

Regulation of Microtubule Dynamics by Ca^{2+} /Calmodulin-Dependent Kinase IV/Gr-Dependent Phosphorylation of Oncoprotein 18

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Oncoprotein 18 (Op18; also termed p19, 19K, p18, prosolin, and stathmin) is a regulator of microtubule (MT) dynamics and is phosphorylated by multiple kinase systems on four Ser residues. In addition to cell cycle-regulated phosphorylation, external signals induce phosphorylation of Op18 on Ser-25 by the mitogen-activated protein kinase and on Ser-16 by the Ca^{2+} /calmodulin-dependent kinase IV/Gr (CaMK IV/Gr). Here we show that induced expression of a constitutively active mutant of CaMK IV/Gr results in phosphorylation of Op18 on Ser-16. In parallel, we also observed partial degradation of Op18 and a rapid increase of total cellular MTs. These results suggest a link between CaMK IV/Gr, Op18, and MT dynamics. To explore such a putative link, we optimized a genetic system that allowed conditional coexpression of a series of CaMK IV/Gr and Op18 derivatives. The result shows that CaMK IV/Gr can suppress the MT-regulating activity of Op18 by phosphorylation on Ser-16. In line with these results, by employing a chemical cross-linking protocol, it was shown that phosphorylation of Ser-16 is involved in weakening of the interactions between Op18 and tubulin. Taken together, these data suggest that the mechanism of CaMK IV/Gr-mediated suppression of Op18 activity involves both partial degradation of Op18 and direct modulation of the MT-destabilizing activity of this protein. These results show that Op18 phosphorylation by CaMK IV/Gr may couple alterations of MT dynamics in response to external signals that involve Ca^{2+} .

Cellular tubulin exists in a highly regulated equilibrium between free tubulin dimers and microtubules (MTs). The function of MTs differs during various phases of the cell cycle. MTs are implicated in determination of cell shape, cell polarity, cell motility, and intracellular transport and movement of cell surface receptors during the interphase period and chromosome segregation during mitosis (for reviews, see references 5 and 15). The plethora of MT functions makes it likely that MT dynamics are regulated by many distinct mechanisms which may be responsive to either internal or external signals.

MTs undergo variable phases of assembly and disassembly in a process referred to as dynamic instability (29). Oncoprotein 18 (Op18; also termed p19, 19K, p18, prosolin, and stathmin) is a conserved cytosolic phosphoprotein which has recently been shown to interact with tubulin and to regulate MT dynamics *in vitro* by increasing the frequency of catastrophes (transitions from growing to shrinking) (1). Op18 is also a potent regulator of MT dynamics in transfected cell lines (24); even modest overexpression of the protein caused a 90% decrease in total polymerized tubulin, which is compatible with the catastrophe activity described for Op18 *in vitro* (1). Moreover, the activity of Op18 is down-regulated during mitosis by phosphorylation on two Ser residues by cyclin-dependent protein kinases (CDKs) (24). Replacement of these two Ser residues with Ala results in a mitotic block (25) due to the constitutive activity of this Op18 mutant (24). These results indicated that it is essential to down-regulate the activity of Op18 during mitosis, which in turn suggests that the MT-regulating properties of Op18 may be important during the interphase portion of the cell cycle.

Four Ser residues of Op18 are subject to phosphorylation in intact cells, namely, Ser-16, Ser-25, Ser-38, and Ser-63 (2, 17, 21, 22). Interestingly, phosphorylations of all four Ser residues fluctuate during the cell cycle and CDKs have been identified as the kinase system involved in cell cycle-regulated phosphorylation of Ser-25 and Ser-38 (3, 20, 22). Besides cell cycle-regulated phosphorylation of Op18, two distinct protein kinases have been identified that phosphorylate Op18 in response to external signals. These kinases are members of the mitogen-activated protein (MAP) kinase family that phosphorylates Ser-25 (22) and the Ca^{2+} /calmodulin-dependent kinase (CaMK) IV/Gr that phosphorylates Ser-16 (23). The CaMK IV/Gr, which is expressed predominantly in neurons and T cells, is activated in response to stimulation of the T-cell antigen receptor (10) and has been shown to regulate transcription in response to Ca^{2+} signals (7, 12, 26, 40).

In the present study, we have addressed the following questions. (i) Is Ser-16 of Op18 a target for other members of the CaMK family? (ii) Is Op18 phosphorylation on Ser-16 involved in coupling of external signals to regulation of MT dynamics? (iii) Does this phosphorylation event regulate association of Op18 with tubulin dimers? To approach these questions, we have optimized a genetic system that allows conditional coexpression of a series of CaMKs and Op18 derivatives.

MATERIALS AND METHODS

DNA constructs and transfections. DNA manipulations were performed by standard recombinant techniques (37). Construction of the mutant Op18-S25,38E cDNA, in which the codons for Ser-25 and Ser-38 are exchanged with Glu, has been described previously (23). The FLAG epitope tag (13) was fused to the COOH-terminal end of Op18-S25,38E by employing the strategy previously described for other Op18 cDNA derivatives (25). The resulting FLAG epitope-tagged Op18-S25,38E cDNA was excised from pBluescript SK(+) as a *Bam*HI-to-*Hind*III fragment and cloned into the corresponding sites in the polylinker of the episomal Epstein-Barr virus-based expression vector pMEP4 (Invitrogen) (8). A series of kinase derivatives under the control of the human metallothionein IIa (hMTIIa) promoter were constructed by cloning appropriate

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fragments into the polycloning site of pMEP4 (8). The CaMK IV/Gr series of FLAG epitope-tagged derivatives consists of wild-type CaMK IV/Gr [CaMK IV/Gr (wt)] (31), inactive CaMK IV/Gr [CaMK IV/Gr (i)] with the conserved Lys-75 codon in the ATP-binding site of wild-type kinase exchanged for a Glu codon, constitutively active CaMK IV/Gr [Δ CaMK IV/Gr (c)] with the Gln-318 codon of CaMK IV/Gr (wt) replaced with a stop codon, and double mutant CaMK IV/Gr [Δ CaMK IV/Gr (i)] containing both mutations (12). The human wild-type [CaMK II (wt)] form and the constitutively active mutant [CaMK II (c)] form of CaMK II- γ_B were kind gifts from H. Schulman (Stanford University) (33). The pMEP4-based shuttle vectors described above were transfected by electroporation, and hygromycin-resistant cell lines were selected in a medium specifically designed to support cell growth under conditions that minimize expression from the hMTIIa promoter as described in detail elsewhere (25). To avoid selection for fast-growing clones, cells were used between 5 and 6 days postelectroporation.

Analysis of MT polymerization status, SDS-PAGE, Western blotting, [35 S]methionine labeling, and immunoprecipitation. Preparation of total cellular proteins, precipitation of FLAG epitope-tagged Op18, and separation of proteins by 10 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously (25). The extent of MT polymerization was determined by extracting soluble tubulin in an MT-stabilizing buffer and then quantifying tubulin in the particulate and soluble fractions as previously described (24, 28). Affinity-purified anti-Op18, specific for the COOH terminal (anti-Op18:34-149), or an anti-FLAG M2 antibody (Kodak) was used for Western blot analysis as previously described (4). 125 I-labeled protein A or the ECL detection system (Amersham), as indicated in Results, was used to reveal bound antibodies. PhosphorImager analysis of radioactive bands was used for quantification. As a control for equal loading, the relevant part of a filter was routinely probed with rabbit anti-triose-phosphate isomerase (4). Pulse-chase experiments were performed by labeling cells (3×10^6 /ml) with 50 μ Ci of [35 S]methionine per ml for 2 h in methionine-free RPMI 1640 medium supplemented with 2 mg of bovine serum albumin per ml. Cells were washed thereafter and recultured in methionine-containing RPMI 1640 medium supplemented with either EDTA (50 μ M) or Cd^{2+} (0.4 μ M) for the times indicated in Results. Cellular proteins were solubilized in lysis buffer containing Triton X-100, and Op18 was immunoprecipitated by using affinity-purified rabbit antibodies raised against *Escherichia coli*-produced Op18 protein as described in detail elsewhere (22). After separation of Op18 by SDS-PAGE, PhosphorImager analysis of radioactive Op18 bands was used for quantification.

Partial purification of expressed CaMKs and in vitro phosphorylation. K562 cells were transfected with pMEP4 plasmids expressing either CaMK IV/Gr (wt) or CaMK II (wt) and grown in hygromycin and EDTA for 7 days. To maximize expression, cells (50×10^6) were induced with 1.5 μ M Cd^{2+} (instead of 0.1 to 0.25 μ M Cd^{2+}) for 8 h. To obtain an active CaMK IV/Gr preparation, transfected cells were stimulated for 1 min with 5 μ g of ionomycin per ml prior to harvesting (23). Cells were subsequently lysed, and the expressed CaMKs were partially purified by ammonium sulfate precipitation and calmodulin-Sepharose 4B (Pharmacia) under previously described conditions (30). Purification was monitored by an 125 I-labeled calmodulin overlay assay after separation by SDS-PAGE (11). Analysis of the final preparation revealed that the expressed CaMK corresponded to more than 95% of the calmodulin-binding proteins present in the preparation. In vitro phosphorylation was performed in the presence or absence of 5 mM Ca^{2+} and 600 nM calmodulin as previously described (11).

Preparation of Op18 and cross-linking of Op18-tubulin complexes. Purified *E. coli*-derived Op18 was prepared as previously described (4). In vivo-phosphorylated FLAG epitope-tagged Op18 was prepared as follows. Transfected cells were lysed and enriched for Op18 by heat precipitation, followed by absorption to DE52 as previously described (9). FLAG epitope-tagged Op18 was thereafter affinity purified by using anti-FLAG M2 affinity gel (Kodak), and bound protein was eluted in H_2O containing 1 mM β -glycerophosphate by heating to 75°C. This protocol resulted in a final preparation that contained undetectable levels of coexpressed FLAG epitope-tagged Δ CaMK IV/Gr (c). Purified bovine tubulin was obtained from cytoskeleton. For cross-linking studies, Op18 (1 μ M) and tubulin (10 μ M) were incubated in 30 μ l of G-PEM buffer [80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 1 mM EDTA, 1 mM Mg^{2+} , 2 mM GTP, pH 6.8]. After 120 min on ice, 3.3 μ l of the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC; 120 mM; Sigma) was added and the sample was incubated at 18°C. The reaction was quenched at various times by addition of 1 volume of 2-mercaptoethanol (10%)–glycine (0.2 M) and precipitated in 66% acetone, and the cross-linked Op18-tubulin complexes were analyzed by SDS-PAGE.

RESULTS

Induced expression of CaMKs from the pMEP4 shuttle vector. For conditional expression of a series of CaMK derivatives, we employed the episomal Epstein-Barr virus-based vector pMEP4. By use of a specifically formulated growth medium, the hMTIIa promoter of this vector can be efficiently suppressed by nontoxic levels of EDTA or induced for high-

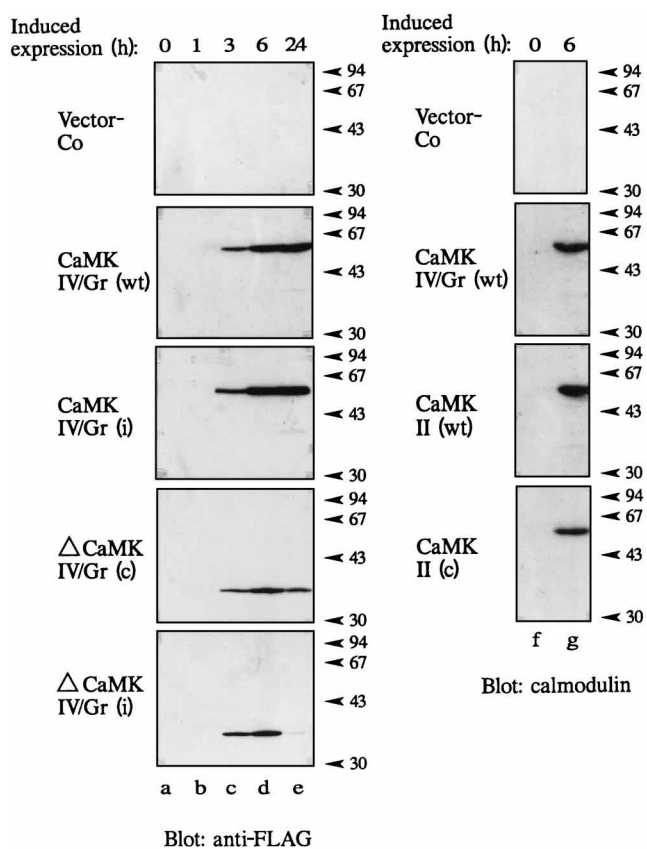


FIG. 1. Regulated expression of CaMKs from the hMTIIa promoter. K562 cells were transfected with the indicated pMEP4-CaMK constructs (see text), and stable transfectants were selected by cultivation with hygromycin and EDTA for 5 days as described in Materials and Methods. Expression from the hMTIIa promoter was then induced with Cd^{2+} (0.25 μ M) for the indicated times. Cell lysates were resolved by SDS-PAGE, proteins were transferred to a nitrocellulose filter, and ectopic expression of epitope-tagged CaMK IV/Gr derivatives was analyzed by Western blotting with anti-FLAG and 125 I-labeled protein A (left). To analyze expression of CaMK II derivatives, a 125 I-labeled calmodulin overlay assay was used and CaMK IV/Gr (wt) was included for comparison (right). As expected, full-length CaMK IV/Gr and CaMK II migrated at about 60 kDa and the truncated CaMK IV/Gr derivatives (Δ CaMK IV/Gr) migrated at about 36 kDa. The values to the right of each panel are molecular masses in kilodaltons.

level expression with Cd^{2+} (see Materials and Methods). Figure 1 shows that FLAG epitope-tagged CaMK IV/Gr (wt), migrating at 60 kDa, can be efficiently induced in erythroleukemic K562 cells as detected by either a FLAG epitope-directed antibody (left panel) or by binding of calmodulin (right panel). The kinetics of expression are rapid (maximal levels are reached within 6 h), and quantification of expression levels in the presence of 0.2 μ M Cd^{2+} reveals 30- to 50-fold induction (see below).

Besides CaMK IV/Gr (wt), regulated expression of the following FLAG epitope-tagged derivatives of this kinase is shown on the left side of Fig. 1: an inactive derivative with its ATP-binding site mutated [CaMK IV/Gr (i)], a constitutively active derivative with its C-terminal calmodulin-binding domain deleted [Δ CaMK IV/Gr (c), migrating at 36 kDa], and an inactivated mutant of the derivative with the C-terminal domain deleted [Δ CaMK IV/Gr (i)]. The results reveal that while all of the CaMK IV/Gr derivatives were efficiently induced, the C-terminally deleted proteins are expressed at somewhat lower levels. The difference in expression level between full-length and truncated CaMK IV/Gr is most evident 24 h after induc-

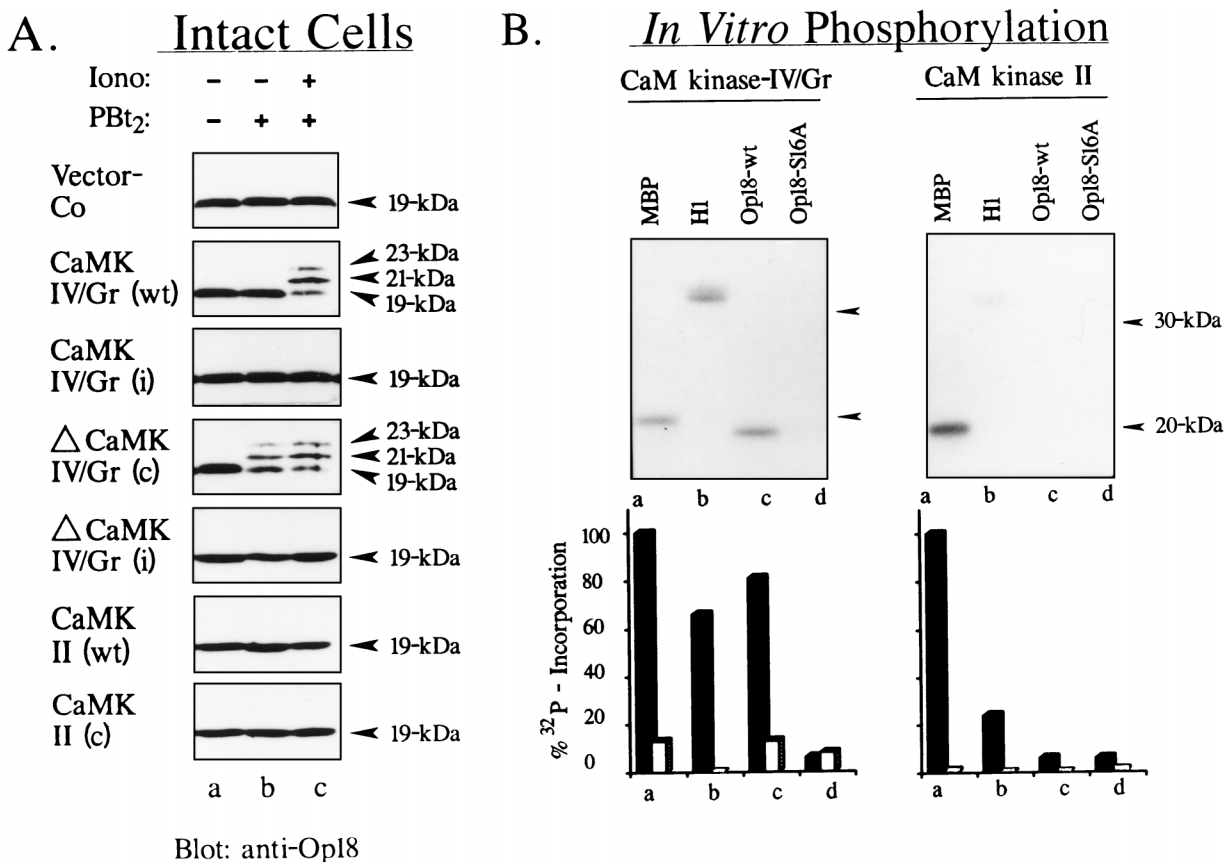


FIG. 2. CaMK-mediated phosphorylation of endogenous Op18 in intact cells and in vitro phosphorylation. (A) K562 cells were transfected with the indicated pMEP4-CaMK derivatives and selected as described in the legend to Fig. 1. Cells were induced with Cd^{2+} ($0.25 \mu\text{M}$) for 6 h and then stimulated for 7 min with solvent alone (lane a), 40 ng of phorbol ester (PBT_2) per ml (lane b), or 2 μg of ionomycin (iono) per ml together with phorbol ester (c). Cell lysates were analyzed by Western blotting with anti-Op18 and ECL detection as described in Materials and Methods. The positions of the 19-, 21-, and 23-kDa phosphoisomers of Op18 are indicated. (B) Partially purified preparations of recombinant CaMK IV/Gr (wt) (left) or CaMK II (wt) (right) were derived from transfected K562 cells. In vitro kinase assays were performed by using the substrate myelin basic protein (a), histone H1 (b), Op18-wt (c), or the Op18-S16A mutant (d). ^{32}P -labeled substrates phosphorylated in the presence of Ca^{2+} /calmodulin were resolved by SDS-PAGE, and selective exposures of autoradiographs are presented. The histograms below show quantification of ^{32}P incorporation into each substrate in the presence (black bars) or absence (open bars) of Ca^{2+} /calmodulin.

tion, which indicates that the C-terminal deletion results in destabilization of the polypeptide.

Expression of a wild-type [CaMK II (wt)] form and a constitutively active mutant [CaMK II (c)] form of CaMK II- γ_{B} , which is an isoform of CaMK II that is expressed in most tissues (33), is also shown in Fig. 1. Since these derivatives lack the epitope tag, expression was revealed by calmodulin binding (right side of Fig. 1). For comparison, the 0- and 6-h time points of induced CaMK IV/Gr (wt) expression were also included in the analysis. The results demonstrate similar expression levels of all three CaMK derivatives. This analysis also shows that the ectopically expressed CaMKs are the predominant calmodulin-binding proteins in transfected cells.

Expression of CaMK IV/Gr, but not that of CaMK II- γ_{B} , results in phosphorylation of Op18 on Ser-16. Ca^{2+} ionophore stimulation of Jurkat cells results in Op18 phosphorylation on Ser-16 by CaMK IV/Gr (21, 23). Erythroleukemic K562 cells do not express endogenous CaMK IV/Gr, and Op18 is not phosphorylated on Ser-16 in response to the Ca^{2+} ionophore ionomycin (10, 23). Hence, K562 cells provide a suitable system for evaluation of the effects of mutant CaMK derivatives on Op18 Ser-16 phosphorylation. To obtain an estimate of Ser-16-specific phosphorylation of Op18 by expressed CaMKs, we took advantage of previous determinations of the mobility

of all observed Op18 phosphoisomers on SDS-PAGE (21). Thus, single-site phosphorylation of Ser-16, or any other residue of Op18, does not result in major shifts in mobility, but if cells are stimulated with phorbol ester (which results in MAP kinase-dependent phosphorylation of Op18 at Ser-25 in lymphoid/hematopoietic cell lines [22]), additional phosphorylation of Ser-16 is readily resolved as specific phosphoisomers. Hence, phosphorylation of Ser-16 and Ser-25 results in a phosphoisomer of 21 kDa, while additional phosphorylation of Ser-38 (Ser-38 is the main constitutively phosphorylated amino acid of Op18 in a proliferating cell line [22]) results in an Op18 phosphoisomer migrating at 23 kDa.

Figure 2A shows that costimulation of CaMK IV/Gr (wt)-transfected K562 cells with phorbol ester and ionomycin generates both the 21- and 23-kDa phosphoisomers of Op18. It is also shown that, as expected, stimulation of $\Delta\text{CaMK IV/Gr}$ (c)-expressing cells with phorbol ester alone is sufficient to generate the same phosphoisomers. Generation of these Op18 phosphoisomers is clearly dependent on expression of active CaMK IV/Gr, as revealed by analysis of cells transfected with either vector-Co or inactive kinase derivatives. Thus, expressed CaMK IV/Gr (wt) phosphorylates Ser-16 of Op18 in response to ionomycin treatment while the $\Delta\text{CaMK IV/Gr}$ (c) mutant phosphorylates Op18 constitutively. Interestingly, expression

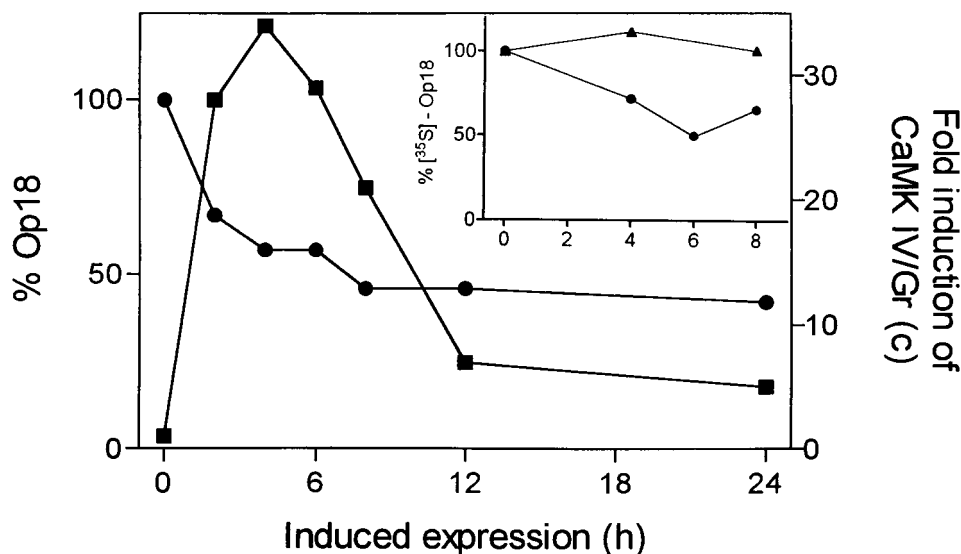


FIG. 3. Phosphorylation of Ser-16 of Op18 is associated with partial degradation of Op18. K562 cells were transfected with the pMEP4 derivatives directing expression of the constitutively active Δ CaMK IV/Gr (c) mutant. Transfected cells were selected as described in the legend to Fig. 1 and then induced with Cd^{2+} (0.25 μM) for the indicated periods. Total cellular proteins were recovered by boiling cells in SDS-PAGE sample buffer, and proteins were resolved by SDS-PAGE. Ectopic expression of the protein kinase (■) and the levels of endogenous Op18 (●) were quantified by Western blotting with specific antibodies and ^{125}I -labeled protein A as described in Materials and Methods. 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 data presented is representative of three independent experiments. The insert shows results of pulse-chase experiments with [^{35}S]methionine-labeled Δ CaMK IV/Gr (c)-transfected K562 cells. After recultivation in the presence of unlabeled methionine, cells were either not induced (\blacktriangle) or induced with Cd^{2+} (\bullet). At the indicated time points, cells were lysed and Op18 was purified by immunoprecipitation. Data shows quantification of Op18-associated radioactivity.

of either wild-type CaMK II- γ_{B} or the constitutively active mutant form of this kinase did not result in phosphorylation of endogenous Op18 on Ser-16.

CaMK IV/Gr and CaMK II have overlapping site preferences *in vitro* (6, 30, 36). To evaluate the preference for Ser-16 of Op18, we performed *in vitro* phosphorylation assays by using partially purified kinases derived from K562 cells overexpressing either CaMK IV/Gr (wt) or CaMK II (wt). Two previously characterized CaMK substrates, myelin basic protein and histone H1 (30), as well as wild-type Op18 and the Op18-S16A mutant, were used in these experiments. Figure 2B shows that CaMK IV/Gr efficiently phosphorylates Op18 on Ser-16 alone. However, Op18 is a poor *in vitro* substrate for CaMK II- γ_{B} and the low-level phosphorylation observed on both the Op18-wt and Op18-S16A substrates shows that Ser-16 is not a significant phosphorylation target for CaMK II. Thus, it appears that Ser-16 of Op18 is a selective target for CaMK IV/Gr.

Association of phosphorylation of Ser-16 of Op18 with partial degradation of Op18 protein and an increase in MT polymer mass. The kinetics of Cd^{2+} -induced Δ CaMK IV/Gr (c) expression and associated changes of Op18 protein levels are shown in Fig. 3. The data reveals a close correlation between the induction of Δ CaMK IV/Gr (c) expression and partial down-regulation of Op18 protein levels. This phenomenon required an active cognate protein kinase, since it was not observed when Δ CaMK IV/Gr (i), CaMK II (wt), or CaMK II (c) was used (data not shown).

Down-regulation appeared to be specific to Op18, since we did not observe changes in the pattern of total cellular proteins or triose-phosphate isomerase (data not shown). Moreover, Op18 is a very stable protein with a half-life well exceeding the time course of the experiment (the half-life of Op18 in K562 cells is more than 36 h), which suggests that decreased Op18 levels are due to induced degradation. To investigate the

mechanism involved, we performed pulse-chase experiments. Accordingly, Op18 was immunoprecipitated from [^{35}S]methionine-labeled Δ CaMK IV/Gr (c)-transfected K562 cells at various times during a chase under either kinase-inducing or -suppressing conditions (Fig. 3, insert). Cd^{2+} -induced expression of the kinase resulted in about a 50% decrease in labeled Op18, while the level of Op18 in EDTA-suppressed cells remained essentially constant. Therefore, the simplest explanation of the data is that phosphorylation of Ser-16 of Op18 is associated with destabilization of the protein.

Since Op18 has a potent MT-destabilizing function, even a modest decrease in the Op18 level may result in a substantial increase of total cellular MTs. We therefore analyzed the level of polymerized tubulin in transfected cells by extracting soluble tubulin in MT-stabilizing buffer. Expression of Δ CaMK IV/Gr (c) resulted in a substantial increase in MT polymer mass (Fig. 4A). This phenomenon required an active cognate protein kinase, since it was not observed when the inactive Δ CaMK IV/Gr (i) mutant or CaMK II (c) was used. The time course of increased MT polymer mass in response to induced expression of Δ CaMK IV/Gr (c) showed a peak after 4 h which correlated with maximal kinase expression (Fig. 4B). Hence, as could be predicted from the MT-destabilizing activity of Op18 (24), down-regulation of Op18 in response to the Δ CaMK IV/Gr (c) mutant is associated with an increase of polymerized tubulin.

Suppression of the MT-regulating activity of Op18 by phosphorylation of Ser-16. A cotransfection system was used to evaluate a direct role of Op18 in regulating MTs in response to Δ CaMK IV/Gr (c)-mediated phosphorylation. In this system, we employed the pMEP4 shuttle vector, which allowed selection of transfected cell lines within 4 to 5 days and rapid coinduction of CaMK and Op18 derivatives. To determine the efficiency of the cotransfection system, we employed a FLAG epitope-tagged mutant of Op18 which contains Glu substitutions instead of Ser-25 and Ser-38 (Op18-S25,38E). This Op18

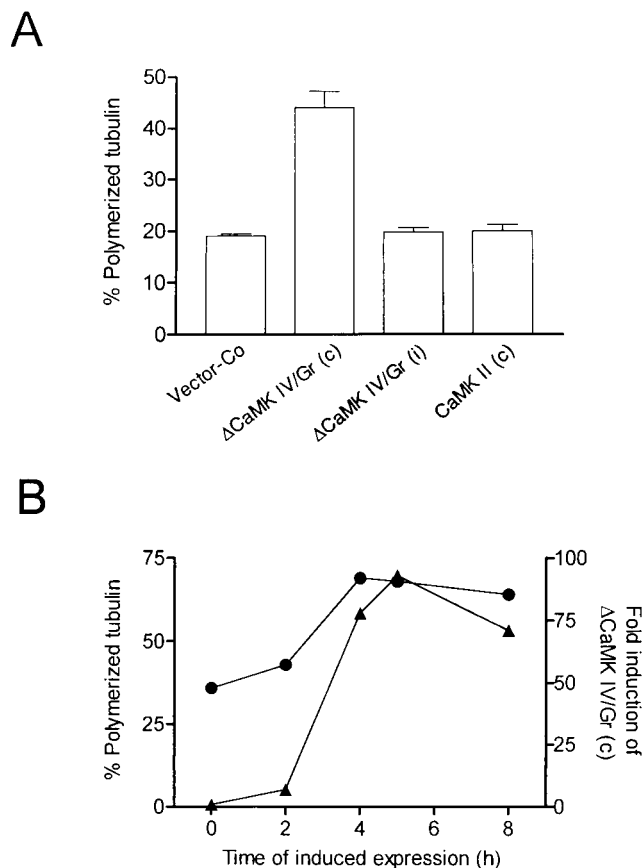


FIG. 4. Induced expression of constitutively active CaMK IV/Gr results in increased levels of polymerized tubulin. (A) K562 cells were transfected with the indicated pMEP4-CaM kinase derivative and selected as described in the legend to Fig. 1. Cells were induced with Cd^{2+} ($0.2 \mu M$) for 5 h, and the level of polymerized tubulin was analyzed as described in Materials and Methods. Data represents means \pm the standard error of the mean of three determinations. (B) Time course of Cd^{2+} ($0.2 \mu M$)-induced expression of Δ CaMK IV/Gr (c) (▲) and changes in MT polymerization status (●).

mutant allows direct determination of the stoichiometry of Ser-16-specific phosphorylation in cotransfected cells. The negatively charged Glu residues at these positions partially mimic the effect of phosphorylation on the corresponding Ser residues and hence result in an electrophoretic mobility shift on SDS-PAGE upon phosphorylation of Ser-16 (23). Hence, Western blot analysis of epitope-tagged Op18-S25,38E can be used to specifically determine the stoichiometry of Ser-16 phosphorylation (21, 23).

Figure 5A shows that cotransfection of vector-Co and the Op18-S25,38E-FLAG mutant results in induced expression of the unphosphorylated protein that migrates at 20 kDa. Induced expression after cotransfection with wild-type CaMK IV/Gr results in a slight increase in the amount of the electrophoretically shifted, Ser-16-phosphorylated 22-kDa form of Op18-S25,38E, which becomes the prominent form upon activation of the expressed CaMK IV/Gr (wt) by ionomycin. Thus, although the selection marker is the same on the cotransfected episomally replicating plasmids, the data demonstrates that both gene products are induced in essentially all transfected cells. Quantification of the data obtained by cotransfection of Op18-S25,38E with selected CaMK derivatives is shown in Fig. 5B. The results show that the constitutive active mutant Δ CaMK IV/Gr (c) efficiently phosphorylates Ser-16 indepen-

dently of ionomycin stimulation. In agreement with previous studies (21, 23), analysis of phosphoisomers showed that the Δ CaMK IV/Gr (c) mutant specifically phosphorylates cotransfected Op18 on Ser-16 (data not shown). Finally, as expected from the result shown in Fig. 2, the data also shows that Ser-16 of Op18-S25,38E remains unphosphorylated in cells cotransfected with either the wild-type form or the constitutively active mutant form of CaMK II- γ_B . Hence, the present system allows regulated expression of two cotransfected gene products and efficient phosphorylation of ectopic Op18 by the Δ CaMK IV/Gr (c) mutant.

Figure 4 shows changes in the level of polymerized tubulin in response to expressed Δ CaMK IV/Gr (c). To explore on which level phosphorylation of endogenous Op18 on Ser-16 may be involved in this response, we cotransfected Δ CaMK IV/Gr (c) with either Op18-wt or the Op18-S16A mutant. Western blot analysis of cotransfected cells showed that these two FLAG epitope-tagged derivatives of Op18 are expressed at similar levels in the presence of $0.2 \mu M$ Cd^{2+} (Fig. 6A). In agreement with the data in Fig. 4, expression of endogenous Op18 is partially down-regulated after induced expression of Δ CaMK IV/Gr (c) (note that Ser-16 phosphorylation results in a minor shift in migration). However, in contrast to the endogenous Op18, we did not detect a significant decrease in the levels of

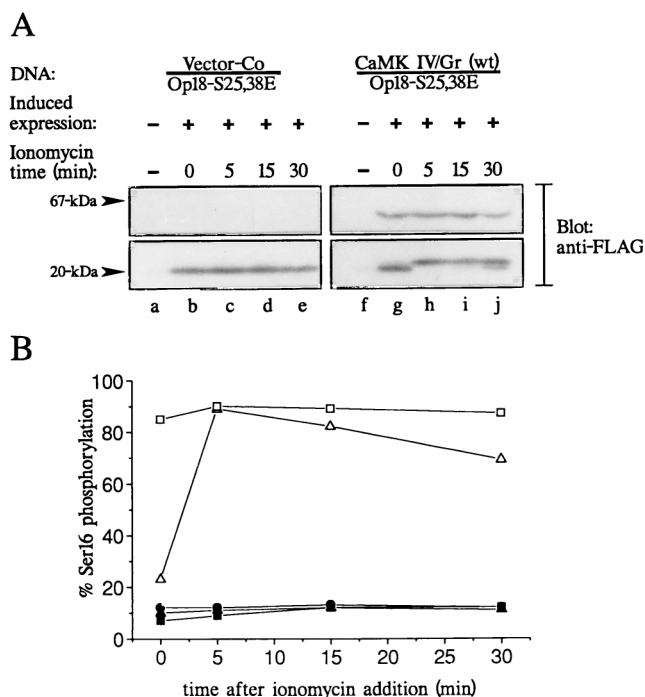


FIG. 5. Conditional coexpression of CaMKs and Op18-S25,38E-FLAG. K562 cells were cotransfected with a panel of pMEP4-CaM kinase constructs ($6 \mu g$) and a pMEP4 vector expressing the FLAG epitope-tagged Op18-S25,38E mutant ($6 \mu g$). Transfectants were selected by cultivation with hygromycin and EDTA for 5 days and then induced with Cd^{2+} ($0.2 \mu M$) for 6 h where indicated (+). (A) Western blot analysis of cells stimulated with ionomycin ($2 \mu g/ml$) for the indicated time. The anti-FLAG antibody was used for detection of both CaMK IV/Gr (wt) (upper) and Op18-S25,38E (lower). The shift in mobility of Op18-S25,38E reveals phosphorylation on Ser-16 (see text). (B) Quantification of the kinetics of Ser-16 phosphorylation of Op18-S25,38E after ionomycin addition in cells cotransfected with Op18-S25,38E and either pMEP4 vector-Co (●), CaMK IV/Gr (wt) (▲), Δ CaMK IV/Gr (c) (□), CaMK II (wt) (■), or CaMK II (c) (▲). The unphosphorylated and phosphorylated forms of Op18-S25,38E were quantified by PhosphorImager analysis of Western blot filters probed with anti-FLAG and ^{125}I -labeled protein A. Data is expressed as a percentage of the total phosphorylated Op18-S25,38E.

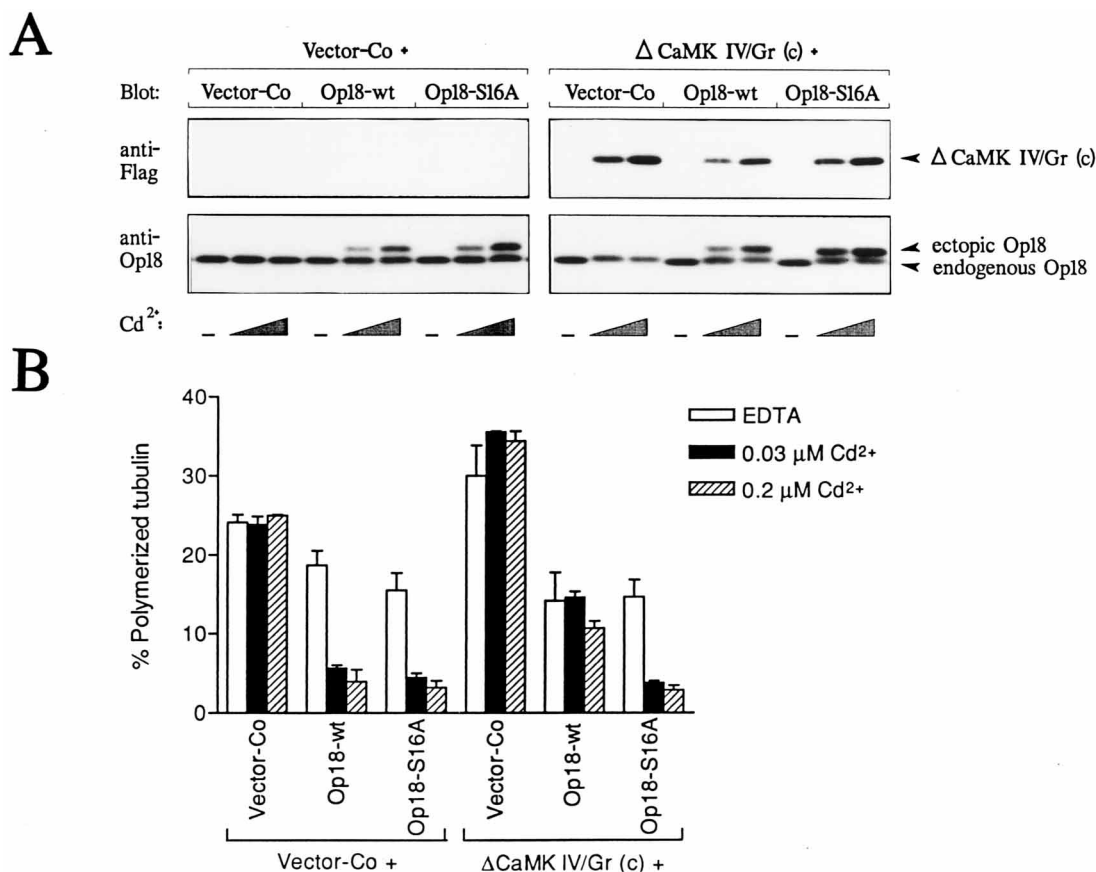


FIG. 6. Phosphorylation of Op18 on Ser-16 suppresses its MT-destabilizing activity. K562 cells were cotransfected with the indicated pMEP4 derivatives, and transfected cell lines were selected as described in the legend to Fig. 5. Expression was either suppressed with EDTA (–) or induced with Cd^{2+} (0.03 or 0.2 μM) for 4 h. (A) Western blot analysis of lysates from cells cotransfected with the indicated pMEP4 constructs. The anti-FLAG antibody was used for detection of Δ CaMK IV/Gr (c), while Op18 was detected by using rabbit anti-Op18 antiserum. The minor shift in mobility of both endogenous and ectopic Op18 after induction of Δ CaMK IV/Gr (c) is caused by phosphorylation on Ser-16. Equal loading of the total cell lysate was confirmed by probing filters with anti-triose-phosphate isomerase (data not shown). (B) MT polymerization status was analyzed as described in the legend to Fig. 4. The mean results of two independent determinations are shown.

ectopic Op18 protein because of its induced rapid expression during the course of the experiment. This result makes the present cotransfection protocol useful for exploration of the role of Ser-16 phosphorylation in the MT-regulatory activity of Op18 with minimal interference on the level of protein degradation.

Our previous report showed that even modest overexpression of Op18 causes depolymerization of MTs (24). In agreement with that study, after 4 h of Cd^{2+} induction of cells cotransfected with either Op18-wt or Op18-S16A, together with vector-Co, dramatic MT depolymerization was evident (Fig. 6B). Most importantly, cotransfection with the Δ CaMK IV/Gr (c) mutant suppressed MT depolymerization caused by Op18-wt but not that caused by the Op18-S16A mutant. This result indicates that phosphorylation of Ser-16 decreases the MT-destabilizing activity of Op18 and provides genetic evidence that expression of the Δ CaMK IV/Gr (c) mutant regulates MT dynamics by phosphorylation of Op18.

Role of phosphorylation of Ser-16 in suppression of interactions between Op18 and tubulin. The results in Fig. 6 suggest that Ser-16 phosphorylation of Op18 per se alters its MT-regulatory function. Op18 has been shown to associate with dimeric, but not polymerized, tubulin, and this property may be of importance for the MT-destabilizing activity of Op18 (1). To analyze Op18 binding to tubulin, we have used a system that

involves chemical cross-linking. Figure 7A shows that cross-linking of tubulin in the presence of Op18 results in efficient generation of a major 71-kDa complex that reacts with both anti- α -tubulin and anti-Op18 antibodies. The 71-kDa complex appears to contain predominantly α -tubulin, since it was poorly recognized by two different anti- β -tubulin antibodies (data not shown). The apparent molecular mass of the 71-kDa complex is in reasonable agreement with the sum of the molecular masses of α -tubulin (55 kDa) and Op18 (19 kDa). In addition to the 71-kDa complex, a minor complex migrating at 83 kDa was also observed.

To determine the role of phosphorylation on complex formation between Op18 and tubulin, the cross-linking protocol was used in conjunction with immunoaffinity-purified, FLAG epitope-tagged Op18 derived from cells cotransfected with either vector-Co or Δ CaMK IV/Gr (c). The bottom of Fig. 7B shows that anti-Op18 detects Op18-FLAG isolated from cells transfected with Op18-wt-FLAG or Op18-S16A-FLAG but not that isolated from vector-Co-transfected cells. The top of Fig. 7B also shows that 71- and 83-kDa complexes were detected with anti-Op18 antibodies after 15 or 30 min of cross-linking in the presence of tubulin. For obscure reasons, the relative intensity of the 83-kDa complex was consistently stronger when Op18-FLAG derived from mammalian cells was used than when *E. coli*-derived Op18 was used (compare Fig. 7A

spective of the exact mechanism, this property made the cotransfection system suitable for direct determination of the role of Op18 Ser-16 phosphorylation in the regulation of its activity.

The cotransfection experiments revealed that Ser-16 phosphorylation suppresses the MT-destabilizing activity of ectopically expressed Op18, which is in line with increased levels of polymerized tubulin in cells induced to express constitutively active CaMK IV/Gr alone. As outlined above, however, the mechanism of CaMK IV/Gr-dependent regulation of MT status may not be identical when the kinase is expressed alone or together with ectopic Op18. Although degradation of endogenous Op18 is likely to be of regulatory importance, it is still clear from the present study that Ser-16 phosphorylation of Op18 has direct consequences for Op18 interaction with tubulin dimers. Thus, we observed about fourfold-decreased efficiency of tubulin cross-linking to isolated Ser-16 phosphorylated protein compared with unphosphorylated controls (Fig. 7). Assuming that interaction between Op18 and tubulin dimers is of importance for the MT-destabilizing Op18 activity, this result supports the conclusion derived from cotransfection experiments (Fig. 6). Hence, we conclude that the activity of Op18 can be suppressed by Ser-16 phosphorylation independently of protein degradation.

The cross-linking experiment whose results are shown in Fig. 7 was performed with Op18-FLAG protein isolated from transfected cells. It should be noted that, independently of coexpression of CaMK IV/Gr, Op18 becomes highly phosphorylated when overexpressed (24). Analysis of Op18-FLAG phosphoisomers revealed that, besides CaMK IV/Gr-mediated phosphorylation on Ser-16, more than 50% of the protein contained additional phosphorylations (data not shown). This is in line with previous results demonstrating that overexpression of Op18 results in hyperphosphorylation of Op18, mostly on Ser-38 (24). This phenomenon is most likely caused by Op18-induced depolymerization of MT, since it has been shown that MT-destabilizing drugs induce rapid activation of a MAP kinase-related kinase (39). Hence, the result of coexpression of Op18 and CaMK IV/Gr is complete phosphorylation of Ser-16 with more than 50% of all Op18 proteins also phosphorylated on additional sites. Thus, while the genetic control of Op18-S16A makes it possible to conclude that Ser-16 phosphorylation is required for the observed regulation of Op18 activity, it is not enough to conclude that Ser-16 phosphorylation is sufficient in itself for the dramatic effects observed in this study.

MTs have complex and specialized functions during both the interphase portion and the mitotic phase of the cell cycle. It is therefore likely that MT dynamics are regulated by many distinct mechanisms that are responsive to either internal or external signals. MT-associated proteins and kinesin-related proteins have been shown to be important for regulation of MT dynamics through the cell cycle (for a review, see reference 27). There is also evidence that MT-dependent processes are regulated in response to external signals, such as growth factors (14, 16, 18). The present study, using transfected cells, suggests yet another MT-regulatory pathway that implicates CaMK IV/Gr and its abundant cytosolic substrate, Op18. Although it still remains to be established, it seems reasonable to assume that this MT-regulatory pathway could also operate under physiological conditions. For example, CaMK IV/Gr is activated in response to T- or B-cell antigen receptor triggering (10, 31), and this may lead to alterations of the MT network. Moreover, both CaMK IV/Gr and its substrate Op18 are abundantly expressed in cells of neural origin, which may have implications for regulation of MT dynamics during outgrowth

of neuronal processes. Op18 may be a member of a family of related proteins. An example of a protein that is highly homologous to Op18, with the CaMK IV/Gr phosphorylation site conserved, is provided by the neuronal SCG10 protein (34). It was recently reported that mice with both alleles of the Op18 gene disrupted by homologous recombination show normal development and no obvious defect during adult life (38). Thus, whatever function Op18 may have in regulating MT dynamics, it is either a nonessential function or, more likely, a function that can be compensated for by a related protein.

The present study shows that CaMK IV/Gr, but not CaMK II, phosphorylates Op18 on Ser-16 and induces an increase in polymerized tubulin. During the course of this study, we have also observed other functional differences between these two CaMKs. For example, in agreement with a previous report (35), induced expression of the constitutively active mutant of CaMK II- γ_B results in a cell cycle block during G₂, while the corresponding mutant of CaMK IV/Gr preferentially caused a cell cycle block at G₁ (data not shown). Earlier studies on these two CaMKs have demonstrated related, but in some cases distinct, substrate preferences (6, 30, 36). In recent studies, phosphorylation and activation of the cyclic AMP-responsive element-binding (CREB) protein by CaMKs were analyzed in detail (7, 26, 40). It was shown that both CaMK IV/Gr and CaMK II phosphorylate the CREB protein on the critical site for transcriptional activation. However, CaMK II also phosphorylated the CREB protein on an additional site that caused suppression of its transcriptional activation. Thus, the physiological consequences of activating either CaMK IV/Gr or CaMK II in response to a Ca²⁺ signal may be completely different. Other examples of functional differences between these two CaMKs are provided by studies of transcriptional regulation of the interleukin-2 enhancer (32) and regulation of the AP-1 and MEF2 families of transcription factors (12, 19). Thus, functional differences between CaMK IV/Gr and CaMK II occur at the level of transcription control and, as shown in the present study, a cytosolic process, namely, MT dynamics, that is specifically regulated by CaMK IV/Gr.

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