Phosphorylation by cdc2-CyclinB1 Kinase Releases Cytoplasmic Dynein from Membranes*

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Movement of various cargoes toward microtubule minus ends is driven by the microtubule motor cytoplasmic dynein (CD). Many cargoes are motile only during certain cell cycle phases, suggesting that CD function may be under cell cycle control. Phosphorylation of the CD light intermediate chain (DLIC) has been suggested to play a crucial role in modulating CD function during the Xenopus embryonic cell cycle, where CD-driven organelle movement is active in interphase but greatly reduced in metaphase. This down-regulation correlates with hyperphosphorylation of DLIC and release of CD from the membrane. Here we investigate the role of the key mitotic kinase, cdc2-cyclinB1, in this process. We show that DLIC within the native Xenopus CD complex is an excellent substrate for purified Xenopus cdc2-glutathione S-transferase (GST) cyclinB1 (cdc2-GSTcyclinB1) kinase. Mass spectrometry of native DLIC revealed that a conserved cdc2 site (Ser-197) previously implicated in the metaphase modulation of CD remains phosphorylated in interphase and so is unlikely to be the key regulatory site. We also demonstrate that incubating interphase membranes with cdc2-GSTcyclinB1 kinase results in substantial release of CD from the membrane. These data suggest that phosphorylation of DLIC by cdc2 kinase leads directly to the loss of membrane-associated CD and an inhibition of organelle movement.

Cytoplasmic dynein (CD) is a multiprotein complex which binds to microtubules and moves along them using energy from ATP hydrolysis. This movement is directed toward microtubule minus ends and allows CD to move and/or position other structures (cargo) within eukaryotic cells (1, 2). CD is implicated in many important processes within eukaryotic cells during interphase, including vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus, centripetal movement of lysosomes, nuclear migration, and maintenance of organelle structure (3). During cell division, it is likely that organelle movement is inactivated (4), while CD continues to function in the formation, alignment and maintenance of mitotic spindles, as well as the movement and positioning of chromosomes (5, 6).

The CD complex consists of four distinct classes of proteins: heavy, intermediate, light-intermediate, and light chains. The ~500-kDa cytoplasmic dynein heavy chain (DHC) is present in the complex as a dimer and is responsible for microtubule binding, ATP hydrolysis, and movement along microtubules. Two or three dynein intermediate chains (DICs) per CD complex bind to a specific region toward the N terminus of the DHC (7). The DIC also binds to the p150Glued protein, a subunit of another multi-protein complex called dynactin (reviewed in Ref. 8), and this interaction is thought to be required for many if not all of the cellular functions of CD (9). A variety of dynein light chains have been identified (10–12) and may have a role in the targeting of particular CD complexes to distinct cargo (13, 14). Four to five dynein light-intermediate chains (DLICs) are present per CD complex, binding to an N-terminal portion of the DHC near to the DIC binding site (15). DLICs show great diversity owing to the presence of two genes (DLIC1 and DLIC2), multiple spliced variants, and differential phosphorylation (15–21).

Little is known yet about the function of the DLICs. They are hyperphosphorylated in extracts made from Xenopus eggs arrested in meiotic metaphase (19). In a well characterized in vitro motility assay (22), this cell cycle-specific hyperphosphorylation of the DLIC correlated with a reduction in CD-driven membrane movement and a reduction in the amount of CD (and its accessory complex dynactin) on cargo membranes (19). On the basis of these data it has been postulated that DLICs have the following regulatory role. Mitotic hyperphosphorylation of the DLICs is proposed to cause CD to detach from cargo membranes, thus down-regulating membrane movement during mitosis. Dell et al. (23) recently demonstrated that purified cdc2 and cyclinB1 could phosphorylate recombinant chicken DLIC1 in vitro. They identified the in vitro phosphorylation site, a conserved cdc2-cyclinB1 phosphorylation motif, and showed that mutation of the phosphorylated serine residue caused the recombinant DLIC1 to become refractory to phosphorylation in meiotic cytosol (23).

Here we provide further evidence to support the hypothesis that phosphorylation of DLIC by a mitotically active kinase plays a key role in metaphase inhibition of membrane movement (19). First, we show that incubation with purified Xenopus cdc2-GSTcyclinB1 kinase removes CD from Xenopus interphase membranes in vitro. Second, we demonstrate for the first time that Xenopus DLIC within the native CD complex is

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF517841.

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V. J. A. 1 The abbreviations used are: CD, cytoplasmic dynein; DHC, cytoplasmic dynein heavy chain; DIC, cytoplasmic dynein light intermediate chain; DLIC, cytoplasmic dynein light intermediate chain; CSF, cytostatic factor; GST, glutathione S-transferase; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; PI, protease inhibitor; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis.
specifically phosphorylated by Xenopus cdc2-GSTcyclinB1 kinase. Finally, we find that this phosphorylation occurs at a site distinct from that proposed by Dell et al. (29). Our results therefore implicate alternative conserved phosphorylation sites in the cell-cycle regulation of CD-driven membrane movement.

**EXPERIMENTAL PROCEDURES**

Cloning of Xenopus DLIC1 cDNA—Degenerate primers DEG21, 5'-cggatgatcag/a/g/aga/gtt/c/tct/g/a/tg/t/c/tg/t/c/c/a/tg/cc-3' and DEG4REV1 and 5'-gagctttctc/a/t/g/g/agt/c/aga/t/agt/c/t/g/t/t/c/t/t/g/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/c/t/t/
TABLE I

| DLIC1 | HLDIC1 | GDLIC1 | RDLIC1 | HLDIC2 | GDLIC2 | RDLIC2 |
|-------|--------|--------|--------|--------|--------|--------|
| 82    | 82     | 82     | 82     | 64     | 65     | XDLIC1 |
| 94    | 85     | 85     | 65     | 66     | 65     | XDLIC1 |
| 94    | 65     | 65     | 66     | 66     | 65     | XDLIC1 |
| 94    | 65     | 65     | 66     | 65     | 65     | XDLIC1 |
| 94    | 65     | 65     | 66     | 65     | 65     | XDLIC1 |
| 94    | 65     | 65     | 66     | 65     | 65     | XDLIC1 |
| 94    | 65     | 65     | 66     | 65     | 65     | XDLIC1 |

DLIC sequences from a Xenopus oocyte cDNA library (24). The resulting polymerase chain reaction product, which showed high similarity to published DLIC sequences, was used to screen the same cDNA library. Four independent clones were identified, all of which encoded part or all of the same sequence. This sequence, which was most closely related to DLICs from other organisms (Table I), was called Xenopus DLIC (XDLIC). The XDLIC1 sequence is presented in Fig. 1 and is available under GenBank™ accession number AF317841.

Multiple Phosphorylation of Xenopus DLIC Proteins and Release from Membranes—We have previously observed a single DLIC species by SDS-PAGE that migrates more slowly by SDS-PAGE in metaphase than interphase samples (19), probably owing to increased levels of phosphorylation. Here we describe how we have used modified electrophoresis conditions that separate multiple DLIC bands and immunoblotting with an antibody to XDLIC1. Interphase extracts, prepared either by activating a meiotically arrested CSF extract (aCSF) or by making extracts from interphase eggs (Fig. 2, left lane, top panel), showed three major bands from ~63 to 67 kDa, with the middle band being the major species. This complex pattern is similar to that observed in species which have two DLIC isoforms, for example bovine (17), chick (16), and rat (17). This may indicate the presence of two Xenopus DLIC isoforms even though a Xenopus DLIC2 was not found in our library screen.

CSF extracts (arrested in meiotic metaphase II) showed a major DLIC band at ~70–71 kDa and a minor band at ~73 kDa (Fig. 2A, CSF). Interphase Xenopus egg extracts can be driven into metaphase by the addition of recombinant GSTcyclinB1, which leads to an activation of cdc2 kinase within 60 min, as monitored by histone kinase activity (Fig. 2B, aCSF), and a shift in molecular weight of GSTcyclinB1 (see faint uppermost band shown on the left in kDa). Asterisks mark the position of GSTcyclinB1.

Histone kinase activity (HK) of total extract is indicated in pmol of phosphate incorporated into histone per µl of extract per min.

**Fig. 1. Sequence analysis of XDLIC1.** cDNA sequence for XDLIC1. Stop codons (\*), XDLIC1 open reading frame (uppercase), amino acid numbers (left), and nucleotide numbers (right) are indicated. cdc2 consensus phosphorylation sites are boxed, and the cdc2 consensus P-loop sequence is underlined. The XDLIC1 protein sequence is available under GenBank™ accession number AF317841.

**Fig. 2.** **Time course of DLIC molecular weight shift and loss of CD from membranes.** A, a meiotic metaphase extract was incubated for 45 min with buffer (CSF) or with calcium (aCSF) to trigger entry into interphase, and then both samples were immunoblotted with antibodies to XDLIC. B, interphase Xenopus egg extract was incubated with recombinant GSTcyclinB1. After the indicated times, total extracts (E) and floated membranes (M) were analyzed by immunoblot for DLICs. Molecular mass markers are shown on the left in kDa. Asterisks mark the position of GSTcyclinB1. Histone kinase activity (HK) of total extract is indicated in pmol of phosphate incorporated into histone per µl of extract per min.

Because we observed a 68-kDa DLIC form after incubation of interphase extracts with GSTcyclinB1 (Fig. 2B) but not in CSF extracts (Fig. 2A), we wanted to determine whether the CD containing this phosopho isoform was released from the membrane. Floating membrane samples were therefore prepared at various times after GSTcyclinB1 addition and analyzed by immunoblotting for DLICs. Thirty minutes after the addition of GSTcyclinB1, CD remained on the membrane, with a completely interphase-like pattern of phosphorylation (Fig. 2B). By 60 min, histone kinase activity was high, DLIC migrated as ~71 kDa and 68 kDa bands in the total extract (Fig. 2B, upper panel), and both forms of DLIC had been lost from the membranes (Fig. 2B, lower panel). This indicated that release of CD requires cdc2 kinase activity and rules out a requirement for
any other kinases that are active in meiotic but not in mitotic extracts.

Xenopus cdc2-GSTcyclinB1 Kinase Phosphorylates Xenopus DLICs in the CD Complex and Removes CD from Membranes in Vitro—We next tested specifically whether Xenopus cdc2-GSTcyclinB1 kinase could phosphorylate Xenopus DLICs while they were part of the intact CD complex. Active cdc2-GSTcyclinB1 kinase was incubated with interphase CD (both purified from Xenopus egg extracts) in the presence of γ-32P-labeled ATP. Intact CD complex was immunoprecipitated from this reaction mixture using a monoclonal antibody to DIC and examined by SDS-PAGE. Fig. 3A shows that a strongly radioactive protein band was detected at ~71 kDa corresponding to DLICs that had incorporated labeled phosphate. Despite possessing potential cdc2 kinase phosphorylation sites (see “Discussion”), the other CD subunits were not substrates for cdc2-GSTcyclinB1 kinase in vitro. Immunoblotting for DLICs after incubation of CD with cdc2-GSTcyclinB1 kinase (Fig. 3B) revealed two new bands at around 71 and 68 kDa, very likely the pattern observed 60 min after GSTcyclinB1 was added to interphase extract (Fig. 2B). None of the lowest mobility interphase DLIC remained, whereas some DLIC still co-migrated with the upper interphase DLIC form.

We wondered whether this phosphorylation of DLICs in the CD complex by cdc2 kinase could be directly responsible for removal of CD from membranes as extracts enter metaphase. To test this in vitro we incubated active Xenopus cdc2-GSTcyclinB1 with membranes isolated by flotation from interphase Xenopus egg extracts. We then pelleted membranes through a sucrose cushion and immunoblotted for theDIC (to show the amount of CD) and ribophorin (an integral membrane protein to control for equal amounts of membrane) using [35S]methionine-labeled secondary antibodies. We quantitated the blot signal very carefully versus a dilution series of membrane samples loaded on the same gel to provide a standard curve for both DIC and ribophorin antibody signals (see “Experimental Procedures.”)

To try to determine whether any of these sites are phosphorylated by cdc2, we isolated native CD from metaphase and interphase extracts either by classical microtubule affinity and sucrose-gradient purification or by immunoprecipitation. The DLIC proteins (and bacterially expressed His6-XDLIC1 as a control) were excised from silver-stained gels and processed for analysis by MALDI-MS. Bacterially expressed His6-XDLIC1 gave a consistent pattern of tryptic peptides, most of which were identifiable in both metaphase and interphase preparations. However, we found that DLICs from both interphase and metaphase CD lacked the peptide containing site 1 (2124 Da), which was obvious in the control bacterially expressed sample (Fig. 5). Instead, both traces showed a peptide 80 Da larger than the expected size (2208–2211 Da) that was absent from the control, corresponding to the addition of one phosphate to the site 1 peptide (37). We conclude that site 1 (Fig. 4A) is phosphorylated in both interphase and metaphase and therefore cannot be responsible for the regulation of CD function in this system. We therefore propose that cell cycle-dependent regulation of CD-driven membrane movement is mediated by phosphorylation of DLICs by cdc2 kinase at an

FIG. 3. Purified cdc2-GSTcyclinB1 kinase phosphorylates DLIC and removes CD from membranes in vitro. A, Xenopus CD and cdc2-GSTcyclinB1 were incubated together in an in vitro phosphorylation experiment as described under “Experimental Procedures” (lane 1). Controls were as follows: lane 2, no antibody for immunoprecipitation from the reaction mixture; lane 3, no cdc2-GSTcyclinB1; lane 4, no cdc2-GSTcyclinB1 or antibody; lane 5, no CD; lane 6, no dynein or antibody. A 5–20% gradient gel was used to ensure resolution of all CD components, but under these conditions DLIC migrates as one band. B, a similar in vitro phosphorylation experiment run on an 8% gel was immunoblotted with anti-XDLIC1 antibody. The equivalent to lanes 3 (−cdc2 and 1 (+cdc2) in panel A are shown. Sizes of protein molecular weight markers are indicated in kDa. C, immunoblotting of membranes pelleted after incubation with (+) or without (−) cdc2-GSTcyclinB1 kinase was carried out using antibodies to DIC, with ribophorin (R) as a membrane-loading control. A representative experiment is shown. The amount of CD on membranes in this experiment (as a percentage of the buffer only control) is displayed (CD) and was calculated after normalization to the amount of membrane present, as described under “Experimental Procedures.”

FIG. 4. Conserved potential cdc2 phosphorylation sites in DLICs. Accession numbers and abbreviations for sequences are as for Table I except for: DDLIC, Drosophila DLIC (GenBank™ accession number AAF48011); MDLIC1, mouse DLIC1 (partial translation from GenBank™ accession number AA146205); and MDLIC2, mouse DLIC2 (partial translation from GenBank™ accession number A253855). The following consensus sequences were used to identify potential cdc2 phosphorylation sites: (S/T)P(X/K/R); (S/R)(T/P); and (S/T)P(K/R) (36). A, the region corresponding to amino acids 184–210 of XDLIC1 contains site 1, SPQR, which is conserved in all DLICs (SPVK in Drosophila). B, the region corresponding to amino acids 379–405 of XDLIC1 contains site 2 SP, which is conserved in all DLICs (SPAR in Drosophila), site 3 SPR (SPLR in Drosophila) which is conserved in all DLICs, and site 4 TPxR, which is conserved in all DLICs.

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cdc2 Removes Dynein from Membranes

Interestingly, DLIC was the only CD subunit phosphorylated by cdc2 kinase either in vitro (Fig. 3A) or in metaphase extracts (19) despite the fact that DHC and DIC sequences contain multiple potential cdc2 kinase phosphorylation sites. We can therefore conclude that there is no phosphate turnover and therefore no $^{32}$P incorporation in vitro at any of these sites in DHC or DIC in metaphase or interphase extracts. Whether these sites are unused or whether they are stably phosphorylated is not known. The latter phenomenon has been elegantly demonstrated for DIC, which becomes hyperphosphorylated upon entry into meiotic metaphase I in Xenopus oocytes and remains stably phosphorylated through multiple interphases and metaphases, at least up to stage X embryos (40). The p150Glued subunit of dynactin also behaved in this unusual manner (40), and it along with the rest of the dynactin complex is a poor substrate for cdc2 kinase in vitro.3 These data raise interesting questions about potential differential regulation of CD phosphatases during oogenesis, early embryogenesis, and the somatic cell cycle.

A key question is which if any of the four obvious potential cdc2 kinase phosphorylation sites in DLIC is used? Dell et al. (23) demonstrate that site 1 in recombinant chick light intermediate chain was efficiently phosphorylated in metaphase Xenopus egg extracts but not interphase extracts and was also phosphorylated by purified cdc2 kinase. However, when we used MALDI-MS to investigate native CD isolated from both interphase and metaphase extracts, we found that site 1 (Fig. 4) is phosphorylated regardless of cell cycle status (Fig. 5). That this site remains phosphorylated in interphase, when very little DLIC phosphate turnover takes place (19), strongly suggests that site 1 is stably phosphorylated in the native molecule and therefore cannot be responsible for the regulation of CD binding to membranes. Whether this phosphate turnover during meiotic metaphase II or whether it becomes stably phosphorylated during meiosis I, as is the case for DIC (40), remains to be established. In addition, although site 1 in recombinant protein is used by cdc2 kinase, it is also a consensus site for MAP kinase (36), and so at present, we can not conclude which kinase is responsible for its phosphorylation in the native complex in CSF extracts, which have high MAP kinase activity (35).

If site 1 can be ruled out, then what about the other three sites? cdc2 kinase sites 2–4 lie in regions that unfortunately have not generated suitable peptides for MALDI-MS with any of the proteases tested so far, so we do not yet know which site in DLIC is responsible for the observed cell cycle regulation. It is interesting to note, however, that Drosophila has only a single DLIC that appears equally related to DLIC1s and DLIC2s and that only possesses sites 1 and 3 (Fig. 4), suggesting that site 3 is the most likely candidate. One method for testing the importance of sites 2–4 is site-directed mutagenesis, but the difference between our results and those of Dell et al. (23) raises concerns over that approach. First, bacterially expressed DLIC may not be in a native conformation, either because of folding problems or because it is not assembled as part of the CD complex. Second, our preliminary results suggest that recombinant DLIC added to extracts does not become incorporated into existing Xenopus CD molecules.2 Third, bacterially expressed DLIC will be completely unphosphorylated, which may leave sites accessible that are normally stably phosphorylated. Fourth, it is well known that some phosphorylation events require other sites to be phosphorylated first, which could complicate interpretation of mutagenesis studies. It seems likely then that a combination of experimental approaches will be needed to investigate fully the regulation of CD

DISCUSSION

Surprisingly little is known about how microtubule-based motors are regulated. Even in melanosomes, where phosphorylation controls the direction of organelle movement, the molecular details of how this is achieved are unclear (38, 39). One example where motor phosphorylation has been directly correlated with altered motor function is the cell cycle regulation of CD-driven ER movement in Xenopus egg extracts (19). On conversion from interphase to metaphase, both ER motility and the amount of membrane-associated CD decreased 10-fold, whereas

Fig. 5. Analysis of trypsin-generated fragments of recombinant and native Xenopus DLICs. Peptide peaks from the 1900–2500-Da region of a MALDI-MS trace are shown from recombinant His$_x$-XDLIC1 (A), interphase DLICs prepared by immunoprecipitation (B), and metaphase DLICs prepared by microtubule affinity (C). Peaks are labeled with their molecular mass in Da, and relative intensity of peaks is displayed at the side. The asterisk marks a peak of around 2113 that was also present in lanes containing antibody alone.

alternative conserved potential phosphorylation site (Fig. 4B). The close proximity of these potential phosphorylation sites (Fig. 4B) and the lack of convenient protease sites in this portion of the XDLIC1 meant that we were unable to determine which of these alternative sites is phosphorylated using MALDI-MS.

2 V. Allan and P. Mayr, unpublished results.
CD activity by DLIC phosphorylation.

Inhibition of ER movement in metaphase egg extracts correlates with the loss of CD from the membrane (19). We show here that incubating interphase egg membranes with Xenopus cdc2-cyclinB1 kinase leads to the release of about half of the CD, suggesting that cdc2 kinase is directly involved in the cell cycle regulation of ER motility in Xenopus early embryogenesis. The simplest model is that DLIC phosphorylation by cdc2 kinase triggers the release of CD, although it is formally possible that DLIC phosphorylation prevents re-binding of any CD that detaches from membranes as part of an equilibrium. How cdc2 activity also leads to the loss of membrane-associated dynactin (19) is unclear, particularly since the dynactin complex itself is not a substrate for this kinase. However, we have not yet tested whether cdc2-dependent phosphorylation of any membrane components is also important. The fact that treatment of interphase membranes with cdc2 kinase releases ~50% of CD, whereas metaphase membranes have lost ~90% of their CD, may simply suggest that the assay with membranes and kinase alone is not fully optimized. This is quite likely, since not all of the DLIC underwent complete molecular weight shifts when purified CD was incubated with cdc2 kinase (Fig. 3B) compared with when the kinase was activated by adding GSTcyclinB1 to interphase extracts (Fig. 2B). It is also possible that other cytosolic factors are needed for full release.

The simple binding or release of CD from membranes is clearly not the only mechanism for regulating organelle movement however. For instance, a variety of organelles that are moving using plus end-directed microtubule motors during interphase also possess CD, presumably inactive, on their surface (31, 39, 41, 42). Moreover, CD-driven ER movement in interphase Xenopus egg extracts can be greatly stimulated without recruiting any extra motors (22).

One interesting question is whether the regulatory mechanism we have characterized operates during mitosis in somatic cells (rather than through the embryonic cell cycle, as assessed here and previously), as there are conflicting reports of whether CD remains associated with its membranous cargoes (43, 44). Since both DIC and p150<sup>Glued</sup> do incorporate phosphate during somatic mitosis but not during embryonic cycles (40), it seems likely that the regulatory system is more complex in the former, perhaps because there are extra functions for dynein in somatic cell division. It should be stressed that although the membrane movement functions of CD may be down-regulated in mitosis, it seems certain that there are other roles for this motor (see Ref. 9 for a review) that have to be stimulated on entry into mitosis, such as the interaction with NuMA (45) and those like the interaction between LIC1 and pericentrin, which may be active at all times (46, 47). To add to this complexity, there may be distinct CD complexes that contain specific assortments of subunits, which may be responsible for different CD functions (44, 47) and which could be differentially regulated. Other microtubule motors, such as Eg5 and MKLP1, may also be regulated by cdc2 kinase and other cell cycle-regulated kinases (48–51). Understanding how the cell integrates the function of these motors to generate different cellular processes and architecture throughout the cell cycle will require knowledge of the regulation of a whole range of motors, and Xenopus egg extracts offer an excellent model system for such investigations.

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