Dynactin Is Required for Coordinated Bidirectional Motility, but Not for Dynein Membrane Attachment

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Submitted August 11, 2006; Revised February 9, 2007; Accepted March 2, 2007
Monitoring Editor: Yixian Zheng

Transport of cellular and neuronal vesicles, organelles, and other particles along microtubules requires the molecular motor protein dynein (Mallik and Gross, 2004). Critical to dynein function is dynactin, a multiprotein complex commonly thought to be required for dynein attachment to membrane compartments (Karki and Holzbaur, 1999). Recent work also has found that mutations in dynactin can cause the human motor neuron disease amyotrophic lateral sclerosis (Puls et al., 2003). Thus, it is essential to understand the in vivo function of dynactin. To test directly and rigorously the hypothesis that dynactin is required to attach dynein to membranes, we used both a Drosophila mutant and RNA interference to generate organisms and cells lacking the critical dynactin subunit, actin-related protein 1. Contrary to expectation, we found that apparently normal amounts of dynein associate with membrane compartments in the absence of a fully assembled dynactin complex. In addition, anterograde and retrograde organelle movement in dynactin deficient axons was completely disrupted, resulting in substantial changes in vesicle kinematic properties. Although effects on retrograde transport are predicted by the proposed function of dynactin as a regulator of dynein processivity, the additional effects we observed on anterograde transport also suggest potential roles for dynactin in mediating kinesin-driven transport and in coordinating the activity of opposing motors (King and Schroer, 2000).

INTRODUCTION

Dynein is the major molecular motor protein responsible for a variety of microtubule-based minus-end–directed movements of vesicles and organelles as well as several steps in mitosis (Karki and Holzbaur, 1999; Mallik and Gross, 2004; Pilling et al., 2006). A key gap in our understanding of dynein function is how this motor protein interacts with membrane compartments. One candidate factor proposed to link dynein to membrane compartments is a multiprotein complex called dynactin (for review, see Schroer, 2004). Although the original work on dynactin suggested that highly purified dynein could mediate vesicle attachment to microtubules in the absence of dynactin, a more recent, and relatively small number of in vitro experiments have led to the generally accepted model that the attachment of dynein to membrane vesicles requires dynactin (Waterman-Storer et al., 1997; Karki and Holzbaur, 1999; Muresan et al., 2001). An alternative model suggests that dynein light and intermediate chain (IC) subunits may link dynein to other membrane-associated proteins independently of dynactin (Tai et al., 1999; Tynan et al., 2000; Yano et al., 2001). In this competing view, dynactin plays a role in regulating or coordinating dynein functions such as processivity (King and Schroer, 2000). Understanding the role of dynactin in dynein function has recently become more important with the realization that these proteins may be targets in human neurodegenerative diseases (Hafezparast et al., 2003; Puls et al., 2003). Yet, in spite of the importance of this issue, definitive evidence on the in vivo role of dynactin in dynein attachment to membranes does not exist, and no direct experiment testing whether dynactin is required for linking dynein to membranes in vivo has been reported.

The actin-related protein Arp1 is the most abundant subunit of the dynactin multiprotein complex. This 45-kDa protein forms a filament composed of eight to 13 monomers, which is capped by the p37 and p32 capping proteins on one end (also known as CapZ) and the p62 subunit of dynactin on the other end. These proteins in addition to some smaller subunits, such as p25, p27, and Arp11 form a scaffold upon which p150Glued binds to the Arp1 filament through an interaction mediated by another protein called dynamitin (for review, see Schroer, 2004). Dynine interacts with the dynactin complex via binding of the dynein IC to the...
S2 Cell Culture

S2 cells were grown and maintained in Schneider’s Drosophila medium (Invitrogen, Carlsbad, CA)/10% fetal bovine serum at room temperature. dsRNA was generated using the Megascript RNA interference (RNAi) kit (Ambion, Austin, TX) from a 500-base pair polymerase chain reaction (PCR) product that is a substrate for the RNase II enzyme. The RNA was treated with RNase-free DNaseI (Ambion, Austin, TX) from a 500-base pair polymerase chain reaction (PCR) product that is a substrate for the RNase II enzyme. The RNA was treated with RNase-free DNaseI (Ambion, Austin, TX). The RNA was then treated with RNase-free DNaseI (Ambion, Austin, TX) and incubated at 37°C for 15 min to degrade any remaining DNA. The final concentration of dsRNA was 5 μg/ml.

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Materials and Methods

Identification and Mapping of arp1 Mutants

Homozygous lethal ethyl methanesulfonate (EMS) mutant lines were obtained from the laboratory of Dr. Charles Zuker (University of California, San Diego, La Jolla, CA). The mutant third instars were examined for sluggish crawling and tail flip phenotypes. The arp1 mutants (previously called gridlock) were mapped to cytological position 87C on the third chromosome of Drosophila. A restriction fragment containing the arp1 gene was sequenced from homozygous third instars.

Immunostaining

Larval segmental nerve immunostaining was performed as described previously (Hurd and Saxton, 1996) and observed using a Bio-Rad MRC1024 confocal microscope (Gundhär et al., 1998).

Antibodies

Antidynein heavy chain antibody (P1H4) (a generous gift from Tom Hays, Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55104), antidynein intermediate chain (Chemicon International, Temecula, CA), and anti-diabolin (Calbiochem, San Diego, CA) were used as described.

Arp1 Fusion Protein and Antibody Preparation

Seven Arp1 expressed sequence tag clones were found in the Berkeley Drosophila Genome Project database. Only one contained a complete Arp1 cDNA (clone GH177B). This clone was purchased from Research Genetics (Huntsville, AL), fully sequenced, and then subcloned into the pET-23b vector (Novagen, Madison, WI), which includes a 6x His tag at the C terminus. The expressed fusion protein was purified using nickel-nitrilotriacetic acid agarose resin (Qiagen, Valencia, CA), electrophoretically isolated, and used to raise antiserum in rabbits (Lampire Biological Laboratories, Pipersville, PA). The specificity of the Drosophila Arp1 antibody was confirmed by Western blotting: a 1:10,000 dilution of the serum detected 5 ng of the recombinant protein and also detected Arp1 in 10 μg of total protein from larval brain extracts. We concluded that the Arp1 antibody cross-reacts with actin, based on the observation that reduction of Arp1 reactivity after Arp1 double-stranded RNA (dsRNA) treatment is not obvious in the high-speed supernatant where actin is abundant, whereas reduction of Arp1 in the high-speed pellet fraction where no actin can be detected is obvious (Figure 2A). In addition, sucrose density centrifugation analyses of high-speed supernatants showed a peak at ~8S with sedimentation behavior that is identical to actin in both green fluorescent protein (GFP) dsRNA and arp1 dsRNA-treated S2 cells (Figure 2, C and D). This peak is not observed in the high-speed pellet fraction where no actin can be detected (Figure 2, E and F).

S2 Cell Culture

S2 cells were grown and maintained in Schneider’s Drosophila medium (Invitrogen, Carlsbad, CA)/10% fetal bovine serum at room temperature. dsRNA was generated using the Megascript RNA interference (RNAi) kit (Ambion, Austin, TX) from a 500-base pair polymerase chain reaction (PCR) product that is a substrate for the RNase II enzyme. The RNA was treated with RNase-free DNaseI (Ambion, Austin, TX) and incubated at 37°C for 15 min to degrade any remaining DNA. The final concentration of dsRNA was 5 μg/ml. After 30 min, 3 ml of complete medium was added to each well. Cells were harvested for analysis after 5 d. For reverse transcriptase-quantitative PCR (RT-qPCR), total RNA from S2 cells was prepared using the RNeasy kit (Qiagen). The RNA was subsequently treated with DNaseI by using the DNA-free kit (Ambion). First-strand cDNA was generated using SuperScript III (Invitrogen) and primers for RT-PCR and quantitative PCR was performed in an Mx3000P qPCR tool (Stratagene, La Jolla, CA) by using Brilliant SYBR Green qPCR Master Mix. The percentage reduction of arp1 mRNA was determined by normalizing to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Primers used for arp1 were forward, tccgaactgaagaaacactcg and reverse, ctgtccctcctcctcgtg. For GAPDH were forward, aatagggcgaagtctggca and reverse, aagcgactcgtcctcag.

Sucrose Density Gradient

For sucrose density gradient analysis of Drosophila larval brains, 250 arp1 larval brains and 80 wild-type brains were dissected and homogenized in PMEG buffer (0.1 M piperazine-N,N’-bis(2-ethanesulfonic acid), pH 6.9, 5 mM EGTA, 0.9% glycerol, 5 mM MgSO4, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), and protease inhibitors) (Hay et al., 1994). The high-speed supernatant was prepared by centrifugation at 50,000 g for 40 min in a TLA105 rotor (Beckman Coulter, Fullerton, CA), and then overlaid on a continuous 25–90% sucrose gradient and centrifuged at 35,000 rpm for 16 h in a SW41 rotor (Beckman Coulter). For sucrose density gradient analysis of S2 cells, arp1 dsRNA-treated cells and GFP dsRNA-treated cells were homogenized in buffer A (50 mM Tris, 150 mM NaCl, pH 7.4, and 0.5 mM EDTA) plus protease inhibitors, centrifuged sequentially 10 min at 10,000 g, 10,000 g, and 50 min at 100,000 g. The high-speed supernatant (HSS) was collected and the high-speed pellet (HSP) was resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH 7.4, and 0.5 mM EDTA). HSS and HSP were overlaid on a continuous 5–20% sucrose gradient prepared in buffer A and centrifuged at 35,000 rpm for 16 h in a SW41 rotor. Then, 0.5 ml fractions were collected, and total proteins were precipitated and analyzed by gel electrophoresis (PAGE) and Western blot. Gradients loaded with the markers carbonic anhydrate, bovine serum albumin, alcohol dehydrogenase, β-amylose, catalase, apoferritin, and thyroglobulin were run in parallel as standards to determine 2.8S, 4.2S, 7.5S, 9S, 11S, 17S and 19S, respectively.

Membrane Flotation Assay

Three instars were individually hand dissected, and ~250 brains were collected into dissection buffer (2X stock contains 128 mM NaCl, 4 mM CaCl2, 4 mM MgCl2, 2 mM KCl, 5 mM HEPES, and 36 mM sucrose, pH 7.2). The brains were then homogenized in acetone (10 vol of 10 mM HEPES K-acetate, 150 mM sucrose, 5 mM EGTA, 3 mM Mg-acetate, 1 mM DTT, and protease inhibitors). Debris was removed by centrifugation at 1000 g for 7 min, and the resulting postnuclear supernatant (PNS) was brought to 40% sucrose, bottom loaded, and overlaid with two cushions of 35% and 85% sucrose. The gradient was centrifuged at 50,000 rpm in a TLS55 rotor (Beckman Coulter, Fullerton, CA) for 1 h. Light membranous organelles and membrane-associated proteins floated to the 35/8 interface, whereas heavier membranes and mitochondria are found in the pellet. Equal amounts of protein from each fraction were analyzed by SDS-PAGE and Western blotting. For flotation analysis of S2 cells, arp1 dsRNA-treated cells and GFP dsRNA-treated cells were homogenized in homogenization buffer (HB, 8% sucrose and 3 mM imidazole, pH 7.4). A PNS was prepared and membrane fractionation was performed as described above. Fractions were collected, and equal amounts of proteins were analyzed by SDS-PAGE and Western blotting.

Real-Time Movies and Quantification

Amylid precursor protein (APP) was C-terminally tagged with yellow fluorescent protein (YFP) and expressed under GAL4/UAS control using the P(Gal4)SG26.1 line (Kaether et al., 2010; Gunawardena et al., 2003). Crosses were made and female larvae with the genotype UAS-APP-YFP+/; P(Gal4)SG26.1/+; arp1/arp1 were filled in a calcium free dissection buffer. Larvae were positioned with the ventral ganglion on the left and the segmental nerves projecting to the right. Movies were recorded using an Eclipse TE-2000-U inverted microscope (Nikon, Tokyo, Japan) at 100× magnification at 10 frames per second and a CoolSNAP HQ cooled charge-coupled device camera driven by a MetaMorph imaging system version 5.0 (Molecular Devices, Sunnyvale, CA). We recorded 150 frames for each movie at 10 frames/s and 2× 2 binning. For mitochondria, the lead peptide plus two amino acids (23 amino acids in toto) of human cytochrome oxidase subunit 8 was fused to GFP and expressed under GAL4/UAS control using P(Gal4)BD2.1 line. Because many mitochondria are stationary, a zone of bleached fluorescence was generated, and mitochondrial movement into the bleached zone was assessed using an MRC600 confocal microscope to collect 300 frames at a rate of 1 frame/s. To use APP-YFP, kymographs were generated from movies using MetaMorph software and analyzed using MetaMorph and MATLAB 7.0 (MathWorks, Natick, MA). We determined particle populations, velocities, pause frequencies, and reversal frequencies based on the coordinates of each tracked particle. Net velocity was used as the net distance traveled divided by the time elapsed during an entire movie (15 s). Segment velocity was defined as the total distance traveled by a particle during a particular run, divided by the duration of the run. A run could be...
Figure 1. Axonal transport and the dynactin complex are disrupted in arp1 mutant larvae. (A and B) In contrast to wild-type larvae arp1 mutant animals exhibit the "tail-flip" phenotype common to axonal transport mutants in Drosophila. (C and D) Immunofluorescence with antibodies recognizing the synaptic cysteine string protein revealed abundant large accumulations within the segmental nerves of the arp1 mutant, whereas wild-type animals exhibited typical background staining. (E) Larval brain extracts from wild-type larvae and homozygous arp1 larvae were analyzed by Western blotting. The blots were probed with antibodies to different subunits of dynein and dynactin complexes. Tubulin antibody was used as a loading control. The p150Glued and p50 subunits are obviously reduced in the arp1 mutant larvae. (F) The dynactin complex is disrupted in the arp11 mutant. Third instar brains were homogenized, and a high-speed supernatant was sedimented on a 5–20% sucrose gradients. In wild type, sedimentation of the dynactin subunits peaks at ~17S, whereas in the arp11 mutant, both Arp1 and p50 sediment at ~45–8S. Asterisk (*) points to dynamitin in the Arp1 blot (the same blot has been reprobed). The peak of Arp1 reactivity detected at ~45 in addition to the expected peak at 17S is due to cross reactivity of our Arp1 antibody with actin (see Materials and Methods and Figure 2).

terminated by a pause, reversal, or the end of the movie, and a run was a minimum of 10 frames (1 s) in length. Although run lengths were used to calculate velocities and determine pause or reversal points, we do not report explicit run lengths (cf. Gross et al., 2002) because of significant inaccuracies resulting from runs already underway at the beginning of a movie, or observations that are prematurely terminated at the end of a movie. Instead, we assume that pauses reflect the dissociation of a motor from a microtubule after some 3. Thus, we presume that this mutant affects a regulatory region whose sequence we have not delineated. Because both mutant alleles had similar phenotypes, we focused on the arp1 allele. To confirm that the arp11 larval phenotype is due to an axonal transport defect, we stained larval segmental nerves with antibodies to synaptic proteins such as cysteine string protein. In wild-type larvae, this antibody stains axons uniformly (Figure 1C), whereas in arp11 mutant larvae, synaptic proteins accumulate in axons (Figure 1D). The presence of these accumulations, previously observed in other axonal transport mutants (Bowman et al., 2000), confirms that axonal transport is disrupted in the arp1 mutants.

To ask whether dynactin is required for all dynein functions in Drosophila, we evaluated whether the consequences of loss of dynein and dynactin differ by comparing the phenotypes caused by loss of Arp1 to loss of DHC. Because both dynein and dynactin are thought to play essential roles at multiple stages in the cell cycle, we evaluated cell cycle defects in larval brains by using 5-bromo-2'-deoxyuridine (BrdU) incorporation, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and by directly examining dividing neuroblasts stained with 4',6-diamidino-2-phenylindole. Observation and quantitation revealed that although the exact frequencies of defects in arp1

Online Supplemental Material

Online supplemental material describes detailed genetics experiments on arp1 mutant and addresses whether dynactin is required for all dynein functions in Drosophila. Movies have been provided of APP–YFP particle motion in wild-type (FigS3videoA.mov) and arp11 mutant (FigS3videoB.mov and FigS3videoC.mov) Drosophila segmental motor neuron axons. Kymographs in Figure 3, A–C, correspond to these videos. Additionally, movies of mitochondrial axon in wild-type (FigS4video1.mov) and arp11 mutant (FigS4video2.mov) axons have been shown. Time-lapse panels from these movies are shown in Supplemental Figure 3, D and E. Imaging parameters are as described in Materials and Methods, with frames collected every 100 ms for 15 s for APP-YFP and every second for 30 s for mitochondria.

RESULTS AND DISCUSSION

Two arp1 mutations were identified in a genetic screen for mutations that disrupt axonal transport in Drosophila mela-
and dhc mutants were not identical, they were similar in character (Table 1 and Supplemental Figures 1 and 2). Similarly, failures in cell viability and oocyte determination evaluated in mitotic clone experiments were similar in arp1 and dhc mutants (Table 1 and Supplemental Figures 1 and 2). These data suggest that dynine requires the dynactin complex for all cellular and developmental functions that we were able to test. We note that arp1 mutant animals survive later in development than do p150Glued mutants; this difference is likely due to different levels of maternal contribution of these two dynactin components.

To determine whether the arp1 mutation destabilizes other dynactin subunits, we probed Western blots of larval brain homogenates with antibodies to different subunits of dynine and dynactin (Figure 1E). Although the amounts of DHC and dynein IC were unchanged in the arp1 mutants compared with wild-type, the p150Glued protein was virtually absent in the arp1 mutant. The level of the dynamitin subunit (p50) was also significantly reduced. The observed reduction of the p150Glued and p50 subunits suggests that the dynactin complex is disrupted in the arp1 mutants. To test whether the p150Glued subunit disrupts the dynactin complex, we performed sucrose density gradient analyses (Figures 1F and 2, C–F). Previous work has shown that p50, p150Glued, and Arp1 all sediment in a major peak at 17S–8S, indicating that they exist as a complex (Paschal et al., 1993; Clark and Meyer, 1994; Eckley et al., 1999). We observed that in wild-type larval brains, the dynactin complex subunits p50 and Arp1 sediment in a major peak at ~185, indicating that they exist as a complex (Paschal et al., 1993; Clark and Meyer, 1994; Eckley et al., 1999). We took advantage of the Gal4/UAS system to induce expression of a vesicular or a mitochondrial protein fused to GFP and GFP, respectively. For vesicles, we studied axonal transport of the vesicular APP fused to GFP (Kaether et al., 2000; Gunawardena et al., 2003) by using the P(Gal4)SG26.1 driver line, which is only expressed in a few motor neurons in the segmental nerves of third instars, allowing us to observe movements clearly. For mitochondria, we used a fusion of the leader peptide of human cytochrome c oxidase subunit 8 to GFP to target mitochondria (Horiuchi et al., 2003) by using the P(GawB)D42 driver line, which is expressed in all motor neurons. If dynactin has a role in enhancing the processivity of dynein, we expected that in an arp1 mutant background, APP-containing vesicles and mitochondria would move toward the cell body with shorter runs and more frequent pauses. In control animals, kymograph analysis revealed many movements of fluorescent APP particles toward and away from the cell body (Figure 3A), suggesting that these vesicles are powered by both anterograde and retrograde motors. In the arp1 mutant times were required. In both the soluble and membrane fractions, arp1 dsRNA treatment caused p50 and p150Glued subunits to shift from ~17S to ~8S, whereas DHC and KHC remained unchanged, peaking at 17S and 8S, respectively, in both conditions (Figure 2, C–F). In the control, GFP dsRNA sample, the soluble dynactin subunits did not completely overlap in one single peak, whereas the membrane associated dynactin subunits displayed partial cofractionation such that ARPL primarily cofractionated with DHC, and p150Glued primarily cofractionated with p50. Why the two sets of proteins have slightly different sedimentation profiles is unclear and might be caused by the stringency of the experimental conditions used or by a previously unsuspected instability of the soluble dynactin pool in S2 cells. Importantly, however, loss of Arp1 causes a clear disruption of the dynactin complex, such that the fractions that do contain DHC do not overlap with fractions containing dynactin subunits. Thus, similar to dynamitin overexpression (Echeverri et al., 1996) (Eckley et al., 1999), our results show that Arp1 mutation or deletion leads to disruption of the dynactin complex, in addition to reduction in the levels of both p150Glued and p50. Our results also suggest that the membrane-associated dynactin complex is more stable than the soluble dynactin complex in S2 cells.

To test the hypothesis that dynactin is required to enhance the processivity of dynein, we investigated the effects of loss of Arp1 function on in vivo vesicle and organelle motility. We took advantage of the Gal4/UAS system to induce expression of a vesicular or a mitochondrial protein fused to YFP and GFP, respectively. For vesicles, we studied axonal transport of the vesicular APP fused to YFP (Kaether et al., 2000; Gunawardena et al., 2003) by using the P(Gal4)SG26.1 driver line, which is only expressed in a few motor neurons in the segmental nerves of third instars, allowing us to observe movements clearly. For mitochondria, we used a fusion of the leader peptide of human cytochrome c oxidase subunit 8 to GFP to target GFP to mitochondria (Horiuchi et al., 2005; Pilling et al., 2006) and used the P(GawB)D42 driver line, which is expressed in all motor neurons. If dynactin has a role in enhancing the processivity of dynein, we expected that in an arp1 mutant background, APP-containing vesicles and mitochondria would move toward the cell body with shorter runs and more frequent pauses. In control animals, kymograph analysis revealed many movements of fluorescent APP particles toward and away from the cell body (Figure 3A), suggesting that these vesicles are powered by both anterograde and retrograde motors. In the arp1 mutant

Table 1. Summary of phenotypic comparisons between arp1 and dhc mutants

<table>
<thead>
<tr>
<th>Process</th>
<th>Assays</th>
<th>arp1°/arp1°</th>
<th>dhc64C°-10/dhc64C°-10</th>
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<tr>
<td>Cell cycle</td>
<td>Mitotic index and phenotype</td>
<td>Reduced mitotic index</td>
<td>Altered mitotic distribution</td>
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<td>BrdU incorporation</td>
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<td>Somatic cell viability</td>
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<td>Complete lethality</td>
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Methods and data supporting this table are in Supplemental Material.
tant, however, we observed significant numbers of station-
ary particles and organelle accumulations (Figure 3, B and C,
and Supplemental Figure 3, B and C), as we had seen pre-
viously with immunofluorescence studies (Figure 1D). Sur-
prisingly, we saw very little movement of APP particles in
either direction in the axons, even in regions where no
organelle accumulations were present (Figure 3D). In almost
half of the axons studied (16 of 33 movies; see
Materials and Methods), axons showed no directed movements of APP
vesicles; moreover, we frequently observed that many par-
ticles, especially within accumulations, underwent apparent
Brownian motion indicative of motor detachment from mi-
crotubules.

To quantify in vivo motor function in the remaining axons
that did display limited particle motion (17 movies), we
evaluated four parameters: net velocity, segment velocity,
pause frequency, and reversal frequency. Net velocity was
defined as the net distance traveled by a particle divided by
the time elapsed during an entire movie. Segment velocity
was defined as the distance traveled by a particle during a
run (i.e., unbroken by a pause, reversal, or end of a movie)
divided by the time elapsed during that run. Run lengths
were not explicitly reported due to inaccuracies resulting
from runs already underway at the beginning of a movie, or
prematurely terminated at the end of a movie. Rather, given
the implicit correlation between shortened run lengths and
increased likelihood of pausing, we used pause frequency,
defined as the number of pauses occurring per micrometer
of particle movement, as an indirect measure of processivity
(see Materials and Methods). Reversals were indicated by a
sustained change in direction of motion for at least 1 s.

The moving particles in the arp1T mutant animals had a
much slower segment velocities than in wild type and trav-
ered shorter distances (i.e., displayed slower net velocities)
(Figure 3, E–G). Processivity was also affected in both direc-
tions, because there was an increased number of pauses per micrometer
of particle movement, as an indirect measure of processivity
(see Materials and Methods). Reversals were indicated by a
sustained change in direction of motion for at least 1 s.

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type were longer than 13 μm and 5% were 25 μm or greater. No retrograde runs in the *arp1* mutant animals were longer than 19 μm, whereas 11% of retrograde runs in wild-type were longer than 19 μm. These quantitative analyses suggest that the slower net velocities in both anterograde and retrograde particle pools are a result of both slower segment velocities as well as increased pause frequency. (Figure 3, B, C, D, and H). Our analysis of a limited number of particle reversals indicates a trend toward increased reversal from anterograde to retrograde movement in *arp1* mutant axons, suggesting an additional role of dynactin in coordinating the activity of opposing motors on the same particle (Figure 3I).

Anterograde and retrograde mitochondrial movement was also reduced in the *arp1* mutant compared with wild type (Supplemental Figure 3, D and E). This is in contrast with the recent observation that a dominant negative allele of p150Glued showed an increase in anterograde and retrograde movement of mitochondria (Pilling et al., 2006). Thus, although our biochemical analyses demonstrate that *arp1* mutations lead to disruption of the dynactin complex, heterozygous expression of a dominant-negative allele of p150Glued must retain sufficient function for mitochondrial transport (McGrail et al., 1995).

A trivial cause of the lack of particle movement within the axons of *arp1* mutant larvae could be that *arp1* mutant neurons or animals are sick or dying. Two observations argue, however, that the motility defects observed are specifically caused by loss of dynactin function on vesicles or organelles. First, in previous work we found that intentionally inducing neuronal cell death did not cause defects in vesicle transport (Gunawardena et al., 2003). Second, we observed that *arp1* mutant siblings that were not dissected for live imaging survived at least another 48 h. Thus, although we cannot fully exclude the possibility of transport defects secondary to global neuronal toxicity, it seems likely that the loss of a functional dynactin complex has a direct effect on organelle processivity and motility.

To test directly whether dynactin is required to link dynein to membranous organelles, we examined the amount of membrane-bound dynein in *arp1* mutant animals and in *arp1* dsRNA-treated S2 cells. We performed subcellular frac-
tion of larval brains or S2 cell extracts by membrane flotation on sucrose step gradients (Figure 4A). We observed no significant difference in the amount of dynein that was associated with the membranes enriched in the 35/8 sucrose interface, whereas mitochondria (cyt c) are enriched in the heavy membrane fraction. Importantly, as seen in A, levels of membrane-associated dynein are equivalent in both control and arp1 RNAi-treated cells. Note that actin is present in all fractions and is thus masking the Arp1 reduction detected with the Arp1 antibody in the arp1 dsRNA-treated cells (see Figure 2 and Materials and Methods).

Our results suggest that an intact dynactin complex is not required for dynein to bind to membranous compartments. This proposal is supported by previous work suggesting that dynein subunits can interact directly with different types of cargo proteins (Almenar-Queralt and Goldstein, 2001). Our finding that arp1 mutants exhibit numerous phenotypic defects that are similar to dynein mutants as well as obvious defects in minus-end–directed movements is consistent with the proposal that dynactin plays an important role in regulating the processivity, or other aspects, of dynein motility (King and Schroer, 2000). However, even though we see apparently unchanged amounts of dynein binding to membranes when dynactin is disrupted, it is formally possible that the mode of attachment of dynein to

Figure 4. Dynein association with membranes is not affected by disruption of the dynactin complex. (A) Schematic representation of the flotation experiment. (B) Subcellular fractionation of third instar membranes on sucrose step gradients indicates that dynein is present in both soluble fraction and membrane fractions (35/8 interface) and that the amount of membrane-associated dynein is not altered in the arp1v mutant larvae. Rab8 and syntaxin are used as loading controls and syntaxin is used as a membrane control. (C) Similar results were obtained when dynein association with membranes is analyzed in arp1 dsRNA-treated S2 cells. Golgi membranes (d120) are enriched in the 35/8 interface, whereas mitochondria (cyt c) are enriched in the heavy membrane fraction. Importantly, as seen in A, levels of membrane-associated dynein are equivalent in both control and arp1 RNAi-treated cells. Note that actin is present in all fractions and is thus masking the Arp1 reduction detected with the Arp1 antibody in the arp1 dsRNA-treated cells (see Figure 2 and Materials and Methods).
cargo influences the characteristics of motility. For example, altered dynactin complex behavior resulting from Arp1 reduction on moving vesicles and organelles might cause incorrect attachment of DHC to membranous organelles, without affecting processivity per se. Similarly, the increased number of stationary organelles observed after Arp1 reduction could be explained by the direct association of dynein with membranes in a nonphysiological manner. The observed decrease in retrograde velocity may also reflect abnormal attachment of the dynein complex to membranes. Alternatively, given that dynein is a cooperative motor, this decrease in velocity may also be a result of poor coordination among dynein complexes attached to a given cargo. We are unable to distinguish between these scenarios based on our data thus far.

Our observation that the arp1mutation also disrupts anterograde movement of APP-containing vesicles and mitochondria does not easily fit the proposal that the regulatory function of dynactin is restricted to the processivity of dynein. Interestingly, depletion of Arp1 may have reduced the levels of KHC on membranes (Figure 4C). Although the degree of reduction is small, it may suggest how dynactin functions in anterograde movement. Whether dynactin in fact plays a direct role in kinesin recruitment to membranes will require further investigation. Nonetheless, our data are consistent with an additional role for dynactin in regulating anterograde motors such as kinesin, or as recently proposed, a role in coordinating plus and minus-end–directed transport (Gross et al., 2000, 2002a,b). In particular, a dominant mutation in p150Gluad severely impaired “anterograde” motion of lipid droplets in Drosophila embryos. Similarly, disruption of dynactin by overexpressing dynamin inhibited movement of endosomes in both directions (Valetti et al., 1999). Finally, biochemical support for a role of dynactin in coordinating plus- and minus-end–directed movements comes from the finding that a subunit of kinesin II can interact with p150Gluad (Deacon et al., 2003) and that dynein interacts directly with kinesin light chain (Ligon et al., 2004). Thus, our data, combined with earlier work, suggests that the in vivo function of dynactin is to regulate and/or coordinate bidirectional motility, but that dynactin may not be required to link dynein to membranes.

ACKNOWLEDGMENTS

We thank Dr. Margaret deCuevas and Rachel Fasnacht for help with the ovarian experiments, Drs. Junyun Ji and Gerold Schubiger for information before publication, and Drs. Erika Holzbaur and Tom Hays for generous gifts of antibodies. This work was funded by a National Institutes of Health grant (to L.S.B.G.), a grant from the American Heart Association Midwest Affiliate (to A.P.), and National Institutes of Health grant GM-46295 to William Saxton. L.S.B.G. is an Investigator of the Howard Hughes Medical Institute.

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