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Mitotic Phosphorylation of the Dynein Light Intermediate Chain is Mediated by cdc2 Kinase

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Cytoplasmic dynein, a large minus-end-directed microtubule motor, performs multiple functions during the cell cycle. In interphase, dynein moves membrane organelles, while in mitosis it moves chromosomes and helps to form the mitotic spindle. The cell-cycle regulation of dynein activity may be controlled, at least in part, by the phosphorylation of its light intermediate chains (DLIC), since a 10-fold increase in light intermediate chain phosphorylation correlates with a decrease in dynein-based membrane transport of similar magnitude in mitosis. In this study, we sought to identify the kinase responsible for this potentially important phosphorylation event. We show that bacterially-expressed chicken light intermediate chain (chDLIC) will undergo mitosis-specific phosphorylation when added to *Xenopus* egg extracts. Mutation of a conserved cdc2 kinase consensus site (Ser197) abolishes this phosphorylation event, and mass spectroscopy analysis confirms that the wild-type DLIC is stoichiometrically phosphorylated at this site when incubated with metaphase but not interphase extracts. We also show that purified cdc2 kinase phosphorylates purified DLICs at Ser197 *in vitro* and that Ser197 phosphorylation is dramatically reduced in metaphase extracts depleted of cdc2 kinase. These results indicate that cdc2 kinase directly phosphorylates dynein and thus may be an important regulator of dynein activity in the cell cycle.

Key words: cdc2 kinase, dynein, organelle transport, mitosis, phosphorylation

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Cytoplasmic dynein is a multi-subunit motor that moves toward the minus ends of microtubules (1–3). Cytoplasmic dynein contains two heavy chains (~500 kDa) that form two large globular domains containing the ATP and microtubule binding sites. This motor also contains several intermediate chains (DIC; ~74 kDa), light intermediate chains (DLIC; 53–59 kDa) and light chains (DLC; 8–22 kDa), which together may form a cargo-binding region of the dynein holoenzyme. In addition to this diversity of subunits, two DLIC genes have been discovered (4,5), and several isoforms of the DIC subunits are likely to exist as well (6,7). The functions of the cytoplasmic dynein polypeptides, other than the heavy chains, are still poorly understood, although the DICs appear to facilitate the binding of dynein to dynactin, a 20S complex that is thought to dock dynein onto subcellular structures (8–10).

Cytoplasmic dynein is required for many different processes throughout the cell cycle. During interphase, dynein is necessary for maintaining the structure and localization of the Golgi apparatus (Golgi), endosomes, and lysosomes, as shown by the analysis of cells derived from a dynein knockout mouse (11). Disruption of dynactin has also revealed dynein's importance in governing membranous organelle distribution (12), as well as endoplasmic reticulum (ER) to Golgi transport (13). During mitosis, however, dynein dissociates from organelles and localizes to the centrosomes, kinetochores, and the mitotic spindles (14–17). Injection of dynein antibodies resulted in the disruption of the mitotic spindle (18), and disruption of dynein–dynactin interaction by the overexpression of dynamitin (the p50 subunit of dynactin) caused prometaphase arrest (16). In *Saccharomyces cerevisiae* cells, dynein is required for the migration of the dividing nucleus into the bud (19,20).

While cytoplasmic dynein's importance throughout the cell cycle is well established, the spatial and temporal regulation of its activity is not well understood. Post-translational modification represents one potential mechanism for regulating dynein function, and cytoplasmic dynein phosphorylation has been documented in several studies (7,21–23). However, the only known cell-cycle regulated phosphorylation of dynein is the metaphase-specific phosphorylation of the DLIC (24). The 10-fold increase in the phosphorylation of DLIC during metaphase correlated with decreases of a similar magnitude in minus-end-directed organelle movement as well as with the amount of dynein associated with membrane organelles. These results suggest that phosphorylation of the DLIC may be important for the cell-cycle regulation of dynein activity.

In this study, we sought to identify the kinase responsible for metaphase-specific phosphorylation of DLIC. Using *Xenopus* egg extracts as a source of metaphase- and interphase-specific proteins and a combination of protein mutagenesis and mass spectroscopy, we identified a cdc2 kinase consensus sequence as the primary mitotic-specific phosphorylation site for DLIC. In addition, purified cdc2 kinase phosphorylated purified DLIC at this site *in vitro*, and a metaphase extract depleted of cdc2 no longer efficiently phosphorylated DLIC. Together these results show that cdc2 acts as a direct dynein kinase and hence, may be involved in the mitotic control of dynein activity.

Results and Discussion

Dynein LIC is phosphorylated in metaphase extracts

When cell-cycle arrested *Xenopus* egg extracts were immunoblotted with an antibody raised against chicken DLIC (chDLIC) (4), polypeptide bands of 62 kDa (Figure 1A, lane I) and 67 kDa (lane M) were recognized in interphase and metaphase extracts, respectively. The slower sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mobility of DLIC in metaphase is consistent with previous results demonstrating that *Xenopus* DLIC is phosphorylated specifically during metaphase (24). To facilitate further experiments to identify the phosphorylation site on DLIC, we engineered a RGS-H_6 tag onto the N-terminus of a chDLIC cDNA (4) for purification and immunoblotting with a commercial antibody directed specifically against this sequence. The chDLIC clone was used, since DLIC is well conserved among several species and since a *Xenopus* clone was not available. We found that bacterially-expressed chDLIC was soluble and could be purified to $>70\%$ purity using metal affinity and ion exchange chromatography (see Materials and Methods).

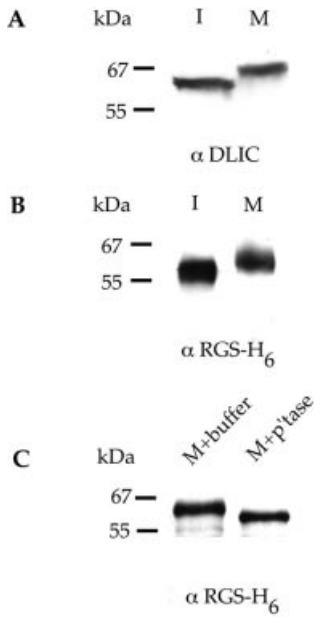

Figure 1: Bacterially-expressed chDLIC is phosphorylated in metaphase HSS. (A) 3 ml of interphase (I) and metaphase (M) high speed supernatant (HSS) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an affinity-purified antibody to DLIC. (B) Bacterially-expressed, purified, chDLIC was incubated with interphase (I) or metaphase (M) HSS and immunoblotted with a RGS-H_6 tag antibody. (C) chDLIC was incubated with metaphase HSS then bound to Ni-NTA beads, washed and incubated with either buffer (50 mM Tris pH 7.8, 2 mM $MgCl_2$, 0.1 mg/ml BSA) (M + buffer) or buffer containing 1 U/ml lambda phosphatase (M + p'tase) for 30 min at room temperature. Protein was eluted from the beads and immunoblotted with a RGS-H_6 antibody. The slower migration of DLIC in metaphase extracts is due to phosphorylation, since it can be reversed by phosphatase treatment.

To investigate whether the purified chDLIC could be phosphorylated in a *Xenopus* metaphase extract, we added the protein to interphase and metaphase extracts and blotted with the RGS-H_6 antibody (Figure 1B, lanes I and M). The chDLIC migrated with an apparent molecular weight of 58 kDa in the interphase extract, but shifted to 62 kDa in the metaphase extract. To ensure that this mobility shift was due to phosphorylation, we recovered the chDLIC following incubation in the metaphase extract by using Ni-NTA beads and then treated the isolated protein with lambda phosphatase or buffer alone (Figure 1C). Phosphatase treatment, but not the buffer control, reversed the mobility shift, demonstrating that the mobility shift must be due to phosphorylation and not another post-translational modification. In addition, we added γ-^{32}P-ATP to interphase and metaphase extracts and measured the incorporation of ^{32}P into chDLIC (Figure 2C). A low level of phosphate incorporation is seen when chDLIC was added to interphase extracts (Figure 2C, lane 1), but when chDLIC was added to metaphase extracts, the level of incorporation increased 22.5 ± 0.8-fold (mean and SD from two independent experiments). Thus, the *Xenopus* metaphase-specific DLIC kinase recognizes the bacterially-expressed chDLIC.

DLIC is phosphorylated at a conserved cdc2 kinase consensus site

When the DLIC sequences from a number of species were compared, we noted the presence of a single conserved cdc2 kinase site (SPXRR; (where X is any amino acid) amino acids 197–201 in chicken; Figure 2A). Recognition sites for other kinases such as cAMP-dependent kinase, MAP kinase, casein kinase II, and glycogen synthase kinase-3 were present in DLIC (not shown). However, either the sites were not conserved or the kinases involved are generally not considered to be cell-cycle regulated. To test if DLIC was phosphorylated at the cdc2 site during metaphase, we mutated the serine in this sequence to an alanine (S197A chDLIC). When bacterially-expressed S197A chDLIC was added to a metaphase extract, it did not undergo a mobility shift as was seen with the wild-type protein (Figure 2B). Furthermore, while wild-type DLIC exhibited a 22-fold increase in phosphate incorporation from γ-^{32}P-ATP when added to metaphase versus interphase extracts, S197A DLIC exhibited only a 4.0 ± 0.6-fold increase in phosphate incorporation (mean and standard deviation from two independent experiments) (Figure 2C).

To confirm that Ser197 of chDLIC is phosphorylated during metaphase, we incubated wild-type chDLIC with interphase or metaphase extracts, purified chDLIC from the extracts by Ni-NTA chromatography, digested the protein with trypsin, and analyzed the resultant tryptic peptide mixture by mass spectrometry (see Materials and methods). The tryptic digestion of interphase-treated chDLIC resulted in a signal at 1056, which corresponds to mass to a charge

ratio (m/z) for the peptide ion DFQEYVEPGEDFPASPQR ($z = 2$ for this peptide). In contrast, the metaphase-treated chDLIC showed a signal at 1096, but none at 1056. The increase in the mass to charge ratio is that expected for phosphorylated peptide ion DFQEYVEPGEDFPA(pS)PQR. The slower mobility in SDS-PAGE combined with the mass spectrometry results indicate that chDLIC becomes quantitatively phosphorylated at Ser197 during metaphase.

Cdc2 kinase phosphorylates DLIC in vitro and in extracts

The results described above suggested that cdc2 kinase may be the kinase that phosphorylates DLIC at metaphase.

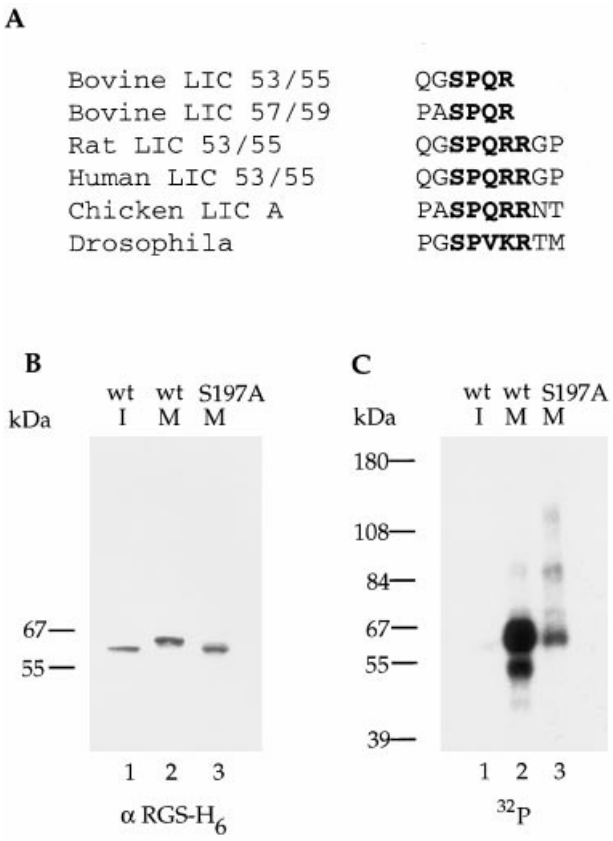

Figure 2: Mutation of a conserved cdc2 kinase recognition site significantly reduces DLIC phosphorylation. (A) Sequence comparison identified a cdc2 kinase recognition sequence, shown in bold, conserved in DLIC from several species. Sequences are from the following sources: bovine DLIC tryptic peptides (5), rat DLIC 53/55 (5), human DLIC 53/55 (Genbank accession number O43237), chicken LIC A (4), and Drosophila (EST LD30603). (B) Wild-type chDLIC was incubated with interphase (lane 1) and metaphase (lane 2) HSS and S197A chDLIC was incubated with metaphase HSS (lane 3), separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with a RGS-H_6 antibody. (C) Incubations were done as in panel B in the presence of γ-^{32}P-ATP. chDLIC was recovered from the reaction on Ni-NTA beads, run on SDS-PAGE, and visualized by autoradiography. The S197A mutation greatly reduces phosphate incorporation into chDLIC in metaphase extracts.

To test this hypothesis *in vitro*, we incubated purified cdc2 kinase (activated by preincubation with cyclin B and cdk-activating kinase (CAK) (25)) with purified chDLIC in the presence of γ-^{32}P-ATP and measured phosphate incorporation. Active cdc2 kinase phosphorylated wild-type DLIC (Figure 3). Phosphate incorporation was not observed either when DLIC was incubated with γ-^{32}P-ATP alone (Figure 3, lane 1) or when the cdc2 kinase had not been activated by cyclin B (data not shown). Thus, phosphorylation is not due to a contaminating kinase in the DLIC preparation. CAK alone also did not phosphorylate DLIC (data not shown). For the S197A chDLIC mutant, phosphorylation by active cdc2 kinase was reduced by 20.8 ± 5.6-fold (mean and standard deviation from two independent experiments) (Figure 3). One possible explanation for this low level of phosphorylation is that chDLIC may contain a cryptic recognition site that becomes phosphorylated by cdc2 kinase *in vitro* in the absence of other substrates. Thus cdc2 kinase phosphorylates DLIC *in vitro* with Ser197 being the preferred site.

To determine whether cdc2 kinase is indeed the kinase that phosphorylates DLIC in cell free extracts, we depleted metaphase extracts of cdc2 by chromatography on beads coupled with p13, a protein that binds to cdc2 kinase with high affinity (26). Mock-depleted extracts were prepared by chromatography on bovine serum albumin (BSA)-coupled beads. p13 chromatography depleted $> 90\%$ of the cdc2 kinase, as determined by immunoblotting the extracts with an antibody to cdc2 (Figure 4A). Chromatography of metaphase extracts on BSA-coupled beads resulted in the loss of some cdc2 protein, likely due to non-specific interactions of the kinase with the column resin. Wild-type chDLIC that was incubated with control metaphase extract or with mock-depleted extracts migrated at a position typical of the phosphorylated protein by SDS-PAGE. However, chDLIC that was incubated with a metaphase extract depleted of cdc2 migrated faster, indicating that it was not phosphorylated (Figure 4B). These results demonstrate that cdc2 is the predominant kinase that phosphorylates DLIC in metaphase extracts.

Conclusion

Dynein-mediated organelle motility and dynein binding to membranes both decrease dramatically when an interphase *Xenopus* egg extract is converted to a metaphase state (24,27). DLIC is the only dynein subunit that shows a strong increase in phosphate incorporation in metaphase (24), which suggests that DLIC phosphorylation may regulate dynein cargo-binding activity during the cell cycle. In this work, we have demonstrated that cdc2 is the primary kinase that phosphorylates DLIC specifically in metaphase. This conclusion is supported by several experiments. First, mutation of the cdc2 kinase site (S197A) blocked the metaphase extract-induced mobility shift of chDLIC on SDS-PAGE and substantially decreased ^{32}P incorporation into chDLIC. Second, mass spectroscopy confirmed that

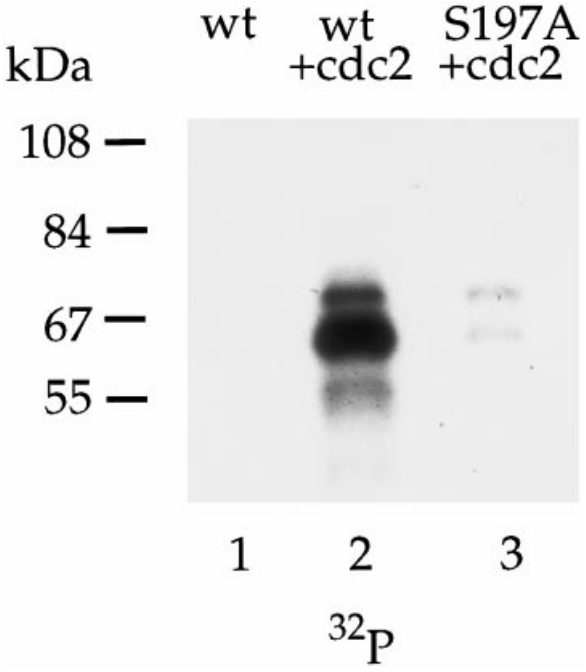

Figure 3: Cdc2 phosphorylates chDLIC in vitro. Wild-type chDLIC alone (lane 1), wild-type chDLIC plus cdc2, cyclin B and CAK (lane 2) or S197A chDLIC plus cdc2, cyclin B and CAK (lane 3) were incubated in the presence of γ-^{32}P-ATP. The reaction was run on SDS-PAGE and visualized by autoradiography. Wild-type chDLIC, but not the S197A mutant, is efficiently phosphorylated by activated cdc2 kinase.

chDLIC was phosphorylated at Ser197 in metaphase but not in interphase extracts. Third, cdc2 kinase phosphorylated DLIC at Ser197 *in vitro*, and finally, cdc2 kinase depleted metaphase extracts did not phosphorylate DLIC efficiently. Taken together, these results establish DLIC as a direct substrate for cdc2 kinase.

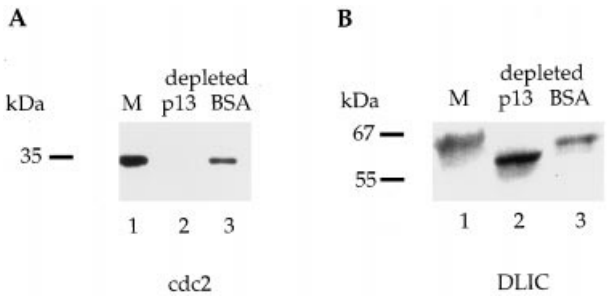

Figure 4: Metaphase HSS depleted of cdc2 kinase does not phosphorylate chDLIC. (A) Control metaphase HSS (lane 1), metaphase HSS depleted of cdc2 by p13 chromatography (lane 2), and metaphase HSS mock depleted by BSA chromatography (lane 3) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an antibody to cdc2 kinase. (B) chDLIC was added to the above supernatants for 30 min and then immunoblotted. chDLIC is not efficiently phosphorylated in the cdc2 kinase depleted extracts.

Our results demonstrate that Ser197 is the major site in chDLIC for metaphase-specific phosphorylation, and mass spectroscopy data indicates that this serine is stoichiometrically phosphorylated in metaphase. However, our results do not rule out the possibility that other mitotic kinases may also be important in regulating dynein activity. Indeed, the finding that the S197A chDLIC is phosphorylated at a 4-fold higher level in the metaphase versus the interphase extract suggests that there is either another cdc2 phosphorylation site or that another mitotically-activated kinase phosphorylates DLIC at a lower level. The possibility of a second cdc2 kinase site in DLIC is raised by the finding that purified cdc2 kinase will phosphorylate purified S197A chDLIC mutant *in vitro*. However, the level of this phosphorylation is very low, and sequence inspection does not reveal a clear cdc2 consensus sequence other than the one containing Ser197.

The Ser197 cdc2 kinase site is conserved in DLICs from a number of organisms and in both DLIC genes identified in rat and chicken (Figure 2A). While such conservation implies that cdc2 phosphorylation of DLIC is important for regulating dynein activity, its precise function is not clear. Dynein is localized to membranous organelles during interphase, but at metaphase it dissociates from membranes and re-localizes to structures in the mitotic spindle (14–17). Thus, it is tempting to speculate that cdc2 phosphorylation of DLIC is involved in the dissociation of dynein from organelles and its relocalization to the spindle. Organelle dissociation does not appear to involve a lower affinity of dynein for dynactin, however, since metaphase and interphase dynein both interact equally well with columns containing bound p150^{glued}, the dynein interacting subunit of the dynactin complex (S Karki, K Dell, E Holzbaur, and R Vale, unpublished results). It is also seems likely that the dramatic redistribution of cytoplasmic dynein in the cell cycle requires post-translational modification of several proteins, and not just the single cdc2 phosphorylation event described in this study.

The activities of many microtubule motor proteins are likely to change during the cell cycle. Regulation by phosphorylation also has been implicated for the bipolar kinesin motor (named Eg5 in *Xenopus* and KLP61F in *Drosophila*). Bipolar kinesin is required for centrosome separation and bipolar spindle formation, and direct cdc2 kinase phosphorylation appears to be necessary for Eg5 to associate with the spindle (28–30). Since dynein and several types of kinesin motors are cooperatively involved in spindle formation and chromosome alignment at the metaphase plate (31), it seems plausible that cdc2 may regulate the activity of multiple microtubule motors in a coordinated manner.

Materials and Methods

Xenopus egg extracts and high speed supernatants

Cytostatic factor arrested (metaphase) extracts were made from *Xenopus* eggs as described by Murray (32). Metaphase extracts were converted to interphase extracts by the addition of 0.5 mM $CaCl_2$ and incubation at room temperature for 45 min followed by the addition of 0.5 mM EGTA. Extracts were made from five or six frogs, and half of the extract was converted to interphase form. Extracts were stored in liquid nitrogen. To make high speed supernatants, 1 ml aliquots of extract were thawed, diluted with 1 volume of acetate buffer (10 mM Hepes pH 7.4, 100 mM K-acetate, 150 mM sucrose, 5 mM EGTA, 3 mM Mg-acetate) plus 0.1 mM PMSF and 10 μg/ml of leupeptin, aprotinin, and pepstatin and then centrifuged at $110000 \times g_{max}$ for 30 min at 2°C in a TLA 120.1 rotor (Beckman, Palo Alto, CA). High speed supernatants, typically 20–30 mg/ml protein concentration, were stored at −80°C for up to 2 months.

Cloning and mutagenesis

The chDLIC (chDLIC) cDNA clone 58.2 (4) was obtained from S Gill and T Schroer (Johns Hopkins University) and was used as a template for further sequence changes using Stratagene's QuikChange protocol (Stratagene USA, La Jolla, CA). An oligonucleotide was designed to add a RGS-H_6 tag immediately downstream of the start methionine and also to add a restriction site that could be used for screening colonies for correctly mutagenized plasmids. A 200 bp Nde1-Ava1 fragment, including the start methionine and the tag, was subcloned back into the original cDNA 58.2, and this region of the clone was sequenced. Similarly, oligonucleotides were designed to create the S197A and S197E mutants, and the histidine-tagged chDLIC was used as a template for creating the mutants. The polymerase chain reaction (PCR) products were sequenced.

Bacterial expression and purification of chDLIC

Freshly transformed BL21 were grown at 37°C in 1 l of Luria Broth with 50 μg/ml ampicillin to an OD_{600} of 0.5 and then induced with 0.4 mM IPTG and grown for 3 h more before harvesting. The chDLIC was partially purified by Ni-NTA chromatography (Qiagen, Chatsworth, CA) as follows. The pelleted bacteria were resuspended in loading buffer (50 mM NaPhosphate pH 8, 300 mM NaCl) with 0.2% Tween 20 and protease inhibitors (10 μg/ml leupeptin, pepstatin, and aprotinin, and 0.1 mM PMSF). Bacteria were lysed by two passes through a Microfluidizer (Microfluidics Corp. Newton, MA) at 140 psi, and the lysate was clarified by centrifugation at $10000 \times g$ for 20 min at 4°C. Imidazole was added to the supernatant to a concentration of 20 mM and the supernatant was equilibrated with 3 ml of Ni-NTA resin on a rotator for 1 h at 4°C. The resin was poured into a column and first washed with 30 ml wash buffer (50 mM NaPhosphate pH 6, 300 mM NaCl) followed by 30 ml wash buffer with 60 mM imidazole. chDLIC was eluted with 400 mM imidazole in wash buffer. chDLIC was then further purified by chromatography on Mono Q resin. The chDLIC pool from the Ni-NTA column was diluted to 50 ml with buffer A (20 mM Tris pH 8, 2 mM $MgCl_2$, 10% w/v glycerol, 0.1% Tween 20) and loaded onto a 2 ml Mono Q column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with 10 ml buffer A and protein was eluted with a gradient of 0–500 mM NaCl in buffer A. chDLIC eluted at ~200 mM NaCl and the peak fraction was usually 0.5 mg/ml protein as determined by Bradford assay.

Polyacrylamide gel electrophoresis and immunoblotting

Protein samples were separated on either 10 or 4–12% gradient polyacrylamide gels following standard Laemmli procedures. For ^{32}P-labeled samples, gels were stained, dried and exposed to X-ray film (Hyperfilm, Amersham Pharmacia Biotech, NJ). Immunoblotting was performed by transferring protein to nitrocellulose membranes at 0.3 A for 1 h. Incubations with primary antibodies were overnight at 4°C at the following dilutions: DLIC (4) 1:500, RGS-H_6 (Qiagen, CA) 1:1000, cdc2 kinase (Santa Cruz Biotechnology, Santa Cruz, CA) 1:500. Blots were then washed and incubated with HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, NJ) for 1 h at room temperature and developed using chemiluminescent reagent (NEN, Boston, MA).

Phosphorylation assays

To detect phosphorylation of chDLIC in high speed supernatants, 1–2 μl of purified, bacterially-expressed chDLIC were incubated with 10–15 μl of interphase or metaphase high speed supernatants, 10 mM $MgCl_2$ and 10 μM ATP in a total volume of 20 μl, for 30 min at 22°C. The reaction was stopped by the addition of sample buffer and boiled for 2 min. The sample was then loaded on SDS-PAGE and any change in the mobility of chDLIC was detected by RGS-H_6 immunoblotting. To measure the incorporation of radioactive phosphate into chDLIC, 20 μl of purified chDLIC was incubated with 70 to 80 μl of high speed supernatant, 10 mM $MgCl_2$ and 10 μCi γ-^{32}P-ATP (Amersham Pharmacia Biotech, NJ) in a volume of 100 μl for 30 min at 22°C. The reaction was stopped by adding 20 μl of Ni-NTA resin equilibrated in 100 μl of cold loading buffer with 10 mM sodium pyrophosphate, 80 mM sodium β-glycerolphosphate, and 1.5 μM microcystin, followed by rotation for 1 h at 4°C. The beads were pelleted and washed with Ni-NTA wash buffer and with wash buffer containing 100 mM imidazole. Labeled protein was eluted from the beads with 10–20 μl 2× sample buffer and boiling to minimize the volume for loading on SDS-PAGE. Gels were dried and phosphorylated proteins visualized by autoradiography.

The *in vitro* phosphorylation of chDLIC by cdc2 kinase was performed as follows. To activate cdc2 kinase, 3 μl of haemagglutinin peptide-tagged cdc2 kinase (20 ng/μl) (C Takizawa and DO Morgan, University of California, San Francisco) were incubated with 1 μl of 6× His, myc-tagged cyclin B (90 ng/μl), for 15 min at 22°C, followed by a second 15 min incubation with 1 μl of cdk-activating kinase (12 ng/μl) and 15 μl of buffer to give a final concentration of 25 mM Hepes, 100 μM ATP, 1 mM DTT and 10 mM $MgCl_2$. 100–200 ng of purified chDLIC were added to the reaction with 1 μl of γ-^{32}P-ATP (10 μCi/ml). After 30 min at 22°C, the reaction was stopped by the addition of sample buffer and proteins were separated by SDS-PAGE. The gel was dried and protein phosphorylation was detected by autoradiography.

For dephosphorylation of chDLIC, lambda protein phosphatase from New England Biolabs (#753) was used at 1 U/ml for 30 min at room temperature. The phosphatase was >95% pure and contained no detectable protease activity.

For quantitation of phosphorylation, autoradiograms images were digitized, and the band intensities were quantitated using NIH Image v. 1.62.

Mass spectroscopy

chDLIC treated with interphase or metaphase extracts was purified by Ni-NTA chromatography (see phosphorylation assays above) and

subjected to SDS-PAGE followed by Coomassie-Blue staining. The protein bands were then excised from the gel, cut into small pieces, and subjected to an in-gel digestion with porcine trypsin (Promega, Madison, WI) (33). After overnight digestion, the tryptic peptides were extracted from the gel and desalted over a 100 nl POROS R2 resin (PE Biosystems, Foster City, CA) in a gel-loader pipette tip. The peptides were eluted from the resin into a nanospray glass capillary (PROTANA, Odense, Denmark) using 1 μl of 50% methanol/5% formic acid (34). The peptide solution was then infused into an LCQ mass spectrometer (FinniganMat, San Jose, CA). For the detection of the peptide ions DFQEYVEPGEDFPASPQR and DFQEYVEPGEDFPA(pS)PQR, zoom scans were performed.

p13 Chromatography

The pRK172p13 cDNA encoding p13 was generously provided by W Dunphy and A Kumagai (California Institute of Technology). p13 protein, which binds with high affinity to cdc2 kinase, was expressed and purified as previously described (35). Following exchange into coupling buffer, p13 was bound to CNBr Sepharose 4B (Amersham Pharmacia Biotech, NJ) following the manufacturers instructions. To deplete cdc2 from metaphase high speed supernatants, 20 μl of supernatant were incubated with 40 μl of p13 beads for 30 min at 4°C. Following a brief centrifugation, the supernatant was removed and added to another 40 μl of beads for 30 min at 4°C. The supernatant from these beads was used as cdc2-depleted metaphase high speed supernatant in standard phosphorylation assays. Although a single incubation with p13 beads removed most of the cdc2 protein (70%), as judged by immunoblotting with cdc2 antibody, we found a second incubation was necessary to remove $>90\%$ of the cdc2. Bovine serum albumin coupled to a CNBr Sepharose 4B column in the manner described above served as a control.

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References

1. Karki S, Holzbaur ELF. Cytoplasmic dynein and dynactin in cell division and intracellular transport. Curr Opin Cell Biol 1999;11: 45–53.
2. Holzbaur EL, Vallee RB. Dyneins: molecular structure and cellular function. Annu Rev Cell Biol 1994;10: 339–372.
3. Hirokawa N. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science 1998;279: 519–526.
4. Gill SR, Cleveland DW, Schroer TA. Characterization of DLC-A and DLC-B, two families of cytoplasmic dynein light chain subunits. Mol Biol Cell 1994;5: 645–654.
5. Hughes SM, Vaughan KT, Herskovits JS, Vallee RB. Molecular analysis of a cytoplasmic dynein light intermediate chain reveals homology to a family of ATPases. J Cell Sci 1995;108: 17–24.
6. Paschal BM, Mikami A, Pfister KK, Vallee RB. Homology of the 74-kD cytoplasmic dynein subunit with a flagellar dynein polypeptide suggests an intracellular targeting function. J Cell Biol 1992;118: 1133–1143.
7. Dillman JFR, Pfister KK. Differential phosphorylation in vivo of cytoplasmic dynein associated with anterogradely moving organelles. J Cell Biol 1994;127: 1671–1681.
8. Vaughan KT, Vallee RB. Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150glued. J Cell Biol 1995;131: 1507–1516.
9. Karki S, Holzbaur ELF. Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. J Biol Chem 1995;270: 28806–28811.
10. Holleran EA, Karki S, Holzbaur EL. The role of the dynactin complex in intracellular motility. Int Rev Cytol 1998;182: 69–109.
11. Harada A, Takei Y, Kanai Y, Tanaka Y, Nonaka S, Hirokawa N. Golgi vesiculation and lysosome dispersion in cell lacking cytoplasmic dynein. J Cell Biol 1998;141: 51–59.
12. Burkhardt JK, Echeverri CJ, Nilsson T, Vallee RB. Overexpression of the dynamitin (p50) subunit of dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J Cell Biol 1997;139: 469–484.
13. Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J. ER-to-Golgi transport visualized in living cells. Nature 1997;389: 81–85.
14. Steuer ER, Wordeman L, Schroer TA, Sheetz MP. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. Nature 1990;345: 266–268.
15. Pfarr CM, Coue M, Grissom PM, Hays TS, Porter ME, McIntosh JR. Cytoplasmic dynein is localized to kinetochores during mitosis. Nature 1990;345: 263–265.
16. Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB. Molecular characterization of the 50 kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. J Cell Biol 1996;132: 617–633.
17. Lin SXH, Collins CA. Immunolocalization of cytoplasmic dynein to lysosomes in cultured cells. J Cell Sci 1992;101: 125–137.
18. Vaisberg EA, Koonce MP, McIntosh JR. Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. J Cell Biol 1993;123: 849–858.
19. Eshel D, Urrestarauzu LA, Vissers S, et al. Cytoplasmic dynein is required for normal nuclear segregation in yeast. Proc Natl Acad Sci USA 1993;90: 11172–11176.
20. Li YY, Yeh E, Hays T, Bloom K. Disruption of mitotic spindle orientation in a yeast dynein mutant. Proc Natl Acad Sci USA 1993;90: 10096–10100.
21. Lin SX, Ferro KL, Collins CA. Cytoplasmic dynein undergoes intracellular redistribution concomitant with phosphorylation of the heavy chain in response to serum starvation and okadaic acid. J Cell Biol 1994;127: 1009–1019.
22. Pfister KK, Salata M, Dillman JF, et al. Differential expression and phosphorylation of the 74-kDa intermediate chains of cytoplasmic dynein in cultured neurons and glia. J Biol Chem 1996;271: 1687–1694.
23. Karki S, Tokito MK, Holzbaur ELF. Casein kinase II binds to and phosphorylates cytoplasmic dynein. J Biol Chem 1997;272: 5887–5891.
24. Niclas J, Allan VJ, Vale RD. Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. J Cell Biol 1996;133: 585–593.
25. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 1997;13: 261–291.
26. Dunphy WG, Brizuela L, Beach D, Newport J. The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell 1988;54: 423–431.
27. Allan VJ, Vale RD. Cell cycle control of microtubule-based membrane transport and tubule formation *in vitro*. J Cell Biol 1991;113: 347–359.
28. Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg E. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation *in vivo*. Cell 1995;83: 1159–1169.
29. Sawin KE, Mitchison TJ. Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. Proc Natl Acad Sci USA 1995;92: 4289–4293.

30. Sharp DJ, McDonald KL, Brown HM, et al. The bipolar kinesin, KLP61F, cross-links microtubules within interpolar microtubule bundles of *Drosophila* embryonic mitotic spindles. J Cell Biol 1999;144: 125–138.
31. Walczak CE, Mitchison TJ. Kinesin-related proteins at mitotic spindle poles: function and regulation. Cell 1996;85: 943–946.
32. Murray AW. Cell cycle extracts. Meth Cell Biol 1991;36: 581–605.
33. Hellman U, Wernstedt C, Gonez J, Heldin CH. Improvement of an in-gel digestion procedure for the microsequencing of internal protein fragments for amino acid sequencing. Anal Biochem 1995;224: 451–455.
34. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal Chem 1996;68: 850–858.
35. Brizuela L, Draettta G, Beach D. p13suc1acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase. EMBO J 1987;6: 3507–3514.