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Transfection-Induced Defects in Dynein-Driven Transport: Evidence that ICs Mediate Cargo-Binding

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Abstract

Cytoplasmic dynein contributes to the localization and transport of multiple membranous organelles, including late endosomes, lysosomes, and the Golgi complex. It remains unclear which subunits of dynein are directly responsible for linking the dynein complex to these organelles, however the intermediate chain (IC), light intermediate chain (LIC) and light chain (LC) subunits are each thought to be important. Based on previous mapping of a dynein IC phosphorylation site (S84), we measured the impact of transfected ICs on dynein-driven organelle transport (Vaughan et al., 2001). Wild-type and S84A constructs disrupted organelle transport, whereas the S84D construct induced no defects. In this study we investigated the mechanisms of transfection-induced disruption of organelle transport. Transfected ICs did not: 1) disrupt the dynein holoenzyme, 2) incorporate into the native dynein complex, 3) dimerize with native dynein ICs or 4) sequester dynein LCs in a phosphorylation-sensitive manner. Consistent with saturation of dynactin as an inhibitory mechanism, truncated ICs containing only the dynactin-binding domain were as effective as full-length IC constructs in disrupting organelle transport, and this effect was influenced by phosphorylation-state. Competition analysis demonstrated that S84D ICs were less capable than dephosphorylated ICs in disrupting the dynein-dynactin interaction. Finally, two-dimensional gel analysis revealed phosphorylation of the wild-type but not S84D ICs, providing an explanation for the incomplete effects of the wild-type ICs. Together these findings suggest that transfected ICs disrupt organelle transport by competing with native dynein for dynactin binding in a phosphorylation-sensitive manner.

Keywords

Cytoplasmic dynein; dynactin; phosphorylation; organelle transport

INTRODUCTION

Cytoplasmic dynein is a large multisubunit motor protein implicated in the transport of membranous organelles, chromosomes and other cargos (Vale 2003; Vallee *et al.* 2004). Linked functionally to dynein, dynactin is an independent multisubunit complex first identified as a dynein cofactor and subsequently shown to contribute to the dynein-driven transport of many cargos (Gill *et al.* 1991; Holzbaur *et al.* 1991; Schroer and Sheetz 1991). The p150^{Glued} subunit is widely recognized as the dynein-binding subunit of dynactin, and has been shown to bind to the IC subunits of dynein (Karki and Holzbaur 1995; Vaughan and Vallee 1995; King *et al.* 2003). This interaction has been proposed as a mechanism to link dynein to cargo in both interphase and mitosis (Paschal *et al.* 1992; Vaughan and Vallee 1995; Echeverri *et al.* 1996; Holleran *et al.* 1996). However, additional dynein subunits have

also been implicated in tethering dynein to cargo, raising the possibility that multiple dynein subunits are responsible for organelle binding. Specifically, the IC, LIC (light intermediate chain) and LC (light chain; Pfister *et al.*, 2005) subunits have each been described as cargo-binding subunits (Niclas *et al.* 1996; Bowman *et al.* 1999; Purohit *et al.* 1999; Tai *et al.* 1999; Yano *et al.* 2001).

Potentially shedding light on this question, the first IC phosphorylation site identified for interphase dynein was shown to regulate dynactin-binding (Vaughan *et al.* 2001). Given the potential role of dynactin in linking dynein to cargo, a regulated interaction between the ICs and p150^{Glued} suggested that the ICs were responsible for cargo-binding. One consequence of mapping this phosphorylation site in the ICs was the generation of S84A and S84D mutants to mimic opposite phosphorylation states (Vaughan *et al.* 2001). These constructs were transfected into COS-7 cells revealing that the S84A mutant was effective at disrupting dynein-based transport whereas the phospho-mimicking S84D mutant was not.

One possibility suggested by these experiments is that the ICs contain cargo-binding activity and are capable of competing with native dynein for cargo-binding. Furthermore, IC phosphorylation regulates cargo-binding and the ability to compete with native dynein. Although this model has the potential to address which dynein subunits are the most important for cargo-binding, other possibilities are supported by the literature. For example, the dynein LICs have been identified as phosphorylation substrates during mitosis (Niclas *et al.*, 1996), and phosphomimetic mutants of the ICs have not displayed changes in binding activity for all cargos (King *et al.* 2003).

To address the significance of IC phosphorylation in interphase dynein, it was important to clarify the mechanisms of transfection-induced disruption of dynein-driven transport. In particular, we compared several ways in which excess ICs could affect dynein activity. We also were interested in effects that were different for phospho- and dephospho-forms of the ICs. Although transfected ICs were not effective at disrupting native dynein complexes, displaying differential binding to dynein LCs or inducing other dynein-specific defects, they did display differential abilities to bind dynactin and disrupt organelle transport. This suggests that the transfected ICs act by competing with native dynein for cargo-binding in a phosphorylation-sensitive manner.

MATERIALS and METHODS

Cell Culture and Cell Harvesting

COS-7 cells and J774A.1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). **Mechanical Lysis:** COS-7 cells were washed 2 times with cold PBS containing a protease inhibitor cocktail (0.01 M benzamidine, 0.01 M NaF, 0.01 M Na vanadate, 0.026 mg/ml leupeptin, 0.026 mg/ml aprotinin, 1 mM PMSF, 0.0026 mg/ml pepstatin; Sigma-Aldrich). Cells were resuspended in RIPAn (RIPA without detergent) buffer (50mM Tris pH 8.0, 150mM NaCl). After incubation for 10 minutes on ice, cells were homogenized using a motorized sterile pestle (Kontes Glass Company, Vineland, NJ) for 30 seconds. **Hypotonic Lysis:** COS-7 cells were washed 2 times with cold HBS buffer plus EDTA (0.15 M NaCl, 10mM Hepes, 5 mM EDTA, pH 7.4). The pellet was resuspended with 1.5 times the pellet volume of Solution A (15 mM KCl, 1.5 mM magnesium acetate, 10 mM Hepes-KOH, pH 7.5) plus protease inhibitor cocktail. After incubation for 10 minutes on ice, cells were homogenized with 30 strokes in a glass tissue grinder (Kontes). Solution B (375 mM KCl, 22.5mM magnesium acetate, 220 mM Hepes-KOH, pH 7.5) was added to the homogenate at a ratio of 1:5. The homogenate was centrifuged at 12,000 RPM, 10 min, and that supernatant was spun at 100,000 × G. Total

protein of the second supernatant was analyzed by Bradford assay using Coomassie Plus Protein Assay reagent (Pierce, Rockford, IL).

Small Scale Sucrose Gradient Centrifugation

Two ml gradients of 5%- 20% sucrose in TBS (50 mM Tris pH 8.0, 150 mM NaCl) were poured using the Jule Gradient Former Model J5 system (Jule Inc., New Haven, CT). Gradients were kept at 4° until sample application. 200 µl of cell lysate (3.26 µg protein per µl) were layered on top of the gradient and centrifuged for 3.5 hours at 50,000 RPM (Optima Tabletop Ultracentrifuge, Beckman Instruments, Palo Alto, CA) after which 200 µl fractions were collected from the top of the gradient. 20 µl samples of each fraction were analyzed by SDS-PAGE and western blot.

Immunoprecipitation

All versions of IC-GFP were immunoprecipitated from either transfected COS-7 extracts or collected sucrose gradient fractions using a 1:50 dilution of BD Living Colors A.V. monoclonal antibody (BD Biosciences, Mountain View, CA) and protein A Sepharose beads (Amersham Pharmacia, Piscataway, NJ). After overnight immunoprecipitation at 4°, beads were washed at 4 times 30 minutes, in RIPA buffer. Proteins were eluted off beads by boiling for 5 minutes in cracking buffer (30% glycerol, 1.5M Tris, 166mM DTT, 0.01% BPB, 3.3% SDS) and analyzed by SDS-PAGE.

Expression Constructs and Site Directed Mutagenesis

RFP-NAGT, p150^{Glued} 1-811, PCIneo-GFP, WT 1-125, and full length IC-GFP, S84D IC-GFP, and S84A IC-GFP PCIneo and pET14b constructs were described previously (Vaughan *et al.*, 2001). pCIneo ΔIC-GFP, S84A ΔIC-GFP, and S84D ΔIC-GFP were produced by mutating nucleotide 377 of rat IC2C from G to A by PCR, thereby creating a novel NcoI site. The 1-100 IC PCR fragments of each parent construct were then gel purified and ligated into the PCIneo-GFP backbone.

Bacterial Protein Expression and Purification

Recombinant proteins were expressed in Rosetta™ cells (Novagen, San Diego, CA) and purified by nickel affinity chromatography using His-Bind resin (Novagen) as described previously (Vaughan and Vallee, 1995).

In Vitro Competition Assay

Recombinant ICs were used to displace endogenous dynein from dynactin immunoprecipitates after purifying dynactin from brain extract using anti-p150^{Glued} antibodies (Vaughan and Vallee, 1995). These immunoprecipitates were incubated with 10 µg of purified recombinant ICs (wild-type or S84D) for one hour at room temperature followed by re-isolation. The abundance of p150^{Glued}, native dynein ICs and recombinant ICs was quantified after western blot analysis with appropriate antibodies (anti-IC- 74.1, Chemicon, Temecula, CA; anti- p150^{Glued} - BD Biosciences, San Jose, CA).

Two-dimensional Gel and Blot Overlay

Two-dimensional gel analysis was performed using a Mini-Protean II 2-D Cell (Bio-Rad, Hercules, CA), according to the manufacturer, with 4-6 ampholytes.

Transfection Analysis and Live Cell Imaging

For cell lysate analysis, COS-7 cells were transfected by nucleoporation (Amaxa, Inc., Gaithersburg, MD). Cells were harvested from the dishes 16 hours after transfection. For

live-cell imaging experiments, cells were transfected using 3 μ g of total plasmid DNA and 9 μ g of LipofectAMINE in 10 cm dishes. In co-transfection experiments, 2.4 μ g of RFP-NAGT and 0.6 μ g of IC-GFP constructs were used. Where indicated, 0.1 mg/ml rhodamine-dextran (Molecular Probes, Carlsbad, CA) was added 16 hours after transfection for 12 hours followed by a 30 minute chase with culture media prior to live cell imaging. Imaging was carried out with a Zeiss Axiovert 100 TV fluorescence microscope using Metamorph software (Molecular Devices, Downingtown, PA) and a Roper Coolsnap HQ digital CCD camera (Roper Corp., Tucson, AZ).

Statistical Analysis

Binding results after ECL detection were recorded on autoradiographic film and digitized using a Fluorchem 8900 (Alpha Innotec, San Leandro, CA). Signal intensity was summed in the area of the bands and the intensity of film background was subtracted. Statistical analysis of organelle distribution assays was performed in Microsoft Excel. P-values of comparisons between control and experimental measurements were determined with a two-tailed t-test assuming unequal variance. Confidence levels were chosen at $p \leq 0.05$.

Supplemental Data

Sucrose gradient sedimentation analysis of native dynein after mechanical or hypotonic lysis is compared in Supplemental Figure 1.

RESULTS

Impact of Dynein IC Transfection on Native Dynein Complexes

To better understand the effect of transfected IC constructs on dynein-based transport, we tested several hypotheses for dynein disruption and the inability of S84D constructs to interfere with dynein activity. As an initial indicator of IC interactions, sucrose density gradient sedimentation was performed on cell extracts. Mechanical lysis and hypotonic lysis protocols were compared to determine which retained a stable dynein complex (Fig. S1). The hypotonic lysis protocol provided more consistent sedimentation of dynein ICs in a single 20S peak and was used for subsequent studies.

Because the full-length wild-type and S84D mutant proteins retain all the domains responsible for incorporating into the dynein complex, it was reasonable to predict that the transfected IC proteins could disrupt endogenous dynein complexes by mass action. To test the effect of IC-GFP expression on dynein integrity, sucrose density gradient analysis was applied to transfected cell lysates (Fig. 1). The IC-GFP fusion protein migrated with a M_r of ~ 100 kD (27kD larger than the endogenous ICs) allowing evaluation of both IC-GFP and native dynein in the same gradients. Western blot analysis of gradient fractions revealed that IC-GFP sedimented as a broad peak near the top of the sucrose gradient (Fig. 1A), whereas native dynein sedimented at 20S similar to previous work (Paschal et al., 1987, Schroer and Sheetz 1991). The sedimentation behavior of native dynein was the same in cells transfected with the S84D mutant IC-GFP construct (Fig. 1A). This suggests that excess ICs do not disrupt dynein assembly globally, and that the S84D mutant does not differ in this respect.

Related to this hypothesis, excess ICs could also dimerize with native dynein ICs, thereby exchanging a fraction of endogenous ICs with the GFP-tagged constructs. To test this possibility, we purified the GFP-tagged constructs by immunoprecipitation and measured the amount of native dynein ICs in the isolates (Fig. 1B). Neither wild-type nor S84D IC-GFP proteins bound appreciable amounts of native dynein, suggesting that the transfected constructs do not dimerize nor incorporate into native dynein under these conditions.

A third potential mechanism of dynein inhibition could reflect the ability of excess ICs to sequester members of the dynein LC families, thereby depleting native dynein of LCs. To test this potential effect, we measured the dynein LC content of immunoprecipitates containing GFP-tagged ICs (Fig. 1C). Western blot analysis was performed using antibodies against LT1 and LL1 LCs (King *et al.* 1996; King *et al.* 1998). The LRB1 dynein LC was not analyzed in this study because of the distance between LRB1 binding sites and the location of the S84D mutation (Susalka *et al.* 2002). In contrast to controls containing only native dynein, neither wild-type nor S84D ICs bound substantial levels of the LL1 dynein LC. Both constructs did display binding to the LT1 dynein LCs, however binding was the same for wild-type and S84D proteins. The latter suggests that sequestration of dynein LCs cannot explain the differences between wild-type and S84D ICs in dynein inhibition.

The P150^{Glued}-binding Domain of ICs is Sufficient for Dominant Negative Phenotypes

Experiments with full-length IC constructs suggest that defects induced by IC-GFP overexpression do not require disruption of native dynein populations. Based on the ability of isolated ICs to bind the p150^{Glued} subunit of dynactin (Karki and Holzbaur 1995; Vaughan and Vallee 1995; King *et al.* 2003) and the impact of IC phosphorylation on p150^{Glued} binding (Vaughan *et al.* 2001), we tested if IC constructs containing only the p150^{Glued} binding domain were capable of disrupting dynein function. For these studies, we prepared a construct encoding the N-terminal 100 amino acids of dynein IC (named ΔIC-GFP). This construct retains the p150^{Glued}-binding region of dynein ICs, but it lacks all other known functional domains of the ICs including binding sites for the dynein LCs and dynein HCs. S84A and S84D mutants were prepared in parallel and all constructs contained a C-terminal GFP-tag because N-terminal tags have been shown to interfere with dynactin binding (Vaughan and Vallee, 1995).

Focusing first on the impact of ΔIC-GFP constructs on Golgi complex integrity, we co-transfected IC constructs with NAGT-RFP similar to previous work (Vaughan *et al.*, 2001). As a control, cells were transfected with NAGT-RFP and GFP alone. In each of the transfections, ΔIC-GFP appeared soluble with no specific localization. Cells expressing wild-type ΔIC-GFP displayed a range of Golgi dispersal phenotypes similar to outcomes with full-length ICs (Fig. 2). Quantitative analysis revealed that 18% of cells displayed no Golgi dispersal, 45% of cells displayed partial dispersal, and 37% of cells displayed complete Golgi dispersal. The variability did not appear to reflect ΔIC-GFP expression level alone (see below).

Because S84A/S84D mutant constructs displayed differences in Golgi dispersal as full-length proteins (Vaughan *et al.*, 2001), the ΔIC-GFP version of each mutant was assessed next. The S84A ΔIC-GFP construct induced a severe Golgi dispersal phenotype, similar to the full-length protein (Fig. 2). In these assays, 9% of cells displayed no Golgi dispersal, 46% displayed partial dispersal, and 44% displayed complete Golgi dispersal. In contrast, cells expressing S84D ΔIC-GFP displayed less Golgi dispersal with 59% displaying no Golgi dispersal, 36% displaying partial dispersal, and 5% displaying complete Golgi dispersal (Fig. 2). This phenotypic shift towards less Golgi dispersal was similar to the results of the full-length S84D construct (Fig. 2).

Late endosomes and lysosomes are also known to be dependent on cytoplasmic dynein for localization and motility (Aniento *et al.* 1993; Burkhardt *et al.* 1997). As shown in previous work (Vaughan *et al.* 2001), expression of IC constructs can induce defects in late endosome and lysosome transport. Live-cell imaging of rhodamine dextran-labeled organelles was performed with the ΔIC-GFP constructs to measure the impact on these organelles. Cells expressing wild-type ΔIC-GFP displayed a range of late endosome and lysosome localizations (Fig. 3). Similar to cells expressing full-length ICs, 25% of ΔIC-GFP

expressing cells displayed no late endosome and lysosome dispersal, 43% displayed partial dispersal, and 32% displayed complete membrane dispersal (Fig. 3). The phenotypic variability in these experiments could not be explained completely by differences in the level of Δ IC-GFP expression (see below).

To test the impact of the S84 phosphorylation, S84A/D mutant Δ IC-GFP constructs were analyzed. Cells expressing the S84A Δ IC-GFP construct displayed a more severe disruption of late endosomes and lysosomes than wild-type ICs (Fig. 3). Binned as above, 11% of transfected cells displayed no late endosome and lysosome dispersal, 32% displayed partial dispersal, and 55% displayed complete endosome and lysosome dispersal (Fig. 3). Similar to the full-length construct, the Δ IC-GFP S84D construct induced less disruption of late endosomes and lysosomes (Fig. 3). 53% of cells displayed no late endosome and lysosome dispersal, 37% displayed partial dispersal, and 10% displayed complete late endosome and lysosome dispersal (Fig. 3). With minor differences, both S84A and S84D Δ IC-GFP constructs mimicked the full-length ICs in their ability to perturb dynein activity.

Impact of Δ IC-GFP Transfection on Native Dynein Complexes

Building on analysis of full-length IC expression (Fig. 1), we measured the ability of Δ IC-GFP constructs to disrupt native dynein complexes (Fig. 4A). As above, COS-7 cells were transfected with Δ IC-GFP constructs and hypotonic lysates were analyzed by sedimentation. Due to the truncation of the ICs, the Δ IC-GFP proteins could be distinguished from native ICs by gel mobility.

Similar to the full-length IC constructs, wild-type Δ IC-GFP was detected at the top of each gradient and displayed little overlap with native dynein (Fig. 4A). When the Δ IC-GFP S84D lysates were analyzed in parallel, the differential sedimentation of Δ IC-GFP and native dynein was also evident (Fig. 4A). Because the two Δ IC-GFP constructs failed to alter the sedimentation behavior of native dynein under these conditions, construct-specific effects on organelle transport do not appear to reflect differential disruption of native dynein.

To assess if the Δ IC-GFP constructs associate with native dynein ICs in solution or bind dynein LCs, we isolated the GFP-tagged constructs by immunoprecipitation and performed western blot analysis for dynein subunits (Fig. 4B). Neither wild-type nor S84D Δ IC-GFP displayed measureable binding to native dynein ICs or LL1/LT1 dynein LCs. The latter is consistent with the loss of LL1 and LT1 binding sites in the Δ IC-GFP constructs. Together, these experiments suggest that transfected Δ IC-GFP constructs do not affect dynein activity by disrupting endogenous dynein populations.

Construct-Specific Disruption of Dynein-Dynactin Interactions

Because Δ IC-GFP constructs retain p150^{Glued}-binding domains but not other known interaction sites (Vaughan and Vallee, 1995; Wilkerson et al., 1995; Lo et al., 2001; Mok et al., 2001; Susalka et al., 2002), we measured the ability of Δ IC-GFP to compete with native dynein for dynactin binding. Dynactin purified from rat brain extract by immunoprecipitation contains measureable levels of dynein (Fig. 5; Vaughan and Vallee, 1995). To test the impact of excess Δ IC-GFP proteins, we incubated these immunoprecipitates with recombinant Δ IC-GFP proteins and re-isolated the dynactin (Fig. 5). Western blot analysis after incubation with wild-type Δ IC-GFP revealed a ~60% reduction in native dynein ICs in these dynactin fractions, and the presence of the recombinant Δ IC-GFP protein reflecting binding to p150^{Glued} (Fig. 5). This suggests that the Δ IC-GFP can bind p150^{Glued} and displace native dynein. In contrast to wild-type Δ IC-GFP, S84D Δ IC-GFP was less capable of binding to p150^{Glued} or displacing native dynein (Fig.

5). This suggests that a consequence of IC phosphorylation is reduced binding to p150^{Glued} and competition with native dynein for dynactin binding.

Phosphorylation of Wild-type But Not Mutant IC Transfection Constructs

Although the experiments described above suggest that transfected ICs disrupt dynein transport by competing with native dynein for cargo binding, they do not resolve the partial effects elicited by the wild-type protein. Wild-type ICs should compete as well as the S84A construct, but failed to do so (Figs. 2&3). One possibility was that the wild-type protein is subject to phosphorylation similar to native dynein and that the transfected protein exists in multiple regulatory states in transfected cells.

To test this possibility, two-dimensional (2D) gel electrophoresis was used to assess the phosphorylation status of the wild-type and S84D constructs (Fig. 6). The presence of both endogenous and transfected constructs in the samples provided internal controls for phosphorylation activity. In both cases, the native ICs were present as two spots (Fig. 6 A&B) corresponding to the phospho- and dephospho-form of the protein observed previously (Vaughan *et al.* 2001). The wild-type IC-GFP construct displayed the same pattern of spots (Fig. 6A), suggesting phosphorylation of the transfected protein. In contrast, analysis of the S84D construct revealed the presence of a single spot for the IC-GFP construct (Fig. 6B) despite the presence of two spots for the native ICs in the same sample. This suggests that the wild-type but not S84D construct is subject to phosphorylation in the extracts of transfected cells. Relevant for the dynein-disruption assays, this provides an explanation for the mixed phenotypes of cells transfected with wild-type IC constructs. The wild-type protein exists in both a phospho-form (which displays reduced binding to dynactin) and a dephospho-form (which displays avid binding to dynactin). The ratio of these two populations would influence how effective the construct would be at competing with native dynein.

DISCUSSION

These studies provide an improved understanding of construct-specific effects of IC transfection on dynein-driven organelle transport. In particular, they suggest that phosphorylation-sensitive binding to the p150^{Glued} subunit of dynactin plays an important role in these assays. Rather than incorporation into and poisoning of native dynein, competitive binding by the ICs appears to explain the effects on the Golgi complex, late endosomes and lysosomes. This has broad implications for the identification of dynein receptors on organelles and the role of dynein ICs in cargo-binding.

Mechanisms of IC-Induced Phenotypes

None of the constructs analyzed in this study displayed the ability to incorporate into or disrupt native dynein complexes. Consistent with this point, the transfected IC constructs did not alter the sedimentation of native dynein, dimerize with native ICs or display differential binding to dynein LCs. Dominant-negative effects on organelle transport appear to result from direct binding to dynactin and competition with native dynein. This suggests that the differential effects of phosphomimetic (S84D) and dephosphorylation (S84A) mutants might simply reflect the regulation of p150^{Glued}-binding by phosphorylation. Advances in the identification of dynein kinases will allow further analysis of this model.

Intermediate Effects After Wild-type IC Transfection

Although the results elicited by our S84 mutants were consistent with the role of dynactin as adaptor for dynein on membranes, the incomplete defects induced by wild-type IC expression were not. Because wild-type ICs shared similar affinity for p150^{Glued} *in vitro*,

one might expect wild-type ICs to compete effectively for organelle binding *in vivo*. The mixed outcomes of experiments with the wild-type protein raise the possibility that transfected ICs act through a different mechanism to perturb membrane transport. However, 2D gel analysis of wild-type and S84D mutant ICs reveals that wild-type IC but not the S84D mutant undergoes phosphorylation in transfected cells. Because IC phosphorylation regulates the ability to bind p150^{Glued}, the degree of phosphorylation would be expected to influence what fraction of transfected ICs retain p150^{Glued}-binding and compete with native dynein. 2D gel analysis suggests that a substantial fraction (ie. 50%) is phosphorylated in these extracts but could be different at the level of individual cells.

Implications for Dynein-Dynactin Interaction Domains

Previous work using biochemical and cell transfection assays has suggested the possibility of multiple interaction sites between the dynein ICs and the p150^{Glued} subunit of dynactin. For the dynein ICs, the N-terminal coiled-coil domain and the adjacent S/T-rich domain containing IC phosphorylation sites have both been implicated (Karki and Holzbaur 1995; Vaughan and Vallee 1995; Vaughan *et al.* 2001; King *et al.* 2003). The role of IC phosphorylation at S84 is a difference between these models however (Vaughan *et al.* 2001; King *et al.* 2003). Whereas the S84D mutation was shown to reduce binding of ICs to p150^{Glued} *in vitro* and to reduce the ability of ICs to compete with native dynein for organelle binding in one study (Vaughan *et al.* 2001), the same mutation behaved similar to wild-type ICs in biochemical and transfection assays in a second study (King *et al.* 2003). One possibility is that the location of the GFP-tag could influence the activity of the IC fragments; King and coworkers placed GFP at the N-terminus (King *et al.* 2003), whereas we placed GFP at the C-terminus because N-terminal tags were shown previously to interfere with the IC-p150^{Glued} interaction (Vaughan and Vallee, 1995; Vaughan *et al.*, 2001). Another possibility is that multiple domains in p150^{Glued} contribute to IC binding. The CC1 (coiled-coil 1) domain of p150^{Glued} has been shown to interfere with microtubule organization when overexpressed (Quintyne *et al.* 1999), a function thought to require dynein activity. CC1 can also bind to IC fragments and cosediment with ICs in sucrose-gradients (King *et al.* 2003), presumably reflecting the presence of coiled-coil sequence in both proteins. Direct protein-protein interactions implicate a region immediately after CC1 in IC binding however (A.A. 600-811; Waterman-Storer and Holzbaur, 1996; Vaughan and Vallee 1995; Deacon *et al.* 2003), and this interaction is sensitive to IC phosphorylation state (Vaughan *et al.*, 2001). These findings could suggest that both interactions contribute to the dynein-dynactin interaction, and that regulation by phosphorylation impacts only one of these activities. Because the S84D mutation only partially mimics the phosphorylated form of the ICs, identification of the kinase/s involved will allow a more thorough analysis of this possibility.

Summary

These findings reinforce the role of the dynein ICs in targeting cytoplasmic dynein to cargo, and implicate dynactin in linking dynein to cargo. Excess ICs compete with dynein for cargo binding and result in defective dynein-based organelle transport after transfection. However, this ability is regulated by IC phosphorylation. These results suggest that the dynein ICs encode much of the targeting activity of the dynein motor and that this targeting is regulated by IC phosphorylation at S84 during interphase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Impact of IC Transfection on Dynein Integrity

(A) Lysates generated from COS-7 cells transfected with full-length wild-type or S84D IC-GFP were analyzed by sucrose density gradient sedimentation. Gel mobilities of GFP-tagged (IC-GFP) and native dynein ICs are indicated. B) 20S fractions were subjected to immunoprecipitation with anti-dynein IC (control) or anti-GFP (GFP-W.T. and GFP-S84D) antibodies and analyzed by western blot with antibodies against native dynein ICs. C) COS-7 cell lysates from transfected cells were subjected to immunoprecipitation with anti-dynein IC (control) or anti-GFP (GFP-W.T. and GFP-S84D) antibodies and probed for LL1 dynein LC and LT1 dynein LC content with anti-dynein LC antibodies

Figure 2. Impact of IC Transfection on Golgi Complex Integrity

A) COS-7 cells were cotransfected with NAGT-RFP (red:lumenal Golgi marker) and GFP-tagged ICs (insets). Phenotypes were binned as “no dispersal”, “partial dispersal”, or “complete dispersal”. Mag. bar = 10 μ m. B) Statistical analysis of cells transfected with either full-length or truncated wild-type ICs. C) Statistical analysis of cells transfected with either full-length or truncated S84A ICs. D) Statistical analysis of cells transfected with either full-length or truncated S84D ICs. Data for full-length constructs are reproduced from Vaughan *et al.*, 2001.

Figure 3. Impact of IC Transfection on Late Endosome/Lysosome Localization

A) COS-7 cells were labeled vitally with rhodamine dextran (red) and transfected with GFP-tagged ICs (insets). Phenotypes were binned as “no dispersal”, “partial dispersal”, or “complete dispersal”. Mag. bar = 10 μ m. B) Statistical analysis of cells transfected with either full-length or truncated wild-type ICs. C) Statistical analysis of cells transfected with either full-length or truncated S84A ICs. D) Statistical analysis of cells transfected with either full-length or truncated S84D ICs. Data for full-length constructs are reproduced from Vaughan *et al.*, 2001.

Figure 4. Impact of Truncated ICs on Dynein Integrity

A) Lysates from cells transfected with wild-type and S84D Δ IC-GFP were analyzed by sucrose density gradient sedimentation and assayed for effects on the sedimentation of endogenous dynein at 20S. Gel mobilities of native dynein ICs and GFP-tagged constructs are indicated. B) Lysates from transfected cells were subjected to immunoprecipitation with anti-IC (control) or anti-GFP (Δ IC-GFP and Δ IC-GFP -S84D) antibodies and probed for dynein LC (LL1 and LT1) content with anti-dynein LC antibodies. Anti-dynein IC antibodies detect both native and transfected ICs in cell extracts (control).

Figure 5. Impact of Excess ICs on Dynein-Dynactin Interaction

A) Dynactin was isolated by immunoprecipitation with anti-p150^{Glued} antibodies and probed for native dynein with anti-dynein IC antibodies. Dynactin precipitates were incubated with buffer (none) or excess wild-type (WT) or S84D (S84D) ICs and then re-isolated and probed by western blot. The presence of the excess recombinant ICs was also analyzed in the precipitate fractions (Recomb. IC). B) Statistical analysis of scanned autoradiogram densitometry quantifying band intensity. The difference in native dynein content between control and wild-type ICs was significant ($p=.0067$) whereas the difference between control and S84D was not ($p=.6552$).

Figure 6. Differential Phosphorylation of Transfected ICs

A) 2D gel analysis of COS-7 cell extracts containing GFP-tagged wild-type ICs. Both native ICs and transfected ICs display 2 spots consistent with a phospho- and dephospho-form of each protein. B) 2D gel analysis of COS-7 cell extracts containing GFP-tagged S84D ICs. Whereas native ICs display 2 spots consistent with a phospho- and dephospho-form of this protein, the S84D construct displays a single spot suggesting only the dephospho-form of the protein.