Cytoplasmic Dynein, the Dynactin Complex, and Kinesin Are Interdependent and Essential for Fast Axonal Transport

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In axons, organelles move away from (anterograde) and toward (retrograde) the cell body along microtubules. Previous studies have provided compelling evidence that conventional kinesin is a major motor for anterograde fast axonal transport. It is reasonable to expect that cytoplasmic dynein is a fast retrograde motor, but relatively few tests of dynein function have been reported with neurons of intact organisms. In extruded axoplasm, antibody disruption of kinesin or the dynactin complex (a dynein activator) inhibits both retrograde and anterograde transport. We have tested the functions of the cytoplasmic dynein heavy chain (*cDhc64C*) and the p150^{Glued} (*Glued*) component of the dynactin complex with the use of genetic techniques in *Drosophila*. *cDhc64C* and *Glued* mutations disrupt fast organelle transport in both directions. The mutant phenotypes, larval posterior paralysis and axonal swellings filled with retrograde and anterograde cargoes, were similar to those caused by kinesin mutations. Why do specific disruptions of unidirectional motor systems cause bidirectional defects? Direct protein interactions of kinesin with dynein heavy chain and p150^{Glued} were not detected. However, strong dominant genetic interactions between kinesin, dynein, and dynactin complex mutations in axonal transport were observed. The genetic interactions between kinesin and either *Glued* or *cDhc64C* mutations were stronger than those between *Glued* and *cDhc64C* mutations themselves. The shared bidirectional disruption phenotypes and the dominant genetic interactions demonstrate that cytoplasmic dynein, the dynactin complex, and conventional kinesin are interdependent in fast axonal transport.

INTRODUCTION

Neurons depend on fast anterograde axonal transport to move newly synthesized organelles and other macromolecular complexes from the cell body to the axon terminal. Fast retrograde axonal transport is also vital, returning spent organelles and other materials in the endocytic/lysosomal pathway from the terminal to the cell body. Axonal microtubules, 90% of which are oriented with plus ends toward the terminal, provide directional tracks for motor proteins that are attached to the various fast transport cargoes (reviewed by Hirokawa, 1998; Martin *et al.*, 1999). Most kinesin family proteins are anterograde motors, pulling their cargoes specifically toward microtubule plus ends. Other kinesins and cytoplasmic dyneins are retrograde motors, pulling

their cargoes toward the minus ends (reviewed by Vale and Fletterick, 1997; Hirokawa, 1998). It is reasonable to suspect that retrograde motors are carried, in some manner, to the terminal by anterograde motors. In return, any anterograde motors that return from the terminal should be transported by retrograde motors. How organelles or other cargoes that carry opposing motors coordinate their activities to accomplish persistent long-range movement in one direction is not known.

Conventional kinesin is a major anterograde motor that has been found in many metazoan cell types. It is abundant in axoplasm, and function disruption studies suggest that it is required for both fast anterograde and fast retrograde axonal transport (Brady *et al.*, 1990; Hurd and Saxton, 1996; Stenoien and Brady, 1997; Gindhart *et al.*, 1998; reviewed by Martin *et al.*, 1999). Mutations of conventional kinesin in *Drosophila* cause partial posterior paralysis and large axonal swellings filled with organelles normally carried by antero-

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grade and retrograde fast transport (Saxton *et al.*, 1991; Hurd and Saxton, 1996; Gindhart *et al.*, 1998). The composition and distribution of the swellings suggest that a loss of kinesin function increases the frequency of cargo stalling within axons. The stalled kinesin cargoes could cause the equivalent of traffic jams, and hence swellings, by steric hindrance of fast organelle transport (Hurd and Saxton, 1996).

Cytoplasmic dynein is a major fast retrograde motor that is present in all eukaryotic cell types. Drugs or antibodies that inhibit dynein function block fast transport in axoplasmic preparations or dissected axons (e.g., Goldberg, 1982; Ekstrom and Kanje, 1984; Forman *et al.*, 1984; Schnapp and Reese, 1989; Wang *et al.*, 1995), but genetic tests *in vivo* had not been reported until recently (Bowman *et al.*, 1999). The dynactin complex is a large assembly of proteins that appears to be required for normal cytoplasmic dynein function in a number of motility processes (reviewed by Holleran *et al.*, 1998). Antibodies to p150^{Glued}, a major component of the dynactin complex, inhibit organelle movements in extruded axoplasm (Waterman-Storer *et al.*, 1997). In most of these dynein and dynactin inhibition studies, axoplasmic organelle movements in both directions were inhibited. Although the inhibition of bidirectional transport by functional disruption of all three proteins—kinesin, dynein, and dynactin—can be discounted as nonspecific effects, it could be due to functional linkages between the anterograde and retrograde transport systems.

In *Drosophila*, the *Glued* (*Gl*) gene encodes the homolog of the p150 subunit of the vertebrate dynactin complex (Swaroop *et al.*, 1987; Gill *et al.*, 1991; Holzbaur *et al.*, 1991). Previous work has demonstrated that the dominant *Gl*¹ mutation encodes a truncated polypeptide that acts as a poison product (Swaroop *et al.*, 1985; McGrail *et al.*, 1995). In heterozygotes, the *Gl*¹ mutation produces a rough eye phenotype with a severe disruption in the organization of the retina and in the retinal axonal projections to the optic lobe (Meyerowitz and Kankel, 1978). Certain mutations in cytoplasmic dynein heavy chain (*cDhc64C*) also produce rough eyes and interact with *Gl*¹ to suppress or enhance the rough eye phenotype (McGrail *et al.*, 1995). Similarly, mutations in *Kinesin heavy chain* (*Khc*) produce rough eye phenotypes (Brendza and Saxton, unpublished observations) and exhibit dominant interactions with *Gl*¹ (Hays, unpublished observations). Whether these results reflect the coordinated activity of motors in axonal transport or separate cellular functions is not clear (Fan and Ready, 1997).

To test for interactions between anterograde and retrograde transport systems, and to address questions about dynein and dynactin functions in fast axonal transport *in vivo*, we conducted genetic and biochemical tests in *Drosophila*. We analyzed the neuronal phenotypes of mutations in *Gl* and *cDhc64C*, which encodes a ubiquitously expressed cytoplasmic dynein heavy chain (Li *et al.*, 1994; Gepner *et al.*, 1996). Our results indicate that dynein and the dynactin complex are critical for fast axonal transport. We also found dominant genetic interactions between the *Khc*, *Kinesin light chain* (*Klc*), *cDhc64C*, and *Gl* genes. The genetic interactions show that conventional kinesin, cytoplasmic dynein, and the dynactin complex are interdependent and may physically interact in fast axonal transport.

MATERIALS AND METHODS

Fly Stocks, Culture Conditions, Larval Locomotion Analysis, and Genetic Strategies

All fly stocks were maintained at 22–25°C, and crosses were conducted at 25°C with a 12-h light/dark cycle on a previously described yeast-agar-based fly medium (Hurd and Saxton, 1996). The mutant chromosomes used in this research are listed in Table 1. Recessive lethal chromosomes were maintained over one of three balancer chromosomes carrying the third instar marker, *Tubby* (*Tb*) [*TM6B*, *Tb Hu e ca*; *TM6C*, *Tb Sb e cu ca*; or *T(2,3)B3*, *CyO:TM6B*, *Tb Hu*] (Lindsley and Zimm, 1992; Flybase, 1999). The *Tb* mutation allowed recognition of test and control genotypes in larval stages, as described by Saxton *et al.* (1991).

Scoring of the Posterior-Paralytic Phenotype Adult flies were mated in vials and transferred every 24 h to prevent overcrowding of progeny. Late third instar larvae were collected, rinsed with water to remove food and debris, and placed on a clean plate of agar-based fly medium. The crawling behavior of each larva was observed for several minutes. Animals were scored as posterior paralytic if at least two posterior segments curved up away from the substrate during the crawling cycle. In most cases, three or four segments were involved in the tail flip, which equates to approximately one-fourth to one-third of body length. Typically, 25–50 larvae of a test class genotype were scored for the posterior-paralytic phenotype from each cross. Each cross was done at least twice.

Induction of Mutant p150^{Glued} A heat-shock regimen was used to stimulate the production of a truncated, dominant negative p150^{Glued} protein in transgenic flies (Fan and Ready, 1997). Flies homozygous for the transgene (*Phs-Gl*¹) were allowed to mate and deposit embryos in vials for 24 h. Vials with progeny were then subjected to 1-h, 36°C heat shocks once a day for 4 d.

Isolation of *cDhc64C*^{ek1} A new mutation, *E(Khc)ek1*, was isolated in an F₁ screen for dominant enhancers of a *Khc* null mutation. Male flies with isogenized second and third chromosomes (*w*⁻; +; + or *w*⁻; +; *e*¹) were mutagenized with 25 mM ethyl methane sulfonate and mated en masse to virgin *pr Khc*¹⁶/*T(2,3)B3*, *CyO:TM6B*, *Tb* females. Matings were transferred daily to new food until d 4, when the adults were discarded (Saxton *et al.*, 1991). After 5.5 d of development, larvae were harvested by floatation on 3 M NaCl in a separatory funnel (Roberts, 1986). After a distilled water wash, groups of ~50 larvae were placed on the center of 150-mm hard agar plates that had been dyed dark blue with either food coloring or bromophenol blue. The contrast provided between the white larvae and the dark blue plates allowed easier observation of crawling third instar larvae with the naked eye, magnifying glasses, or stereomicroscopes. Test class larvae (heterozygous for both *Khc*¹⁶ and mutagenized chromosomes) that exhibited the posterior-paralytic phenotype and developed into fertile adults were used to establish permanent lines. Of the 60,000 test class larval genomes screened, 10 *E(Khc)* loci were identified and designated *ek1*–*ek10*. The first dominant enhancer recovered from the screen, *E(Khc)ek1*, was mapped by meiotic recombination to the left arm of chromosome 3 at 10 ± 4 centimorgans with the use of the flanking mutations *roughoid* (*ru*) and *hairy* (*h*). Complementation tests with various deletions, *cDhc64C* mutant alleles, and a *cDhc64C* wild-type transgene were performed, as described by Gepner *et al.* (1996), and confirmed that *E(Khc)ek1* is a *cDhc64C* allele. When either of the hypomorphic mutations *cDhc64C*⁶⁻¹⁰ or *cDhc64C*⁶⁻⁶⁻¹⁶ were combined with the small deletion *Df(3L)10H* or the amorphic allele *cDhc64C*⁴⁻¹⁹, lethality occurred during late larval and pupal stages. When *cDhc64C*^{ek1} was combined with either of the hypomorphic alleles, the lethal phase was shifted earlier and a greater proportion of deaths occurred during the third larval instar. Thus, in these assays, *cDhc64C*^{ek1} is more severe than a null mutation.

Table 1. *Drosophila* chromosomes used in this research

Stock	Description	Source
<i>mwh cDhc64C⁴⁻¹⁹ jv ca</i>	Amorphic allele, recombinant chromosome	Gepner <i>et al.</i> , 1996
<i>cDhc64C⁶⁻¹⁰ P{FRT w⁺}</i>	Hypomorphic allele, recombinant chromosome	Gepner <i>et al.</i> , 1996
<i>cDhc64C⁶⁻¹⁶h st p^p ss e^s</i>	Hypomorphic allele, recombinant chromosome	J. Gepner and T. Hays
<i>Df(3L)10H st e</i>	Deletes <i>cDhc64C</i> (64B10-12;64C5-9)	Garbe <i>et al.</i> , 1993; Gepner <i>et al.</i> , 1996
<i>Df(3L)GN24</i>	Deletes <i>cDhc64C</i> (63F04-07;64C13-15)	Bloomington Stock Center (Bloomington, IN)
<i>w, P{Dhc⁺}^x</i>	Genomic <i>cDhc64C⁺</i> transgene	Gepner <i>et al.</i> , 1996
<i>cDhc64C^{ek1}</i> (synonym = <i>E(Khc)ek1⁻</i>)	Antimorphic allele, original chromosome	This study
<i>ru cDhc64C^{ek1} sr e & cDhc64C^{ek1} h th</i>	Recombinant chromosomes	This study
<i>st cu sr e Pr ca</i>		
<i>Gl¹ & Gl¹ Sb¹</i>	Antimorphic allele, original and recombinant chromosomes	Bloomington Stock Center
<i>Df(3L)fz-GF3b</i>	Deletes <i>Glued</i> (70B;70C6)	Bloomington Stock Center
<i>Gl^{1-hs}</i>	Transgenic, inducible antimorphic allele	Fan and Ready, 1997
<i>pr Khc¹⁶</i>	Null allele	C. Regan, D. Rose, K. Brenda, and W. Saxton
<i>b Khc²⁷ lf & c Khc²⁷ Elp</i>	Null allele, parental and recombinant chromosomes	D. Rose, K. Brenda, and W. Saxton
<i>P{Khc⁺, w⁺} PK15a, PK4c & PK9a</i>	Genomic <i>Khc⁺</i> transgene, 3 independent lines	Saxton <i>et al.</i> , 1991
<i>Df(3L)8ex94</i>	Deletes <i>Klc</i>	Gindhart <i>et al.</i> , 1998
<i>Df(3L)34ex5</i>	Deletes <i>Klc</i> and other nearby genes	Gindhart <i>et al.</i> , 1998
<i>Df(3L)8ex34</i>	Deletes <i>Klc</i> and other nearby genes	J. Gindhart
<i>P{GEN-KLC⁺}PC4</i>	Genomic <i>Klc⁺</i> transgene	Gindhart <i>et al.</i> , 1998
<i>Df(3R)ry615</i>	Deletes <i>Arp87C</i> and other genes	Bloomington Stock Center
<i>Df(3L)ZN47</i>	Delete <i>Klp64D</i> and other genes	Bloomington Stock Center
<i>Df(3L)vin7 and Df(3L)vin5</i>	Delete <i>Klp68D</i> and other genes	Bloomington Stock Center

Video Imaging and Frame Capturing

Larvae were videotaped with a Dage (Michigan City, IN) 68 Newvicon camera (Hurd and Saxton, 1996). The camera was fitted with three Nikon (Garden City, NY) Vivitar automatic extension tubes (68 mm total lens extension) and a Nikon microNikkor 55-mm lens. Single video frames were digitized for processing in NIH Image 1.62b7 (National Institutes of Health, Bethesda, MD) and Adobe (Mountain View, CA) Photoshop 5.0.

Sample Preparation and Microscopy

Larval dissection, fixation, immunohistochemistry, and confocal microscopy were as described previously (Hurd and Saxton, 1996) with the following exception. Before reactions with the β 1 tubulin mAb, formaldehyde-fixed larvae were postfixed in -20°C methanol for 15 min and then washed in PBS with 0.01% Tween 20. The primary antibodies and dilutions used were rabbit polyclonal anti-*Drosophila* synaptotagmin (1:500) (Littleton *et al.*, 1993), mouse monoclonal anti-*Drosophila* cysteine string protein (1:250) (Zinsmaier *et al.*, 1990), rabbit polyclonal anti-*Drosophila* HOOK (1:100) (Kramer and Phistery, 1996), mouse monoclonal anti- β 1 tubulin (1:100) (Dettman *et al.*, 1996), rabbit polyclonal anti-*Drosophila* kinesin heavy chain (1:100) (Saxton *et al.*, 1988), and mouse monoclonal anti-*Drosophila* dynein heavy chain (1:200) (McGrail and Hays, 1997). The secondary antibodies used were goat polyclonal anti-rabbit conjugated to FITC or goat polyclonal anti-mouse conjugated to TRITC, both at 1:500 (Jackson Immunoresearch Laboratories, West Grove, PA). Sample preparation and thin sectioning for transmission electron microscopy were as described by Hurd and Saxton (1996) except that an accelerating voltage of 80 kV was used for imaging.

Immunoprecipitation Experiments

To collect cytosol for immunoprecipitation tests, we used two tissues, fly heads as a source of neural tissue and ovaries because the

female germline is rich in microtubule motors and the dynactin complex. Heads or ovaries from either wild-type flies or flies that expressed DHC tagged with three hemagglutinin epitopes (DHC-3HA, which functions as wild type based on mutant rescue experiments [Silvanovich and Hays, unpublished data]) were homogenized in an equal volume of lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 3.3 U/ml aprotase) supplemented with protease inhibitors (2 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mg/ml pepstatin, 10 mM benzamide) and centrifuged at 42,000 rpm for 40 min in a Ti50 rotor. The pellet was discarded, and the high-speed supernatant was combined with protein A-agarose beads (Sigma Chemical, St. Louis, MO) to remove proteins that would bind nonspecifically. After brief centrifugation, the preadsorbed high-speed supernatant was transferred to a fresh tube. Typically, 50 μl of this supernatant was used for the immunoprecipitation experiments. Mouse monoclonal anti-*Drosophila* DHC antibodies (McGrail and Hays, 1997), mouse monoclonal anti-HA (HA.11) antibodies (Babco, Richmond, CA), or p150^{Glued} antibodies (McGrail *et al.*, 1995) were bound to protein A-agarose beads in the presence of $1\times$ Tris-buffered saline (Sambrook *et al.*, 1989) and collected by pelleting. A total of 50 μl of preadsorbed high-speed supernatant was combined with the antibody-bead complexes and 50 μl of NET-gel (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin, 0.02% sodium azide) supplemented with protease inhibitors (2 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mg/ml pepstatin, 10 mM benzamide). The binding reaction was performed at either 4 or 25°C for 4–6 h. The beads were collected by a brief centrifugation and washed four times with buffer 1 (125 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% sodium deoxycholate, 0.01% SDS, 0.02% sodium azide), two times with buffer 2 (125 mM Tris-Cl, pH 8.0, 1.0 M NaCl, 5 mM EDTA, 0.02% sodium azide), and once with sterile water. The immunoprecipitates and protein controls were analyzed by standard Laemmli denaturing gel electrophoresis (Gallagher and Smith, 1991) and western blotting with a

Figure 1. Mutations in *cDhc64C* cause posterior paralysis and defects in fast axonal transport. (A and B) Single video frames of third instar larvae (~3 mm in length) crawling forward (to the left). (A) The control, heterozygous for a severe *cDhc64C* mutation (*cDhc64C^{ck1}/+*), exhibited wild-type posture. (B) The mutant, carrying a moderate mutation over a deletion [*cDhc64C⁶⁻¹⁰/Df(3L)10H*], exhibited posterior paralysis whereby the distal-most segments curved upward. (C–F) Laser scanning confocal micrographs of control, *P{cDhc64C⁺}/+*; *cDhc64C⁶⁻¹⁰/Df(3L)10H* (C and E), and mutant, *cDhc64C⁶⁻¹⁰/Df(3L)10H* (D and F), larval segmental nerves stained for tubulin (C and D) and synaptotagmin (E and F). Segmental nerves contain motor and sensory axons that connect the ventral ganglia to the body wall muscles and sensory organs in the abdominal segments. These fields of view each cover about half an abdominal segment. (C and D) Microtubules run longitudinally in the nerves and appeared unaffected by the *cDhc64C* mutations. A predominantly longitudinal and striated staining pattern was observed in both control and mutant nerves. The meshwork of single microtubules in the background was in muscle cells underlying the nerves. (E) Synaptotagmin staining was distributed in a punctate pattern along the nerve, presumably representing the distribution of anterograde fast transport vesicles. (F) Synaptotagmin in the mutant nerve was much more abundant and concentrated in large accumulations. n, segmental nerve; m, body-wall muscle. Bars, 10 μ m.

chemiluminescent detection system (Tropix, Bedford, MA) according to the manufacturer's instructions.

RESULTS

cDhc64C and *p150^{Glued}* Are Essential for Axonal Transport in *Drosophila*

To determine if cytoplasmic dynein functions as a fast axonal transport motor in *Drosophila*, we studied larvae that carried mutations in *cDhc64C* (Gepner *et al.*, 1996). Because null or amorphic *cDhc64C* mutants die too early to observe axonal transport phenotypes, we used hypomorphic alleles that allow development to progress through the larval stages (Gepner *et al.*, 1996). The alleles *cDhc64C⁶⁻¹⁰* and *cDhc64C⁶⁻¹⁶*, when combined with a deletion of *cDhc64C*

[*Df(3L)10H*], caused posterior-paralysis phenotypes similar to those caused by conventional kinesin mutations (Figure 1). The penetrance, or percentage of animals that showed the paralytic phenotype, was 100%. Control larvae with the same genotype but carrying a wild-type *cDhc64C* transgene showed no posterior paralysis, indicating that the effect was specific to the loss of cytoplasmic dynein function.

To ascertain if the behavioral phenotypes reflected cellular axonal transport defects, mutant and control larval neuromuscular preparations were examined by immunofluorescence microscopy. The distribution of microtubules in segmental nerves was not altered by the dynein mutations (Figure 1). However, the distribution of the endosome protein HOOK (Kramer and Phistery, 1996, 1999), which we presume to be a fast retrograde cargo, and of cDHC itself

was abnormal. Large accumulations, similar in appearance to the axonal swellings caused by conventional kinesin mutations, were scattered along the lengths of segmental nerves. The fast anterograde vesicle proteins synaptotagmin and cysteine string protein, and the fast anterograde motor KHC, were also present in the accumulations (Figure 1). These results suggest that cytoplasmic dynein mutations, like conventional kinesin mutations, cause organelle jams, i.e., randomly distributed axon swellings filled with organelles carried by both fast anterograde and retrograde axonal transport.

To determine if the abnormal protein accumulations in segmental nerves were indeed organelle jams and, if so, how they compare with those caused by *Khc* mutations, we examined *cDhc64C* mutant nerves by transmission electron microscopy. Nerve cross-sections revealed axon swellings filled with retrograde transport organelles, including lysosomal and multivesicular bodies. They also contained mitochondria, many small vesicles, and smooth tubular membranes (Figure 2). The swellings and their contents were not distinguishable from those caused by *Khc* mutations (Hurd and Saxton, 1996). Thus, cytoplasmic dynein, like conventional kinesin, is required for both retrograde and anterograde fast axonal transport.

To address the possibility that the dynactin complex is also required for fast axonal transport, we disrupted the function of p150^{Glued} with the use of mutations in *Gl*. Available *Gl* alleles, when homozygous, cause lethality too early for assessment of axonal transport phenotypes. To circumvent this effect, we used *Phs-Gl^I*, a dominant negative *Gl* transgene controlled by a heat-shock promoter that expresses a truncated p150^{Glued} protein (Fan and Ready, 1997). C-terminal-truncated forms of p150^{Glued} act in a dominant negative manner, probably by disrupting the association between the dynein motor and the dynactin complex (McGrail *et al.*, 1995). Animals bearing *Phs-Gl^I* were exposed to a heat shock once per day during the embryonic and larval stages. They developed mild posterior paralysis by the late third instar (penetrance ~ 50%) and axonal swellings that contained retrograde and anterograde proteins (penetrance = 100%) (Figure 3). *Phs-Gl^I* animals without heat-shock treatments and wild-type animals subjected to heat shocks did not exhibit defects (Figure 3). Thus, disruption of an anterograde motor (kinesin), a retrograde motor (cytoplasmic dynein), or a retrograde activator complex (dynactin) inhibited fast axonal transport in both directions. The bidirectional effects may all be due to steric hindrance effects, as proposed in the "organelle jam" hypothesis (Hurd and Saxton, 1996). Alternatively or additionally, the bidirectional disruptions may be due to important functional interactions between these three components of the fast axonal transport machinery.

Immunoprecipitation of Either Cytoplasmic Dynein or the Dynactin Complex Does Not Coprecipitate Conventional Kinesin

To test for physical interactions of kinesin with either dynein or the dynactin complex, *Drosophila* cytosol from heads or ovaries was fractionated with either cDHC or p150^{Glued} antibodies conjugated to beads. The conditions used were conducive to coimmunoprecipitation of cytoplasmic dynein

intermediate chain with p150^{Glued} (data not shown), as has been described for vertebrate homologues (Vaughan and Vallee, 1995). Western blots of the soluble and immunoprecipitated fractions were probed with anti-KHC antibodies. No coprecipitation of kinesin was detected with either cDHC (Figure 4) or p150^{Glued} (data not shown). Thus, if there are physical interactions between kinesin and dynein or p150^{Glued}, they may be labile or restricted to a small fraction of motors, e.g., those bound to subsets of cargo organelles.

Kinesin, p150^{Glued}, and Cytoplasmic Dynein Mutations Exhibit Genetic Interactions

To test for functional interactions between kinesin and dynein/dynactin in axonal transport, we looked for non-complementation between mutations in *Khc*, *Klc*, *cDhc64C*, and *Gl*. This sort of dominant genetic enhancer test can reveal functional interactions that are not detected by biochemical fractionation (e.g., Huffaker *et al.*, 1987; Kaiser and Schekman, 1990). As one positive control, we tested for noncomplementation between null mutations in *Khc* and *Klc*, whose proteins are known to physically interact. Larvae that were heterozygous for either single mutation showed no posterior paralysis and only a few axonal swellings per nerve. In contrast, larvae that were doubly heterozygous (*Khc^{null}/+*; *Klc^{null}/+*) developed the posterior-paralytic phenotype (~50–70% penetrance) and had more axonal swellings (Figures 5–7). This dominant interaction of *Khc* and *Klc* was gene specific in that multiple null alleles from different genetic backgrounds interacted and single transgenic copies of either *Khc⁺* or *Klc⁺* suppressed the interaction (Figure 6). In addition, we found that deletions that remove the *Klp64D* and *Klp68D* genes fully complemented *Khc*. *Klp64D* and *Klp68D* are expressed in the larval nervous system and encode homologues of the mammalian and sea urchin motor subunits that form the heterotrimeric kinesin II motor (Stewart *et al.*, 1991; Pesavento *et al.*, 1994). Thus, at least one neuronal motor does not show genetic interactions with kinesin.

A functional interaction between the kinesin and dynein pathways in axonal transport was first revealed through genetic interactions with *Gl*. *Gl^I/+* larvae exhibited no paralytic phenotypes and very few axonal swellings (Figure 6). However, larvae that were *Khc^{null}/+*; *Gl^I/+* exhibited dramatic posterior paralysis (penetrance ~ 50–70%) (Figure 5) and had abundant axonal swellings (Figure 6). A deletion of the *Gl* locus [*Df(3L)fz-GF3b*] also failed to complement *Khc^{null}/+*. Not surprisingly, the interaction phenotypes were milder and less penetrant (~30%) than those caused by the *Gl^I* allele, which is antimorphic. We also documented similar specific genetic interactions between mutations in *Gl* and *Klc* (Figure 7). Additionally, a deletion that removes a potential homologue of the dynactin complex ARP1 gene (Fyrberg *et al.*, 1994) failed to complement severe *Khc* mutations. Thus, the functions of conventional kinesin and the dynactin complex are interdependent in fast axonal transport.

A second line of evidence for the interdependence of dynein and kinesin was obtained from a genetic screen for new dominant enhancers of *Khc*. The first mutation isolated was an allele of *cDhc64C* that we have named *cDhc64C^{ek1}*. Larvae that were heterozygous for *cDhc64C^{ek1}* showed no posterior paralysis or axonal swellings. However, the pos-

Figure 2. Mutations in *cDhc64C* cause axonal swellings filled with fast retrograde and anterograde organelles. Transmission electron micrographs of heterozygous control, *Df(3L)10H/+* (A and C), and mutant, *cDhc64C⁶⁻¹⁰/Df(3L)10H* (B and D), larval segmental nerve cross-sections. (A) One-fourth of a large nerve containing a central cluster of axons (arrows) supported by glial cells (g) and a layer of extracellular matrix (e) is shown. A peripheral glial cell nucleus is also in view (gn). (C) A view of the same section at higher magnification; the predominant axonal organelles were mitochondria and small vesicles. The two axons from A are indicated by triplets of arrows. (B) The overall organization of the mutant nerve and its axons was normal (same magnification as in A). However, some axons were swollen (arrowheads). (D) This higher-magnification view of the largest swollen axon from B (same magnification as in C) contained many prelysosomal bodies (p), multivesicular bodies (b), mitochondria (m), and vesicles. Normal-sized axons are indicated by arrows. Bars, 500 nm.

Figure 3. Dominant negative mutation of *Glued* causes defects in fast axonal transport. Laser scanning confocal micrographs of larval segmental nerves from animals homozygous for *Phs-Gl^l* stained for synaptotagmin are shown. (A) A control animal expressing wild-type p150^{Glued} exhibited normal synaptotagmin staining. (B) A test animal expressing a dominant negative form of p150^{Glued} exhibited accumulations of synaptotagmin that denote axonal swellings that contain fast anterograde cargoes. n, segmental nerve; t, tracheal tube; m, muscle. Bar, 10 μ m.

terior-paralysis and axonal-swelling phenotypes of *Khc^{null}/+*; *cDhc64C^{ek1}/+* larvae were as severe as those generated by the *Khc-Klc* and *Khc-Gl^l* interactions described above. The *Khc-cDhc64C^{ek1}* interaction was specific; a *cDhc64C⁺* transgene rescued the posterior-paralysis and axonal-swelling phenotypes. *Klc^{null}* and *cDhc64C^{ek1}* also failed to complement, and the phenotypes generated were very similar to those caused by the double heterozygous *Khc-cDhc64C^{ek1}* combination.

To determine if the cDHC-kinesin interactions were specific to the *cDhc64C^{ek1}* allele, we tested for genetic interactions between *Khc* or *Klc* and other *cDhc64C* mutations. Both a small deletion of the *cDhc64C* region, *Df(3L)10H*, and an amorphic (null-like) allele, *cDhc64C⁴⁻¹⁹*, failed to complement *Khc^{null}* and *Klc^{null}* alleles (Figure 7). Thus, the dominant genetic interactions between kinesin and cDHC are a general property of severe *cDhc64C* mutations. The interaction phenotypes caused by *Df(3L)10H* or *cDhc64C⁴⁻¹⁹* were milder than those caused by *cDhc64C^{ek1}* (Figures 6 and 7). This was probably due to the fact that *cDhc64C^{ek1}* conveyed some genetic properties that were more severe than a null mutation (see MATERIALS AND METHODS). These results confirm that conventional kinesin and cytoplasmic dynein are functionally interdependent in axonal transport.

To complete the investigation of dynein function in axonal transport, we asked if mutations in *cDhc64C* and *Gl* failed to complement one another. Strong dominant genetic interactions between mutations in these genes have been shown previously in *Drosophila* eye development (McGrail *et al.*, 1995), in which dynein and the dynactin complex are

Figure 4. Immunoprecipitation of cDHC does not coprecipitate KHC. *Drosophila* cytosol fractions were separated by gel electrophoresis and transferred to a membrane. The membrane was stained first for cDHC (A) and subsequently for KHC (B). Thus, the cDHC bands are present in both panels. The experiment shown here was conducted with ovary cytosol from DHC-3HA-expressing flies immunoprecipitated with anti-HA mAb-agarose bead complexes. However, comparable results were obtained with DHC-3HA head extracts and either ovary or head wild-type extracts with the use of DHC mAbs for the immunoprecipitation. (Lanes 1) Proteins that pelleted from cytosol with taxol-stabilized microtubules in the absence of ATP. cDHC but not KHC was efficiently bound to microtubules under these conditions. (Lanes 2) KHC but not DHC was readily detected in cytosol. (Lanes 3) The pellet from cytosol that was incubated with unconjugated agarose beads. (Lanes 4 and 5) The pellets from cytosol that was incubated with beads conjugated with antibodies to cDHC via the 3HA tag; incubation was at either 4°C (lanes 4) or 25°C (lanes 5). (Lanes 6 and 7) The supernatants of the pellets shown in lanes 4 and 5, respectively. KHC remained in the immunoprecipitation supernatants, and cDHC was detected in the pellets. IgG, immunoglobulin G.

thought to participate in mitosis and nuclear positioning (McGrail *et al.*, 1995; Gepner *et al.*, 1996; Fan and Ready, 1997). In our tests of axonal organelle transport, some *Gl* and *cDhc64C* mutations did fail to complement, causing increases in axonal swellings. However, the interactions were mild relative to those generated by the kinesin-cytoplasmic dynein or the kinesin-dynactin interactions (Figures 6 and 7). Even when the most severe alleles were combined (*Gl^l/+* + *cDhc64C^{ek1}*), no posterior paralysis was observed. It is striking that the cytoplasmic dynein and dynactin genes, whose products associate and are each individually critical for axonal transport, interact more strongly with conventional kinesin genes than with one another.

DISCUSSION

To determine if dynein and the dynactin complex have important functions in fast axonal transport and to investigate possible interactions between anterograde and retrograde transport systems in vivo, we conducted genetic and

Figure 5. Mutations in *Khc* interact with mutations in *Klc*, *cDhc64C*, and *Gl* to produce the posterior-paralysis phenotype. Single video frames of wandering third instar larvae crawling forward (toward the left) are shown. All of the animals were heterozygous for a *Khc* null allele (*Khc*^{16/+}). The animals in B, C, and D were also heterozygous for severe mutations in *Klc* [*Df(3L)34ex5/+*], *cDhc64C* (*cDhc64C*^{ek1/+}), and *Gl* (*Gl*^{1/+}), respectively. The larva in A showed normal locomotion, whereas the double heterozygous larvae all showed a synthetic posterior-paralysis phenotype, as indicated by the upturned tails.

biochemical tests in *Drosophila*. The neuronal phenotypes of mutations in *cDhc64C* and *Gl* indicate that dynein and the dynactin complex do indeed have important functions in axonal transport. The organelle-filled axonal-swelling and posterior-paralysis phenotypes observed are similar to those caused by conventional kinesin mutations. Both retrograde and anterograde fast transport cargoes accumulate in the swellings, despite the fact that dynein is a retrograde motor. The same bidirectional axonal transport phenotypes are generated by pair-wise heterozygous combinations of mutations in *Khc*, *Klc*, *cDhc64C*, and *Gl*. These dominant genetic interactions and the shared single-mutant phenotypes indicate that the kinesin and dynein motor systems are interdependent during fast axonal transport.

It is curious that disruption of either the kinesin or the dynein motor system inhibits both anterograde and retrograde fast axonal transport. We consider three nonexclusive models to explain the bidirectional effects. The first model is based on evidence that known microtubule motors are unidirectional and that >90% of microtubules in axons have uniform polarity, i.e., plus ends toward the terminal (Burton and Paige, 1981; Heidemann *et al.*, 1981; Baas *et al.*, 1988; Topp *et al.*, 1994). Therefore, most minus end-directed motors such as dynein, which are synthesized in the cell body, are probably carried into the axon as fast anterograde cargoes before they are used to drive retrograde transport. If kinesin transports dynein down the axon, then kinesin mutations should reduce the concentration of dynein in the axon. Thus, retrograde as well as anterograde transport would suffer. The weakness in this model is that it does not explain why *cDhc64C* and *Gl* mutations disrupt anterograde transport. Because kinesin is presumably degraded at the terminal (Hirokawa *et al.*, 1991), kinesin levels should not be affected directly by changes in retrograde transport. In a second model proposed by Hurd and Saxton (1996), the stalling of an organelle as a result of faulty kinesin activity hinders the passage of other anterograde and retrograde motor-cargo complexes, encouraging them to detach from

their microtubule tracks at that same point. Detached cargoes then accumulate and generate axonal swellings. The bidirectional transport inhibition caused by cytoplasmic dynein or dynactin complex mutations could also be attributed to this sort of steric hindrance.

In a third model, the activities of conventional kinesin, cytoplasmic dynein, and the dynactin complex are linked through physical contacts. Thus, in neurons without dynein, kinesin does not function normally and vice versa. This model is interesting because such a linkage could ensure proper coordination of opposing motor activities and provide an even distribution of motors along the axon. To date, there is no biochemical evidence for a direct dynein-kinesin interaction. However, this does not eliminate the possibility of such interactions. For example, although it is generally accepted that the dynactin complex and cytoplasmic dynein have important physical contacts *in vivo*, strong evidence of such contacts remained elusive for a number of years. Now, a demonstration of binding of p150^{Glued} to a dynein intermediate chain, combined with shared phenotypes and genetic interactions between dynactin complex and dynein mutations, provide a compelling argument for substoichiometric but important direct interactions (Muhua *et al.*, 1994; Plamann *et al.*, 1994; Karki and Holzbaur, 1995; McGrail *et al.*, 1995; Vaughan and Vallee, 1995; Bruno *et al.*, 1996; Tinsley *et al.*, 1996). There have been several observations that are consistent with an association between dynein, the dynactin complex, and kinesin in fast axonal transport. Kinesin and dynein localize to fast anterograde organelles (Hirokawa *et al.*, 1990, 1991). In extruded axoplasm and intact axons, individual organelles can move in both directions on microtubules (e.g., Allen *et al.*, 1985; Schnapp *et al.*, 1985; Overly *et al.*, 1996). Some purified plus end-directed vesicles bind both plus end- and minus end-directed motors (Muresan *et al.*, 1996). Inhibition of kinesin in extruded squid axoplasm with specific mAbs impairs both anterograde and retrograde vesicle motility (Brady *et al.*, 1990; Stenoien and Brady, 1997). Likewise, p150^{Glued} antibodies that disrupt the interaction of dynein and the dynactin complex inhibit vesicle motility in both directions (Waterman-Storer *et al.*, 1997). Organelle motility on peripheral single microtubules and interior groups of microtubules was affected similarly in those antibody disruption studies. Thus, it seems unlikely that simple steric hindrance between anterograde and retrograde organelle movement is the explanation for bidirectional inhibition (Brady *et al.*, 1990; Stenoien and Brady, 1997; Waterman-Storer *et al.*, 1997). Our finding that single and double heterozygous *cDhc64C* and *Gl* mutations exhibit less severe phenotypes than single or double heterozygous combinations that include kinesin mutations might be explained by a greater flux of anterograde cargoes versus retrograde cargoes. However, in light of the previous studies discussed above, our current genetic observations support the idea of a physical linkage between anterograde and retrograde fast transport motors. This linkage may be indirect, such as both motors binding the same cargo but at distinct sites. Or the linkage could be direct in the form of physical or regulatory contacts between kinesin, dynein, and the dynactin complex at the same or closely placed cargo-binding sites.

To determine the validity and relative influences of the three models for anterograde-retrograde linkage discussed

Figure 6. Mutations in *Khc* interact with mutations in *Klc*, *cDhc64C*, and *Gl* to produce axonal swellings. Laser scanning confocal micrographs of segmental nerves from third instar larvae stained for synaptotagmin are shown. The genotypes are as follows: (A) wild type; (B) *Khc⁶/+* (hypomorphic allele); (C) *Khc¹⁶/+* (null allele); (D) *Df(3L)34ex5/+* (*Klc* deletion); (E) *Khc¹⁶/+*; *Df(3L)34ex5/+*; (F) *Khc¹⁶/+*; *Df(3L)34ex5, +/+*, *P{Khc⁺}*; (G) *cDhc64C^{ek1}/+*; (H) *Khc¹⁶/+*; *cDhc64C^{ek1}/+*; (I) *Khc¹⁶/+*; *cDhc64C^{ek1}, +/+*, *P{Khc⁺}*; (J) *Gl¹/+*; (K) *Khc¹⁶/+*; *Gl¹/+*; (L) *Khc¹⁶/+*; *Gl¹, +/+*, *P{Khc⁺}*; (M) *Df(3L)10H/+* (*cDhc64C* deletion); (N) *Df(3L)10H, +/+*, *Gl¹*; and (O) *cDhc64C^{ek1}, +/+*, *Gl¹*. (A, B, G, J, and M) Axonal swellings were rarely seen in wild type, heterozygous hypomorphic *Khc* mutations, or animals heterozygous for severe *cDhc64C* or *Gl* alleles. (C and D) Heterozygotes for severe *Khc* and *Klc* mutations did have axon swellings in some regions but showed no swellings in other regions. (E, H, and K) Combination of *Klc*, *cDhc64C*, or *Gl* mutations with *Khc* in double heterozygotes caused dramatic increases in the number of axon swellings relative to the respective single heterozygous animals (compare C and D with E; compare C and G with H; compare J and K with M). In contrast, combination of the most severe alleles of *cDhc64C* and *Gl* led to only a slight increase in axon swellings (compare J and M with N and then compare G and M with O). (F, I, and L) The axon swellings caused by double heterozygous combinations with *Khc* null mutations were rescued by a single transgenic copy of wild-type *Khc*. Bar, 10 μ m. b, boutons of an underlying neuromuscular junction; n, segmental nerve; t, tracheal tube. All micrographs are at the same magnification.

	+	Khc ⁻	Klc ⁻	Gl ¹
Khc ⁻	J	JJJJ	JJJ	JJJ
Klc ⁻	J		JJJJ	JJJ
Gl ¹	+			
Dhc ⁻	+	a JJ	a JJ	+
Dhc ^{ek1}	+	JJJ	JJJ	J

Figure 7. Summary of the dominant genetic interactions observed between *Khc*, *Klc*, *cDhc64C*, and *Gl*. The gene symbols at the top and left represent heterozygous mutant contributions. The internal rectangles contain information on the double heterozygous combinations indicated by the respective row and column. Khc⁻, Klc⁻, and Dhc⁻ represent multiple severe alleles and deletions tested for each of these genes. In each box, the upper left-hand symbol indicates the presence or absence of the posterior-paralytic phenotype in third instar larvae. The lower right-hand symbol indicates the abundance of axon swellings typically found with each genotype (with the use of anti-synaptotagmin): +, just a few axon swellings per animal (overlaps wild type, i.e., most nerves have no swellings); J, multiple nerves have a few axon swellings; JJ, all nerves have axon swellings but some parts of nerves are free from swellings; JJJ, all nerves have axon swellings, and there are a few small areas of wild-type staining; JJJJ, large swellings predominate the entire width and length of nerves. These designations are qualitative and represent the typical phenotype from each genotype, although variation generated individuals with more or fewer swellings. a, the tail-flipping phenotype was expressed at low penetrance (10–20% compared with ≥50% for the other genotypes).

above, more experimentation will be needed. Direct measurements of organelle motility in wild-type and mutant *Drosophila* provide an excellent approach to such questions and the more general issues of motor regulation and coordination. Analysis of single-lipid-droplet motility in wild-type and mutant embryos suggests that both dynein and a plus end-directed kinesin are active and that the dynactin complex is important for coordinating their activities (Welte *et al.*, 1998; Gross, Welte, Block, and Wieschaus, personal communication). We are developing tests of single-organelle motility in wild-type and mutant *Drosophila* axons that should prove very informative. Continued biochemical and genetic investigations of motors and their associations should identify new regulatory and cargo-linkage proteins. The integration of results obtained from these differing approaches will lead to a definitive understanding of the rela-

tionship between kinesin, dynein, and the dynactin complex in fast axonal transport.

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