Molecular interactions of Polo-like-kinase 1 with the mitotic kinesin-like protein CHO1/MKLP-1

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Summary

Polo-like kinases and kinesin-like motor proteins are among the many proteins implicated in the execution of cytokinesis. Polo-like-kinase 1 (Plk1) interacts with the mitotic kinesin-like motor protein CHO1/MKLP-1 during anaphase and telophase, and CHO1/MKLP-1 is a Plk1 substrate in vitro. Here, we explore the molecular interactions of these two key contributors to mitosis and cytokinesis. Using the transient transfection approach, we show that the C-terminus of Plk1 binds CHO1/MKLP-1 in a Polo-box-dependent manner and that the stalk domain of CHO1/MKLP-1 is responsible for its binding to Plk1. The stalk domain was found to localize with Plk1 to the mid-body, and Plk1 appears to be mislocalized in CHO1/MKLP-1-depleted cells during late mitosis. We showed that Ser904 and Ser905 are two major Plk1 phosphorylation sites. Using the vector-based RNA interference approach, we showed that depletion of CHO1/MKLP-1 causes the formation of multinucleate cells with more centrosomes, probably because of a defect in the early phase of cytokinesis. Overexpression of a non-Plk1-phosphorylatable CHO1 mutant caused cytokinesis defects, presumably because of dominant negative effect of the construct. Finally, CHO1-depletion-induced multinucleation could be partially rescued by cotransfection of a non-degradable hamster wild-type CHO1 construct, but not an unphosphorylatable mutant. These data provide more detailed information about the interaction between Plk1 and CHO1/MKLP-1, and the significance of this is discussed.

Key words: Plk1, CHO1/MKLP-1, Cytokinesis, Interaction

Introduction

The Polo-like kinase (plk) family has emerged as a key player in many cell-cycle-related events. In addition to the N-terminal kinase domain, all plk family members (including mammalian Plk1, Snk and Fnk/Prk, Xenopus laevis Plx1, Drosophila Polo, fission yeast Plo1 and budding yeast Cdc5) have a distinctive highly conserved region in the C-terminal non-catalytic domain, denoted the Polo box (Clay et al., 1993; Glover et al., 1998). Genetic and biochemical experiments with several different organisms have documented that Polo-like kinases are involved in many aspects of mitosis. At the onset of mitosis, Xenopus Plo1 phosphorylates and activates Cdc25C, which subsequently activates Cdc2. Thus, Plo1 was proposed to be a trigger kinase for cells to enter mitosis (Kumagai and Dunphy, 1996). Both Drosophila polo and fission yeast Plo1 mutants show the phenotype of monopolar spindles, indicating a role for plks in centrosome assembly and separation during the formation of bipolar mitotic spindles (Ohkura et al., 1995; Sunkel and Glover, 1998). During the metaphase-anaphase transition, Plk phosphorylates and activates subunits of the anaphase-promoting complex (Kotani et al., 1998). Depletion of Cdc5 in budding yeast leads to an arrest in cytokinesis, and overexpression of the Polo-box region results in connected cells with incomplete septa, which share cytoplasm (Song and Lee, 2001). Genetic analysis in Drosophila also demonstrates that Polo is required for cytokinesis. A polo mutant in Drosophila fails to form the correct mid-zone and mid-body structures at telophase during meiotic divisions (Carmena et al., 1998). Using the RNA interference (RNAi) technique, we also observed the failure of cytokinesis in Plk1-depleted mammalian cells (Liu and Erikson, 2002). The protein level, activity and subcellular localization of Plk1 are tightly regulated during the cell cycle. Plk1 is undetectable during G1 phase, starts to accumulate during S and G2 phases, and reaches a peak during mitosis. It was recently demonstrated that the centrosomal localization of Plk1 results from the recognition and interaction of the Polo-box domain with phosphorylated peptides (Elia et al., 2003a; Elia et al., 2003b).

During mitosis, sister-chromatid segregation and subsequent cytokinesis require the functions of motor proteins. Several kinesin-like motor proteins have been reported and they are divided into different subfamilies (Miki et al., 2001). The mitotic kinesin-like protein 1 (MKLP-1) subfamily includes hamster CHO1 (Sellitto and Kuriyama, 1988), human MKLP-1 (Nislow et al., 1992), Caenorhabditis elegans ZEN-4 (Raich et al., 1998) and Drosophila Pav-KLP (Adams et al., 1998). These subfamily members all contain a plus-end-directed N-terminal motor domain, α-helical coiled-coil central stalk domain and a C-terminal globular tail domain. Owing to alternative splicing, one form of CHO1/MKLP-1 has an additional ~100 amino acids in the tail domain. Thus, CHO1/MKLP-1 and a truncated isoform are co-expressed in the same cell (Kuriyama et al., 2002). The
motor function of CHO1/MKLP-1 was originally proposed to be necessary in chromosome segregation and spindle elongation during cytokinesis (Nislow et al., 1992). However, genetic experiments in Drosophila emphasize the essential role of Pav-KLP during cytokinesis. Cells of pav mutant embryos develop an abnormal telophase spindle and fail to undergo cytokinesis. This is accompanied by a failure correctly to localize a septin (Pav-KLP), actin and the actin-associated protein Anillin (Adams et al., 1998). More recently, using RNAi, it was shown that CHO1/MKLP-1 is required for formation of the mid-body matrix in mammalian cells (Matuliene and Kuriyama, 2002). Although CHO1/MKLP-1 functions mainly during mitosis and cytokinesis, it is clearly detected during interphase. Furthermore, immunofluorescent studies showed that endogenous CHO1/MKLP-1 localizes to punctate spots within the nucleus before nuclear envelope breakdown (Nislow et al., 1992).

The interaction between Plk1 and CHO1/MKLP-1 was first reported in mammalian cells. These two proteins both localize to the interzone during anaphase and the mid-body during telophase and cytokinesis. In addition, these two proteins co-immunoprecipitate, and CHO1/MKLP-1 is phosphorylated by Plk1-associated kinase activity in vitro (Lee et al., 1995). Subsequent genetic analysis in Drosophila provided more information about the interaction of these two gene products. Pav-KLP protein co-immunoprecipitates with Polo from Drosophila embryo extracts. The colocalization of Polo with Pav-KLP to centrosomes and the central spindle is disrupted in pavarotti mutant embryos (Adams et al., 1998), and, conversely, a mutation in polo prevents the formation of a proper spindle mid-zone and disturbs the correct localization of Pav-KLP (Carmena et al., 1998). Therefore, the two proteins appear to be mutually dependent for their correct localization, yet there is little information on the molecular details of their interactions.

In this article, we further explore the interaction between Plk1 and CHO1/MKLP-1 in mammalian cells. Using the transient transfection approach, we have found that the C-terminal Polo-box domain of Plk1 influences its association with CHO1/MKLP-1 and that the stalk domain of CHO1/MKLP-1 is responsible for Plk1 binding. We mapped two serine residues, Ser904 and Ser905, to be Plk1 phosphorylation sites. Using vector-based RNAi, we found that depletion of CHO1/MKLP-1 significantly inhibits cell division, indicating the essential role of CHO1/MKLP-1 during cytokinesis. Finally, the CHO1-depletion-induced phenotype was partially reversed by reintroduction of wild-type hamster CHO1 but not a Plk1-unphosphorylatable mutant.

Materials and Methods

RNA preparation

Double-stranded 21-nucleotide RNAs were synthesized by Dharmacon Research (Lafayette, CO). The targeting sequence of human CHO1/MKLP-1 (accession no. X67155) was AAAGCCATGCGGCAAGGAGCTCTT, corresponding to the coding region 2625-2647 relative to the first nucleotide of the start codon.

Vector construction

To deplete endogenous CHO1/MKLP-1 specifically in HeLa cells, plasmid pBS/U6-CHO1 was constructed as described previously (Sui et al., 2002). The targeting sequence of human MKLP-1 (accession no. X67155) was GGGCTTTCTGATGACAAGTG, corresponding to the coding region 105-125 relative to the first nucleotide of the start codon. Plasmid pBS/U6-CHO1-1st half (sense strand) was used as a control vector. This control vector produces RNA that cannot form a hairpin structure to generate interfering RNA. Plasmid pBS/U6-GFP-CHO1 targets the same site as pBS/U6-CHO1 except with a green fluorescent protein (GFP) marker. Plasmid pBS/U6-GFP-Plk1 is similar to pBS/U6-Plk1 as described previously except with a GFP marker (Liu and Erikson, 2003). Both pBS/U6-GFP-CHO1 and pBS/U6-GFP-Plk1 express GFP independently of hairpin RNAs, so only GFP-positive cells express small interfering RNAs (siRNAs).

Cell culture and synchronization

HeLa and Cos-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in 8% CO2. To obtain mitotically synchronized HeLa cells, cells were treated with 50 ng/ml nocodazole for 8 hours. After floating cells were gently washed away with PBS, mitotic cells were mechanically shaken off the plates, washed three times with cold DMEM over a 1 hour period and then seeded onto polylysine-coated coverslips in prewarmed DMEM containing 10% FBS. To obtain the maximum numbers of cells in telophase, cells were released for 75 minutes and fixed as described below. Alternatively, HeLa cells were first treated with 0.3 μM mimosine for 18 hours to block the cells at late G1 phase and then released into normal growth medium supplemented with 24 μM deoxycoxytidine and 100 μM monastral for 12 hours to arrest the cells at prometaphase. After three washes with PBS, the cells were further incubated for an additional 75 minutes to obtain the maximum number of cells in telophase (Kapoor et al., 2000).

DNA transfections

For binding experiments, Cos-7 cells were transfected with GenePorter transfection reagent (GTS, San Diego, CA). Cells were seeded at 80% confluence in 10-cm dishes on the day before transfection. 10 μg plasmid DNA was diluted with 0.5 ml serum-free DMEM and mixed with 0.5 ml diluted GenePorter reagent. The mixture was incubated at room temperature for 30 minutes and then resuspended in 4 ml DMEM. After the cells were incubated in this mixture for 4 hours, 5 ml DMEM containing 20% serum was added. About 30 hours after transfection, 100 ng/ml nocodazole was added and the culture was further incubated for 16 hours. For phenotype analysis of CHO1/MKLP-1 depletion, HeLa cells were co-transfected with pBS/U6-CHO1 and pBabe-Puro at a ratio of 9:1 using GenePorter reagents. After 2 days of selection for transfection-positive cells using 2 μg/ml puromycin, floating cells were removed and the remaining cells were incubated in the presence of puromycin until harvested for phenotype analysis. To transfect GFP-fusion vectors, PolyFect reagents (Qiagen) were used.

Immunoprecipitation and immunoblotting

Transfected Cos-7 cells were lysed in TBSN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1.5 mM EDTA, 5 mM EGTA, 0.5% Nonidet P-40, 0.5 mM Na3VO4) supplemented with phosphatase and proteasome inhibitors (20 mM p-nitrophenyl phosphate, 1 mM peflabclo, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotonin), and the lysates were clarified by centrifugation at 15,000 g for 30 minutes. Cell lysates were incubated with either anti-Plk1 (Zymed, South San Francisco, CA) or anti-CHO1/MKLP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody for 1.5 hours at 4°C, followed by a 1 hour incubation with Protein-A/Sepharose beads. Immunocomplexes were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Co-immunoprecipitated proteins were detected by
western blotting using anti-hemagglutinin (HA) and anti-FLAG primary antibodies.

Immunofluorescence staining
For localization of CHO1/MKLP-1 domains at late M phase, HeLa cells were transfected and synchronized as described above. Cells were fixed with paraformaldehyde and permeabilized with methanol. After three washes with 0.1% Triton X-100 in PBS, coverslips were blocked with 10% goat serum in PBS for 30 minutes, stained with 10 μg ml⁻¹ anti-Plk1 primary antibody for 2 hours at room temperature, followed by incubation for 30 minutes with Cy3-conjugated antimouse secondary antibody. Finally, DNA was stained with DAPI. For phenotype analysis of CHO1/MKLP-1 depletion, HeLa cells were harvested at 3 days after transfection with pBS/U6-CHO1. The fixed cells were either double stained with anti-α-tubulin antibody and DAPI or triple stained with anti-α-tubulin and anti-γ-tubulin antibodies, and DAPI.

Recombinant protein purification and kinase reactions
Various domains of CHO1/MKLP-1 were amplified by PCR, subcloned into pGEX-KG vector and overexpressed in Escherichia coli BL21 (DE3). Expression was induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 5 hours after the cell density had reached 0.5 at 600 nm. Glutathione-S-transferase (GST) fusion proteins were affinity purified using glutathione-agarose beads. Purified GST-CHO1/MKLP-1 was incubated with recombinant GST-Plk1 from Hi5 insect cells in TBMD buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 2 mM EGTA, 0.5 mM sodium vanadate, 20 mM p-nitrophenyl phosphate) supplemented with 25 μM ATP and 50 μCi of [γ-³²P]ATP. The reaction mixtures were incubated at 30°C for 30 minutes and resolved using sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue, dried and subjected to autoradiography.

Metabolic labeling
HeLa cells were treated with nocodazole (200 ng ml⁻¹) for 10 hours and labeled for 4 hours with [³²P] orthophosphate (ICN) at 1 mCi ml⁻¹ in phosphate-free DMEM. Cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 10% glycerol, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1% NP-40, 50 mM NaF, 0.2 mM sodium vanadate and proteinase inhibitors). Cell lysates were microcentrifuged for 30 minutes at 14,000 rpm and the supernatants were incubated with anti-CHO1 or anti-GFP antibodies followed by Protein-A/Sepharose beads.

Results
C-terminal Polo-box domain of Plk1 is responsible for its binding to endogenous CHO1/MKLP-1. (A) FLAG-tagged Plk1 constructs used in the transfection experiment. The hatched and filled bars indicate the positions of kinase domain (residues 53-305) and Polo box (residues 410-440), respectively. Stars indicate the positions of three point mutations within the polo box (W414F, V415A, L427A). (B,C) Cos-7 cells were transiently transfected with 10 μg of the indicated Plk1 constructs per 10-cm Petri dish. 30 hours after transfection, cells were treated with 100 ng ml⁻¹ nocodazole for 16 hours and harvested. Cell lysates were subjected to anti-CHO1/MKLP-1 antibody immunoprecipitation, and anti-FLAG western blotting. As a control, the same quantities of cell lysates were incubated with Protein-A beads only. About 5% of the total cell lysates was used to assess expression. (D) Different amounts of Plk1 constructs (2.5 μg, 5 μg or 10 μg of DNA per 10-cm dish) were used for transfection as described above. Cell lysates were immunoprecipitated with anti-CHO1/MKLP-1 antibody, followed by anti-FLAG immunoblotting.
different amounts of full-length wild-type and FAA mutant (W414F, V415A, L427A) constructs. The binding of wild-type Plk1 with CHO1/MKLP-1 was easily detected at a low level (as low as 2 μg of DNA per 10-cm dish), whereas interaction was not detected until a much higher level of FAA mutant was expressed (Fig. 1D).

Stalk domain of CHO1/MKLP-1 is responsible for its binding to Plk1

Endogenous CHO1/MKLP-1 and Plk1 were previously shown to co-immunoprecipitate from HeLa cells (Lee et al., 1995). To examine which domains of CHO1/MKLP-1 are required for its association with endogenous Plk1, we transiently transfected HA-tagged hamster CHO1 domains (Fig. 2A) into Cos-7 cells. Lysates from the transfected cells were analysed to determine the expression of the various constructs (Fig. 2B, leftmost six lanes). Cell lysates were then subjected to immunoprecipitation with anti-Plk1 antibody and the immunoprecipitates were probed with anti-HA antibody (Fig. 2B, rightmost six lanes). Regions containing the stalk domain (full, C-term and stalk) were co-immunoprecipitated with anti-Plk1 antibody, whereas the N-terminal motor domain and the tail domain were not. Thus, the stalk domain of hamster CHO1/MKLP-1 is required for its binding to Plk1.

Colocalization of the stalk domain with Plk1 at the mid-body during telophase

Double-immunofluorescence staining shows that CHO1/MKLP-1 colocalizes with Plk1 in the interzone in anaphase and the mid-body during telophase and cytokinesis (Lee et al., 1995). We further tested whether the stalk domain itself is sufficient for localization to the mid-body during telophase. In order to observe cells in cytokinesis, HeLa cells transfected with hamster GFP-CHO1/MKLP-1 fusion domains for 30 hours were treated with nocodazole for 8 hours to arrest the cells at prometaphase. A subpopulation of mitotic cells was detached from the culture dish, extensively washed and seeded onto coverslips for 75 minutes to obtain telophase cells. As shown in Fig. 2C, the motor domain was widely distributed throughout the entire cell, whereas the stalk domain colocalized with Plk1 to the mid-body (indicated by the appearance of yellow color in the merge panel). Although most tail-domain-expressing cells showed a pattern similar to that of the motor domain, a subset of the tail domain colocalized with chromosomes, suggesting that this domain has DNA-binding capacity. These data support the co-immunoprecipitation results presented above.

Mislocalization of Plk1 in CHO1/MKLP-1-depleted cells during late mitosis

The colocalization of Plk1 with CHO1/MKLP-1 to mitotic structures has been documented previously (Lee et al., 1995). We wished to determine whether the localization of Plk1 would
be affected in CHO1/MKLP-1-depleted cells by using RNAi. As described previously, Plk1 normally localizes with CHO1/MKLP-1 to the mid-zone during anaphase (Fig. 3A, top) and then to the mid-body during telophase at the onset of cytokinesis (Fig. 3A, bottom). However, after CHO1/MKLP-1 RNAi treatment, rather than a concentrated staining in the mid-zone and mid-body, a diffuse Plk1 immunostaining was observed throughout the entire cells (Fig. 3B). These data suggest that the correct localization of Plk1 during late mitosis requires the presence of CHO1/MKLP-1. It should be realized that these cells were examined shortly after CHO1/MKLP-1 depletion and thus were able to commence cytokinesis.

Whether CHO1 localization depends on Plk1 was also tested using the vector-based RNAi technique (see below). As shown in Fig. 3C, transfection of pBS/U6-GFP-Plk1 efficiently depleted Plk1, whereas the level of Erk2 was not changed. Immunofluorescence staining indicated that CHO1 still localizes to mitotic structures in the absence of Plk1 (Fig. 3D).

Ser904 and Ser905 are two major Plk1 phosphorylation sites

CHO1/MKLP-1 has been shown to be a Plk1 substrate (Lee et al., 1995). To investigate whether Plk1-mediated phosphorylation might regulate CHO1/MKLP-1 function, we mapped the Plk1 phosphorylation sites. We first subcloned the three major domains of hamster CHO1 (motor, stalk and tail) into a GST fusion vector, expressed them in bacteria and purified the proteins on glutathione-agarose beads (Fig. 4A, leftmost three lanes). These hamster CHO1/MKLP-1 proteins were then phosphorylated by Plk1 purified from recombinant baculovirus-infected insect cells and the phosphorylation signal was detected by autoradiography (Fig. 4A, rightmost three lanes). Only the tail domain yielded a strong specific signal (arrowhead on the right). To identify the phosphorylation site(s), shorter fragments of the tail domain were cloned (Fig. 4B,C). Robust phosphorylation was detected with regions containing the last 53 C-terminal residues (Tail 643-953, Tail 801-953, Tail 901-953) and we concluded that residues 901-953 contain major Plk1 phosphorylation sites. There is, however, at least one additional minor site(s) within the sequence 643-800. Phosphoamino acid analysis had previously shown that serine is the major residue phosphorylated by Plk1 in full-length CHO1/MKLP-1 (data not shown). Thus, a series of serine to alanine mutants was constructed that covered all the possible combinations. A representative experiment is shown in Fig. 4D; only the Ser904, Ser905 double mutant failed to be phosphorylated by Plk1. Based on sequence similarity, we tested whether Ser805 and Ser807 of the 801-900 region are also Plk1 phosphorylation sites. As shown in Fig. 4E, the weak phosphorylation signal of the 801-900 region was abolished after the introduction of Ser805Ala and Ser807Ala mutations. Finally, we performed the kinase assay using the constructs in the longer sequence context (Fig. 4E, right). Again, wild-type tail region 643-953 was easily phosphorylated by Plk1, whereas the 4A mutant (Ser805Ala, Ser807Ala, Ser904Ala, Ser905Ala) was no longer a good Plk1 substrate. The S805/807, S904/905 clusters from CHO1 cells correspond to S812/814, S911/912 of MKLP-1 from human origin. To rule out the possibility that CHO1 phosphorylation was caused by other contaminating kinases during Plk1 purification, we also used both kinase-dead (KM) and wild-type (WT) GST-Plk1 in the kinase assay. Wild-type, but not kinase-defective, GST-Plk1 phosphorylates CHO1 (Fig. 4F).

We also analysed the in vivo phosphorylation states of CHO1 (Fig. 4G-I). Nocodazole treatment induced an increase in Plk1 levels, whereas the CHO1 level was not obviously affected. CHO1 phosphorylation increased about fourfold in

**Fig. 3. Mislocalization of Plk1 in CHO1/MKLP-1-depleted cells during late mitosis.** (A,B) HeLa cells on coverslips were first synchronized at late G1 phase with double-thymidine block. Upon release from the second thymidine block, cells were directly transfected with synthetic 21-nucleotide double-stranded RNA targeting CHO1/MKLP-1. Cells were fixed 10 hours later and subjected to double staining with anti-CHO1 and anti-Plk1 antibodies. DNA was stained with DAPI. (C,D) HeLa cells were transfected withpBS/U6-GFP-Plk1. 48 hours after transfection, cells were harvested and subjected to anti-Plk1 western blot (C) or anti-CHO1 staining (D). Scale bar, 10 μm.
mitotic cells, compared with that of randomly growing cells (Fig. 4G). CHO1 phosphorylation was attenuated by 60% after Plk1 depletion, whereas the protein level of CHO1 was not affected (Fig. 4H), indicating that Plk1 is a CHO1 kinase in vivo. The residual CHO1 phosphorylation signal of Plk1-deficient cells might be due to the additional protein kinases. We have reported that Cdc2 activity increased in Plk1-depleted cells caused by the stabilization of cyclin B (Liu and Erikson, 2002). Indeed, CHO1 was previously proposed to be a Cdc2 substrate (Nislow et al., 1992). Furthermore, GFP-fused hamster CHO1 wild-type or 4A mutant was transfected into HeLa cells, and the phosphorylation states of overexpressed CHO1 were determined. The mutation significantly reduced (about 25% of wild type) but did not completely eliminate CHO1 phosphorylation in vivo (Fig. 4I).

Depletion of CHO1/MKLP-1 leads to the formation of multinucleated cells with amplified centrosome numbers. To examine the function of endogenous human CHO1/MKLP-1, we took advantage of the recently developed vector-based RNAi technology to deplete CHO1/MKLP-1 specifically in HeLa cells (Sui et al., 2002). The targeting sequence of human CHO1/MKLP-1 is the coding region 105-125 relative to the first nucleotide of the start codon. The vector pBS/U6-CHO1 was transfected into HeLa cells and the cells were cultured for

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**Fig. 4.** Identification of Ser-904 and Ser-905 of CHO1/MKLP-1 as two major Plk1 phosphorylation sites. (A) GST-fused CHO1/MKLP-1 domains were purified (left) and subjected to in vitro kinase reactions with purified Plk1 (right). Only the tail domain (residues 643-953) was strongly phosphorylated by Plk1, indicated by the arrowhead on the right. (B,C) Different regions of the tail domain fused with GST were purified and subjected to in vitro kinase reactions with Plk1. The stars indicate the positions of the proteins based on Coomassie Blue staining. The size markers are indicated on the left. The region 901-953 contains Plk1 phosphorylation sites. (D) Wild-type or mutant GST fusion proteins of the 901-953 region were subjected to kinase assays with recombinant Plk1. (E) Wild-type or mutant GST fusion proteins of the 801-900 region (left) or the 643-953 region (right) were subjected to kinase assays with recombinant Plk1. The 2A mutant contains the mutations Ser805Ala and Ser807Ala; the 4A mutant contains the mutations Ser805Ala, Ser807Ala, Ser904Ala and Ser905Ala. The arrow on the left indicates the position of GST-Plk1, and the stars indicate the positions of the proteins based on Coomassie Blue staining. (F) Purified GST-fused CHO1 C-terminus (residues 406-953) was subjected to kinase assay with either kinase defective (KM) or wild-type (WT) GST-Plk1. (G) CHO1 is highly phosphorylated during mitosis. Asynchronous or mitotic (treated with 200 ng ml–1 nocodazole for 10 hours) HeLa cells were labeled with [32P]-orthophosphate for 4 hours. Cell lysates were either directly analysed on an anti-Plk1 western blot or immunoprecipitated with anti-CHO1 antibody first, then detected with anti-CHO1 antibody. Phosphorylated CHO1 was visualized by autoradiography and quantified using a phosphorimager. (H) HeLa cells were transfected with pBS/U6-Plk1 to deplete Plk1 first, then treated with nocodazole for 10 hours and labeled in vivo as in (G). (I) Cells were transfected with GFP-CHO1 wild-type or 4A mutant as indicated. 30 hours after transfection, cells were incubated with nocodazole for 10 hours and labeled with [32P]-orthophosphate for additional 4 hours. Cell lysates were immunoprecipitated with anti-GFP antibody and phosphorylated GFP-CHO1 was quantified using a phosphorimager.
48 hours. Cell lysates were prepared and standard western blotting was performed. CHO1/MKLP-1 was efficiently depleted by siRNA (Fig. 5A, top), whereas the level of Erk2 was unchanged (Fig. 5A, bottom). The level of CHO1/MKLP-1 protein was reduced by at least 90% 48 hours after transfection, suggesting that the vector-based RNAi approach can efficiently deplete CHO1/MKLP-1 in mammalian cells. We developed a protocol to select the transfection-positive cells to circumvent the variation of transfection efficiency in different experiments. As described in Materials and Methods, pBabe-Puro, which expresses a puromycin-resistance gene, was co-transfected with pBS/U6-CHO1 to select the transfected cells. After 2 days of selection, most untransfected cells were dead, and the surviving cells were used for phenotype analysis. We first determined whether CHO1/MKLP-1 is required for cell growth. Transfection with the control vector did not affect the growth rate of HeLa cells, whereas transfection with pBS/U6-CHO1 and pBabe-Puro strongly inhibited cell proliferation (Fig. 5B). Co-transfection of pBS/U6-CHO1 with non-degradable hamster GFP-CHO1 partially rescued cell growth (Fig. 5B).

Next, we analysed the effect of CHO1/MKLP-1 depletion on cell cycle progression using fluorescence-activated cell sorting (FACS). As shown in Fig. 5C, the transfection of control vectors did not affect the cell cycle profile, whereas CHO1/MKLP-1 depletion induced an obvious increase in the proportion of cells with 4N and 8N DNA content, suggesting that CHO1/MKLP-1-depleted cells cannot complete cytokinesis. To confirm this phenotype, we analysed the CHO1/MKLP-1-depleted cells by confocal microscopy (Fig. 5D). Three days after transfection, about 85% of CHO1/MKLP-1-depleted cells were multinucleated compared with less than 3% of control cells (Fig. 5E). This is consistent with the increase of cell population with 4N and 8N DNA contents observed in the FACS profiles (Fig. 5C).

The fact that aberrations in centrosome numbers have been implicated in aneuploidy and tumorigenesis led us to analyse the centrosome numbers in CHO1/MKLP-1-depleted cells. As shown by immunofluorescent staining with anti-γ-tubulin antibody, transfection of cells with

![Fig. 5. Depletion of CHO1/MKLP-1 causes multinucleation.](image)
control vector did not significantly affect centrosome numbers, whereas cells with depleted CHO1/MKLP-1 had multiple centrosomes (Fig. 5F). Upon careful inspection of CHO1/MKLP-1-depleted cells, we noticed that almost every multinucleate cell also had an increased number of centrosomes. Indeed, about 95% of multinucleate cells had four centrosomes, whereas less than 5% of multinucleated cells had 1-2 centrosomes (Fig. 5G). This strongly suggests that amplified centrosome numbers are probably due to the defect in cell division. We were also able to detect mitotic CHO1/MKLP-1-depleted cells in which multiple spindle poles had formed (Fig. 5H).

Cytokinesis defect in CHO1-deficient cells
To analyse the involvement of CHO1 during cytokinesis directly, we depleted human CHO1 with pBS/U6-GFP-CHO1, the same vector used above except GFP is expressed as an independent marker in transfected cells. HeLa cells were transfected with pBS/U6-GFP-CHO1 or a control vector. Twenty-four hours after transfection, cells were fixed and subjected to anti-α-tubulin staining. As shown in Fig. 6A,B, CHO1 depletion did not affect chromosome condensation, sister chromatid separation or spindle formation. However, the tubulin staining pattern was different during late mitosis for control and CHO1-deficient cells. A clear mid-body matrix was detected in control cells during anaphase and telophase (Fig. 6A, arrows). The decrease of tubulin intensity in this region is caused by the presence of large amounts of other proteins that are required for cytokinesis (Saxton and McIntosh, 1987). By contrast, tubulin staining in the CHO1-deficient cells was still apparent in the mid-body matrix region (Fig. 6B, arrows), presumably because of the absence of other antigens in this region, such as Plk1 (Fig. 3). To confirm the requirement for CHO1 for cytokinesis, CHO1 depletion was coupled to cell synchronization (Fig. 6C,D). For control cells, about 45% of telophase/cytokinesis cells were GFP positive, consistent with the transfection efficiency under this experimental condition. In the case of CHO1-deficient cells, only 15% of telophase/cytokinesis cells were GFP positive, indicating that CHO1 depletion inhibits cell division at an early phase of cytokinesis.

Multinucleation induced by overexpression of an unphosphorylatable CHO1 mutant
To determine the functional significance of CHO1 phosphorylation by Plk1, we ectopically expressed GFP-fused CHO1 constructs in HeLa cells and their subcellular localizations were determined (Fig. 7A-E). Endogenous CHO1 has been reported to localize to both centrosome and nucleus during interphase (Sellitto and Kuriyama, 1988), and this is also the case for ectopically expressed GFP-CHO1 (Fig. 7A, top, middle). However, GFP-CHO1-4A was observed only in the nucleus, not in the centrosome, during interphase (Fig. 7A, bottom). Careful inspection at high resolution indicated that GFP-CHO1 showed a speckled pattern that varied in extent and intensity within the nucleus, whereas a much more uniform distribution pattern was detected for the GFP-CHO1-4A mutant (Fig. 7B). Another striking localization of GFP-CHO1 wild type (GFP-CHO1-WT), but not the 4A mutant, is to the

![Fig. 6. Cytokinesis defect in CHO1-depleted cells. (A,B) HeLa cells were transfected with control vector (A) or pBS/U6-GFP-CHO1 (B), in which GFP was independently expressed as a marker for transfected cells. Only the GFP-positive cells produced hairpin RNA to induce CHO1 depletion. 30 hours after transfection, cells were fixed and subjected to anti-α-tubulin staining. Arrows indicated the regions of mid-zone/mid-body structure. Scale bar, 10 μm. (C) The protocol used to deplete CHO1 in well-synchronized cells. (D) Histogram quantifying the results.](image-url)
intercellular bridge (Fig. 7C,D). Both GFP-CHO1 and GFP-CHO1-4A localize to typical mitotic structures like endogenous CHO1 during mitosis (Fig. 7E). Neither overexpression of GFP-CHO1-WT (Fig. 7F) nor GFP-CHO1-4A (data not shown) affects Plk1 localization. However, 30% of GFP-CHO1-4A-expressing cells were found to be multinucleate (Fig. 7G,H).

Cytokinesis defect in GFP-CHO1-4A overexpressing cells
To test directly whether GFP-CHO1-4A-expression-induced multinucleation was caused by a defect in cytokinesis, we transfected GFP-CHO1 constructs into well-synchronized cells (Fig. 8). Following double-thymidine block, cells were released into fresh medium for 13.5 hours to obtain the maximum number of cells at telophase/cytokinesis. For cells transfected with GFP-CHO1-WT, about 80% of telophase/cytokinesis cells were GFP positive, indicating the normal progression of cell division of these cells. However, only 40% of telophase/cytokinesis cells were GFP positive for cells transfected with GFP-CHO1-4A, suggesting the dominant negative effect of this construct for cytokinesis. In fact, a significant proportion of GFP-CHO1-4A-expressing cells were binucleate (Fig. 8C).

Rescue of CHO1-depletion-induced phenotype by co-transfection with hamster CHO1/MKLP-1
Data presented above suggested an essential function of
CHO1/MKLP-1 during cytokinesis. Because of the specificity of the RNAi technique, we attempted to rescue CHO1/MKLP-1 depletion in human cells with hamster MKLP-1. A sequence alignment indicates that four nucleotides within the RNAi targeting site we selected are different for human and hamster CHO1/MKLP-1 (Fig. 9A). Therefore, the hamster CHO1/MKLP-1 mRNA was not expected to be a target of human RNAi. HeLa cells were co-transfected with pBS/U6-CHO1, GFP-CHO1 (hamster) and pBabe-Puro at 4.5:4.5:1 ratio using GenePorter reagent, and puromycin was added the day after transfection. 3 days after transfection, the floating cells were removed and the remaining cells were fixed and subjected to FACS to analyse the rescue effect of hamster CHO1 in CHO1-depleted HeLa cells (Fig. 9B). As shown in Fig. 9C, 66% of CHO1-depleted cells had an 8N DNA content, compared with less than 5% of control cells. By contrast, only 22% of HeLa cells co-transfected with hamster GFP-CHO1/MKLP-1 had an 8N DNA content. Moreover, up to 59% of cells were not able to complete cytokinesis after co-transfection with hamster GFP-CHO1-4A, in which four phosphorylation sites are converted to alanines (S805A, S807A, S904A, S905A). The similar experiments were performed with cells on coverslips; representative images are shown in Fig. 9D. Consistent with the data obtained with FACS, hamster GFP-CHO1 can efficiently rescue the CHO1-depletion phenotype, whereas hamster GFP-CHO1-4A did not (Fig. 9E).

Next, we examined the Plk1 localization pattern in these cells. Plk1 was found to localize to mitotic structures in GFP-CHO1-WT-expressing cells in the absence of endogenous CHO1 (Fig. 10A). It was very difficult to detect the limited numbers of late mitotic cells after endogenous CHO1 depletion and hamster GFP-CHO1-4A expression. Nevertheless, Plk1 seemed to localize normally in the few cells examined (Fig. 10B). Therefore, Plk1 localization was not CHO1-phosphorylation dependent. Finally, we also compared the ability of different constructs to rescue the CHO1-depletion-induced cytokinesis defect (Fig. 10C-E). Although the introduction of GFP-CHO1-WT resulted in CHO1-deficient cells completing cytokinesis, the expression of GFP-CHO1-4A in CHO1-deficient cells did not allow the cells to complete cytokinesis and they became multinucleate.

**Discussion**

Interaction of Plk1 with CHO1/MKLP-1 was first demonstrated in mammalian cells. CHO1/MKLP-1 immunoprecipitates with Plk1 and the two proteins are both localized to the mid-zone during anaphase and the mid-body during telophase and cytokinesis (Lee et al., 1995). The Polo-box-dependent localization of Plk1 at mitotic structures has been documented previously (Lee et al., 1998; Jang et al., 2002; Elia et al., 2003a; Elia et al., 2003b). Here, we provide evidence that the Polo box influences Plk1 association with CHO1/MKLP-1 (Fig. 1). The normal localization of Polo kinase to centrosomes and the central spindle is disrupted in a pav (an MKLP-1 ortholog) mutant of *Drosophila* (Adams et al., 1998). Our data indicate that sequences in the Polo-box domain of Plk1 and the stalk domain of CHO1/MKLP-1 are responsible for the interaction of the full-length proteins with mitotic structures. This interaction might then facilitate the phosphorylation of CHO1/MKLP-1 by Plk1. In addition, this interaction might permit CHO1/MKLP-1 to deliver Plk1 to other substrates.

To examine the interaction of Plk1/CHO1 in more detail, we ectopically expressed HA-tagged CHO1/MKLP-1 domains in Cos-7 cells. Because both Plk1 and CHO1/MKLP-1 are microtubule-interacting proteins (Feng et al., 1999), nocodazole treatment before harvest was used to depolymerize microtubules and hence to minimize the potential for indirect interaction. The stalk domain of CHO1/MKLP-1 was found...
to be responsible for interaction with Plk1 by the co-immunoprecipitation approach (Fig. 2). This result is consistent with co-localization of the GFP-stalk and Plk1 to the mid-body during telophase/cytokinesis (Fig. 2C). During the preparation of this manuscript, a paper describing the subcellular distribution of the *Drosophila* Pav-KLP domains, was published (Minestrini et al., 2002). In agreement with our data, the stalk domain alone of Pav-KLP was found to be sufficient for the exclusive association of Pav-KLP to the ring canals, actin-rich cytokinetic structures.

*Drosophila* Polo associates with Pav-KLP and shows a similar localization pattern during late mitosis, but this localization is disrupted in *pav* mutants (Adams et al., 1998). Pav-KLP accumulates at the spindle poles during meiosis in some *polo* mutants but fails to associate with the spindle midzone (Carmena et al., 1998). Thus, Pav-KLP and Polo appear to be mutually dependent for correct localization. Similarly, we show here that mammalian Plk1 mislocalized and is not detected on mitotic structures during late mitosis after the depletion of CHO1/MKLP-1. Therefore, the depletion of CHO1/MKLP-1 might prevent Plk1 from interacting with its substrates during cytokinesis. By contrast, CHO1/MKLP-1 localization is not dependent on Plk1, because CHO1 still localizes to mitotic structures in the Plk1-deficient cells.

The function of mitotic kinesin-like motor protein has been controversial in different organisms. The mammalian CHO1/MKLP-1 can cross-bridge and slide antiparallel microtubules in vitro, so it was proposed to function in spindle elongation (Nislow et al., 1992). In line with its function during karyokinesis, microinjection of CHO1/MKLP-1 antibodies was shown to cause mitotic arrest in mammalian cells (Nislow et al., 1990) and sea-urchin embryos (Wright et al., 1993). However, genetic studies in *Drosophila* and *C. elegans* revealed essential functions of CHO1/MKLP-1 homologs during cytokinesis. It was suggested that *Drosophila* PAV-KLP is required to establish the structure of the telophase spindle, to provide a framework for the assembly of the contractile ring and to mobilize mitotic regulator proteins (Adams et al., 1998). *C. elegans* ZEN-4 was also shown to be required for mid-zone microtubule organization and cytokinesis (Raich et al., 1998).

Using direct transfection of synthetic 21-nucleotide double-stranded RNA to knock down MKLP-1 in CHO cells, it was reported that CHO1/MKLP-1 is required for the organization of the central spindle and the formation of mid-body matrix...
This report supports the CHO1/MKLP-1-depletion-induced polyploidy phenotype we observed here using the vector-based RNAi approach in HeLa cells. Furthermore, analysis of CHO1 depletion in well-synchronized cells here also indicates the requirement of CHO1 in the early phase of cytokinesis. In contrast to the antibody microinjection experiment, CHO1/MKLP-1 depletion by RNAi did not lead to mitotic arrest. Indeed, when the CHO1/MKLP-1-depleted cells are selected with puromycin, they clearly underwent multiple rounds of DNA synthesis, as demonstrated here, during which we monitored the shift in ploidy by FACS analysis and the increase in chromosome and centrosome numbers by immunostaining (Fig. 5). Taken together, it appears that mammalian CHO1/MKLP-1 is required for cytokinesis but not karyokinesis. It should be realized that, in the previous report with siRNA experiments, CHO1/MKLP-1-depleted cells were not selected or examined for several days.

Malignant tumors are frequently characterized by aneuploidy (abnormal numbers of chromosomes) (Seckinger et al., 1989). Moreover, centrosome defects, such as excessive numbers of centrosomes and centrosomes with aberrant structures, are also a common feature of malignant tumors and have been implicated in genetic instability in cancer (Pihan et al., 1998). Thus, centrosomal anomalies correlate well with aneuploidy and cancer development (Pihan and Doxsey, 1999). However, whether centrosome anomalies constitute a cause or consequence of aneuploidy is controversial. Centrosome defects can alter the normal assembly and function of mitotic spindles, leading to the mis-segregation of chromosomes. By contrast, extra copies of centrosomes can also be the result of defects in mitotic progression or failure of cytokinesis. In the case of CHO1/MKLP-1-depleted cells, centrosome hyperamplification is likely to be a byproduct, not the cause, of polyploidy, owing to the cytokinesis failure of these cells. However, the aberrant centrosome numbers in CHO1/MKLP-1-depleted cells might subsequently lead to chromosome mis-segregation owing to the presence of multiple spindle poles if a subpopulation of these cells undergoes division (Fig. 5H).

Although D/E-X-S/T-φ has been proposed to be a consensus motif for Plk1 phosphorylation (Nakajima et al., 2003), Plk1 phosphorylation sites do not always fit D/E-X-S/T-φ in the reported Plk1 substrates. For example, T203, S205, S206 and T207 within the context of the 201-PPTLSSTV-208 motif of BRCA2 have been reported to be Plk1 phosphorylation sites (Lin et al., 2003). Plk1 phosphorylates cyclin B1 at Ser147 within the context QAFSDV (Toyoshima-Morimoto et al., 2001). In the case of Scc1 sites phosphorylated by Cdc5, nine serine residues were identified by mass spectrometry. The sites that do not fit D/E-X-S/T are Ser 183 (RRFSPD); Ser194 (NNLS); Ser273 and Ser276 (GESIMS); Ser325 (NTKS); and Ser389 (YESL). Ser263 (DNSV) was not identified by mass spectrometry (Alexandru et al., 2001). We have recently identified another centrosomal component as a new Plk1 substrate. Extensive analysis indicated that its Plk1 phosphorylation sites do not fit the D/E-X-S/T sequence (T. Zhou et al., unpublished).

The cytokinetic function of CHO1/MKLP-1 is potentially regulated by Plk1-associated kinase activity was supported by two lines of evidence. Ectopic expression of GFP-CHO1-4A caused cytokinesis defects, most likely caused...
by the dominant negative effect of the mutant protein to inhibit the function of endogenous CHO1 during cell division. Moreover, only wild-type GFP-CHO1, not the Plk1-unphosphorylatable mutant GFP-CHO1-4A, can rescue the CHO1-depletion-induced formation of multinuclei (Fig. 9). An essential function of polo kinase has been implicated in different organisms. In budding yeast, ectopic expression of Cdc5 causes the formation of additional septin ring structures (Lee and Erikson, 1997), and an obvious cytokinetic defect was observed in cells overexpressing the Polo-box domain of Cdc5, presumably owing to a dominant negative effect (Song and Lee, 2001). Drosophila Polo kinase was also demonstrated to be required for cytokinesis (Carmena et al., 1998). Cytokinetic defects in the premitotic divisions are supported by reduced numbers of ring canals and enlarged cells in cysts of primary spermatocytes. It is of interest that mislocalization of Pav-KLP and the septin Peanut was also observed in polo mutants (Carmena et al., 1998). In mammalian cells, we previously reported that 15% of cells showed cytokinesis arrest 48 hours after Plk1 was depleted using siRNA (Liu and Erikson, 2002). The cytokinetic function of Polo kinase is probably mediated by its substrates through phosphorylation.

In addition to CHO1/MKLP-1, our lab recently identified another motor-associated protein, NudC, as a Plk1 target during cytokinesis (Zhou et al., 2003). Downregulation of NudC by RNAi results in cells arrested at telophase, which are rescued by ectopic expression of wild-type NudC but not an unphosphorylatable mutant. Moreover, Plk1 is mislocalized in both NudC-depleted and NudC-overexpressing cells. Thus, Plk1-dependent-phosphorylation might be a general mechanism for the normal function of motor proteins during cytokinesis. To support this concept, it was recently reported that phosphorylation of MKLP-2 by Plk1 is also required for cytokinesis (Neef et al., 2003). MKLP-2, formerly known as rabbkinesin-6, is involved in normal cell division. It was demonstrated that Plk1 was mislocalized from the central spindle in MKLP-2-depleted cells, and the wild type, but not an unphosphorylatable mutant of MKLP-2, resulted in normal Plk1 localization to the central spindle. Therefore, multiple motors (NudC, MKLP-1, MKLP-2) are required to act together to recruit Plk1 to the mid-zone/mid-body structures for regulation of cytokinesis. In their report, Neef et al. claimed that Plk1 fails to phosphorylate MKLP-1 (Neef et al., 2003) and we have no explanation for this apparent discrepancy. Further studies with an exchange of reagents will be necessary to resolve this issue.

One possible reason for the involvement of multiple motors in Plk1 localization is that different motors act in the different phases of late mitosis (Ohkura, 2003). So far, interaction of Plk1 with its substrates can be categorized into three types. Phosphorylation of Cdc25C by Cdc2/cyclin-B might generate a binding site for the Plk1 Polo-box domain to facilitate subsequent activation of Cdc25C by Plk1 (Elia et al., 2003a; Elia et al., 2003b). In the case of MKLP-2, priming phosphorylation by Plk1 itself further enhances its binding and phosphorylation at the stalk region (Neef et al., 2003). Plk1/MKLP-1 interaction does not seem to be phosphorylation dependent, because Plk1 phosphorylates the tail domain, whereas it interacts with the stalk domain of MKLP-1.

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