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Identification of the T-complex protein as a binding partner for newly synthesized cytoplasmic dynein intermediate chain 2

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ABSTRACT

Cytoplasmic dynein is a macromolecular motor complex with diverse functions in eukaryotic cells. Dynein plays essential roles in intracellular transport of organelles and mitosis, mediated in part by interactions between the dynein intermediate chain 2 (IC-2) subunits and adapter proteins that bind specific cargos. In experiments to identify phosphorylation-dependent binding partners for IC-2 we instead identified a phosphorylation-independent binding partner, the cytosolic chaperonin containing T complex protein 1 (CCT). CCT consists of eight subunits (CCT1-8) and facilitates folding of a subset of newly synthesized proteins. We confirmed interactions between IC-2 and CCT5 and CCT8 in co-immunoprecipitation experiments and determined that the C-terminal half of IC-2 is necessary and sufficient to bind CCT8. Interestingly, co-immunoprecipitation of IC-2 and CCT is abolished by prior cycloheximide treatment of cells, suggesting that CCT participates in folding of nascent IC-2. *In vitro* translation experiments employing recombinant CCT complex demonstrated that CCT is able to bind newly synthesized IC-2 after release from the ribosome consistent with a role in folding of IC-2.

1. Introduction

Dyneins are minus-end-directed motor proteins that use ATP to transport cargos along microtubules to perform diverse cellular functions. There are two classes of dyneins: cytoplasmic dynein (dynein 1, DYNC1H1) and axonemal dynein (dynein 2, DYNC2H1; also called flagellar or ciliary dynein) [1,2]. Cytoplasmic dynein mediates several cellular processes in eukaryotes, including Golgi apparatus localization [3], transport of lysosomes [4], endosomes [4] and autophagosomes [5], and mitotic spindle assembly [6]. Each dynein complex is composed of two 530-kDa heavy chains (HCs), two 74-kDa intermediate chains (ICs), four 53- to 59-kDa light intermediate chains (LICs) and several 10- to 14-kDa light chains (LCs). The HCs have ATPase activity and are responsible for the movement of dynein complexes along microtubules. The cytoplasmic intermediate chain (DYNC1I) binds to many cargos via its N-terminal region.

We have previously shown that epidermal growth factor and nerve growth factor stimulate the ERK phosyphorylation of cytoplasmic dynein intermediate chain 2 (IC-2) on Serine 81 [7,8]. It is known that retrograde delivery of Trk-receptor endosomes by dynein along microtubules is crucial to transmit survival signals in neurons. Mitchell et al. showed that IC-2 phosphorylation by ERK on Serine 81 promotes the association of dynein with Trk signaling endosomes [8] and is necessary for neuronal survival after Trk receptor stimulation [8]. The p150^{Glued} subunit of dynactin bridges many cargos to dynein through its direct interaction with the N-terminus of IC-2 [9]. Vaughan et al. reported that phosphorylation of the intermediate chain at Serine 84 regulates binding of p150^{Glued} [10], and similarly phosphorylation at Threonine 89 disrupts the interaction between IC and p150^{Glued} [11]. However, Serine 81 phosphorylation of IC-2 had no discernible effect on the interaction between IC-2 and p150^{Glued} [7]. These data suggested that Serine 81 phosphorylation might regulate cargo binding through a different cargo adapter.

We performed experiments to identify novel IC-2 binding partners regulated by Serine 81 phosphorylation. We used A375

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melanoma cells since they exhibit constitutively elevated ERK signaling resulting from a B-Raf mutation. We transfected these cells with mRFP-tagged wild type or phosphorylation-deficient (S81A) IC-2, and compared the spectrum of proteins co-immunoprecipitated with the tagged IC-2 from membrane fractions. We detected one candidate protein band more abundant in S81A than in wild type IC-2 immunoprecipitates and mass spectrometry identified five subunits of the cytosolic chaperonin containing T-complex protein 1 (CCT) within this band. CCT consists of eight subunits (CCT1-8) and facilitates folding of a subset of newly synthesized proteins or misfolded proteins to achieve their native conformation. We confirmed these interactions by blotting independent mRFP-IC-2 immunoprecipitates for endogenous CCT5 and CCT8 proteins, and by demonstrating that exogenous HA-tagged CCT8 could be co-precipitated with mRFP-tagged IC-2. We also showed that endogenous IC-2 interacts with endogenous CCT8. The C-terminus of IC-2 is necessary and sufficient to bind CCT8. However, additional experiments did not confirm phosphorylation-regulated binding of CCT8 to IC-2. Interestingly, the binding of CCT8 to mRFP-IC-2 was abolished by prior treatment of cultures with cycloheximide, suggesting that CCT binds to newly synthesized IC-2. *In vitro* translation experiments demonstrated that recombinant CCT complex is able to associate with newly synthesized IC-2 after its release from ribosomes. These data are consistent with a role for CCT in folding newly synthesized IC-2.

2. Materials and methods

2.1. Cell culture and drug treatment

A375 melanoma cells were obtained from ATCC. REF52 rat embryo fibroblasts were obtained from Dr. Tom Parsons (University of Virginia). After transfection with appropriate vectors, A375 cells were treated with 25 μM UO126 (Sigma) for 3 h, 50 μg/ml cycloheximide (CHX) (Sigma) for 5 h, or appropriate vehicle controls.

2.2. Plasmids

Rat intermediate chain 2C constructs were used in this study. mRFP-tagged IC-2C constructs are described in Mitchell et al., 2012 [8]. IC-2C was HA tagged at the C-terminus using standard techniques (Machida, unpublished). HA-tagged CCT8 is described by Machida et al. [12]. Myc-tagged IC-2 constructs are described in Lo et al. [13].

2.3. Transfection

The day before transfection A375 melonoma cells (5.8×10^6) were seeded in 100 mm dishes. 8 μg of indicated plasmids (mRFP, mRFP-tagged wild type, mRFP-tagged S81A, mRFP-tagged S81D, mRFP-tagged S81E IC-2, HA-tagged CCT8, Myc-tagged IC-2 and pcDNA3.1) were transfected according to manufacturer's instructions using Lipofectamine 2000 (Life Technologies).

For transfection of REF52, 8×10^5 cells were plated in 100 mm dishes. 2.5 μg of indicated plasmids (mRFP, mRFP-tagged wild type, mRFP-tagged S81A) were transfected according to manufacturer's instructions using Superfect Reagent (Qiagen).

2.4. Subcellular fractionation

To isolate the membrane fraction cells were scraped into media and pelleted at 500 rpm for 5 min. Pellets were re-suspended in ice-cold CB buffer (38 mM each of the potassium salts of aspartic, gluconic, and glutamic acids; 20 mM MOPS; 5 mM reduced glutathione; 10 mM potassium bicarbonate; 0.5 mM magnesium carbonate; 1 mM EGTA; 1 mM EDTA, pH 7.1, 1 mM PMSF, 3 mM benzamidine, 10 μg/ml each of leupeptin and pepstatin, 10 nM microcystin LR, 1 mM sodium vanadate, 1 μM calyculin). Cells were homogenized with 8 passes through a ball bearing homogenizer (Isobiotec; 8 micron clearance). After centrifuging the extract twice at 1000 × g for 10 min, the supernatant was centrifuged at 100,000 × g for 60 min at 4 °C. The pellet was resuspended with CB buffer.

2.5. Immunoprecipitation and immunoblotting

For whole cell lysates cells were scraped into media and centrifuged at 500 rpm for 5 min. Pellets were lysed by pipeting in Flag buffer (50 mM Tris–HCl pH 7.3, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM EDTA and 0.5 mM EGTA) containing protease and phosphatase inhibitors (1 mM PMSF, 3 mM benzamidine, 10 μg/ml each of leupeptin and pepstatin, 10 nM microcystin LR, 1 mM sodium vanadate, 1 μM calyculin). Membrane fraction or whole cell lysate was immunoprecipitated with appropriate antibody for 2.5 h at 4 °C. After antibody incubation, samples were rotated for 45 min with Dynabeads Protein G (Invitrogen) at 4 °C. After washing, proteins were eluted using 0.2 M glycine pH2 for 30 min. Immunoprecipitates were separated by 10% SDS-PAGE and then transferred to Immobilon-P membrane. After blocking, membranes were incubated with anti-CCT5 (Abcam), anti-CCT8 (Abcam), anti-RFP (Abcam), anti-IC 74.1 [14], anti-$p150^{Glued}$ (BD), or anti-HA (12CA5) primary antibodies. Membranes were probed with appropriate HRP-conjugated secondary antibodies and developed using chemiluminescence reagents (Pierce).

2.6. Identification of IC-2 partners

Crude membrane fractions were immunoprecipitated with anti-RFP antibody after transfection with mRFP-tagged wild type (WT), S81A IC-2 or empty vector. Immunoprecipitates were resolved using a 8–15% gradient SDS-PAGE gel, stained with Coomassie Blue and candidate bands were excised for Mass Spectrometry and SWISS-PROT 112011 database interrogation using techniques described in Ref. [7].

2.7. In vitro translation of IC-2 and CCT binding assays

To examine whether newly synthesized IC-2 polypeptide is able to bind CCT complex *in vitro*, we translated IC-2C using a reconstituted eukaryotic translation system constructed using purified human translation factors [15]. Briefly, C-terminal HA-tagged IC-2 was synthesized in the reconstituted translation system in the presence or absence of purified human recombinant CCT complex (0.48 μM final concentration) [12] for 4 h at 32 °C. Puromycin was added to a final concentration of 1 mM to stop translation and release newly synthesized peptides from ribosomes. After 30 min incubation at 32 °C, CCT was added to those reaction mixtures not containing CCT. After a further 30 min incubation at 32 °C, each reaction mixture was divided into two aliquots. One aliquot was resolved by SDS-PAGE (12.5%) and the other was separated by Native-PAGE (3% stacking gel and 6% running gel containing 25 mM Tris–HCl pH8.8, 190 mM Glycine and 0.1 mM ATP) followed by Western blotting with anti-HA antibody.

3. Results

3.1. T-complex protein 1 interacts with cytoplasmic dynein intermediate chain 2C

Previous study has shown that IC-2 is phosphorylated by ERK at