Specific Phosphorylation of Nucleophosmin on Thr^{199} by Cyclin-dependent Kinase 2-Cyclin E and Its Role in Centrosome Duplication*

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The kinase activity of cyclin-dependent kinase 2 (CDK2)-cyclin E is required for centrosomes to initiate duplication. We have recently found that nucleophosmin (NPM/B23), a phosphoprotein primarily found in nucleolus, associates with unduplicated centrosomes and is a direct substrate of CDK2-cyclin E in centrosome duplication. Upon phosphorylation by CDK2-cyclin E, NPM/B23 dissociates from centrosomes, which is a prerequisite step for centrosomes to initiate duplication. Here, we identified that threonine 199 (Thr^{199}) of NPM/B23 is the major phosphorylation target site of CDK2-cyclin E in vitro, and the same site is phosphorylated in vivo. NPM/T199A, a nonphosphorylatable NPM/B23 substitution mutant (Thr^{199} → Ala) acts as dominant negative when expressed in cells, resulting in specific inhibition of centrosome duplication. As expected, NPM/T199A remains associated with the centrosomes. These observations provide direct evidence that the CDK2-cyclin E-mediated phosphorylation on Thr^{199} determines association and dissociation of NPM/B23 to the centrosomes, which is a critical control for the centrosome to initiate duplication.

The centrosome, a major microtubule-organizing center of the animal cells, directs the formation of bipolar mitotic spindles, which is essential for accurate chromosome segregation to daughter cells (for reviews, see Refs. 1–3). Since each daughter cell inherits one centrosome upon cytokinesis, the centrosome must duplicate prior to the next mitosis and do so only once. Thus, centrosome duplication must take place in coordination with other cell cycle events including DNA synthesis. In mammalian cells, the centriole, the core component of the centrosome, initiates duplication at the G_{1}/S boundary (reviewed in Refs. 4–6). Activation of cyclin-dependent kinase 2 (CDK2)1-cyclin E has recently been found to be essential for the centrosome to initiate duplication (7, 8). The activity of CDK2-cyclin E is regulated by the temporal expression of cyclin E, which normally occurs in late G_{1} (9, 10), and it has been known that active CDK2-cyclin E complexes are required for initiation of DNA replication (11, 12). These observations indicate that the late G_{1}-specific activation of CDK2-cyclin E plays a key role for the coordinated initiation of centrosome and DNA duplication. Indeed, we have shown that constitutive activation of CDK2-cyclin E by cyclin E overexpression in cultured mammalian cells results in uncoupling of the initiation of centrosome and DNA duplication; in these cells, the centrosomes initiate duplication in early G_{1} long before the onset of DNA synthesis (13). Unlike the initiation of DNA synthesis, which can only be triggered by CDK2-cyclin E after completion of a series of necessary events (14, 15), the initiation of centrosome duplication appears to depend primarily on the activation of CDK2-cyclin E. Thus, the late G_{1}-specific activation of CDK2-cyclin E may serve as a checkpoint control for timely initiation of centrosome duplication.

We have recently identified nucleophosmin (NPM/B23) as a substrate of CDK2-cyclin E in the initiation of centrosome duplication (16). NPM/B23, also called numatrin or NO38, was originally identified as a major nucleolar phosphoprotein localized in granular regions of the nucleolus and has been shown to be associated with preribosomal particles (17–19). To date, NPM/B23 has been implicated in several distinct cellular functions, including assembly and/or intranuclear transport of preribosomal particles, cytoplasmic/nuclear trafficking, the regulation of DNA polymerase α activity, and centrosome duplication (16–21). NPM/B23 has also been shown to possess molecular chaperoning activities, including preventing protein aggregation, protecting enzymes during thermal denaturation, and facilitating renaturation of chemically denatured proteins (22). We have shown that NPM/B23 associates specifically with unduplicated centrosomes, and this association is controlled by CDK2-cyclin E-mediated phosphorylation, in which NPM/B23 loses its affinity to centrosomes in its phosphorylated form (16). Dissociation of the centrosomal NPM/B23 is essential for the centrosome to initiate duplication (16). For instance, microinjection of the anti-NPM/B23 monoclonal antibody, which blocks the CDK2-cyclin E-mediated phosphorylation of NPM/B23, inhibits centrosome duplication. Moreover, ectopic expression of this NPM/B23 deletion mutant (NPMΔ186–239), which is unable to be phosphorylated by CDK2-cyclin E, results in suppression of centrosome duplication. These results demonstrate that dissociation of centrosomal NPM/B23 by CDK2-cyclin E-mediated phosphorylation is critical for initiation of centrosome duplication and that the site(s) of NPM/B23 phosphorylated by CDK2-cyclin E lies within the sequence between amino acid residues 186 and 239.

We here show that Thr^{199} of NPM/B23 is specifically phosphorylated by CDK2-cyclin E in vitro, and this phosphorylation...
is also observed in vivo. When NPM/B23 mutant with a substitution of this specific threonine residue to alanine (nonphosphorylatable) is expressed in cells, it affects centrosome duplication in a dominant negative fashion, resulting in suppression of centrosome duplication. These observations provide direct evidence that the CDK2-cyclin E-mediated phosphorylation of NPM/B23 on Thr¹⁹⁹ is critical for dissociation of centrosomal NPM/B23 and initiation of centrosome duplication.

**EXPERIMENTAL PROCEDURES**

**Cells and Transfection—**Swiss 3T3 and HeLa cells were maintained in complete medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) in an atmosphere containing 10% CO₂.

For generation of HeLa cells overexpressing cyclin E, a plasmid encoding a human cyclin E gene was co-transfected with a plasmid encoding a neomycin-resistant gene into HeLa cells by the calcium phosphate protocol. As a negative control, a vector was transfected. G418-resistant colonies that arose in the medium containing G418 (800 µg/ml) at 2–3 weeks after transfection were subcloned and analyzed for cyclin E expression. One cell line that overexpressed cyclin E (HeLa/CycE) and one vector-transfected G418-resistant cell line (HeLa/Vec) were maintained.

For transient transfection of wild-type and mutant NPM/B23 sequences, Swiss 3T3 cells were co-transfected with plasmids encoding either a FLAG-tagged wild-type or substitution mutant (Thr¹⁹⁹ → Ala) NPM/B23 with a puromycin resistance gene plasmid (pBabe/puro) at a molar ratio of 20:1 by the calcium phosphate protocol. After transfection in 37 °C for 8 h, cells were fed with fresh complete medium for 16 h. The cells were then treated with complete medium containing puromycin (4 µg/ml) for 36 h. The puromycin-resistant cells were pooled and replated on coverslips and further cultured in fresh complete medium for 24 h.

**Plasmid Construction and Purification of GST-NPM/B23—**Mutant as well as wild-type NPM/B23 cDNA sequences were fused to glutathione S-transferase using a two-step polymerase chain reaction as described. The polymerase chain amplified products were then inserted in frame into a pGEX-4T-1 vector using BamHI and EcoRI restriction sites. GST-NPM fusion proteins were bacterial purified according to the protocol provided by the manufacturer (Amersham Pharmacia Biotech). Briefly, cells were induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h before harvesting. Clarified bacterial lysates were passed over a Sepharose 4B column, and GST-NPM proteins were eluted. The concentration of the eluted GST-NPM was estimated by comparison with bovine serum albumin with a known concentration run in parallel on SDS-PAGE.

**Immunoblot Analysis—**Cells were lysed in SDS/Nonidet P-40 lysis buffer (1% SDS, 1% Nonidet P-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 4 mM Pefabloc SC, 2 µg/ml leupeptin, 2 µg/ml pepstatin). Bacterial lysates were kept on ice for 5 min and then centrifugated at 10,000 g for 10 min at 20 °C for immunoblotting or a 10-min centrifugation at 20,000 × g at 4 °C. The supernant was further denatured at 95 °C for 5 min in sample buffer (2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, 0.01% bromphenol blue). Samples were resolved by SDS-PAGE and transferred onto Immobilon-P (Millipore Corp.) sheets. The blots were first incubated in blocking buffer (5% (w/v) nonfat dry milk in Tris-buffered saline plus Tween 20) for 1 h. The blots were then incubated with primary antibodies for 2 h, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h. All of the procedures were performed at room temperature. The antibody-antigen complex was visualized by ECL chemiluminescence (Amersham Pharmacia Biotech).

**Indirect Immunofluorescence—**Cells grown on coverslips were fixed with 10% formalin, 10% methanol for 10 min at room temperature. The cells were permeabilized with 1% Nonidet P-40 in phosphate-buffered saline for 5 min, followed by incubation with blocking solution (10% normal goat serum in phosphate-buffered saline) for 1 h. Cells were then probed with primary antibodies for 1 h, and antibody-antigen complexes were detected with either rhodamine- or FITC-conjugated goat immunoglobulin G antibodies. Immunoprecipitation using anti-cyclin E antibody (sc-198; Santa Cruz Biotechnology) was performed in 10 mM PIPES buffer in the presence of 0.1% Triton X-100 at 4 °C for 15 min and at 37 °C for an additional 15 min. The samples were resolved by SDS-PAGE, and the gel was dried and autoradiographed. For both histone H1 and GST-NPM kinase assays, ³²P incorporation was quantitated by scanning with a Fuji 1000 phosphoimager.

**BrdU Incorporation Assay—**Cells were transfected with a BrdUrd labeling kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Briefly, cells were fixed in 70% ethanol in 50 mM glycine (pH 2.0) for 20 min at −20 °C, incubated in the blocking buffer for 1 h at room temperature, and then probed with anti-γ-tubulin polyclonal and anti-BrdUrd monoclonal antibodies for 30 min at 37 °C. Antigen-antibody complexes were detected by FITC-conjugated sheep anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG antibodies. The preparation of cells is described in the legend to Fig. 5.

**Phosphoamino Acid Analysis—**GST-NPM/B23 phosphorylated in vitro by CDK2-cyclin E in the presence of [³²P]-γ-ATP was resolved by SDS-PAGE. ³²P-Labeled NPM/B23 was eluted from the gel and subjected to acid hydrolysis. The phosphorylated amino acids were separated by two-dimensional electrophoresis on a thin layer cellulose gel plate as described previously (27).

**Two-dimensional Tryptic Phosphopeptide Mapping—**For preparation of in vitro ³²P-labeled NPM/B23, HeLa/CycE cells were labeled for 2.5 h in phosphate-free medium containing 1 mCi/ml ³²P-orthophosphate and 2% dialyzed fetal bovine serum. Cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris (pH 8.0), 50 mM β-glycerophosphate, 50 mM sodium fluoride, 50 mM NaCl, 0.1% sodium deoxycholate, 4 mM Pefabloc SC, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 4 µM Microcystin-LR). After preclearing with protein G-conjugated agarose, the supernatant was subjected to immunoprecipitation with anti-NPM/B23 monoclonal antibody. Antibody-antigen complexes were collected by protein G-conjugated agarose and were resolved by 10% SDS-PAGE. The gel was dried and autoradiographed. The in vitro ³²P-labeled NPM/B23 proteins were eluted from the gel. In vitro ³²P-labeled GST-NPM/B23 fusion proteins were prepared as described above for phosphoamino acid analysis. Two-dimensional tryptic phosphopeptide mapping was performed as described previously (27). Briefly, both in vitro labeled GST-NPM/B23 and in vivo labeled NPM/B23 proteins were oxidized in performic acid and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (Worthington). Each sample was loaded onto a thin layer cellulose gel plate and run for 1 h at 1200 V at 4 °C. The plates were dried and subjected to chromatography (35% n-butanol, 25% pyridine, 7.5% acetic acid) in the vertical direction.

**RESULTS**

**Identification of the Site of NPM/B23 Specifically Phosphorylated by CDK2-Cyclin E in Vitro—**We have previously shown that NPM/B23 is a direct centrosomal protein substrate of the CDK2-cyclin E serine/threonine kinase complex in centrosome duplication. NPM/B23 deletion mutant (Δ186–239) fails to be phosphorylated by CDK2-cyclin E and acts as a dominant-negative when expressed in cells (16), indicating that the CDK2-cyclin E-mediated phosphorylation site(s) lie between amino acids 186 and 239. The sequence analysis of human NPM/B23 revealed that there are several serine and threonine residues within this region. By phosphoamino acid analysis, we first tested whether serine or threonine residue(s) (or both) are phosphorylated by CDK2-cyclin E in vitro.

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Phosphorylation of NPM/B23 by CDK2-Cyclin E

 Fig. 1. CDK2-cyclin E phosphorylates NPM/B23 in vitro on threonine residue(s). A, wild-type NPM/B23 and NPM mutant fused to GST (GST-NPM/wt and GST-NPM[Δ186–239], respectively) as well as GST were subjected to in vitro kinase reactions with CDK2-cyclin E. The reaction conditions were run on 10% SDS-PAGE (pH 8.8), and autoradiographed (left panel). The right panel shows the Coomassie blue-stained gel. Lane 1, GST; lane 2, GST-NPM[Δ186–239]; lane 3, GST-NPM/wt. B, the 32P-labeled GST-NPM/wt proteins as shown in A were purified from the gel and subjected to acid hydrolysis. The phosphorylated amino acids were then separated by two-dimensional electrophoresis and visualized by autoradiography as described previously (27). The positions of the migrations of phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) standards, detected by ninhydrin staining, are indicated by circles. +, the sample loading origin.

reaction of wild-type NPM/B23 fused to GST (GST-NPM/wt) was performed using baculovirally purified active CDK2-cyclin E in the presence of [γ-32P]ATP. As negative controls, GST proteins as well as GST-NPM[Δ186–239] were used as substrates. The kinase reaction samples were resolved by SDS-PAGE and autoradiographed (Fig. 1A). CDK2-cyclin E did not phosphorylate the GST moiety (lane 1). As shown previously, GST-NPM[Δ186–239] deletion mutant failed to be phosphorylated by CDK2-cyclin E (lane 2), while GST-NPM/wt was phosphorylated at a readily detectable level (lane 3). The phosphorylated GST-NPM/wt proteins were eluted from the gel and subjected to phosphoamino acid analysis (Fig. 1B). We found that NPM/B23 was phosphorylated exclusively on threonine residue(s) in vitro by CDK2-cyclin E.

There are four possible CDK2 phosphorylation consensus sequences within this region (Fig. 2A, indicated by arrowheads). To identify which threonine residue(s) are phosphorylated by CDK2-cyclin E, each of these four threonine residues (Thr199, Thr219, Thr234, and Thr237) was replaced with alanine (nonphosphorylatable amino acid). These mutants fused to GST (GST-NPM/T199A, GST-NPM/T219A, GST-NPM/T234A, and GST-NPM/T237A, respectively) were subjected to an in vitro kinase assay with CDK2-cyclin E (Fig. 2B, top panel). GST and GST-NPM/wt were included as controls in the experiment. Three GST-NPM mutants (T219A, T234A, and T237A) were phosphorylated (lanes 4–6) at levels similar to GST-NPM/wt (lane 2). However, GST-NPM/T199A showed dramatically reduced phosphorylation (lane 3), suggesting that Thr199 is the primary phosphorylation site of NPM/B23 by CDK2-cyclin E in vitro.

CDK2 is known to be activated also by association with cyclin A, which is up-regulated during S and G2 phases (9, 10, 28–30). We thus examined whether CDK2-cyclin A could phosphorylate Thr199. GST-NPM fusion proteins were subjected to an in vitro kinase assay with baculovirally purified CDK2-cyclin A (Fig. 2B, bottom panel). CDK2-cyclin A could phosphorylate all of the mutants except GST-NPM/T199A at an efficiency similar to GST-NPM/wt, demonstrating that CDK2-cyclin A can also specifically phosphorylate Thr199 in vitro.

It has previously been shown that CDK1-cyclin B, a CDK-cyclin complex specifically activated during mitosis (reviewed in Ref. 31), phosphorylates NPM/B23 (32). However, the phosphorylation target site(s) of CDK1-cyclin B had not been identified. We thus tested whether CDK1-cyclin B phosphorylates the same threonine residue that is phosphorylated by CDK2-cyclin E. GST-NPM substitution mutants described above as well as GST-NPM/wt were subjected to an in vitro kinase assay with immunopurified CDK1-cyclin B (Fig. 2C). CDK1-cyclin B phosphorylated GST-NPM/T199A (lane 2) at an efficiency similar to GST-NPM/wt (lane 1), indicating that Thr199 is not the target site of CDK1-cyclin B. In contrast, the levels of [32P] incorporation of both GST-NPM/T234A (lane 4) and GST-NPM/T237A (lane 5) were reduced to less than 50% of GST-NPM/wt. When both Thr234 and Thr237 were replaced with alanine residues (GST-NPM/T234A/T237A), the level of [32P] incorporation became almost undetectable (lane 6). This result indicates that CDK1-cyclin B phosphorylates both Thr234 and Thr237 in vitro.
Moreover, CDK2-cyclin E and CDK1-cyclin B phosphorylate different sites of NPM/B23.

Thr^{199} of NPM/B23 is phosphorylated in Vivo—We next examined whether Thr^{199} is phosphorylated in vivo by two-dimensional tryptic peptide mapping of NPM/B23 prepared from metabolically labeled cells with [^{32}P]orthophosphate. Since CDK2-cyclin E is normally activated only in late G_{1}, we assumed that CDK2-cyclin E-mediated phosphorylation of NPM/B23 might not be efficiently detected if the exponentially growing cells are used. In addition, it is not known whether CDK2-cyclin A-mediated phosphorylation of NPM/B23 on Thr^{199} occurs in vivo in a similar manner as in vitro. To circumvent these problems, we first generated HeLa cells overexpressing cyclin E by transfecting human cyclin E together with a plasmid encoding the neomycin resistance gene as a selection marker. It has been shown that overexpression of cyclin E results in constitutive activation of CDK2-cyclin E (9, 13–15). The G418-resistant colonies were subcloned and examined for cyclin E expression in HeLa cells stably transfected with human cyclin E (HeLa/CycE) and HeLa/Vec, respectively. The extracts prepared from these cells (50 μg of total protein) were subject to immunoblot analysis using anti-human cyclin E polyclonal antibody (sc-198; Santa Cruz Biotechnology). HeLa/CycE cells express ~5-fold more cyclin E proteins than HeLa/Vec cells. B, histone H1 kinase activity of HeLa/CycE cells. The cell extracts derived from exponentially growing HeLa/CycE and the control HeLa/Vec cells were immunoprecipitated with anti-human cyclin E antibody, and the immunoprecipitates were subjected to an in vitro histone H1 kinase assay as described previously (13). C, tryptic phosphopeptide mapping of in vitro phosphorylated NPM/B23 by CDK2-cyclin E and in vivo phosphorylated NPM/B23 in HeLa/CycE cells. GST-NPM/wt phosphorylated in vitro by CDK2-cyclin E was prepared as described in the legend to Fig. 1. For preparation of in vivo phosphorylated NPM/B23, HeLa/CycE cells were metabolically labeled in the presence of [^{32}P]orthophosphate. The lysates were immunoprecipitated with anti-NPM/B23 monoclonal antibody, and the immunoprecipitates were resolved by 10% SDS-PAGE. [^{32}P]-Labeled NPM/B23 proteins were eluted from the gel. The in vitro [^{32}P]-labeled GST-NPM/wt (a) and in vivo [^{32}P]-labeled NPM/B23 (b) were oxidized with performic acid and digested with trypsin as described previously (27). Each tryptic digestion sample was loaded onto a thin layer cellulose gel plate and subjected to electrophoresis (horizontal dimension), followed by ascending chromatography. Mixed map was generated by loading equal counts of trypsin-digested in vitro [^{32}P]-labeled GST-NPM/wt and in vivo [^{32}P]-labeled NPM/B23 (c). +, the origin of the sample placement. The arrows indicate the [^{32}P]-labeled tryptic fragment that is observed both in the in vitro and in vivo samples.
CDK2-cyclin E, it does not exclude the possibility that the deleted sequence other than the phosphorylation site may be also important for the regulation of centrosome duplication. We thus examined whether the nonphosphorylatable NPM/T199A mutant acts as a dominant negative in centrosome duplication in fashion similar to NPM(Δ186–239). If expression of NPM/T199A mutant results in suppression of centrosome duplication, the phosphorylation of Thr^{199} is most likely a sole event necessary for the NPM/B23-dependent control of centrosome duplication. The FLAG epitope-tagged wild-type NPM/B23 (NPM/wt) and NPM/T199A were placed in eukaryotic expression vectors and transfected into Swiss 3T3 cells together with a plasmid encoding a puromycin-resistant gene. As a control, the vector was transfected. The puromycin-resistant cells selected by puromycin treatment for 36 h were replated and cultured for additional 24 h. Cells were first examined for the level of expression of transfected NPM/B23 by immunoblot analysis using anti-FLAG antibody (Fig. 4A). Both NPM/wt and NPM/T199A transfecteds expressed similar levels of transfected NPM/B23.

Cells were examined for centrosomes by immunostaining of γ-tubulin, a major component of the pericentriolar material of the centrosome (reviewed in Ref. 35). The graph in Fig. 4B shows the centrosome profiles of the vector, NPM/wt, and NPM/T199A transfectants. In the vector-transfected cells, ~40% of the cells contained one centrosome, and ~60% contained two centrosomes. The cells transfected with NPM/wt showed centrosome profiles similar to the vector transfectants. In contrast, the majority (>80%) of cells transfected with NPM/T199A contained one centrosome, indicating that ectopic expression of NPM/T199A results in suppression of centrosome duplication.

To verify whether the anti-γ-tubulin antibody-reactive signals (dots) represented intact centrosomes with a pair of centrioles, cells were also immunostained for the centrioles. Since α-tubulin is one of the major constituents of centrioles, immunostaining of α-tubulin allows visualization of a centriole pair within the centrosome. Cells were subjected to cold treatment (which depolymerizes microtubules nucleated at the centrosomes), followed by a brief extraction prior to fixation (see “Experimental Procedures”), and co-immunostained with anti-γ-tubulin polyclonal and anti-α-tubulin monoclonal antibodies (Fig. 4C). Each dot detected by anti-γ-tubulin antibody (panels a and c) was resolved to a pair of dots (representing a centriole pair) by anti-α-tubulin antibody at a higher magnification (panels b and f, panels I–VIII). All of the anti-γ-tubulin antibody-reactive dots were co-immunostained by anti-α-tubulin antibody as doublets. Thus, the doublets detected by anti-γ-tubulin antibody represent duplicated centrosomes. The centrosome profiles determined by anti-α-tubulin antibody were similar to those determined by anti-γ-tubulin antibody (data not shown).

To eliminate the possibility that inhibition of centrosome duplication by NPM/T199A is due to a general cell cycle arrest, Swiss 3T3 cells were transiently transfected with either a vector or a NPM/T199A mutant plasmid along with a plasmid encoding a puromycin resistance gene as a selection marker. The puromycin-resistant cells selected among 36-h puromycin treatment were cultured for an additional 24 h. During the final 3 h of culturing, BrdUrd was added to the medium to monitor cell cycling. Cells were co-immunostained with anti-γ-tubulin polyclonal and anti-BrdUrd monoclonal antibodies (Fig. 5A). Approximately 10% of vector-transfected and ~6% of NPM/T199A-transfected cells were BrdUrd-positive, suggesting that the expression of NPM/T199A may be partially cytotoxic. A similar observation was previously made for the NPM(Δ186–239) deletion mutant (16). Examination of centro-
somes revealed that all of the BrdUrd-positive vector-transfected cells contained duplicated centrosomes, while the majority (>80%) of NPM/T199A-transfected BrdUrd-positive cells contained a single centrosome (Fig. 5, B and C). Thus, dominant-negative activity of NPM/T199A specifically targets the centrosome duplication process.

**Aberrant Mitoses with Monopolar Spindles Resulting from Expression of NPM/T199A Mutant**—The finding that the expression of NPM/T199A mutant results in the suppression of centrosome duplication but not DNA duplication predicts that NPM/T199A-transfected cells should progress through the cell cycle to mitosis without centrosome duplication. This should lead to mitosis with monopolar instead of bipolar spindles. To test this prediction, Swiss 3T3 cells were transiently transfected with either a plasmid encoding FLAG epitope-tagged NPM/T199A or a control vector. For each transfection, pBabe/puro was co-transfected as a selection marker. Puromycin was added to medium 16 h after transfection. Puromycin-resistant cells at 36 h after the addition of puromycin were replated and further cultured for 24 h. During the final 3 h of culturing, BrdUrd was added to the medium. Cells were then processed for co-immunostaining with anti-BrdUrd monoclonal (B, panels a and b) and anti-γ-tubulin polyclonal antibodies (B, panels a’ and b’). First, the percentage of cells that had incorporated BrdUrd was determined through examination of >300 cells (A). In vector-transfected cells, virtually all of the BrdUrd-positive cells contained two anti-γ-tubulin antibody-reactive dots (duplicated centrosomes) (B, panels a and a’, indicated by arrows), while the majority of BrdUrd-positive NPM/T199A contained a single dot (unduplicated centrosome) (B, panels b and b’, indicated by an arrow). The number of centrosomes per cell in the BrdUrd-positive cells was scored by fluorescence microscopy. For each transfecant, >100 BrdUrd-positive cells were examined, and the results from three independent experiments are shown in C.

**NPM/T199A Mutant Associates with the Centrosomes**—We have previously shown that NPM/B23 dissociates from centrosomes upon CDK2-cyclin E-mediated phosphorylation (16), which implies that the NPM/T199A mutant should associate with centrosomes. We thus examined the localization of transfected FLAG epitope-tagged NPM/T199A by co-immunostaining with anti-γ-tubulin polyclonal and anti-FLAG monoclonal antibodies (Fig. 7). No anti-FLAG antibody staining was observed in the vector-transfected cells (Fig. 7B). In contrast, anti-FLAG antibody detected a single dot adjacent to nucleus in the NPM/T199A-transfected cells (Fig. 7F), which overlap with the dot detected by anti-γ-tubulin antibody (Fig. 7, E and H). Thus, NPM/T199A mutant physically associates with centrosomes.
DISCUSSION

Centrosome hyperamplification, which leads to formation of aberrant mitotic spindles, is now well accepted as one of the major causes of chromosome instability in human cancers (36–39). In normal cells, the centrosome duplication cycle is tightly regulated. Coordinated initiation of centrosome and DNA duplication is one of the major regulatory checkpoints for proper progression of the centrosome duplication cycle, and it is established at least in part by the late G1-specific activation of CDK2-cyclin E (7, 8).

Activation of CDK2-cyclin E plays a major role in the initiation of DNA synthesis through phosphorylation of retinoblastoma susceptibility protein (pRb). When pRb is phosphorylated, it releases the pRb-bound E2F transcriptional factor, which then stimulates the transcription of a number of genes required for DNA synthesis (for reviews, see Refs. 40 and 41). The requirement for E2F has also been implicated in the initiation of centrosome duplication (42), suggesting that E2F-dependent expression of specific protein(s) may be needed for centrosome duplication. Indeed, it has been shown that, in Chinese hamster ovary cells, synthesis of certain centrosomal proteins during G1 is necessary for the initiation of centrosome duplication (43). In addition, CDK2-cyclin E has been shown to directly act on a centrosomal protein to initiate centrosome duplication. NPM/B23 binds specifically to unduplicated centrosomes and loses its centrosome binding activity when phosphorylated by CDK2-cyclin E. Dissociation of the centrosomal NPM/B23 appears to be a prerequisite for the centrosomes to initiate duplication, since centrosome duplication is blocked by microinjection of anti-NPM/B23 antibody, which prevents CDK2-cyclin E-mediated phosphorylation and dissociation of centrosomal NPM/B23 (16).

We have previously shown that NPM/B23 deletion mutant (NPM(D186–239)), which fails to be phosphorylated by CDK2-cyclin E, acts as a dominant negative when expressed in cells, resulting in suppression of centrosome duplication (16). In this study, we identified Thr199 as the CDK2-cyclin E phosphorylation target site of NPM/B23 both in vitro and in vivo. The NPM/B23 mutant with an alanine substitution at this site (NPM/T199A) acts as a dominant negative when expressed in cells and suppresses centrosome duplication, similar to the NPM(D186–239) deletion mutant. The initial stage of centrosome duplication consists of a series of distinct steps: loss of orthogonal configuration and physical separation of the centriole pair, which is followed by synthesis of a procentriole next to each preexisting centriole. Co-immunostaining of centrosomes

![Graphical representation of the percentage of mitotic cells with the indicated number of poles.](image)

**Fig. 6.** Expression of NPM/T199A mutant results in a high frequency of monopolar mitosis. Swiss 3T3 cells were transiently co-transfected with either a plasmid encoding FLAG epitope-tagged NPM/T199A or a control vector along with pBabe/puro as a selection marker. Puromycin-resistant cells were selected as described in the legend to Fig. 4. Cells were immunostained with anti-α- and β-tubulin antibodies (B, panels a and f) and anti-γ-tubulin polyclonal antibody (B, panels c and e). Cells were also counterstained with DAPI (B, panels c and g). Antigen-antibody complexes were visualized by FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG antibodies. The mitotic cells were first identified by condensed chromosomes under a fluorescence microscope, and the number of spindle poles in each mitotic cell was determined (A). For each transfectant, >50 mitotic cells were examined. Representative immunostaining images are shown in B. Panels d and h are overlay images of panels a–c and e–g, respectively. The arrows point to the spindle pole. Scale bar, 10 μm.
CDK2-cyclin E-mediated phosphorylation of NPM/B23 comprises one of the key events in the initiation of centrosome duplication. At present, the molecular basis of the centrosome-binding property of NPM/B23 (i.e. which centrosomal protein(s) the unphosphorylated form of NPM/B23 associates with) is unknown. The identification of the CDK2-cyclin E-mediated phosphorylation site will, however, expedite the elucidation of the particular centrosomal component(s) with which NPM/B23 directly associates.

Another important issue of the inhibition of centrosome duplication by expression of NPM/T199A mutant is the consequence of monopolar spindle formation. Considering the role of the centrosomes (spindle poles) in cytokinesis (47), it is safe to assume that monopolar mitotic cells do not undergo cytokinesis. If these cells enter the next cell cycle without cytokinesis, we should expect an increase in the number of cells with abnormal amplification of genome. However, the flow cytometric analysis of the NPM/T199A-transfected cells failed to detect any noticeable increase in the number of cells with abnormally amplified genome (data not shown), suggesting that formation of monopolar spindles probably leads to cell death. However, the mechanism of how cell death is induced in the monopolar mitotic cells remains to be clarified.

NPM/B23 associates with and dissociates from centrosomes in a cell cycle stage-specific manner (16, 44). During early to middle G1, NPM/B23 associates with the unduplicated centrosomes. In late G1, NPM/B23 dissociates from the centrosomes upon phosphorylation by CDK2-cyclin E. During S and G2 phases, association of NPM/B23 with the duplicated centrosomes is not detected. However, during mitosis, NPM/B23 reassociates with the centrosomes. This cell cycle stage-dependent dissociation and reassociation of NPM/B23 with centrosomes may be controlled by differential phosphorylation by CDK-cyclin complexes. CDK2-cyclin E activity peaks during late G1, triggering dissociation of the centrosomal NPM/B23. Upon entry into S phase, cyclin E expression becomes halted. Since cyclin E is intrinsically unstable, cyclin E-dependent CDK2 activity becomes minimal during S phase (9, 10). In contrast, the level of cyclin A is low in late G1 but increases during S and G2 phases (27–30). Thus, during S and G2 phases of the cell cycle, CDK2-cyclin A activity is high. We found that CDK2-cyclin A could also phosphorylate NPM/B23 specifically on Thr199 in vitro at an efficiency similar to CDK2-cyclin E. Thus, it is possible that the continual presence of active CDK2-cyclin A is responsible for preventing the reassociation of NPM/B23 to centrosomes during S and G2. Moreover, NPM/B23 has previously been shown to be phosphorylated by CDK1-cyclin B, a mitotic CDK-cyclin complex (32). We found that CDK1-cyclin B specifically phosphorylates Thr234 and Thr237 in vitro, which are different from CDK2-cyclin E (and cyclin A)-mediated phosphorylation sites. It remains to be investigated whether phosphorylation of Thr234 and/or Thr237 by CDK1-cyclin B is required for reassociation of NPM/B23 with the centrosomes during mitosis. These questions are currently addressed in our laboratory.

NPM/BNPM/B23 has been shown to participate in various cellular events that are to all appearances unrelated to each other, including ribosome assembly, intracellular trafficking, DNA polymerase activity, and centrosome duplication. These diverse functions of NPM/B23 are perhaps attributed to its molecular chaperoning activity as reported previously (22). All of the cellular events in which NPM/B23 has been shown to function involve either large multiprotein complexes or organelles consisting of many different proteins in a crowded condition. Thus, association/dissociation of NPM/B23 may dramatically influence the centrosome proper and thus determine the structural as well as functional state of the centrosome. Moreover, the CDK2-cyclin E-mediated functional modification of NPM/B23 may target other cellular event(s) as well, since
the BrdUrd incorporation assay showed that expression of NPM/T199A mutant partially blocks (or slows down) the cell cycle progression.

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