Interphase-specific Phosphorylation-mediated Regulation of Tubulin Dimer Partitioning in Human Cells[□]

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The microtubule cytoskeleton is differentially regulated by a diverse array of proteins during interphase and mitosis. Op18/stathmin (Op18) and microtubule-associated protein (MAP)4 have been ascribed opposite general microtubule-directed activities, namely, microtubule destabilization and stabilization, respectively, both of which can be inhibited by phosphorylation. Here, using three human cell models, we depleted cells of Op18 and/or MAP4 by expression of interfering hairpin RNAs and we analyzed the resulting phenotypes. We found that the endogenous levels of Op18 and MAP4 have opposite and counteractive activities that largely govern the partitioning of tubulin dimers in the microtubule array at interphase. Op18 and MAP4 were also found to be the downstream targets of Ca^{2+} - and calmodulin-dependent protein kinase IV and PAR-1/MARK2 kinase, respectively, that control the demonstrated counteractive phosphorylation-mediated regulation of tubulin dimer partitioning. Furthermore, to address mechanisms regulating microtubule polymerization in response to cell signals, we developed a system for inducible gene product replacement. This approach revealed that site-specific phosphorylation of Op18 is both necessary and sufficient for polymerization of microtubules in response to the multifaceted signaling event of stimulation of the T cell antigen receptor complex, which activates several signal transduction pathways.

INTRODUCTION

Microtubules (MTs) are polar polymers of α/β tubulin heterodimers that, besides forming the mitotic spindle during cell division, have diverse cell type-specific functions. MTs are dynamic and switch stochastically between phases of polymerization and depolymerization, a unique phenomenon that has been called dynamic instability (for review, see Desai and Mitchison, 1997). During interphase and mitosis, the MT system is differentially regulated by regulatory proteins (for review, see Cassimeris, 1999). One such protein, Op18/stathmin (Op18), seems to destabilize the MT system by two distinct mechanisms, namely, by promoting transition from a growing to a shrinking MT (Belmont and Mitchison, 1996), termed catastrophe; and by forming a ternary tubulin sequestering complex (Jourdain *et al.*, 1997; for review, see Cassimeris, 2002). The physiological relevance of the MT-destabilizing activity of Op18 has been shown by increased MT polymer content and reduced catastrophe promotion in new lung cells depleted of Op18 (Howell *et al.*, 1999).

Phosphorylation of Op18 inhibits its function (Marklund *et al.*, 1996), and analysis of Op18 phospho-isomers in metaphase-blocked human cells revealed stoichiometric phos-

phorylation of the two cyclin-dependent kinase target sites, Ser-25 and -38, as well as abundant phosphorylation of the remaining two sites, Ser-16 and -63 (Larsson *et al.*, 1995). This suggests that Op18 is phosphorylation inactivated during spindle assembly (Larsson *et al.*, 1997), which would be consistent with the reported lack of a mitotic phenotype in cell lines extensively depleted of Op18 by RNA interference (Holmfeldt *et al.*, 2006). Op18 is predominantly unphosphorylated during interphase, but activation of diverse signal-transducing kinase systems results in specific combinations of phosphorylation of the four serine residues, which result in various degrees of inactivation. These kinase systems include microtubule-associated protein (MAP) kinase and the Ca^{2+} - and calmodulin-dependent protein kinase IV (CaMKIV), which are both activated by triggering of the T cell antigen receptor (TCR)/CD3 complex, and the cAMP-dependent kinase (PKA) (for review, see Lawler, 1998). Moreover, the actin regulatory Rac protein has been suggested to modulate the MT system, at least in part by activation of kinase systems that phosphorylate Op18 (Daub *et al.*, 2001; Wittmann *et al.*, 2004).

The MT-stabilizing proteins include MAP2, MAP4, and tau, which belong to a well-characterized family of microtubule-associated proteins (MAPs). Although MAP2 and tau are only found in neural tissues, MAP4 seems to be abundantly expressed in all tissues (for review, see Cassimeris, 1999). MAP4 and other MAPs promote assembly and stabilization of MTs by a mechanism that involves binding to the polymer (for review, see Drewes *et al.*, 1998). The phenotype of partial depletion of MAP4 by antisense RNA expression has suggested that there is a significant MT-stabilizing function in the epithelial HeLa cell line (Nguyen *et al.*, 1999). MAP4 is phosphorylated by mitotic protein kinase systems, which apparently reduce MAP4 activity during mitosis (Ookata *et al.*, 1995; Shiina and Tsukita, 1999; Kitazawa *et al.*, 2000). Studies on MAP4-depleted cells have failed to reveal

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Abbreviations used: CaMKIV, Ca^{2+} - and calmodulin-dependent protein kinase IV; MT, microtubule; Op18, oncoprotein 18/stathmin; PAGE, polyacrylamide gel electrophoresis; shRNA, short hairpin RNA.

mitotic defects (Wang *et al.*, 1996; Holmfeldt *et al.*, 2005), which suggests that MAP4 has no essential role during mitosis.

MAP4 is a target for the PAR-1/microtubule affinity-regulating kinases (MARK) kinase family, which are critical for cell polarity as well as other cellular functions in species ranging from yeast to mammals (for review, see Drewes *et al.*, 1998). These kinases phosphorylate and thereby inactivate the MT-stabilizing activity of classical MAPs such as MAP2, MAP4, and tau (Drewes *et al.*, 1997). Ectopic expression of mammalian MARK1 and MARK2 in mammalian cells has been shown to cause a dramatic destabilization of interphase MTs, which is associated with phosphorylation of MAP4 (Ebner *et al.*, 1999). MARK kinases are activated by phosphorylation by the tumor suppressor kinase LKB1 (Goransson *et al.*, 2006), but these kinases seem to be constitutively active in cells, and the role of this level of regulation is still unclear.

In vitro studies and the phenotypes of cells expressing ectopic proteins have established that phosphorylation has the potential to inhibit the MT-directed activities of both Op18 and MAP4. However, the significance of phosphorylation-mediated inhibition of the endogenous gene products is still unexplored. This issue concerns both the significance of the endogenous activities of Op18 and MAP4 and the anticipated phosphorylation-regulated interplay between the opposing activities of these ubiquitously expressed proteins. Here, we address these questions by using human cells and interfering hairpin RNA-based systems for gene product depletion and inducible gene product replacement.

MATERIALS AND METHODS

DNA Constructs

The pMEP4 shuttle vector directing inducible expression of Flag epitope-tagged (Op18-F) and kinase-target site deficient derivatives of Op18 (Op18-S16,63A-F, Op18-S16A,25-F) have been described previously (Marklund *et al.*, 1994b). These derivatives were made resistant to short hairpin RNA (shRNA)-mediated suppression by introducing seven silent mutations within the 19-nucleotide (nt)-targeting sequence, and the coding sequence of the polymerase chain reaction (PCR)-generated fragment was confirmed by nucleotide sequence analysis (sequence information will be provided on request). Derivatives of pMEP4 directing inducible expression of Flag-tagged constitutively active CaMKIV(c)-F, CaMKII γ (c), and myc-tagged PKA-myc have been described previously (Melander Gradin *et al.*, 1997; Gradin *et al.*, 1998). The pMEP-MARK2-HA derivative, which directs expression of hemagglutinin (HA)-tagged rat MARK2 kinase, was generated by PCR by using pEUHATagMARK2 (Drewes *et al.*, 1997) as template. The general strategy for construction of replicating Epstein-Barr virus (EBV)-based shuttle vectors for constitutive expression of short hairpin RNA (shRNA) has been described (Holmfeldt *et al.*, 2004). The targeting sequences [AA(19 nt)TT] used in the present study were as follows: MAP4 (accession U19727): (shRNA-MAP4-41) GAT AGT CCC AGC CAA GGA T; (shRNA-MAP4-10) CTG GCC AGA AGA TAC CAA C; Op18 (accession NM_005563): (shRNA-Op18-443) CGT TTG CGA GAG AAG GAT A; (shRNA-Op18-OE) CGA GAC TGA AGC TGA CTA A. A BLAST search of the National Center for Biotechnology Information database ensured specific targeting of the cognate mRNA.

Transfections and Cell Culture

Single transfections and cotransfections of K562 by using the EBV-based replicating shuttle vectors and subsequent selection of hygromycin-resistant cell lines were performed in a medium specifically designed to support cell growth under conditions that minimize expression from the human metallothionein IIa (hMTIIa) promoter, as described in detail previously (Gradin *et al.*, 1998; Holmfeldt *et al.*, 2004). The same protocol was used for Jurkat T cells and DG75 B cells but with the modification that electroporation was done in serum-free RPMI 1640 medium containing 8% Ficoll. Conditional expression and coexpression in K562 cells was induced from the hMTIIa promoter by addition of 0.1 μ M Cd²⁺. For efficient expression in DG75 and Jurkat cells, the protocol was modified such that 5 μ M Cd²⁺ was added for a 3-h period followed by recultivation in fresh medium without Cd²⁺. For expression of ectopic protein kinases, cells were transfected with 6 μ g of pMEP kinase DNA mixed with 10 μ g of vector-CoDNA, and for coexpression 6 μ g of each pMEP kinase derivative was mixed with 4 μ g of vector-Co. Due to the stringent

replication control of the EBV-based vector, the ratio of transfected DNAs is stable during the time course of the experiment (Melander Gradin *et al.*, 1997). Transfection of replicating shuttle vectors that direct constitutive synthesis of specific interfering shRNA was performed according to the same basic protocol as described for pMEP vectors, with 2 μ g of each shRNA producing constructs mixed with empty pMEP vector up to a total quantity of 16 μ g of DNA. For inducible ectopic expression in shRNA-synthesizing cells, we used three basic protocols in which the amount of shRNA-resistant pMEP-Op18-F DNA was adjusted to result in an induced expression level close to the level of endogenous Op18. First, in functional gene product replacement experiments in which Flag-tagged Op18 was expressed in K562 cells depleted of Op18, 2 μ g of shRNA-Op18 was mixed with 2 μ g of pMEP-Op18-F derivative and empty pMEP vector up to a total quantity of 16 μ g of DNA. Second, in functional gene product replacement experiments in which Flag-tagged Op18 was expressed in Jurkat cells depleted of Op18, 2 μ g of shRNA-Op18 was mixed with 6 μ g of pMEP-Op18-F derivative and empty pMEP vector up to a total of 16 μ g of DNA. Third, in experiments in which Flag-tagged Op18 was coexpressed with a protein kinase in Op18-depleted K562 cells, 2 μ g of shRNA-Op18 was mixed with 2 μ g of pMEP-Op18-F derivative, 6 μ g of the pMEP kinase derivative, and empty pMEP vector to a total quantity of 16 μ g of DNA.

Quantification of Op18 and Polymer-Monomer Partitioning of Tubulin Dimers

Expression levels of Op18 were determined by immunoblotting by using affinity-purified rabbit antibodies raised against an internal peptide sequence of Op18, corresponding to residues 46–58 (Holmfeldt *et al.*, 2003a). Analysis of cellular MT polymer content by flow cytometry (>90% of all cells were included in the acquisition gate and >150,000 cells were collected) was performed using a FACSCalibur instrument (BD Biosciences, San Jose, CA) with modifications allowing determination of MT polymer content at interphase and mitotic phases of the cell cycle, as detailed in Holmfeldt *et al.* (2003b). This flow cytometry-based procedure faithfully reproduced the results obtained by quantification of soluble and particulate tubulin by Western blot analysis. To determine the total amount of polymerizable tubulin dimers, cells were treated with the polymerization-promoting drug Taxol (paclitaxel) at 2 μ g/ml for 2 h, which was found by quantitative Western blotting to cause essentially complete polymerization (<3% soluble tubulin dimers) and allowed calculation of the percentage of tubulin dimers in polymers under the different experimental conditions.

RESULTS

Op18 and MAP4 Function as Opposing Interphase-specific Regulators of Monomer-Polymer Partitioning of Tubulin Dimers in Three Different Cell Models

We have previously found that destabilization of MTs by ectopic catastrophe-promoting Op18 derivatives is counteracted by ectopic MAP4 (Holmfeldt *et al.*, 2002). To evaluate the physiological significance of this finding, the effects of depleting cells of MAP4 and Op18, alone or in combination, were determined. Our approach was based on a shuttle vector system that confers hygromycin resistance and directs the expression of shRNA (Holmfeldt *et al.*, 2005). After 5 d of counterselection with hygromycin to eliminate untransfected K562 cells, Op18 and MAP4 expression was found to be reduced by >95 and ~85%, respectively, without having detectable effects on the total amount of tubulin dimers or the proliferating cell nuclear antigen (PCNA) loading control (Figure 1A). Consistent with unaltered levels of tubulin dimers, it can be seen in Figure 1B that addition of the MT-polymerizing drug Taxol revealed similar maximum levels of polymerizable tubulin dimers in control cells and cells that were specifically depleted of Op18 and/or MAP4. This demonstrates that the total amounts of tubulin dimers in K562 cells remain relatively constant during 5 d of Op18 and/or MAP4 depletion.

It can be seen in Figure 1C that extensive depletion of Op18 is associated with an increase in interphase MT polymers from ~58 to ~92% of the total tubulin dimer pool, which implies a drastic decrease in monomeric tubulin dimers. Moreover, it is also apparent that MAP4 depletion reduces MT polymer levels. Significantly, codepletion of MAP4 and Op18 results in interphase MT polymer levels

Figure 1. Interphase-specific opposing effects of Op18 and MAP4. K562 cells were transfected as described in *Materials and Methods* with the indicated mixtures of shuttle vector-Co, shRNA-Op18-443, or shRNA-MAP4-10. Untransfected cells were counterselected with hygromycin for 5 d. (A) Immunoblots of total lysates of transfected cells by using the indicated antibody for detection. Quantification was achieved by serial dilution, which revealed >96% specific depletion of Op18, ~85% depletion of MAP4, but no alterations in tubulin levels. The PCNA protein was used as loading control. (B) Immunoblots of total protein from cells that expressed the indicated shRNA derivatives for 5 d. The MT polymer content of interphase cells was determined as described in *Materials and Methods*, and data are expressed as percentage of polymerized tubulin. (C) Total amount of polymerizable tubulin dimers in specifically depleted cells was determined by addition of Taxol for 2 h (2 μ g/ml) followed by determination of MT polymer content by flow cytometry. Data are expressed as percentage of the MT polymer content of vector-Co cells at interphase. To confirm that Taxol promotes close to maximal polymerization under the present conditions, soluble tubulin dimers were analyzed by immunoblotting, which revealed that <3% was unpolymerized tubulin (data not shown). (D) Mitosis-specific MT polymer content of cells described in C was determined by flow cytometry, which allowed gating of mitotic cells as outlined under *Materials and Methods* (primary histograms are shown in Figure Supplement S2). Data are expressed as percentage of the MT polymer content of mitotic vector-Co cells. The data are representative of at least three independent transfection experiments.

that are close to those of control cells, thus revealing counteractive activities at the level of tubulin dimer partitioning. However, despite these major effects of Op18 and MAP4 depletion on the level of MT polymer content, in all cases the organization of the interphase MT array seemed normal by epifluorescence analysis of MTs (data not shown). The data in Figure 1 were obtained using K562 erythroleukemia cells, but opposite and counteractive activities were also observed in the two lymphocytic cell lines, namely, DG75 B cells and Jurkat T cells (Supplemental Figure S1), and they were subsequently confirmed using two independent shRNAs for both MAP4 and Op18 (data not shown).

Previous studies of Op18- or MAP4-depleted cells monitored over a 9-d period have not revealed any phenotype during spindle assembly or growth rate defect (Holmfeldt et al., 2005, 2006). Consistent with these results, we found that single depletion or codepletion of Op18 and MAP4 did not significantly alter the average MT polymer content of mitotic spindles (Figure 1D) or the distribution of spindle MT content within the mitotic population (Supplemental Figure S2), and it did not detectably interfere with spindle formation, cell division, growth rate, or the basal frequency of apoptotic cells, as studied in K562, DG75, and Jurkat cells (data not shown). This suggests that these two proteins specifically counteract the activities of each other in interphase cells only, which is consistent with the reported phosphorylation-inactivation of both of these MT regulatory proteins by mitotically active kinase systems (Ookata et al., 1995; Larsson et al., 1997). Thus, MAP4 and Op18 have prominent interphase-specific counteractive effects on the partitioning between soluble and polymeric tubulin in human cells.

Phosphorylation-mediated Regulation of the Interphase MT System Is Op18 and MAP4 Dependent

Op18 and MAP4 have both been shown to be phosphorylated inactivated by several different kinase systems. For example, ectopic constitutively active derivatives of CaMKIV and PKA have been reported to inactivate Op18 by phosphorylation (Melander Gradin et al., 1997; Gradin et al., 1998), and as shown in Figure 2, A and B, induced expression of either of these two kinases results in a concomitant increase in the amount of interphase MT polymer. This was not observed in cells expressing constitutively active CaMKII γ_B (c), which has a related site specificity to CaMKIV(c) but has been shown to be incapable of phosphorylating Op18 in intact cells or in vitro (Melander Gradin et al., 1997). Moreover, as expected from previous reports (Drewes et al., 1997; Ebner et al., 1999), overexpression of the MARK2 kinase that phosphorylates classical MT-stabilizing proteins such as MAP4 has the opposite effect, namely, a decrease in MT polymer levels (Figure 2, A and B). It should be noted that further increases in ectopic MARK2 levels over those shown did not cause a further reduction in MT polymers (data not shown), which suggests that a fraction of all MT polymers in K562 cells is resistant to MARK2-mediated destabilization.

To investigate possible counteractive effects from the action of kinases with opposing MT-directed activities, MARK2 was coexpressed with CaMKIV(c). This analysis revealed efficient counteraction, in that cells coexpressing MARK2 and CaMKIV(c) had an MT polymer content that was almost normal (Figure 2C). These opposite and counteractive activities of ectopic kinases were also observed in the DG75 B cell and Jurkat T cell models (Supplemental Figure S3). It is noteworthy that expression of MARK2 or CaMKIV(c) alone, or in combination, did not detectably alter the MT polymer

Figure 2. MT regulation in response to induced expression of protein kinases. K562 cells were transfected with the pMEP shuttle vector derivative indicated, counterselected with hygromycin for 5 d, and ectopic expression was induced for the indicated times. (A) Immunoblots of total proteins of cells induced to express the protein kinase indicated. (B) The MT polymer content of interphase cells described in A, determined as in Figure 1. (C) Immunoblots of total proteins from cells induced for 8 h to express CaMKIV(c)-F or MARK2-HA, alone or in combination. The histogram shows the MT polymer content of cells at interphase. (D) Mitosis-specific MT content, determined as in Figure 1. The data are representative of at least three independent transfection experiments.

content of mitotic spindles (Figure 2D); nor did it interfere with spindle formation or cell division in any other way (data not shown). Moreover, at the expression levels used, we did not observe any adverse effects of ectopic CaMKIV(c) or MARK2 on the degree of cell viability over a 24-h period (data not shown). These phenotypes of ectopic protein kinases illustrate the ability of MARK2 and CaMKIV(c) to have prominent opposing effects on the state of assembly of interphase MTs.

As shown above, depletion of Op18 alone in K562 cells caused close to maximal MT polymerization, which pre-

Figure 3. The significance of Op18 and MAP4 for phosphorylation-mediated regulation of interphase MT polymers by CaMKIV and MARK2 kinases. K562 cells were transfected as in Figures 1 and 2 with the indicated combinations of shuttle vector shRNA and pMEP derivatives. (A) Immunoblots of total proteins of singly or doubly depleted cells induced for 8 h to express CaMKIV(c)-F. The MT polymer content of interphase cells was determined as in Figure 1. (B) MT regulation by the MARK2-HA kinase, shown using the same shRNA derivatives and conditions as in A. The data are representative of at least two independent transfection experiments.

cludes detection of further polymerization in response to an ectopic Op18-specific kinase. To circumvent this problem, the significance of CaMKIV(c)-mediated phosphorylation of Op18 for MT polymerization was evaluated in cells depleted of both Op18 and MAP4. The results showed that the MT polymer level in such doubly depleted cells, which was close to normal, was essentially unaltered by induced expression of CaMKIV(c) (Figure 3A). The same type of analysis also revealed that cells depleted of both Op18 and MAP4 did not respond to ectopic MARK2 (Figure 3B). Hence, although the expressed protein kinases are likely to phosphorylate several cellular proteins, our data demonstrate that Op18 and MAP4 are essential for the observed phosphorylation-regulation of tubulin dimer partitioning. This is consistent with Op18 and MAP4 acting as the downstream phosphorylation targets of CaMKIV and MARK2, respectively. And furthermore, show that phosphorylation-mediated regulation of both Op18 and MAP4 can have a major interphase-specific effect on monomer-polymer partitioning of tubulin dimers.

Figure 4. Importance of physiological Op18 levels in control of tubulin dimer partitioning. K562 cells were transfected as described in *Materials and Methods* with the indicated mixtures of EBV-based shuttle vector-Co, shRNA-Op18-443, and a Flag-tagged Op18 derivative (Op18-F), which was resistant to shRNA. Untransfected cells were counterselected with hygromycin for 5 d. (A) Immunoblot analysis of Op18 levels in cells transfected with the indicated shuttle vector derivative and induced to express ectopic Op18-F for the times indicated. Migration of endogenous Op18 (arrows) and Flag epitope-tagged Op18-F (arrowheads) is indicated. (B) The MT polymer content of interphase cells described in A was determined at the indicated time points, as in Figure 1. The data are representative of at least three independent transfection experiments.

Physiological Op18 Levels Have an Immediate Effect on Monomer-Polymer Partitioning

Extensive depletion of a long-lived gene product requires several days of RNA interference, which means that shRNA-Op18 expression may cause an adaptive response and thus have indirect effects on the regulation of the MT system. To determine the immediate effect of physiologically relevant Op18 levels, we developed a system for inducible Op18 expression in Op18-depleted cells, which involved cotransfection of cells with replicating vectors directing either shRNA-Op18 expression or inducible expression of Op18-F. The system was tuned such that the ectopic Op18-F protein was induced within 4–6 h to levels similar to those of endogenous Op18 (Figure 4A, arrowheads indicate migration of Op18-F), which correspond to ~0.1% of all cytosolic proteins in a cell line that expresses average Op18 levels such as K562 (Brattsand *et al.*, 1993). Analysis of interphase MT polymer content showed that induced Op18 expression in Op18-depleted cells caused a 30–40% reduction in MT polymers, resulting in an MT content close to that of control cells (Figure 4B). Induced Op18-F expression in undepleted cells, which doubled the level of Op18, caused a proportional decrease in MT polymer content. These immediate effects of induced Op18-F expression suggest that a period of 5 d of gradual Op18 depletion does not cause adaptations that alter the responsiveness of MT polymers to the destabilizing activity of Op18. Thus, endogenous levels of Op18 have a direct effect on tubulin dimer polymer-monomer partitioning in K562 cells and the level of monomer is very low in the absence of Op18.

Site-specific Phosphorylation of Op18 Is the Cause of MT Polymerization in Response to Ectopic Protein Kinases

A stringent test of the importance of Op18 for phosphorylation-mediated regulation of interphase MTs would require gene product replacement with phosphorylation site-deficient mutants. Such mutants of Op18 are, however, constitutively active during mitosis, blocking spindle formation and causing accumulation of aberrant mitotic cells (Marklund *et al.*, 1996). We circumvented this problem by using the inducible gene product replacement system outlined in Figure 4, in which the phenotype is scored shortly after induced expression of the complementing Op18 protein and the ectopic kinase. Thus, this system allowed us to critically assess the significance of Op18 phosphorylation in response to phosphorylation signals. As expected, expression of shRNA-Op18 and PKA, alone or in combination, resulted in close to maximal MT polymerization (Figure 5A, shaded bars indicate induced PKA expression). Simultaneous induction of PKA and wild-type Op18-F in Op18-depleted cells resulted in high MT polymer content, which is consistent with phosphorylation-inactivation of Op18-F. Significantly, PKA expression had no effect on MT polymers if the complementing Op18 gene was mutated such that the two PKA serine target sites were exchanged to nonphosphorylatable alanine residues (Op18-S16,63A-F). The corresponding analysis was also performed with Op18-depleted cells induced to coexpress CaMKIV(c) together with either Op18-F or Op18-S16,63A-F (Figure 5B). In this case, also, the results demonstrated that CaMKIV(c)-mediated regulation of MT polymer content requires the cognate phosphorylation sites on Op18. Thus, site-specific phosphorylation of Op18 is the cause of the dramatic MT polymerization that coincides with induced expression of either CaMKIV(c) or PKA.

T Cell Receptor/CD3 Complex Mediates MT Polymerization by a Pathway That Is Dependent on Phosphorylation of Op18

The significance of site-specific phosphorylation of Op18 in response to a multifaceted signaling event was explored by analyzing MTs in T cells stimulated by the TCR/CD3 complex. A multitude of Tyr- and Ser/Thr-specific kinase systems, including mitogen-activated protein kinase (MAPK) and CaMKIV, are activated by TCR/CD3 stimulation (for review, see Cantrell, 2002), which is associated with high stoichiometric phosphorylation of Op18 on MAPK and CaMKIV target sites in the Jurkat T cell model (Marklund *et al.*, 1993, 1994a). Consistent with the expected result of phosphorylation-inactivation of Op18, we found that stimulation of the TCR/CD3 complex with an anti-CD3 antibody resulted in increased levels of MT polymers in Jurkat cells, which was not observed in the presence of a nonstimulatory CD2-specific antibody (Figure 6A).

The significance of phosphorylation-inactivation of Op18 activity in response to TCR/CD3 complex stimulation was addressed by analyzing the effect of Op18 depletion in the presence and absence of anti-CD3. As shown in Figure 6, B and C, Op18 also exerts a prominent MT-destabilizing effect in this cell system, although it is not as prominent as in K562 (Figure 1) and DG75 cells (Supplemental Figure S1). Significantly, in contrast to vector control cells, anti-CD3 stimulation has very little effect on Op18-depleted cells. To directly address the MT-regulatory importance of site-specific Op18 phosphorylation, we performed inducible gene product replacements using the same general strategy as presented above for K562 cells. Thus, the consequence of CaMKIV- and MAPK-specific phosphorylation was determined by com-

Figure 5. The significance of Op18 phosphorylation as evaluated by inducible gene product replacement. K562 cells were transfected as in Figure 1 with the indicated combinations of shuttle vectors. (A) Immunoblots of total proteins of control cells or cells expressing shRNA-Op18-443 induced for 8 h to express PKA and/or Flag-tagged Op18 derivatives either with the wild-type amino acid sequence (Op18-F) or with Ser-16 and Ser-63 substituted by Ala residues (Op18-S16,63A-F). The MT polymer content of interphase cells was determined after 8 h of induced expression as in Figure 1. (B) MT regulation by the CaMKIV(c)-F kinase is shown using the same shRNA and Op18 derivatives as in A. The data are representative of at least three independent transfection experiments.

paring phenotypes of Op18-F with that of the Op18-S16, 25A-F derivative, which contained Ala substitutions at the two major target sites of these two protein kinases, i.e., Ser-16 and Ser-25, respectively. The results showed that although TCR/CD3 stimulation by anti-CD3 caused the expected increase in MT polymer content in Op18-depleted cells induced to express Op18-F, stimulation had no effect on Op18-depleted cells expressing the protein kinase target site-deficient Op18-S16, 25A-F derivative (Figure 6, B and C). These gene product replacement experiments demonstrate that TCR/CD3 stimulation causes MT polymerization by a mechanism that is dependent on phosphorylation-inactivation of Op18 at the CaMKIV target site (Ser-16) and the MAPK target site (Ser-25).

DISCUSSION

Here, we explored phosphorylation-regulated interplay between Op18 and MAP4 and the physiological significance of site-specific Op18 phosphorylation during signal transduction events. Our approach relied on replicating vectors directing either constitutive expression of shRNAs or inducible ectopic expression, which provided a system for inducible gene product replacement. Given that interphase phenotypes could be scored within a few hours of induced

expression, the spindle-disrupting activity during mitosis of phosphorylation-target site-deficient Op18 derivatives did not significantly obstruct the present analysis. Our results show that 1) Op18 and MAP4 are major interphase-specific and counteracting phosphorylation-responsive regulators of monomer-polymer partitioning in all three cell models analyzed; 2) site-specific phosphorylation of Op18 is the cause of the drastic MT polymerization observed in response to cognate ectopic kinases; and 3) Op18 phosphorylation at Ser-16 and Ser-25, which have been shown previously to be MAPK and CaMKIV target sites in T cells, is the direct cause of MT polymerization observed after stimulation of the TCR/CD3 complex.

Based on the present findings, we conclude that Op18 and MAP4 are major regulators of tubulin dimer partitioning in cells of hematopoietic origin, which seems likely to apply to all cell types that express significant levels of Op18 and MAP4. Op18 levels are known to vary greatly between cell lines and tissues, with particularly high protein levels in neural and embryonic tissues and in diverse diagnostic groups of tumors (for review, see Mori and Morii, 2002). It thus seems reasonable to assume that the impact of Op18 phosphorylation will vary in different cell types, depending on the levels of Op18 and the actual stoichiometry of phosphorylation. Compared with many leukemia cell lines, K562

Figure 6. The significance of Op18 phosphorylation in response to T cell receptor/CD3 complex stimulation. (A) Jurkat T cells were stimulated as indicated with either the anti-CD3 antibody UCHT-1 (8 μ g/ml) or the control anti-CD2 antibody OKT11 (8 μ g/ml). MT polymer content was determined as in Figure 1 at the indicated time points. (B) Immunoblots of total proteins of Jurkat T cells expressing Vector-Co or shRNA-Op18-443, induced for 8 h to express either Op18-F or Op18-S16,25A-F. (C) The MT polymer content of interphase cells was determined without anti-CD3 stimulation (open bars) or with anti-CD3 stimulation (black bars, 8 μ g/ml for 10 min) of the cells. The data are representative of three independent transfection experiments.

cells have modest levels of Op18 (1.1 μ g Op18/mg total protein), which is comparable to the Op18 levels in untransformed cells such as primary proliferating human T cells (0.66 μ g Op18/mg total protein) and less than the levels in Jurkat cells (2 μ g Op18/mg total protein) (Brattsand *et al.*, 1993). We have shown that phosphorylation of Op18 causes a pronounced change in tubulin dimer partitioning in both K562 and Jurkat cells (Figures 1, 2, and 4–6 and Supplemental Figures S1 and S3); together, the present study indicates that phosphorylation-mediated regulation of Op18 activity represents a significant mechanism of MT regulation in all cell types that express adequate levels of Op18.

Depletion experiments and expression of ectopic MARK has shown that phosphorylation-mediated regulation of MAP4 activity may have a major effect on the MT system at interphase. MAP4 has been shown to be phosphorylated by several protein kinase systems *in vitro*, such as cyclin-dependent kinases, MAPK family members, glycogen synthase kinase 3, and MARK family members (for review, see Drewes *et al.*, 1998). Phosphorylation of MAP4 during mitosis is mediated by cyclin-dependent kinases, which inhibit its MT-stabilizing activity *in vitro* (Ookata *et al.*, 1995). Kinase target site-deficient mutants of MAP4 have been shown

to cause mitotic defects, suggesting that phosphorylation-mediated inactivation is essential for spindle formation (Shiina and Tsukita, 1999). There is, however, no current evidence that MARK family members phosphorylate MAP4 during specific phases of the cell cycle or that these kinases are activated in response to signal transduction events. Moreover, studies on MAP4 phosphorylation in response to growth factors have shown increased phosphorylation only 12–24 h after addition; this response is much too delayed to reflect phosphorylation by signal-transducing kinase systems such as MAPK family members (Srsen *et al.*, 1999). Thus, despite that there have been extensive studies, there is currently no evidence that MAP4 activity is regulated by phosphorylation in response to signal transduction events.

One implication of the present demonstration of prominent opposing effects of Op18 and MAP4 is that the activities of these MT regulators should show a mutual dependence in maintaining the proper monomer-polymer partitioning of tubulin dimers. Although many papers have reported large variations in Op18 levels in various cell lines and tissues, the corresponding analysis of MAP4 is still lacking. Because both Op18 and MAP4 are subject to phosphorylation-mediated inactivation, there may be only a weak correlation between activity and protein levels. Moreover, it has been suggested that there is an additional level of control of MAP4 activity by regulation of MT stability through binding of mammalian septins to MAP4 (Kremer *et al.*, 2005). Using the HeLa cell line, it was shown by depletion experiments that the MT-stabilizing activity of MAP4 is substantially inhibited by constitutive binding of septins. Thus, given this complexity of regulation of MAP4 activity, the protein ratios of Op18 and MAP4 cannot be expected to allow prediction of polymer-monomer partitioning in a given cell type.

The present study shows that Op18 and MAP4 are not required for spindle assembly during mitosis of K562 cells, which is indeed consistent with previous reports (Wang *et al.*, 1996; Holmfeldt *et al.*, 2006). The present data have also shown that removal of both of these two MT regulators has no detectable consequences during mitosis (Figure 1 and Supplemental Figure S2). In addition, ectopic expression of CaMKIV and MARK2, which inactivates Op18 and MAP4, respectively, does not cause detectable interference with spindle assembly (Figure 2), which we have confirmed using DG75 and Jurkat cells (data not shown). Thus, the counteractive activities of Op18 and MAP4 seem to be interphase specific. This contrasts with the antagonizing activities described for the MT regulatory proteins MCAK/XKCM1 and TOGp/XMAP215 (for review, see Kinoshita *et al.*, 2002), which are evident in *Xenopus* egg extracts in both interphase and mitosis (Tournebise *et al.*, 2000), and also in mitotic human cells (Cassimeris and Morabito, 2004; Holmfeldt *et al.*, 2004). Interestingly, the antagonizing activities of MCAK and TOGp cannot be detected in human cells that are in interphase (Holmfeldt *et al.*, 2004), which reveals a clear-cut difference between the interphase-specific activities of Op18 and MAP4 described in the present study.

The TCR/CD3 complex has been shown to activate a multitude of signaling pathways (Cantrell, 2002), but it is still apparent from the present data that phosphorylation of Op18 is both sufficient and necessary for the demonstrated increase in MT polymers in TCR/CD3-stimulated T cells. It is known that stimulation of the TCR/CD3 complex by an antigen-presenting cell leads to formation of an immunological synapse, translocation of the MT organizing center, and polarized secretion of effector molecules (for review, see Krummel and Macara, 2006). Interestingly, dynein is recruited to immunological synapses of Jurkat T cells stimu-

lated by antigen presenting cells, which has been shown to be essential for MT polarization (Combs *et al.*, 2006). Given the evidence that Cdc42 is essential for polarization of T cells (Stowers *et al.*, 1995), it seems that T cell polarization is controlled by the same general signaling pathways described for astrocytes, in which Cdc42 has been shown to require the MT motor dynein to induce MT polarization (Etienne-Manneville and Hall, 2001). In this article, we have shown that stimulation of Jurkat cells with anti-CD3 results in a dramatic increase in MT polymers as a result of Op18 phosphorylation. To facilitate synchronous activation in a homogenous cell population, we used a soluble anti-CD3 antibody, which precludes analysis of polarization—because formation of an immunological synapse requires activation by an antigen-presenting cell. It seems conceivable, nevertheless, that the Op18-dependent pathway responsible for MT polymerization would greatly facilitate dynein–MT interactions at immunological synapses. As proposed for both astrocytes (Etienne-Manneville and Hall, 2001) and Jurkat T cells (Combs *et al.*, 2006), recruited dynein would be expected to create tension on MTs and thereby translocate the MT-organizing center toward the direction of polarization. Thus, we speculate that phosphorylation-mediated inactivation of Op18 activity and consequent polymerization of MTs may facilitate interactions with dynein at sites of polarization in several cell types.

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