kak. Axons in the anterior commissure (ac) and posterior commissure (pc), as well as motor axons (ma) and longitudinal axons, contain Shot/Kak. Scale bar, 10 μ m. *D*, A confocal section showing Shot/Kak (red, arrowhead) protein in the sensory axons emanating from the dorsal cluster of sensory neurons in each abdominal hemisegment. The antiserum also recognizes Shot protein in epithelial cells and a nonspecific epitope in tracheal lumen. The epithelial and sensory axon staining disappears in *shot* mutants, but the tracheal lumen staining does not. Scale bar, 10 μ m. *E*, The dorsal cluster sensory neurons and their axons in the same section revealed with mAb 22C10 (Fujita et al., 1982). *F*, Merge of *D* and *E*.

to later stages of embryonic development when synapse formation occurs (Fig. 4*A*). Intriguingly, mRNAs encoding the actin binding domain containing isoforms A and B are expressed in the CNS (Fig. 4*A*), but mRNAs encoding isoforms C and D are not detectably expressed in the CNS (Fig. 4*B*). The mRNAs encoding isoforms A and B are also transiently expressed in the developing PNS at stage 13 (data not shown). This difference in the expression of the actin binding and non-actin binding isoforms parallels the differences observed in the expression of neuronal, actin binding and epidermal, non-actin binding isoforms of BPAG1/dystonin. Otherwise, the mRNAs encoding the N-terminal isoforms reported here, and those identified previously (Strumpf and Volk, 1998), are expressed in identical patterns in the embryo: in the epidermis at low levels and in muscle attachment cells at high levels.

An antiserum against Kak (Strumpf and Volk, 1998) was used to investigate whether the Shot/Kak proteins are expressed in developing axons. This antibody is raised against epitopes present in the 22 triple helical repeats of the long isoforms of Shot/Kak. Therefore it does not detect the shorter isoforms of Shot that we

have identified but recognizes a major band of at least 400 kDa on Western blots (Strumpf and Volk, 1998). In embryos, Shot/Kak protein can be detected in axons in the CNS and PNS (Fig. 4*C–F*). Although the expression level in growth cones is difficult to determine because Shot is expressed in surrounding epidermal cells, Shot is present in CNS axons as early as stage 12 (data not shown) and can be detected in the intersegmental nerve (ISN). In addition, it accumulates in cortical regions of the neuronal cell bodies of chordotonal and dorsal cluster sensory neurons (Fig. 4*D–F*), consistent with an association with the actin cytoskeleton.

Shot/Kak protein expression in neurons, epidermis, and epidermal muscle attachment cells cannot be detected in *shot*³, *shot*^{P1}, *shot*^{P2}, and *kak*^{P2} mutant embryos. *shot*¹ and *shot*² mutants still express normal levels of Shot protein, consistent with their relatively weaker mutant phenotypes. The phenotypes of *shot*^{P1} and *shot*^{P2} insertion mutants are also somewhat weaker than the phenotypes of *shot*³ and *kak*^{P2} insertion mutants, suggesting that Shot proteins may perhaps be present in the nervous system in these mutants at levels not detected by this antibody. The Kak antiserum also reacts strongly with an antigen present in the wall of the tracheal lumen (Fig. 4*D*); this cross-reactivity remains in all of the *shot* mutant alleles tested.

Short stop is required for the continued extension of all sensory axons, rather than their navigation

We identified *shot* in a screen for mutations affecting sensory axon morphology (Kolodziej et al., 1995). *shot*³ mutant embryos stained with mAb 22C10, a reagent that labels all sensory neurons and their axons (Fujita et al., 1982), are revealed to contain only rudimentary sensory axons (Kolodziej et al., 1995). Sensory axon bundles are more variably affected in *shot*² mutants, and defects cannot be detected with the mAb 22C10 antibody in *shot*¹ mutants (Kolodziej et al., 1995). *shot* mutants appear normal with respect to other aspects of neuronal differentiation and viability.

To gauge the relative severity of the *shot* mutations more precisely, we compared the length of a bundle of four sensory axons in wild-type and mutant embryos. mAb 49C4 specifically recognizes four of the five lateral chordotonal (LCH) neurons and their axons (Kolodziej et al., 1995). In wild-type embryos, the LCH sensory axons grow anteriorly, turn ventrally when they join the ISN, and extend into the CNS by stage 16. In *shot*¹ embryos, the LCH axons arrest at variable points within the ISN (Kolodziej et al., 1995). As quantitated here, the LCH axons in *shot*² and *shot*³ are more severely affected (Table 1), and the LCH axons in *shot*³ mutants almost always stall before advancing far along the ISN (93%). In these severe alleles, the LCH axons never extend to the CNS border. The P-element insertions *kak*^{P1}/*kak*^{P2}, *shot*^{P1}, and *shot*^{P2} affect LCH axon growth on average less severely than the *shot*² and *shot*³ alleles but show a similar range of stall phenotypes (Table 1). Although the long form of Shot protein cannot be detected in these P-element mutant embryos (Fig. 4 and data not shown), these slight differences in phenotype may reflect low levels of the long Shot proteins or other isoforms still present in some of the P-element alleles.

The shorter axons observed in *shot* mutants could reflect a delay in axonogenesis or a defect in the ability of growth cones to advance. Dye filling of the chordotonal neurons allowed us to resolve details of the timing of axonogenesis and the morphology of individual axons and growth cones in wild-type and mutant embryos. In wild-type embryos, axonogenesis in the LCH neurons occurs before stage 15 ($n = 14$) (Fig. 5*A*). In *shot*²/*shot*³ mutant embryos, the LCH neurons also extend a growth cone

Table 1. Percentages of wild-type and truncated LCH axons in early stage 16 wild-type and *shot* mutant embryos

Allele	Dorsal ^a	ISN border ^b	ISN ^c	CNS border ^d	CNS ^e
<i>WT</i>	0 (<i>n</i> = 98) ^f	0	0	0	100
<i>WT</i>	0 (<i>n</i> = 21) ^g	0	0	5	95
<i>kak</i> ^{P1}	0 (<i>n</i> = 56) ^f	27	55	18	0
<i>shot</i> ^{P2}	2 (<i>n</i> = 91) ^f	50	46	2	0
<i>shot</i> ^{P1}	1 (<i>n</i> = 91) ^f	40	69	0	0
<i>shot</i> ²	0 (<i>n</i> = 112) ^f	68	32	0	0
<i>shot</i> ² / <i>shot</i> ³	0 (<i>n</i> = 13) ^g	90	7	0	0
<i>shot</i> ³	0 (<i>n</i> = 98) ^f	93	7	0	0
<i>shot</i> ³	0 (<i>n</i> = 7) ^g	63	47	0	0

^aDorsally directed axon bundles. Numbers in parentheses refer to hemisegments examined.

^bThese axon bundles extend anteriorly toward the ISN and stall or fail to reach the ISN.

^cThese axon bundles traveled a significant distance in the ISN.

^dThese axon bundles reached the CNS but did not appear to penetrate it.

^eWild-type axon bundles entered the CNS and formed arborizations.

^fThese axon bundles were visualized using mAb 49C4.

^gThese axons were visualized by dye-filling single LCH neurons.

toward the ISN by early stage 15 (*n* = 6) (Fig. 5*D*). By early stage 16, the LCH axons in wild-type embryos reach the CNS (Table 1, Fig. 5*C*). By early stage 16, no LCH axons in *shot*^{P2}, *shot*³, or *shot*²/*shot*³ mutant embryos reach the CNS (Table 1, Figs. 5*E,F*). Therefore, the stalled axon phenotype detected in stage 16 *shot* embryos does not reflect a delay in axonogenesis.

The inability of growth cones to advance in *shot* mutant embryos could reflect a defect in the formation of actin-based structures known to be important for growth cone motility, such as filopodia or lamellopodia. We found that the LCH axon morphologies observed in *shot* mutant embryos are indistinguishable from those of wild-type embryos at the same point in the axon trajectory (Fig. 5). LCH growth cones in wild-type embryos extend numerous fine filopodia and have a wide lamellopodium before they join the ISN (Fig. 5*A*). As they advance along the ISN, they become narrower and less complex (Fig. 5*B*). These morphological transitions are also observed in *shot* mutant embryos, with the growth cones adopting the general morphology of wild-type growth cones at the place where they stall (Figs. 5*E,F*). Growth cones that fail to contact the ISN are more complex than those that stall within the ISN. The direction of axon growth is also unaffected in *shot* mutant embryos. Thus, growth cones form normally and orient correctly in *shot* mutant embryos but fail to continue advancing. Although growth cone morphology appears to be unaffected, we often observe defects in LCH dendrite morphology in *shot*^{P2}, *shot*³, and *shot*²/*shot*³ mutant embryos (*n* = 8/30) (Fig. 5*D,E*). This is consistent with the description of dendrite detachment reported in *kak* mutants (Prokop et al., 1998).

Short stop is required for the continued extension of all motor axons, rather than their navigation

In the abdominal hemisegments of wild-type embryos, 31 motor axons innervate 30 muscle fibers in a stereotyped pattern (Sink and Whittington, 1991; Landgraf et al., 1997). These motor axons are organized into nerve bundles that innervate different muscle fields. The main ISN motor axons innervate the dorsal muscles, the intersegmental nerve b (ISNb) motor axons innervate ventral muscles, and the segmental nerve a (SNa) motor axons innervate ventrolateral muscles (Sink and Whittington, 1991).

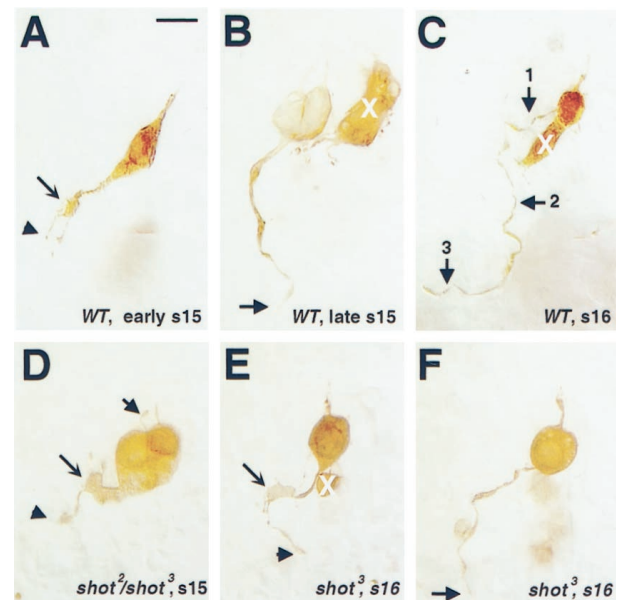


Figure 5. Lateral chordotonal sensory axons are truncated in *shot* mutants. *A*, An LCH5 axon in a very early stage 15 wild-type embryo (*WT*, early *s15*) extends anteriorly before reaching the ISN (very early stage 15) and makes a characteristically wide growth cone (arrow) with extensive filopodia (arrowhead). LCH5 refers to the fifth most anterior chordotonal neuron in the cluster of five LCH neurons. *B*, LCH1 and LCH2 axons in a stage 15 wild-type embryo (*WT*, late *s15*) join the ISN and extend ventrally. The growth cones are narrower and less complex during growth in the ISN (arrow). *C*, An LCH5 axon in a stage 16 wild-type embryo (*WT*, *s16*) extends anteriorly (arrow 1), turns ventrally and extends along the ISN (arrow 2), and enters the CNS and turns anteriorly (arrow 3). *D*, LCH4 and LCH5 axons in a very early stage 15 *shot*²/*shot*³ mutant embryo also display wide growth cones (arrow) with several filopodia (arrowhead). Neither the timing of axonogenesis nor the morphology of the growth cones is affected (compare *D* with *A*). However, the dendrites (short arrow) are abnormally short and bent compared with wild type. *E*, *F*, LCH5 axons in stage 16 *shot*³ mutant embryos. Axons stall either before reaching the ISN (*E*) or after a short ventral projection within the ISN (*F*). Growth cone morphology is normal (compare *E* with *A*, and *F* with *B*). All images are photomontaged from in-focus regions of dye-filled LCH5 neurons. Anterior is to the left; dorsal is up. Labeled non-neuronal cells are marked with a white X. Scale bar, 10 μ m.

Table 2. Percentages of normal and defective motor axon bundles in wild-type and *shot* mutant embryos

Allele ^a	WT ISN ^b	1 absent ^c	1–2 absent	1–3 absent ^d	Early stall ^e	ISN crossover
<i>WT</i>	100 (<i>n</i> = 87)	0	0	0	0	0
<i>shot</i> ³	4 (<i>n</i> = 79)	3	68	24	1	0
<i>shot</i> ² / <i>shot</i> ³	1 (<i>n</i> = 77)	6	48	43	0	1
<i>shot</i> ³ / <i>Df</i>	0 (<i>n</i> = 112)	0	40	42	17	1

Allele	WT SNa ^f	One branch missing ^g	Both branches absent	Early stall ^h	Bypass or misrouted ⁱ
<i>WT</i>	100 (<i>n</i> = 86)	0	0	0	0
<i>shot</i> ³	11 (<i>n</i> = 76)	33	38	17	1
<i>shot</i> ² / <i>shot</i> ³	25 (<i>n</i> = 63)	46	28	0	0
<i>shot</i> ³ / <i>Df</i>	4 (<i>n</i> = 114)	24	60	8	4

Allele	WT ISNb ^j	Stall in ventral muscle field ^k	Stall at muscle field border	Absent	One connection missing ^l	Bypass or misrouted ^m
<i>WT</i>	82 (<i>n</i> = 92)	0	0	0	18	0
<i>shot</i> ³	4 (<i>n</i> = 77)	75	14	5	0	1
<i>shot</i> ² / <i>shot</i> ³	2 (<i>n</i> = 65)	57	30	6	0	5
<i>shot</i> ³ / <i>Df</i>	0 (<i>n</i> = 105)	61	20	7	0	12

^aThe deficiency is *Df(2R)CX1*.

^bThe wild-type ISN forms three terminal arborizations over the dorsal muscles. All neuromuscular connections were smaller in size in the rare cases in which innervation was observed.

^cThe most dorsal arborization is absent. The numbers in the other column headings refer to the dorsal arborizations, numbered from most dorsal (1) to most ventral (3).

^dThe ISN stalls in the dorsal muscle field and fails to form any arborizations.

^eThe ISN stalls just before entering the dorsal muscle field.

^fThe wild-type SNa forms two branches, which innervate lateral muscles. In mutant embryos, we scored the SNa as wild type if it formed two branches.

^gThe dorsal branch was missing in the vast majority of these cases, and the lateral branch grew along the border of the ventral muscles.

^hThe SNa stalled over the ventral muscles or was absent.

ⁱThe SNa innervated the adjacent segment or showed an abnormal branching pattern.

^jThe wild-type ISNb forms connections with ventral muscles 12, 13, 6/7. These connections were reduced in size when they were observed in *shot* mutants.

^kThe ISNb stalls somewhere between muscle 6 (the ventral border of the ventral muscle field) and muscle 13.

^lOne of the normal ISNb connections, usually the 6/7 connection, could not be seen.

^mThe ISNb failed to defasciculate from the ISN, or it innervated the lateral muscles or the wrong part of the ventral muscles.

shot was first identified as a gene specifically required for the outgrowth of the ISN and ISNb motor axons; SNa development was reported to be normal in *shot*¹ mutants (Van Vactor et al., 1993). ISN motor axons in *shot*¹ mutant embryos generally arrest near the dorsal trunk of the trachea, just before they would make their two most dorsal arborizations. The ISNb motor axons arrest in the middle of the ventral muscle field. Thus, *shot* was suggested to be required for the extension of only a subset of motor axons beyond a well defined point in their development (Van Vactor et al., 1993).

This previous analysis of *shot*'s role in motor axon development is based on *shot*¹, which still expresses Shot protein (data not shown) and has a weaker effect on sensory axon development than the other *shot* alleles (Kolodziej et al., 1995). Other mutations in *kak* also affect motor axon development but affect synapse formation without affecting axon outgrowth (Prokop et al., 1998). Thus, *kak* was proposed to be specifically involved in synapse formation and not to be required earlier in motor axon development. To investigate motor axon development in a stronger allele, we therefore analyzed motor axons in *shot*³ and *shot*³/*Df(2R)CX1* mutant embryos, because *shot*³ appears to affect the LCH axons most severely among the alleles that lack detectable Shot proteins.

In late stage 16/ stage 17 wild-type embryos stained with mAb 1D4, the motor axons in the ISN pathway have reached the dorsal muscles and formed three terminal arborizations. The motor

axons in the SNa pathway have reached the ventrolateral muscles and bifurcated into two branches, a lateral branch that extends posteriorly along the border of the ventral muscle field and a dorsal branch that extends more dorsally into the ventrolateral muscle field. The ISNb motor axons have defasciculated from the ISN and have formed contacts with muscles 12, 13, and 6/7.

In late stage 16/ stage 17 *shot*³ mutant embryos, 90–100% of the motor axons (Table 2) in all three major pathways (ISN, SNa, and ISNb) stall prematurely (Figs. 6, 7). The ISN motor axons not only lack terminal arborizations (Prokop et al., 1998), but do not reach the muscles that they normally innervate (Fig. 6). Although SNa development appears normal in *shot*¹ embryos, the SNa motor axons in stronger *shot* mutants generally fail to form both branches, and they often stall at the entry to the ventrolateral muscles (Figs. 6, 7). The ISNb motor axons defasciculate normally from the ISNb and correctly target the ventral muscle field, but they stall in the ventral muscle field or before they enter it (Figs. 6, 7). The place where the motor axons in a given pathway stall is variable and does not suggest as well defined an arrest point as proposed earlier from studies of the *shot*¹ allele (Van Vactor et al., 1993). Some SNa and ISNb motor axon bundles fail to exit the CNS successfully or stall earlier than the muscle field entry points, and some ISN motor axon bundles (17%) stall well before the dorsal muscles in *shot*³/*Df(2R)CX1* embryos (Table 2). Thus, *shot* may also be required for motor axon extension before entry into the muscle fields. The rare axons that reach their

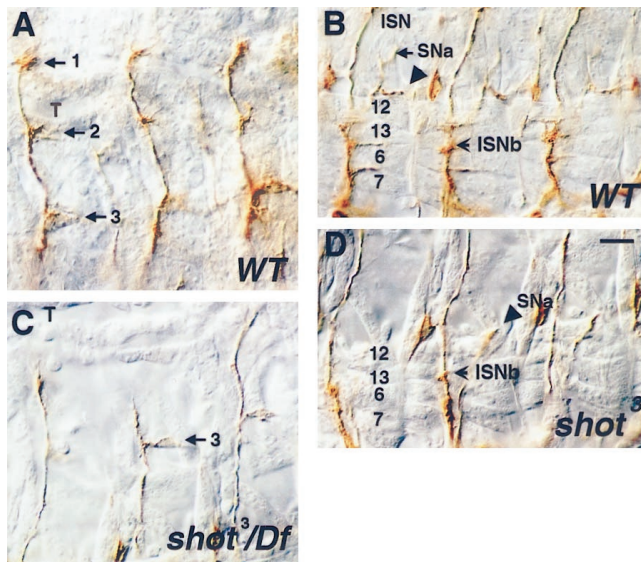


Figure 6. Motor axons stall in *shot* mutant embryos. *A*, A dorsal section of three abdominal hemisegments in a late stage 16 embryo stained with mAb 1D4 (Van Vactor et al., 1993) to visualize the motor axons. Motor axons in the ISN (arrows) form three terminal arborizations over the dorsal muscles. *T*, Trachea. *B*, A ventrolateral section of three abdominal hemisegments in a late stage 16 embryo stained with mAb 1D4. The ISN extends dorsally. The SNa motor axons contact lateral muscles and bifurcate into dorsal (arrow) and lateral (arrowhead) branches. The ISNb motor axons (concave arrow) make contacts with muscles 12 and 13 and the cleft between muscles 6 and 7. *C*, ISN motor axons (arrow) typically stall at variable positions in the dorsal muscle field in *shot³/Df(2R)CX1* mutant embryos, but generally form the third arborization. *D*, The SNa (arrowhead) and ISNb motor axons (concave arrow) stall before reaching their muscle targets in a late stage 16 *shot³* mutant embryo. *WT*, Wild type.

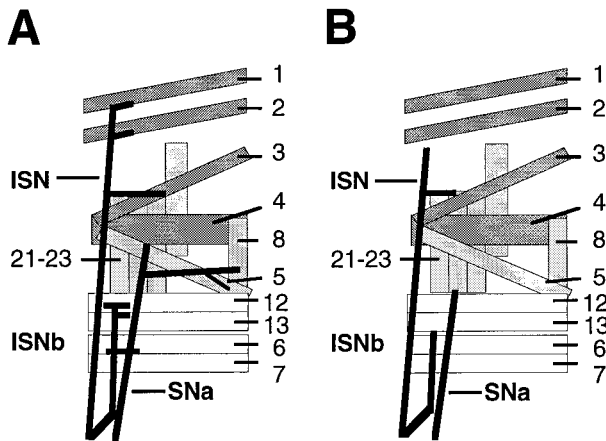


Figure 7. A schematic of the pattern of muscle innervation in wild-type (*A*) and *shot* mutant embryos (*B*). Motor axons in *shot* mutants stall before reaching their muscle targets. Muscle fibers are numbered and shaded according to whether they are dorsal (dark gray), lateral (light gray), or ventral (white). Motor axon bundles (ISN, ISNb, and SNa) are in black.

muscle targets generally fail to form the arborizations indicative of neuromuscular junction formation, or they form arborizations of reduced size, as seen in weaker *kak* mutants (Prokop et al., 1998). *shot* does not appear to be required for motor axons to choose the appropriate pathways or for selective fasciculation.

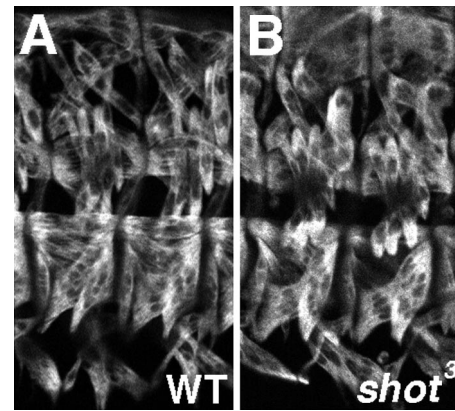


Figure 8. Muscle patterning is normal in *shot* null mutant embryos. *A*, A confocal section showing the ventral and lateral muscles in two abdominal hemisegments of a stage 16 wild-type embryo (*WT*) stained with a monoclonal antibody against myosin heavy chain. *B*, A similar section showing the ventral and lateral muscles in two abdominal hemisegments of a *shot³* null mutant embryo.

Motor axons in *shot* mutant embryos complete a substantially greater portion of their trajectory than do sensory axons. In wild-type embryos, sensory neurons in the dorsal and lateral PNS clusters extend axons along the ISN motor axon pathway and fasciculate with the ISN motor axons. Because the ISN motor axons extend beyond the lateral chordotonal cluster in *shot* mutant embryos, it is unlikely that defects in the extension of the motor axons explain defects in the growth of sensory axons in *shot* mutant embryos. The ability of sensory and motor axons to fasciculate appears normal in *shot* mutant embryos.

The same range of defects is seen in *shot³/Df(2R)CX1* mutant embryos as compared with *shot³* mutants alone, and the frequency of the more severe phenotypes is only modestly enhanced (Table 2). These data, taken together with the absence of detectable long isoforms of Shot in homozygous *shot³* mutant embryos, suggest that *shot³* is a null allele. Thus, Shot is only essential in motor axon development for the later steps of outgrowth. If *shot* were required for the initial stages of motor axon outgrowth, one would expect to see some examples of segments with no mAb1D4-stained axon bundles in the most severe alleles.

It has been reported previously that *shot* mutants are defective in muscle attachment because of defects in the formation of epidermal attachment cells. Moreover, Shot appears to be important for stabilizing the cytoskeleton of these attachment cells against contractile forces exerted by muscle. On becoming contractile (stage 17 embryos or later), muscles rip loose from their attachment sites. Because defects in muscle organization could affect motor axon growth and targeting, we examined muscle morphology in *shot³* null mutant embryos using an antibody against myosin. Muscle number and organization were normal in (10 of 10) late stage 16 *shot³* mutant embryos (Fig. 8), a stage at which motor axons have reached their target muscles in wild-type embryos. Muscles are also normal in embryos homozygous for the weaker *shot¹* allele (Van Vactor et al., 1993). Moreover, the expression of connectin, which marks a subset of lateral muscles (Gould and White, 1992; Nose et al., 1992), is also normal in *shot* mutant embryos (data not shown). We conclude that defects in muscle development probably do not contribute to the motor axon growth defects observed.

DISCUSSION

Short stop proteins comprise a novel, neuronally expressed plakin subfamily required for motor and sensory axon growth

Shot/Kak proteins contain an N-terminal domain, a central rod domain, and a C-terminal globular domain. We have shown that the predicted actin binding properties of the N-terminal domains, the length of the rod, and the structure of the C-terminal domain are all varied by the use of different transcriptional start points or alternative splicing. The diversity in these three key structural features, the selective expression of isoforms containing only complete predicted actin binding domains in the nervous system, and the Shot axon growth defects together suggest that a complex mixture of Shot proteins may function to organize other proteins spatially with respect to the actin cytoskeleton during sensory and motor axon extension.

Shot/Kak proteins are most closely related to the plakin family (Ruhberg and Watt, 1997). They also share some structural features with dystrophin (Gregory and Brown, 1998; Strumpf and Volk, 1998), the rod-like actin binding protein altered in Duchenne muscular dystrophy (Koenig et al., 1988) that links the actin cytoskeleton in muscle to the membrane (Michalak and Opas, 1997). Plakins comprise a family of rod-like proteins that cross-link actin and intermediate filaments and are required for the integrity of skin and sensory axons (Guo et al., 1995; McLean et al., 1996; Fuchs and Cleveland, 1998). These proteins (BPAG1, plectin, desmoplakin, and envoplakin) localize to membrane attachment sites for intermediate filaments and some also bind microtubules (Fuchs and Cleveland, 1998). In their overall structure, and as we show here, the presence of isoforms with varied predicted actin binding properties, Shot/Kak proteins resemble other plakins.

Variations in the predicted actin binding properties in additional isoforms of Shot identified here likely contribute significantly to Shot's functions in different cell types. Isoforms C and D lack either part or all of the predicted N-terminal actin binding domain. Shot isoforms C and D appear to be epithelial and muscle attachment cell specific and are not detectably expressed in the nervous system (Fig. 4). Conceivably, the N-terminal domains of these isoforms may interact with cytoskeletal proteins other than actin or may bind to the actin binding isoforms, by analogy to other plakins (Ruhberg and Watt, 1997; Yang et al., 1999). The apparent absence of the C and D isoforms from the nervous system suggests that Shot proteins may interact differently with the neuronal and epithelial actin cytoskeletons. These differences may reflect cell type-specific differences in Shot function, enabling neuronal growth cones to be motile and enabling the cortical actin cytoskeleton in epithelial cells to resist mechanical stress.

Shot/Kak proteins and their vertebrate and *C. elegans* homologs are distinguished from other plakins in that the longest isoforms contain a signature C-terminal domain with homology to the Gas2 protein (Schneider et al., 1988; Strumpf and Volk, 1998). The function of this domain is unknown, but by analogy to the other plakins, it may interact with other cytoskeletal proteins to organize them relative to the actin cytoskeleton. We have determined that this C-terminal domain is also alternatively spliced, which may further enhance the diversity of such interactions. Two EF-hand Ca^{2+} -binding motifs are also found in the C-terminal domain of the long Shot isoforms, as well as in dystrophin.

shot also encodes shorter rod-like proteins that contain different C-terminal domains. Variation of the rod length likely alters the spacing of different complexes containing the N-terminal and C-terminal domains, and varying the C-terminal domain likely alters the nature of these complexes. These short Shot proteins lack the 22 heptad repeats found in the long isoforms (Strumpf and Volk, 1998) but are predicted to contain considerable coiled-coil forming sequence (Lupas, 1997). Their C-terminal domain most closely resembles that of plectin (Liu et al., 1996), but the functional significance of this homology is unknown.

Short stop is required for sensory and motor axon extension, rather than navigation

We show here that *shot* is required for the development of all motor and sensory axons, a process that occurs normally in weaker *kak* alleles (Prokop et al., 1998). The structure of Shot and its expression in axons suggest that *shot* is required in neurons for axon extension, consistent with observations that *shot* acts cell autonomously in mushroom body neurons to promote axon fasciculation and growth (Lee and Luo, 1999). Although defects in substrate cells could conceivably contribute to the severity of the phenotypes observed, muscles do not detectably express Shot and appear to differentiate normally during motor axon growth in *shot* mutant embryos. Although neuronal isoforms of BPAG1 are involved in maintaining the axon cytoskeleton (Brown et al., 1995a,b), Shot is the first plakin-like molecule to have a demonstrated developmental role in axon extension.

Our results suggest that *shot* is involved in selected later axon extension events rather than in early steps of growth cone formation or axon outgrowth per se. In *shot* mutant embryos lacking detectable Shot proteins, chordotonal neurons form growth cones, extend at the appropriate time, and grow toward the ISN, but they either fail to join the nerve or stall after a short ventral extension. Motor axons arrest near their target muscle fields but generally leave the CNS, select the appropriate pathways, and defasciculate normally. The defects are more severe than previously reported for weaker alleles but are consistent with a general defect in the ability of motor axons to grow into or perhaps recognize muscle target fields. Axon tracts in the CNS of *shot* mutants are only modestly reduced in diameter (Prokop et al., 1998), further suggesting that Shot plays a role in only a subset of axon growth processes.

Growth cone advance is disrupted in *shot* mutants, yet the morphology of stalled growth cones in mutants appears similar to motile growth cones in wild-type embryos. Thus, Shot is unlikely to be essential for actin polymerization, or its regulation by Rho family GTPases, because these processes are required for the generation of filopodia and lamellopodia (Hall, 1998). Despite the lack of obvious defects in such actin-based structures, Shot's interactions with actin are nonetheless likely to be critical for growth cone motility, because only isoforms with predicted actin binding domains are detectably expressed in neurons. Mutants in profilin, an actin binding protein, also show defects in growth cone motility, but form filopodia and lamellopodia (Wills et al., 1999). Defects in actin-based motility are therefore not always reflected in growth cone morphology.

Short stop may organize proteins in the growth cone during axon development

The plakin-like structures of Shot proteins suggest that these proteins may link actin to proteins that interact with the C-terminal domains. In particular, Shot may help coordinate the

actin and microtubule components of the cytoskeleton during sensory or motor axon growth cone advance. Microtubule arrays in epithelial muscle attachment cells are abnormally organized with respect to the actin cytoskeleton in *shot* mutants (Prokop et al., 1998). Moreover, the mouse *ACF7* gene has been reported in a recent GenBank submission (accession number AAD32244) to encode an actin/microtubule cross-linking protein that contains all of the key structural features of the long isoforms of Shot. In the nervous system, Shot appears to be distributed along axons, where it could interact with the microtubule-filled core of the axon cytoskeleton, and the C-terminal of the long Shot isoform is efficiently targeted to axons (S. Lee and P. Kolodziej, unpublished data).

Functional redundancy with other proteins that may coordinate actin and microtubule dynamics in response to guidance cues could explain why sensory and motor axons exhibit normal growth cone morphologies and can partially extend in *shot* mutants. Several other proteins have recently been discovered to link actin and microtubules (Andra et al., 1998; Togel et al., 1998; de Hostos, 1999; Yang et al., 1999), although they have not yet been implicated in axon growth. Shot's proposed interactions with actin and microtubules could be a target of many, but not all, guidance systems. A combination of functional redundancy in such a key cell biological role and involvement in a subset of guidance systems could explain why some features of axon development are normal in *shot* mutants, yet the phenotypes are more penetrant and less cell type-specific than those of mutations in known *Drosophila* guidance receptors (Desai et al., 1996; Kolodziej et al., 1996; Krueger et al., 1996; Hoang and Chiba, 1998; Kidd et al., 1998).

Alternatively, defects in Shot's interactions with other proteins may underlie the *shot* axon growth phenotypes. Shot may localize or modulate selected signaling molecules permissive for continued sensory and motor axon growth. Other classes of proteins that may also interact with Shot include membrane proteins and components of membrane ruffles, actin-based structures involved in cell motility. Epidermal attachment cells in *shot* mutants fail to localize Vein, an extracellular matrix protein whose localization depends on surface receptors expressed by these cells (Strumpf and Volk, 1998). Gas2, which shares limited homology to the Shot C-terminal domain, partially colocalizes to membrane ruffles in cultured fibroblasts on serum induction (Brancolini and Schneider, 1994).

The length of the long Shot isoforms suggests that they could play a central role in spacing protein assemblies in the growth cone or axon. Based on comparison of the lengths of the coiled-coil regions of Shot and dystrophin (Lupas, 1997), and the known 175 nm lengths of dystrophin determined by electron microscopy (Pons et al., 1990), Shot proteins could be as long as 220 nm, a substantial fraction of the 500 nm width of a *Drosophila* axon. Shot proteins also contain Ca^{2+} -binding domains and may therefore represent another cytoskeletal target for the action of Ca^{2+} -transients, waves of calcium release that appear to regulate growth cone motility (Gomez and Spitzer, 1999). In any event, *shot* mutants provide a powerful tool for identifying a crucial link in the molecular chain from reception of environmental stimuli to axon extension.

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