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Microtubule Regulation in Mitosis: Tubulin Phosphorylation by the Cyclin-dependent Kinase Cdk1

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The activation of the cyclin-dependent kinase Cdk1 at the transition from interphase to mitosis induces important changes in microtubule dynamics. Cdk1 phosphorylates a number of microtubule- or tubulin-binding proteins but, hitherto, tubulin itself has not been detected as a Cdk1 substrate. Here we show that Cdk1 phosphorylates β-tubulin both in vitro and in vivo. Phosphorylation occurs on Ser172 of β-tubulin, a site that is well conserved in evolution. Using a phosphorylation peptide antibody, we find that a fraction of the cell tubulin is phosphorylated during mitosis, and this tubulin phosphorylation is inhibited by the Cdk1 inhibitor roscovitine. In mitotic cells, phosphorylated tubulin is excluded from microtubules, being present in the soluble tubulin fraction. Consistent with this distribution in cells, the incorporation of Cdk1-phosphorylated tubulin into growing microtubules is impaired in vitro. Additionally, EGFP-β3-tubulinS172D/E mutants that mimic phosphorylated tubulin are unable to incorporate into microtubules when expressed in cells. Modeling shows that the presence of a phosphoserine at position 172 may impair both GTP binding to β-tubulin and interactions between tubulin dimers. These data indicate that phosphorylation of tubulin by Cdk1 could be involved in the regulation of microtubule dynamics during mitosis.

INTRODUCTION

In eukaryotic cycling cells, interphase microtubules form a dynamic network that is essential for cell polarity and intracellular traffic. When cells enter into mitosis, the interphase microtubule network rearranges into a mitotic spindle that is responsible for faithful chromosome segregation between daughter cells. Microtubule dynamics and turnover increase strikingly as cells progress from interphase to mitosis, with a microtubule half-life of 5–10 min in interphase and of 60–90 s in mitosis (Wittmann et al., 2001).

Microtubule dynamics in cells rely in part on the intrinsic properties of the microtubule building block, the α-β-tubulin dimer, and its ability to bind and hydrolyze a GTP nucleotide (Mitchison and Kirschner, 1984). The tubulin dimer is subject to posttranslational modifications such as glutamylation, tyrosination, and acetylation (MacRae, 1997; Westermann and Weber, 2003). There is little evidence for a role of these modifications in the regulation of microtubule dynamics. Tubulin can also be phosphorylated by several kinases (Westermann and Weber, 2003). However, tubulin phosphorylation has not been connected with the cell-cycle-dependent regulation of microtubule dynamics.

Microtubule dynamics are also regulated by a number of microtubule effectors, including microtubule-associated proteins (MAPs), molecular motors such as kinesins, the Ras-like GTPase Ran-GTP, microtubule plus end-directed proteins and tubulin-binding proteins (Andersen, 1999, 2000; Carazo-Salas et al., 2001; Cassimeris, 2002; Heald and Nogales, 2002; Kinoshita et al., 2002; Galjart and Perez, 2003). These microtubule- or tubulin-associated proteins are themselves under the control of a balance of protein phosphatases and kinases.

The cyclin-dependent kinase Cdk1 (or Cdc2), associated with its cognate partner cyclin B, is a key enzyme for entry in mitosis (Nigg, 2001) and is essential for spindle morphogenesis. Cdk1 up-regulates microtubule dynamics when added in cell-free Xenopus extracts (Verde et al., 1990, 1992). Cdk1 inactivation is necessary for proper anaphase spindle dynamics and for cytokinesis (Wheatley et al., 1997). Large amounts of Cdk1 induce the depolymerization of interphase microtubules when injected into mammalian cells (Lamb et al., 1990) and the destabilization of microtubule arrays when added on lysed mammalian cells (Lieuvin et al., 1994).

Among Cdk1 substrates are a number of microtubule effectors (Ubersax et al., 2003). MAP4 has been shown to be phosphorylated by Cdk1 in vivo (Ookata et al., 1997), and other nonneuronal MAPs like E-MAP115 or XMAP215/TOG exhibit consensus sequences for this kinase (Masson and Kreis, 1995; Vasquez et al., 1999; Charrasse et al., 2000). Furthermore, MAP4 and XMAP215/TOG interact with cyclin B and this interaction could target Cdk1 to microtubules (Ookata et al., 1995; Charrasse et al., 2000). Phosphorylation of MAPs either dissociates them from the microtubule lattice...
Mishima et al. (1995; Kreis et al., 1995). Other substrates of Cdk1 are microtubule motors, such as the kinesin-related proteins Eg5, Kid, or MKL1 (Blangy et al., 1995; Sawin and Mitchison, 1995; Ohsugi et al., 2003; Mishima et al., 2004). These microtubule motors are clearly implicated in different steps of mitosis. Phosphorylation by Cdk1 has been shown to regulate their localization in the mitotic spindle (Blangy et al., 1995; Ohsugi et al., 2003; Mishima et al., 2004). Likewise, Op18/stathmin, a protein that sequesters tubulin dimers and destabilizes microtubules during interphase, is phosphorylated on two serine residues by Cdk1 at the onset of mitosis (Cassimeris, 2002). Phosphorylation turns off the microtubule destabilizing activity of Op18/stathmin.

In the present study, we show that, in addition to regulating microtubule effectors, Cdk1 can directly phosphorylate β-tubulin in vitro and in mitotic cells, and that this phosphorylation impairs tubulin incorporation into microtubules. We suggest that the phosphorylation of tubulin by Cdk1 may represent a means by which mitotic cells regulate microtubule dynamics.

MATERIALS AND METHODS

Antibodies

Anti-phospho-peptide P172 polyclonal antibody (Ab) was made by Eurogentec (Seraing, Belgium). Two rabbits were immunized with phosphopeptide Ac-VVVpSPKVSDTVVEC-CONH2, and serum was affinity-purified against H11002. Transient transfection was carried out with 1 μg of plasmid DNA using Lipofectamine plus reagent (Invitrogen) according to manufacturer’s instructions. Cells were maintained in the presence of DNA for 30 h. For analysis with HeLa extracts, cell extract (i.e., cell extract) was stored at −80°C. To perform soluble and insoluble protein extracts, 100-mm plastic dishes were removed from the top of the glycerol cushions and kept for SDS-PAGE. Cushions were discarded, and pellets were washed once with PEM buffer and were resuspended in PEM buffer instead of kinase. Phosphorylation reactions were stopped with Laemmli buffer supplemented with 10 mM EDTA, and samples were processed for SDS-PAGE and autoradiography or analysis by Western blotting (Molecular Image FX; Bio-Rad, Hercules, CA). Relative amounts of incorporated radioactivity were quantified using Quantity One software (Bio-Rad).

For phosphorylation reactions on peptides, peptides mapping the human β1-tubulin sequence, RRMTfSVPfSPKVDTVYEP (pep172) and RRMTfSVPfSPKSVDTVVEP (pep172-P), were synthesized by Eurogentec. Note that two Arg residues were added at the N-terminus of each peptide to allow their binding onto P81 phosphocellulose (see below). Each peptide (150 μg) was phosphorylated in the same conditions as above. Reactions were stopped and amounts of radioactivity incorporated in peptides were quantified by peptide binding onto P81 phosphocellulose paper as described (Filil et al., 1991).

Cell Culture and Transfection

HeLa S3 cells were grown at 37°C in suspension cultures in MEM Eagle Joklik’s formulation (Cambrex, Walkersville, MD), supplemented with 10% horse serum (Invitrogen, Cergy, France) and 1% penicillin/streptomycin (Invitrogen). Adherent HeLa cells were grown in RPMI medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin on 10-cm plastic dishes for cell extract preparation or on glass coverslips in 30-mm plastic dishes for transfection and immunofluorescence. Transient transfection was carried out with 1 μg of plasmid DNA using Lipofectamine plus reagent (Invitrogen) according to manufacturer’s instructions. Cells were maintained in the presence of DNA for 30 h. For analysis of G1/S phase or to M phase by a 19-h treatment with 0.5 μM nocodazole, respectively. At this concentration of nocodazole, cells were blocked in mitosis with no net microtubule disassembly (Jordan et al., 1997). For experiments with taxol, taxol was added progressively to a final concentration of 35 μM during the polymerization step. Samples were then layered on PEM-60% glycerol (soluble) and centrifuged at 135°C and ultracentrifuged at 200,000 × g for 20 min at 35°C. Supernatants (100 μL), containing nonpolymerized tubulin, were removed from the top of the glycerol cushions and kept for SDS-PAGE. Cushions were discarded, and pellets were washed once with PEM buffer and were resuspended in 100 μL of PEM buffer with 10 mM β-glycerophosphate and 0.5 μM microcystine (and supplemented with 5 mM CaCl2 and 50 mM KCl for experiments with taxol) to depolymerize microtubules. Pellets were then resuspended by ultracentrifugation at 200,000 × g for 10 min at 4°C and were processed for SDS-PAGE.

Tubulin Extraction from Synchronized Cells

Drugs used were purchased from Sigma, and stock solutions were in dimethyl sulfoxide (DMSO). HeLa S3 or HCT116 cells were synchronized to G1/S phase or to M phase by a 19-h treatment with 0.5 μM nocodazole or 0.3 μM nocodazole, respectively. At this concentration of nocodazole, cells were blocked in mitosis with no net microtubule disassembly (Jordan et al., 1997). Synchronization was controlled by flow cytometry analysis. For no-cocodazole-release experiments, mitotic cells were washed twice with phosphate-buffered saline (PBS) and released into normal medium for 30 or 60 min, until most of them had reached metaphase (Sauer et al., 2005). For experiments with roscovitine and nocodazole, cells were treated for 2 h with 30 μM roscovitine or with DMSO alone and then treated for 3 h with 0.3 μM nocodazole, as adapted from Meijer et al. (1997). To perform tubulin extraction, 106 cells were washed in PBS and lysed with 1 mL of cold OPT buffer supplemented with 10 mM NaF, 10 mM β-glycerophosphate, 0.5 μM microcystine, 1 μM pepstatin, and 400 μM phenylmethylsulfonyl fluoride (PMSF). After a 15-min incubation on ice with shearing through a pipette tip, lysed cells were ultracentrifuged at 200,000 × g for 10 min at 4°C, and supernatant (i.e., cell extract) was stored at −80°C. To perform soluble and insoluble tubulin fractions, the protocol was as above except that cells were extracted with 1 mL of OPT at 35°C for 2 min (soluble fraction) and then washed twice with 40 mL of OPT buffer at 37°C, before being incubated 15 min on ice with 1 mL of cold OPT buffer (insoluble fraction). Eighteen milligrams of cell extract was used for each fraction. Pellets were incubated for 1 h at 4°C with 300 μL YL1/2-coupled Sepharose 4B previously washed with PEM supplemented with 10 mM NaF and β-glycerophosphate. Bound tubulin was eluted with peptide VGVDSTVEGEGEEGEGEGE at 0.1 mg/mL in same buffer, and fractions were analyzed by SDS-PAGE and Western blotting using P172 Ab. For analysis with anti-phosphorylated vimentin 4A4 Ab, 10 μL treated cells were washed once with PBS, lysed in 100 μL of 1% SDS, and sonicated before SDS-PAGE and Western blotting.
**RESULTS**

**In Vitro Phosphorylation of β-Tubulin by Cdk1**

We have previously shown that Cdk1 induces the destabilization of microtubule networks when added on lysed mammalian cells in culture (Lieuvin et al., 1994). Subsequently, we have designed a method for the isolation, in biochemical quantities, of proteins associated with HeLa cell microtubules (Fourrest-Lieuvin, 2005). In a survey of Cdk1 substrates in these cell extracts, we identified a band comigrating with tubulin (unpublished data).

To test directly whether Cdk1 could phosphorylate tubulin, we incubated purified HeLa cell tubulin with or without the human Cdk1-cyclin B1 complex (referred below as Cdk1), in the presence of γ32P-ATP as described in *Phosphorylation Reactions* (see above). The stoichiometry of transferred ATP per mole of β-tubulin was calculated after SDS-PAGE using a phosphorimager and Quantity One software (Bio-Rad). In parallel, aliquots from the same phosphorylation reaction were blotted together with tubulin from mitotic cell extracts. The blot was stained with Ponceau red and then incubated with the P172 Ab. The quantity of cell extract tubulin was evaluated from the Ponceau red staining by comparison with the known amounts of bovine brain tubulin, using Quantity One software (Bio-Rad). Therefore, it was possible to calculate the approximate stoichiometry of phosphorylation in mitotic cells from the ratio [P172 staining/amount of tubulin] for mitotic cell extract tubulin.

**Phosphorylation of β-Tubulin by Cdk1**

Figure 1. Cdk1 phosphorylates native and recombinant β-tubulin in vitro. (A) Purified HeLa cell tubulin was incubated in the presence of γ32P-ATP either with (+) or without (−) Cdk1-cyclin B. Samples were then processed for SDS-PAGE (top) and analyzed by autoradiography (bottom). (B) Maltose-binding protein-CK2 fusion protein, recombinant β-tubulin, and recombinant α-tubulin were incubated with Cdk1-cyclin B in the presence of γ32P-ATP. Samples were processed for SDS-PAGE (left) and analyzed with a phosphorimager (PI, right).

**Evaluation of the Proportion of Phosphorylated Tubulin in Mitotic Cell Extracts**

Purified bovine brain tubulin was phosphorylated in vitro with Cdk1 in the presence of γ32P-ATP as described in *Phosphorylation Reactions* (see above). The stoichiometry of transferred ATP per mole of β-tubulin was calculated after SDS-PAGE using a phosphorimager and Quantity One software (Bio-Rad). In parallel, aliquots from the same phosphorylation reaction were blotted together with tubulin from mitotic cell extracts. The blot was stained with Ponceau red and then incubated with the P172 Ab. The quantity of cell extract tubulin was evaluated from the Ponceau red staining by comparison with the known amounts of bovine brain tubulin, using Quantity One software. Likewise, the P172 signal was quantified. With the in vitro-phosphorylated tubulin, it was possible to link the ratio [P172 staining/amount of tubulin] to a stoichiometry of phosphorylation. Therefore, it was possible to calculate the approximate stoichiometry of phosphorylation in mitotic cells from the ratio [P172 staining/amount of tubulin] for mitotic cell extract tubulin.
is the main Cdk1 phosphorylation site in recombinant /H9252-tubulin.

**Anti-Phospho-Peptide P172 Antibody**
To study /H9252-tubulin phosphorylation in cells, an anti-phospho-peptide polyclonal Ab directed against the phosphorylated S172PK site was raised (P172 Ab; see Materials and Methods). We first tested whether the P172 Ab was specific for phosphorylated tubulin, compared with unphosphorylated tubulin, using both recombinant /H9252-tubulin and bovine brain tubulin. Western blot analysis showed that P172 Ab reacted with /H9252-tubulin phosphorylated by Cdk1 (Figure 3A, lanes 1 and 3), not with unphosphorylated tubulin (Figure 3A, lanes 2 and 4). To test whether the P172 Ab was specific of the phosphorylated Ser172 residue on /H9252-tubulin, we tested the antibody on recombinant /H9252-tubulin point mutants incubated or not with Cdk1. Results showed that only mutants with phosphorylated Ser172 residue reacted with the antibody (Figure 3B, lanes 5 and 7). We conclude that the P172 Ab reacts specifically with phosphorylated Ser 172, when tubulin is exposed to active Cdk1.

**Phosphorylation on Ser 172 of /B-Tubulin in Mitotic Cells**
To examine the /B-tubulin phosphorylation status in interphase or mitotic cells, we used cell extracts from aphidicolin- or nocodazole-arrested HeLa S3 cells, respectively. The phosho-specific P172 Ab was blotted on affinity-purified tubulin from these cell extracts. Tubulin from nocodazole-arrested cells was distinctly labeled with P172 Ab, whereas tubulin extracted from cells arrested in G1/S using aphidicolin yielded a barely detectable signal (Figure 4A, bottom panel). The low concentration of nocodazole used in these experiments has been shown to block microtubule dynamics (Jordan et al., 1992). To test whether anomalies in spindle dynamics could interfere with tubulin phosphorylation, nocodazole-arrested cells were released into fresh medium for 30 and 60 min to allow recovery of spindle functionality before extraction (Sauer et al., 2005). The extent of tubulin phosphorylation was comparable in nocodazole-released cell extracts as in nocodazole-arrested cell extracts (Figure 4A). Similar results were obtained for adherent HeLa or HCT116 cells (unpublished data). When cells were pretreated with roscovitine, an inhibitor of Cdk1 (Meijer et al., 1997), before exposure to nocodazole, the P172 Ab signal was strongly diminished on immunoblots (Figure 4B, middle panel). These results were consistent with /B-tubulin being phosphorylated by Cdk1 in mitotic cells, but did not preclude the possibility that /B-tubulin was phosphorylated by another kinase at a site cross-reacting with the P172 Ab.

To test this possibility, equal amounts of WT and S172A purified recombinant /B-tubulins were transferred onto nitrocellulose membranes, which were incubated with mitotic
cell extracts at 37°C in the presence of ATP and MgCl₂ to activate mitotic kinases. Membranes were then washed and immunoblotted with P172 Ab (Figure 4C). WT H9252-tubulin reacted strongly with P172 Ab after phosphorylation by mitotic extracts, whereas S172A H9252-tubulin did not. This showed that P172 Ab was specific of the phosphorylated Ser172 in H9252-tubulin, even when tubulin was exposed to a panel of mitotic kinases. Taken together, all these experiments demonstrate that H9252-tubulin is phosphorylated on Ser172 in mitotic cells, presumably by Cdk1 itself.

We then evaluated the proportion of phosphorylated tubulin versus total tubulin in mitotic cell extracts (see Materials and Methods). This evaluation indicated that phosphorylated tubulin represented ~0.2–0.5% of the total tubulin in M-phase extracts.

Localization of Phosphorylated Tubulin in Cells

We used both immunofluorescence microscopy and cell fractionation to localize phosphorylated β-tubulin. In immunofluorescence studies, adherent HeLa cells were either labeled with P172 Ab alone (unpublished data) or sequentially double-labeled with P172 Ab and anti-β-tubulin TUB2.1 mAb (Figure 5, A and B). The P172 Ab stained mitotic cells, from metaphase to telophase, but not interphase cells (Figure 5A). Microtubule structures such as spindles or midbodies in mitotic cells were not labeled, as assessed by confocal microscopy analysis (Figure 5B). The P172 labeling was the same with single stain, in every cell type tested (Rat 2, MDCK, and HCT116 cells), whatever the method of fixation used. The P172 Ab may recognize other phospho-proteins than tubulin in whole mitotic cells, but, conservatively, our results demonstrate an absence of detectable incorporation of phosphorylated tubulin in microtubules in cells.

To substantiate this conclusion, we analyzed the soluble (corresponding to the free tubulin) and the insoluble (corresponding to microtubules) tubulin fractions from nocodazole-arrested HeLa S3 cells, by Western blotting with P172 Ab. Results showed that free tubulin was much more labeled by P172 Ab than polymerized tubulin (Figure 5C). A semiquantification of the P172 signal showed that ~77% of the phospho-tubulin was in the free tubulin pool (Figure 5D). Taken together, these results indicate that the bulk of phosphorylated tubulin remains unpolymerized in mitotic cells.

Assembly Properties of Phosphorylated Tubulin

To test the effect of phosphorylation on tubulin polymerization directly, we performed in vitro experiments. Bovine brain tubulin was phosphorylated or not with Cdk1 in the presence of γ²P-ATP and then allowed to polymerize (see Materials and Methods). After polymerization, samples were centrifuged to separate microtubule pellets from unpolymerized tubulin. Supernatants and pellets were analyzed.
both with a phosphorimager (PI) and by Western blotting with P172 Ab (Figure 6A). Results showed that the bulk (58%) of total tubulin was in pellets (Figure 6A, lane 4 of Coomassie and Ponceau panels, and Figure 6C, top panel, /H11002 taxol histograms). In contrast, the majority (81%) of phospho-tubulin was present in the supernatant and thus was unpolymerized (Figure 6A, lane 3 of PI and P172 panels, and Figure 6C, bottom panel, /H11002 taxol histograms).

The poor incorporation of phosphorylated tubulin in microtubules could reflect either an impairment of tubulin polymerization by phosphorylation or the presence of a large proportion of denatured tubulin among phospho-tubulin molecules. To test these possibilities, taxol was added during the polymerization reaction, to increase total tubulin polymerization. Taxol addition induced an increase in the quantity of phosphorylated tubulin in the microtubule pellet (Figure 6B, P172 panel), which amounted to ~63% of total phospho-tubulin (Figure 6C, bottom panel, + taxol histograms). These experiments indicate that the bulk of phospho-tubulin is not denatured but has an impaired polymerization capacity.

**Behavior of EGFP-β-Tubulin<sup>S172A</sup> and EGFP-β-Tubulin<sup>S172D/E</sup> Mutants in Cells**

To test whether, in cells as in vitro, phosphorylation of tubulin on Ser172 is sufficient to impair tubulin polymerization, we examined the incorporation, in cell microtubules, of various EGFP-tubulin mutants. These mutants mimicked either unphosphorylated tubulin (S172A mutation) or phosphorylated tubulin (S172E or S172D mutations). The WT and mutant constructs were transfected into adherent HeLa cells. Each of the constructs was expressed full-length as assessed in a control experiment (Supplementary Data, Figure S1A). After transfection, cells were either fixed directly (Figure 7A) or lysed before fixation to improve the visualization of microtubules (Figure 7B). Whole cells and lysed cells were either stained only with anti-GFP Ab (unpublished data), or double-stained with anti-GFP Ab and with anti-α-tubulin mAb (Figure 7).

When cells were not lysed before fixation, a GFP staining was observed in the cytoplasm of transfected cells, for every construct tested. However, it was very difficult to distinguish microtubules against the fluorescent background, probably because HeLa cells are not very flat (Figure 7A). When cells were lysed before fixation, we observed a colocalization of GFP staining with the α-tubulin staining in the case of cells transfected with EGFP-β-tubulin<sup>WT</sup> (Figure 7B, left panels). Concerning EGFP-β-tubulin<sup>S172A</sup>, a colocalization of the GFP staining with microtubules, although less extensive than in the case of the WT protein, was also evident (Figure 7B, middle panels). In contrast, when cells were transfected with either EGFP-β-tubulin<sup>S172E</sup> (unpublished data) or EGFP-β-tubulin<sup>S172D</sup> (Figure 7B, right panels), no GFP staining was observed on microtubules after cell
In this study, we find that Cdk1 can phosphorylate β-tubulin. We provide evidence that tubulin phosphorylation by Cdk1 occurs in mitotic mammalian cells and impairs tubulin incorporation into microtubules both in cells and in vitro. It is known that protein phosphatases and kinases affect the overall dynamic properties of cellular microtubules during interphase as well as mitosis (Verde et al., 1990; Lieuvin et al., 1994; Yoshida et al., 2003; Schaar et al., 2004; Tranchzek et al., 2004). However, the literature stipulates that kinases and phosphatases act through the phosphorylation/dephosphorylation of effector proteins associated with microtubules or with tubulin. In this context, our findings are new, showing a direct interplay between a kinase and tubulin, not mediated by an associated effector.

We find that phosphorylation by Cdk1 mainly occurs on the Ser172 residue of β-tubulin. In the 3-D structure of β-tubulin, the Ser172 residue is located in the T5 loop, which binds the ribose of GDP or GTP, at the exchangeable nucleotide binding site of tubulin (Hesse et al., 1987; Lowe et al., 2001). As shown in our model (Figure 8B), addition of a phosphate group to Ser172 can interfere with nucleotide binding. Nucleotide binding and exchange are important for tubulin assembly, and interference with nucleotide affinity or binding kinetics could logically be involved in the observed inhibition of tubulin assembly capacity by Ser172 phosphorylation. Additionally, residues in the T5 loop are involved in contacts between dimers along protofilaments (Lowe et al., 2001) and modification of these contacts in phosphorylated tubulin might also perturb tubulin assembly. A comprehensive study of the mechanisms through which phosphorylation affects tubulin assembly will require biochemical amounts of isolated phosphorylated tubulin.

Our finding that phosphorylation inhibits tubulin polymerization is consistent with previous observations of microtubule disassembly in cells injected with large amounts of Cdk1 or in lysed cells incubated with Cdk1 (Lamb et al., 1990; Lieuvin et al., 1994). However, we find that <1% of total tubulin is phosphorylated in mitotic cells, and this raises questions concerning the mechanisms through which such a low stoichiometry of tubulin phosphorylation could influence microtubule dynamics.

One possibility is that phosphorylated tubulin interacts with microtubule plus ends in cells. In a mitotic cell, considering that polymerized tubulin represents ~50% of total tubulin, and based on an average microtubule length of 5 μm and on a tubulin dimer size of 8 nm (Caudron et al., 2002), the ratio of the number of microtubule plus ends to the number of microtubule dimers is ~1/16,000. For a stoichiometry of phosphorylated tubulin/total tubulin of 0.2% (20 phospho-polymerized dimers vs. 10,000 dimers), there is a 30-fold excess of phosphorylated tubulin dimers compared with microtubule plus ends. Additionally, Cdk1 may be specifically targeted to microtubule plus ends. For instance, in the yeast S. cerevisiae, the Cdk1–mitotic cyclin Clb4 complex has been found to be localized to the plus ends of astral microtubules by the protein Kc9, which is related to the mammalian tumor suppressor adenomatous polyposis coli (APC; Maekawa et al., 2003; Maekawa and Schiebel, 2004). In Δclb4 yeast cells, astral microtubule dynamics are altered, showing a moderate increase of growth and shrinkage rates (Maekawa and Schiebel, 2004). Maybe Cdk1 localization mechanisms exist in higher eukaryotic cells. Indeed, in mammalian cells, a population of Cdk1 is associated with spindles (Bailly et al., 1989; Riabowol et al., 1989; Rattner et al., 1990; Andreassen and Margolis, 1994). The Cdc14...
Phosphatase also associates with spindles and is essential for microtubule bundling and stabilization during anaphase and exit from mitosis (Cho et al., 2005; Higuchi and Uhlmann, 2005). Therefore, it would be of great interest to examine whether phosphorylated tubulin is a substrate for Cdc14.

Phosphorylated tubulin at microtubule ends could conceivably affect microtubule dynamics. In previous studies, Verde et al. (1990) reported that Cdk1 had no effect on pure tubulin polymerization on isolated centrosomes. A requirement for APC or for other factors capable of targeting Cdk1 at microtubule plus ends may account for this negative result.

Because the S172PK site and the T5 loop are both very well conserved in evolution and are present in the yeast S. cerevisiae (Figure 2A), yeast genetics should provide powerful tools to investigate the effects of tubulin Ser172 phosphorylation on the cell cycle and on microtubule dynamics.

Figure 7. Transfected EGFP-β-tubulinS172D remains soluble, whereas EGFP-β-tubulinWT and EGFP-β-tubulinS172A colocalize with microtubules. Adherent HeLa cells were transfected with EGFP-β-tubulinWT, EGFP-β-tubulinS172A, or EGFP-β-tubulinS172D. Cells were either fixed directly (A) or lysed before fixation (B). Whole cells and lysed cells were double-stained with GFP Ab and α-tubulin AG mAb. In the case of EGFP-β-tubulinS172D (B, right panel), the GFP staining had been overexposed to visualize lysed transfected cells. The data presented here are representative of an analysis of 100 transfected cells from three independent experiments. Bars, 20 μm.
Phosphorylation of β-Tubulin by Cdk1

Figure 8. Ser172 is located near the nucleotide in β-tubulin, and phosphorylated Ser172 might interfere with GTP/GDP binding and turnover. (A) Localization of the Ser172 residue in the 3-D structure of the tubulin heterodimer (accession number PDB#1JFF, Lowe et al., 2001). Note that Ser172 is numbered Ser174 in PDB#1JFF. The serine residue is in red, the GDP nucleotide in β-tubulin is in green, and the GTP nucleotide in α-tubulin is in yellow. (B) Modeling of a phosphoserine (P-Ser) at the position 172, using the graphics package TURBO-FRODO. Some of the possible interferences between the phosphate group and the ribose of GDP are depicted with blue dotted lines.

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REFERENCES
Bailly, E., Doree, M., Nurse, P., and Bornens, M. (1989). p34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO J. 8, 3985–3995.


