Association of polo-like kinase with α -, β - and γ -tubulins in a stable complex

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The polo-like kinase (Plk) family has been shown to have an important role in the regulation of the cell-division cycle, especially in organization of the spindle structure, in species from fungi to humans. Recent reports have demonstrated that in mammalian cells Plk is associated with components of the anaphase-promoting complex and a peptidyl-prolyl isomerase, Pin1. To characterize a putative Plk-containing complex, we fractionated mitotic cell lysates on a gel-filtration column. The Plk complex was eluted from the column at molecular sizes ranging from 669 to 2500 kDa in the presence of detergent and high concentrations of salt. Specific associations of Plk with α -, β - and γ -tubulins in both interphase and mitotic cells were shown

by reciprocal immunoprecipitations and immunoblottings and were independent of the microtubule polymerization state, whereas binding assays *in vitro* indicated that Plk interacts with α - and β -tubulins directly. In addition, mitotic Plk was able to phosphorylate associated tubulins *in vitro*. Finally, we show that the kinase domain of the Plk molecule is both required and sufficient for its binding to tubulins *in vivo*. The specific interaction between Plk and tubulins might provide a molecular basis for the physiological functions of Plk in regulating the cell cycle, particularly in establishing the normal bipolar spindle.

Key words: cell-cycle regulation, kinases, spindle formation.

INTRODUCTION

Strict regulation of the cell division cycle is essential for the normal development of organisms and functions of tissues. Before cell division, genetic material must be replicated once, and only once. The interphase microtubule lattice must be reorganized into the mitotic spindle so that parental chromosomes can be properly aligned to the midplane and then separated precisely into the two daughter cells. The well-conserved cyclin-dependent kinase ('CDK') family has been proved to have a pivotal role in regulating multiple checkpoints of the cell division cycle in diverse species from fungi to mammals. Recently, increasing evidence has accumulated supporting a critical role for the polo-like kinase (Plk) in mitosis.

The *polo* gene of *Drosophila melanogaster* was the first member of the Plk family to be identified [1,2]. *Polo*-related kinase genes were later found in other organisms, *Plo1* + in *S. pombe* [3], *CDC5* in *S. cerevisiae* [4], *Plk* in human and mouse [5–9], *Plx1* in *Xenopus* [10] and *tbplk* in *Trypanosoma brucei* [11]. In addition, two less-conserved mammalian *polo* family members have been described that are both early serum response genes, e.g. *SNK* (serum-inducible kinase) [12] and *FNK/PRK* (fibroblast growth factor-inducible kinase/proliferation-related kinase) [13,14]. The *polo* family genes encode serine/threonine-specific protein kinases. Amino acid sequences of polo family members share 50–65% identity in their catalytic domains near the N-termini. The C-terminal portions exhibit a lower degree of identity; however, a small region called the polo-box within the C-terminal halves of the molecules has a surprisingly high 90% identity.

The expression of Plk seems to correlate with actively proliferating cells. Mouse *Plk* mRNA levels are high in fetal and newborn tissues but low in adults except for ovaries and testes [9]. The level of Plk expressed in cancers might be correlated with

clinical course. Lower levels of *Plk* transcript in non-small-cell lung carcinoma are associated with better 5-year survival after surgery [15]. The overexpression of Plk in NIH 3T3 cells leads to a malignant transformation of cells [16], suggesting that Plk might influence the development of some tumours.

Plk protein levels fluctuate in a cell cycle-dependent manner, low in G₁, increasing in S and G₂/M, and rapidly decreasing at the end of mitosis [17,18], possibly through ubiquitin-mediated proteolysis [19]. The kinase activity of Plk is even more tightly regulated during the cell cycle. Mitotic Plk displays kinase activity severalfold higher than its interphase counterpart; the phosphorylation of Plk itself is an important mechanism in regulating its activity [17,18,20].

The functional significance of the Plks was first revealed by genetic studies in *Drosophila* and *S. pombe*. Mutations in the *Drosophila polo* gene cause the appearance of abnormal mitotic cells, characterized by monopolar spindles or bipolar spindles with one of the poles broadened [2]. *S. pombe* lacking *Plo1* + function exhibits similar abnormalities [3], suggesting a potential role for Plks in spindle organization. This notion was further supported by microinjection studies with Plk antibodies in mammalian cells. The introduction of a Plk-specific antibody into HeLa cells [21] suppressed centrosome separation during the G₂/prophase transition and decreased γ -tubulin and MPM2 staining intensity in centrosomes. Despite these observations, the mechanisms underlying these abnormalities remain unclear.

Biochemical studies indicate that Plk might also have other functions during the cell cycle. The microinjection of Plk sense mRNA into NIH 3T3 cells stimulated DNA synthesis, suggesting a potential role in S-phase [8], although that result could reflect the acquisition of an abnormal G₁/S-phase function. Yeast Cdc5 regulates adaptation to the DNA damage checkpoint [22]. Furthermore, *Xenopus* Plx1 is able to phosphorylate and activate

Abbreviations used: DTT, dithiothreitol; HEK, human embryonic kidney; Plk, polo-like kinase.

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Cdc25c, in turn activating the cyclin B/cdc2 complex; further evidence that Plk is required for the onset of mitosis [10]. Several recent reports have shown that Plk might be involved in the completion of mitosis through its interaction with the anaphase-promoting complex [23,24]. It has also been reported that Plk interacts with Pin1, a peptidyl-prolyl isomerase [25,26] thought to be involved in protein folding and unfolding.

Multiple roles for Plk during mitosis and cytokinesis are also supported by its dynamic pattern of localization. In interphase cells, Plk is mainly localized at the centrosomes. During mitotic progression, a fraction of Plk is redistributed to spindle fibres, whereas some remains at the spindle poles. During later stages of mitosis, Plk is concentrated in the midzone [27]. In addition, *Drosophila* polo is able to phosphorylate purified tubulins *in vitro* [28]. The dynamic distribution pattern of Plk, together with the results of previous functional studies, implies that Plk might have a close relationship to microtubules. Nevertheless, at present, knowledge of how Plk regulates spindle organization is lacking. Elucidation of the role of Plk in regulating cell-cycle progression will in part depend on identifying other proteins with which Plk interacts.

In the present study we investigated the nature of the Plk complex in mammalian cells and have partly purified a large Plk-containing complex. We found that α -, β - and γ -tubulins were stably associated with Plk in both interphase and mitotic cells, and that all three tubulins were phosphorylated by Plk *in vitro*. Furthermore, the kinase domain of Plk alone was necessary and sufficient for the interaction with the tubulins.

EXPERIMENTAL

Cell culture

Mouse mammary carcinoma FT210 cells were maintained in RPMI1640 medium (Biowhitaker, Walkersville, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin. Prometaphase cells were obtained by synchronization with 100 ng/ml nocodazole for 16 h. Cells at the G₁/S boundary were prepared by a 2.5 mM thymidine double block (16 h block, 8 h release and 16 h block).

Preparation of cell lysates

Cells were washed three times with cold PBS, then lysed in TCSD buffer [50 mM Tris/HCl (pH 7.4)/1% (w/v) CHAPS/300 mM NaCl/1 mM dithiothreitol (DTT)/10 mM NaF/1 mM PMSF/1 μ g/ml leupeptin/1 μ g/ml aprotinin] on ice for 1 h, with occasional shaking. The lysate was then centrifuged at 100 000 g and 4 °C for 1 h. The supernatant (S100 fraction) was recovered for chromatography and immunoprecipitation experiments.

FPLC gel filtration

Mitotic FT210 cell lysates (S100 fraction) were concentrated with a Centricon-10 (Amicon, Beverly, MA, U.S.A.). Aliquots of 0.5 ml (approx. 3 mg of total protein) were loaded on a Superose 6 column (Pharmacia, Uppsala, Sweden) that had been pre-equilibrated with TCSD buffer. The column was eluted with the same buffer at 0.5 ml/min; 1 ml fractions were collected. Alternatively, the S100 fraction was concentrated with (NH₄)₂SO₄ (75% satn.) and the precipitate was resuspended in TCSD buffer. The residual (NH₄)₂SO₄ was removed by passing the sample through a Sephadex G-25 column. The Superose 6 column was calibrated with molecular mass standards of 669, 443, 200, 150 and 66 kDa. We determined the maximum loading capacity of the column as approx. 10 mg.

Ion-exchange chromatography

Cell lysates were made as above except that the TCSD buffer contained 25 mM NaCl. The cell lysate was loaded on an anion-exchange Resource Q column (Pharmacia). The column was washed with low-salt buffer until the A₂₈₀ of the flowthrough returned to the baseline. Column-bound proteins were eluted with a 25–500 mM NaCl linear gradient at 0.5 ml/min; 1.5 ml fractions were collected.

Analysis of FPLC fractions

Fractions collected from each column were precipitated with 0.15% (w/v) sodium deoxycholate and 72% (w/v) trichloroacetic acid. The precipitate was re-dissolved in SDS sample buffer and separated by SDS/PAGE [7.5% (w/v) gel]; gels were transferred to Immobilon-P membranes. The membranes were probed with specific antibodies and developed by enhanced chemiluminescent detection reagents (NEN Life Science Products, Boston, MA, U.S.A.).

Immunoprecipitation

Equal aliquots of the S100 fractions were incubated with appropriate antibodies and Protein G–Sepharose 4B beads (Sigma, St. Louis, MO, U.S.A.) at 4 °C overnight. The Sepharose 4B beads were washed five times with TCSD buffer. Proteins bound on the beads were released by being boiled in SDS sample buffer for 5 min, then loaded on polyacrylamide gels. A polyclonal antibody against the C-terminal 13-residue oligopeptide of murine and human Plk (Zymed, San Francisco, CA, U.S.A.) was used at 5 μ g/ml to immunoprecipitate Plk. Tubulin monoclonal antibodies specific to α -tubulin (clone DM1A), β -tubulin (clone 2-28-33) and γ -tubulin (clone GTU-88) (Sigma) were used for immunoprecipitations at 15 μ g/ml of lysate.

Preparation of epitope-tagged Plk constructs

The HA epitope sequence (YPYDVPDYA) derived from the human influenza haemagglutinin was encoded at the N-terminus of the full-length murine Plk (Th10 clone) [8] by using PCR. The sense and anti-sense oligonucleotides used as primers were 5'-ACG ATG TAT CCA TAT GAT GTT CCA GAT TAT GCA GCG GCC AAA GCT-3' and 5'-ATC TCA TTA TGC GTC TGA GAT CTC GAA-3' respectively. The PCR product was cloned into pGEM-T vector (Promega, Madison, WI, U.S.A.); the *SalI/XhoI* DNA fragment encoding the HA-epitope and residues 3–12 of mPlk was isolated and ligated into the BlueScript vector (Stratagene, La Jolla, CA, U.S.A.), which already contained the residual 3' region of wild-type Plk (residues 13–603), resulting in the tagged full-length HA-Plk. The C-terminal truncated mutant HA-Plk356 was generated by the digestion of HA-Plk with *SmaI* restriction enzyme. HA-Plk264 was generated by PCR with anti-sense oligonucleotides as primers: 5'-ATC TCA TTA CTT GAT CCG GAG GTA GGT-3'. To generate the kinase domain deletion construct HA- Δ KD Plk, an *XhoI* site was inserted at position 960 of the mPlk cDNA (corresponding to codon 312 of the protein) by PCR with primers 5'-CTG GCTCGAGCA CCA GCC CGT CTC CCT ATT-3' (spanning codons 10–14 plus 312–316). A restriction digestion of the PCR product and HA-Plk was then performed with *XhoI*; the two fragments (N-terminus of HA-Plk and C-terminus of the PCR product) were ligated. The catalytically inactive mutant (single amino acid mutation K82R) HA-K82R Plk was constructed as described by Golsteyn et al. [27].

For mammalian expression, all constructs were subcloned into pcDNA3neo vector (Invitrogen, San Diego, CA, U.S.A.) under the transcriptional control of the CMV promoter. All constructs were verified for orientation and proper construction by restriction enzyme analysis and DNA sequencing.

Transfections

Human embryonic kidney (HEK) 293 cells were transfected with HA-tagged Plk constructs in accordance with the protocol for transient transfection of adherent cells (Qiagen, Valencia, CA, U.S.A.). Cells were seeded at 60% confluence in DMEM with 10% (v/v) fetal bovine serum on the day before transfection. For each HA-tagged Plk fragment, 2.5 µg of DNA was used for a 10 cm plate. DNA was mixed with 150 µl of serum-free DMEM and 15 µl of SuperFect transfection reagent. The mixture was incubated at room temperature for 10 min and then resuspended in 3 ml of DMEM growth medium (with serum). Cells were incubated in the new mixture at 37 °C for 3 h, then rinsed with PBS and fresh growth medium was added. Approx. 36 h after transfection, nocodazole was added and the culture was incubated for an additional 16 h to obtain prometaphase cells. Mitotic 293 cells (mitotic index more than 90%) were lysed in TCSD buffer; the S100 fraction was recovered as described above.

Kinase reactions

The mitotic Plk immunoprecipitates were washed extensively with TCSD buffer, and then washed three times with kinase buffer [20 mM Hepes (pH 7.4)/150 mM KCl/10 mM MgCl₂/1 mM EGTA/0.5 mM DTT/5 mM NaF/0.1 mM orthovanadate]. Kinase reactions were initiated by the addition of 20 µl of kinase buffer containing 10 µCi of [γ -³²P]ATP. After incubation for 30 min at 37 °C, the reactions were terminated with 5×SDS sample buffer and boiled for 5 min. Alternatively, the reaction mixtures were boiled in 1% (w/v) SDS for 5 min, then diluted 1:30 with Nonidet P40 buffer [50 mM Tris/HCl (pH 7.4)/1% (v/v) Nonidet P40/350 mM NaCl/5 mM NaF/5 mM EGTA]. Reimmunoprecipitations were performed immediately with anti-tubulin antibodies. Samples were resolved by SDS/PAGE [7.5% (w/v) gel]; ³²P-labelled proteins were detected by autoradiography.

Phosphoamino acid analysis

The ³²P-labelled protein bands were excised from the Immobilon-P membranes. Phosphoamino acid analysis was performed by thin-layer electrophoresis by the method of Boyle et al. [29].

Translation *in vitro* and binding assay

Plk was translated *in vitro* by using plasmid pcDNA3-Th10 [8] and TNT T7 Coupled Reticulocyte Lysate system (Promega). [³⁵S]Methionine was included to label translated protein.

For the binding assay, 5 µl of the final translation reaction mixture was incubated with 4 µg of purified HeLa cell tubulins (Cytoskeleton, Denver, CO, U.S.A.) in Pipes buffer (80 mM Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP, 1 mM DTT, 5% glycerol, 1% Triton X-100) at 4 °C for 4 h. Tubulins were then immunoprecipitated with anti-(β -tubulin) antibody. Fluorography of ³⁵S was performed to detect coprecipitated Plk. Alternatively, Plk was immunoprecipitated from 5 µl of the translation mixture diluted in 1 ml Pipes buffer. After extensive washing, the immunoprecipitation beads were incubated with HeLa tubulins at 4 °C for 4 h. Plk-bound tubulin was detected by Western blotting. Translation reaction mixture with plasmid DNA omitted was used as a control.

RESULTS

Co-elution of Plk and tubulins in gel-filtration and anion-exchange fractions

We found that mouse mammary gland carcinoma FT210 cells express relatively high levels of Plk; therefore mitotic FT210 cells were used as the Plk source in chromatography studies. Cells were lysed in TCSD buffer containing 1% (w/v) CHAPS; the S100 fractions of the lysates were loaded on a Superose 6 gel-filtration column. The column was eluted with the same lysis buffer. Fractions were subjected to SDS/PAGE as described in the Experimental section; immunoblotting was performed with a Plk polyclonal antibody, 8847, raised against the N-terminal peptide of murine Plk [17]. Only a small percentage of Plk was eluted in fraction 13, a volume corresponding to the expected molecular mass (65–67 kDa) of monomeric Plk (Figure 1A). Most Plk was detected in fractions 3–7, corresponding to a molecular mass range of 2500–669 kDa, indicating that Plk might be a component of a large complex. The total amount of protein loaded was well within the capacity of the column, so the broad profile was not a result of overloading. During sample preparation and migration through the column, some proteins might have dissociated from the core complex, leading to the broad elution profile, or alternatively Plk might be a member of multiple complexes. When the lysate was precipitated with 75% satd. (NH₄)₂SO₄ before loading, the Plk elution profile remained the same, implying that the complex(es) is/are stable in high concentrations of salt. The two minor bands in fraction 5 were cross-reacting proteins enriched in this fraction, because they were not detected by blotting of total cell lysates with the 8847 antiserum.

The same blot was stripped and reprobed with antibodies specific for β - and γ -tubulins (Figures 1B and 1C). Most of the β -tubulin was present in early fractions; very little β -tubulin was detected in the fraction corresponding to 110 kDa, which is the size of dimers of α - and β -tubulin. Similarly, γ -tubulin was eluted in fractions corresponding to a much larger form than 52 kDa. Multiprotein complexes containing α -, β - and γ -tubulins

Figure 1 Co-elution of Plk and tubulins in Superose 6 gel-filtration column fractions

The S100 supernatant made from mitotic FT210 cells was loaded on a Superose 6 column, which was then eluted with lysis buffer. Fractions were concentrated by precipitation with deoxycholate and trichloroacetic acid. The precipitates were dissolved in SDS sample buffer and separated by SDS/PAGE [7.5% (w/v) gel]. Immunoblotting was performed with Plk polyclonal antibody 8847 (A), anti-(β -tubulin) (B) and anti-(γ -tubulin) (C) antibodies. Arrowheads at the top show positions at which protein standards were eluted from the Superose 6 column. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 2 Co-elution of Plk and tubulins in Resource Q anion-exchange fractions

Lysates were made from mitotic FT210 cells in lysis buffer containing 25 mM NaCl. The S100 fraction was loaded on a pre-equilibrated Resource Q column, then washed with lysis buffer until the A_{280} of the flowthrough returned to the baseline. Bound proteins were eluted with a linear 25–500 mM NaCl gradient. Fractions were processed as described in the legend to Figure 1 for immunoblotting with anti-Plk (A), anti-(β -tubulin) (B) and anti-(γ -tubulin) (C) antibodies.

have been reported [30–32]; therefore some of the tubulins in fractions 3–7 might have represented the tubulin pool associated with these complexes, although some might also have been in tubulin oligomeric forms.

FT210 cell lysates were fractionated on a Resource Q anion-exchange column; bound proteins were eluted with an increasing salt gradient. Plk was eluted between 315 and 400 mM (Figure 2), indicating that Plk (and its complex) bound well to the positively charged matrix. Reprobed blots showed that levels of β - and γ -tubulins paralleled Plk in the fractions. The two cross-reacting bands detected in Figure 1(A) were in fractions that were eluted at low salt concentrations (approx. 100 mM) (results not shown).

Figure 3 Co-immunoprecipitation of tubulins with Plk from interphase and mitotic FT210 cells

S100 fractions of interphase and mitotic FT210 cells synchronized with a double thymidine block (I) or a nocodazole block (M) were subjected to immunoprecipitation with a Plk antibody (Zymed). The immunoprecipitates were separated by SDS/PAGE [7.5% (w/v) gel] and transferred to Immobilon-P. The membrane was probed with 8847 Plk antibody to show the Plk protein. Duplicate blots were also probed with antibodies specific to α -tubulin, β -tubulin and γ -tubulin. H, rabbit IgG heavy chain; IP, immunoprecipitation; IB, immunoblotting. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 4 Co-immunoprecipitation of PLK with tubulins from interphase and mitotic FT210 cells

Immunoprecipitations were performed with anti-(α -tubulin), anti-(β -tubulin) or anti-(γ -tubulin) antibodies, and precipitates were separated by SDS/PAGE as described in the legend to Figure 3 for immunoblotting with the 8847 Plk polyclonal antibody. IP, immunoprecipitation; IB, immunoblotting. The positions of molecular mass markers are indicated (in kDa) at the right.

Co-immunoprecipitation of Plk and tubulins in both interphase and mitotic FT210 cells

The similar elution profiles from both a sizing column and an anion-exchange column indicated that Plk and tubulins might be associated members of the same complex. We tested this idea by a series of immunoprecipitation experiments. S100 fractions made from synchronized G₁/S and prometaphase cells were first subjected to immunoprecipitation with a Plk polyclonal antibody raised against a C-terminal peptide (Zymed). This antibody precipitated Plk very efficiently (Figure 3). In a lighter exposure of the blot, it was clear that less Plk precipitated from the interphase than from the mitotic sample (results not shown). Probing of duplicate blots showed that all three tubulins were present in the Plk immunoprecipitates. Less α - and β -tubulins were found associated with Plk in interphase samples than in mitotic samples, probably reflecting changes in Plk levels during the cell cycle. In contrast, the amounts of γ -tubulin in the two immunoprecipitates were almost identical. Because the level of Plk is higher in mitosis, these results suggest a change in the molar ratio between Plk and γ -tubulin in the complex during mitosis. Although Plk was depleted from the supernatant by immunoprecipitation, an examination of tubulin proteins remaining in the supernatants after immunoprecipitation indicated that only a very small amount (less than 0.5%) of the total soluble tubulin was observed in Plk immune complexes (results not shown).

Reciprocal immunoprecipitations were also performed (Figure 4). Monoclonal antibodies against α -, β - and γ -tubulins specifically precipitated each of the three tubulins. More importantly, Plk was found in precipitates of α -, β - and γ -tubulins. Again generally, more Plk was detected in mitotic samples than in interphase samples. The antibody against α -tubulin repeatedly bound more Plk than other anti-tubulin antibodies from the same amount of cell lysate. Both immunoprecipitation experiments were performed with lysis buffer containing detergent and high concentrations of salt; therefore non-specific binding between Plk and tubulins seems unlikely.

Figure 5 Phosphorylation of tubulins by the Plk-associated kinase activity

Plk immunoprecipitates from mitotic FT210 cells were incubated with [γ - 32 P]ATP. Left panel: lane 32 P, autoradiography of the kinase reaction; lanes β and γ , immunoblottings of the same membrane with anti-(β -tubulin) (β) and anti-(γ -tubulin) (γ) antibodies. Right panel: autoradiography of reimmunoprecipitates with the indicated antibodies after kinase reactions. IP, immunoprecipitation; IB, immunoblotting. The positions of molecular mass markers are indicated (in kDa) in the centre.

Figure 6 Plk is responsible for the phosphorylation of tubulins in the kinase reaction *in vitro*

HEK 293 cells were transiently transfected with vector DNA (V), HA-tagged wild-type Plk (Plk) or catalytically inactive HA-K82R construct with a single amino acid mutation at the ATP-binding site (K82R). Tagged proteins were precipitated from nocodazole-blocked cells with anti-(HA tag) antibody. Kinase reaction of the immune complex *in vitro* was performed as described in the legend to Figure 5. The autoradiogram was overexposed to detect any weakened kinase activity. The positions of molecular mass markers are indicated (in kDa) at the left.

Mitotic Plk phosphorylates co-immunoprecipitated tubulins on serine and threonine

Because we had established that a strong association exists between tubulins and Plk, we next tested whether murine Plk can phosphorylate the associated tubulins. We performed kinase assays with mitotic Plk immune complexes from nocodazole-treated cells. Autoradiography of the kinase reactions showed that two proteins at 52 and 54 kDa were heavily phosphorylated (Figure 5, lane 32 P). The weakly phosphorylated 66 kDa protein was Plk. The same membrane was then probed with anti-tubulin antibodies. The 54 kDa band in the first lane co-migrated with both α - and β -tubulin. The γ -tubulin band co-migrated with the 52 kDa phosphoprotein. To clarify the identity of the phosphorylated proteins further, reimmunoprecipitations with anti-tubulin antibodies were performed immediately after the kinase reaction. As shown in the right three lanes of Figure 5, the anti-tubulin antibodies specifically reprecipitated phosphorylated α -,

Figure 7 Plk binds directly to tubulins

Left panel: Plk labelled with 35 S, translated *in vitro*, was preincubated with purified HeLa cell tubulins; tubulin was precipitated with anti-(β -tubulin) antibody. Co-precipitated Plk was detected by fluorography. Right panel: Plk was first isolated from the *in vitro* translation mixture by immunoprecipitation, and then incubated with HeLa tubulins. Plk-bound tubulins were detected by Western blotting with anti-(β -tubulin) antibody. The control sample (C) in both assays was a mock translation reaction without plasmid DNA. IP, immunoprecipitation; IB, immunoblotting. The positions of molecular mass markers are indicated (in kDa) at the left.

β - and γ -tubulin. The intensities of the autoradiographic signals for the tubulins do not necessarily accurately reflect differences in phosphorylation because the relative efficiencies of the antibodies used in the reimmunoprecipitation would also have affected the result. A very small amount of autophosphorylated Plk was also detected in reprecipitated samples, probably owing to renaturation and reassociation during the overnight reprecipitation.

The phosphorylated tubulins were excised from the membrane and hydrolysed in 6 M HCl. Thin-layer electrophoresis and autoradiography demonstrated that all three tubulins were phosphorylated on serine and threonine residues, but not tyrosine (results not shown).

The kinase activity detected in the Plk immune complex might have derived from two sources: Plk itself or other kinase(s) associated with Plk. To distinguish between these two possibilities, we transfected HEK 293 cells with Plk constructs, one encoding HA-tagged full-length Plk and the other encoding the catalytically inactive form K82R. HA-tagged kinase was immunoprecipitated from prometaphase cells with anti-(HA tag) antibody (BabCO, Richmond, CA, U.S.A.). The kinase activity associated with the immune complex was then detected by incubation with [γ - 32 P]ATP. As shown in Figure 6, a prolonged exposure of autoradiography demonstrated that the HA-Plk immune complex heavily phosphorylated associated tubulins, whereas the HA-K82R immune complex had almost no kinase activity. This result strongly indicates that Plk, rather than other associated kinase(s), is responsible for the phosphorylation of tubulins during the kinase reaction *in vitro*.

Plk binds directly to purified tubulins

The Plk complex shown in Figure 1 is large and undoubtedly contains other proteins in addition to tubulins; an important question is therefore whether Plk interacts with tubulins directly or indirectly. We used *in vitro* translated, radioactively labelled Plk and purified HeLa cell tubulins to test for direct interactions. Tubulin was preincubated with Plk and later immunoprecipitated with anti-tubulin antibody. Co-precipitated Plk was detected by fluorography (Figure 7, left panel). In the reciprocal experiment (Figure 7, right panel), Plk was first isolated from the *in vitro*

Figure 8 Diagram of HA-tagged Plk constructs used in the transfection experiment

The circle stands for the HA tag, the hatched bar for the kinase domain (residues 53–305) and the black box for the polo-box (residues 410–440). The size of each protein deduced from its amino acid sequence is: Plk, 67 kDa; Δ KD, 38 kDa; K82R (mutant at the ATP-binding site), 67 kDa; Plk264, 30 kDa; Plk356, 40 kDa. Amino acid numbers are based on murine Plk sequences.

translation preparation by immunoprecipitation, and then incubated with the purified HeLa tubulins. The immunoprecipitated Plk efficiently bound α - and β -tubulins, whereas in the control sample only a negligible amount of tubulin was detected. Because the reticulocyte lysate used to translate the Plk protein contained neither detectable endogenous Plk or tubulins, nor any detectable microtubule-associated proteins, these experiments support the notion that the purified tubulins interacted directly with the Plk translated *in vitro*.

Kinase domain of Plk is required for its binding to tubulins

To determine which domains of Plk are required for its association with tubulins, we transiently transfected several different HA epitope-tagged Plk constructs (Figure 8) into HEK 293 cells. Lysates were made from the transfected cells; expression of each Plk construct was first examined with the anti-(HA tag) antibody (Figure 9, left panel). Full-length Plk, the kinase domain-deleted Plk (Δ KD) and the catalytically inactive Plk (K82R) were all well expressed, as shown by the strong HA-specific bands at the corresponding positions. Both full-length and K82R Plk migrated at approx. 67 kDa, and Δ KD Plk at approx. 38 kDa. The bands indicated by arrowheads were non-specific proteins cross-reacting with the anti-HA antibody, because they were also present in empty-vector-transfected samples. The low-molecular-mass bands (asterisked) at approx. 50 and approx. 40 kDa were probably proteolytic degradation products derived from full-length Plk and K82R Plk.

Immunoprecipitation was performed first with the monoclonal antibody against α -tubulin. Co-immunoprecipitated Plk was detected with the HA-tag-specific antibody (Figure 9, right panel). Both full-length Plk and K82R Plk were highly enriched in α -tubulin immunoprecipitates, whereas only a trace amount of Δ KD Plk was detected. These results demonstrate the importance of the kinase domain, but not the kinase activity of Plk, for binding to α -tubulin. Duplicate immunoprecipitations were performed with antibodies against β -tubulin and γ -tubulin, with essentially the same results. Deletion of the polo box from full-length HA-Plk had little effect on the association of tubulin with Plk (results not shown).

Knowing that the kinase domain of Plk protein was required for its efficient interaction with tubulins, we then tested whether

Figure 9 Kinase domain of Plk is required for its binding to tubulins

HEK 293 cells were transiently transfected with HA-tagged Plk constructs as indicated; 36 h after transfection, cells were synchronized with nocodazole for 16 h and collected. Total cell lysates (left panel) or immunoprecipitates with anti-(α -tubulin) antibody from transfected cells (right panel) were subjected to immunoblotting with anti-HA antibody (12CA5). IP, immunoprecipitation; IB, immunoblotting; V, vector; Plk, full-length; Δ KD, deletion of the kinase domain; K82R, ATP-binding site mutant; H, heavy chain. Arrowheads at the left indicate proteins cross-reacting with the anti-HA antibody; asterisks in the centre indicate degradation products from Plk and K82R; exogenously expressed Plk fragments are indicated by bars at the centre. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 10 Kinase domain of Plk is sufficient for its binding to tubulins

HEK 293 cells were transiently transfected with Plk constructs and synchronized as described in the legend to Figure 7. Lysates (left panel) or immunoprecipitates with anti-(α -tubulin) antibody (right panel) were probed with anti-HA antibody. IP, immunoprecipitation; IB, immunoblotting; V, vector; Plk, full-length Plk; 264, residues 1–264 of Plk; 356, residues 1–356 of Plk; H, heavy chain; L, light chain. Different Plk fragments are indicated by bars at the centre. The positions of molecular mass markers are indicated (in kDa) at the left.

the kinase domain alone was sufficient for binding. HEK 293 cells were transfected with plasmids carrying different lengths of the kinase domain of Plk, Plk356 and Plk264 (see Figure 8). Immunoprecipitations with the antibody against α -tubulin were analysed with anti-HA antibody (Figure 10). Full-length Plk and vector transfections were included as positive and negative controls. Both Plk356 and Plk264 were expressed moderately (Figure 10, left panel), and both were co-immunoprecipitated with tubulins (Figure 10, right panel). Shorter fragments (Plk173

and Plk133) were also tested; however, their expression levels were too low for the analysis to be completed. These results demonstrate that the kinase domain of Plk is important in tubulin binding but that the kinase activity is irrelevant. Therefore the kinase domain is both necessary and sufficient for tubulin binding.

DISCUSSION

We have investigated the nature of the Plk complex in mouse FT210 and HEK 293 cells and demonstrated the association of Plk with all three members of the tubulin family. The size of the Plk complex ranged from 669 to 2500 kDa, possibly indicating that Plk is a member of multiple stable complexes or that a very large complex is subject to partial dissociation during the purification. The complex bound strongly to the anion-exchange resin, suggesting that it might have a net negative charge on the surface at neutral pH, unlike Plk itself, which has an isoelectric point of 9.2 [7].

The Plk complex proved to be quite stable considering the buffer conditions used in this study [1% (w/v) CHAPS/300 mM NaCl]. Even 75%-satd. (3.6 M) $(\text{NH}_4)_2\text{SO}_4$ precipitation did not disrupt the complex. Interestingly, when the detergent was dialysed from the S100 fraction of cell lysates, most of the Plk was converted into the monomeric form, as indicated by its elution profile from the sizing column. This result suggests that the complex is probably held together mainly by hydrophobic forces rather than by ionic binding (results not shown).

Plk has been localized to components of the spindle throughout the cell cycle [25]; it seems to interact with a kinesin-like microtubule motor protein, CHO1/MKLP-1 [18]. The stable interaction between Plk and tubulins in the presence of nocodazole was rather surprising, because after 16 h of nocodazole treatment all microtubules should have depolymerized. In contrast, interphase cells synchronized by a double thymidine block had intact microtubules and the association between Plk and tubulins remained. These results indicate that the interaction between Plk and tubulins is independent of microtubule polymerization states and suggest an intimate association of Plk with microtubule subunits. Because the kinase activity of Plk is regulated by the cell cycle, the removal of Plk from interphase microtubules seems unnecessary. Instead, the stable association might permit the rapid phosphorylation of tubulins at the G_2/M transition. Therefore the constitutive association between Plk and tubulins in mitotic and interphase cells does not necessarily undermine the notion of a direct role for Plk in regulating spindle organization. All three members of the tubulin family were phosphorylated by Plk during the kinase reactions *in vitro*. Such phosphorylation of α - and β -tubulins is believed to be one of the principal mechanisms adjusting the stability of microtubules [33,34]. The Plk-mediated phosphorylation of tubulins might speed up the turnover rate of microtubule and shorten the half-life of spindle microtubules, which is the characteristic of spindle microtubules in contrast with their interphase counterpart. This view is supported by changes both in Plk activity and in its localization along different parts of the spindle during cell cycle [8,18,27]. In addition to tubulins, Plk also phosphorylates a microtubule-associated protein and a microtubule-based motor molecule, CHO1/MKLP-1 [18,28]. This being so, the association between Plk and tubulins, by functioning as a scaffold, might help to hold all the substrates and kinase together. Although Tavares et al. [28] demonstrated that exogenously added α - and β -tubulins could serve as substrates for *Drosophila* polo *in vitro*, this study is the first to show that α -, β - and γ -tubulins are associated with Plk in a stable complex. Our binding assay *in*

vitro indicates that the interaction between Plk and α - and β -tubulins is direct.

During the G_2/M transition, the size and phosphoprotein content of the centrosomes increase markedly and are reflected in increased MPM2 antibody staining [35]. These changes are believed to be essential steps for centrosomal maturation and required for the nucleation of spindle microtubules during mitosis. Because *Xenopus* Plx1 has been reported to phosphorylate Cdc25c and thereby render it MPM2 reactive [10], it seems likely that Plk contributes to the MPM2 staining of the centrosome. Consistently with this notion, microinjection of a Plk-specific antibody has been shown to decrease MPM2 staining intensity in centrosomes [21]. Alternatively, or in addition, the high-affinity association between Plk and tubulins reported here might help to recruit newly expressed Plk as well as γ -tubulin, the key component of the microtubule organization center [36,37], from the cytoplasm to the centrosome/spindle poles at the onset of mitosis. Plk antibody-injected cells often had unseparated spindle poles and monopolar spindles [21], suggesting that Plk might affect the formation of the normal bipolar spindle through its action on γ -tubulin and MPM2 antigens. It is also possible that the accumulation of Plk and γ -tubulin in the centrosome might prime the centrosome such that other centrosomal proteins can be attracted from the cytoplasm and modified. In an early study, a centrosomal protein CP190 (or Bx63) failed to localize to centrosomes in *polo* mutant *Drosophila* embryos [1]. Studies in *Drosophila polo* or homologue mutants of *S. pombe* and *S. cerevisiae* have repeatedly found abnormalities in the establishment of bipolar spindles and separation of chromosomes [2,3]; however, the mechanism remains unknown. Our finding that Plk is stably associated with γ -tubulin might provide a partial explanation underlying these changes in antibody-injected cells and *polo* mutants.

Our understanding of the different functional domains of the Plk protein is still developing. Mundt et al. [20] have reported that the C-terminal 20–28-residue region of Plk has an inhibitory effect on its kinase activity. We found that the Plk kinase domain was both necessary and sufficient to form stable associations with tubulins. The catalytically inactive Plk mutant K82R still bound to tubulins, indicating that it is the kinase domain sequence, not the kinase activity, that is required for the binding. It also indicates that autophosphorylation of Plk is not required for its association with tubulins. This was consistent with co-immunoprecipitation experiments in which we found that both interphase Plk (with low kinase activity) and mitotic Plk (with high kinase activity) were associated with tubulins. It will be interesting to know whether the same region is also involved in the interaction of Plk with other proteins such as Pin1 and components of the anaphase-promoting complex.

On the basis of these findings, we propose that Plk affects the organization of the spindle in part through its association with, and phosphorylation of, tubulins. We are currently purifying the Plk complex and trying to identify other components. Studies are also under way to assess the sites on tubulin phosphorylated by Plk and to study the biology of cells transfected with mutant tubulins that cannot be phosphorylated.

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