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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 phosphopeptide 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-tubulin S172D/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 special 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 into 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., late labeling microtubule effectors, Cdk1 can directly phosphorylate during interphase, is phosphorylated on two serine residues implicated in different steps of mitosis. Phosphorylation by et al., Mishima et al., 1995; Sawin and Mitchison, 1995; Ohsugi et al., 1995; Ohsugi et al., France), or on a Leica TCS-SP2 confocal microscope (Wetzlar, Germany). visualized either on a Zeiss Axioscop conventional microscope (Le Pecq, medium complemented with 10% fetal calf serum and 1% penicillin/streptomycin. Anti-phosphopeptide and then depleted against the same unphosphorylated peptide. Anti-phospho-peptide P172 polyclonal antibody (Ab) was made by Eurogenetec. 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 μM) was phosphorylated in same conditions as above. Reactions were stopped and amounts of radioactive incorporation in peptides were quantified by peptide binding onto P81 phosphocellulose paper as described (Filil et al., 1991).

Assembly Assays after Tubulin Phosphorylation
To allow proper tubulin assembly, we used a source of γ2-R plasma-ATP without any additives (Perkin-Elmer Cetus; reference BLU/NEG/002A). Bovine brain tubulin (35 μM) was phosphorylated for 20 min at 30°C with 20 μM γ2-ATP, 100 μM ATP, 5 mM MgCl2, 10 mM β-glycerophosphate, 0.5 μM microcystine, 200 U (10 μL) Cdk1-cyclin B in 100 μL of PEM buffer (100 mM Pipes, pH 6.7, 1 mM EGTA, 1 mM MgCl2). Controls were performed with 2 μl of kinase storage buffer instead of kinase. Afterward, samples were incubated for 10 min on ice, to depolymerize any tubulin structure that could have assembled during the phosphorylation reaction. GTP (1 mM) was added and each mixture was allowed to polymerize for 30 min at 35°C. 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% (vol/vol) glycerol cushions and kept for SDS-PAGE. Cushions were discarded, and pellets were washed once with PEM buffer at 35°C before being incubated for 15 min at 4°C 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 cleaved 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 10 μM nocodazole and 0.3 μM nocoazode, respectively. At this concentration of nocoazode, cells were blocked in mitosis with no net microtubule disassembly (Jordan et al., 1992). Synchronization was controlled by flow cytometry analysis. For nocodazole-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 50 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 phenylmethylsulfonylfluoride (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 two times 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 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 VGVDSVEGECEGEEGEY at 0.1 mg/ml in same buffer, and fractions were analyzed by SDS-PAGE and Western blotting using P172 Ab. For analysis of anti-phosphorylated vimentin 4A4 mAb, 104 treated cells were washed once with PBS, lysed in 100 μl of 1% SDS, and sonicated before SDS-PAGE and Western blotting.

Materials and Methods
Antibodies
Anti-phospho-peptide P172 polyclonal antibody (Ab) was made by Eurogentec (Seraing, Belgium). Two rabbits were immunized with phosphopeptide Ac-VPYpSPKSVSDTVEC-CONH2, and serum was affinity-purified against phosphopeptide and then depleted against the same unphosphorylated peptide. Anti-α-tubulin AG monoclonal antibody (mAb) and anti-α-tubulin antibody YL1/2 rat mAb were gifts of Laurence Laplanche (CEA, Grenoble, France). Anti-β-tubulin TUB21 Ab was from Sigma (La Verpillière, France), anti-GFP polyclonal Ab (A11122) from Molecular Probes, and anti-phosphotyrosine from RMMNTF5SVPVSKPSDVTEYVE (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 μM) was phosphorylated in same conditions as above. Reactions were stopped and amounts of radioactive incorporation 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 Bioscience, 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 100-mm plastic dishes for cell 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 GFP constructs by Western blot, transfected cells were scraped in Laemmli buffer. For immunofluorescence, cells were then either lysed in OPT buffer (80 mM Pipes, pH 6.7, 1 mM EGTA, 1 mM MgCl2, 0.5% Triton X-100, 10% glycerol) 5 min before fixation, or fixed directly for 10 min in methanol at –20°C. Cells were then treated for immunofluorescence and visualized either on a Zeiss Axiovert conventional microscope (Le Peq, France), or on a Leica TCS-SP2 confocal microscope (Wetzlar, Germany).

Native and Recombinant Tubulin Purification
HeLa cell tubulin and bovine brain tubulin were purified as described (Caudron et al., 2000; Fourest-Lievew, 2005). Recombinant mouse α2- and β5-tubulins were produced from PET3a plasmids provided by Ronald Melki (LEBS, Gil-sur-Yvette, France). As adapted from Melki et al. (1997), recombinant tubulins were overexpressed in milligram quantities in Escherichia coli BL21(DE3), isolated as inclusion bodies from lysed cells using Bugbuster protein extraction reagent (Novagen, Darmstadt, Germany), dissolved in 20 mM Tris, pH 7.5, 10 mM dithiothreitol, 7.5 M urea, dialyzed against 20 mM Tris, pH 7.5, 0.1 mM dithiothreitol, and stored at –80°C until use.

Mutagenesis
Point mutations were performed on mouse β-tubulin-PET3a (same as above) and EGF-Mouse β-tubulin constructs, using the QuickChange kit from Stratagene (La Jolla, CA). The EGF-Mouse β-tubulin construct was a generous gift from Jurgen Wehland and coworkers (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany).

Phosphorylation Reactions
HeLa cell tubulin (20 pmol), bovine brain tubulin (20 pmol), recombinant α- and β-tubulin (10 pmol), or casein (using P172 Ab. For analysis of anti-phosphorylated vimentin 4A4 mAb, 104 treated cells were washed once with PBS, lysed in 100 μl of 1% SDS, and sonicated before SDS-PAGE and Western blotting.
Incubation of Blotted Recombinant Tubulins with Mitotic Cell Extracts

Equal amounts of wild-type (WT) and S172A recombinant β-tubulins were processed for SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were saturated overnight at 37°C in PBS plus 5% Tween-20 and 2% gelatin and were washed twice with kinase buffer (see below) before incubation with extracts. Mitotic cell extracts with kinase activity were performed as adapted from Matsumoto-Taniura et al. (1996); Nocodazole-arrested cells were washed twice with PBS and were then homogenized in a Dounce grinder with two volumes of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 0.5 μM microcinine, 1 μM pepstatin, and 400 μM PMSF). Extracts were ultracentrifuged at 250,000 × g for 15 min at 4°C and were supplemented with 1 mM ATP and 5 mM MgCl$_2$ just before incubation for 30 min at 37°C with tubulin-blotted and -saturated membranes. Membranes were then washed 20 min in PBS-0.5 M NaCl, 20 min in stripping buffer (0.75% glycine, 0.1% Nodinet-P40, 1% SDS, pH 2.2) and washed three times in PBS-0.1% Tween. All washings were performed in the presence of 10 mM NaF and 10 mM β-glycerophosphate. Membranes were finally immunoblotted with P172 Ab.

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 γ$^{32}$P-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 processed for SDS-PAGE and transferred onto nitrocellulose membranes. The blots were 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.

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 (Lieuvrin et al., 1994). Subsequently, we have designed a method for the isolation, in biochemical quantities, of proteins associated with HeLa cell microtubules (Fourest-Lieuvin, 2005). In a survey of Cdk1 substrates in these cell extracts, we identified a band co-migrating 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 γ$^{32}$P-ATP. Results showed a phosphorylation of β-tubulin (Figure 1A). In the same manner, bovine brain β-tubulin was phosphorylated by Cdk1 (see for example, Figure 6A). An additional phosphorylated peptide was observed above β-tubulin (Figure 1A), not comigrating with α-tubulin but apparently corresponding to cyclin B1, which is a known substrate for Cdk1 (Labbè et al., 1989). To test directly whether β-tubulin, and not α-tubulin, was a Cdk1 substrate, we incubated purified recombinant β- and α-tubulins with Cdk1. As a positive control, we included a recombinant form of protein kinase CK2, which is a well-established substrate for Cdk1 in vitro and in vivo (Mulner-Lorillon et al., 1990; Litchfield et al., 1992; Bosc et al., 1995). Both CK2 and β-tubulin were phosphorylated by Cdk1 at similar levels, whereas α-tubulin was not phosphorylated (Figure 1B, right panel). These results indicate that β-tubulin, not α-tubulin, is a bona fide Cdk1 substrate in vitro.

Figure 1. Cdk1 phosphorylates native and recombinant β-tubulin in vitro. (A) Purified HeLa cell tubulin was incubated in the presence of γ$^{32}$P-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 γ$^{32}$P-ATP. Samples were processed for SDS-PAGE (left) and analyzed with a phosphorimager (PI, right).

Determination of the Cdk1 Phosphorylation Site in β-Tubulin Sequence

Analysis of β-tubulin sequences of diverse origins revealed the presence of two putative sites for phosphorylation by Cdk1. One site, S$_{172}$PK, was a good candidate for phosphorylation by Cdk1 (Holmes and Solomon, 1996). This site is conserved among β-tubulin genes of different species, from the yeast Saccharomyces cerevisiae to humans (Figure 2A). It is also conserved between human β-tubulin isoforms. The other putative site, T$_{219}$P, is very short and less conserved (Figure 2A).

To test whether the S$_{172}$PK site could indeed be phosphorylated by Cdk1, we tested two synthetic peptides containing this site, pep172 and pep172-P. These peptides matched the human β1- tubulin sequence and were indistinguishable, except that a phosphate was incorporated at the Ser 172 residue of pep172-P. When incubated with Cdk1 and γ$^{32}$P-ATP, pep172 incorporated large amounts of radioactivity, whereas pep172-P did not (Figure 2B). This experiment indicates that the S$_{172}$PK site, and no other amino-acid in pep172, can be phosphorylated by Cdk1.

To test whether the S$_{172}$PK site was the preferred phosphorylation site in β-tubulin, we performed point mutations in recombinant β-tubulin. S172A and T219A point mutants and a S172A/T219A double mutant were assayed for phosphorylation by Cdk1 along with WT β-tubulin (Figure 2C). The S172A point mutation strongly inhibited the phosphorylation of β-tubulin by Cdk1 (Figure 2C, lane 6), whereas the T219A point mutation did not (Figure 2C, lane 7). Phosphorylation was also inhibited in the case of the double mutant (Figure 2C, lane 8). These results indicate that the S$_{172}$PK site
is the main Cdk1 phosphorylation site in recombinant β-tubulin.

**Anti-Phospho-Peptide P172 Antibody**

To study β-tubulin phosphorylation in cells, an anti-phospho-peptide polyclonal Ab directed against the phosphorylated Ser172 residue 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 β-tubulin and bovine brain tubulin. Western blot analysis showed that P172 Ab reacted with β-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 for the phosphorylated Ser172 residue on β-tubulin, we tested the antibody on recombinant β-tubulins 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 β-Tubulin in Mitotic Cells**

To examine the β-tubulin phosphorylation status in interphase or mitotic cells, we used cell extracts from aphidicolin- or nocodazole-arrested HeLa S3 cells, respectively. The pho-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 pre-treated 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 β-tubulin being phosphorylated by Cdk1 in mitotic cells, but did not preclude the possibility that β-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 β-tubulins were transferred onto nitrocellulose membranes, which were incubated with mitotic

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**Figure 2.** Cdk1 phosphorylates β-tubulin on the serine 172 residue. (A) The sequence of human β1-tubulin (accession number PO7436 in NCBI protein database) from residues 161–190 and 204–233 was aligned with β-tubulin sequences of other organisms, using the software ClustalW (Infobiogen, Evry, France). Conserved residues are shown on a gray background, S172PK and T219P sites are in bold, and T5 loop is underlined. (B) Each of two synthetic peptides, matching human β1-tubulin sequence from residue 164 to residue 182, were incubated with Cdk1-cyclin B and γ32P-ATP. One peptide, pep172-P, was synthesized with a phosphate on Ser172. As a control, a mixture of the two peptides was incubated with γ32P-ATP but without Cdk1. After incubation, incorporated radioactivity in each peptide was counted. (C) Wild-type (WT) or point-mutated recombinant β-tubulins were incubated in the presence of γ32P-ATP with (lanes 5–8) or without (lanes 1–4) Cdk1-cyclin B. Mutants tested were as follows: Ser172 to Ala (S172A) β-tubulin (lanes 2 and 6), Thr219 to Ala (T219A) β-tubulin (lanes 3 and 7), and S172A/T219A double-mutated β-tubulin (lanes 4 and 8). After incubation, samples were processed for SDS-PAGE (top) and analyzed with a phosphorimager (bottom).
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 phosphorylated-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 phosphorylated 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 phosphorylated tubulin is not denatured but has an impaired polymerization capacity.

**Behavior of EGFP-β-Tubulin*^{S172A}* and EGFP-β-Tubulin*^{S172D/E}* 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-β-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 β-tubulin staining in the case of cells transfected with EGFP-β-tubulinWT (Figure 7B, left panels). Concerning EGFP-β-tubulinS172A, 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-tubulinS172E (unpublished data) or EGFP-β-tubulinS172D (Figure 7B, right panels), no GFP staining was observed on microtubules after cell

**Figure 5.** P172 Ab stains mitotic cells, but phosphorylated tubulin is not detected in the microtubules of mitotic cells. (A) Adherent HeLa cells were double-stained with P172 Ab (left) and with anti-β-tubulin TUB 2.1 mAb (middle). DNA was stained with Hoechst 33258 (right). Pictures were taken on a conventional fluorescence microscope. (B) Adherent HeLa cells were labeled as in A and analyzed on a confocal microscope. Stacked images were merged (right). Bars, 20 μm. (C) HeLa S3 cells were arrested in M-phase with nocodazole, and the free tubulin fraction and the polymerized tubulin fraction (corresponding to microtubules in cells) were extracted. These two fractions were analyzed by Western blotting with P172 Ab (bottom). Ponceau red staining of the blot is shown (top). (D) Semiquantification of the phosphorylated tubulin in the free and polymerized tubulin fractions. For each tubulin fraction, quantities of phosphorylated tubulin (P172 staining relative to Ponceau staining) were evaluated and results are given as percentages of total phosphorylated tubulin (free + polymerized).
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; Trinczek 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 phosphorylated 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 Kar9, 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 subpopulation of Cdk1 is associated with spindles (Bailey et al., 1989; Riabowol et al., 1989; Rattner et al., 1990; Andreassen and Margolis, 1994). The Cdc14

**DISCUSSION**

**Localization of the Cdk1 Phosphorylation Site in the Tubulin 3-D Structure**

In an attempt to explain why phosphorylation of β-tubulin by Cdk1 may inhibit polymerization, we looked at the position of Ser172 in the 3-D structure of the tubulin. This serine residue lies in the T5 loop of β-tubulin, which is near the ribose of the GDP or GTP nucleotide (Figure 8A). Additionally, this loop is at the interface between two tubulin dimers within a protofilament (Lowe et al., 2001). We also modeled a phosphoserine at position 172 in the tubulin structure using the graphics package TURBO-FRODO (Roussel and Cambillau, 1991), to examine the possible incidence of a phosphate group on the binding of the GDP/GTP nucleotide. As shown in our model (Figure 8B), the presence of a bulky negatively charged phosphate group could induce modifications in the nucleotide-binding area that may impair GDP/GTP binding and turnover, thereby impairing tubulin assembly.

**Figure 6.** Tubulin phosphorylated in vitro by Cdk1 incorporates very poorly into microtubules. (A) Bovine brain tubulin was incubated in the presence of γ32P-ATP with (lanes 3 and 4) or without (lanes 1 and 2) Cdk1-cyclin B. Afterward, tubulin was allowed to polymerize and centrifuged to separate microtubule pellet (P) from unpolymerized tubulin (S). Equal volumes of each sample were processed for SDS-PAGE and were either Coomassie stained and analyzed with a phosphorimager (PI) or blotted with P172 Ab (bottom). Ponceau red staining of the blot is shown. (B) Same experiment as in A, except that the polymerization step was done in the presence of taxol. (C) Semiquantification of the proportions of unpolymerized tubulin (S). Equal volumes of each sample were processed for SDS-PAGE and were either Coomassie stained and analyzed with a phosphorimager (PI) or blotted with P172 Ab (lanes 1 and 2) Cdk1-cyclin B. Afterward, tubulin was allowed to polymerize and centrifuged to separate microtubule pellet (P) from unpolymerized tubulin (S). Equal volumes of each sample were processed for SDS-PAGE and were either Coomassie stained and analyzed with a phosphorimager (PI) or blotted with P172 Ab (P, bottom panel).
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 S<sub>172</sub>PK site and the T5 loop are both very well conserved in evolution and are present in the yeast <i>S. cerevisiae</i> (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-tubulin<sup>S172D</sup> remains soluble, whereas EGFP-β-tubulin<sup>WT</sup> and EGFP-β-tubulin<sup>S172A</sup> colocalize with microtubules. Adherent HeLa cells were transfected with EGFP-β-tubulin<sup>WT</sup>, EGFP-β-tubulin<sup>S172A</sup>, or EGFP-β-tubulin<sup>S172D</sup>. 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-tubulin<sup>S172D</sup> (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.

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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