

Regulation of Microtubule Dynamics by Extracellular Signals: cAMP-dependent Protein Kinase Switches Off the Activity of Oncoprotein 18 in Intact Cells

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Abstract. Oncoprotein 18 (Op18, also termed p19, 19K, metastasin, stathmin, and prosolin) is a recently identified regulator of microtubule (MT) dynamics. Op18 is a target for both cell cycle and cell surface receptor-coupled kinase systems, and phosphorylation of Op18 on specific combinations of sites has been shown to switch off its MT-destabilizing activity. Here we show that induced expression of the catalytic subunit of cAMP-dependent protein kinase (PKA) results in a dramatic increase in cellular MT polymer content concomitant with phosphorylation and partial degradation of Op18. That PKA may regulate the MT system by downregulation of Op18 activity was evaluated by a genetic system allowing conditional co-expression of

PKA and a series of kinase target site-deficient mutants of Op18. The results show that phosphorylation of Op18 on two specific sites, Ser-16 and Ser-63, is necessary and sufficient for PKA to switch off Op18 activity in intact cells. The regulatory importance of dual phosphorylation on Ser-16 and Ser-63 of Op18 was reproduced by *in vitro* assays. These results suggest a simple model where PKA phosphorylation downregulates the MT-destabilizing activity of Op18, which in turn promotes increased tubulin polymerization. Hence, the present study shows that Op18 has the potential to regulate the MT system in response to external signals such as cAMP-linked agonists.

MICROTUBULES (MTs)¹ are important for many cellular processes, for example in organelle transport, organizing the cytoplasm, and for intracellular movement of cell surface receptors (for review see Cole and Lippincott-Schwartz, 1995). During cell division, large arrays of MTs form the mitotic spindle, which segregates condensed chromosomes (for review see Hyman and Karsenti, 1996). The remarkable dynamic behavior of MTs, termed “dynamic instability,” can be described as stochastic transitions at MT ends between phases of growth and shrinkage, with transitions from shrinking to growing called “rescues,” and the reverse called “catastrophes” (for review see Desai and Mitchison, 1997).

The multitude of cellular functions involving MTs and their dynamic behavior makes regulation of MT assembly a central cell biological issue. Little is known, however,

about regulation of MT dynamics in response to external and internal cellular signals. Most studies on this topic have been concerned with microtubule-associated proteins (MAPs), a group of proteins that regulate MT dynamics by direct binding (for review see Mandelkow and Mandelkow, 1995). Many reports have described how phosphorylation of MAPs by various protein kinases, including the cAMP-dependent protein kinase (PKA), decreases their MT-binding activities. However, the significance of these findings for regulation of MT dynamics in intact cells remains to be established (for review see Maccioni and Cambiasso, 1995).

Studies on intact cells indicate the presence of MT-regulatory factors that oppose the MT-stabilizing activity of MAPs by promoting catastrophes and thereby increasing the dynamics of MTs (for review see McNally, 1996). Two such factors that increase the catastrophe rate have recently been identified, namely a kinesin-like protein, XKCM1 (Walczak et al., 1996) and a cytosolic protein, oncoprotein 18 (Op18; Belmont and Mitchison, 1996). Op18 has been given many names in the literature (e.g., p19, 19K, metastasin, prosolin, and stathmin) due to its complex pattern of phosphorylation and its elevated expression in a variety of human malignancies (for review see Belmont et al., 1996). By using a conditional expression

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1. *Abbreviations used in this paper:* MAP, mitogen-activated protein; MAPs, microtubule-associated proteins; MT, microtubule; Op18, oncoprotein 18; PKA, cAMP-dependent protein kinase; wt, wild-type.

system and kinase target site-deficient mutants of Op18, we have previously demonstrated that Op18 is a phosphorylation-responsive regulator of MT dynamics in intact cells; and furthermore, that multisite phosphorylation during mitosis downregulates the MT-destabilizing activity of Op18 (Marklund et al., 1996). These findings were extended in a subsequent study, where it was shown that mitosis-specific phosphoisomers of Op18 have severely reduced in vitro activities toward purified tubulin (Larsson et al., 1997). Taken together, the combined genetic, biochemical, and morphological data indicates that downregulation of Op18 activity is essential for formation of the mitotic spindle.

Studies in brain tissue and various cell lines have shown that Op18 is phosphorylated on four residues, namely Ser-16, Ser-25, Ser-38, and Ser-63 (Labdon et al., 1992; Beretta et al., 1993; Marklund et al., 1993a; Wang et al., 1993). All four phosphorylation sites are subject to regulation by external signals during the cell cycle. During mitosis, Op18 is phosphorylated on all four sites to high stoichiometry; Ser-25 and Ser-38 are phosphorylated by members of the cyclin-dependent kinase family, and Ser-16 and Ser-63 by an as yet unidentified protein kinase (Brattsand et al., 1994; Larsson et al., 1995). Op18 is also phosphorylated by several kinase systems in response to external signals. Thus, Op18 is phosphorylated on Ser-25 by members of the mitogen-activated protein (MAP) kinase family in response to phorbol esters, T cell antigen receptor stimulation, and nerve growth factor (Beretta et al., 1993; Marklund et al., 1993a,b). Op18 is also phosphorylated by Ca^{2+} /calmodulin-dependent kinase IV/Gr (CaMK IV/Gr) on Ser-16 in response to calcium signals in specific cells that express this kinase (Marklund et al., 1993a; Melander Gradin et al., 1997). Finally, cAMP-linked agonists have been shown to increase Op18 phosphorylation (Schubart et al., 1987), and subsequent studies have shown PKA-mediated in vitro phosphorylation of Op18 on both Ser-16 and Ser-63 (Beretta et al., 1993).

As outlined above, recent studies have shown that phosphorylation-mediated downregulation of Op18 activity is essential in human cell lines during formation of the mitotic spindle. These results imply that Op18 activity is of primary importance in regulating MT dynamics during the interphase of the cell cycle, and not during mitosis. It follows that kinase systems regulated by external signals, such as MAP kinase, PKA, and CaMK IV/Gr, may have important functions in regulating the MT-destabilizing activity of Op18. We have recently addressed this question by studying the consequences of CaMK IV/Gr-mediated phosphorylation of Op18. This study demonstrated that CaMK IV/Gr-mediated phosphorylation of Ser-16 suppresses Op18 activity in intact cells (Melander Gradin et al., 1997). However, the data did not exclude regulatory importance of constitutive phosphorylation on additional sites of Op18. Importance of additional sites appears likely, because subsequent in vitro studies in our laboratory have suggested that phosphorylation on multiple sites of Op18 synergize in downregulation of Op18 activity. PKA has the potential to phosphorylate two specific sites of Op18 and may, therefore, be a very efficient regulator of Op18 activity and consequently also of the MT system. The present study addresses this possibility by evaluating the MT-regulatory

role of all four potential phosphorylation sites of Op18 in response to PKA activity.

Materials and Methods

DNA Constructs and Transfections

The coding region of a cDNA encoding a c-myc epitope-tagged $\text{C}\alpha$ subunit of PKA (NcoI to ApaI fragment; Orellana and McKnight, 1992), was cloned into pMEP4 (Groger et al., 1989) as a BamHI to HindIII fragment after linking to the 5' untranslated region of Op18. The pMEP4-based Op18 derivatives and $\Delta\text{CaMK IV/Gr}$ used in this study has been described together with standard and co-transfection protocols for the K562 leukemia cell line (Marklund et al., 1994; Larsson et al., 1995; Melander Gradin et al., 1997). 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 (Groger et al., 1989). Conditional expression of various Op18 derivatives was achieved by using the hMTIIa promoter, which can be suppressed by low concentrations of EDTA (50 μM) and induced by Cd^{2+} (0.03–0.2 μM ; Marklund et al., 1994). Transfected cells were cultured in a medium containing EDTA (50 μM) that has been specifically designed to support cell growth under conditions that minimize expression from the hMTIIa promoter (Marklund et al., 1994). About 50–70% of all pMEP4-transfected cells surviving electroporation were resistant to hygromycin B (0.25 mg/ml; Boehringer Mannheim, Mannheim, Germany) and mock-transfected cells were killed within 3 d. Experiments were routinely performed 5–6 d after transfection.

Analysis of MT Polymerization Status, Op18 Phosphoisomers, SDS-PAGE, and Western Blot

Preparation of total cellular proteins and separation of proteins by 10–20% gradient SDS-PAGE has been described (Marklund et al., 1994). The cellular content of MT polymers was determined by extracting soluble tubulin in an MT-stabilizing buffer followed by quantification of tubulin in the particulate and soluble fraction as described (Minotti et al., 1991; Marklund et al., 1996). Affinity-purified anti-Op18, specific for the COOH-terminal (anti-Op18:34–149) or NH_2 -terminal (anti-Op18:2–33), was prepared and used for Western blot analysis as described (Brattsand et al., 1993). For detection of epitope-tagged proteins, an anti-c-myc antibody (AB-1, Oncogene Science Inc., Manhasset, NY) or the anti-Flag-M2 antibody (Kodak) was used. ^{125}I -protein A or the enhanced chemiluminescence detection system (Amersham Buchler GmbH, Braunschweig, Germany), were used to reveal bound antibodies, as indicated. PhosphorImager analysis of radioactive bands was used for quantification. As a control for equal loading, the relevant part of filters was routinely probed with rabbit anti-triose-phosphate isomerase (Brattsand et al., 1993). To analyze Op18 phosphoisomers, we used a native PAGE system that separates Op18 according to the charge differences introduced by each of the four identified phosphorylations (Marklund et al., 1993b).

In Vitro Phosphorylation of Op18, Cross-linking of Op18-Tubulin Complexes and Determination of the MT-destabilizing Activity of Op18

Wild-type (wt) and kinase target site-deficient mutant derivatives of purified *Escherichia coli*-derived Op18 were prepared as described (Brattsand et al., 1993). Purified Op18 was in vitro phosphorylated to high stoichiometry with PKA (New England BioLabs, Inc., Beverly, MA) by incubating 2.8 U of kinase/ μg of Op18 in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 and 500 μM ATP for 3 h at 30°C. Reactions were terminated by heating to 75°C for 10 min and precipitated overnight at -20°C with 6 vol of MeOH containing 1% sucrose. The precipitate was washed twice with 75% MeOH containing 1% sucrose, dried under vacuum, and resuspended in PEM buffer (80 mM Pipes, 1 mM EDTA, 1 mM Mg^{2+} , pH 6.8). To remove PKA that remained insoluble after MeOH precipitation, the final preparation of phosphorylated Op18 was clarified by centrifugation (15 min, 14,000 g). The concentration of the resulting phosphorylated Op18 was calculated using SDS-PAGE comparison with a standard recombinant Op18 preparation. As controls, in vitro phosphorylations were also performed either in the absence of ATP or by using mutants of Op18 lacking the specific phosphorylation sites. In these cases, tubulin binding and MT-

destabilizing activity of Op18 was unaffected. Therefore, the observed effects of Op18 phosphorylation could be attributed to addition of phosphate groups to specific Ser residues. Purified bovine tubulin was obtained from Cytoskeleton (Denver, Colorado). For cross-linking studies, Op18 (1 μ M) and tubulin (10 μ M) was incubated in 30 μ l of PEM buffer with 1 mM GTP. After 120 min on ice, 6 μ l of glutaraldehyde (0.3%) was added and the sample was incubated at 18°C. The reaction was quenched at various times by addition of 1 vol 2-mercaptoethanol (10%)/glycine (0.2 M), precipitated in 66% acetone, and the cross-linked Op18-tubulin complexes analyzed by SDS-PAGE. In vitro assembly of tubulin (4 μ M) in the presence of various amounts of Op18 was performed in assembly buffer (25 μ l of PEM buffer with 1 mM GTP, containing an additional 4 mM Mg²⁺, 10% glycerol and 4 μ g/ml taxol) as previously described (Larsson et al., 1997). Determination of tubulin and Op18 protein mass, by analysis of amino acid composition, was performed as described (Brattsand et al., 1993).

Immunofluorescence and Flow Cytometric Analysis

Cells were extracted with MT-stabilizing buffer (see above) containing 0.05% saponin and 10 μ g/ml RNase. Cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 15 min followed by quenching with NaBH₄, and thereafter stained with anti- α -tubulin (clone B-5-1-2, Sigma Chemical Co., Poole, UK). Bound antibodies were revealed by fluorescein conjugated rabbit anti-mouse Ig and DNA was stained with 0.1 μ g/ml propidium iodide. Cells were mounted using 1 mg/ml of *p*-phenylenediamine in PBS with 80% glycerol and analyzed by epifluorescence. MT fluorescence was also analyzed by flow cytometry as described (Roos et al., 1993).

Results

Regulation of MT Polymerization Status by Ectopic Expression of Op18, CaMK IV/Gr, and PKA

To investigate MT-regulatory properties of Op18 and two of its cognate protein kinases, we expressed epitope-tagged derivatives of Op18-wt, a constitutively active mutant of CaMK IV/GR (Δ CaMK IV/Gr), and the catalytic α subunit of PKA in K562 leukemia cells using the Cd²⁺-inducible pMEP4 shuttle vector system. Western blot analysis of lysates from transfected cells reveals induced expression of the indicated gene products within 4 h of Cd²⁺ addition (Fig. 1 A, note that the band migrating below endogenous Op18 is caused by proteolysis). Quantification of data from several experiments shows that Cd²⁺ treatment results in 5–20-fold induction of Op18 and 30–100-fold induction of Δ CaMK IV/Gr and PKA.

Previous studies have shown that CaMK IV/Gr phosphorylates Op18 on a single site, namely Ser-16, both in vitro and in intact cells. Previous in vitro experiments have shown that PKA phosphorylates Op18 on two sites, namely Ser-16 and Ser-63. To analyze Op18 phosphoisomer composition in cells induced to express either Δ CaMK IV/Gr or PKA, we used a native gel system (Marklund et al., 1993b). The data shows, in agreement with a previous report (Melander Gradin et al., 1997), that essentially all endogenous Op18 molecules are phosphorylated on a single site in cells induced to express Δ CaMK IV/Gr (Fig. 1 B). In agreement with the site preference of PKA in vitro (Bertetta et al., 1993), it is also shown that overexpression of this kinase results in phosphorylation of two sites of Op18.

We have recently shown that overexpression of Op18 causes destabilization of interphase MTs and that this activity of Op18 can be suppressed by phosphorylation (Marklund et al., 1996; Melander Gradin et al., 1997). The response to ectopic Op18 expression, or two of its cognate

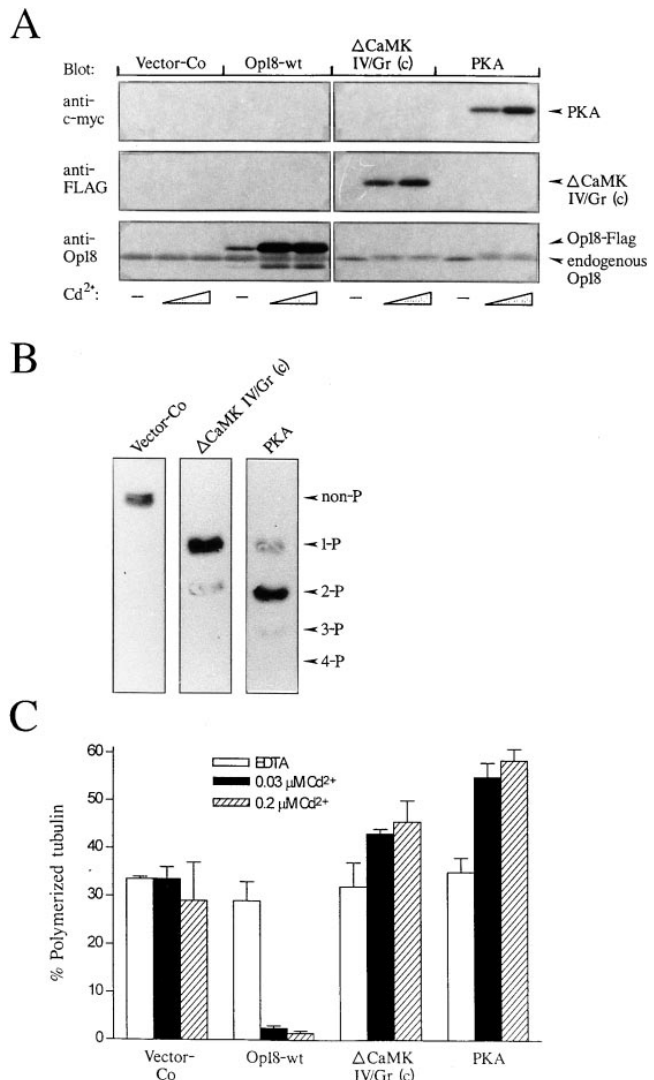


Figure 1. (A) Regulated expression of PKA, Δ CaMK IV/Gr, and Op18 from the hMTIIa-promoter. K562 cells were transfected with the indicated pMEP4 constructs (see text) and “stable” transfectants were selected by cultivation with hygromycin and EDTA for 5 d as described in Materials and Methods. Expression from the hMTIIa-promoter was either suppressed with EDTA or induced as indicated with Cd²⁺ (0.03 and 0.2 μ M) for 4 h. Cell lysates were resolved by SDS-PAGE, proteins transferred to nitrocellulose filter, and ectopic expression of c-myc epitope-tagged catalytic subunit of PKA and Flag epitope-tagged Δ CaMK IV/Gr was detected with the appropriate epitope-tag specific mouse monoclonal antibody and rabbit anti-mouse Ig. To visualize both endogenous Op18 and Flag epitope-tagged Op18, rabbit anti-Op18:2–33 was used. Bound antibodies were revealed by ¹²⁵I-protein A. (B) K562 cells expressing the indicated pMEP4 constructs were induced with Cd²⁺ (0.2 μ M) for 4 h. Op18 was partially purified from cell lysates and resolved by native PAGE. After transfer to nitrocellulose filter, Op18 phosphoisomers were detected by rabbit anti-Op18. The arrows indicate the migration of non-phosphorylated Op18, as well as migration forms of Op18 with various numbers of phosphate groups (1-P, 2-P, 3-P, and 4-P). The assignment of the arrows is based on the migration of recombinant Op18 with defined number of phosphorylated sites. (C) Cellular content of MT polymers was analyzed in K562 cells transfected with the indicated pMEP4 constructs. Expression from the hMTIIa-promoter was regulated as described for A.

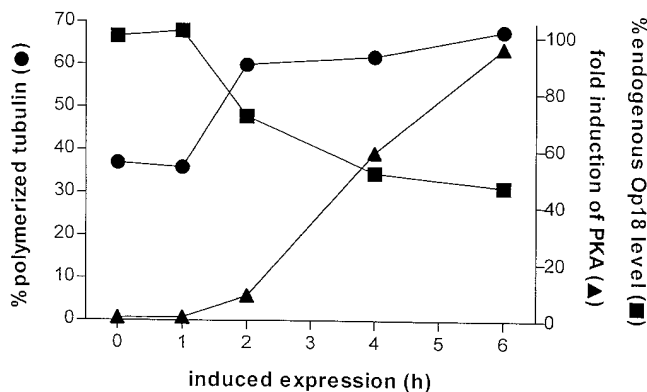


Figure 2. Time course of induced PKA expression increased cellular content of MT polymers and partial degradation of endogenous Op18. K562 cells were transfected with a pMEP4 derivative directing expression of the catalytic subunit of PKA, and drug-resistant cells were selected as in Fig. 1. Cells were thereafter induced with Cd^{2+} ($0.2 \mu\text{M}$) for the indicated period and total cellular proteins were resolved by SDS-PAGE. Ectopic expression of the protein kinase (\blacktriangle), the levels of endogenous Op18 protein (\blacksquare) and cellular content of MT polymers (\bullet), were quantified by Western blot using specific antibodies and ^{125}I -protein A as described under Materials and Methods. The Op18 content in non-induced cells was arbitrarily set to 100%. Calculation of Op18 content was internally controlled by probing the relevant part of the Western blot filter with anti-triose-phosphate isomerase, which does not vary under the experimental conditions used. The presented data is representative for three independent experiments.

kinases, on the level of cellular content of MT polymers is shown in Fig. 1 C. In agreement with our previous reports, ectopic expression of Op18 results in depolymerization of MTs, whereas expression of $\Delta\text{CaMK IV/Gr}$ results in increased MT polymers. Most importantly, it is also shown that expression of PKA increased cellular content of MT polymers with even higher efficiency than $\Delta\text{CaMK IV/Gr}$.

Induced expression of $\Delta\text{CaMK IV/Gr}$ has been shown to result in a partial ($\sim 50\%$) degradation of endogenous Op18. Both the phosphorylation of Op18 on Ser-16 and the associated partial degradation of Op18 are likely to contribute to the observed increase of cellular content of MT polymers in response to $\Delta\text{CaMK IV/Gr}$ expression (Melander Gradin et al., 1997). Fig. 2 shows the time course of Cd^{2+} -induced PKA expression and the consequences of induced expression on endogenous Op18 protein levels and cellular content of MT polymers. The data reveal that similarly to $\Delta\text{CaMK IV/Gr}$, PKA-mediated phosphorylation induces a partial degradation of Op18. Moreover, it is also evident that the cellular content of MT polymers increases in parallel with Op18 degradation. Cd^{2+} induces a 10-fold increase of PKA expression within 2 h. At this early time point, both downregulation of Op18 and increased MT polymers are readily observed.

PKA-mediated Phosphorylation Switches Off the MT-destabilizing Activity of Op18

To dissect the function of PKA-specific Op18 phosphorylation at distinct sites, we used a previously described co-transfection protocol (Melander Gradin et al., 1997). This

protocol allows regulated expression of two co-transfected gene products and efficient phosphorylation of ectopic Op18 by a cognate protein kinase. It is shown by Western blot analysis in Fig. 3 A that PKA can be efficiently co-expressed with either Op18-wt or Op18-S16,63A. In agreement with the data in Fig. 1 A, levels of endogenous Op18 are decreased after induced expression of PKA alone (note that phosphorylation results in a minor shift in migration). However, in contrast to the endogenous Op18, we do not detect a significant decrease in the levels of ectopic Op18 protein because of rapid-induced expression during the time course of the experiment. Hence, the present co-transfection protocol is useful to explore the effect of specific regulatory phosphorylations of Op18 on the MT-regulatory activity of Op18, with minimal interference on the level of protein degradation.

To determine if PKA phosphorylation regulates Op18 activity, we analyzed alterations in cellular content of MT polymers in co-transfected cells. As expected, Cd^{2+} treatment of cells transfected with vector-Co alone was without effect, whereas co-transfection of vector-Co with either Op18-wt or Op18-S16,63A results in Cd^{2+} -induced depolymerization of MTs (Fig. 3 B). Moreover, as predicted from the data shown above, co-transfection of vector-Co and PKA results in a Cd^{2+} -induced increase in MT polymers. Most significantly, co-transfected PKA completely suppresses the MT-depolymerizing activity of Op18-wt, but not of the Op18-S16,63A PKA target site-deficient mutant.

The simplest interpretation of the results outlined above is that PKA-mediated phosphorylation of Ser-16/Ser-63 switches off the MT-regulatory function(s) of Op18. However, the overexpressed and PKA phosphorylated Op18 protein may have some aberrant MT-regulatory properties that are not detected by analysis of total cellular content of MT polymers. Therefore, we analyzed the appearance of MTs in co-transfected cells by immunofluorescence. The analysis was performed on cells extracted with an MT-stabilizing buffer before fixation, and nuclear DNA was stained with propidium iodide. The immunofluorescence shown in Fig. 4 A demonstrates a characteristic interphase network of MTs in cells induced to express PKA either alone or together with Op18-wt. Moreover, cells induced to express any of the two Op18 derivatives without PKA, or Op18-S16,63A together with PKA, contained very few MTs. The spherical shape of the K562 leukemia cells used in this study is not optimal for morphological studies of MTs and it is not possible to analyze the length or number of individual MTs. Nevertheless, detailed examination of cells using high magnifications failed to reveal any morphological differences in the MT network displayed in cells expressing PKA alone or together with Op18-wt.

To quantify MT-specific immunofluorescence and to analyze heterogeneity within the cell population, we used flow cytometry. The resulting histograms show that expression of PKA alone results in a 1.8-fold homogeneous increase in mean fluorescence of the major cell population (Fig. 4 B, note log scale). It is also shown that induced expression of Op18 results in an almost 10-fold decrease in MT specific fluorescence. Most significantly, PKA restores the fluorescence in cells co-transfected with Op18-wt, but not Op18-S16,63A.

Taken together, the result in Fig. 4 suggests that PKA

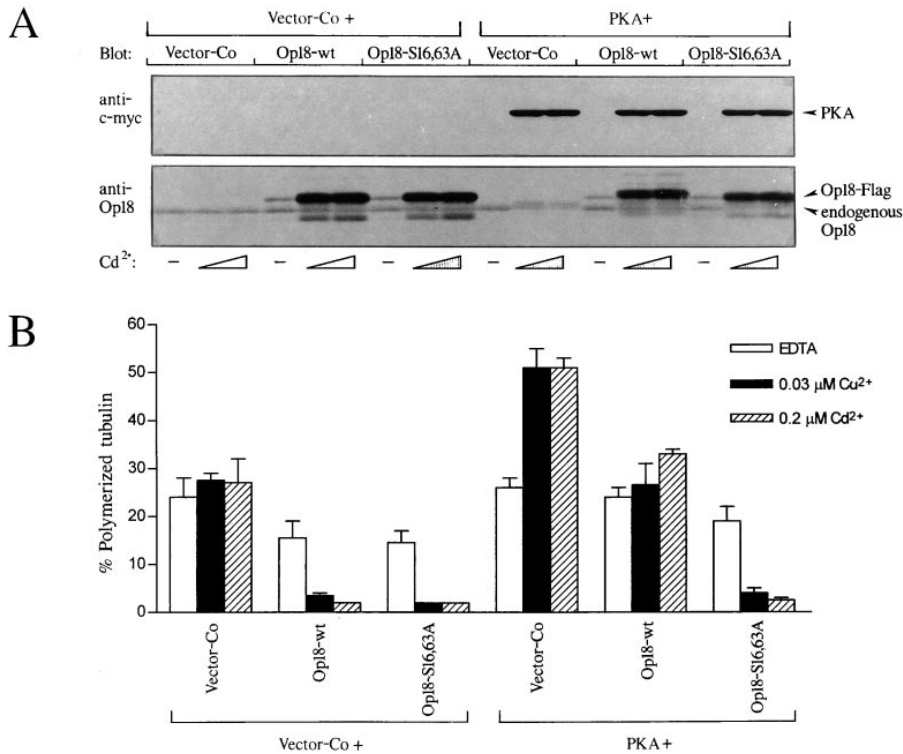


Figure 3. Regulatory effects on the MT system by conditional co-expression of PKA and Op18. K562 cells were co-transfected with the indicated pMEP4 derivatives (6 μg DNA of each derivative) and transfected cell lines were selected as in Fig. 1. Expression was either suppressed with EDTA (–) or induced with Cd²⁺ (0.03 or 0.2 μM) for 4 h. (A) Western blot analysis of cells co-transfected with the indicated pMEP4 constructs. The anti-c-myc antibody was used for detection of PKA, whereas Op18 was detected using rabbit anti-Op18:2–33. The minor shift in mobility of both endogenous and ectopic Op18 protein after induction of PKA is caused by phosphorylation. Equal loading of total cell lysate was confirmed by reprobing filters with anti-triose-phosphate isomerase (not shown). (B) Cellular content of MT polymers of the co-transfected cells treated as in A. The mean result of two independent determinations is shown.

expression induces a homogeneous increase of cellular content of MT polymers without gross alterations in MT morphology. Moreover, cells overexpressing Op18 in its PKA phosphorylated form show no detectable phenotype on the level of MT morphology. These results are compatible with a simple model where PKA promotes increased tubulin polymerization by phosphorylation of Op18, which switches off the MT-destabilizing activity of Op18.

The Role of Individual Phosphorylation Sites for PKA-mediated Modulation of Op18 Activity

Previous studies have shown that both Ser-16 and Ser-63 are phosphorylated by PKA in vitro. This is in line with the appearance of di-phosphorylated endogenous Op18 in transfected cells induced to express a catalytic subunit of PKA (Fig. 1 B). An earlier study has shown that overexpression of Op18 for 24 h results in hyper-phosphorylation of Op18, mostly on Ser-38 (Marklund et al., 1996). This phenomenon is most likely caused by Op18-induced MT depolymerization, because it has been shown that drug-induced depolymerization results in activation of a MAP kinase-related kinase (Shinohara-Gotoh et al., 1991). In the present study, we induced expression for a short time period (4–5 h). This results in a more modest phosphorylation level of the overexpressed Op18-wt protein, as compared to 24 h of induced expression, with ~20% of all Op18 phosphorylated on a single site (Fig. 5). However, by inducing expression of Op18-wt in the presence of PKA, ~85% of all cellular Op18 is phosphorylated on one or more sites (Fig. 5). Hence, unlike the situation of endogenous Op18 in cells induced to express PKA alone (Fig. 1), it is evident that not all ectopic Op18 molecules are phosphorylated on two sites in cells that also overexpress PKA.

To analyze the site-specificity of PKA-mediated phosphorylation of Op18, we determined the phosphorylation status of a panel of phosphorylation site-deficient Op18 mutants co-expressed with either vector-Co or PKA (Fig. 5). Op18 can be phosphorylated on four specific sites in K562 cells (Larsson et al., 1995). The Op18-S16,25,38,63A derivative has all of these four sites mutated and the expressed protein is, as expected, essentially unphosphorylated if expressed together with PKA. It should be noted that Op18 derivatives without epitope-tag were expressed in this experiment, and the low level of phosphoisomers observed for Op18-S16,25,38,63A is due to the endogenous gene product. From the analysis shown in Fig. 5, it is evident that Ser-63 is the predominant site phosphorylated by PKA, because almost 75% of the Op18-S16,25,38A mutant protein is phosphorylated on a single site. In contrast, only ~30% of the Op18-S25,38,63A mutant protein is phosphorylated on a single site.

To dissect the functional role of specific phosphorylation sites during PKA-mediated suppression of Op18 activity, we determined cellular content of MT polymers in cells co-transfected with PKA and the panel of kinase target site-deficient mutants of Op18 presented in Fig. 5. The results in Fig. 6 show that phosphorylation of Ser-16/Ser-63 is both sufficient and necessary for efficient PKA-mediated suppression of Op18 activity (compare Op18-wt and Op18-S25,38A). Single-site phosphorylation on Ser-63 only results in a partial suppression of Op18 activity and single-site phosphorylation of Ser-16 has even less effect (compare Op18-S25,38,63A with Op18-S16,25,38A). Finally, and as expected, PKA has no effect on the MT-destabilizing activity of the Op18-S16,25,38,63A mutant protein.

In interpreting the result shown in Fig. 6, the stoichiometry of phosphorylation shown in Fig. 5 should be consid-

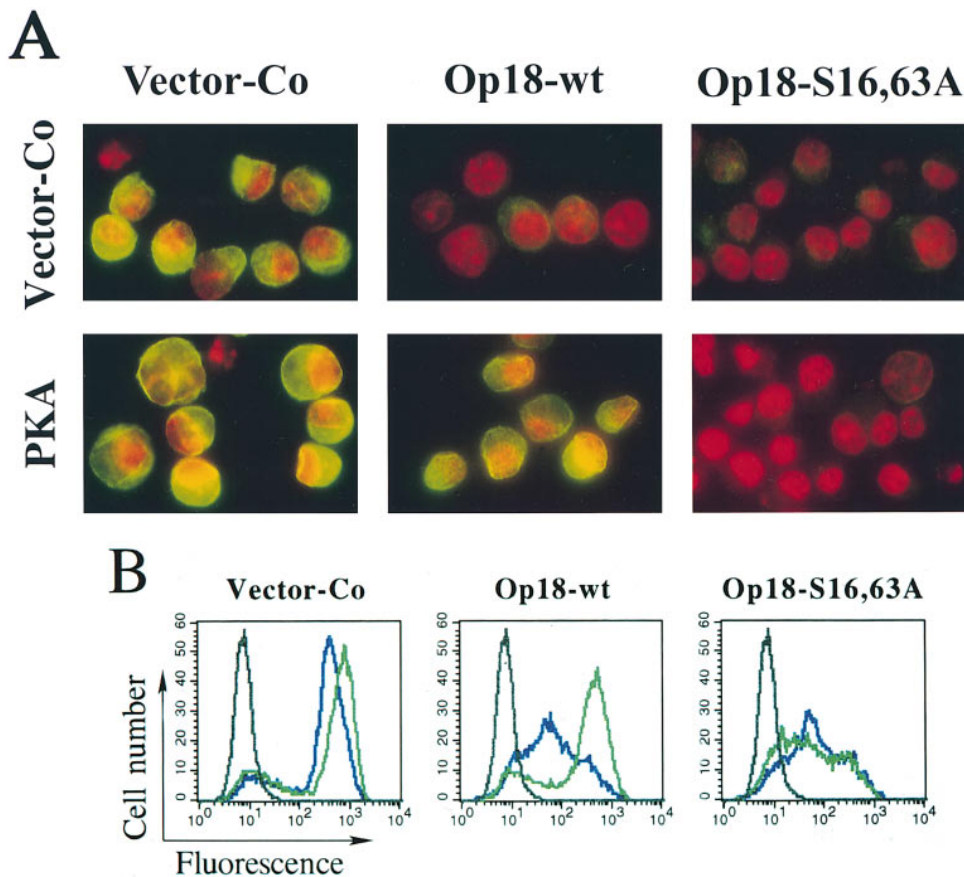


Figure 4. Immunodetection of MTs in co-transfected cells. K562 cells co-transfected with the indicated pMEP4 based constructs were induced with Cd^{2+} (0.2 μM) for 4 h. Cells were thereafter extracted with MT-stabilizing buffer, fixed and double stained with anti- α -tubulin (green) and propidium iodide-DNA (red). (A) MTs and DNA staining of representative interphase cells analyzed by epifluorescence. (B) Flow cytometric analysis of cellular MT content of cells shown in A. Black lines show control staining in the absence of anti- α -tubulin but in the presence of fluorescein conjugated rabbit anti-mouse immunoglobulin. Blue lines depict α -tubulin-specific fluorescence of cells co-transfected with vector-Co and the pMEP4 constructs indicated above each panel. Green lines depict α -tubulin-specific fluorescence of cells co-transfected with PKA and the pMEP4 constructs indicated above each panel.

ered. Thus, only $\sim 30\%$ of the overexpressed Op18-S25, 38,63A protein is phosphorylated on its remaining site, Ser-16. Nevertheless, the results suggest that dual phosphorylation on Ser-16 and Ser-63 is of importance for efficient PKA-mediated suppression of Op18 activity in intact cells.

Kinetics of Dual Phosphorylation by PKA

PKA phosphorylates both Ser-16 and Ser-63 of Op18 in intact cells, and both mono- and di-phosphorylated Op18 are generated (Fig. 5). To evaluate potential cooperativity during PKA-mediated phosphorylation of Ser-16 and Ser-63, the time course for generation of multi-phosphorylated Op18 species in the presence of purified PKA was determined (Fig. 7 A). The data reveals transient accumulation of mono-phosphorylated Op18 and subsequent accumulation of di-phosphorylated Op18, until a time point where Op18 is predominantly phosphorylated on two sites. Only low levels of tri-phosphorylated Op18 are generated, which indicates that Ser-16 and Ser-63 are the sole physiological PKA target sites of Op18. Because $>50\%$ of the available Op18 substrate is mono-phosphorylated at early time points, before significant amounts of di-phosphorylated Op18 are present, the data shows that Ser-16 and Ser-63 are not simultaneously phosphorylated by PKA.

To further address if Ser-16 and Ser-63 of Op18 are independently phosphorylated by PKA, the rate of in vitro phosphorylation of the Op18-S63A and Op18-S16A mutants was compared to that of Op18-wt. The results show

that Ser-63 is phosphorylated three- to fourfold faster than Ser-16 (Fig. 7 B). Because the sum of the rates of phosphorylation on each site ($21 + 73 = 94$ pmol PO_4 /min) is close to the phosphorylation rate observed using the wild-type Op18 substrate (96 pmol PO_4 /min), the results suggest that Ser-16 and Ser-63 are independently phosphorylated by PKA. This, together with the site preference of PKA in vitro, is in line with the distribution of Op18 phosphoisomers observed in cells co-transfected with PKA and a panel of kinase target site-deficient mutants (Fig. 5). Finally, native gel analysis shows that extensive PKA-mediated phosphorylation of an Op18-wt substrate, or the indicated mutated substrates, results in essentially homogeneous preparations of specific Op18 phosphoisomers (Fig. 7 B).

Dissection of the Regulatory Role of Ser-16 and Ser-63 Phosphorylation for Op18 Activities towards Purified Tubulin

Op18 has been shown to bind to tubulin, an interaction that is likely to be of functional importance (Belmont and Mitchison, 1996). To analyze the effect of Op18 phosphorylation on tubulin binding, we used glutaraldehyde to preserve Op18-tubulin complexes. As shown by SDS-PAGE separation in Fig. 8 A, addition of glutaraldehyde preserves an 83-kD complex that is recognized by antibodies against Op18. As expected, this complex is also recognized by anti- α -tubulin (data not shown). We have previously reported that cross-linking with the zero-length cross-linker 1-ethyl-3-(3-[dimethylamino]propyl)carbodiimide, re-

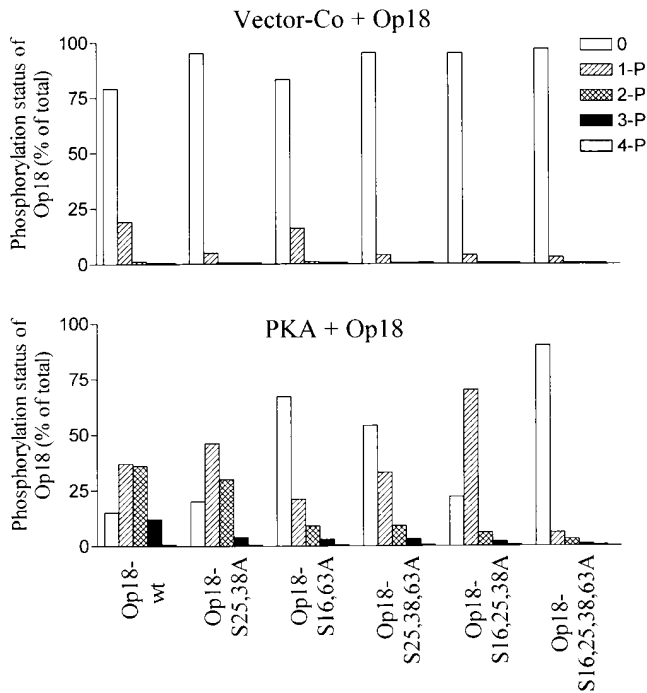


Figure 5. Distribution of Op18 phosphoisomers in cells induced to express a panel of kinase target site-deficient mutants of Op18 together with PKA. K562 cells co-transfected with either vector Co (*top*) or PKA (*bottom*) and the indicated pMEP4 derivatives of Op18 were induced with Cd²⁺ (0.2 μ M) for 4 h. Phosphoisomers of Op18 were analyzed by a native PAGE system as in Fig. 1 and phosphorimager analysis of radioactive bands was used for quantification. It should be noted that the Op18 derivatives used in this experiment do not contain the Flag epitope-tag. The present co-transfection protocol results in Op18 derivatives expressed at 5–10-fold higher levels than the endogenous Op18 protein. Hence, the data primarily reflects the phosphorylation status of the recombinant Op18 gene product, but phosphorylation of endogenous Op18 is still notable.

sults in two distinct Op18–tubulin complexes migrating at 71 and 83 kD (Larsson et al., 1997). As shown in Fig. 8, cross-linking with glutaraldehyde reproduces the 83-kD but not the 71-kD complex. However, both protocols seem to generate predominantly Op18 cross-linked to α -tubulin, because the complexes react poorly with two different anti- β -tubulin antibodies (Larsson et al., 1997; and data not shown). It should be noted that cross-linking of Op18 to tubulin seems to be a very specific process, as non-related proteins do not interfere with the process, and Op18 is not cross-linked to tubulin that has been made polymerization incompetent by 5 min at 60°C (data not shown).

For functional dissection of individual phosphorylation sites, Op18-S63A, Op18-S16A, and Op18-wt substrates were phosphorylated to essentially complete stoichiometry by PKA (see native gel shown in Fig. 7 B). As shown in Fig. 8 A, single-site phosphorylation on either Ser-16 or Ser-63 results in a partial reduction of the Op18–tubulin complex (\sim 50%) as compared to the nonphosphorylated Op18 protein. Moreover, and in agreement with our previous study (Larsson et al., 1997), phosphorylation on both sites essentially abolished complex formation. Similar effects of mono- and diphosphorylation of Op18 on Ser-16/

Ser-63 were observed by using the 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide cross-linker (data not shown). Hence, single-site phosphorylation on either Ser-16 or Ser-63 have significant functional consequences on Op18–tubulin complex formation but the effect of di-phosphorylation is clearly synergistic.

We also determined functional consequences of Ser-16 and Ser-63 phosphorylation of Op18 during MT polymerization. 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. As shown in Fig. 8 B, unphosphorylated Op18 completely inhibits MT polymerization at \sim 4 μ M concentration, whereas dual phosphorylation on Ser-16 and Ser-63 neutralizes the inhibitory activity of Op18. The effect of single-site phosphorylation of either Ser-16 or Ser-63 alone was small and barely detectable in this assay system. Taken together, dual phosphorylation on Ser-16 and Ser-63 blocks both tubulin complex formation and the inhibitory activity during MT polymerization, whereas the effect of single-site phosphorylation could only be detected with certainty by analyzing tubulin complex formation.

Discussion

By using a conditional expression strategy, we show that the catalytic subunit of PKA mediates a rapid and dramatic increase in cellular MT-polymer content in parallel with dual phosphorylation and partial degradation of Op18. PKA is a multi-functional kinase with a multitude of cytosolic and nuclear protein substrates. To evaluate a potential role of Op18 phosphorylation for PKA regulation of the MT system, a panel of kinase-target-deficient mutants of Op18 were co-transfected with PKA. The result demonstrated that PKA switches off the MT-destabilizing activity of Op18 in intact cells by dual phosphorylation of Ser-16/Ser-63. In vitro studies confirmed the importance of dual phosphorylation of these two sites in switching off Op18 activity. Hence, the model system used in this study demonstrated the potential of PKA to regulate the MT system via Op18 phosphorylation.

Immunofluorescence analyses suggest that PKA expression induces a homogeneous increase of cellular content of MT polymers without gross alterations in MT morphology. Moreover, cells overexpressing Op18-wt together with PKA show no detectable phenotype on the level of MT morphology (Fig. 4). Hence, it appears that Op18 in its PKA-phosphorylated form is devoid of all of its MT-regulatory properties. These results are compatible with a simple model where PKA promotes increased tubulin polymerization by phosphorylation of Op18, which in turn directly switches off the MT-destabilizing activity of Op18.

This study demonstrates that dual phosphorylation of Ser-16 and Ser-63 inactivates both tubulin-binding activity and MT polymerization inhibitory activity of Op18. This may appear contradictory to a recent study (Horwitz et al., 1997), in which the potential mimic of phosphorylation provided by Asp substitutions at phosphorylated sites was analyzed. In this study the authors concluded that specific Asp substitutions decreased the MT-destabilizing activity of a panel of glutathione S-transferase–Op18 fusion protein derivatives as analyzed by micro-injection of a cell line.

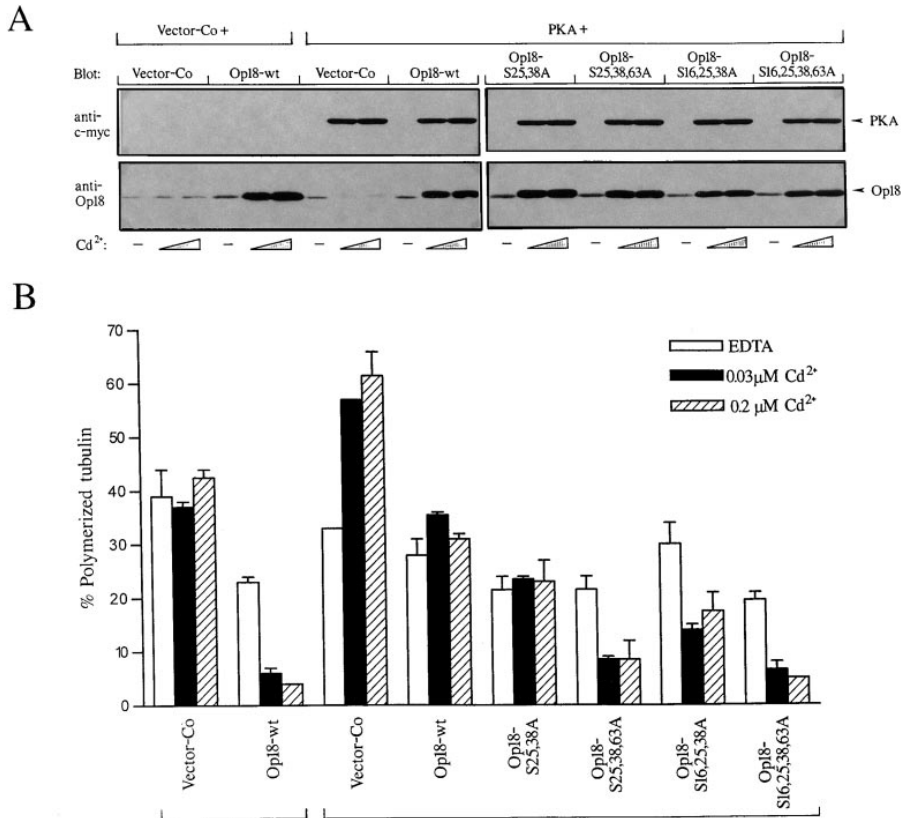


Figure 6. Dual phosphorylation of Ser-16 and Ser-63 is both sufficient and necessary for efficient PKA-mediated modulation of the MT-destabilizing activity of Op18. K562 cells were co-transfected with the indicated pMEP4 derivatives (6 μg DNA of each derivative). Expression was either suppressed with EDTA (-) or induced with Cd²⁺ (0.03 or 0.2 μM) for 4 h. (A) Western blot analysis of cells co-transfected with the indicated pMEP4 constructs. The anti-c-myc antibody was used for detection of PKA and Op18 was detected by rabbit anti-Op18:34–149. The Op18 derivatives used in this experiment do not contain the Flag epitope tag and, consequently, co-migrates with endogenous Op18. (B) Cellular content of MT polymers of the co-transfected cells treated as in A. The mean result of two independent determinations is shown.

However, *in vitro* assays failed to reveal any effect by Asp substitutions on the MT polymerization inhibitory activity of the Op18 fusion proteins. To explain these results, the authors proposed that cellular factors are required to reveal the inhibitory effect of Op18 phosphorylations. Because our studies show that phosphorylation on specific sites of the native Op18 protein clearly inhibits its *in vitro* activity, it appears that Asp substitutions at phosphorylation sites of a fusion protein only partially mimic the regulatory effects of phosphorylation.

As outlined in the introduction section, Op18 has been independently identified by several groups. In at least three cases, Op18 was initially identified as a major phosphorylation event in response to increased cAMP levels, induced by either specific hormones or drugs (Brattin and Portanova, 1981; Schubart, 1982; Sobel and Tashjian, 1983). This together with *in vitro* phosphorylation studies, and site-specific phosphorylation of Op18 in response to ectopic PKA expression shown herein, suggest that Op18 is a physiological substrate for PKA. However, Op18 is not phosphorylated in response to PKA activation in all cell types investigated. Hence, published results suggest that, whereas Op18 is clearly a likely major cytosolic substrate for PKA in cell types of neural origin, Op18 phosphorylation is not significantly increased in response to cAMP-linked agonists in hematopoietic/lymphoid cell types (Mary et al., 1989; Strahler et al., 1992). The reason for these differences is not clear, but it seems possible that PKA activity is less abundant in some cell types, and/or that most of the catalytic subunits are translocated to the nucleus upon activation. Nevertheless, Op18 is clearly efficiently phosphorylated in response to cAMP signaling in many cell

types, which the present study predicts has implications for regulation of the MT system.

PKA has previously been implicated in the regulation of the MT systems. For example, the two neuronal MAPs, MAP2 and tau, are phosphorylated on the same sites in intact cells in response to cAMP-linked agonists as they are by PKA *in vitro* (Jefferson and Schulman, 1991; Fleming and Johnson, 1995). It is generally believed that different signal transduction cascades may control MT dynamics by phosphorylation of MAPs. In functional studies, phosphorylation has been shown to inhibit to various extents the MT-stabilizing *in vitro* activity of MAPs (Jameson and Caplow, 1981; Lindwall and Cole, 1984; Drechsel et al., 1992; Hoshi et al., 1992; Masson and Kreis, 1995; Ookata et al., 1995; Trinczek et al., 1995). Hence, from these studies it can be predicted that phosphorylation of MAPs in intact cells should result in clearly visible disruption of MTs. Although many studies have been concerned with phosphorylation of MAPs by PKA, there is to our knowledge no study demonstrating PKA-mediated disruption of MTs in intact cells.

If, as outlined above, PKA modulates the MT system by phosphorylation of MAPs, previous studies predict that activation of the kinase should destabilize MTs. Using a conditional overexpression strategy, the present study shows the opposite result, namely, that PKA activity mediates an increase in cellular MT polymer content. Moreover, PKA can efficiently switch off the potent MT-destabilizing activity of Op18 in intact cells, provided that the Ser-16 and Ser-63 Op18 target sites are intact, suggesting an Op18-dependent mechanism. Another indication that PKA regulation of MTs is primarily mediated by Op18 in K562

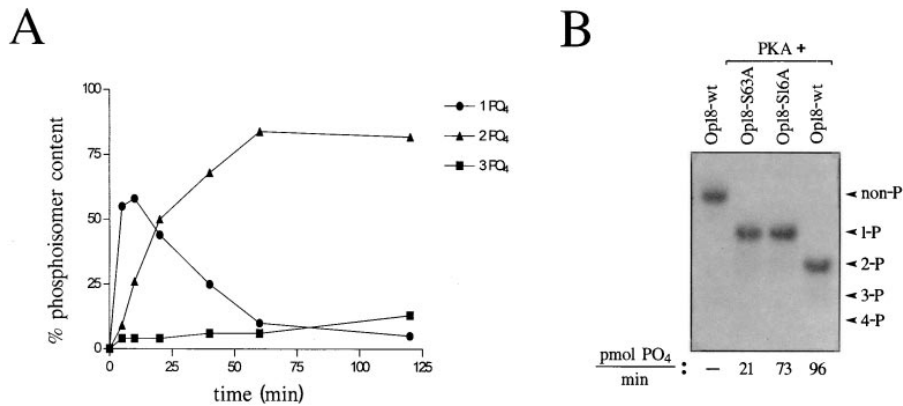


Figure 7. PKA-mediated in vitro phosphorylation of Op18. (A) Op18-wt was mixed with PKA (2.8 U of kinase per μg Op18) using the conditions described in Materials and Methods. Specific Op18 phosphoisomers were thereafter quantitated at the indicated time points by separation on a native gel system. Mono- (●), di- (▲), and tri- (■) phosphorylated Op18 isomers are shown. (B) Op18-wt and the indicated kinase target site-deficient mutants of Op18 were in vitro phosphorylated by PKA as above. Op18 phosphoisomers were thereafter analyzed by native gels together with nonphosphorylated

Op18. After transfer to nitrocellulose filter, Op18 phosphoisomers were revealed by rabbit anti-Op18 and ^{125}I -protein A followed by autoradiography. The rate of PO_4 incorporation of the indicated Op18 derivatives is presented below the autoradiograph.

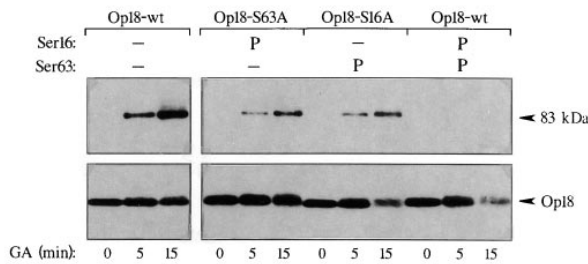
cells comes from the observation that specific Op18 mutants can completely abolish the MT-stabilizing effect of PKA. If PKA activation would mediate increased MT polymers primarily by activating MT-stabilizing proteins (e.g., MAPs), it would be anticipated that the resulting MTs would show increased stability, and therefore be partially resistant to the destabilizing activity of specific mutants of Op18. Co-transfection experiments show that this is not the case because PKA expression is without any effect on the MT system in the presence of the Op18-mutants with Ser-16 and Ser-63 to Ala substitutions (Figs. 3 and 6). However, although Op18 appears to be a major regulator of the MT system in the K562 erythroleukemia cell line, this does not need to be the case in all tissues/cell lines. It is also possible that other phosphorylation responsive proteins, functionally related to Op18, are involved in regulation of the interphase MT network. If this is the case, redundancy may explain why mice with both alleles of the Op18 gene disrupted by homologous recombination survive and show normal development (Schubart et al., 1996).

The MT system seems, in general, less intimately associated with second messenger systems than is the actin system, and little is known about its regulation in response to external signals (Mitchison, 1992). However, the present and a previous study by us show that the recently identified MT-regulating protein, Op18, links the MT system and at least two second messengers, namely cAMP and Ca^{2+} . In our previous study, a constitutively active mutant of CaMK IV/Gr was shown to suppress the MT-destabilizing activity of Op18-wt by phosphorylating Ser-16 (Melander Gradin et al., 1997). However, the importance of constitutive phosphorylations on additional sites for suppression of Op18 activity was not addressed. Thus, overexpressed Op18 shows significant levels of constitutive phosphorylation on Ser-38, which is likely to have a synergistic effect together with CaMK IV/Gr-mediated Ser-16 phosphorylation (Larsson et al., 1997). In the present study, the MT-regulatory role of all four potential phosphorylation sites of Op18 was determined in response to induced expression of PKA. The result shows that PKA phosphorylates both Ser-16 and Ser-63 in intact cells, with preference for the latter site. Moreover, it was also demonstrated that

PKA-mediated dual phosphorylation of Op18 on these two Ser residues is both necessary and sufficient for efficient regulation of the MT system. As predicted from the dual specificity of PKA, comparison in the same experiment reveals that PKA is more potent than CaMK IV/Gr in inducing increase in cellular content of MT polymers (Fig. 1). Furthermore, the importance of dual phosphorylation of Op18 was also evident in co-transfection experiments in which Op18 was overexpressed. Hence, although CaMK IV/Gr mediates complete phosphorylation on Ser-16 of overexpressed Op18, the resulting MT-destabilizing activity of Op18-wt was not as efficiently suppressed as observed in this study by co-transfection of PKA (Fig. 3). This is despite the observation that ectopic PKA did not mediate complete phosphorylation of its two target sites of Op18, as indicated by quantification of Op18 phosphoisomers (Fig 5). Because the kinase activity of ectopic PKA was not sufficient to dual phosphorylate overexpressed Op18 to completion, the data does not provide any information on the relative MT-regulatory importance of Ser-16 versus Ser-63 in intact cells (Figs. 5 and 6). However, in vitro experiments suggested similar effects of phosphorylation of either of the two Ser residues and a clear-cut synergistic effect of phosphorylating both sites (Fig. 8).

The effects of phosphatase/kinase inhibitors on MT dynamic instability was recently investigated in living newt lung epithelial cells (Howell et al., 1997). Significant and rapid (within 1 min) alterations of the dynamic behavior of individual MTs was observed in response to all inhibitors tested. The authors proposed that both MAPs and Op18 are likely mediators of the MT-regulatory response to drug-induced alterations of protein phosphorylation. Moreover, a previous report has also shown that phorbol ester treatment of macrophages results in a rapid (detectable within minutes) increase in MT length and number (Robinson and Vandre, 1995). Phorbol esters are specific activators of protein kinase C, which is an upstream activator of the MAP kinase in leukocytes (Cantrell, 1994). Because Op18 is a major cytosolic substrate of the MAP kinase (Marklund et al., 1993b), it seems possible that the reported effect is at least in part due to phosphorylation-mediated inactivation of Op18. In addition, studies on murine embryological fibroblasts have shown that stimulation with thrombin,

A



B

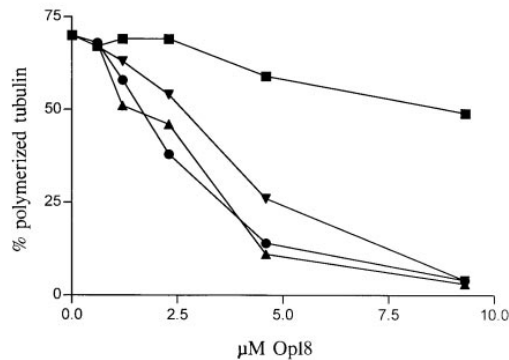


Figure 8. Functional consequences of Ser-16 and Ser-63 phosphorylation for Op18 activities towards purified tubulin. (A) Analysis of Op18-tubulin complexes. Op18 was phosphorylated by PKA to high stoichiometry on the indicated Ser-residues. Op18-wt or the indicated kinase target site-deficient Op18 mutants were used as substrates. Phosphorylated Op18 (1 μ M) was mixed with tubulin (10 μ M) and complexes were preserved by addition of glutaraldehyde (0.05% final concentration) for the indicated time. After separation on SDS-PAGE, complexes were revealed by Western blotting with anti-Op18:33-149. The position of the 83-kD complexes of Op18-tubulin is indicated. (B) Analysis of MT polymerization. Phosphoisomers of Op18 were analyzed for inhibitory activity during in vitro assembly of tubulin in the presence of taxol. Nonphosphorylated (●), Ser-16 phosphorylated (▲), Ser-63 phosphorylated (▼), and Ser-16/Ser-63 phosphorylated (■) Op18 is shown. Data in A and B are representative for at least four separate experiments using three independent preparations of in vitro phosphorylated Op18.

epidermal growth factor, and phorbol esters increases the cellular content of MT polymers (Ball et al., 1992). Because Op18 is likely to be phosphorylated in response to all of these three mitogens, it seems possible also in these cases that inactivation of Op18 contributes to increased MT polymers.

One potential mechanism for increased cellular content of MT polymers in response to external signals may be stimulation of tubulin synthesis. In the studies on macrophages and fibroblasts, mentioned above, changes in the total pool of tubulin were not quantified. However, because both studies reported significant responses within an hour after stimulation, it seems unlikely that an increase in the total pool of tubulin is responsible for the observed increase of cellular MT polymers. In the present study, alterations of MT polymers in response to overexpressed PKA, which was detected within 2 h (Fig. 2), was determined by

quantification of both polymerized and soluble tubulin. Over a time course of 12 h we did not observe any detectable alteration in the total pool of tubulin by overexpressing PKA or Op18 (data not shown). Hence, in the present study, and most likely in the two studies mentioned above, external signals appear to induce an early increase in MT polymers in the absence of alterations of the tubulin pool size. In a longer time perspective, however, the rate of tubulin synthesis is most likely changed because the level of tubulin heterodimers is regulated by an autoregulatory mechanism, that is related to the pool size of unpolymerized tubulin heterodimers (Cleveland et al., 1981; Pachter et al., 1987).

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