Decreasing oncoprotein 18/stathmin levels reduces microtubule catastrophes and increases microtubule polymer in vivo

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SUMMARY

Oncoprotein 18/stathmin (Op18) has been identified recently as a protein which destabilizes microtubules. To characterize the function of Op18 in living cells, we used microinjection of anti-Op18 antibodies or antisense oligonucleotides to block either Op18 activity or expression in interphase newt lung cells. Anti-tubulin staining of cells microinjected with anti-Op18 and fixed 1-2 hours after injection showed an increase in total microtubule polymer. In contrast, microinjection of either non-immune IgG or anti-Op18 preincubated with bacterially-expressed Op18 had little effect on microtubule polymer level. Cells treated with Op18 antisense oligonucleotides for 4 days had ≥50% reduced levels of Op18 with no change in the soluble tubulin level. Measurement of MT polymer level in untreated, antisense or nonsense oligonucleotide treated cells demonstrated that reduced Op18 levels resulted in a 2.5-fold increase in microtubule polymer. Next, the assembly dynamics of individual microtubules at the peripheral regions of living cells were examined using video-enhanced contrast DIC microscopy. Microinjection of antibodies against oncoprotein 18 resulted in a 2.2-fold reduction in catastrophe frequency and a slight reduction in plus end elongation velocity compared to uninjected cells or cells microinjected with non-immune IgG. Preincubation of anti-Op18 antibody with recombinant Op18 greatly diminished the effects of the antibody. Similarly, treatment of cells with antisense oligonucleotides reduced catastrophes 2.5- to 3-fold compared to nonsense oligonucleotide treated or untreated cells. The other parameters of dynamic instability were unchanged after reducing Op18 with antisense oligonucleotides. These studies are consistent with Op18 functioning to regulate microtubule catastrophes during interphase in vivo.

Key words: Cytoskeleton, Microtubule assembly, Dynamic instability, Newt lung cell

INTRODUCTION

Microtubules (MTs) are dynamic cytoskeletal polymers; in vivo these polymers continually exchange subunits with a soluble pool of tubulin heterodimers through mechanisms of dynamic instability and tubulin flux or treadmilling within MTs (reviewed by Desai and Mitchison, 1997; Waterman-Storer and Salmon, 1997b). Dynamic instability, the stochastic switching between phases of MT growth and shortening, has been observed most clearly at the peripheral regions of cells (Cassimeris et al., 1988; Sammak and Borisy, 1988). Treadmilling of tubulin subunits within MT polymers has also been observed in vivo, and both dynamic instability and treadmilling likely contribute to MT turnover in the cell (Rodionov and Borisy, 1997; Waterman-Storer and Salmon, 1997a; Yvon and Wadsworth, 1997).

Each of these MT turnover pathways may be regulated by accessory proteins (Simon et al., 1992; Rodionov et al., 1999). For example, the properties of purified brain tubulin are insufficient to generate the fast elongation and the frequent catastrophes of plus end MT dynamic instability measured in vivo (Simon et al., 1992), suggesting that accessory proteins are necessary to destabilize MTs and increase turnover. One protein which could speed MT turnover is oncoprotein 18/stathmin (Op18; also termed p19, metablastin, and prosolin; Belmont et al., 1996), a recently identified protein which destabilizes MTs (Belmont and Mitchison, 1996). Studies with purified components in vitro have demonstrated that Op18 can stimulate MT catastrophes (the switch from growth to shortening; Belmont and Mitchison, 1996) or sequester tubulin dimers (Curmi et al., 1997; Jourdain et al., 1997). These studies are not contradictory because we find that Op18 has both these activities, and that they can be separated in vitro by small changes pH or deletion of regions within Op18 (Howell et al., 1999). Either of these functional activities could contribute to MT destabilization in vivo.

The ability of Op18 to destabilize MTs in cells has been measured primarily by changes in total MT polymer after increasing or decreasing the level or activity of Op18. Increasing Op18 levels, by over-expression or microinjection,
resulted in decreased MT polymer levels during interphase, consistent with Op18 destabilizing MTs (Marklund et al., 1996; Horowitz et al., 1997). During mitosis, MT polymer level was unaffected by increasing Op18 levels, suggesting that the phosphorylation of Op18 observed during mitosis turns off the MT destabilizing activity of Op18 (Larsson et al., 1997). Phosphorylation was also used to demonstrate the consequences of Op18 inactivation during interphase. Overexpression of a constitutively active Ca/calmodulin dependent protein kinase IV/G or the catalytic subunit of PKA resulted in phosphorylation of Op18 and an increased level of MT polymer (Melander Gradin et al., 1997, 1998). In both these phosphorylation studies, mutants of Op18 lacking specific phosphorylation sites were used to demonstrate that Op18 inactivation was responsible for the increased MT polymer. In summary, studies of MT polymer level in vivo are consistent with a MT destabilizing function for Op18: excess Op18 reduces MT polymer level, while Op18 inactivation raises MT polymer level.

To date, the consequences of Op18 inactivation on MT assembly dynamics have not been measured in living cells. Here we describe changes in MT polymer level and MT plus end dynamic instability in newt lung cells after inactivation or depletion of Op18 and demonstrate that Op18 regulates both MT polymer level and plus end catastrophes in vivo.

**MATERIALS AND METHODS**

**Cell culture**

Primary cultures of newt lung cells were grown on coverslips in chambers (see Rose et al., 1958) at room temperature (23°C) as previously described (Rieder and Hard, 1990; Howell et al., 1997). For microinjection studies, cells were typically used four to six days previously described (Rieder and Hard, 1990; Howell et al., 1997). For microinjection studies cells were typically used four to six days after each culture had begun. LLC-PK1 and NIH 3T3 cells were grown in DMEM supplemented with 10% fetal calf serum (LLC-PK1) or calf serum (NIH 3T3). Cells were maintained at 37°C in a 5% CO2 environment.

**Preparation of recombinant Op18**

All DNA isolations and manipulations were performed as previously described (Sambrook et al., 1989). All restriction enzymes, T4 DNA ligase, alkaline phosphatase and T4 polynucleotide kinase were obtained from Boehringer Mannheim and/or New England Biolabs and used according to the specifications of the manufacturer. Taq polymerase was obtained from Perkin Elmer/Cetus and Vent polymerase was purchased from New England Biolabs.

The DNA encoding the open reading frame (ORF) of the Xenopus Op18 homolog XO35A (Maucuer et al., 1993) was obtained from a λUni-ZAP Xenopus ovary cDNA library (Stratagene, La Jolla, CA) using PCR. Specifically, a fragment encoding the XO35A ORF was amplified with Taq polymerase using the primers 5¢-CTCCAGCTCTTTCACCTG-3¢ and 5¢-CCTTGACTGTAGTTCTGG-3¢. The resulting products were then reamplified using Vent polymerase and primers corresponding to the N and C termini of the ORF. The primers also introduced the unique restriction sites necessary for subsequent manipulations. The products were digested with BamHI and EcoRI and ligated into pBSKS+II creating the plasmid pBSKS+·XO35A. The insert was sequenced and shown to be identical to that of the published XO35A sequence. The pGEX-2T expression vector (Phar-macia) was modified for insertion of the Op18 ORF. A fragment was removed from pGEX-2T by digestion with BamHI and EcoRI, and the polylinker 5¢-GATCTCATATGGCCTCCGGGAGT-3¢ was inserted into that site creating pGEX-2T(NP). The ORF of Op18 was then retrieved from pBSKS+·XO35A by digestion with NdeI and EcoRI and ligated in frame into pGEX-2T(NP) creating pGEX2T(NP)-XO35A. GST-Op18 was expressed in the E. coli strain DH5-α(BRC) carrying pGEX2T(NP)-XO35A and GST-Op18 was purified using published protocols (Auszubel et al., 1996). For antibody inhibition experiments, thrombin-cleaved (Sigma) protein was loaded onto a HiTrap Q column (Phar-macia) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and >99% pure Op18 was recovered in the flow through. For affinity purification (below), the ORF was retrieved from pBSKS+·XO35A by digestion with NdeI and EcoRI and ligated in frame into the expression vector PET28 to create pET28-XO35A. His6-Op18 was expressed in the E. coli strain BL21(pLysS) (Auszubel et al., 1996) and purified on nickel resin according to the specifications of the manufacturer (Qiagen).

**Preparation of rabbit antiserum and affinity purification of anti-Op18 sera**

Rabbits were immunized with 1 mg recombinant GST-Op18 fusion protein every third week (Berkeley Antibody Company). Antibodies were affinity purified against His-Op18 fusion protein coupled to Affi-gel-10 (Bio-Rad) using standard procedures (Harlow and Lane 1988). The eluted antibodies were dialyzed against 1× PBS. For use in microinjection, the antibodies were simultaneously exchanged into microinjection buffer (20 mM sodium glutamate, 1 mM EGTA, 0.5 mM MgSO4) and concentrated to 3 mg/ml in a Centricon-30 (Amicon).

**Microinjection**

Newt lung cells at the peripheral regions of the cell sheet were microinjected with one of the following: affinity-purified anti-Op18 (2.5-3 mg/ml); purified rabbit non-immune IgG (4.2 mg/ml), or anti-Op18 preincubated with recombinant Xenopus Op18 protein at a molar ratio of 1 antibody:2 antigen. Cells were injected on an Olympus CK2 inverted microscope with attached Narishige MN-151 manipulator, using a capillary holder and prepulled glass microtins (ID 0.5 μm purchased from either World Precision Instruments, Inc. Sarasota, FL or Eppendorf, Inc, Madison, WI). A 60 cm3 syringe was used to provide air pressure. Cells were injected, incubated for 1-2 hours, and then individual MTs were followed for 2-5 minutes using video-enhanced differential-interference contrast (VE-DIC) light microscopy, as described below. We have not measured the amount of material injected into cells, but injected volumes are likely consistent with previous estimates of 5-10% of cell volume (Grassman et al., 1980; Saxton et al., 1984). The cells injected with non-immune rabbit IgG likely received a greater intracellular concentration of IgG since this was present at a higher needle concentration than the anti-Op18 antibody.

**Antisense depletion of Op18**

Newt lung cells were depleted of Op18 using an antisense oligonucleotide sequence used previously (Di Paolo et al., 1996). The nonsense sequence used as a control was also identical to that used previously by Di Paolo et al. (1996). Primary cultures were allowed to grow on coverslips for 4-7 days and then treated with 10 μM HPLC-purified Op18 antisense (5¢-CTCCAGCCTTCCTTCCAG-3¢) or nonsense (5¢-CTCCAGCTTCCTTCCAG-3¢) phosphorothioate oligonucleotides (PONs) for 24 hours. 5 μM PONs were then added every 24 hours to respective cultures for 3 additional days (Di Paolo et al., 1996). Di Paolo et al. (1996) previously demonstrated that this 4 day time course was necessary to reduce Op18 by 75% in the antisense-treated cells. After this 4 day treatment, coverslips were then processed for anti-tubulin immunofluorescence and measurement of MT polymer (below). For some coverslips, MT dynamic instability was measured using video-enhanced DIC light microscopy (VE-DIC, below) in living cells, prior to fixation and anti-tubulin immunofluorescence.
To detect changes in Op18 or soluble tubulin levels after antisense or nonsense PONs treatment, lung tissue pieces (identical to those used for culture on coverslips) were incubated in culture medium with the same concentrations of PONs described above and examined by immunoblotting (described below). The soluble tubulin fraction was isolated by lysis in a cytoskeleton stabilizing buffer (Marklund et al., 1996; Minotti et al., 1991), the cytoskeletons were pelleted (12,000 rpm, 10 minutes) and the soluble fraction was used for immunoblotting.

**Video enhanced DIC light microscopy**

Coverslips were mounted onto glass slides using small pieces of paraffin as spacers, the chamber was then filled with culture medium and sealed with valap (1:1:1 mixture of petrolatum, lanolin, and paraffin). VE-DIC microscopy was used to visualize individual MTs in newt lung cells as previously described (Howell et al., 1997; Vasquez et al., 1994).

**Immunofluorescence**

After MTs were visualized in living cells, coverslips were removed from the microscope slide and rinsed in phosphate-buffered saline (PBS: 0.171 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4, pH 7.3). Coverslips were fixed for 5 minutes in 0.5% glutaraldehyde/PEM (100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM MgSO4), lysed in 0.5% NP-40/PEM for 15 seconds, and then washed in PBS. For further extraction, the coverslips were incubated in −20°C methanol for 5 minutes, and −20°C acetone for 1 minute, rehydrated in PBS, and then blocked with 10% fetal bovine serum (in PBS) for 30 minutes in a humid chamber at 37°C. Coverslips were rinsed with PBS and incubated for 2 hours at 37°C with 45 μl of a 1:20 dilution of a mouse monoclonal anti-beta tubulin antibody, either E7 (Developmental Studies Hybridoma Bank, Univ. of Iowa, Iowa City, IA) or DM1A (Sigma). The coverslips were then rinsed with PBS and incubated for 2 hours at 37°C with 45 μl of a 1:10 dilution of rhodamine-conjugated goat anti-mouse IgG to stain MTs. For some cells, injected antibodies were localized with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Coverslips were rinsed again with PBS and mounted with Mowiol (Calbiochem). Cells were photographed with a Nikon 60×/1.4 NA Plan Apo objective on a Nikon Optiphot microscope equipped for epifluorescence. Additional coverslips were fixed and stained without prior VE-DIC observations.

For quantitation of MT polymer, untreated or PONs treated cells were lysed prior to fixation and then examined by anti-tubulin immunofluorescence, with the following modifications: 20% donkey serum (Molecular Probes) was used to block nonspecific binding and a 1:20 dilution of the rhodamine-conjugated donkey anti-mouse IgG Cy3, (Jackson Labs) was used as the secondary antibody. Cells were examined with a 63×/1.4 NA objective on an inverted Leica DMIRB microscope (Leica Mikroskopie und Systeme GmbH, Germany). The excitation light was attenuated to 50% using neutral density filters and the images were projected to a cooled CCD camera (Precision Instruments, NJ) driven by Image-1/MetaMorph imaging software (Universal Imaging Corporation, PA). The images were acquired and stored as 16 bit images. Images were then used to measure relative amounts of MT polymer by measuring pixel intensity within a 30 × 30 μm box. Four measurements were made for each cell and 43-57 cells were measured for each treatment. Background intensity was estimated from several intensity measurements in regions of the coverslip that did not contain any cells and this intensity was always less than 10% of the intensity within stained cells. Background intensity was subtracted from each measurement and the data is reported as mean intensity ± s.d.

**Comparison of MT polymer levels by immunofluorescence and immunoblotting**

To determine whether fluorescence intensity measurements from single cells (above) detects changes in MT polymer level, changes in MT polymer were measured after incubation with taxol by both the immunofluorescence method described above and by anti-tubulin immunobots of isolated cytoskeletons. NIH 3T3 cells were incubated overnight in medium supplemented with 4 nM taxol. Cells were then either lysed and fixed for immunofluorescence (above) or cytoskeletons were isolated using methods described previously (Marklund et al., 1996; Minotti et al., 1991). The cytoskeletons were resuspended in SDS sample buffer and proteins separated by SDS-PAGE. Methods for immunoblotting are described below. Measurement of immunofluorescent intensity was performed essentially as described above, except that the boxes were smaller (10 μm × 10 μm) due to the decreased size of NIH 3T3 cells compared to newt lung cells. Cells were examined with a 60×/1.4 NA objective and 1.5× optivar on a Nikon Microphot-FXA. Imaging methods were identical to those described above except that a Hamamatsu cooled CCD camera (#C4880) was used. To compare changes in total polymer by the two methods (Fig. 6C), intensity measurements were normalized to 100 for untreated cells.

**MT tracking and data analysis**

MT growth and shortening velocities were measured by tracking the ends of MTs over time using the RTM software written by Salmon and colleagues (Glikson et al., 1992). MT lengths were measured relative to an arbitrary point on the video screen and therefore do not represent total MT length from the centrosome. The growth and shortening velocities were determined from the slopes of the length vs. time plots. Velocities were compared using analysis of variance (ANOVA) provided by Microsoft Excel. Growth and shortening times were determined from the total time spent in growth or shortening for a particular treatment, divided by the number of catastrophes or rescues observed (Walker et al., 1988). The inverse of this number is typically reported as the catastrophe or rescue frequency (Walker et al., 1988). Standard deviations for transition frequencies were determined from the catastrophe and rescue frequency divided by the square root of the number of transitions observed (Walker et al., 1988). Since only a mean and standard deviation are determined for transition frequencies, we compared means using a Student’s t-test for two means with unequal variance (95% confidence level; Pollard, 1977). This calculation assumes a Poisson distribution of growth times (Pollard, 1977).

**Tubulin and anti-Op18 binding to immobilized Op18**

6×His-Op18 (1.5 μg) was incubated with 3 μl TALON metal affinity resin (Clontech Laboratories, Palo Alto CA) for 30 minutes at room temperature. This quantity of resin was sufficient to bind at least 10-fold more 6×His-Op18 (not shown). Tubulin (8 μg) and/or anti-Op18 (14-27 μg) were added to the reaction mixture and samples incubated an additional 30 minutes at room temperature. The final volume for each mixture was 100 μl and the molar ratio of the proteins was approximately 1 mole Op18: 0.5 moles tubulin: 1-2 moles anti-Op18. All samples were prepared in P/M buffer (100 mM Pipes, pH 6.8, 1 mM MgSO4) lacking EGTA to avoid chelation of the metal ions of the affinity resin. Bound proteins were separated by pelleting (1.5 minutes, 12,000 rpm, room temperature) through a 400 μl glycerol cushion (25%, v/v, glycerin in P/M). Sedimented beads were heated in SDS-sample buffer, eluted proteins were separated on 10% SDS-PAGE and visualized by staining with Coomassie blue.

**SDS-PAGE and immunoblotting**

Whole cell extracts from newt lung tissue were prepared by mincing the lungs, rinsing them 3× with PBS, and then lysing on ice for 30 minutes in lysis buffer (PEM buffer supplemented with 0.05% NP-40 and 0.5 mM PMSE). The lysate was pelleted for 15 minutes at 12,000 rpm, 15°C and the protein concentration of the soluble fraction determined using the Bio-Rad Protein Assay reagent and IgG as a standard. Proteins were separated on 10% or 3-12.5% SDS-PAGE,
transferred to PVDF membranes at 200 mA for 1 hour (for Op18) or 2 hours (for tubulin), and blocked 1 hour overnight in 5% nonfat dry milk/TrisBuffered Saline Tween (0.9% NaCl, 0.1% Tris, 2% Tween-20). The membrane was incubated for 5 hours-12 hours with either rabbit anti-
Op18 (1:1000 dilution) or mouse anti-α-tubulin (DM1A (Sigma) 1:3000 dilution). Membranes were rinsed in TBS-Tween, and incubated for 2 hours with either anti-mouse or anti-rabbit peroxidase conjugated antibodies. After several washes in TBS-Tween, the membranes were developed using ECL (Amersham). The staining intensity of Op18 was compared to that produced by a concentration range of bacterially expressed Op18 (10-400 ng; concentration determined by amino acid composition; Brattsand et al., 1993). The detection limit was 10 ng Op18. The integrated intensities of immuno-reactive bands were also measured using a Foto/Analyst Image Analysis System (Fotodyne) and Scion Image software (NIH Image and Scion Corp.) run on a Power Macintosh 7100 computer.

RESULTS

Op18 is expressed in newt lung tissue

We determined whether cells from newt lung tissue expressed Op18 by immunoblotting with anti-Op18 antibodies. Fig. 1A shows a silver-stained gel of newt lung proteins and bacterially expressed recombinant Xenopus Op18. As shown in the corresponding immunoblot (Fig. 1B), the anti-Op18 antibody recognized both the recombinant protein and an 18 kDa band in newt lung cells. The antibody also recognized an approximate 50 kDa band in newt lung cells but the nature of this cross-reactive protein is unknown. Xenopus extracts probed with this antibody also show a cross-reacting band of approximately the same size (HD, unpublished observations). In Xenopus, the 50 kDa antigen neither co-pellets with MTs nor co-fractionates with tubulin in a sucrose gradient (HD, unpublished observations) suggesting that this 50 kDa antigen does not modify MT assembly. Immunoblots were used to estimate the Op18 concentration in newt lung tissues. On average, lung tissue contained approximately 250 ng Op18 per mg total soluble protein (±140 ng/mg). This estimate is qualitative since the amount of Op18 was only 2-3 times the detection limit (10 ng). The estimated value is similar to previous measurements of Op18 concentration in non-transformed, non-embryonic cells (120-330 ng/mg for several different cell types; Brattsand et al., 1993), but lower than the Op18 level in Xenopus egg extracts (1000 ng/mg; Belmont and Mitchison, 1996).

Neither secondary antibody alone (data not shown) nor purified rabbit IgG (also used for microinjection studies) recognized the recombinant Op18 protein or any other protein in the lung tissue (Fig. 1B).

Microinjection of anti-Op18 into newt lung cells increases MT polymer

To examine the consequences of Op18 inactivation on MT polymer in interphase cells, we first microinjected antibodies to Op18 and examined MT polymer by anti-tubulin immunofluorescence. Additional cells were injected with either purified rabbit IgG or anti-Op18 preincubated with bacterially expressed Op18. Cells at the periphery of the cell sheet were microinjected with antibodies, incubated at room temperature for 1-2 hours, fixed, and stained with a mouse monoclonal antibody to tubulin. Injected cells were identified by one of two ways: by their position relative to the tissue explant, or by localization of the injected rabbit IgG with fluorescently labeled anti-rabbit IgG (see Fig. 3B). Control experiments demonstrated that the microinjection procedure and the presence of non-immune rabbit IgG did not alter MT organization or MT polymer level in cells (Fig. 2A-D).

Microinjection of anti-Op18 resulted in an increase in MT polymer compared to uninjected cells (Fig. 2). The increase in MT polymer is especially evident at the peripheral regions of injected cells, but a large increase in fluorescence was also noted around the nuclei. Often it is not possible to distinguish individual MTs near the nucleus, but the bright fluorescence likely represents a high density of MTs since the staining pattern is unaltered in cells lysed prior to fixation. Occasionally we observed that MTs in anti-Op18 injected cells appeared more rigid since they were often straighter than the curved MTs of uninjected cells. Injected cells were both epithelial cells (attached to each other in a cell sheet) and fibroblasts (Rieder and Hard, 1990), but the increase in MT polymer after anti-Op18 injection was observed in both cell types. Microinjection of anti-Op18 preincubated with bacterially-expressed Op18 resulted in MT polymer levels similar to that observed in uninjected cells (data not shown). Finally, the observed increase in MT polymer after anti-Op18 injection was not unique to newt cells since LLC-PK epithelial cells injected with anti-Op18 also showed an increase in MT polymer (data not shown).

Anti-Op18 inhibits tubulin binding to Op18

Op18 binds to tubulin dimers in vitro (Belmont and Mitchison 1996; Curmi et al., 1997; Jourdain et al., 1997; Howell et al., 1999). The anti-Op18 antibody blocks this interaction between Op18 and tubulin. 6×His-Op18 was first bound to a metal affinity resin and then incubated with purified tubulin or tubulin plus anti-Op18. The bound proteins were then isolated by a centrifugation (Materials and Methods). Tubulin bound
Oncoprotein 18 regulates catastrophes in vivo

the immobilized 6×His-Op18 at a maximum binding of approximately 2 moles tubulin to 1 mole Op18 (data not shown), consistent with several previous studies (Curmi et al., 1997; Jourdain et al., 1997; Howell et al., 1999). Anti-Op18 also bound the immobilized 6×His-Op18 and could prevent tubulin association with Op18 (Fig. 3A). It has been suggested that Op18 may also interact transiently with MT ends (Howell et al., 1999; McNally, 1999) but this has not been detected and it is not known whether the antibody could block this potential interaction. When injected into cells, the antibody did not show localization to any cellular structures and instead appeared soluble (Fig. 3B).

Op18 levels are reduced by antisense PONs

The anti-Op18 antibody used above also reacts with a second protein on denaturing gels. Although this second protein does not

Fig. 2. Injection of antibodies to Op18 increases MT polymer. Newt lung cells were injected with anti-Op18 and fixed 1-2 hours later. Anti-tubulin immunofluorescence micrographs of uninjected cells (A,B), cells injected with non-immune rabbit IgG (C,D) or injected with anti-Op18 (E,F). Arrowhead in F denotes an uninjected cell. Fibroblasts are shown in A, C and D; epithelial cells in B, D and F. Micrographs from the same experiment were printed with identical exposures (A and E; B and F). Micrographs C and D were printed to best match the contrast of the other micrographs. Bar, 50 μm.

Fig. 3. (A) Anti-Op18 blocks tubulin binding to Op18. 6×His-Op18 was bound to TALON metal affinity resin and incubated with tubulin (0.5 mole tubulin: 1 mole Op18) with or without anti-Op18 (1 mole Op18: 1 mole Op18) as indicated. Bound proteins were separated by centrifugation through a 25% glycerol cushion and analyzed by SDS-PAGE followed by detection of proteins by Coomassie blue staining. Positions of Op18, antibody heavy chain (HC) and α tubulin are shown. Positions of mw markers are given in kDa. (B) Microinjected anti-Op18 is soluble. Cells were fixed 1-2 hours after anti-Op18 injection and stained with fluorescein-labeled anti-rabbit IgG. The cell shown was also stained for tubulin (see Fig. 2E). Bar, 50 μm.
Depletion of Op18 by antisense PONs increases MT Polymer

Antisense PONs decrease Op18 expression in newt lung tissue. (A) Anti-Op18 immunoblot of newt lung tissue prepared for electrophoresis after 4 day incubations with antisense or nonsense PONs as described in Materials and Methods. The positions of Op18 and a second cross-reactive band at 50 kDa are marked. Each lane was loaded with 80 μg protein. (B) Corresponding anti-tubulin immunoblot of the soluble tubulin pool. Each lane was loaded with 17 μg total protein. Untreated (U), antisense PONs (AS) and nonsense PONs (NS) treated samples are as indicated for both A and B.

The amount of MT polymer in antisense and nonsense PONs treated newt lung cells was then determined by measuring fluorescence intensity within stained cells. Images were acquired and the mean fluorescence intensity was then measured within four 30 μm × 30 μm boxes (see Materials and Methods). For each cell, three measurements were made at random sites within the lamellar region and one measurement was made closer to the nucleus as depicted in Fig. 6A. Based on the average fluorescence intensity within each cell, antisense treatment resulted in a 2.5-fold increase in MT polymer compared to untreated or nonsense treated cells. Similar increases in MT polymer were obtained by separately comparing intensity measurements at sites near the nucleus, or at sites in the lamella region. This suggests that the increase in MT polymer occurred throughout the cell.

Since the microscopic method used above had not been used previously to measure MT polymer level, we compared the microscopic method to a more conventional assay using immunoblotting to measure the amount of tubulin in the cytoskeleton fraction. For these experiments, cells were incubated overnight in medium supplemented with 4 nM taxol prior to fixation or isolation of cytoskeletons (Materials and Methods). We used the NIH 3T3 cell line for these experiments because it was difficult to isolate a cytoskeleton fraction from lung tissue. As shown in Fig. 6C, the two methods gave similar results and showed that 4 nM taxol generated an approximate 2.5-fold increase in MT polymer.

Real-time analysis of microtubule dynamics in newt lung cells

In newt lung cells, individual MTs at the periphery of epithelial or fibroblast cells can be visualized using VE-DIC microscopy (Cassimeris et al., 1988; Howell et al., 1997). We examined MT dynamic instability in this region of the cell after injection with antibodies or incubation with PONs. Microinjection of non-immune rabbit IgG had little effect on MT assembly in these cells since the parameters of dynamic instability were virtually unchanged compared to un.injected cells (Table 1). Most importantly, catastrophe frequency is unaffected by non-immune rabbit IgG since MTs underwent a catastrophe, on average, once every 63 seconds, similar to the frequency observed in un.injected cells (69 seconds). Note that the mean elongation velocity is also equivalent in untreated cells and cells injected with non-immune IgG (4.9 μm/minute vs 5.1 μm/minute; Table 1). These results demonstrate that neither the microinjection procedure, nor the presence of rabbit IgG, affected MT assembly dynamics.

In contrast, MT dynamic instability was altered in cells microinjected with anti-Op18 1-2 hours prior to observation. The most striking change in dynamic instability was the decreased frequency of catastrophe in anti-Op18 injected cells compared to either un injected cells or cells injected with non-
immunoglobulin G (IgG) (Table 1). Anti-Op18 injection decreased catastrophe frequency approximately 2.2-fold, from 1 catastrophe every 69 seconds in uninjected cells to 1 catastrophe every 154 seconds in anti-Op18 injected cells. The difference in these means is statistically significant ($P<0.05$). Microinjection of anti-Op18 also decreased elongation velocity to a mean of 3.5 mm/minute (Table 1). This reduction is statistically significant ($P<0.05$) when compared to either uninjected cells or cells injected with non-immune IgG. The other parameters of dynamic instability were affected to a lesser extent by the presence of the antibody.

To confirm that the above observations were due to decreased availability of Op18, we microinjected additional cells with antibody to Op18 that had been preincubated with bacterially-expressed Op18 (Ab + Ag). Preincubation of anti-Op18 with recombinant Op18 greatly reduced the effects of the antibody on dynamic instability (Table 1). Microinjection of Ab + Ag resulted in average MT growth times of 87 seconds, slightly longer than average growth times in control and non-immune IgG injected cells (63-69 seconds), but the average 87 second growth time is still approximately 2 times shorter than the 154 second average growth time in anti-Op18 injected cells

### Table 1. Reduced Op18 alters dynamic instability in newt lung cells

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<td>0.0115</td>
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<td>s.d.</td>
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<td>Kres (s⁻¹)</td>
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<tr>
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<td>12</td>
<td>11</td>
<td>9</td>
<td>14</td>
<td>9</td>
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Parameters of MT dynamic instability were measured at the periphery of living cells as described in Materials and Methods. Ve, elongation velocity; Vrs, shortening velocity; Kcat, catastrophe frequency; Kres, rescue frequency; n, number of MTs or transitions; Time obs, total time MTs observed in elongation or shortening.
Preincubation of the antibody with Op18 also resulted in elongation velocities, shortening velocities, and rescue frequencies similar to uninjected cells (Table 1).

Finally, we followed MT dynamic instability in cells treated with antisense or nonsense PONs. Incubation with nonsense PONs had little effect on MT dynamic instability, although catastrophes were reduced to once every 82.6 seconds compared to 69 seconds in untreated cells (Table 1). Treatment with antisense PONs had a much larger effect on MT catastrophe frequency; catastrophes were only observed once every 204 seconds (Table 1). This average growth time was significantly different from that measured in untreated or nonsense PONs treated cells ($P<0.05$). MT elongation and shortening velocities in cells treated with antisense PONs were comparable to untreated or cells treated with nonsense PONs (Table 1). Similarly, rescue frequency in the antisense PONs treated cells was similar to that measured in untreated or nonsense PONs treated cells.

**DISCUSSION**

**Reduced Op18 resulted in increased MT polymer**

Microinjection of antibodies to Op18, or incubation with Op18 antisense PONs, resulted in an increase in interphase MT polymer (Figs 2, 5 and 6). Measurement of relative MT polymer levels in untreated, antisense and nonsense PONs treated cells showed that reduction in Op18 level resulted in a 2.5-fold increase in MT polymer (Fig. 6). Our studies are consistent with converse experiments showing a decrease in MT polymer upon over-expression or microinjection of Op18 (Marklund et al., 1996; Horwitz et al., 1997). Our observations are also consistent with several recent studies that inactivated Op18 by over-expression of specific kinases, resulting in increased MT polymer (Melander Gradin et al., 1997, 1998). Measurement of MT polymer levels showed that expression of a constitutively active PKA in K562 cells increased MT polymer by 1.8-fold (Marklund, 1998). The similar increase in MT polymer in K562 leukemia and newt lung cells is unexpected because Op18 levels are 4-fold higher in K562 leukemia cells ($1100$ ng/mg; Brattsand et al., 1993). The difference in Op18 concentration in these different cell types suggests that other factors may contribute to limit the maximum MT polymer gain per cell. This interpretation is limited since we measured Op18 concentration in lung tissue and therefore do not know whether different cell types within the tissue express Op18 to different levels.

**Op18 promotes MT plus end catastrophes in interphase newt lung cells**

Microinjection of interphase newt lung cells with affinity-purified anti-Op18 resulted in a 2.2- to 2.4-fold decrease in MT catastrophe frequency compared to uninjected cells or cells injected with non-immune rabbit IgG (Table 1). Consistent with the microinjection studies, treatment of newt lung cells with antisense PONs resulted in a 2.5- to 3-fold decrease in catastrophe frequency compared to untreated or nonsense PONs treated cells (Table 1). Since two independent methods to reduce either Op18 availability or Op18 protein level yielded similar results, it is likely that each set of observations resulted from Op18 inactivation/depletion. These changes in catastrophe frequency measured in tissue cells are consistent with observations in meiotic *Xenopus* egg extracts; immunodepletion of Op18 resulted in a 1.5- to 5-fold decrease in catastrophe frequency (Tournebize et al., 1997).

Inactivation or depletion of Op18 had little affect on the other parameters of dynamic instability, with the exception of elongation velocity (Table 1). Microinjection of antibodies to Op18 resulted in a small, but statistically significant, reduction in MT elongation velocity. In contrast, no decrease in elongation velocity was observed after Op18 antisense PONs treatment and it is possible that the different time courses of the two experiments were responsible for the different effects on elongation rates. Microinjected cells were examined 1-2 hours after injection and these cells show increased MT polymer at this time. It is possible that a portion of the tubulin pool had been depleted to generate the additional MT polymer within this short time interval. A reduction in the tubulin pool may result in changes in MT growth rate (although it is not known whether MT elongation rates in vivo are dependent on soluble tubulin concentration as discussed below). In contrast, the antisense PONs treatment lasted four days and the cells had a tubulin pool similar to untreated or nonsense PONs treated cells. In agreement with the Op18 antisense PONs experiments, immunodepletion of Op18 from meiotic *Xenopus* egg extracts also did not result in any change in MT elongation rate (Tournebize et al., 1997).

Based on observations of MT dynamic instability in living cells or egg extracts, it appears that Op18 functions to increase MT catastrophes in vivo with little or no change in MT growth rate. The mechanism responsible for Op18-mediated catastrophes could result either from a direct stimulation of catastrophes at MT tips (Belmont and Mitchison, 1996; Howell et al., 1999) or through a tubulin sequestering mechanism (Curmi et al., 1997; Jourdain et al., 1997). Differentiating between these mechanisms is difficult since either mechanism would predict an increase in MT polymer and a decrease in MT catastrophes after Op18 inactivation. The two mechanisms can be distinguished in vitro based on changes in elongation rate: a sequestering protein would bind tightly to tubulin dimers and result in a slower rate of elongation, while a catastrophe promoter would not bind tightly to tubulin dimers and therefore would not slow elongation (Howell et al., 1999). It is important to note that Op18 has both tubulin sequestering and MT catastrophe promoting activities in vitro; these functional activities can be separated by small changes in pH or deletion of regions within Op18 (Howell et al., 1999). MT growth rates cannot be used to differentiate between these mechanisms in cells because several recent experiments have suggested that MT growth rate does not depend on the tubulin subunit concentration in either *Xenopus* egg extracts (Parsons and Salmon, 1997) or mammalian cytoplasm (Rodionov et al., 1999). Although we cannot rule out Op18 sequestering of tubulin dimers, in vivo Op18 functions as catastrophe promoter; Op18 stimulates catastrophes without significantly altering MT growth rate.

Several other experiments provide additional evidence that Op18 likely functions to specifically promote MT catastrophes in cells. Microinjection of a truncated Op18 (lacking amino acids 100-147) into living cells resulted in a loss of MT polymer and many of the remaining of MTs appeared shorter (Larsson et al., 1999). This truncated Op18 stimulates MT
catastrophes in vitro, but has lost the ability to sequester tubulin and binds tubulin weakly (Howell et al., 1999). Thus, MT depolymerization produced by microinjection of this truncated Op18 most likely occurred by a specific promotion of MT catastrophes and not through a tubulin sequestering mechanism.

Our VE-DIC imaging methods do not allow detection of MT minus ends, so it is not known whether Op18 also modifies MT treadmilling or MT minus end dynamics in cells. Loss of Op18, acting through either a tubulin-sequestering or MT catastrophe-promoting mechanism, could increase the stability of MT minus ends and contribute to the increase in MT polymer. This minus end stabilization could contribute to the high density of MTs we observe near the nucleus, but it not clear whether changes in the dynamics of these MTs would make a significant contribution to regulating MT polymer level since treadmilling has been observed only for a subset of MTs in cells (Waterman-Storer and Salmon, 1997a; Rodionov and Borisy, 1999; Yvon and Wadsworth, 1997).

**Additional catastrophe-promoters are likely active in newt lung cells**

Based on the assembly properties of purified tubulin, previous studies have estimated that interphase cells must contain factors to increase MT catastrophes 10- to 20-fold (Cassimeris et al., 1988; Simon et al., 1992). In contrast, we find that injection of anti-Op18 or incubation with antisense PONs reduced catastrophes by approximately 2.5-fold and similar results were obtained after Op18 immunodepletion from *Xenopus* egg extracts (Tournèbize et al., 1997). Therefore, our data suggest that additional catastrophe promoters must be present in newt lung cells. The identity of other catastrophe promoters in these cells is unknown, but may be related to XKCM1, a kinesin-like protein isolated from *Xenopus*, which also promotes catastrophes (Walczak et al., 1996).

**Op18 and cell morphogenesis**

Op18 appears necessary for differentiation of PC-12 cells stimulated with NGF since neurite outgrowth is reduced in cells incubated with antisense PONs (Di Paolo et al., 1996). The studies by Di Paolo et al. (1996) were conducted before the role of Op18 in regulating MT assembly were identified, and therefore any changes in MT assembly were not measured. Given the role of Op18 in regulating MT assembly during interphase, the phenotype of antisense-treated PC-12 cells should be similar to the antibody-injected or antisense PONs-treated cells examined here: less dynamic MTs and increased MT polymer. We suggest that failure of neurite outgrowth in antisense PONs-treated PC-12 cells results from global, rather than localized inactivation of Op18. Localized Op18 inactivation via phosphorylation would lead to increased MT assembly and stability, and presumably lead to extension of a neuronal-like process. In contrast, global Op18 inactivation may result in a large increase in MT polymer throughout the cell, which does not result in localized cell extension. This local Op18 inactivation model is similar to the ‘selective stabilization’ model proposed by Kirschner and Mitchison (1986), except that here ‘stabilization’ results from inactivation of a destabilizing protein, rather than activation of a MT capping protein.

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