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Control of Microtubule Dynamics by Oncoprotein 18: Dissection of the Regulatory Role of Multisite Phosphorylation during Mitosis

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Oncoprotein 18 (Op18; also termed p19, 19K, metablastin, stathmin, and prosolin) is a conserved protein that regulates microtubule (MT) dynamics. Op18 is multisite phosphorylated on four Ser residues during mitosis; two of these Ser residues, Ser-25 and Ser-38, are targets for cyclin-dependent protein kinases (CDKs), and the other two Ser residues, Ser-16 and Ser-63, are targets for an unidentified protein kinase. Mutations of the two CDK sites have recently been shown to result in a mitotic block caused by destabilization of MTs. To understand the role of Op18 in regulation of MT dynamics during mitosis, in this study we dissected the functions of all four phosphorylation sites of Op18 by combining genetic, morphological, and biochemical analyses. The data show that all four phosphorylation sites are involved in switching off Op18 activity during mitosis, an event that appears to be essential for formation of the spindle during metaphase. However, the mechanisms by which specific sites down-regulate Op18 activity differ. Hence, dual phosphorylation on the CDK sites Ser-25 and Ser-38 appears to be required for phosphorylation of Ser-16 and Ser-63; however, by themselves, the CDK sites are of only minor importance in direct regulation of Op18 activity. Subsequent phosphorylation of either Ser-16, Ser-63, or both efficiently switches off Op18 activity.

Microtubules (MTs) are polymeric components of the cytoskeleton found in all eukaryotes and are composed of heterodimers of α- and β-tubulin. In nondividing cells and during the interphase of the cell cycle, MTs are important for organizing the cytoplasm, for organelle transport, and for intracellular movement of cell surface receptors (for a review, see reference 7). During mitosis, large arrays of MTs, termed the mitotic spindle, segregate the condensed chromosomes (for a review, see reference 17).

Tubulin exists in a dynamic equilibrium between free tubulin dimers and MTs. The dynamic instability model describes the dynamic behavior of MTs in terms of the following four parameters: the rates of growth and shrinkage of tubulin polymers and the frequencies of catastrophes (transitions from growth to shrinkage) and rescues (transitions from shrinkage to growth) (30). The dynamic instability of MTs is in part regulated by MT-associated proteins (MAPs), most of which are phosphoproteins (for a review, see reference 15). It is thought that signal transduction cascades that regulate cell proliferation and differentiation control MT dynamics by phosphorylation of MAPs. The consensus in the field is that MAPs stabilize MTs by direct binding and that phosphorylation of MAPs decreases the binding affinity toward MTs and thereby weakens their stabilizing effect (10, 36).

The multitude of MT functions makes it likely that MT dynamics are regulated by several mechanisms that are responsive to both internal and external signals. An analysis of MT dynamics in intact cells suggests the presence of MT regulatory factors that oppose the action of MAPs by inducing catastrophes and thereby increasing the dynamics of MTs (for a review, see reference 28). Oncoprotein 18 (Op18) was recently identified as a protein that destabilizes MTs both in vitro (3) and in intact cells (26). Contrary to MAPs, Op18 was found to bind tubulin heterodimers but not MTs, and it was demonstrated that Op18 activity increases the frequency of catastrophes during in vitro assembly of tubulin (3). The catastrophe-inducing activity of Op18 is in line with the results from intact cells, namely, that overexpression of Op18 dramatically shifts the equilibrium between free tubulin dimers and MTs in the direction of free tubulin dimers (26).

Op18 is a cytosolic protein that has been independently studied by several groups under different names (e.g., p19, 19K, metablastin, prosolin, and stathmin) (for a review, see reference 1). This protein was initially identified due to its complex pattern of phosphorylation in response to a multitude of signals (8, 9, 13, 34, 37) and due to its high expression in a variety of human malignancies (14, 33). Op18 is variably phosphorylated on four distinct Ser residues in intact cells, namely, Ser-16, -25, -38, and -63 (4, 19, 23). All four Ser residues are phosphorylated to high stoichiometry during mitosis. Cyclin-dependent kinases (CDKs), mainly CDK1 associated with cyclin B, have been identified as the kinase system that phosphorylates Ser-25 and Ser-38, but the protein kinase involved in phosphorylation of Ser-16 and Ser-63 during mitosis remains to be identified (5, 21). Besides cell cycle-regulated phosphorylation, Op18 is phosphorylated by members of the mitogen-activated protein kinase family on Ser-25 (22–24), by the Ca^{2+}/calmodulin-dependent kinase IV/Gr (CaMK IV/Gr) on Ser-16 (25), and by the cyclic AMP-dependent protein kinase (PKA) on Ser-16 and Ser-63 (4). Hence, Op18 is a target for multiple protein kinases, which are regulated either through the cell cycle or by signal transduction cascades.

Prior to the identification of the MT-regulatory activity of Op18, the function of this protein and the significance of its complex phosphorylation were addressed by an approach involving conditional overexpression of wild-type Op18 (Op18-wt) and mutated kinase target site-deficient derivatives of

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Op18 (21, 27). The data demonstrated that although overexpression of Op18-wt did not result in a significant cell cycle phenotype, an Ala substitution at any of the four kinase target sites resulted in accumulation of cells blocked in mitosis prior to metaphase. To search for the mechanism responsible, MTs were analyzed in cells induced to overexpress either Op18-wt or a CDK target site-deficient mutant of Op18 (Op18-S25,38A) (26). The data revealed that expression of either of these two Op18 derivatives depolymerized MTs during the interphase of the cell cycle but that only the Op18-S25,38A mutant interfered with formation of the mitotic spindle. Hence, phosphorylation of overexpressed Op18 is essential to allow spindle formation during mitosis and subsequent cell division.

The study outlined above showed that Op18 regulates MT dynamics in response to phosphorylation signals. Moreover, the data suggested that a CDK target site-deficient mutant is constitutively active, which in turn suggests that Op18 activity is switched off during mitosis by phosphorylation on the CDK sites. Such data seemingly contradict a model proposed by Belmont and Mitchison (3), which infers activation of Op18 at the onset of mitosis by phosphorylation to explain the increase in MT dynamics that occurs during formation of the mitotic spindle (2). Since Op18 is multisite phosphorylated by two distinct protein kinase systems during mitosis, it remains possible that various combinations of Ser-16 and Ser-63 phosphorylation either modulate or antagonize the effect of CDK phosphorylation of Ser-25 and Ser-38. Therefore, to understand the physiological function of Op18, it is essential to determine how phosphorylation on all four kinase target sites controls its activity. In this study, we analyzed the regulatory effect of multisite phosphorylation of Op18 during mitosis. The roles of all four individual phosphorylation sites of multisite-phosphorylated Op18 were dissected in terms of phenotypes of kinase target site-deficient mutants, cooperatively between phosphorylation events, binding activity to tubulin, and inhibition of MT polymerization.

MATERIALS AND METHODS

DNA transfection and cell culture condition. The pMEP4-based Op18 derivatives and transfection protocols used in this study have previously been described (21, 27). The pMEP4 shuttle vector contains the Epstein-Barr virus origin of replication and the *EBNA-1* gene to allow high-copy episomal replication and the *hph* gene, which confers hygromycin B resistance in mammalian cells (12). Conditional expression of various Op18 derivatives was obtained by employing the hMTIIa promoter, which can be suppressed by low concentrations of EDTA (25 μM) and induced by Cd^{2+} (0.03 to 0.1 μM) (27). Transfected cells were cultured in a medium containing EDTA (25 μM) which has been specifically designed to support cell growth under conditions that minimize expression from the hMTIIa promoter (27). About 50 to 70% of all pMEP4-transfected cells that survived electroporation were resistant to hygromycin B (0.25 mg/ml; Boehringer Mannheim), and mock-transfected cells were killed within 3 days. All experiments were routinely performed at 5 to 6 days postelectroporation.

Analysis of MT polymerization status, Western blotting, and analysis of Op18 phosphoisomers. The fraction of polymerized MTs in transfected cells was determined by extracting soluble tubulin in an MT stabilizing buffer {0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.9], 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100, 4 mM taxol} containing 5 μg of pefablock per ml and 5 μg of leupeptin per ml. Tubulin contents were analyzed in the particulate cytoskeleton and soluble fractions by sodium dodecyl sulfate-polyacrylamide (10 to 20% gradient) gel electrophoresis (SDS-PAGE) followed by quantitative Western blot analysis as previously described (26). Affinity-purified anti-Op18, specific for the COOH terminus (anti-Op18:34-149), was used together with ^{125}I-protein A for Western blot analysis as previously described (6). As a control for equal loading, the relevant parts of filters were routinely probed with rabbit anti-triose-phosphate isomerase. To analyze Op18 phosphoisomers, we employed a native PAGE system that separates Op18 according to the charge differences introduced by each of the four identified phosphorylations (24).

In vitro phosphorylation of Op18. *Escherichia coli*-derived Op18 derivatives were purified and treated with cyclic AMP-dependent protein kinase (PKA; New England BioLabs, Inc.) and/or CDKs affinity purified on $p13^{suc1}$-coated beads as previously described (24). Phosphorylation of Ser-16 and Ser-63 by PKA was achieved by incubating 2.8 U of kinase/μg of Op18 in 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–500 μM ATP for 3 h at 30°C. Thereafter, additional kinase (2.8 U of PKA/μg of Op18) and ATP (500 μM) were added and the sample incubated for an additional 3 h. Reactions were terminated by heating to 75°C for 10 min and precipitated overnight at −20°C with 6 volumes of MeOH containing 1% sucrose. The precipitate was washed twice with 75% MeOH containing 1% sucrose, dried under vacuum, and resuspended in 80mM piperazine-*N*,*N*′-bis (2-ethanesulfonic acid) (PIPES)–1 mM EDTA–1 mM Mg^{2+} (pH 6.8). To remove PKA, which remained insoluble after MeOH precipitation, the final preparation of phosphorylated Op18 was clarified by centrifugation (15 min, 14,000 × *g*). Phosphorylation of Op18 on Ser-25 and Ser-38 by CDKs was performed with 0.8 μl of $p13^{suc1}$-coated beads (absorbed with lysate from 6×10^6 nocadozole-arrested Jurkat cells) per μg of Op18 with 500 μM ATP in 50 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–5 mM EGTA–1 mM dithiothreitol–a cocktail of phosphatase and protease inhibitors as previously described (24). Reaction mixtures were incubated for a total of 8 h at 30°C with the addition of fresh ATP (500 μM) after 1.5 and 5 h. Op18-wt, Op18-S63A, and Op18-S16A were used in sequential double-kinase incubations to obtain derivatives phosphorylated on three or all four of the Ser target sites. In these cases, the CDK reaction was initiated and the two additions of PKA (2.8 U of PKA/μg of Op18) and ATP (500 μM) were incorporated at 3 and 5 h into the 8-h incubation. Reaction mixtures were adjusted to 0.5% Triton X-100–0.3 M NaCl, centrifuged to remove beads prior to termination, and precipitated as described above. The concentration of the resulting phosphorylated Op18 was calculated by SDS-PAGE comparison with a standard recombinant Op18 preparation for which the protein mass had been determined by amino acid analysis as previously described (6). As controls, in vitro phosphorylation was also performed either in the absence of ATP or with mutants of Op18 that lack the specific phosphorylation sites. In these cases, the tubulin binding and MT-destabilizing activities of Op18 were unaffected. Therefore, the observed effects of Op18 phosphorylation could be attributed to the addition of phosphate groups to specific Ser residues.

Preparation of Op18, cross-linking of Op18-tubulin complexes, and determination of the MT-destabilizing activity of Op18. Op18-wt and kinase target site-deficient mutant derivatives of purified *E. coli*-derived Op18 were prepared as previously described (6). Partially purified Op18 was prepared from S- or M-phase-blocked HeLa cells as previously described (24). Purified bovine tubulin was obtained from Cytoskeleton (Denver, Colo.). For cross-linking studies, Op18 and tubulin were incubated at the indicated concentrations in 30 μl of G-PEM buffer (80 mM PIPES, 1 mM EDTA, 1 mM Mg^{2+}, 2 mM GTP [pH 6.8]). After 2 h on ice, the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC; Sigma) was added (final concentration, 12 mM), and the samples were incubated at 18°C. The reaction was quenched at various times by the addition of 1 volume of 2-mercaptoethanol (10%)–glycine (0.2 M) and precipitated with 66% acetone, and cross-linked Op18-tubulin complexes were analyzed by SDS-PAGE. In vitro assembly of tubulin (4 μM) in the presence of various amounts of Op18 was performed in assembly buffer (25 μl of G-PEM containing an additional 4 mM Mg^{2+}, 10% glycerol, and 4 μg of taxol per ml). After 1 h at 37°C, samples were diluted eightfold in assembly buffer and centrifuged for 30 min at 48,000 × *g*, and the pellet of polymerized tubulin was washed in assembly buffer. Unpolymerized tubulin recovered in the supernatant was precipitated with acetone (66%) and solubilized in 0.1% SDS. The protein contents of polymerized and unpolymerized tubulin were analyzed with bicinchoninic acid protein assay reagent (Pierce). The Op18 protein is poorly detected by bicinchoninic acid assay, which results in minimal interference with quantification of tubulin protein. In control experiments, the activities of *E. coli*-derived Op18 and mammalian Op18 were compared and found to be indistinguishable under the conditions used for the tubulin assembly assay. The activities of Op18 mutants with Ala substitutions for the Ser residues at positions 16, 25, 38, and 63 were also found to be indistinguishable. Determinations of tubulin and Op18 protein masses by analysis of amino acid composition were performed as previously described (6).

Immunofluorescence and flow cytometric analysis. Cells were extracted with MT stabilizing buffer (see above) containing 0.05% saponin and 10 μg of RNase per ml. Cells were fixed in 4% paraformaldehyde–0.5% glutaraldehyde for 15 min, quenched with $NaBH_4$, and stained with anti-α-tubulin (clone B-5-1-2; Sigma). Bound antibodies were revealed by fluorescein-conjugated rabbit anti-mouse immunoglobulin, and DNA was stained with 0.1 μg of propidium iodide per ml. Cells were mounted with 1 mg of *p*-phenylenediamine per ml in phosphate-buffered saline with 80% glycerol and analyzed either by epifluorescence or by using a Nikon Diaphot equipped with a Molecular Dynamics confocal imager system. An analysis of DNA content was performed by flow cytometry of propidium iodide-stained cells as previously described (27).

RESULTS

The phenotype of kinase target site-deficient Op18 mutants in interphase and mitosis. A recent study investigated the consequences of overexpression of Op18-wt and the CDK target site-deficient mutant Op18-S25,38A on the level of cell

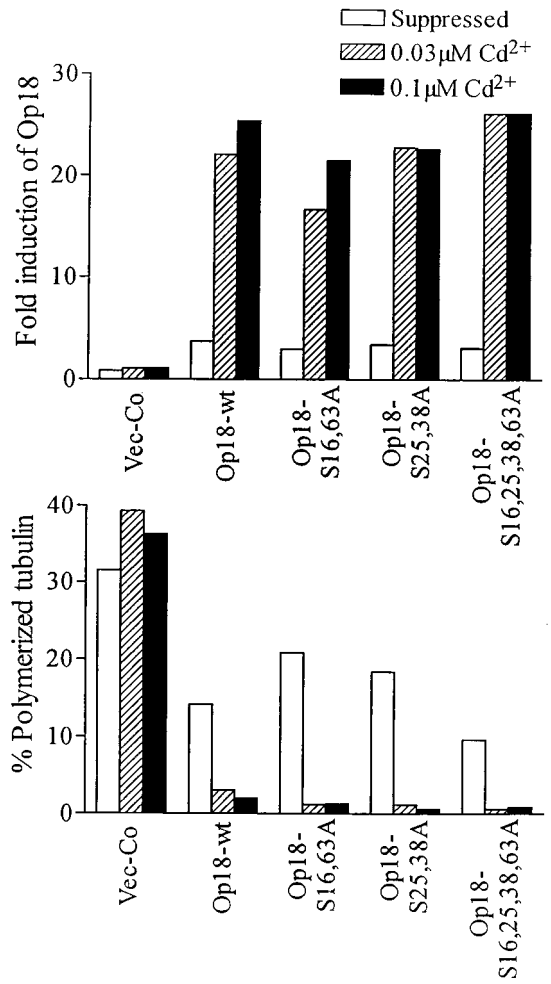

FIG. 1. Depolymerization of MTs by expression of either Op18-wt or kinase target site-deficient mutants of Op18. K562 cells were transfected with either vector control (Vec-Co) or the indicated pMEP4 derivatives, and hygromycin B-resistant cell lines were selected as described in Materials and Methods. Expression from the hMTIIa promoter of pMEP4 was either suppressed by the presence of 25 μM EDTA or induced for 5 h with the indicated concentrations of Cd^{2+}. (Top) Op18 levels were determined by Western blot analysis with rabbit anti-Op18 and ^{125}I-protein A, and data are expressed as fold inductions over the endogenous level. (Bottom) Cells were extracted with an MT stabilizing buffer, and the amounts of tubulin recovered in the particulate and soluble fractions were determined by quantitative Western blot analysis as described in Materials and Methods. Data are the percentages of total cellular tubulin content polymerized.

cycle regulation and MT polymerization status (26). The phenotype of the Op18-S25,38A mutant indicated that phosphorylation on Ser-25 and Ser-38 suppresses the MT-destabilizing activity of Op18 and that this suppression is an essential event to allow normal mitosis. Besides these two CDK target sites, Op18 is phosphorylated on Ser-16 and Ser-63 during mitosis by an as-yet-unidentified protein kinase system (21). To investigate the regulatory role of the complex mitotic phosphorylations of Op18, we expressed either Op18-wt, Op18-S25,38A, Op18-S16,63A, or Op18-S16,25,38,63A in K562 leukemia cells by using the Cd^{2+}-inducible pMEP4 shuttle vector system. The data in Fig. 1 show that Op18-wt and the phosphorylation site-deficient mutants were all expressed and induced to similar levels by Cd^{2+}. Moreover, induced expression of any of these Op18 derivatives resulted in depolymerization of MTs within 5 h of Cd^{2+}-induced expression (Fig. 1). Prolonged expression of these Op18 mutants results in a mitotic block (21), but during the time course of this experiment (5 h), most cells were still in interphase. It follows that the data in Fig. 1 primarily reflect the activity of the expressed protein in interphase cells, i.e., in the absence of mitosis-specific phosphorylations. Hence, the data show that contrary to the situation during mitosis, the substitution of Ala for any of the four Ser residues of Op18 does not dramatically interfere with the MT-destabilizing activity of the expressed Op18 protein during the interphase of the cell cycle.

We recently reported that in contrast to Op18-wt, overexpression of the Op18-S25,38A mutant interfered with formation of the mitotic spindle (26). This indicated the regulatory importance of phosphorylation on the two CDK target sites during mitosis and is consistent with the Op18-S25,38A mutant being constitutively active. To extend the analysis to all four phosphorylation targets, the morphology of K562 cells blocked in mitosis by the Op18-S25,38A, Op18-S16,63A, or Op18-S16,25,38,63A mutant was analyzed. In agreement with earlier results (21), an analysis of the DNA profile shows that 24 h of induced expression of mutated Op18, but not Op18-wt, resulted in a mitotic block (Fig. 2A). DNA and MT staining of mitotic cells revealed that all the mutated Op18 derivatives caused a block in mitosis by interfering with spindle formation. However, the mutants appeared to differ with respect to the severity of the mitotic phenotype. While all cells that expressed mutant Op18 exhibited an overlapping array of aberrant spindles, a predominant spindle abnormality was elicited by the different Op18 derivatives (Fig. 2A).

To quantify the heterogeneity of spindle abnormalities, aberrant mitotic figures were classified into three categories, types I to III. Type I mitotic figures contained less dense kinetochore MTs than those of normal spindles, and only a few chromosomes were aligned at the metaphase plate. Type II mitotic figures lacked kinetochore MTs, and MTs appeared as small star-like asters. Most cells contained two asters, suggesting that the centrosomes were separated. In addition, all the condensed chromosomes appeared to be aggregated. Type III mitotic figures exhibited the most severe defects since MT structures were undetectable and the condensed chromosomes were aggregated. As expected, an examination of transfected cells revealed that overexpression of Op18-wt caused only a minor increase in the frequency of abnormal mitotic spindles (Fig. 2A). Expression of Op18-S16,63A caused mainly type I defects, and Op18-S25,38A expression caused mainly type II defects. Mutations of all four kinase target sites (Op18-S16,25,38,63A) resulted in the most severe interference with the spindle, and most mitotic cells were of type III. However, there was clear heterogeneity and overlap in the distribution of spindle abnormalities in the cell populations that expressed distinct Op18 mutants (Fig. 2A). Hence, it seems that although all three Op18 mutants interfere with spindle formation by the same mechanism, the order of the severity of the disorders caused by the mutants is as follows: Op18-S16,25,38,63A > Op18-S25,38A > Op18-S16,63A.

We have previously shown that blocking of Op18 phosphorylation by the introduction of specific Ser-to-Ala substitutions can have secondary effects on phosphorylation of the remaining sites in intact cells (21). Therefore, during analysis of phenotypes, it is important to consider the effects of the introduced Ser-to-Ala substitutions on the total phosphorylation status of the expressed protein. Endogenous Op18 is phosphorylated at low levels during interphase; during mitosis, the two CDK sites, Ser-25 and Ser-38, are phosphorylated to completion together with high-stoichiometry phosphorylation on

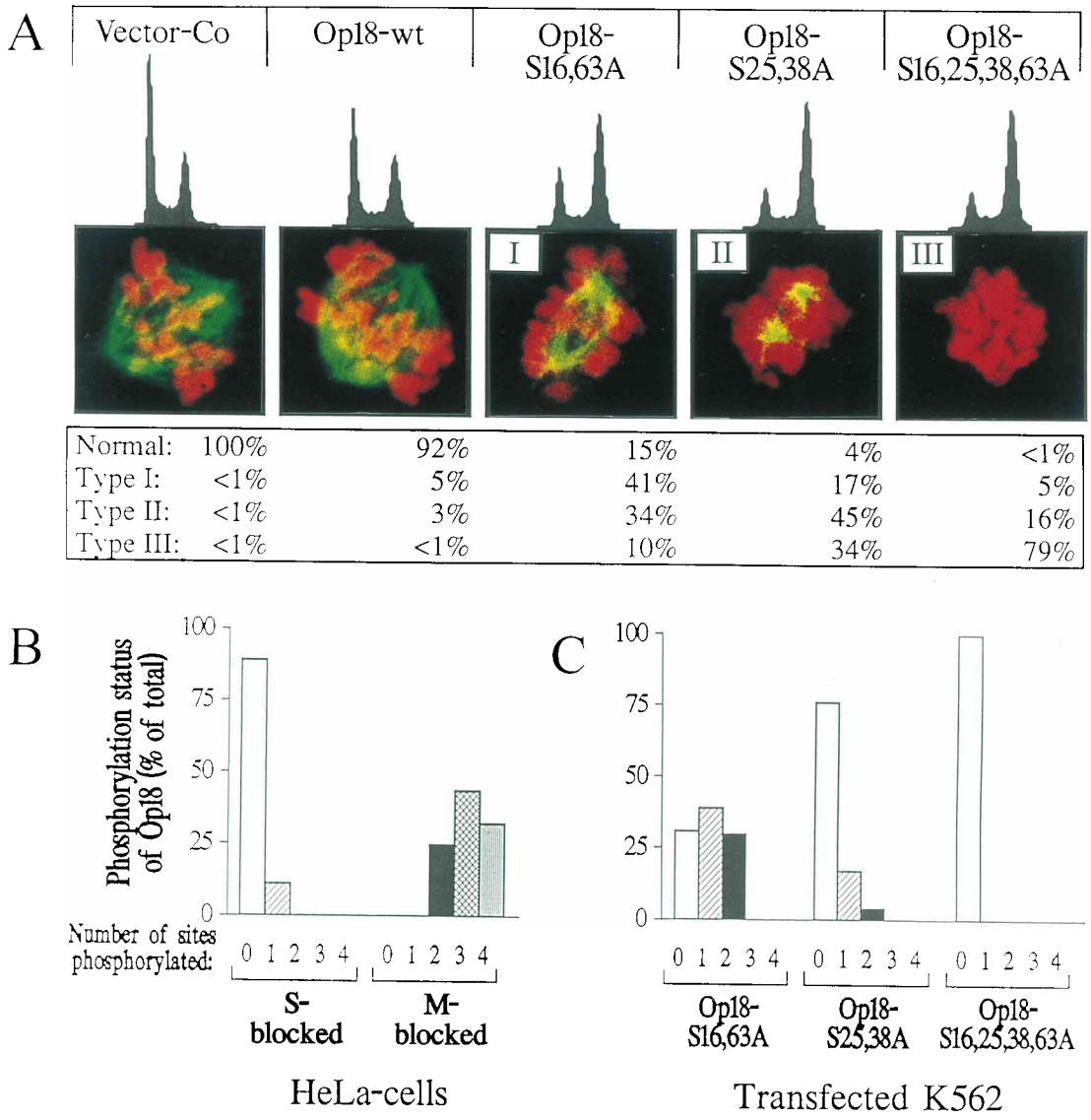

FIG. 2. Mitotic phenotypes of Op18-wt and kinase target site-deficient mutants of Op18. K562 cells were transfected, and hygromycin B-resistant cell lines were selected as described in the legend to Fig. 1. (A) Cells were treated with Cd^{2+} (0.1 μM) for 24 h, fixed, and double stained with anti-α-tubulin (green) and propidium iodine-DNA (red). Shown are DNA profiles and representative examples of M-phase cells expressing the indicated pMEP4 derivatives, as observed by confocal microscopy. Distributions of the three types of spindle abnormalities (types I to III) were calculated from data derived from observations of at least 300 mitotic cells. The results shown are representative for two independent experiments. (B) Exponentially growing HeLa cells were treated with thymidine (1 mM) (S-blocked) or nocodazole (0.5 μM) (M-blocked) for 16 h. Op18 was purified, and phosphoisomers of Op18 were resolved and quantitated by a native PAGE system as described in Materials and Methods. (C) K562 cells were induced to express the indicated Op18 derivatives for 24 h, and the phosphoisomer contents of the expressed proteins were analyzed as in described for panel B. Since the transfected Op18 derivatives were expressed at 10- to 20-fold-higher levels than that of the endogenous Op18 protein, the data primarily reflect the phosphorylation status of the recombinant Op18 gene product. The data shown in panels B and C are representative for five independent experiments.

both Ser-16 and Ser-63 (5, 21) (Fig. 3). The resulting distribution of di-, tri-, and tetraphosphorylated Op18 isomers in HeLa cells, which were used for an analysis of Op18 in a homogeneous population of cells blocked in mitosis prior to metaphase, is shown in Fig. 2B. The phosphoisomer distribution after 24 h of induced expression of the indicated Op18 mutants in K562 cells was also analyzed, and the data reveal clear differences among the mutants (Fig. 2C). As expected, the mutant protein associated with the most severe phenotype, Op18-S16,25,38,63A, was completely unphosphorylated. Only low levels of the Op18-S25,38A protein were diphosphorylated, and around 30% of the Op18-S16,63A protein was diphosphorylated. It should be noted that K562 cells have poor spindle assembly checkpoint control; therefore, interference with the mitotic spindle results only in a transient block in metaphase. Therefore, the frequency of mitotic cells in the transfected-cell population is only about 25 to 30% (26). Hence, the observed fraction (30%) of diphosphorylated Op18-S16,63A protein in transfected cells most likely corresponds to complete phosphorylation on both Ser-25 and Ser-38 in all of the cells blocked in mitosis. Taken together, the results in Fig. 2 suggest some correlation between the phosphorylation status of expressed Op18 mutants and the severity of the phenotypes on the level of spindle abnormalities. However, the

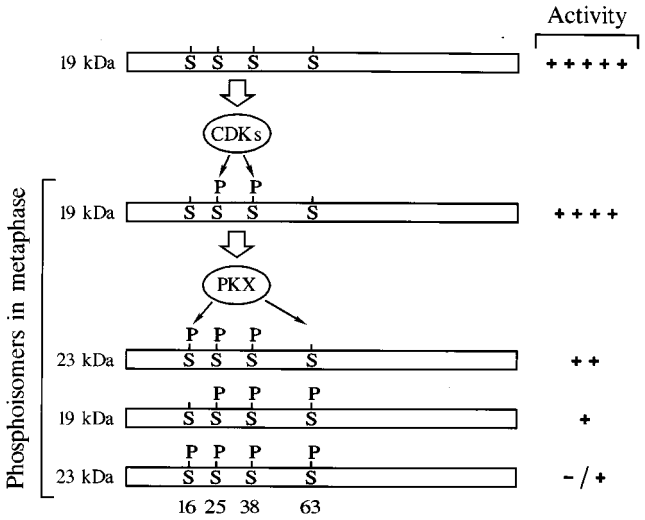

FIG. 3. Model of sequential phosphorylation during mitosis and regulation of Op18 activity. The phosphoisomers that can be detected in cells blocked in mitosis prior to metaphase, together with the proposed dependency of Ser-16 and Ser-63 phosphorylation (targets for an unidentified protein kinase [PKX]) upon phosphorylation of Ser-25 and Ser-38 (CDK targets), are illustrated. The activities of Op18 phosphoisomers, as determined by tubulin association and inhibition of MT polymerization, are also shown. +++++, unphosphorylated Op18 (arbitrarily assigned). The activities given for the indicated phosphoisomers are estimates from the data shown in Fig. 6 and 7.

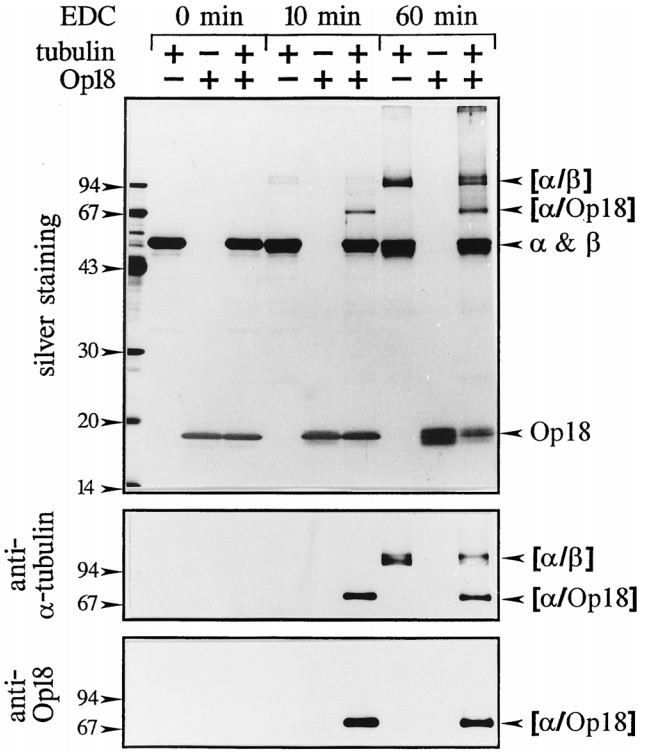

FIG. 4. Physical interactions between Op18 and tubulin revealed by a zero-length cross-linker. Bovine tubulin (20 μM) and Op18 (60 μM) were mixed in the indicated combinations. Complex formation was analyzed by the addition of the zero-length cross-linker EDC for the indicated times, as described in Materials and Methods. The generated complexes were analyzed by silver staining of SDS-PAGE gels or by Western blotting with the indicated antibodies. The positions of Op18, α- and β-tubulin, and specific complexes are indicated. +, presence; −, absence. Molecular mass standards (in kilodaltons) are given on the left.

results obtained with the Op18-S16,63A mutant show that a high level of dual phosphorylation on the two CDK target sites Ser-25 and Ser-38 is insufficient to allow transfected cells to form a functional mitotic spindle. Together with the poor phosphorylation of the Op18-S25,38A protein, which is associated with a severe phenotype, this indicates that phosphorylation of Ser-16 and Ser-63 is important for the regulation of Op18 activity during mitosis. In addition, the low levels of Ser-16 and Ser-63 phosphorylation of the Op18-S25,38A protein suggest that Ser-16 and Ser-63 phosphorylation is dependent upon phosphorylation of Ser-25 and Ser-38, as previously reported (21) (Fig. 3).

Interaction of Op18 phosphoisomers with tubulin heterodimers. Op18 associates with tubulin heterodimers, but not polymerized tubulin, and this interaction may be of importance for the MT-destabilizing activity of Op18 (3). To analyze a potential role for Op18 phosphorylation in regulating the association with tubulin heterodimers, a system that employs the zero-length cross-linker EDC was used. As shown in Fig. 4, the addition of EDC to a mixture of Op18 and tubulin generated a 71-kDa complex (α-tubulin–Op18) that reacted with both anti-α-tubulin and anti-Op18 antibodies. This 71-kDa complex appeared to contain predominantly α-tubulin, since it reacted poorly with two anti-β-tubulin antibodies (data not shown). Moreover, N-terminal sequence analysis of the 71-kDa complex indicated that 80 to 90% of the tubulin cross-linked to Op18 was α-tubulin and that 10 to 20% was β-tubulin, which agrees with the serological data. The size of the 71-kDa complex is close to the sum of the molecular masses of α-tubulin (55 kDa) and Op18 (19 kDa). In addition to the 71-kDa α-tubulin–Op18 complex, a 110-kDa complex corresponding to the α- and β-tubulin heterodimer was generated after 60 min of cross-linking of tubulin alone or in the presence of Op18. To control for the specificity of Op18-tubulin cross-linking, we analyzed complex formation with tubulin that was inactivated by five successive freeze-thawing cycles or by 5 min at 60°C. In both cases, EDC failed to cross-link Op18 and tubulin (data not shown), which shows that cross-linking of this complex requires native and assembly-competent tubulin heterodimers.

Ser-25 and Ser-38 of Op18 are phosphorylated to completion during mitosis, which in combination with Ser-16 and Ser-63 phosphorylation generates the four phosphoisomers of Op18 that have previously been identified in cells arrested in mitosis prior to metaphase (5, 21) (Fig. 2B and 3). To generate each of the phosphoisomers, Op18-wt and specific mutants were in vitro phosphorylated to high stoichiometry on specific target sites by using the appropriate combinations of CDKs and PKA. The site specificities of these protein kinases have previously been determined by a combination of tryptic phosphopeptide mapping and site-specific mutants of Op18 (4, 24, 25). Figure 5 shows that specific Op18 phosphoisomers of high stoichiometry could be generated in vitro. Further analysis of these phosphoisomers by EDC-dependent cross-linking to tubulin is also shown in Fig. 5. The results reveal various degrees of phosphorylation-dependent decreases of both the major Op18–α-tubulin complex migrating to 71 kDa and a minor complex migrating to 83 kDa (the 83-kDa complex is present but not visible at the exposure of the autoradiograph shown in Fig. 4). Cophosphorylation on all four kinase target sites of Op18 essentially abolished complex formation, but phosphorylation on the two CDK target sites, Ser-25 and Ser-38, had only a modest effect. In combination with the two CDK target sites, phosphorylation on either Ser-16 or Ser-63 resulted in a

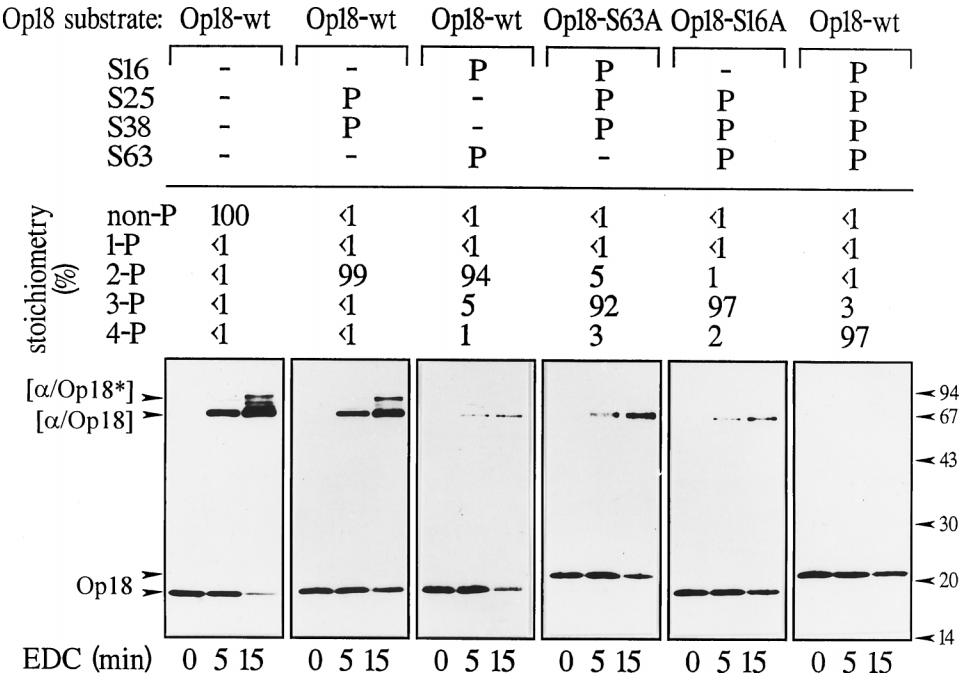

FIG. 5. Dissection of the regulatory role of Op18 phosphorylation for complex formation with tubulin. Op18 was phosphorylated in vitro (P) on the indicated Ser residues by using combinations of Op18 mutants and specific protein kinases as described in Materials and Methods. The stoichiometries of the resulting Op18 phosphoisomers were quantitated by native PAGE and Western blotting with anti-Op18. The data presented are the percentages of each phosphoisomer present in the indicated Op18 preparations. Phosphorylated Op18 (1 μM) was mixed with tubulin (10 μM), and complex formation was revealed by the addition of the zero-length cross-linker EDC for the indicated times. After separation by SDS-PAGE, complexes were revealed by Western blotting with anti-Op18. The positions of Op18 phosphoisomers migrating to 19 and 23 kDa are indicated on the left. The positions of the 71-kDa (α/Op18) and 83-kDa (α/Op18*) complexes of α-tubulin–Op18 are also indicated. Molecular mass standards (in kilodaltons) are given on the right. Data are representative for five separate experiments with two independent preparations of *in vitro*-phosphorylated Op18.

marked decrease in complex formation, with Ser-63 phosphorylation having the strongest effect. Op18 phosphoisomers with single or dual phosphorylations on Ser-16 and Ser-63 in the absence of phosphorylation of both CDK target sites are not found in mitotic cells (21). Nevertheless, dual phosphorylation of Ser-16 and Ser-63 was sufficient to strongly inhibit complex formation with tubulin (Fig. 5). However, single-site phosphorylation of either Ser-16 or Ser-63 had only a minor effect (data not shown). The results in Fig. 5 suggest that phosphorylation of Op18 interferes with the Op18-tubulin interaction per se, a conclusion supported by the results of MT polymerization experiments described below, but do not exclude the possibility that Op18 phosphorylation interferes with the cross-linking process. However, cross-linking with glutaraldehyde reproduced the data obtained with the zero-length cross-linker EDC, rendering the latter possibility unlikely (data not shown).

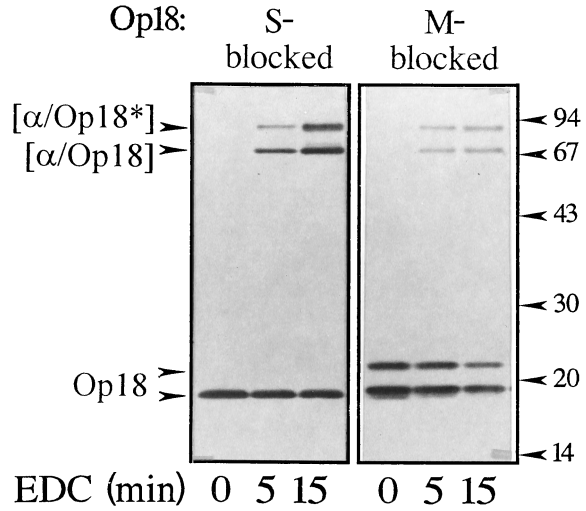

FIG. 6. In vitro complex formation of tubulin with Op18 from cell cycle-manipulated HeLa cells. Tubulin (10 μM) was mixed with partially purified Op18 (1 μM), derived from HeLa cells treated with thymidine (1 mM) (S-blocked) or nocadozole (0.5 μM) (M-blocked) for 16 h, and complex formation was analyzed as described in the legend to Fig. 5. The positions of Op18 migrating to 19 kDa and the tri (Ser-16, Ser-25, and Ser-38)- or tetraphosphorylated form of Op18 migrating to 23 kDa are indicated on the left. The positions of the 71-kDa (α/Op18) and 83-kDa (α/Op18*) complexes of α-tubulin–Op18 are also indicated. Molecular mass standards (in kilodaltons) are given on the right.

Taken together, our analysis of mitotic phosphoisomers of Op18 shows that dual phosphorylation of the two CDK target sites, Ser-25 and Ser-38, has only a modest effect compared to that of dual phosphorylation of Ser-16 and Ser-63. However, in combination with phosphorylation on either Ser-16 or Ser-63, phosphorylation of the CDK target sites strongly inhibits complex formation with tubulin. The modest effect of diphosphorylation on Ser-25 and Ser-38 is in line with the failure of Op18-S16,63A-expressing cells to form functional spindles, since Op18 was diphosphorylated on Ser-25 and Ser-38 in such cells (Fig. 2). Moreover, the regulatory importance of Op18 phosphorylation on Ser-16 and Ser-63, as revealed by the data shown in Fig. 5, is compatible with the severe phenotype of the Op18-S25,38A derivative, which is poorly phosphorylated on Ser-16 and Ser-63 (Fig. 2C).

The results in Fig. 5 show complex formation between essentially pure Op18 phosphoisomers and tubulin. However, Op18 derived from HeLa cells blocked in mitosis contains a mixture of phosphoisomers, as outlined in Fig. 2B and 3. It was

therefore of interest to analyze tubulin complex formation of the normal mixture of the Op18 phosphoisomers present in S- and M-phase-blocked HeLa cells. The results in Fig. 6 reveal that Op18 derived from HeLa cells generated both 71- and 83-kDa complexes with tubulin. For as-yet-unknown reasons, the relative intensity of the 83-kDa complex was stronger with Op18 from mammalian cells compared to that with *E. coli*-derived Op18. Most importantly, however, multisite-phosphorylated Op18 derived from mitotic cells generated substantially lower amounts of both 71- and 83-kDa complexes compared with those generated with Op18 derived from S-phase cells. It should be noted that about 25% of the Op18 phosphoisomer content in M-phase-blocked cells was diphosphorylated on Ser-25 and Ser-38 (21) (Fig. 2B), which had only a modest effect on Op18-tubulin complex formation (Fig. 5). It follows that approximately 25% of the Op18 content of M-phase cells should be only slightly inhibited in its tubulin association activity, which agrees with the residual complex-competent Op18 observed in Fig. 6.

Activities of Op18 phosphoisomers in an MT polymerization inhibition assay. As outlined above, the phosphorylation site specificity of regulation of Op18 activity, as evaluated by the ability to form complexes with tubulin heterodimers, is in line with the data obtained by transfecting cells with kinase target site-deficient Op18 mutants. The simplest interpretation of these results is that phosphorylation on Ser-16 and/or Ser-63 turns off the activity of Op18, whereas phosphorylation of Ser-25 and Ser-38 has only a modest effect on the level of Op18 activity but is critical to allow multisite phosphorylation on Ser-16 and Ser-63. It follows that Ser-25 and Ser-38 phosphorylation may exert its major effect indirectly through modulation of Ser-16 and Ser-63 phosphorylation. To analyze the regulation of Op18 on an additional level, we determined the activities of a panel of Op18 phosphoisomers during MT polymerization (Fig. 7A). The assay was performed in the presence of taxol, and the activity of Op18 is therefore likely to reflect inhibition of polymerization rather than destabilization of MTs. At about 3.5 μM, unphosphorylated Op18 completely inhibited MT polymerization, but dual phosphorylation on Ser-16 and Ser-63 neutralized the inhibitory activity of Op18 (Fig. 7B). The activity of each M-phase-specific phosphoisomer is shown in Fig. 7C. The data demonstrate that simultaneous phosphorylation of all four kinase target sites efficiently neutralized the inhibitory effect of Op18 on MT polymerization, whereas the effect of dual phosphorylation on the two CDK target sites was barely detectable. Moreover, in combination with phosphorylation of the two CDK target sites, phosphorylation on either Ser-16 or Ser-63 had a marked inhibitory effect on Op18 activity, with Ser-63 phosphorylation having the strongest effect. Consistent with the data for Op18-tubulin complex formation, single-site phosphorylation of either Ser-16 or Ser-63 had only minor inhibitory effects (data not shown). Hence, the activities of distinct Op18 phosphoisomers during MT polymerization are down-regulated by multisite phosphorylation during mitosis and the degree of down-regulation correlates with the ability of Op18 to form complexes with tubulin, as shown in Fig. 5. Since specific combinations of phosphorylated Ser residues have the same regulatory effects on both Op18 association with tubulin and Op18 activity during MT polymerization, the data suggest that phosphorylation regulates Op18 activity on the level of Op18-tubulin complex formation.

A

	Sites phosphorylated				Phosphoisomers
	S16	S25	S38	S63	in metaphase
Op18-wt	-	-	-	-	-
Op18-wt	-	P	P	-	+
Op18-wt	P	-	-	P	-
Op18-S63A	P	P	P	-	+
Op18-S16A	-	P	P	P	+
Op18-wt	P	P	P	P	+

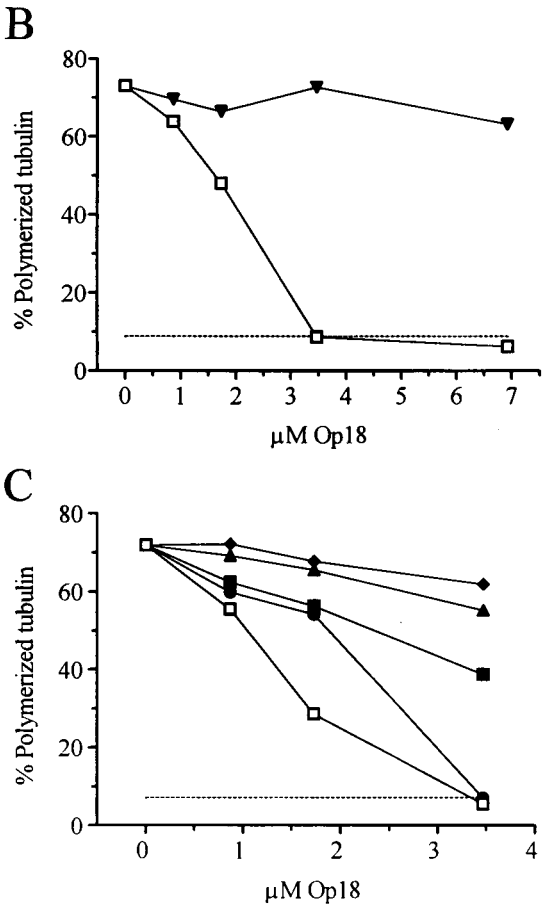

FIG. 7. Dissection of the regulatory role of Op18 phosphorylation on the level of MT polymerization. (A) Op18 derivatives were in vitro phosphorylated (P) on the indicated Ser residues as described in the legend Fig. 5. The phosphoisomers that can be detected in cells blocked in mitosis prior to metaphase are indicated (+). (B and C) Phosphoisomers of Op18 were analyzed for inhibitory activity during in vitro assembly of tubulin in the presence of taxol, as described in Materials and Methods. The symbol for each phosphoisomer is given in panel A. Dashed lines indicate the background levels of tubulin sedimented after incubation in ice. Data are representative for at least four separate experiments with two independent preparations of in vitro-phosphorylated Op18.

DISCUSSION

To understand the cellular function of Op18, it is important to understand the role of regulatory phosphorylations. Our previous report showed that a CDK target site-deficient mutant of Op18 blocked cells in mitosis prior to metaphase, and it was demonstrated that phosphorylation on the two CDK

sites, Ser-25 and Ser-38, is essential to allow formation of the mitotic spindle (26). Moreover, the data suggested that the CDK target site-deficient mutant of Op18 is constitutively active rather than dominant negative as previously proposed (27). However, Op18 is multisite phosphorylated on four Ser residues during mitosis and distinct kinase target sites are likely to differ in their regulatory roles. In the present study, we performed a detailed analysis of cells induced to express the Op18-S25,38A, Op18-S16,63A, and Op18-S16,25,38,63A mutant derivatives. The data suggest that all three Op18 mutants interfere with spindle formation by the same mechanism but that the different mutations mediate differences in the severity of the phenotype in the following order: Op18-S16,25,38,63A > Op18-S25,38A > Op18-S16,63A (Fig. 2A). Previous analysis of these mutants in terms of DNA profiles, frequency of M-phase-blocked cells, and endoreduplication after prolonged expression (more than 48 h) suggested that they interfere with cell division by the same mechanism (21). However, since MTs were not analyzed and the tested parameters did not differ among mutants, the differences in the severity of the phenotype were not previously detected.

In HeLa cells blocked prior to metaphase by nocodazole, endogenous Op18 was phosphorylated to completion on both Ser-25 and Ser-38 and to high stoichiometry on Ser-16 and Ser-63 (21) (Fig. 2B). Due to mitotic slippage, K562 tumor cells are only transiently blocked in mitosis by the interference of MTs, and it follows that only a fraction of all cells are blocked in M phase at a given time point (18, 26). With K562 cells, we routinely observed about 25 to 30% of M-phase cells after 24 h in the presence of either MT-depolymerizing drugs or ectopically expressed Op18 mutants (21, 26). Nevertheless, since only mitotic cells contain multisite-phosphorylated Op18 (5) (Fig. 2B), the M-phase-specific phosphoisomers of Op18 can still be readily resolved and analyzed in a mixed population of cells. An analysis of expressed Op18 mutants revealed striking differences in the stoichiometry and distribution of phosphoisomers that resulted from expressing distinct kinase target site-deficient Op18 mutants (Fig. 2C). As expected, mutations of all four available kinase target sites resulted in a nonphosphorylated protein; the expressed Op18-S16,63A protein contained the amount of Ser-25- and Ser-38-diphosphorylated phosphoisomers expected from the frequency of cells blocked by the mutant in M phase. Hence, it seems likely that dual phosphorylation on Ser-25 and Ser-38 is the result of the mitotic block caused by the Op18-S16,63A mutant. Interestingly, however, the Op18-S25,38A mutant also blocked cells in mitosis, but in this case, the recombinant protein contained low levels of dual phosphorylation on the two remaining sites, i.e., Ser-16 and Ser-63. These results suggest that phosphorylation of Ser-25 and Ser-38 is a prerequisite for efficient multisite phosphorylation on Ser-16 and Ser-63 (Fig. 3). Although Op18-S25,38A showed a more severe phenotype than that of Op18-S16,63A, it was still evident that substitutions for Ser-16 and Ser-63 are sufficient to block formation of a functional mitotic spindle (Fig. 2A). Since the Op18-S16,63A protein showed the expected level of dual phosphorylation on Ser-25 and Ser-38, these data demonstrate that phosphorylation on the two CDK target sites is not sufficient to allow spindle formation (i.e., to down-regulate the MT-destabilizing activity of Op18).

Taken together, the results derived from analysis of intact cells suggest that Ser-16 and Ser-63 phosphorylation is the main regulator of Op18 activity, whereas Ser-25 and Ser-38 phosphorylation has a modest effect on the level of Op18 activity but is critical for allowing multisite phosphorylation on Ser-16 and Ser-63. Hence, the regulatory phenotype of the Op18-S25,38A mutant may primarily be indirect, through modulation of the level of Ser-16 and Ser-63 phosphorylation. The morphology of the spindles in cells that express any of the kinase target site-deficient mutants of Op18 predicts that all four phosphorylated Ser residues are either directly or indirectly involved in switching off the MT-destabilizing activity of Op18 during mitosis (Fig. 2A). Belmont and Mitchison demonstrated that Op18 interacts with tubulin heterodimers, but not with MTs, and proposed that this interaction is of relevance for the catastrophe-inducing activity of Op18 observed during in vitro assembly of MTs (3). In this study, we functionally characterized each of the four Op18 phosphoisomers that have been identified in M-phase-blocked cells by two distinct biochemical approaches, namely, determination of tubulin binding and inhibition of MT polymerization. The results demonstrated a close correlation between these two assays of Op18 function, namely, the ability to bind tubulin correlates with the ability to inhibit MT polymerization (Fig. 5 and 7). Furthermore, this biochemical analysis confirmed that the function of phosphorylation is to down-regulate Op18 activity. Interestingly, biochemical analysis of Op18 function also fulfilled important predictions made from studies of intact cells concerning the functions of the individual phosphorylation sites. Hence, dual phosphorylation on Ser-16 and Ser-63 was essentially as efficient in down-regulation of Op18 activity as was phosphorylation on all four kinase target sites of Op18 (Fig. 5 and 7). Moreover, dual phosphorylation on Ser-25 and Ser-38 had only minor effects unless it was combined with phosphorylation on Ser-16 and/or Ser-63. Besides tubulin binding by Op18, there are at present no clues concerning the mechanism(s) by which Op18 regulates MT polymerization and dynamics. Thus, future mechanistic studies are required before the significance of phosphorylation-dependent regulation of Op18 activity can be fully understood.

By combining the results from genetic, morphological, and biochemical experiments of Op18 function, we have derived the model outlined in Fig. 3. In this model, dual phosphorylation on the CDK sites Ser-25 and Ser-38 is required for phosphorylation of Ser-16 and/or Ser-63; however, by themselves, the CDK sites are of only modest regulatory importance in direct regulation of Op18 activity. Subsequent phosphorylation on either Ser-16, Ser-63, or both efficiently switches off Op18 activity, which is required for formation of the mitotic spindle. With respect to the regulatory influence of Op18 activity by phosphorylation on distinct sites, this model has strong support from the experimental data. However, since the identity of the cell cycle-regulated Ser-16 and Ser-63 kinase is still unknown, we have not been able to directly investigate the dependency of Ser-16 and Ser-63 phosphorylation upon phosphorylation on Ser-25 and Ser-38 in in vitro experiments. Hence, there exist alternative explanations for our experimental results; e.g., Ser-25 and Ser-38 phosphorylation may regulate the phosphorylation status of Ser-16 and Ser-63 by modulating their sensitivity to phosphatases rather than protein kinases.

To extend the present study, it is essential to identify the kinase that phosphorylates Ser-16 and Ser-63 of Op18 during mitosis. The sequence surrounding Ser-16 (KRASGQA) and Ser-63 (RRKSHEA) lacks the Pro residues and other features that are essential for phosphorylation by CDKs, but both sites are good in vitro targets for PKA (25). There is recent evidence that PKA has an essential role during mitosis in the *Xenopus* embryological cell cycle (11). PKA has also been implicated in mitosis of the somatic cell cycle of mammalian cells. However, in the latter case, the results indicate that it is essential to down-regulate the activity of PKA during passage through mitosis (20). Thus, more studies are required before PKA can

be implicated in Ser-16 and Ser-63 phosphorylation of Op18 during mitosis.

A recent report by Horwitz et al. suggested negative regulation of Op18 activity by phosphorylation (16), which is in line with the results in our previous study (26). The strategy of Horwitz et al. was based on the potential mimic of phosphorylation provided by Asp substitutions at phosphorylated sites of a glutathione *S*-transferase–Op18 fusion protein. Their results revealed that the Ser-63-to-Asp substitution alone, but not Ser-25- and Ser-38-to-Asp substitutions, is sufficient to abolish the MT-destabilizing activity of the fusion protein observed in microinjected interphase cells. These results suggest that Ser-63 is of particular importance for negative regulation of Op18 activity by phosphorylation and are in line with the results in the present study. However, they observed a major discrepancy. Although site-specific neutralizing effects of Asp substitutions on Op18 activity were readily observed in microinjected cells, the same Asp substitutions did not alter the ability of the Op18 fusion protein to block MT assembly in vitro. To rationalize the results, they proposed that regulation of Op18 activity by phosphorylation requires additional cellular factors. However, the present study shows that phosphorylation can directly neutralize the in vitro activity of Op18 in systems composed of purified components; furthermore, the results obtained from in vitro assays were the same as predictions from observations made with intact cells. Hence, it appears that Asp substitutions at phosphorylation sites of a glutathione *S*-transferase–Op18 fusion protein only partially reproduces the effects of phosphorylation of the native Op18 protein.

The dynamics of MTs in mitotic cells is about 10-fold more rapid than that in interphase cells; this can be accounted for by an increase in the frequency of catastrophes (transitions from growing to shrinking) during mitosis (2). In a search for factors responsible for increased dynamics during mitosis, Op18 was identified by Belmont and Mitchison as a protein that increases the catastrophe rate of MTs during in vitro assembly and as a regulator of MT dynamics in *Xenopus* egg extracts (3). The identification of Op18 as a catastrophe factor is certainly in line with the observed spindle morphology in cells that expressed constitutively active mutants of Op18 (i.e., kinase target site-deficient mutants). Hence, as can be predicted by an increased catastrophe rate, the spindles appeared underdeveloped with symmetrical arrays of short MTs extending from spindle poles. To provide a model of how Op18 may specifically increase the frequency of catastrophes during mitosis, Belmont and Mitchison proposed that Op18 activity is increased by phosphorylation at the onset of mitosis (3). This model is clearly incompatible with the result of the present study, in which we have analyzed the activities of all phosphoisomers of Op18 that have been identified in M-phase cells. Hence, contrary to the prediction of their model, our data demonstrate that it is essential to switch off the activity of Op18 during mitosis and that the constitutive activities of all the kinase target site-deficient mutants of Op18 tested cause a cell cycle block prior to metaphase. Moreover, an analysis of a mixture of Op18 phosphoisomers derived from cells blocked in mitosis prior to metaphase showed the expected decrease in Op18 activity, as determined by competence to associate with tubulin heterodimers (Fig. 6). Finally, cells that overexpressed Op18-wt divided with normal spindle morphology, but their interphase MTs were almost completely depolymerized. Thus, it seems very unlikely that any phosphoisomer of Op18 has increased activity during a specific stage of mitosis, since it would be predicted to result in aberrant mitosis in cells that overexpressing the Op18-wt protein.

The present study demonstrates that phosphorylation-mediated down-regulation of Op18 activity is an essential event for proceeding through mitosis. However, the data suggest that Op18 activity is not completely turned off in cells blocked at M phase. A significant fraction of the endogenous Op18 in nocodazole-blocked cells are diphosphorylated on the CDK target sites (21) (Fig. 2B), which has been shown in this study to be insufficient to inactivate Op18 (Fig. 6 and 7). It would clearly be important to determine the phosphorylation status of Op18 at each specific stage of mitosis in the absence of drugs, but this is not feasible at present. However, an analysis of HeLa cells selected by mitotic shake off showed that the stoichiometry of Op18 phosphorylation in this population of mitotic cells was even higher than that anticipated from an analysis of nocodazole-selected M-phase cells. Hence, the abundance of diphosphorylated Op18 was very low relative to those of tri- and tetraphosphorylated phosphoisomers in mitotic cells selected in the absence of drugs (5). It is therefore possible that Op18 is completely switched off by multisite phosphorylation at a discrete stage(s) of mitosis. In any case, the phenotypes of Op18-wt and mutated derivatives, combined with biochemical data, clearly demonstrate that down-regulation of Op18 activity by phosphorylation is an essential event during formation of a mitotic spindle.

Since Op18 activity is switched off by phosphorylation during cell division, it seems unlikely that the main cellular function of Op18 is to regulate MT dynamics during mitosis. It seems more likely that the primary physiological role of Op18 is to regulate MT dynamics in response to external signals during the interphase of the cell cycle. If this is the case, it follows that Op18 phosphorylation by kinase systems, such as MAP kinase, PKA, and CaMK IV/Gr, should result in alterations of MT dynamics in response to cell surface receptor stimulation. We recently addressed this question by evaluating the consequences of CaMK IV/Gr-dependent phosphorylation of Op18 on the level of MT dynamics (29). The results showed that CaMK IV/Gr can suppress the MT-destabilizing activity of Op18 by phosphorylation, which results in increased levels of cellular MTs. These results provide evidence of a role for Op18 in coupling external signals to alterations of the interphase MT network. The neuronal SCG10 protein is highly homologous to Op18, with all of the phosphorylation sites conserved (31). As expected from sequence homology to Op18, this protein also inhibited MT assembly in vitro (32). Hence, a family of Op18-related proteins may be involved in regulation of the interphase MT network in response to phosphorylation signals. If this is the case, functional redundancy of related proteins may explain why mice with both alleles of the *Op18* gene disrupted by homologous recombination survived and showed normal development with no obvious defect during adult life (35).

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