The Kinesin-associated Protein UNC-76 Is Required for Axonal Transport in the *Drosophila* Nervous System

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Kinesin-I is essential for the transport of membrane-bound organelles in neural and nonneural cells. However, the means by which kinesin interacts with its intracellular cargoes, and the means by which kinesin–cargo interactions are regulated in response to cellular transport requirements are not fully understood. The C terminus of the *Drosophila* kinesin heavy chain (KHC) was used in a two-hybrid screen of a *Drosophila* cDNA library to identify proteins that bind specifically to the kinesin tail domain. UNC-76 is an evolutionarily conserved cytosolic protein that binds to the tail domain of KHC in two-hybrid and copurification assays, indicating that kinesin and UNC-76 form a stable complex in vivo. Loss of *Drosophila Unc-76* function results in locomotion and axonal transport defects reminiscent of the phenotypes observed in kinesin mutants, suggesting that UNC-76 is required for kinesin-dependent axonal transport. *Unc-76* exhibits dosage-sensitive genetic relationships with *Khc* and *Kinesin light chain* mutations, further supporting the hypothesis that UNC-76 and kinesin-I work in a common transport pathway. Given the interaction of FEZ1, the mammalian homolog of UNC-76, with protein kinase Cζ, and the role of FEZ1 in axon outgrowth, we propose that UNC-76 helps integrate kinesin activity in response to transport requirements in axons.

INTRODUCTION

Kinesin-I is a plus-end–directed microtubule motor that facilitates the movement of vesicles, messenger ribonucleoproteins (mRNPs), and organelles. It was first identified as a protein in squid axoplasm that facilitates ATP-dependent vesicle movement along microtubules (Brady, 1985; Vale *et al.*, 1985). Subsequent molecular, genetic, and biochemical studies have shown that kinesin-I is required for intracellular transport in eukaryotes in many cellular contexts (reviewed in Martin *et al.*, 1999b; Goldstein, 2001; Stebbings, 2001). The requirement for kinesin-based transport is especially acute in elongated cell types, such as neurons, in which long-distance transport from the cell body to the synapse must occur. Loss of function of either kinesin subunit results in transport defects in the nervous system, including organelle aggregation, defects in synapse formation, and electrophysiological defects (Saxton *et al.*, 1991; Gho *et al.*, 1992; Hurd and Saxton, 1996; Gindhart *et al.*, 1998).

Kinesin-I is the founding member of a superfamily of motor proteins that share a conserved mechanochemical domain with microtubule-stimulated ATPase activity, but have different cargo binding domains that are specialized for specific cellular functions (Vale and Fletterick, 1997; Hirokawa, 1998; Goldstein and Philip, 1999). For example, the kinesin-I tail domain is composed of two light chains and the C-terminal region of two heavy chains. Biochemical studies and sequence motif predictions helped assemble a model of kinesin–cargo interactions in which protein–protein interaction motifs in the kinesin tail domain bind to proteins on the surface of vesicles, mitochondria, and mRNPs (reviewed in Kamal and Goldstein, 2002; Karcher *et al.*, 2002). In addition to its role in cargo attachment, the kinesin tail domain is also required for negative regulation of the basal ATPase activity of the motor domain (Coy *et al.*, 1999; Stock *et al.*, 1999; Hackney and Stock, 2000). By identifying proteins that bind to the kinesin tail domain, it is possible to understand the

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nature of kinesin–cargo interactions and how kinesin activity is regulated in response to signal transduction pathways, thus modifying kinesin activity to meet cellular transport requirements.

A number of genetic and biochemical studies have begun to unravel the protein–protein interactions required for kinesin-dependent transport events. These proteins fall into three classes: transmembrane proteins on the vesicle surface that are cargo-bound receptors, scaffold proteins that indirectly link kinesin to cargos, and regulatory proteins that phosphorylate the kinesin tail domain or, like hsp70, remove kinesin from the cargo surface (reviewed in Verhey *et al.*, 2001; Kamal and Goldstein, 2002; Karcher *et al.*, 2002). However, genetic studies have been completed for only two kinesin-associated proteins (KAPs). Sunday driver (SYD) and UNC-16 are the *Drosophila* and *Caenorhabditis elegans* homologs of mammalian JIP3/JSAP1, a scaffold protein that binds vesicles, KLC, and components of the jun N-terminal protein kinase (JNK) signaling cascade (Bowman *et al.*, 2000; Byrd *et al.*, 2001); therefore, SYD and its homologs may coordinate regulation of vesicle transport by kinesin with JNK signaling. Amyloid precursor protein-like (APPL) is the *Drosophila* homolog of amyloid precursor protein (APP), a vesicle protein that, when mutated, causes familial Alzheimer's disease (Luo *et al.*, 1990; Luo *et al.*, 1992). APPL and APP are kinesin cargo receptors that bind to KLC to facilitate vesicle transport (Torroja *et al.*, 1999; Kamal *et al.*, 2000; Gunawardena and Goldstein, 2001; Kamal *et al.*, 2001).

We have completed yeast two-hybrid screens for *Drosophila* proteins that bind to the kinesin tail domain. One of these proteins, UNC-76, binds to the KHC tail domain, and mutational analysis shows that UNC-76 is essential for axonal transport in the *Drosophila* nervous system. These results support a model in which the interaction of UNC-76 and kinesin is essential for intracellular transport. UNC-76 homologs are essential for nervous system function in *C. elegans*, and the mammalian UNC-76 homolog FEZ1 is a direct target of protein kinase C (PKC)ζ-dependent signaling and axon outgrowth (Bloom and Horvitz, 1997; Kuroda *et al.*, 1999). We propose that UNC-76 may directly or indirectly facilitate the regulation of kinesin activity in vivo.

MATERIALS AND METHODS

Yeast Two-Hybrid Analysis

Yeast growth conditions and manipulations were done using established protocols (Ausubel *et al.*, 1997; Parchaliuk *et al.*, 1999). The LexA bait vector pEG202, prey vector pJG4-5, and *lacZ* reporter plasmid pSH18-34 (Zervos *et al.*, 1993) were used in yeast strain EGY191 (MATα trp1, his3, ura3, and lexAops-LEU2) to express fragments of kinesin heavy chain (KHC) and kinesin light chain (KLC). The bait fusion pEG-KST, containing aa 675–975 of *Drosophila* KHC, was used to screen the RFLY1 (Finley *et al.*, 1996) *Drosophila* embryonic cDNA library. Putative KHC interactors were characterized by polymerase chain reaction (PCR) amplification by using primers BCO1 and BCO2 (Ausubel *et al.*, 1997) and restriction fragment length polymorphism mapping of cDNA inserts by using *Hin*fI and *Hae*III, and controls were performed to demonstrate that observed interactions were bait and prey specific. The pEG-stalk and pEG-tail constructs encode aa 675–850 and 850–975 of *Drosophila* KHC, respectively. They were PCR amplified from pJG-KST (Bowman *et al.*, 2000) by using the primers KHC stalk (5′ CCG-GCTCGAGCTGGCGGAGTGATCCACCGTCCTC 3′) and BCO1 to amplify the pEG-stalk insert, and KHC tail (5′ GGCCGAATTCATC-

CGAAAGAATGTCGTAAACGAG 3′) and BCO2 to amplify the pEG-tail insert; PCR products were subcloned into the *Eco*RI and *Xho*I sites of pEG202.

Sequence Analysis

Clones representing each restriction fragment length polymorphism class were sequenced by the University of Massachusetts Boston Sequencing Facility. BLASTN comparison of interactor KAPH4 to the *Drosophila* genome database showed that KAPH4 encodes aa 277–474 of the annotated *Drosophila* gene CG3981. The cDNA for CG3981 corresponds to expressed sequence tag project clone LD08195 (GenBank accession no. AY069376), which is 2963 base pairs in length and encodes a 474 amino acid polypeptide. Alignment of *Drosophila* UNC-76, *C. elegans* UNC-76, and FEZ1 was performed using ClustalW 1.8 (Jeanmougin *et al.*, 1998) and Boxshade 3.21. Secondary structure and sequence motif prediction were performed using COILS (Lupas *et al.*, 1991), Garnier secondary structure prediction (Garnier *et al.*, 1978), PSORT (Nakai and Horton, 1999), InterPro (Apweiler *et al.*, 2001), and PhosphoBase (Kreegipuu *et al.*, 1999).

In Vitro Association of Unc-76 and Kinesin

Epitope-tagged versions of UNC-76 were generated by subcloning the fragment of UNC-76 encoded by the prey plasmid pJG-H4 (aa 277–474; pET-H4) and full-length UNC-76 (pET-UNC-76) into the bacterial expression vector pET30a (Novagen, Madison, WI). The encoded fusion protein contains a 6xHIS-tag for protein purification and an S-tag for fusion protein detection on Western blots. Both fusion proteins were soluble and stable under standard bacterial expression and purification conditions (QIAGEN, Valencia, CA).

His-tag copurification assays were done according to Micheva *et al.* (1997) with some modifications. Approximately 300 wild-type (ORE-R) or MYC-KLC (Gindhart *et al.*, 1998) flies were homogenized in NP-40 buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl, pH 8.0, 100 μg/ml phenylmethylsulfonyl fluoride) on ice. The homogenate was centrifuged 2×10 min at $10,000 \times g$ in an Eppendorf microcentrifuge at 4°C to pellet insoluble debris. The crude supernatant was incubated for 30 min with 25 μl of Ni^{2+}-NTA agarose beads (QIAGEN) at 4°C with rocking to preclear the supernatant. The beads were pelleted by centrifuging for 2 s at $10,000 \times g$, washed three times with NP-40 buffer, and resuspended in 1× SDS-PAGE sample buffer. His-tagged pET-H4 or pET-UNC-76 were coupled to Ni^{2+}-NTA agarose beads by incubating 50 μl of NP-40 washed beads with 10 μg/ml His-tagged fusion protein for 20 min at room temperature. The beads were pelleted, washed twice in NP-40 buffer, and resuspended in 40 μl of NP-40 buffer. Coated beads (25 μl) were added to the precleared lysate and incubated for 30 min at 4°C with rocking. The beads were pelleted by centrifuging 2 s at $10,000 \times g$, washed three times with NP-40 buffer, and resuspended in 1× SDS-PAGE sample buffer. Gel samples of the crude supernatant and copurification supernatant fractions were diluted with an equal volume of 2× SDS-PAGE sample buffer.

Antisera Production and Immunochemistry

UNC-76 aa 277–474 (pET-H4) was expressed in *Escherichia coli*, purified, and used to generate polyclonal antisera 1347 and 1348 in guinea pigs (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Antiserum 1348 was affinity purified using bacterially expressed pGEX-H4, which encodes aa 277–474 of UNC-76 fused to glutathione *S*-transferase in plasmid pGEX-2T (Guan and Dixon, 1991). For affinity purification of UNC-76 antisera, purified pGEX-H4 protein was cross-linked to glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to established protocols (Bar-Peled and Raikhel, 1996). Affinity purified UNC-76 antisera were used at a 1:100 dilution for immunostaining and a 1:1000 dilution for Western blots. Immunostaining of *Drosophila* embryos and lar-

vae was performed using standard protocols (Hurd and Saxton, 1996; Gindhart *et al.*, 1998; Rothwell and Sullivan, 2000). KLC antisera (Gindhart *et al.*, 1998) were used at a 1:100 dilution, and synaptotagmin (SYT) antisera (Littleton *et al.*, 1993) were used at a 1:500 dilution. Secondary antibodies conjugated to alkaline phosphatase horseradish peroxidase, fluorescein isothiocyanate (FITC), and Texas Red were obtained from Sigma-Aldrich (St. Louis, MO) and Jackson Immunoresearch Laboratories (West Grove, PA), and used according to manufacturers' instructions. To control for staining variability, fixation and antibody staining of mutant and wild-type larvae (e.g., *Unc-76⁻* and *Unc-76/+*) were performed simultaneously in the same sylgard-coated Petri dish. Each genotype was analyzed on multiple occasions, with two or three larvae of a particular genotype dissected per experiment. Segmental nerves showing representative staining patterns were selected for imaging by confocal microscopy. Antibody detection on Western blots was performed on a Storm 840 PhosphorImager (Amersham Biosciences), by using the ECF chemifluorescence or ECL Plus kit (Amersham Biosciences). Quantitation of bands on Western blots was performed using ImageQuant software (Amersham Biosciences). Duplicate gels were stained with Coomassie Blue and analyzed using NIH Image.

Drosophila Genetics

The *Khc* and *Klc* alleles used in this work were described previously (Gindhart *et al.*, 1998; Martin *et al.*, 1999a; Brendza *et al.*, 2000). *Khc¹⁶* is a null allele, and *Df(3L)34ex5* is a small deletion that removes the *Klc* transcription unit. The *Unc-76* P[*lacW*] alleles *l(1)G0360*, *l(1)G0158*, and *l(1)G0310*, and the *Unc-76* duplication chromosomes *Dp(1;2;Y)w⁺* (duplication of 2C9-3D1 on Y chromosome) and *Tp(1;3)wᵖᶜᵒ* (transposition of 2C1-3C5 onto chromosome 3) were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). The P element insertion site of each *Unc-76* allele was identified by plasmid rescue and DNA sequencing of genomic DNA flanking each insertion site (Huang *et al.*, 2000). To generate *Unc-76* revertants and deletion mutants, P[*lacW*]*l(1)G0310*, *w/FM7c*, *B* females were crossed to *w/Dp(1;2;Y)w⁺*; *Dr/TMS*, *Sb* Δ2-3 males. Dysgenic F₁ male progeny were crossed to *Sxl/Binsinscy*, *w B* females, and white-eyed female F₂ progeny were crossed to *Binsinscy* males to establish individual lines and screen for X-linked lethality. Putative excision mutants were tested for complementation of *l(1)G0310*; 33% of the excision events (42/125) failed to complement *l(1)G0310*. Small deletions within *Unc-76* were identified by PCR amplification of *Unc-76ᵉˣᶜⁱˢⁱᵒⁿ/+* genomic DNA by using primers flanking the P[*lacW*] *l(1)G0310* insertion site. Using this approach, a small deletion (*Df(1)ex107*) that removes *Unc-76* sequences was identified. To precisely map the deletion breakpoints, PCR products were subcloned into pBS SK+ (Stratagene, La Jolla, CA) and sequenced. The lethal phase of *Unc-76* alleles was determined according to Gindhart *et al.* (1998).

Brightfield and Fluorescence Microscopy

Embryos were examined using an Olympus BX60 microscope with differential interference contrast optics and a 20× objective. Brightfield images were obtained using an Olympus C-3040 digital camera. Epifluorescence images were captured using an Olympus BX60 microscope, 60× oil immersion objective, FITC filter set, CCD-300T camera (Dage-MTI, Michigan City, IN), Scion AG-5 frame grabber, and Scion Image software (Scion, Frederick, MD). Confocal images were obtained using a Leica TCS-NT confocal imaging system and a Leica DM IRBE inverted microscope with a 40× oil immersion objective. Colocalization of UNC-76 and SYT in segmental nerves was performed using secondary antisera coupled to FITC and Texas Red. Optical sections of 2 μm were collected, and stacks of optical sections were obtained using Leica LCS confocal microscope software. Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).
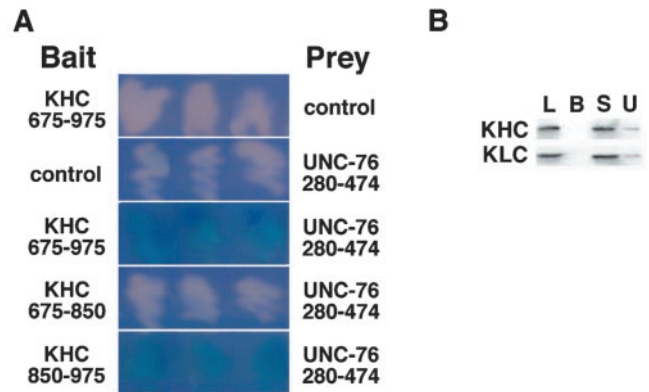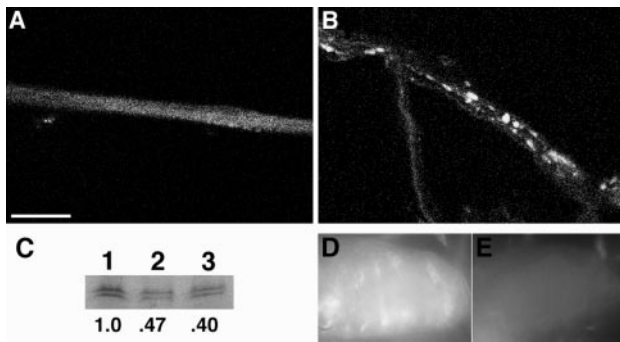

Figure 1. UNC-76 interacts with the KHC tail domain in the yeast two-hybrid assay and in copurification assays. (A) Yeast cells containing a *lacZ* reporter gene and various combinations of *LexA* DNA binding domain (baits, left column) and B42 activation domain (preys, right column) fusion proteins were grown on CM Gal/Raff Xgal plates. Colonies in which reporter gene activation is enhanced by specific bait-prey interactions are blue, whereas colonies in which a bait-prey interaction do not occur are white. Colonies that contain the KHC stalk and tail domains (aa 675-976) or the KHC tail domain (aa 850-975) bait fusions and the UNC-76 prey fusion enhance reporter gene transcription, but colonies containing the KHC stalk domain (aa 675-850) and UNC-76 do not. Control, *LexA* DNA binding domain bait or B42 activation domain prey. (B) Western analysis of protein fractions from UNC-76 copurification assay. 6xHIS-tagged full-length UNC-76 was bound to Ni²⁺-NTA agarose beads and incubated with detergent-soluble extracts of adult flies containing a transgenic copy of myc epitope-tagged KLC. KHC and KLC copurify with UNC-76 beads (lane U), but not with Ni²⁺-NTA agarose beads alone (lane B). KHC, blot probed with anti-KHC antibody; KLC, blot probed with anti-myc antibody to detect transgenic KLC; L, detergent-soluble lysate; B, proteins that copurify with Ni²⁺-NTA beads; S, supernatant from UNC-76 copurification assay; U, proteins that copurify with 6xHIS-UNC-76 beads.

RESULTS

UNC-76 Binds to the Tail Domain of Drosophila KHC

We performed a yeast two-hybrid screen, by using the carboxy-terminal 300 aa of *Drosophila* KHC to screen a *Drosophila* embryonic cDNA library for genes that encode KAPs. Screening 1.8 × 10⁷ transformants (Ura⁺, His⁺, Trp⁺) resulted in the isolation of 170 putative KHC interactors (Leu⁺, LacZ⁺). Six clones identified in the screen encode aa 277–474 of annotated gene CG3981 (Adams *et al.*, 2000). This clone binds to the *Drosophila* KHC tail domain (aa 850–975) but not to the stalk domain, the KLC repeats, or to control bait proteins (Figure 1A; our unpublished data). CG3981 is similar to the *C. elegans* protein UNC-76 and rat FEZ1 (Bloom and Horvitz, 1997; Kuroda *et al.*, 1999); therefore, we will refer to gene CG3981 as *Unc-76*. ClustalW alignment reveals three blocks of sequence conservation among the homologs at aa 140–202, 345–413, and 421–474 of *Drosophila* UNC-76 (Figure 2A). The KHC-interacting region of UNC-76 includes the second and third conserved domains. Structure and domain predictions show that UNC-76 is acidic, primarily α-helical, and may contain a small α-helical coiled-coil between aa 350–388, but did not reveal any other

A

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DmUNC-76    1 MRDLGTKMAELKFEAPLAKFEETDEWGGCDFISSQNAINDTLNLNLKDSS
CeUNC-76    1 -------------------------------------------------
FEZ1        1 -------------------------------------------------

DmUNC-76   51 AGGKPDATKLRLLEDAVRDAHVSKNGGGVVGGGAGSISPNCNTLQGGSLI
CeUNC-76    1 ----------MRFGEVAMEAADLRVPDIPLASCDDDDIDSNKNLSN----
FEZ1        1 -----------MEAPLVSLDEEFEDIRPCCTEDPEEKPQSLYGTSP----

DmUNC-76  101 EIGLSDVGLVPGEGAGVGLDGLEKRSLAVGDHVDNFTETFGGSLEDLVNT
CeUNC-76   37 ----------------HSS--D--EKHHCNSNSDEERLHDEFSGSLEDLVGN
FEZ1       36 -------------------HHLEDPSISELENFSSEIISFK-SMEDLVNE

DmUNC-76  151 FDEKITKCFGNYEENVEELAPVQ----VRSQEEIMNECQMWWTITGNFGN
CeUNC-76   69 FDEKIAACLKDHSVTTADIAPVQ----IRTQEEVMNESQTWWTLTGNFGN
FEZ1       66 FDEKLNVCFRNYNAKTENLAPVKNQLQHQEEEETLRDEEVWDALTDNYIP

DmUNC-76  197 ILPIDWSKSYTRQMHMPTLN---LGQNHTKQQQQNRNQQQQLHNQSHQAY
CeUNC-76  115 IQPLDFGTSSICKKMAAALDSDSLKDDASTRRSMTNSDDEDLLRQQMDVH
FEZ1      116 SLSEDWRDPNIEALNGNSSD---------------------TEIHEKE

DmUNC-76  244 PHTNGLGSGSGSGLDAQTPGDEFNDLTSBDEAVANDLDMHALILNGLNGD
CeUNC-76  165 QMIGHHHGSTDTGG--ETPPOTADQVIEEIDEMLQSCDFTGSMMT-----
FEZ1      143 EEDEFIEKSENDSGINPEPLLTADQVIEEIEEMMQNSPDPEEEVEVLE--

DmUNC-76  294 MDDQPIKTVEEVIKEIDDIMDEAESPLDEPETCDSEVIEKAREVLGAPLY
CeUNC-76  208 ---------DRTMBSVDSMYSSMRSPFPS-SIQSSDADIKLRSAQALVSN
FEZ1      191 ---------EBDGGEISSQADSVLLQ-------EMQALTQTFNNNWS

DmUNC-76  344 AEKLQYLTTTQLMELYMEMEVLIQELSETLINELALRDELEFEKELKNSF
CeUNC-76  248 PDNLQELSYSKLVTLCAEMEQLIRVYNESLVDELAHRDELDYEKEMKNSF
FEZ1      222 YEGLRHMSGSELTELLDQVEGAIRDFSEELVHQLARRDELEFEKEVKNSF

DmUNC-76  394 ISLLLAVQNKRRQYHVEKKR-----------------------------
CeUNC-76  298 ISLLLAIQNKRRVVANDRKRKVGK-------------------------
FEZ1      272 ITVLIEVQNKQKEQRELMKKRRKEKGLSLQSSRIEKGNQMPLKRFSMEGI

DmUNC-76  414 -----------GKFQGPEPKYLTTVIPYHLENGTPNNQSLQVLIKILKAI
CeUNC-76  322 -----------ASDASQLPQYLTATIPYNDHQHIDN-ASIASLIKILRAI
FEZ1      322 SNILQSGIRQTFGSSGADRQYLNTVIPYEKKSSPPSVEDLQMLTNILFAM

DmUNC-76  453 NEDSPTVPALLTDYILKVLCPT----
CeUNC-76  360 HDDNTTVPTLLTDYILTHVCPKNISC
FEZ1      372 KEDNEKVPTLLTDYILKVLCPT----
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B

Figure 2. Alignment of *Drosophila* UNC-76 with its homologs and genetic map of *Unc-76* locus. (A) CLUSTALW 1.8 alignment of *Drosophila* UNC-76, *C. elegans* UNC-76, and rat FEZ1 is shown. Identical aa in two of three homologs are shaded black, conserved amino acid substitutions are shaded gray, and gaps are represented by dashed lines. Three regions of high sequence similarity are boxed, and the region of similarity with NF1-binding protein SCHIP-1 is underlined. The sequence of *Drosophila* UNC-76 is available from GenBank under accession no. NP_569981. (B) Map of the *Unc-76* genetic interval and location of *Unc-76* mutations used in this analysis. The *Unc-76* gene is transcribed from left to right in this diagram; the gene is oriented so *Unc-76* is transcribed toward the centromere of the X chromosome. Exons are represented as boxes; coding regions are filled, whereas the 5' and 3'-untranslated region are not. The location of P element insertions *l(1)G0360, l(1)G0158*, and *l(1)G0310* are shown; they are inserted at +9, +28, and +172 of the *Unc-76* transcript, respectively. The portion of the *Unc-76* transcript deleted by *Df(1)ex107* (+3 to +581) is shown by a solid line under *Unc-76* exon 1. *Df(1)ex107* deletes the *Unc-76* translation start site. Bar, 1 kb.

notable structural motifs. The second conserved domain (aa 347–407) is similar to the schwannomin (NF2) binding domain in SCHIP-1 (60.1% conserved residues; Goutebroze *et al.*, 2000). NF2 is an evolutionarily conserved tumor suppressor protein involved in tight junction formation (Trofatter *et al.*, 1993).

To test whether *Drosophila* UNC-76 can bind to native kinesin, bacterially expressed 6xHIS-tagged UNC-76 was incubated with Ni^{2+}-NTA agarose beads, and then the 6xHIS-UNC-76–coated beads were used in a copurification assay with detergent-soluble extracts from MYC-KLC *Drosophila* adults (Gindhart *et al.*, 1998). Western analysis of supernatant and bead fractions with antibodies to KHC and KLC confirm that endogenous fly kinesin binds to 6xHIS-UNC-76 beads, but not control beads lacking protein (Figure 1B) or containing protein encoded by the bacterial expression vector alone. Similar results were observed using the portion of UNC-76 encoded by the two-hybrid clone, thus confirming that the kinesin binding site is located between UNC-76 aa 277–474. The copurification of KLC with 6xHIS-UNC-76 indicates that UNC-76 binds to native kinesin containing both light and heavy chains, because the UNC-76 binding site is in the tail domain of the heavy chain (Figure 1A). These results support two-hybrid data demonstrating the direct interaction of KHC and UNC-76.

Unc-76 Is Essential for Drosophila Development

To determine the in vivo function of UNC-76, mutations in the *Unc-76* gene were identified, and the resulting phenotypes were studied at the organismal and cellular level. The *Unc-76* gene is located at cytological interval 2C9 on the X chromosome (Adams *et al.*, 2000), distal to the embryonic polarity gene *corkscrew* (*csw*; Figure 2B). A collection of lethal P[lacW] insertion mutations, mapped to this interval by chromosomal in situ hybridization, was mapped precisely by cloning and sequencing genomic DNA flanking the insertion site. Three P[lacW] insertions were identified in the *Unc-76* 5'-untranslated region. All three insertions are recessive lethal, fail to complement each other and deletion mutations that remove *Unc-76*, but are complemented by genetic duplications containing *Unc-76*. The P[lacW] insertion mutation *l(1)G0310* was used to generate *Unc-76* deletions and revertants. Flies heterozygous for *l(1)G0310* were crossed to flies containing a stable source of P transposase to induce excision of the P[lacW] transposon. Precise excision events restore wild-type gene structure, but imprecise excision events often result in small deletions flanking the original insertion site. More than 100 *l(1)G0310* excisions were tested by complementation and PCR analysis. Precise excisions of *l(1)G0310* are viable and complement lethal *Unc-76* alleles, whereas imprecise excisions, such as the small deletion *Df(1)ex107* (Figure 2B), are recessive lethal and do not complement other *Unc-76* alleles. Collectively, these results indicate that the lethality associated with the P element insertions in *Unc-76* is caused by loss of *Unc-76* function. More importantly, these results demonstrate that *Unc-76* is essential for *Drosophila* development.

Unc-76 Mutations Exhibit Phenotypes Similar to Khc and Klc Mutations

The loss of *Unc-76* function results in lethality at the transition between second and third instar at approximately 5 d of

Figure 3. Unc-76 mutations cause axonal transport defects in the *Drosophila* nervous system. Larvae heterozygous (A) or homozygous (B) for *Unc-76* null allele *l(1)G0310* were incubated with antisera specific for the synaptic vesicle precursor marker SYT to compare the accumulation pattern of fast anterograde transports in wild-type and *Unc-76* mutant backgrounds. Note that large aggregates of SYT immunoreactivity are found in the segmental nerves of *Unc-76* mutants (B), but SYT accumulation is diffuse in heterozygotes (A). Bar, 25 μm. (C) UNC-76 protein concentration is reduced in *Unc-76* heterozygous adults. Whole cell extracts of wild-type (ORE-R, lanes 1 and 2) and *Unc-76* heterozygote (*l[1]G0310/+*, lane 3) were electrophoresed, blotted, and incubated with UNC-76 antisera. The equivalent of one adult female is present in lanes 1 and 3, whereas 0.5 fly equivalent is present in lane 2 as a control. The numbers below each lane represent the ratio of the pixel volume of each lane divided by the pixel volume of lane 1 (wild-type). (D and E) UNC-76 antisera staining of wild-type (D) and *Unc-76⁻* (E) second instar CNS. Note that UNC-76 staining is more intense in ventral nerve cord of wild-type larva, whereas staining is at background levels in *Unc-76⁻* ventral nerve cord. Epifluorescence images were captured at 60× magnification. Anterior is to the left.

Figure 4. Immunolocalization of UNC-76 in the embryonic and larval nervous system. (A–D) UNC-76 is found in axons of the embryonic CNS. Anterior is to the left. (A and B) Lateral (A) and horizontal (B) views of a stage 14 embryo. (A) Shows that UNC-76 protein is restricted to the CNS (arrows) at this stage of development, whereas (B) shows that UNC-76 accumulation is detectable in both longitudinal (arrows) and commissural (asterisks) axons. (C and D) Lateral and horizontal views of UNC-76 staining at stage 17. UNC-76 is found within longitudinal axons of CNS (arrows) late in embryogenesis, but commissural axon staining is greatly reduced (D) compared with earlier in development (B). (E and F) UNC-76 staining in segmental nerves of wild-type third instars. (E) A projection of 29 optical sections, each 2 μm in thickness, whereas F is one optical section from the projection. Note that UNC-76 accumulation is diffuse within segmental nerves. Bar (E and F), 25 μm.

development. The P[lacW] alleles alone or in combination with *Df(1)ex107* die at the L2-L3 transition, suggesting that these mutations are null alleles of *Unc-76*. Individuals lacking *Unc-76* function exhibit a progressive paralysis phenotype reminiscent of the paralysis observed in null alleles of *Khc* and *Klc* (Saxton *et al.*, 1991; Hurd and Saxton, 1996; Gindhart *et al.*, 1998). First instars look normal and do not exhibit impaired locomotion or feeding behavior. However, progressive loss of vigor is observed during the second instar, resulting in complete paralysis and death at the onset of L3. The longest-living *Unc-76* mutants have mouthparts characteristic of third instars, but lack the ability to shed their second instar cuticle. This "L3 trapped in an L2 body" phenotype is also observed in *Klc* null mutants. The "tail flipping" locomotion defect that is the hallmark of weak loss-of-function *Khc* and *Klc* alleles (Hurd and Saxton, 1996; Gindhart *et al.*, 1998; Martin *et al.*, 1999a) is not observed in *Unc-76* mutants, although uncoordinated crawling behavior is seen before onset of paralysis. However, the locomotion defects characteristic of moderate *Khc* and *Klc* alleles are readily observed only in L3 larvae. Because the available *Unc-76* mutations are null alleles that die before L3, the ability of *Unc-76* mutations to evoke tail flipping cannot be tested directly. The similarity between the organismal phenotypes of *Unc-76* and *Khc* is also observed in *C. elegans*, in which *unc-76* and the KHC homolog *unc-116* both exhibit an uncoordinated phenotype (Patel *et al.*, 1993; Bloom and Hor-

vitz, 1997; Byrd *et al.*, 2001). Unlike *Drosophila*, however, nematode *unc-76* and *unc-116* are not required for viability.

Loss of function mutations in *Drosophila* fast axonal transport motor complexes kinesin-I, kinesin-II, and dynein result in defects in the trafficking of axonal cargos (Hurd and Saxton, 1996; Gindhart *et al.*, 1998; Bowman *et al.*, 1999; Martin *et al.*, 1999a; Ray *et al.*, 1999). These trafficking defects include axon clogs, which are aggregates of membrane-bounded anterograde cargos such as synaptic vesicle precursors, mitochondria, and prelysosomal vesicles (Hurd and Saxton, 1996). Because *Unc-76* mutations cause neuromuscular phenotypes similar to those observed when axonal microtubule motor function is disrupted, we examined neuromuscular preparations of *Unc-76* mutant and control larvae for disruption of axonal transport. Immunostaining with antisera to the synaptic vesicle precursor marker SYT shows that the segmental nerves of *Unc-76* mutant larvae contain aggregates of SYT immunoreactivity, whereas *Unc-76/+* control larvae have a diffuse, punctate staining pattern (Figure 3). The aggregates of SYT staining observed in *Unc-76* mutant larvae are similar to those observed in *Khc* and *Klc* mutant larvae and seem to be axon clogs correlated with the disruption of transport in the *Drosophila* nervous system. Like kinesin mutants, the segmental nerves of *Unc-76* mutants contain aggregates of KLC immunoreactivity, but tubulin, which is a component of slow axonal transport

(Terada *et al.*, 2000), is not found in SYT-containing aggregates (our unpublished data). The similarity of the *Unc-76*, *Khc*, and *Klc* neuromuscular and organelle jam phenotypes, in conjunction with two-hybrid and copurification experiments demonstrating the physical association of UNC-76 and KHC, strongly suggests that UNC-76 is an important component of the kinesin transport pathway in the *Drosophila* nervous system.

UNC-76 Is Not Associated with Axon Clogs in the Drosophila Nervous System

An important question is whether UNC-76 functions as a kinesin-cargo adaptor. Proteins that help kinesin attach to cargos should be transported down axons by kinesin-dependent fast axonal transport. To test this hypothesis, we generated specific antisera to UNC-76, and studied its accumulation in embryos and larvae. The UNC-76 antisera recognize a doublet at ~70 kDa, the intensity of which is reduced by half in adults heterozygous for the *Unc-76* mutation *l(1)G0310* (Figure 3C) and the deletion mutant *Df(1)ex107* (our unpublished data). UNC-76 antisera specificity for immunostaining experiments was demonstrated by incubating wild-type and *l(1)G0310* second instar neuromuscular preparations with affinity-purified UNC-76 antisera; wild-type larvae display UNC-76 staining in the larval central nervous system (CNS) (Figure 3D), but only background staining is observed in *l(1)G0310* individuals (Figure 3E). Specific accumulation of UNC-76 is first observed in the developing nervous system at stage 14 of embryogenesis, persisting through the end of embryogenesis (Figure 4). This result is consistent with RNA in situ hybridization experiments that showed *Unc-76* transcript localization in the embryonic CNS (our unpublished data). UNC-76 accumulation is observed within neurons but is not found in glial cells. Accumulation is observed in both the longitudinal (parallel to embryo midline) and commissural (cross midline) axons (Figure 4, A and B); later in development, however, UNC-76 is observed only in longitudinal axons and the brain lobes (Figure 4, C and D). The localization of UNC-76 to axon tracks of the CNS during embryonic development suggests that the interaction between UNC-76 and kinesin is restricted to the nervous system and that kinesin-UNC-76 interactions can occur in axons.

The accumulation of UNC-76 in axons is consistent with the model that UNC-76 is a kinesin-cargo adapter. A prediction of this model is that UNC-76 should be found associated with membrane-bound cargos in axons. The segmental nerves of the *Drosophila* larva provide an excellent model system for studying axonal transport, because the nonmyelinated segmental nerves consist of 30–40 sensory and motor axons that enervate each hemisegment. Axon clogs observed when vesicle transport is disrupted contain both anterograde and retrograde membrane cargos, as well as cargo-bound motor proteins. Presumably, if UNC-76 is a kinesin-cargo adapter in axons, then UNC-76 should colocalize with axon clogs. To test this hypothesis, we compared the localization pattern of UNC-76 and SYT in segmental nerves. Previous findings demonstrated that the presynaptic vesicle markers SYT and cysteine string protein colocalize with kinesin, kinesin-II, and dynein in axon clogs (Gindhart *et al.*, 1998). Intriguingly, UNC-76 staining in segmental nerves is diffuse (Figure 4, E and F) and is not enriched in
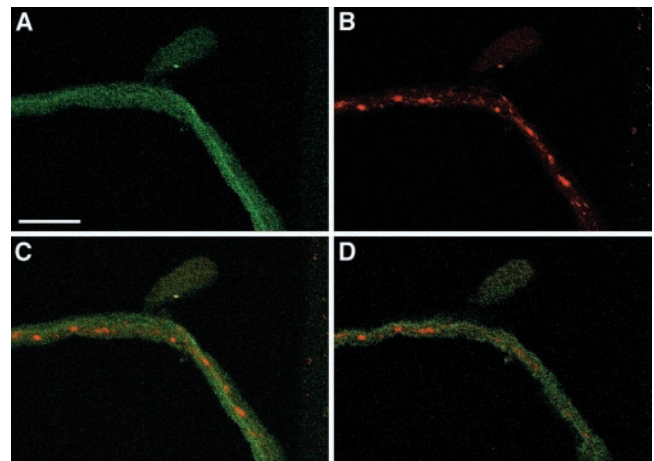

Figure 5. UNC-76 and SYT do not colocalize in segmental nerves. (A) Shows the accumulation of UNC-76 in a nine-section optical projection (each section is 2 μm) of a segmental nerve from a *Khc16/+; Df(3L)34ex5/+* individual that exhibits locomotion defects and SYT axon clogs (B). Note that UNC-76 accumulation is fairly uniform, whereas SYT is found in axon clogs (B). (C) Overlay of images in A and B shows that UNC-76 and SYT are largely non-overlapping; upon viewing a single 2-μm optical section (D), it is apparent that UNC-76 is not detected in axon clogs. Bar, 25 μm.

SYT-containing axon clogs of *Khc/+; Klc/+* individuals (Figure 5), suggesting that UNC-76 is not associated with membrane cargos transported by fast axonal transport. Although it is difficult to determine the specific location of UNC-76 within axons by using immunofluorescence, it seems to be distributed uniformly, suggesting it is in the axoplasm.

Dosage-dependent Interactions between Unc-76, Khc, and Klc

To more fully understand the functional relationship between UNC-76 and kinesin in axons, we studied the effect of altering *Unc-76* dosage in *Khc* and *Klc* heterozygotes. The reduction of KHC or KLC function by 50% does not have a noticeable effect on axonal transport or locomotion of *Drosophila* larvae. However, larvae with half the normal *Khc* or *Klc* dosage provide a sensitized genotype for the identification of mutations that disrupt axonal transport (Martin *et al.*, 1999). To test whether altering *Unc-76* dosage enhances *Khc* or *Klc*, males of the genotype *Unc76⁻/Dp(Unc-76⁺)* were crossed to *Khc⁻/+* or *Klc⁻/+* females, progeny larvae were scored for locomotion defects, and the presence of axonal clogs in larvae of each genotypic class was assessed by SYT immunolocalization of neuromuscular preparations (Figure 6). Two genetic duplications were used: *Dp(1;2;Y)w⁺* is a duplication of segment 2C-3D onto the Y chromosome, and *Tp(1;3)wᵛᶜᵒ* is a transposition of 2C-3C onto chromosome 3. This approach ensured that *Unc-76* would be expressed in the appropriate spatiotemporal domain, but at a higher level than wild type, thus minimizing the potential for pleiotropic effects caused by ectopic *Unc-76* expression. No enhancement of *Khc* or *Klc* was observed when *Unc-76* dosage was reduced by half (Figure 6C; our unpublished data). In con-
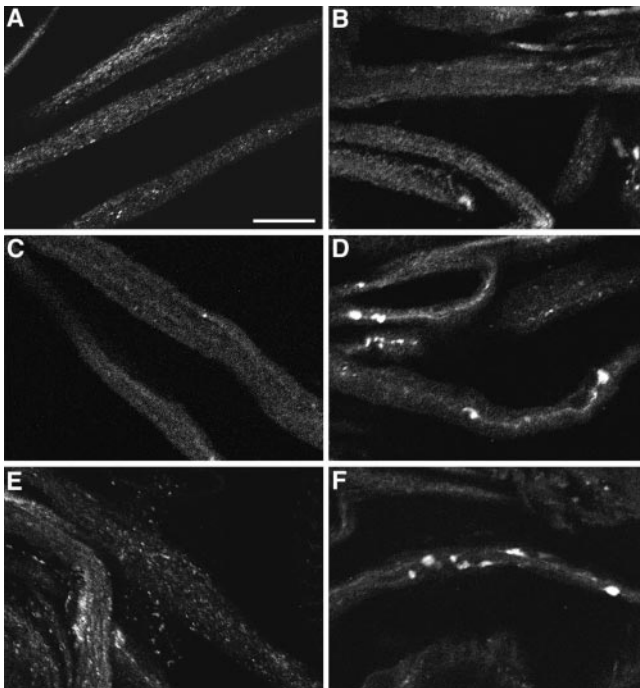
Figure 6. Increasing the genetic dosage of Unc-76 enhances Klc and Khc mutant phenotypes. SYT accumulation in segmental nerves of larvae of the following genotypes: (A) *Dp(1;2;Y)w⁺/+; (Unc-76* duplication); (B) *Df(34ex5)/+ (Klc* deletion); (C) *l(1)G0310/+ (Unc-76* null allele); *Df(34ex5)/+;* (D) *Dp(1;2;Y)w⁺/+; Df(34ex5)/+;* (E) *Khc¹⁶/+ (Khc* null allele); and (F) *Df(1;2;Y)w⁺/+; Khc¹⁶/+.* Bar, 25 μm.

trast, increasing the dosage of *Unc-76*, in combination with reducing *Khc* or *Klc* dosage, results in strong axon clog and locomotion phenotypes (Figure 6, D and F). This interaction is observed using both *Unc-76* duplications. The *Unc-76* duplication on the Y chromosome has a stronger effect, because it elicits both locomotion and axon clog phenotypes in *Khc* heterozygotes, whereas the chromosome 3 duplication causes only the axon clog phenotype. The enhancement of *Khc* by increased *Unc-76* dosage was more pronounced than its enhancement of *Klc*; this is similar to observations for *Appl* (Gunawardena and Goldstein, 2001). The genetic interaction between *Dp(Unc-76)* and kinesin mutations was more pronounced when the *Khc* or *Klc* mutant allele was inherited maternally. This result is not unexpected, given the large maternal contribution of kinesin and the perdurance of maternally supplied kinesin for several days.

DISCUSSION

A number of studies have demonstrated unequivocally that the microtubule motor kinesin-I binds to its intracellular cargos by protein–protein interactions and that kinesin transport is regulated by phosphorylation-dependent signaling pathways. We have used the yeast two-hybrid system to identify KAP-encoding genes that potentially mediate or regulate kinesin–cargo interactions and then used reverse genetic and cell biology tools available in *Drosophila* to reveal

the in vivo relationship between KAPs and kinesin. We have identified an evolutionarily conserved protein, UNC-76, that binds specifically to the KHC tail domain in the yeast two-hybrid system and copurification assays. Immunostaining and genetic analyses demonstrate that UNC-76 function is required for axonal transport in the *Drosophila* nervous system. The dosage-dependent enhancement of kinesin mutant phenotypes by *Unc-76* suggests that the binding of KHC by UNC-76 is relevant in vivo. Collectively, these results support a model in which UNC-76 has an essential function in kinesin transport pathways.

UNC-76 Function in the Nervous System

The analysis of UNC-76 function in other model systems is consistent with our observation that it has an essential role in the *Drosophila* nervous system. The *C. elegans* gene *unc-76* was identified by the uncoordinated crawling behavior of loss-of-function *unc-76* mutants (Hedgecock *et al.*, 1985; Bloom and Horvitz, 1997). The UNC-76 protein accumulates in all *C. elegans* neurons at all stages of development (Bloom and Horvitz, 1997). Mutants have subtle defects in axon outgrowth and bundling in axon fascicles, but the growth and differentiation of individual axons along the body wall are unaffected. We have not observed a requirement for *Unc-76* in *Drosophila* nervous system development, because locomotion defects in larvae lacking zygotic *Unc-76* are not observed until mid-second instar, ~3 d posthatching. Similar locomotion defects in *Khc* and *Klc* mutants do not become apparent until the second instar, presumably due to the perdurance of maternally supplied kinesin; it is possible that the maternal contribution of UNC-76 mRNA or protein is sufficient for embryogenesis and early larval development. UNC-76 protein accumulates in a dynamic pattern in axons during embryonic CNS development but is not detected in motor or sensory axons of the embryo. However, by the second instar, UNC-76 is detected in segmental nerves, which contain both motor and sensory axons. We cannot eliminate the possibility that UNC-76 accumulates in axons of the embryonic peripheral nervous system, because other proteins, such as PTP69D, clearly have a role in embryonic motor neuron development but cannot be detected in motor neurons of wild-type embryos (Desai *et al.*, 1996).

The mammalian homolog of UNC-76, FEZ1, was identified in a yeast two-hybrid screen for proteins that bind the regulatory domain of rat PKCζ (Kuroda *et al.*, 1999). FEZ1 is expressed at high levels in the nervous system of embryos and adults. In COS-7 cultured cells, FEZ1 is found associated with both cytosolic and membrane fractions, but its phosphorylation by PKCζ causes the redistribution of membrane-bound FEZ1 to the cytosol. In addition, the phosphorylation of FEZ1 by PKCζ stimulates neurite outgrowth in PC12 cultured cells, suggesting that PKCζ-dependent neuron differentiation may require FEZ1. Interestingly, antisense inhibition experiments in cultured rat hippocampal neurons demonstrate that kinesin is required for full extension of neurites (Ferreira *et al.*, 1992), thus suggesting a possible relationship between PKCζ signaling, FEZ1 activity, and kinesin-dependent neurite outgrowth. Growth of axons occurs rapidly during *Drosophila* larval development, especially between L2 and L3, when the larva increases in length severalfold (Jan and Jan, 1976). Perhaps *Unc-76* mutant lethality at the L2-L3 transition indicates that its function is

required for axon outgrowth in the developing larval nervous system.

Is UNC-76 a Cargo Adapter or Kinesin Regulator?

Atypical PKCs (ι/λ and ζ) are essential for nerve growth factor-dependent neurite outgrowth and mitogen-activated protein kinase activation in PC12 cells (Reinhold and Neet, 1989; Lloyd and Wooten, 1992; Wooten *et al.*, 1994). Perhaps UNC-76 acts as a control point for the regulation of kinesin activity by PKCζ and its effectors. Posttranslational modification of UNC-76 by PKCζ could regulate UNC-76 binding to KHC, or UNC-76 could recruit regulatory proteins or cargos to the kinesin tail domain, as has been suggested for the KLC-binding scaffold proteins JIP1, JIP2, and JIP3/JSAP1 (SYD), which bind kinesin and members of the JNK signaling cascade (Ito *et al.*, 1999; Yasuda *et al.*, 1999; Bowman *et al.*, 2000; Kelkar *et al.*, 2000; Verhey *et al.*, 2001). Another plausible model is that UNC-76 is a cargo adapter for cytosolic nonmembrane cargos that escape entrapment in axon clogs. Analysis of kinesin-dependent axon clogs has shown that they are composed of membrane-bound organelles (Hurd and Saxton, 1996; Martin *et al.*, 1999); the presence of soluble nonmembrane cargos, such as mRNPs or protein complexes, was not tested directly. Recent experiments show that KHC homolog KIF5B is present in soluble complexes with NF1 (neurofibromin) and NF2 (merlin) in HeLa cells (Hakimi *et al.*, 2002), supporting a model in which kinesin is required for NF1 and NF2 transport to the cell periphery. UNC-76 contains a domain similar to the NF2 binding domain of SCHIP-1 (Goutebroze *et al.*, 2000); an intriguing possibility is that UNC-76 is a bipartite cargo adaptor that mediates kinesin–NF2 interactions. Overexpression of UNC-76 may enhance kinesin mutant phenotypes by reducing the kinesin pool available for vesicle transport. UNC-76 may target kinesin for inactivation by signal transduction pathways (regulatory model), or mask the kinesin tail domain's ability to bind membrane cargo receptors (titration model).

The in vivo relationship between UNC-76 and PKCζ in *Drosophila* is unclear. The closest *Drosophila* homolog to PKCζ is encoded by the *DaPKC* gene (Wodarz *et al.*, 2000). During embryogenesis, *DaPKC* is expressed in epithelial tissues and developing neuroblasts. DaPKC protein accumulates at the apical cortex of polarized cells, and is part of a protein complex with bazooka (BAZ) essential for apical-basal epithelial polarity; however, there is no known role for DaPKC in axonal transport. We are currently investigating the nature of UNC-76 interactions with DaPKC and other regulatory proteins, and possible functional relationships between those interactions and kinesin activity. *Drosophila* UNC-76 contains a 120 aa N-terminal region that is not evolutionarily conserved (Figure 2); perhaps this region can serve as an interface for *Drosophila*-specific regulatory interactions.

Parallels and Orthogonals between UNC-76, APPL, and SYD

The *Unc-76* loss-of-function phenotype is similar to those observed for *Appl* and *syd*, which encode proteins that bind to the kinesin tail domain (Torroja *et al.*, 1999; Bowman *et al.*, 2000; Gunawardena and Goldstein, 2001). Mutations in all three genes result in the disruption of axonal transport in larval segmental nerves, resulting in aggregates of membrane-bound cargos. APPL is the homolog of APP, a transmembrane protein that is a kinesin cargo receptor through its interaction with the tetratricopeptide (TPR) repeats of KLC (Kamal *et al.*, 2000, 2001). SYD is the *Drosophila* homolog of JIP3/JSAP1 and has the dual role of kinesin cargo receptor through KLC binding and scaffold for components of the JNK signaling cascade. UNC-76 differs from APPL and SYD in that it binds to the tail domain of KHC, not the TPR repeats of KLC. Furthermore, UNC-76 does not seem to be a membrane cargo receptor, because it is not a transmembrane protein and is not enriched in axon clogs in the segmental nerves of kinesin mutant larvae. However, the possibility that UNC-76 is a receptor, adapter, or facilitator of kinesin binding for cargos that do not accumulate in axon clogs cannot be eliminated. Interestingly, it has been shown that the first 119 aa of *C. elegans* UNC-76 are sufficient for targeting to axons, and fusions of the first 197 aa of UNC-76 to β-gal or green fluorescent protein are directed to axons in *C. elegans* (Bloom and Horvitz, 1997). The part of *Drosophila* UNC-76 that binds to KHC does not include this region. Perhaps there are multiple binding sites for kinesin in UNC-76, or UNC-76 can be transported into neurons by another motor protein or through indirect association with kinesin. The axon targeting domain of UNC-76 is intriguing in light of the recent identification of mouse GRIP1, which binds to KHC homologs KIF5a/b/c and targets kinesin to dendrites (Setou *et al.*, 2002). The amino terminus of UNC-76 may have an analogous role in axon targeting of kinesin or other motor proteins.

The similar phenotypes and dosage-dependent genetic interactions of *Unc-76*, *Khc*, and *Klc* support a model in which UNC-76 is an important component of the kinesin transport pathway. Individuals lacking UNC-76 display locomotion phenotypes and axon clogs identical to the phenotypes displayed by strong alleles of either kinesin subunit. Furthermore, increasing the genetic dosage of *Unc-76* enhances the phenotype of *Khc* and *Klc* mutants. This may be caused by the titration of free kinesin away from other binding partners, such as APPL and SYD. There are several similarities between the genetic interactions of *Unc-76* and *Appl* with *Khc* and *Klc*. Overexpression of APPL and UNC-76 enhances *Khc* more than *Klc*, enhancement of the *Khc* and *Klc* phenotypes is more pronounced when the kinesin mutant allele is inherited maternally, and reduction of *Appl* or *Unc-76* dosage does not enhance *Khc* or *Klc* (Torroja *et al.*, 1999; Gunawardena and Goldstein, 2001). The phenotype of *Khc* mutants may be more severe than *Klc* because all kinesin cargos require the activity of the KHC-encoded kinesin motor domain, whereas some cargos may bind directly to the KHC tail, thus not requiring KLC for transport. Similarly, loss of *Appl* or *Unc-76* function may disrupt the transport of a subset of kinesin cargos. The increase of *Unc-76* and *Appl* dosage could reduce the concentration of soluble kinesin available for cargo binding to reduce kinesin-dependent transport to subthreshold levels. Although it is possible that another gene in the *Unc-76* duplication enhances kinesin mutant phenotypes, this seems unlikely given the small number of genes identified in previous genetic screens for mutations causing larval locomotion defects (Martin *et al.*, 1999a; Gunawardena and Goldstein, 2001). However, it is important to define more precisely the functional domains

of UNC-76 that interact with kinesin and other proteins, such as PKCζ.

REFERENCES

Adams, M.D., *et al*. (2000). The genome sequence of *Drosophila melanogaster*. Science *287*, 2185–2195.

Apweiler, R., et al. (2001). The InterPro database, an integrated documentation resource for protein families, domains and functional sites. Nucleic Acids Res. *29*, 37–40.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1997). Current Protocols in Molecular Biology, New York: John Wiley & Sons.

Bar-Peled, M., and Raikhel, N.V. (1996). A method for isolation and purification of specific antibodies to a protein fused to the GST. Anal. Biochem. *241*, 140–142.

Bloom, L., and Horvitz, H.R. (1997). The Caenorhabditis elegans gene *unc-76* and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. Proc. Natl. Acad. Sci. USA *94*, 3414–3419.

Bowman, A.B., Kamal, A., Ritchings, B.W., Philip, A.V., McGrail, M., Gindhart, J.G., and Goldstein, L.S. (2000). Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein. Cell *103*, 583–594.

Bowman, A.B., Patel-King, R.S., Benashski, S.E., McCaffery, J.M., Goldstein, L.S., and King, S.M. (1999). *Drosophila* roadblock and *Chlamydomonas* LC 7, a conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis. J. Cell Biol. *146*, 165–180.

Brady, S.T. (1985). A novel brain ATPase with properties expected for the fast axonal transport motor. Nature *317*, 73–75.

Brendza, R.P., Sheehan, K.B., Turner, F.R., and Saxton, W.M. (2000). Clonal tests of conventional kinesin function during cell proliferation and differentiation. Mol. Biol. Cell *11*, 1329–1343.

Byrd, D.T., Kawasaki, M., Walcoff, M., Hisamoto, N., Matsumoto, K., and Jin, Y. (2001). UNC-16, a JNK-signaling scaffold protein, regulates vesicle transport in *C. elegans*. Neuron *32*, 787–800.

Coy, D.L., Hancock, W.O., Wagenbach, M., and Howard, J. (1999). Kinesin's tail domain is an inhibitory regulator of the motor domain. Nat. Cell Biol. *1*, 288–292.

Desai, C.J., Gindhart, J.G.J., Goldstein, L.S.B., and Zinn, K. (1996). Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. Cell *84*, 599–609.

Ferreira, A., Niclas, J., Vale, R.D., Banker, G., and Kosik, K.S. (1992). Suppression of kinesin expression in cultured hippocampal neurons using antisense oligonucleotides. J. Cell Biol. *117*, 595–606.

Finley, R.L., Jr., Thomas, B.J., Zipursky, S.L., and Brent, R. (1996). Isolation of *Drosophila* cyclin D, a protein expressed in the morphogenetic furrow before entry into S phase. Proc. Natl. Acad. Sci. USA *93*, 3011–3015.

Garnier, J., Osguthorpe, D.J., and Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. *120*, 97–120.

Gho, M., McDonald, K., Ganetzky, B., and Saxton, W.M. (1992). Effects of kinesin mutations on neuronal functions. Science *258*, 313–316.

Gindhart, J.G., Jr., Desai, C.J., Beushausen, S., Zinn, K., and Goldstein, L.S. (1998). Kinesin light chains are essential for axonal transport in *Drosophila*. J. Cell Biol. *141*, 443–454.

Goldstein, L.S. (2001). Kinesin molecular motors: transport pathways, receptors, and human disease. Proc. Natl. Acad. Sci. USA *98*, 6999–7003.

Goldstein, L.S., and Philip, A.V. (1999). The road less traveled: emerging principles of kinesin motor utilization. Annu. Rev. Cell Dev. Biol. *15*, 141–183.

Goutebroze, L., Brault, E., Muchardt, C., Camonis, J., and Thomas, G. (2000). Cloning and characterization of SCHIP-1, a novel protein interacting specifically with spliced isoforms and naturally occurring mutant NF2 proteins. Mol. Cell. Biol. *20*, 1699–1712.

Guan, K.L., and Dixon, J.E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. *192*, 262–267.

Gunawardena, S., and Goldstein, L.S. (2001). Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*. Neuron *32*, 389–401.

Hackney, D.D., and Stock, M.F. (2000). Kinesin's IAK tail domain inhibits initial microtubule-stimulated ADP release. Nat. Cell Biol. *2*, 257–260.

Hakimi, M.-A., Speicher, D.W., and Shiekhattar, R. (2002). The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway for neurofibromatosis. J. Biol. Chem. *277*, 36909–36912.

Hedgecock, E.M., Culotti, J.G., Thomson, J.N., and Perkins, L.A. (1985). Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. Dev. Biol. *111*, 158–170.

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science *279*, 519–526.

Huang, A.M., Rehm, E.J., and Rubin, G.M. (2000). Recovery of DNA sequences flanking P-element insertions: inverse PCR and plasmid rescue. In: *Drosophila* Protocols, ed. W. Sullivan, M. Ashburner, and R.S. Hawley, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 429–437.

Hurd, D.D., and Saxton, W.M. (1996). Kinesin mutations cause motor neuron disease phenotypes by disrupting fast axonal transport in *Drosophila*. Genetics *144*, 1075–1085.

Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K.I. (1999). JSAP1, a novel jun N-terminal protein kinase (J.N.K)-binding protein that functions as a scaffold factor in the JNK signaling pathway. Mol. Cell. Biol. *19*, 7539–7548.

Jan, L.Y., and Jan, Y.N. (1976). Properties of the larval neuromuscular junction in *Drosophila* melanogaster. J. Physiol. *262*, 189–214.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., and Gibson, T.J. (1998). Multiple sequence alignment with Clustal X. Trends Biochem. Sci. *23*, 403–405.

Kamal, A., Almenar-Queralt, A., LeBlanc, J.F., Roberts, E.A., and Goldstein, L.S. (2001). Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature *414*, 643–648.

Kamal, A., and Goldstein, L.S. (2002). Principles of cargo attachment to cytoplasmic motor proteins. Curr. Opin. Cell Biol. *14*, 63–68.

Kamal, A., Stokin, G.B., Yang, Z., Xia, C.H., and Goldstein, L.S. (2000). Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron *28*, 449–459.

Karcher, R.L., Deacon, S.W., and Gelfand, V.I. (2002). Motor-cargo interactions: the key to transport specificity. Trends Cell Biol. *12*, 21–27.

Kelkar, N., Gupta, S., Dickens, M., and Davis, R.J. (2000). Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. Mol. Cell. Biol. *20*, 1030–1043.

Kreegipuu, A., Blom, N., and Brunak, S. (1999). PhosphoBase, a database of phosphorylation sites: release 2.0. Nucleic Acids Res. *27*, 237–239.

Kuroda, S., Nakagawa, N., Tokunaga, C., Tatematsu, K., and Tanizawa, K. (1999). Mammalian homologue of the *Caenorhabditis elegans* UNC-76 protein involved in axonal outgrowth is a protein kinase C zeta-interacting protein. J. Cell Biol. *144*, 403–411.

Littleton, J.T., Bellen, H.J., and Perin, M.S. (1993). Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. Development *118*, 1077–1088.

Lloyd, E.D., and Wooten, M.W. (1992). pp42/44MAP kinase is a component of the neurogenic pathway utilized by nerve growth factor in PC12 cells. J. Neurochem. *59*, 1099–1109.

Luo, L., Tully, T., and White, K. (1992). Human amyloid precursor protein ameliorates behavioral deficit of flies deleted for Appl gene. Neuron *9*, 595–605.

Luo, L.Q., Martin-Morris, L.E., and White, K. (1990). Identification, secretion, and neural expression of APPL, a *Drosophila* protein similar to human amyloid protein precursor. J. Neurosci. *10*, 3849–3861.

Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. Science *252*, 1162–1164.

Martin, M., Iyadurai, S.J., Gassman, A., Gindhart, J.G., Jr., Hays, T.S., and Saxton, W.M. (1999a). Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport. Mol. Biol. Cell *10*, 3717–3728.

Martin, M.A., Hurd, D.D., and Saxton, W.M. (1999b). Kinesins in the nervous system. Cell Mol. Life Sci. *56*, 200–216.

Micheva, K.D., Kay, B.K., and McPherson, P.S. (1997). Synaptojanin forms two separate complexes in the nerve terminal. Interactions with endophilin and amphiphysin. J. Biol. Chem. *272*, 27239–27245.

Nakai, K., and Horton, P. (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. *24*, 34–36.

Parchaliuk, D.L., Kirkpatrick, R.D., Agatep, R., Simon, S.L., and Gietz, R.D. (1999). Yeast two-hybrid system: part C-characterizing positives. Tech. Tips Online, P01714.

Patel, N., Thierry-Mieg, D., and Mancillas, J.R. (1993). Cloning by insertional mutagenesis of a cDNA encoding *Caenorhabditis elegans* kinesin heavy chain. Proc. Natl. Acad. Sci. USA *90*, 9181–9185.

Ray, K., Perez, S.E., Yang, Z., Xu, J., Ritchings, B.W., Steller, H., and Goldstein, L.S. (1999). Kinesin-II is required for axonal transport of choline acetyltransferase in *Drosophila.* J. Cell Biol. *147*, 507–518.

Reinhold, D.S., and Neet, K.E. (1989). The lack of a role for protein kinase C in neurite extension and in the induction of ornithine decarboxylase by nerve growth factor in PC12 cells. J. Biol. Chem. *264*, 3538–3544.

Rothwell, W.F., and Sullivan, W. (2000). Fluorescent analysis of *Drosophila* embryos. In: *Drosophila* Protocols, ed. W. Sullivan, M. Ashburner, and R.S. Hawley, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 141–157.

Saxton, W.M., Hicks, J., Goldstein, L.S., and Raff, E.C. (1991). Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. Cell *64*, 1093–1102.

Setou, M., Seog, D.H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M., and Hirokawa, N. (2002). Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. Nature *417*, 83–87.

Stebbings, H. (2001). Cytoskeleton-dependent transport and localization of mRNA. Int. Rev. Cytol. *211*, 1–31.

Stock, M.F., Guerrero, J., Cobb, B., Eggers, C.T., Huang, T.G., Li, X., and Hackney, D.D. (1999). Formation of the compact conformer of kinesin requires a COOH-terminal heavy chain domain and inhibits microtubule-stimulated ATPase activity. J. Biol. Chem. *274*, 14617–14623.

Terada, S., Kinjo, M., and Hirokawa, N. (2000). Oligomeric tubulin in large transporting complex is transported via kinesin in squid giant axons. Cell *103*, 141–155.

Torroja, L., Chu, H., Kotovsky, I., and White, K. (1999). Neuronal overexpression of APPL, the *Drosophila* homologue of the amyloid precursor protein (APP), disrupts axonal transport. Curr. Biol. *9*, 489–492.

Trofatter, J.A., *et al.* (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell *72*, 791–800.

Vale, R.D., and Fletterick, R.J. (1997). The design plan of kinesin motors. Annu. Rev. Cell Dev. Biol. *13*, 745–777.

Vale, R.D., Reese, T.S., and Sheetz, M.P. (1985). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell *42*, 39–50.

Verhey, K.J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B.J., Rapoport, T.A., and Margolis, B. (2001). Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. J. Cell Biol. *152*, 959–970.

Wodarz, A., Ramrath, A., Grimm, A., and Knust, E. (2000). *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. J. Cell Biol. *150*, 1361–1374.

Wooten, M.W., Zhou, G., Seibenhener, M.L., and Coleman, E.S. (1994). A role for zeta protein kinase C in nerve growth factor-induced differentiation of PC12 cells. Cell Growth Differ. *5*, 395–403.

Yasuda, J., Whitmarsh, A.J., Cavanagh, J., Sharma, M., and Davis, R.J. (1999). The JIP group of mitogen-activated protein kinase scaffold proteins. Mol. Cell. Biol. *19*, 7245–7254.

Zervos, A.S., Gyuris, J., and Brent, R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell *72*, 223–232.