

# Dynactin–membrane interaction is regulated by the C-terminal domains of p150<sup>Glued</sup>

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Dynactin has been proposed to link the microtubule-associated motor cytoplasmic dynein with membranous cargo; however, the mechanism by which dynactin–membrane interaction is regulated is unknown. Here we show that dynein and dynactin exist in discrete cytosolic and membrane-bound states in the filamentous fungus *Neurospora crassa*. Results from *in vitro* membrane-binding studies show that dynein and dynactin–membrane interaction is co-dependent. p150<sup>Glued</sup> of dynactin has been shown to interact with dynein intermediate chain and dynactin Arp1 filament; however, it is not known to play a direct role in membrane binding. In this report we describe our analysis of 43 p150<sup>Glued</sup> mutants, and we show that C-terminal deletions which remove the terminal coiled-coil (CC2) and basic domain (BD) result in constitutive dynactin–membrane binding. *In vitro* addition of recombinant p150<sup>Glued</sup> CC2+BD protein blocks dynactin–membrane binding. We propose that the C-terminal domains of p150<sup>Glued</sup> regulate dynactin–membrane binding through a steric mechanism that controls accessibility of the Arp1 filament of dynactin to membranous cargo.

## INTRODUCTION

Cytoplasmic dynein is a multisubunit, microtubule-associated force-producing enzyme required for a wide range of cellular processes (Allan and Schroer, 1999; Karki and Holzbaur, 1999). Dynein-dependent movement of cargo requires a number of steps such as cargo attachment, motor activation, translocation of cargo, motor inactivation, cargo release, and then possibly motor reactivation for return transport. The mechanisms by which these steps are regulated are not understood. Dynactin, an additional multisubunit complex, has been proposed to link dynein with membranous organelles, and activate motor and regulate its processivity (Schroer *et al.*, 1996; Holleran *et al.*,

1998; King and Schroer, 2000; Kumar *et al.*, 2000). The most abundant subunit of dynactin is actin-related protein 1 (Arp1), which forms a short (37 nm) filament that has been proposed to interact with a spectrin-like cytoskeleton associated with membranous cargo.

The largest subunit of dynactin, p150<sup>Glued</sup>, has been shown to mediate dynein/dynactin interaction; however, it has not been shown to function directly in membrane binding. p150<sup>Glued</sup> is predicted to contain five major structural domains, with three globular regions alternating with two  $\alpha$ -helical coiled-coil domains (Schroer, 1996). A microtubule-binding domain is located in the first globular domain, and interaction of p150<sup>Glued</sup> with dynein intermediate chain occurs through the central region of p150<sup>Glued</sup> (Vaughan and Vallee, 1995). Residues within the second coiled-coil (CC2) domain have been shown to bind to Arp1 (Waterman-Storer *et al.*, 1995). The function of the C-terminal globular basic domain (BD) is unknown.

As with vertebrates, conventional kinesin and cytoplasmic dynein of filamentous fungi have been shown to be required for anterograde and retrograde transport of membranous cargo, respectively (Seiler *et al.*, 1999). In contrast to vertebrates, these transport systems are not essential for viability, and this makes filamentous fungi excellent genetic models for the study of microtubule-dependent, long-range transport of membranous organelles. We have developed a screen that allows the isolation of hundreds of *Neurospora crassa* mutants (referred to as ‘ropy’) that are defective in dynein/dynactin function (Bruno *et al.*, 1996). Here, we examine dynein– and dynactin–membrane interaction in wild-type *N. crassa* and specific p150<sup>Glued</sup> mutants. Our results suggest that the C-terminal domains of p150<sup>Glued</sup> regulate the interaction of membranous cargo with the Arp1 filament of dynactin.

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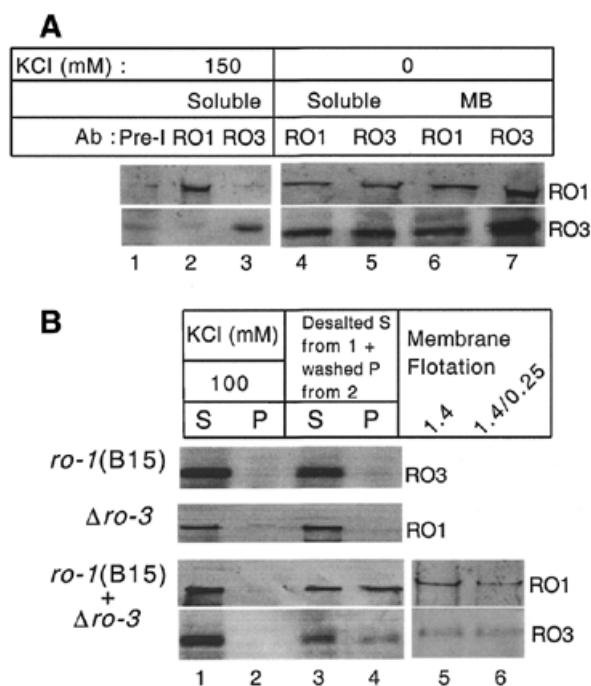
### Dynein and dynactin are co-dependent for membrane interaction

Dynein and dynactin do not generally co-immunoprecipitate with each other (Paschal *et al.*, 1993), and it has been suggested that dynein and dynactin supercomplexes may represent the activated state of the motor that is capable of cargo interaction (Faulkner *et al.*, 2000). To determine whether membrane association in *N. crassa* correlates with stabilized dynein–dynactin interaction, we immunoprecipitated dynein and dynactin from both soluble and membrane-bound pools. We found that dynein and dynactin do not show appreciable co-immunoprecipitation in the presence of 150 mM KCl (Figure 2A, lanes 2 and 3). However, in the absence of salt, dynein and dynactin efficiently co-immunoprecipitate regardless of whether they are from the soluble or membrane-bound pools (Figure 2A, lanes 4–7). These results suggest that the triggering event for membrane interaction is unlikely to be the stabilization of dynein and dynactin association.

Dynein alone has been shown to bind to membrane *in vitro* (Lacey and Haimo, 1994; Tai *et al.*, 1999); therefore, we examined the dependency of dynein and dynactin interaction with membrane by using *ro-1* (lacking dynein heavy chain) and  $\Delta ro-3$  mutants (lacking dynactin p150<sup>Glued</sup>). Consistent with dynactin’s proposed membrane-linker function, we find that dynein can not bind to membrane when dynactin is removed genetically (Figure 2B, lanes 3 and 4). Unexpectedly, we find that dynactin also can not bind to membrane when dynein is removed genetically (Figure 2B, lanes 3 and 4). We also mixed equal amounts of cell extracts from *ro-1* and  $\Delta ro-3$  mutants followed by membrane-binding analysis. We found that in mixed extracts dynein and dynactin were capable of binding to membrane (Figure 2B, lanes 3 and 4). These data suggest that dynein– and dynactin–membrane interaction is co-dependent. Addition of a 3-fold excess of extract from either the *ro-1* or the  $\Delta ro-3$  mutant did not result in increased membrane binding for the respective dynein or dynactin complexes (data not shown). The inability of excess dynactin to drive all dynein into the membrane pellet or excess dynein to drive all dynactin into the membrane pellet suggests that even in *ro-1* and  $\Delta ro-3$  mutants the respective dynein and dynactin complexes exist in two discrete states: one that is capable of binding to membrane and one that is not.

### C-terminal domains of p150<sup>Glued</sup> are involved in dynactin–membrane interaction

Previously it had been proposed that membranous organelles bind directly to dynactin by way of an organelle-associated spectrin binding to the dynactin Arp1 filament (Holleran *et al.*, 1996; Muresan *et al.*, 2001). A region within the CC2 domain of p150<sup>Glued</sup> has been shown to interact with Arp1 filament (Waterman-Storer *et al.*, 1995); however, it is not known whether this domain is required for assembly of the dynactin complex or regulation of Arp1 filament–membrane interaction. To explore a possible role of p150<sup>Glued</sup> in dynactin–membrane interaction, we examined dynein– and dynactin–membrane binding in a large collection of *ro-3* mutants. To identify *ro-3* mutants that produce polypeptides which are defective in dynactin function, and not production of RO3 protein, we screened 43 independently isolated *ro-3* mutants for the

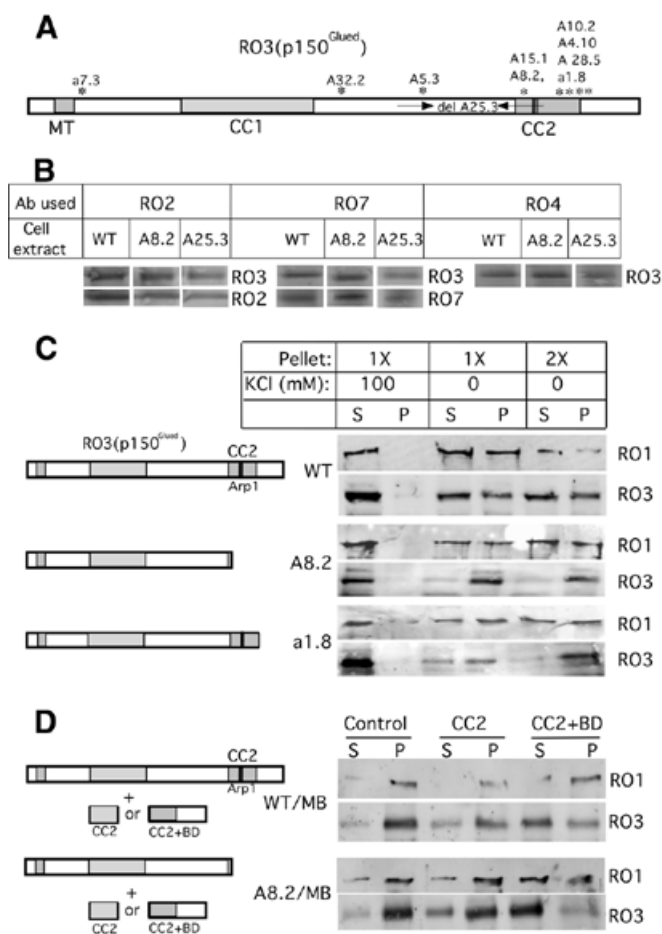


**Fig. 2.** Dynein and dynactin are co-dependent for membrane interaction. (A) Salt-sensitive co-immunoprecipitation of dynein and dynactin from soluble and membrane-bound pools. RO1 (dynein heavy chain) and RO3 (dynactin p150<sup>Glued</sup>) were detected by western analysis and are labeled on the right. Lanes 1, 2 and 3 are immunoblots of immunoprecipitated proteins from the soluble (S100) fraction of wild type using pre-immune serum (pre-I), anti-RO1 and anti-RO3 antibodies, respectively, in the presence of 150 mM KCl. Lanes 4 and 5 are immunoprecipitations of dynein and dynactin from the desalted soluble pool using anti-RO1 and anti-RO3 antibodies, respectively. Lanes 6 and 7 are immunoprecipitations of dynein and dynactin from the salt-released membrane-bound (MB) pool using anti-RO1 and anti-RO3 antibodies, respectively. (B) Effects of dynein and dynactin mutations on salt-dependent pelleting and flotation of dynein and dynactin with membrane. *Neurospora crassa* strains used are labeled on the left. Lanes 1 and 2 are supernatant and pellet, respectively, following 100 000 g centrifugation of 100 mM KCl-treated low-speed cell extracts. Lanes 3 and 4 are supernatant and pellet, respectively, following 100 000 g centrifugation of desalted supernatants from lane 1 that were recombined with washed membrane pellet from lane 2. Lanes 5 and 6 are 1.4 M sucrose and 1.4/0.25 M sucrose interface layers following flotation of membrane pellet (lane 4 resuspended in 2.0 M sucrose).

presence of RO3. We identified 10 *ro-3* mutants that produced RO3 polypeptides which ranged in size from 90 to 145 kDa. The sites of the respective *ro-3* mutations are presented in Figure 3A. Nine of the *ro-3* mutations were nonsense, frameshift or deletion mutations that resulted in the production of truncated polypeptides. Only one of the mutants contained a missense mutation (near the N-terminal microtubule-binding domain). Interestingly, of the nine mutants producing truncated polypeptides, five remove residues contained within the CC2 domain that have been shown to interact with Arp1. In each of these mutants, the sedimentation value of dynactin was not altered. In addition, the truncated RO3 proteins co-immunoprecipitated with the dynactin subunits RO2 (p62), RO4 (Arp1) and RO7 (Arp11) (Figure 3B). These results indicate that the Arp1-binding site in CC2 is not required for incorporation of RO3 into the dynactin complex.



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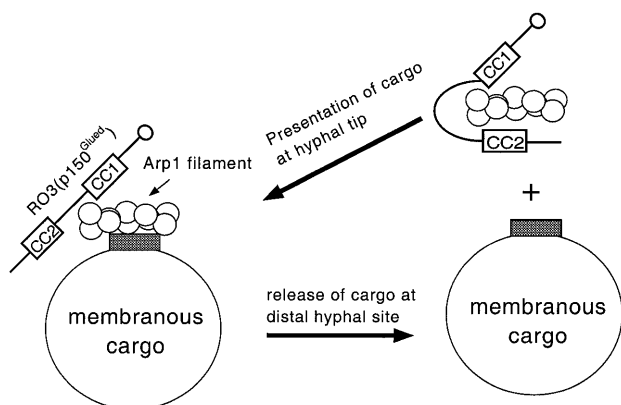
**Fig. 3.** C-terminal domains of p150<sup>Glued</sup> are involved in dynein–membrane interaction. **(A)** Sites of mutations present in 10 independent alleles of *ro-3*. With the exception of the a7.3 missense mutation, the asterisks represent the sites of individual mutations that either introduce a nonsense or frameshift mutation, which results in the production of truncated RO3 protein. Mutant A25.3 possesses an internal deletion in the *ro-3* gene. **(B)** Co-immunoprecipitation of dynein subunits from wild type and C-terminal deletion mutants of *ro-3*. Proteins immunoblotted are labeled on the right of the blot, and cell extracts and anti-protein antibodies are labeled on top of the blot. **(C)** Salt-dependent pelleting of dynein and dynactin from wild type and C-terminal deletion mutants of *ro-3*. The relative lengths of RO3 (p150<sup>Glued</sup>) polypeptides from wild type and the C-terminal deletion mutants A8.2 and a1.8 are presented on the left. The proteins detected by western analysis are labeled on the right. The first two lanes represent supernatant (S) and pellet (P) fractions following centrifugation at 100 000 g of low-speed extracts in the presence of 100 mM KCl. The last four lanes are where the respective supernatants from the first lane were desalted, mixed with either 1× or 2× washed membrane pellet, and then centrifuged at 100 000 g. **(D)** Salt-dependent pelleting of membrane-bound dynein and dynactin from wild type and the A8.2 mutant in the absence and presence of recombinant p150<sup>Glued</sup> C-terminal domains. The methodology of the experiment is presented schematically on the left. Membrane-bound (MB) dynein and dynactin from wild type and the A8.2 mutant lacking p150<sup>Glued</sup> CC2+BD are labeled on the left. Membrane-bound dynein and dynactin were first released by 100 mM KCl, desalted by gel filtration, and then incubated with either 100 μg of CC2 or CC2+BD for 20 min followed by incubation with washed membrane pellet for 1 h and centrifugation at 100 000 g. Control represents the addition of 100 μg of bovine serum albumin in place of the recombinant proteins.

interaction, we conducted salt-dependent pelleting and flotation experiments. The results from two representative mutants, A8.2 and a1.8, which remove CC2+BD or the BD alone, respectively, are presented (Figure 3C). In the A8.2 mutant, nearly all the dynactin binds to membrane in a salt-sensitive fashion, while in the a1.8 mutant there is only a slight increase in the proportion of dynactin associated with membrane relative to the wild-type control. However, if double the amount of membrane pellet is used in the pelleting assay of the a1.8 mutant, almost all the dynactin is present in the membrane fraction. As before, increasing the amount of membrane pellet used in the assay of wild type does not affect the amount of dynein and dynactin present in the membrane-bound state (Figures 1B and 3C). The results indicate that the removal of CC2+BD results in constitutive dynein–membrane binding, while removal of only the BD domain results in a dynactin complex that can be completely driven into the membrane-bound state by the addition of excess membrane fraction. Interestingly, dynein–membrane interaction is relatively unaffected by these *ro-3* mutations. This result is consistent with our extract mixing experiments (described above), which show that in the absence of dynactin, dynein still exists in two discrete states: one that is capable of binding to membrane and one that is not.

The results suggest that p150<sup>Glued</sup> CC2 and BD may regulate dynein–membrane interaction by sterically affecting the accessibility of the Arp1 filament to membranous cargo. To test this hypothesis, we expressed in *Escherichia coli* His-tagged p150<sup>Glued</sup> recombinant polypeptides containing either CC2+BD or only CC2 and then determined whether these polypeptides could block dynein–membrane interaction *in vitro*. As shown in Figure 3D, the addition of the recombinant CC2+BD protein to salt-released dynein from wild type or the A8.2 mutant was able to effectively block the re-association of dynein with membrane. The His-tagged polypeptide containing only the CC2 domain had only a slight effect on dynein–membrane rebinding. To determine whether these recombinant proteins are likely to affect dynein–membrane interaction through a direct physical interaction with dynactin, we examined whether a column containing the His-tagged polypeptides could retain dynactin from extracts of wild type and the A8.2 mutant. Our results showed that columns containing either CC2+BD or CC2 bound dynactin from wild type and the A8.2 mutant (data not shown). Our results suggest that the C-terminal domains of p150<sup>Glued</sup> function in regulating dynein–membrane interaction through controlling the accessibility of the Arp1 filament to membranous cargo (Figure 4).

We propose that soluble and membrane-bound states of dynein result directly from two different conformational states of the C-terminal domains of p150<sup>Glued</sup> (Figure 4). In the soluble state, the CC2+BD domains of p150<sup>Glued</sup> bind to the Arp1 filament and sterically block its interaction with membranous cargoes. (Our calculation of the length of CC2+BD is ~30 nm, sufficient to cover the 37 nm Arp1 filament and thereby prevent interaction with membranous cargo.) In the membrane-bound state, these domains fold-back (i.e. either bind to N-terminal segments of p150<sup>Glued</sup> or the p24/p50 dynamitin subunits contained within the shoulder/sidearm subcomplex of dynactin) and allow Arp1–membrane interaction. Alternatively, in both states the CC2 domain of p150<sup>Glued</sup> may remain associated with the Arp1 filament and regulate Arp1 filament–membrane

To determine whether the mutations removing the RO3 (p150<sup>Glued</sup>) CC2 and BD or only the BD affect dynein–membrane



**Fig. 4.** Model for the regulation of dynein–membrane interaction in *N. crassa*. For simplicity, only the dynein Arp1 filament and a single p150<sup>Glued</sup> subunit are shown. The C-terminal CC2+BD of p150<sup>Glued</sup> interacts with Arp1 filament and prevents dynein–membrane binding (i.e. dynein is in the soluble state). Upon interaction with membranous cargo, the C-terminal domains of p150<sup>Glued</sup> release from the Arp1 filament to allow cargo to bind to the Arp1 filament. Following translocation to the cargo release site, the membranous cargo dissociates from the Arp1 filament, the C-terminal domains of p150<sup>Glued</sup> reassociate with the Arp1 filament, and dynein and dynactin return to the hyphal tip to initiate another round of transport.

interaction in a manner similar to tropomyosin-based regulation of myosin–actin interaction in muscle tissue (Huxley, 1972). A further understanding of dynein–membrane interaction will require identification of the switch that regulates the conformation state of the C-terminal domains of p150<sup>Glued</sup>.

## METHODS

**Strains, growth conditions and genetic techniques.** The *N. crassa* wild-type (74-OR23-1A; FGSC 987) and *ro-1* (B15) (FGSC 4352) strains were obtained from the Fungal Genetic Stock Center (FGSC), Department of Microbiology, University of Kansas Medical Center, Kansas City, KS. Strains deleted for the *ro-3* gene have been described (Tinsley *et al.*, 1996). Media, growth conditions and sexual crosses were as described (Davis and de Serres, 1970).

**Sequence analysis of *ro-3* mutants.** Genomic DNA from *ro-3* mutants was isolated using the Dneasy Plant Mini Kit (Qiagen Inc., Santa Clarita, CA). The *ro-3* gene was amplified from the mutants by PCR using pfu Turbo DNA polymerase from Stratagene (La Jolla, CA). The entire DNA sequence of the *ro-3* gene was determined for each mutant *ro-3* allele.

**Expression of RO3 recombinant proteins.** DNA fragments encoding *ro-3* CC2+BD and CC2 domains were amplified by PCR using a wild-type copy of the *ro-3* gene. The PCR-generated fragments were inserted into a modified pQE41 vector to produce RO3-His<sub>6</sub>-tagged constructs (Qiagen). The DNA sequence of both constructs was determined to ensure maintenance of the proper reading frame and the absence of any mutations. The constructs were transformed into *E. coli*, high-level expression was induced, and recombinant His-tagged CC2+BD and CC2 proteins were purified using the QIA Expression Kit (Qiagen).

**Immunoprecipitation and immunoblotting.** Anti-RO1, anti-RO2, anti-RO3, anti-RO4 and anti-RO7 antibodies were produced as described (Minke *et al.*, 1999, 2000; Lee *et al.*, 2001). Immunoprecipitation was performed as described (Beckwith *et al.*, 1998; Kumar *et al.*, 2000). For immunoblotting, proteins resolved by SDS–PAGE were electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and then probed with anti-RO1 and anti-RO3 antibodies at 1:1000 dilution followed by goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase at 1:15000 dilution (Promega, Madison, WI). Western blot processing was performed as described (Promega).

**Dynein/dynein–membrane binding assay.** Frozen mycelia (0.5 g each) were suspended in 1.5 ml of extraction buffer (EB; 50 mM PIPES pH 7.0, 50 mM HEPES, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml TAME, 1 μg/ml pepstatin A and 10 μg/ml soybean trypsin inhibitor) and the hyphae were ground with zirconium beads using a mortar and pestle. Debris were removed by centrifugation at 5000 g for 10 min. Extracts were cleared by centrifugation at 28 000 g for 10 min. Supernatant (0.5 ml) was incubated with 100 or 200 mM KCl (to remove loosely bound membrane-associated proteins) for 60 min, overlaid over a 7.5% sucrose cushion (0.2 ml in EB), and then centrifuged at 100 000 g in a Beckman Ti 100.3 rotor for 45 min to pellet down membranous organelles and membrane-associated proteins. The supernatant was desalted using Sephadex gel filtration column NAP (Amersham Pharmacia Biotech, Piscataway, NJ). The pellet was washed in 0.3 ml of 7.5% sucrose in EB and centrifuged at 100 000 g for 45 min. The pellet was then resuspended with desalted supernatant (0.5 ml), incubated for 60 min, overlaid over a 7.5% sucrose cushion (0.2 ml in EB), and recentrifuged at 100 000 g for 45 min. The pellet obtained from high-speed centrifugation was resuspended to the same volume as supernatant. Samples (60 μl) from each supernatant and pellet were analyzed by SDS–PAGE followed by western blotting. Membrane flotation experiments were performed as described (Niclas *et al.*, 1996).

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