Regulation of Microtubule Destabilizing Activity of Op18/Stathmin Downstream of Rac1*§

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In the leading edge of migrating cells, a subset of microtubules exhibits net growth in a Rac1- and p21-activated kinase-dependent manner. Here, we explore the possibility of whether phosphorylation and inactivation of the microtubule-destabilizing protein Op18/stathmin could be a mechanism regulating microtubule dynamics downstream of Rac1 and p21-activated kinases. We find that, in vitro, Pak1 phosphorylates Op18/stathmin specifically at serine 16 and inactivates its catastrophe promoting activity in biochemical and time lapse microscopy microtubule assembly assays. Furthermore, phosphorylation of either serine 16 or 63 is sufficient to inhibit Op18/stathmin in vitro. In cells, the microtubule-destabilizing effect of an excess of Op18/stathmin can be partially overcome by expression of constitutively active Rac1(Q61L), which is dependent on Pak activity, suggesting that the microtubule cytoskeleton can be regulated through inactivation of Op18/stathmin downstream of Rac1 and Pak in vivo. However, in vivo, Pak1 activity alone is not sufficient to phosphorylate Op18, indicating that additional pathways downstream of Rac1 are required for Op18 regulation.

Tight regulation of cytoskeletal dynamics in living cells is essential for many cellular behaviors such as mitosis or motility. Rho family GTPases are well characterized regulators of actin cytoskeleton reorganization in response to extracellular signals (1). However, recent evidence has also linked Rho GTPases to regulation of the dynamics of the microtubule cytoskeleton (2). For example, RhoA and its downstream effector, mDia, have been implicated in the formation of a stable, detyrosinated microtubules (3, 4), whereas Cdc42, together with cytoplasmic dynein, is involved in the orientation of the microtubule-organizing center toward the leading edge of migrating cells (5, 6). In addition, activation of Rac1 promotes microtubule growth into advancing lamellipodia of migrating cells (7).

In cells and in vitro, microtubules exhibit a nonequilibrium polymerization behavior known as dynamic instability, the stochastic switching between phases of growth and shortening (8). Dynamic instability can be described by four parameters: the rates of growth and shortening and the transition frequencies from growth to shortening (catastrophe frequency) and from shortening to growth (rescue frequency). In vivo, dynamic instability is regulated by microtubule-associated proteins, microtubule plus end-binding proteins, and soluble proteins, creating a balance between microtubule stabilizing and destabilizing factors that promote the rapid turnover of microtubules required for cellular morphogenesis (9–11).

One soluble microtubule-destabilizing protein is Op18/stathmin (Op18) (12, 13). Op18 binds tubulin dimers, inhibits tubulin polymerization, promotes catastrophes, and modulates tubulin GTP hydrolysis (14–18). Although the mechanism by which Op18 induces microtubule catastrophes is not entirely understood, it appears that this activity is at least partially separable from its ability to sequester tubulin dimer (16, 17). Mammalian Op18 is regulated by phosphorylation at four serine residues by a number of protein kinases, and dual phosphorylation particularly of serines 16 and 63 inhibits the microtubule destabilizing activity of Op18 (19–22).

Op18 becomes phosphorylated in various cancer cell lines in response to treatment with epidermal growth factor (EGF) (23–25), and it has recently been shown that EGF-stimulated phosphorylation at serine 16 requires the activity of Rac1 and p21-activated serine/threonine kinases (Paks) (23, 26). However, the physiological significance of this is unknown. In migrating cells, a subset of microtubules exhibits increased growth into the advancing cell edge because of a decreased catastrophe frequency and an increased time microtubules spend in the growth phase (7, 27). Recently, we have found that expression of constitutively active Rac1(Q61L) results in similar microtubule net growth in nearly all cellular microtubules and that this depends on Pak kinase activity (7). One possibility is that this Rac1-mediated promotion of microtubule growth could be by Pak kinase regulation of the microtubule destabilizing activity of Op18. We find here that Pak1 can directly phosphorylate Op18 at serine 16 and inhibit its catastrophe promoting activity in vitro, although in vivo Pak kinase activity alone is not sufficient to phosphorylate Op18. In addition, inhibition of Paks downstream of Rac1(Q61L) increases microtubule destabilizing activity of Op18 in PK1 cells, indicating that Op18 activity can be regulated by Paks in vivo.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and Constructs—pcDNA3 mammalian expression vectors (Invitrogen) encoding EGFP-Rac1(Q61L) or EGFP-Rac1(Q61L)

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§ The online version of this article (available at http://www.jbc.org) contains video files.

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The abbreviations used are: Op18, Op18/stathmin; EGF, epidermal growth factor; Pak, p21-activated kinase; PKA, cAMP-dependent kinase; GST, glutathione S-transferase; Pipes, 1,4-piperazinediethanesulfonic acid.
Rac1(T12N) were a kind gift from Klaus Hahn (The Scripps Research Institute). Full-length GST-Pak1 and GST-PBD/ID(H83L) were expressed and purified by glutathione affinity chromatography as described (28, 29). Plasmids containing wild-type and mutant Op18, samples of untagged Op18 and related proteins, and anti-Op18 antibodies were generous gifts of Martin Gullberg (University of Umeå, Umeå, Sweden). The antiserum specific for phosphorylated serine 16 was from Annette Sobel (INSERM U7440, Paris, France).

The Op18 open reading frame was amplified by PCR with Vent DNA polymerase (New England Biolabs) introducing NcoI and BamHI restriction sites at the 5′- and 3′-end, respectively, and cloned into the pHAT2 vector (30). The resulting protein contains the peptide MSHHHHHHHH fused to the N-terminus of human Op18. Histidine-tagged Op18 proteins were expressed in E. coli (BL21/DE3/plyss) cells (Stratagene) and purified on TALON metal affinity resin (Clontech) according to the manufacturer’s instructions.

**In Vitro Phosphorylation and Microtubule Polymerization Assay—** Op18 or related proteins were phosphorylated with GST-Pak1 in 25 mM Na-HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.5 mM dithiothreitol, and 1 mM ATP or with PKA (New England Biolabs) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM ATP at 30 °C. The reactions were stopped at 70 °C for 10 min and analyzed by native gel electrophoresis on 4–15% gradient gels (Bio-Rad) or SDS-PAGE and autoradiography. For use in microtubule polymerization assays, 35 μM Op18 was phosphorylated in 30-μl reactions with 1 μM GST-Pak1. To achieve stoichiometric phosphorylation, fresh kinase was added after 2 h, and the incubation continued for another 2 h. HeLa cells were transfected with FuGENE 6 (Roche Applied Science). To analyze Op18 phosphorylation on immunoblots, Op18 was quantitatively enriched from the cell lysates by heat precipitation of the heat-stable Op18 from the remaining supernatant with 5–6 volumes methanol and 1% sucrose overnight at −20 °C. The pellet was directly dissolved in a small volume of SDS-PAGE sample buffer. In vitro kinase assays with immunoprecipitated Pak1 were as described (29). The kinase inhibitors were from LC Laboratories.

To measure the inhibitory effect of Op18 on microtubule polymerization, 7 μM phosphocellulose-purified, cyclized bovine brain tubulin (31) was mixed with 1–12 μM Op18 in 30 μl of 80 mM K-Pipes, pH 6.8, 10% glycerol, 1 mM EGTA, 5 mM MgCl2, 1 mM GTP, and 7 μM taxol. The microtubules were polymerized at 37 °C for 1 h and separated from unpolymerized tubulin dimer by centrifugation at 45,000 ×g for 10 min at 37 °C. Equal amounts of supernatant and pellet were subjected to SDS-PAGE. The gels were stained with Coomassie Brilliant Blue and quantified on a personal densitometer (Molecular Dynamics).

**In Vitro Microtubule Dynamics—** Sea urchin axonemes (32) in 80 mM K-Pipes, pH 7.5, 1 mM EGTA, 1 mM MgCl2 were perfused into a 5 μM Op18 in 30 mM K-Pipes, pH 6.8, 10% glycerol, 1 mM EGTA, 5 mM MgCl2, 1 mM GTP, and 7 μM taxol. The chamber was perfused with about 5 volumes of 10 mM K-Pipes, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.5 mM dithiothreitol, and 1 mM ATP, or with PKA (New England Biolabs) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM ATP at 30 °C. The reactions were stopped at 70 °C for 10 min and analyzed by native gel electrophoresis on 4–15% gradient gels (Bio-Rad) or SDS-PAGE and autoradiography. For use in microtubule polymerization assays, 35 μM Op18 was phosphorylated in 30-μl reactions with 1 μM GST-Pak1. To achieve stoichiometric phosphorylation, fresh kinase was added after 2 h, and the incubation continued for another 2 h. HeLa cells were transfected with FuGENE 6 (Roche Applied Science). To analyze Op18 phosphorylation on immunoblots, Op18 was quantitatively enriched from the cell lysates by heat precipitation of the heat-stable Op18 from the remaining supernatant with 5–6 volumes methanol and 1% sucrose overnight at −20 °C. The pellet was directly dissolved in a small volume of SDS-PAGE sample buffer. In vitro kinase assays with immunoprecipitated Pak1 were as described (29). The kinase inhibitors were from LC Laboratories.

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**In Vitro Microtubule Dynamics—** Sea urchin axonemes (32) in 80 mM K-Pipes, pH 7.5, 1 mM EGTA, 1 mM MgCl2 were perfused into a 5–10-μl chamber (consisting of a clean coverslip mounted with two strips of double-sided tape on a microscope slide) on ice and allowed to adhere. The chamber was perfused with about 5 volumes of 10 mM tubulin and 1 μM GTP in the same buffer, sealed, and transferred to the microscope stage that had been prewarmed to 37 °C with an air stream incubator (NevTek). The samples were observed on an inverted TE-300 microscope (Nikon) equipped with differential interference contrast optics, a 100×/1.4 NA Plan Apo objective lens, a 1.4 NA condenser, and a metal halide liquid light guide illuminator (Nikon). The images were acquired in 12-bit mode with an Orca II cooled CCD camera (Hamamatsu) and subarrayed such that a frame rate of less than 60 ms could be achieved using “stream acquisition” in Metamorph software (Universal Imaging). To reduce noise, eight such frames were background-subtracted, averaged, and contrast-stretched for each microtubule image. Such averaged images were acquired every 5 s, and dynamic instability parameters were quantified by measuring microtubule length over time. Plus ends were identified as the longer microtubules growing from the axoneme (31).

**Determination of Tubulin Polymer to Dimer Ratio in Cells—** Pak1 cells were cultured and injected as described (7). The microtubule polymer/dimer ratio was essentially determined as described (30). Cells growing on a coverslip (Eppendorf) were injected with X-rhodamine-labeled tubulin at 2–3 mg/ml. The cells were imaged with a 40×/1.3 NA Plan Fluor objective lens (Nikon) with the Orca II camera before and after extraction. To extract tubulin dimers, the cells were washed briefly with 80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1 mM MgCl2, 10 μM taxol, and extracted in the same buffer containing Oxyrase (O2-releasing) at 0.1 mg/ml and 0.5% Triton X-100. During the whole procedure the slide was kept at 37 °C. The total tubulin fluorescence in cells before and after extraction was determined using Metamorph and after background correction, and the ratio of polymerized tubulin to soluble dimer was calculated. Statistical analysis of the data was performed with Microsoft Excel (Microsoft) and Analyze-it for Excel (Analyze-it Software, Ltd.).

**RESULTS**

**Pak1 Directly Phosphorylates Op18 at Serine 16—** Because Op18 phosphorylation downstream of EGF stimulation is dependent on Pak kinase activity in cells (23), we first wanted to determine whether purified Pak could directly phosphorylate Op18 in vitro. For this purpose, Op18 was incubated with constitutively active GST-Pak1 (28) in the presence of [γ-32P]ATP (Fig. 1A). Although in vitro phosphorylation by GST-Pak1 proceeded relatively slowly, possibly because of the low specific activity of the bacterially expressed kinase, the rate of Op18 phosphorylation was comparable with the rate of GST-Pak1 autophosphorylation, suggesting that Op18 is a physiological substrate of Pak1. The incorporation of radiolabeled phosphate into the GST-Pak1 autophosphorylation assay was dependent on Pak kinase activity in cells (23), we first wanted to determine whether purified Pak could directly phosphorylate Op18 in vitro. For this purpose, Op18 was incubated with constitutively active GST-Pak1 (28) in the presence of [γ-32P]ATP (Fig. 1A). Although in vitro phosphorylation by GST-Pak1 proceeded relatively slowly, possibly because of the low specific activity of the bacterially expressed kinase, the rate of Op18 phosphorylation was comparable with the rate of GST-Pak1 autophosphorylation, suggesting that Op18 is a physiological substrate of Pak1.
Op18 (●) or Op18 phosphorylated with either Pak1 (○) or PKA (□) was mixed with 7 µM tubulin dimer under tubulin polymerization-promoting conditions. Microtubules (P) were separated from soluble tubulin dimer (S) by centrifugation and analyzed by SDS-PAGE. Although nonphosphorylated Op18 clearly inhibited microtubule polymerization at concentrations as low as 2 µM, even at 12 µM, Op18 phosphorylated with either PKA or Pak1 had no effect on tubulin polymerization. B, phosphorylation of serine 16 is required to inactivate Op18. Op18 (●) or Op18(S16A) (○) were phosphorylated with Pak1. The reaction was stopped after increasing periods of time, and the effect of 6 µM of the phosphorylated protein on tubulin polymerization was tested. Although the inhibition of Op18 by Pak1 phosphorylation was apparent after 1 h, prolonged phosphorylation of Op18(S16A) did not affect its inhibition of tubulin polymerization. In A, fresh kinase was added after 2 h to achieve stoichiometric phosphorylation, which explains the slightly lower maximal level of Op18 inhibition in B. wt, wild type.

To further examine the site specificity of Op18 phosphorylation by Pak1, we analyzed in vitro phosphorylated wild-type and mutant Op18 proteins by native gel electrophoresis (Fig. 1C). Op18 and Op18(S63A) were phosphorylated by GST-Pak1 predominantly at a single site. However, Op18(S16A) was essentially not phosphorylated, demonstrating that Pak1 is highly specific for serine 16. Some minor phosphorylation occurred most likely on serine 63 because a minor band corresponding to a second phosphorylation site was present with Op18 or Op18(S16A) as a substrate but was absent with Op18(S63A). As a positive control, PKA phosphorylated Op18 at both serines 16 and 63, as described previously (21) and showed a slight preference for serine 63 over serine 16. Interestingly, we also found that Pak1 and PKA phosphorylated the stathmin-like domain of one neuronal Op18 homologue, SCG10, but not another, RB3, suggesting that phosphorylation of these sites may regulate specific stathmin family members only (Fig. 1, D and E).

**Phosphorylation by Pak1 Inhibits Op18 in Vitro**—We next determined whether phosphorylation by Pak1 affected the ability of Op18 to inhibit the polymerization of purified tubulin in vitro (19). Tubulin dimer was mixed with increasing amounts of Op18, and microtubule polymerization was induced by the addition of GTP and taxol and incubation at 37 °C. Polymerized microtubules were then separated from unpolymerized tubulin dimer by centrifugation. Nonphosphorylated Op18 efficiently inhibited tubulin polymerization, whereas Op18 that had been phosphorylated with Pak1 allowed microtubule polymerization even at Op18 concentrations exceeding the concentration of tubulin present in the reaction (Fig. 2A). As a positive control, phosphorylation with PKA equally inhibited Op18 (21).

To test whether Pak1-mediated inactivation of the microtubule destabilizing activity of Op18 was due to phosphorylation at serine 16, Op18 and Op18(S16A) were phosphorylated with Pak1 for increasing periods of time, and their effects on tubulin polymerization were compared. The inhibition of Op18 correlated with the progress of the phosphorylation reaction, whereas incubation of Op18(S16A) with Pak1 for long periods had no effect on its ability to inhibit microtubule polymerization (Fig. 2B). Together, these results demonstrate that Pak1 phosphorylation at serine 16 is necessary and sufficient to inhibit the microtubule destabilizing activity of Op18 in vitro.

Because such a bulk tubulin polymerization assay does not distinguish between tubulin dimer sequestration and direct catastrophe promotion, we also examined the effect of Pak1 phosphorylation of Op18 on microtubule plus end dynamic instability parameters. The microtubules were nucleated from axonemes at 10 µM tubulin concentration and observed by digitally enhanced differential interference contrast microscopy. This assay was performed at pH 7.5, a level at which binding of Op18 to tubulin dimers is reduced (34–36), and Op18 predominantly increases the plus end catastrophe frequency independent of its effects on dimer sequestration and growth rate (16). Under these conditions, the addition of 0.8 or 1.5 µM Op18 increased the catastrophe frequency ~2- or 3-fold, respectively (Fig. 3 and Table I). We also observed a slight decrease in plus end growth rate that was statistically significant at high Op18 concentrations (p < 0.01 by analysis of variance; Table I). In contrast, concentrations as high as 2.0 µM Pak1-phosphorylated Op18 had no significant effect on either plus end catastrophe frequency or growth rate (Fig. 3 and Table I), showing that phosphorylation by Pak1 alone can regulate
the catastrophe promoting activity of Op18.

How Op18 phosphorylation affects microtubule dynamics in vitro has so far only been analyzed with protein that had been dual phosphorylated on both serines 16 and 63 (35). Because Pak1 primarily phosphorylated Op18 at serine 16, we wanted to examine the relative contribution of phosphorylation at either serine 16 or 63 to Op18 inhibition. For this purpose, we analyzed plus end microtubule dynamic instability in vitro in the presence of either Op18(S16A) or Op18(S63A) phosphorylation site-deficient mutants. At similar concentrations, both nonphosphorylated mutant proteins increased the catastrophe frequency comparable with wild-type Op18. Surprisingly, phosphorylation of both mutant proteins with PKA resulting in single site phosphorylation of either serine 63 or 16, respectively, equally inhibited the catastrophe promoting activity of Op18. This suggests that phosphorylation at either serine 16 or 63 is sufficient to inactivate Op18 in vitro (Fig. 3B and Table II).

**Regulation of Op18 Phosphorylation Downstream of Rac1 in Vivo**—Phosphorylation of Op18 in response to EGF has been observed in cancer cells; however, how this affects microtubules in these cells has not been examined (23–25). To study whether such growth factor-induced Op18 phosphorylation could be important for the regulation of microtubule stability in vivo, we first determined the extent of Op18 phosphorylation after EGF stimulation in PtK1 cells, the same cell type in which we have observed increased microtubule growth downstream of Rac1 activation (7). PtK1 epithelial cells that had been treated with EGF for 5–60 min were lysed and analyzed by immunoblot with an anti-Op18 antibody or an antisemir specific for phosphorylated serine 16 (37). We clearly detected serine 16 phosphorylation 15 min after EGF stimulation, and maximal phosphorylation was reached after 30 min (Fig. 4A). However, the fraction of total Op18 that was phosphorylated was relatively low, as judged by the intensity of bands of lower electrophoretic mobility that appeared after EGF addition and were recognized by the anti-Op18 antibody. In addition, these bands indicate that EGF treatment induces phosphorylation at other sites besides serine 16 as previously shown (23).

We had previously found that expression of constitutively active mutants of Pak1 was not sufficient to induce the net microtubule growth observed in Rac1(Q61L)-expressing cells (7). To clarify this, we wanted to test whether constitutively active Pak1 can phosphorylate Op18 in vivo. For this purpose, we used HeLa cells, because high transfection efficiency could be obtained, and examined phosphorylation of Op18 at serine 16 in cells expressing either dominant negative Rac1(T17N), constitutively active Rac1(Q61L), or two different constitutively active Pak1 mutants, Pak1(T423E) or Pak1(H83L,H86L) (38). This analysis revealed that Op18 phosphorylation at serine 16 was only increased in Rac1(Q61L)-expressing cells but not in cells expressing either of the constitutively active Pak1 mutants (Fig. 4C). To determine whether the Pak1 mutants were active in HeLa cells, we immunoprecipitated Pak1 from transfected cells and tested its activity in a kinase assay. This showed that Pak1(T423E) was clearly active and capable of phosphorylating Op18 in vitro (Fig. 4D).

To test whether other kinases that are known to phosphorylate Op18 at serine 16 are involved, we used different kinase inhibitors and determined their effect on EGF-induced Op18 phosphorylation in PtK1 cells (Fig. 4B) and Rac1(Q61L)-induced Op18 phosphorylation in vivo (Table II).

![Figure 3](image-url) **Phosphorylation of Op18 by Pak1 inhibits its catastrophe promoting activity in vitro.** A, representative images of a digitally enhanced differential interference contrast time lapse sequence of microtubules growing from axonemes in the presence of either 1.5 μM Op18 or 2.0 μM Pak1-phosphorylated Op18. Microtubule growth is inhibited by Op18 but not by Pak1-phosphorylated Op18. Elapsed time is indicated in minutes/seconds. Bar, 10 μm. B, comparison of the catastrophe frequencies measured under different conditions. The error bars indicate the estimated standard deviation assuming a Poisson distribution of microtubule growth times (31).

<table>
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<tr>
<th></th>
<th>Growth rate (μm/min)</th>
<th>Catastrophe frequency (s⁻¹)</th>
<th>Number of catastrophes</th>
<th>Shortening rate (μm/min)</th>
<th>Time observed (in growth) (min)</th>
<th>Number of microtubules</th>
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<tbody>
<tr>
<td>None</td>
<td>1.01 ± 0.22</td>
<td>0.0013</td>
<td>12</td>
<td>−30.1 ± 14.1</td>
<td>159 (156)</td>
<td>22</td>
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<tr>
<td>0.8 μM Op18</td>
<td>0.89 ± 0.23</td>
<td>0.0028</td>
<td>24</td>
<td>−27.1 ± 13.6</td>
<td>151 (145)</td>
<td>27</td>
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<tr>
<td>1.5 μM Op18</td>
<td>0.79 ± 0.15</td>
<td>0.0043</td>
<td>27</td>
<td>−24.7 ± 10.5</td>
<td>109 (105)</td>
<td>29</td>
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<tr>
<td>0.8 μM Op18, Pak1-phosphorylated</td>
<td>1.05 ± 0.25</td>
<td>0.0014</td>
<td>9</td>
<td>−32.2 ± 12.9</td>
<td>113 (110)</td>
<td>19</td>
</tr>
<tr>
<td>2.0 μM Op18, Pak1-phosphorylated</td>
<td>0.99 ± 0.19</td>
<td>0.0013</td>
<td>10</td>
<td>−28.0 ± 12.4</td>
<td>134 (131)</td>
<td>19</td>
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phosphorylated mutant Op18 as indicated. The rates are the means ± S.D. 

<table>
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<tr>
<th>Growth rate</th>
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<td>(μm/min)</td>
<td>(s⁻¹)</td>
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<td>(μm/min)</td>
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<tr>
<td>1.6 μM Op18(S16A)</td>
<td>0.78 ± 0.19</td>
<td>0.0040</td>
<td>25</td>
<td>33.3 ± 15.2</td>
<td>108 (105)</td>
</tr>
<tr>
<td>1.6 μM Op18(S16A), PKA-phosphorylated</td>
<td>0.99 ± 0.12</td>
<td>0.0013</td>
<td>12</td>
<td>33.3 ± 12.6</td>
<td>160 (156)</td>
</tr>
<tr>
<td>1.6 μM Op18(S63A)</td>
<td>0.75 ± 0.12</td>
<td>0.0040</td>
<td>23</td>
<td>26.2 ± 11.1</td>
<td>101 (97)</td>
</tr>
<tr>
<td>1.6 μM Op18(S63A), PKA-phosphorylated</td>
<td>1.00 ± 0.18</td>
<td>0.0015</td>
<td>14</td>
<td>33.2 ± 13.0</td>
<td>162 (158)</td>
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Pak-dependent Phosphorylation Downstream of Rac1 Can Inactivate Op18 in Vivo—Because serine 16 phosphorylation of Op18 downstream of activated Rac1 depended on Pak1 activity (23), we wanted to investigate whether the effects of Rac1 activation on the microtubule cytoskeleton could be linked to Pak-mediated inactivation of Op18 in cells. Specifically, we anticipated that activation of Rac1 might result in inactivation of Op18 and promote an increase in the amount of polymerized tubulin in cells (42). Therefore, we analyzed the ratio of microtubule polymer to tubulin dimer in individual cells (33). Briefly, living PtK1 cells were microinjected with fluorescently labeled tubulin and imaged to determine the total tubulin fluorescence in the cell, comprised of both soluble dimers and assembled microtubules. Tubulin dimers were then extracted with a detergent-containing microtubule-stabilizing buffer, and a second image was acquired to determine the proportion of fluorescent tubulin incorporated into microtubules (Fig. 5A). In control PtK1 cells, 65 ± 5% of the tubulin was incorporated into microtubule polymer, similar to what has been described for interphase porcine kidney epithelial cells (33). Expression of dominant negative Rac1(T17N) had no effect (63 ± 7%), whereas expression of constitutively active Rac1(Q61L) surprisingly somewhat decreased the polymer to dimer ratio (58 ± 5%) (Fig. 5B), likely because Rac1(Q61L) increases microtubule breakage and turnover in PtK1 cells (7). Although Op18 is ubiquitously expressed, its levels are relatively low in many non-neuronal cells. Estimates from immunoblots showed that PtK1 cells contained ~100 ng of Op18/mg of soluble protein (~1:20 to 1:40 Op18:tubulin molar ratio), which is comparable with other cell types (42, 43). Thus, partial inactivation of endogenous Op18 might not be enough to observe a global effect on the microtubule polymer in PtK1 cells.

To determine whether Rac1 was capable of regulating the microtubule cytoskeleton through Op18 in vitro, we therefore increased the intracellular Op18 concentration by microinjection to an estimated concentration of about 10 μM (assuming an average injection volume of 5–10% of the volume of the cell), which is close to the cellular tubulin concentration (Fig. 5C). Injection of such excess Op18 similarly reduced the amount of polymerized tubulin in both control (30 ± 11%) and in active Rac1(Q61L)-expressing cells (36 ± 10%). However, in dominant negative Rac1(T17N)-expressing cells injected with the same amount of Op18, the level of polymerized tubulin was further reduced to only 12 ± 6%, indicating that Op18 is inactivated downstream of Rac1. Furthermore, when we injected active Rac1(Q61L)-expressing cells with phosphorylation site-deficient Op18(S16A), the microtubules were depolymerized to a similar extent (18 ± 8%) as in cells with Op18 and dominant negative Rac1(T17N) (Fig. 5C). This indicates that Rac1 can regulate the microtubule destabilizing activity of Op18 in vitro and that this depends on serine 16.

Based on our in vitro experiments demonstrating that Pak1 phosphorylated and inactivated Op18, we wanted to test whether Pak activity was required downstream of Rac1 to regulate Op18 in vivo. For this purpose, we injected control and phosphorylation in HeLa cells (data not shown) (39). Neither 10 μM KN-62, a selective inhibitor of Ca²⁺/calmodulin-dependent kinases, nor 10 μM H-89, a potent inhibitor of PKA and Rock-II, inhibited EGF-induced Op18 phosphorylation, indicating that these kinases do not phosphorylate Op18 downstream of Rac1. Furthermore, up to 20 μM H-89 had no effect on Rac1(Q61L)-induced microtubule growth in PtK1 cells (data not shown) (7). In addition, 1 mM b-sphosphatidyl serine 16 is not involved.
active Rac1(Q61L)-expressing cells containing fluorescent tubulin with a mutant fragment of Pak1, PBD/ID(H83L), that inhibits the kinase activity of Paks in vivo but cannot sequester activated Rac1 (29). Neither PBD/ID(H83L) alone (61 ± 7%) nor in combination with active Rac1(Q61L) (68 ± 6%) reduced the microtubule polymer level as compared with control cells (Fig. 5D). However, when we injected cells with a mixture of Op18 and PBD/ID(H83L), Pak inhibition decreased the microtubule polymer level in control PtK1 cells to 16 ± 6% and to 20 ± 7% in active Rac1(Q61L)-expressing cells (Fig. 5D), which is significantly less than what we observed in cells injected with Op18 alone and similar to the level in cells with dominant negative Rac1(T17N) and Op18 or active Rac1(Q61L) and Op18(S16A). Together, these results suggest that the microtubule destabilizing activity of Op18 can be inactivated in vivo downstream of Rac1 by Pak-dependent phosphorylation at serine 16.

**DISCUSSION**

In this study, we have investigated the possibility of whether inactivation of the microtubule destabilizing protein Op18 through phosphorylation downstream of Rac1 and Paks could...
be responsible for the Rac1- and Pak-dependent regulation of leading edge microtubule dynamics that we have observed in vitro (7). We find that Op18 is specifically phosphorylated in vitro by Pak1 at serine 16. This specificity of Pak1 in vitro is consistent with the results of Daub et al. (23), who observed Rac1- and Pak-dependent phosphorylation only at serine 16 in EGF-stimulated cells. Phosphorylation by Pak1 is sufficient to down-regulate the inhibitory activity of Op18 on bulk tubulin polymerization as well as its plus end catastrophe promoting activity as demonstrated by measuring dynamic instability parameters of individual microtubules in vitro by digitally enhanced differential interference contrast microscopy.

This differs from other studies in which specific effects of Op18 phosphorylation on microtubule polymerization have only been examined with Op18 protein that had been phosphorylated on multiple sites (19, 21, 35). Specifically, phosphorylation of both serines 16 and 63 by PKA inhibits the catastrophe promoting activity of Op18 in vitro (35). Because Op18 displays a complicated pattern of phosphorylation in vitro (12), it has long been speculated that combinatorial phosphorylation is required to fully inactivate Op18. We find here that, in vitro, monophosphorylation of either serine 16 or 63 is sufficient to inhibit the catastrophe promoting activity of Op18. This is consistent with the observation that overexpression of Ca^2+/calmodulin-dependent protein kinase, which is also specific for serine 16, inhibits the microtubule destabilizing activity of Op18 in cells (20, 44) and that serine 63 phosphorylation of a truncated Op18 reduced its helicity (45). Serine 16 is the only phosphorylation site conserved through all members of the stathmin protein family (12). However, we found that the least conserved of these proteins, neuron-specific RB3, is not phosphorylated by Pak1 or PKA, suggesting that there are functional differences between stathmin-like proteins.

In vivo, we readily detected Op18 phosphorylation at serine 16 downstream of EGF treatment or expression of constitutively active Rac1(Q61L). Constitutively active Pak1(T423E), however, did not phosphorylate Op18 in vivo, although the immunoprecipitated kinase was active in vitro. This suggests that in vivo, additional factors downstream of Rac1 are required for Op18 phosphorylation. This is consistent with our previous observation that constitutively active Pak1 did not have the same effect on microtubule growth as constitutively active Rac1 (7) and observations of other groups that Pak activity alone is often not sufficient to mediate cytoskeletal changes (46, 47). We do not know what additional factors are required for Rac1-mediated Op18 phosphorylation, but our inhibitory studies indicate that PKA and Ca^2+/calmodulin-dependent protein kinase, which can phosphorylate Op18 at serine 16, are not involved. One possibility is that proper localization of activated Pak to the cells leading edge is required (48), which we did not observe with constitutively active forms of Pak.2

Our quantification of cellular microtubule polymer levels showed that Rac1 and endogenous levels of Paks are capable of regulating Op18 activity in vitro. However, we were only able to see Rac1- or Pak-mediated effects on microtubule polymer levels in the presence of excess Op18. At such elevated Op18 levels, similar to the cellular tubulin concentration, tubulin dimer sequestration is likely to be dominant over direct catastrophe promotion, whereas at physiological Op18 concentrations the catastrophe frequency might be regulated very locally through Op18 phosphorylation. Our assay of total cellular microtubule polymer is not sensitive enough to detect such differences. In addition, Rac1 activation also increases microtubule turnover and minus end depolymerization caused by increased retrograde flow and microtubule breakage, effectively lowering the total amount of microtubule polymer (7, 49). This might also explain why the amount of microtubule polymer in the presence of excess Op18 was only slightly increased in Rac1(Q61L)-expressing cells as compared with control cells.

The notion of local Op18 regulation is also consistent with the relatively small fraction of endogenous Op18 that becomes phosphorylated after EGF stimulation (our results and Ref. 23), which would not be expected to have a major impact on global microtubule dynamics. In fact, we were not able to observe an obvious effect of EGF on microtubule dynamics in PK1 cells.2 Interestingly, a novel FRET probe to monitor Op18-tubulin association in living cells has been used to show that less Op18 is bound to tubulin in the leading edge because of locally enhanced phosphorylation.3 Indeed, in a normal migrating cell, only a small subset of microtubules, referred to as “pioneer” microtubules, exhibits a decreased catastrophe frequency resulting in increased net growth into the protruding edge of the cell (7, 27). This suggests that local regulation of a fraction of Op18 by active Rac1 and Pak at the leading edge could lead to regional pioneer microtubule behavior (48, 50). Pioneer microtubule behavior is observed in most microtubules in cells expressing constitutively active Rac1(Q61L) (7) consistent with a more global inactivation of a microtubule polymerization inhibitor such as Op18.

However, other mechanisms downstream of Rac1 and/or Pak are likely involved in the regulation of leading edge microtubule dynamics, because Pak activity is necessary but not sufficient for Rac1-mediated promotion of microtubule growth (7). Dynamic interactions between the microtubule and actin cytoskeleton might be required for pioneer microtubule behavior to occur (11). A different possibility is that Rac1 and Pak-mediated inactivation of Op18 initiates microtubule growth by locally lowering the catastrophe frequency, which could then be sustained by further microtubule stabilization through association of plus end-binding proteins such as CLIP-170, EB1, or APC (40, 51–54). Because inhibitors of protein kinase C and GSK3β, which have been suggested to be involved in the stabilization of microtubules through plus end-binding proteins (40, 41) had no effect on Op18 phosphorylation, it is likely that these are parallel pathways regulating microtubule organization in migrating cells.

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Rac1 Regulation of Op18/Stathmin

The movie shows a direct comparison of in vitro microtubule dynamic instability in the presence of 1.5 uM Op18/stathmin (left) or 2.0 uM Pak1-phosphorylated Op18/stathmin (right). Microtubules were nucleated from sea urchin axonemes at 10 uM tubulin concentration and observed by digitally enhanced differential interference contrast microscopy immediately after the sample was warmed to 37 °C on the microscope stage. In the presence of Op18/stathmin microtubules undergo frequent catastrophes, while they mostly grow when Op18/stathmin was first phosphorylated with Pak1. Elapsed time is indicated in minutes:seconds.