

# Protein Phosphatase 1 Regulates the Cytoplasmic Dynein-driven Formation of Endoplasmic Reticulum Networks In Vitro

Viki Allan

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, United Kingdom

**Abstract.** Interphase *Xenopus* egg extracts form extensive tubular membrane networks in vitro. These networks are identified here as endoplasmic reticulum by the presence of ER resident proteins, as shown by immunofluorescence, and by the presence of single ribosomes and polysomes, as shown by electron microscopy. The effect of phosphorylation on ER movement in interphase was tested using the phosphatase inhibitor, okadaic acid. Okadaic acid treatment resulted in an increase of up to 27-fold in the number of ER tubules moving and in the extent of ER networks formed compared to control extracts. This activation was blocked by the broad-specificity kinase inhibitor 6-dimethylaminopurine. Okadaic acid had no effect, however, on the direction of ER tubule movement,

which occurred towards the minus end of microtubules, and was sensitive to low concentrations of vanadate. Inhibition of phosphatases also had no effect on the speed or duration of ER tubule extensions, and did not stimulate the activity of soluble cytoplasmic dynein. The sensitivity of ER movement to okadaic acid closely matched that of protein phosphatase 1. Although the amount of ER motility was greatly increased by inhibiting protein phosphatase 1 (PPI), the amount of cytoplasmic dynein associated with the membrane was not altered. The data support a model in which phosphorylation regulates ER movement by controlling the activity of cytoplasmic dynein bound to the ER membrane.

**M**ICROTUBULE-BASED movement is required for the positioning of many organelles within the cell, including the ER and the Golgi apparatus (Terasaki et al., 1986; Ho et al., 1989). The ER is a large organelle which forms an intricate polygonal network of membrane tubules and lamellae which extend throughout the cell. In epithelial cells, this structure is thought to be generated initially by the movement of ER membrane tubules along microtubules towards their plus, or rapidly growing ends, in the cell periphery (Lee et al., 1989). Fusion of these ER tubules then produces the polygonal membrane network, and once formed, the ER network continues to undergo microtubule-dependent movements (Lee and Chen, 1988). Microtubule motors are therefore involved in both establishing and maintaining the structure and position of the ER.

Organelle movement towards the plus ends of microtubules is thought to be driven by kinesin (Vale et al., 1985; Hollenbeck and Swanson, 1990; Rodionov et al., 1991) while movement towards the minus ends may be promoted by cytoplasmic dynein (Paschal et al., 1987; Paschal and Vallee, 1987; Schroer et al., 1989). Some organelles may

possess only one motor type whereas others are capable of moving in either direction depending on the prevailing conditions (e.g., Heuser, 1989; Thaler and Haimo, 1990; Morris and Hollenbeck, 1993). The mechanisms controlling the direction of such organelle movements have been best characterized for pigment granules. Kinesin is the plus end-directed pigment granule motor in melanophores from *Gymnocorymbus ternetzi* (Rodionov et al., 1991). In similar cells from *Tilapia mossambica*, plus end directed movement (presumably kinesin-driven) is stimulated by cAMP-dependent protein kinase, whereas movement towards the minus ends is activated by protein phosphatase 2B/calcineurin (Thaler and Haimo, 1990).

Membrane movement may also be regulated in terms of the amount and rate of movement an organelle makes, or in terms of the number of organelles moving. For instance, activation of protein kinase C or over-expression of normal p21<sup>N-ras</sup> in rat fibroblasts increased the rate of organelle movement, without obvious effect on the direction of movement (Alexandrova et al., 1993). The role of phosphorylation in regulating organelle movement has also been investigated using okadaic acid (OA)<sup>1</sup>, which is an inhibitor of

Dr. Allan's present address is Dept. of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Stopford building, Oxford Rd., Manchester M13 9PT, UK.

Address all correspondence to Viki Allan, Dept. of Biochemistry/Molecular Biology, School of Biological Sciences, Univ. of Manchester, Stopford Bldg., Oxford Rd., Manchester M13 9PT, UK. Tel.: 44 161 275 5646. Fax: 44 161 275 5082.

1. *Abbreviations used in this paper:* 6-DMAP, 6-dimethylaminopurine; NO, 1-nor-okadaone; OA, okadaic acid; PDI, protein disulphide isomerase; PPI, protein phosphatase 1; PP2A, protein phosphatase 2a; TRAP $\alpha$ , translocon-associated protein  $\alpha$ ; VE-DIC, video-enhanced differential interference contrast microscopy.

the two main classes of protein phosphatases, PP1 and PP2A (Bialojan and Takai, 1988). Treating quiescent, serum-starved epithelial cells with OA increased the number of organelles moving, the time they spent moving and their rate of movement, again without affecting direction, whereas serum starvation itself was shown to reduce the amount of organelle movement in vivo (Hamm-Alvarez et al., 1993). Interestingly, removing serum or calcium from the culture medium caused the release of cytoplasmic dynein from endosome/lysosome membranes into a soluble pool in rat fibroblasts (Lin and Collins, 1993). Phosphorylation in some situations may also inhibit organelle movement, as was observed in mitotic *Xenopus* egg extracts in the presence of active p34<sup>cdc2</sup> kinase (Allan and Vale, 1991). Phosphorylation may not provide the only regulatory mechanism, however: GTP-binding proteins may also play a role in controlling the amount of movement, as in squid axoplasmic extracts GTP $\gamma$ S inhibited vesicle movement in a phosphorylation-independent manner (Bloom et al., 1993).

The exact mechanisms by which phosphorylation or the activation of GTP-binding proteins regulate the amount or direction of movement are so far not known. For example, although dynactin (Gill et al., 1991; Holzbaur et al., 1991) has been shown to activate the vesicle movement driven by purified cytoplasmic dynein in vitro (Schroer and Sheetz, 1991), it is not clear whether it is involved in any of the examples described above. In addition, although kinesin can be phosphorylated both in vitro (Sato-Yoshitake et al., 1992; Matthies et al., 1993) and in vivo (Hollenbeck, 1993), little is known about how these events relate to control of motor activity in the cell. Recent results suggest that the activity of kinesin may be modulated by the phosphorylation of a group of kinesin-associated proteins rather than the motor itself (McIlvain et al., 1994). The ability to reconstitute a post-translational regulatory system in an in vitro assay should greatly facilitate the characterization of such mechanisms. *Xenopus* egg extracts provide a useful experimental paradigm for such investigations, as not only do they support microtubule-based motility of both vesicles and ER-like networks, but the movement is also regulated posttranslationally according to cell cycle status (Allan and Vale, 1991). Here, the membrane networks which form in these *Xenopus* egg extracts are identified as ER by the presence of ER resident proteins and structural features such as ribosomes and polysomes. Interestingly, although phosphorylation in metaphase extracts was previously shown to inhibit membrane movement (Allan and Vale, 1991), in this study increasing phosphorylation in interphase extracts by inhibiting PP1 resulted in up to a 27-fold activation of ER movement along microtubules. This activation is specific for membrane-associated cytoplasmic dynein, and does not affect the soluble pool of motor proteins. The amount of cytoplasmic dynein on the ER membrane, however, is not altered by okadaic acid treatment, suggesting that phosphorylation in interphase modulates motility by regulating the activity of ER-associated cytoplasmic dynein.

## Materials and Methods

### Materials

Taxol was a gift from Dr. N. Lomax (National Cancer Institute). Sucrose (GIBCO BRL, Gaithersburg, MD) was ultra pure grade and Hepes (BDH;

Poole, Dorset, UK) was biochemical grade. Vanadate-free ATP was purchased from Sigma (Poole, Dorset, UK). Creatine phosphate and the catalytic subunit of cyclicAMP-dependent protein kinase were supplied by Boehringer Mannheim (Lewes, East Sussex, UK). Phosphorylase kinase was purchased from Life Technologies Ltd. (Paisley, Renfrewshire, UK). OA was obtained from Sigma, Life Technologies Ltd. and Calbiochem-Novabiochem (Nottingham, UK). Microcystin LR and the inactive OA analogue 1-nor-okadaone (NO) were purchased from Calbiochem-Novabiochem and Calyculin A was purchased from Sigma. Osmium tetroxide and collodion (nitrocellulose) solutions were purchased from TAAB (Reading, Berks, UK). Rhodamine-conjugated concanavalin A and fluorescein-conjugated wheat germ agglutinin were obtained from Vector Laboratories (Peterborough, Cambs, UK). Fluorescein-conjugated sheep anti-mouse IgG and Texas red-conjugated donkey anti-rabbit IgG were obtained from Amersham (Aylesbury, Bucks, UK) and alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Promega (Southampton, UK). The Western-Light chemiluminescent detection system was obtained from TROPIX Inc. (Bedford, MA). A monoclonal antibody (ID3: Vaux et al., 1990) to protein disulphide isomerase was provided by Dr. S. Fuller (EMBL, Heidelberg, Germany); affinity purified rabbit anti-translocon-associated protein  $\alpha$ (TRAP $\alpha$ : previously called signal sequence receptor) antibodies (Prehn et al., 1990; Hartmann et al., 1993) were provided by Dr. T. Rapoport (Max-Delbrück-Centrum für Molekular Medizin, Berlin, Germany); and affinity-purified rabbit anti-tyrosinated tubulin antibodies were provided by Dr. T. Kreis (University of Geneva, Switzerland). A monoclonal antibody to the intermediate chain of cytoplasmic dynein (clone 70.1, Steuer et al., 1990) was purchased from Sigma. All other reagents were obtained from either Sigma or BDH.

### Preparation of *Xenopus* Egg Cytosol and Membrane Fractions

Laid *Xenopus* eggs are arrested in metaphase II of meiosis and can be induced to enter interphase by electrical activation (e.g., Murray, 1991). Concentrated interphase extracts were prepared 15 min after activation as described previously (Allan and Vale, 1991; Allan, 1993) and stored in liquid nitrogen. Following rapid thawing, extracts were diluted on ice with two volumes of acetate buffer (100 mM K-acetate, 3 mM Mg-acetate, 5 mM EGTA, 10 mM Hepes, pH 7.4, 150 mM sucrose) containing an energy mix for replenishing nucleotide triphosphates (1 mM MgATP and 7.5 mM creatine phosphate). The diluted extracts were then centrifuged in a Beckman TL-100 rotor at 55,000 rpm (117,000  $g_w$ ) for 30 min at 4°C. The supernatant was collected, avoiding the region just above the pellet. The crude organelle layer, which rests on top of the glycogen granule/ribosome pellet after centrifugation, was resuspended in acetate buffer to 40% of the original extract volume. This membrane fraction contains a mixture of organelles, including ER, mitochondria, Golgi apparatus, but is depleted of yolk platelets and pigment granules. This membrane fraction was used without further purification.

### Phosphatase Activity and Inhibitors

OA, NO, calyculin A, and microcystin LR were dissolved in DMSO and subsequently diluted in acetate buffer. Phosphorylated substrates (<sup>32</sup>P-labeled) were prepared as described (MacKintosh, 1993). PP1 activity was assayed in duplicate by adding 5  $\mu$ l of phosphorylated phosphorylase kinase to 10  $\mu$ l of sample (prepared as for video-enhanced differential interference contrast microscopy [VE-DIC] microscopy—see below) and incubating for 30 s at room temperature. After SDS-PAGE on a 5% gel, the amount of phosphate remaining in the  $\beta$  subunit of phosphorylase kinase was determined by cutting out the gel bands for Čerenkov counting (MacKintosh, 1993). PP2A activity was assayed in duplicate by incubating 10  $\mu$ l of phosphocasein with 10  $\mu$ l sample for 10 min at room temp. The release of TCA-soluble <sup>32</sup>P-phosphate was then followed by liquid scintillation counting (MacKintosh, 1993).

### Quantitation of Membrane-associated Cytoplasmic Dynein

Crude interphase extracts (50  $\mu$ l) were diluted with 2 vol of acetate buffer plus energy mix and incubated for 15 min at room temperature  $\pm$  2  $\mu$ M OA. Extracts were placed on ice for 10 min and then centrifuged as described above. The crude organelle layers were resuspended in 10  $\mu$ l acetate buffer plus 1 mM MgATP,  $\pm$  2  $\mu$ M OA, to give  $\sim$ 15  $\mu$ l final volume. These organelle fractions were resuspended well and then mixed thoroughly with 135  $\mu$ l 2.0 M sucrose in acetate buffer plus 1 mM MgATP, protease inhibi-

tors and  $\pm 1 \mu\text{M}$  OA, before being transferred to ultraclear centrifuge tubes ( $5 \times 41$  mm). The samples were then overlaid with  $200 \mu\text{l}$  1.5 M sucrose in acetate buffer (plus 1 mM MgATP, protease inhibitors,  $\pm 1 \mu\text{M}$  OA) followed by  $300 \mu\text{l}$  acetate buffer (containing 150 mM sucrose). The gradients were centrifuged for 3 h at 39,000 rpm in an SW55 rotor with high speed adaptors for  $5 \times 41$ -mm tubes. The membrane fractions were collected from the 150 mM/1.5 M sucrose interface, resuspended by trituration, and then diluted with SDS-PAGE sample buffer or with 4 volumes acetate buffer without sucrose, for UV-vanadate cleavage (see below). The load fraction was also collected for SDS-PAGE analysis and was shown to be depleted of ER membranes by immunoblotting with anti-TRAP $\alpha$  (data not shown).

UV-vanadate cleavage of the cytoplasmic dynein heavy chain present in the membrane fraction was performed by incubating  $100 \mu\text{l}$  of  $5\times$ -diluted membranes with 1 mM MgATP  $\pm 200 \mu\text{M}$  sodium orthovanadate for 10 min at room temperature in a micro-titre plate. The plate was put on ice for 5 min and subsequently exposed at a distance of 2 cm from a Mineralight lamp (Ultraviolet Products Inc., San Gabriel, CA) on the long wavelength setting for 60 min. Irradiated samples were then prepared for SDS-PAGE.

Cytoplasmic dynein heavy chain was identified by silver staining of 4% acrylamide-urea gels. Cytoplasmic dynein intermediate chains and TRAP $\alpha$  were identified by immunoblotting of samples run on 10% polyacrylamide gels. Electrophoretic transfer of proteins onto nitrocellulose was performed using a Genie electroblotter (Research Products International Inc., Mt. Prospect, IL) at 24V for 30 min on ice (transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol, 0.02% SDS). Blocking and antibody incubations were performed in 5% non-fat milk/TBS/0.1% Tween 20, with washes in TBS/0.1% Tween 20. Anti-TRAP $\alpha$  was used at 1/15,000 or 1/60,000 dilution and 70.1 was used at 1/3,000 or 1/12,000 dilution (for chromogenic and chemiluminescent detection, respectively). Second antibodies were detected with either nitro blue tetrazolium/5-bromo-chloro-indolyl phosphate or, according to the Western Light protocol, using the CSPD™ substrate (TROPIX, Inc.) followed by exposure to x-ray film. Quantitation of labeled bands on x-ray film and cytoplasmic dynein heavy chain bands on silver stained gels was performed using a Molecular Dynamics computing densitometer.

### Video-enhanced Differential Interference Contrast (DIC) Microscopy

*Xenopus* egg cytosol ( $8 \mu\text{l}$ ) was combined with  $0.5 \mu\text{l}$  crude organelle fraction,  $0.5 \mu\text{l}$  of  $20 \mu\text{g/ml}$  microtubule seeds (Allan, 1993) and  $1 \mu\text{l}$  acetate buffer or  $1 \mu\text{l}$  acetate buffer containing phosphatase inhibitors. To test the requirement for kinase activity, samples were preincubated with  $0.5 \text{ mM}$  6-dimethylaminopurine (6-DMAP) for 10 min at room temperature before the addition of phosphatase inhibitors. The mixture was introduced into a simple microscope flow cell (Allan and Vale, 1991; Allan, 1993) and incubated at room temperature for 5 min in a humid chamber, during which time microtubules polymerized from endogenous *Xenopus* tubulin. Microtubule seeds were added as nucleation sites to ensure similar numbers of microtubules were obtained regardless of the presence of phosphatase inhibitors. Microtubules then attached to the coverslip surface via extract motor proteins adsorbed onto the glass, and membrane tubule movement was assayed on these microtubules for up to 30 min. The membrane tubules derive from the unfractionated egg membranes added at the start of the assay, and the tubular networks accumulate with time, presumably as a result of further membrane recruitment via vesicle fusion coupled with continuing tubule movement. All steps requiring incubation of the flow cells at room temperature were performed in a humid chamber to prevent the sample drying out. To compare the activation of ER motility with phosphatase activity at various OA concentrations, samples for phosphatase assays were prepared as for motility, except that microtubule seeds were replaced with acetate buffer. Following incubation in microfuge tubes for 10 min at room temperature to ensure phosphatase inhibition, enzyme activity was measured as described above.

Soluble motor protein activity was assayed by preincubating carboxylated beads ( $0.10 \mu\text{m}$ , Polysciences no. 16688, diluted 1/500 from a 2.5% solid stock) in high-speed supernatants for 5 min at  $23^\circ\text{C}$  plus or minus phosphatase inhibitors. The direction of organelle and bead movement was assayed using axoneme-nucleated microtubules, which were prepared by first adsorbing salt-washed sea urchin axonemes (Gibbons and Fronk, 1979) onto the coverslip surface. When *Xenopus* supernatants plus organelles or beads were subsequently added, tubulin polymerized only from the plus ends of the axonemes (Allan and Vale, 1991).

Motility was followed in real time using a Zeiss standard microscope fitted with DIC optics, a plan  $100\times$  1.25 NA lens, and a 1.4 NA achromatic aplanatic oil immersion condenser. Light from a 100 W mercury vapor

lamp was passed through both water and glass heat-filters (LOT-Oriel Ltd., Leatherhead, Surrey, UK) and a 546-nm narrow band interference filter, before being relayed to the microscope via a 1-mm silica optical fiber (Technical Video Ltd., Woods Hole, MA) (Inoué, 1986). DIC images were projected onto a Hamamatsu Newvicon camera (C2400-07) using a  $12\times$  eye-piece. Background subtraction, image contrast enhancement and two-frame rolling averaging were performed in real time using a Hamamatsu Argus 10 image processor. Sequences for subsequent analysis were recorded onto S-VHS tape using a Panasonic S-VHS AG 7330 recorder. Where single frame images were required, the Argus 10 output signal (after background subtraction and contrast enhancement) was digitized using a Quick Capture board (Data Translation, Wokingham, Berks., UK) in an IBM-AT-compatible computer. The digitized images were then imported into NIH-IMAGE (a public domain program) on a Macintosh IIfx for further image processing. Final images were transferred into Aldus Pagemaker and printed using a Fuji Pictography 3000.

### Data Analysis

All data analysis was performed on DIC images recorded onto video tape in real time. Rates of organelle movement were obtained from video tape using an interactive AT-based computer program (Sheetz et al., 1986). To analyze the extent of membrane network formation under each incubation condition, the number of three-way junctions in a membrane network was counted in twenty randomly selected fields recorded on video tape. The number of moving membrane tubules was determined from 10 random fields per sample, recorded for 30 s each (5 min total per incubation). At the membrane concentrations used, it was straight forward to observe and count individual tubule movements. Two general classes of ER tubule motility were observed: the movement of existing tubules (e.g., see Fig. 2, Allan and Vale, 1991); and the extension of new tubules from existing membrane networks. Soluble motor activity was estimated by counting the number of moving carboxylated beads on individual microtubules, and is expressed as the number of movements per minute per linear length (mm) of microtubules analyzed.

### Immunofluorescence Microscopy

Membrane networks for immunofluorescence microscopy were formed in flow cells made using  $12 \times 12$ -mm coverslips with  $0.075$ -mm-thick plastic strips as spacers. After 30 min, excess membrane and cytosol were washed away by gently flowing  $100 \mu\text{l}$  of acetate buffer containing  $20 \mu\text{M}$  taxol through the chamber, leaving the microtubules and membrane networks intact on the glass surface. Fixation was performed by flowing through  $100 \mu\text{l}$  of 0.2% glutaraldehyde in acetate buffer, followed by incubation for 20 min at room temperature. The flow chambers were then rinsed with  $100 \mu\text{l}$  acetate buffer and the coverslips lifted off the spacers with forceps. After blotting with filter paper the networks were post-fixed by gentle immersion in methanol at  $-20^\circ\text{C}$  for 5 min, which served to attach the networks firmly to the glass surface and permeabilize the membrane networks. The coverslips were then incubated in  $1 \text{ mg/ml}$  NaBH $_4$  in PBS for  $3 \times 4$  min. After blocking for 30 min with 0.2% fish skin gelatin/PBS, coverslips were incubated for 20 min with 1D3 anti-protein disulphide isomerase culture supernatant, anti-TRAP $\alpha$  antibodies or anti-tubulin antibodies, diluted 1/5, 1/500 and 1/200, respectively, in fish skin gelatin/PBS. Coverslips were washed with PBS ( $3 \times 5$  min) and incubated for 20 min with fluorescently-conjugated second antibodies diluted 1/40 in fish skin gelatin/PBS. Alternatively, coverslips were stained with rhodamine-conjugated concanavalin A plus fluorescein-conjugated wheat germ agglutinin, diluted 1/500 and 1/200, respectively, in PBS. After  $3 \times 5$ -min washes in PBS, coverslips were mounted in polyvinyl alcohol mounting medium (Osborn and Weber, 1982) containing 25 mg/ml 1,4-diazabicyclo-[2.2.2]octane to reduce photo-bleaching. Samples were observed with a Biorad MRC-600 confocal microscope using the 488DF10 and 568DF10 single channel excitation filters. Identical confocal settings were used for all samples, and similar contrast-enhancement was subsequently applied to all images. Digitized images were processed and printed as described for VE-DIC images.

### Electron Microscopy

EM grids were placed on  $12 \times 12$  mm coverslips and coated with 1% collodion (nitrocellulose) to attach the grids firmly to the coverslips. A flow chamber was made by placing each coated coverslip on a microscope slide with small pieces of  $0.14$ -mm-thick plastic as spacers. The chambers were filled with  $20 \mu\text{l}$  of sample and incubated 10–40 min to allow membrane network formation. The motility observed using nitrocellulose-coated cov-

erslips was very similar to that seen with uncoated glass surfaces. After washing by gently flowing 100  $\mu$ l of acetate buffer containing 20  $\mu$ M taxol through the chamber, the networks were fixed by flowing through 200  $\mu$ l of 2% paraformaldehyde plus 0.25% glutaraldehyde in Pipes buffer (80 mM Pipes/KOH, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 150 mM sucrose, pH 6.8), followed by incubation for 30 min at room temperature. The networks were further stabilized by flowing through 200  $\mu$ l of 1% osmium tetroxide (diluted from a 4% stock with Pipes buffer) and incubating for 20 min at room temperature. Both fixation and osmication of the membrane networks was required to maintain the morphology of both microtubules and membranes prior to negative staining or freeze-drying. The flow cells were disassembled after washing extensively with water, and the grids were carefully removed. Fixed, osmicated samples were frozen in liquid nitrogen and then freeze-dried for either 1.5 h at  $-94^{\circ}\text{C}$  or 3.5 h at  $-86^{\circ}\text{C}$  and then rotary shadowed with platinum/carbon at an angle of  $30^{\circ}$  using a Balzers Freeze Etch Device (BA 360-M). Other samples were negatively stained with 2% uranyl acetate and subsequently were lightly rotary shadowed with platinum/carbon at an angle of  $30^{\circ}$ . Specimens were observed using a Philips 301 electron microscope.

## Results

### Membrane Networks Formed in *Xenopus* Egg Extracts Are Endoplasmic Reticulum

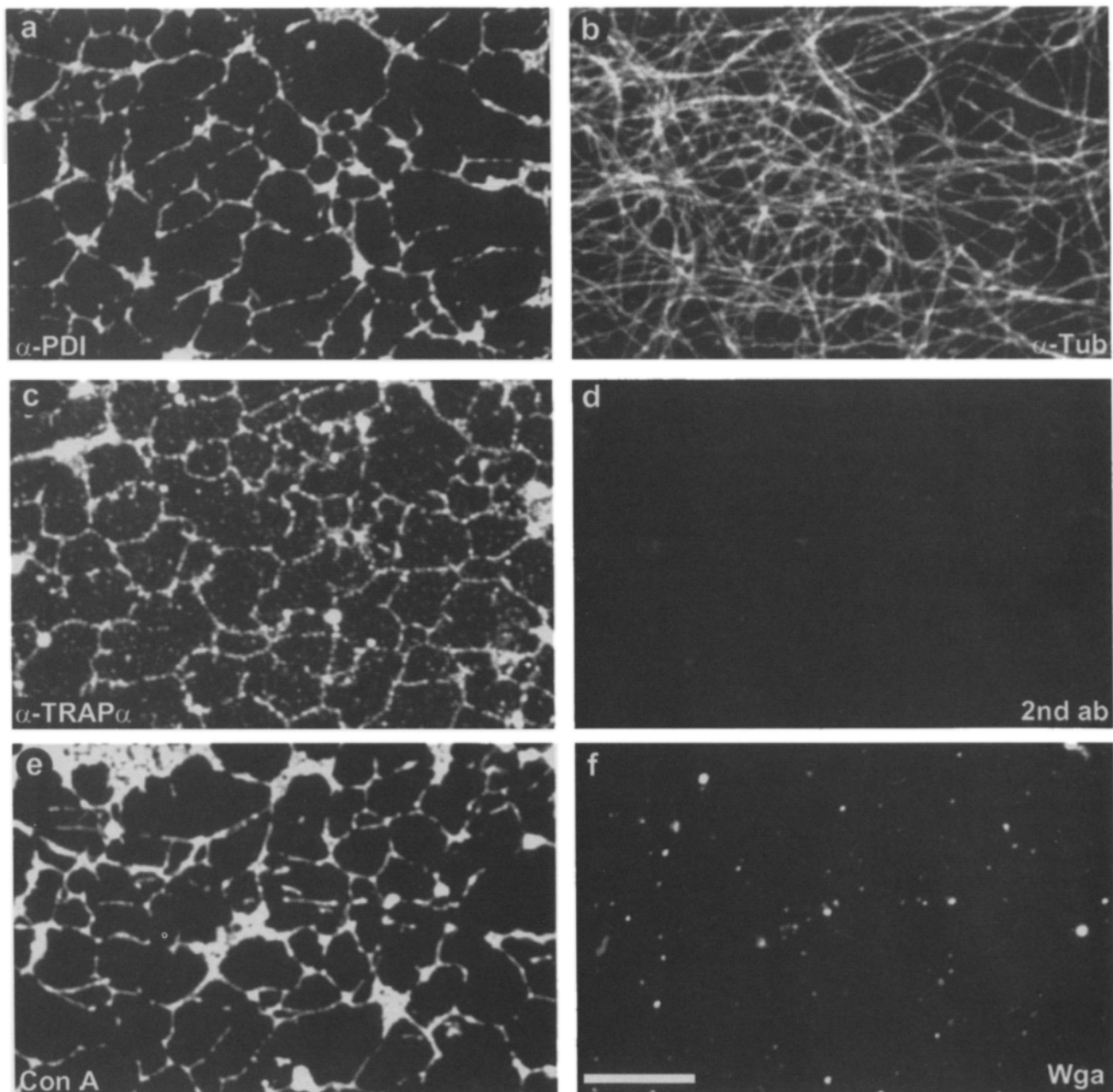
Interphase *Xenopus* egg extracts have previously been shown to form extensive tubular membrane networks which resembled the ER morphologically (Allan and Vale, 1991). As this network formation involves the movement of membrane tubules along microtubules and is under cell cycle control (Allan and Vale, 1991), this system should provide a useful model for studying the regulation of microtubule-based organelle movement and dynamics. To exploit this system further, it was important first to identify the organelle(s) which formed the networks in vitro, since an unfractionated, mixed membrane population was used in these assays. Techniques for fixing and permeabilizing the delicate networks have therefore been developed, as described in the Materials and Methods.

Glutaraldehyde-fixed, methanol-permeabilized networks were probed with a variety of antibodies which recognized *Xenopus* organelles. Monoclonal antibodies (clone 1D3; Vaux et al., 1990) to protein disulphide isomerase (PDI), a resident luminal ER protein, labeled an extensive network of membrane tubules and lamellae (Fig. 1 *a*) that resembled the networks observed using VE-DIC (see Fig. 4 and Allan and Vale, 1991). The underlying layer of microtubules was revealed by double labeling with a polyclonal anti-tubulin antibody (Fig. 1 *b*). It is clear from these images that both membrane and microtubule morphology is preserved under these fixation conditions. The membrane networks also contained the ER resident membrane protein TRAP $\alpha$  (Fig. 1 *c*), previously termed the signal sequence receptor (Prehn et al., 1990; Hartmann et al., 1993). When primary antibodies were omitted, however, very low levels of labeling with fluorescently-conjugated secondary antibodies was observed (Fig. 1 *d*). The membrane networks also bound rhodamine-conjugated con A (Fig. 1 *e*) which recognizes high  $\alpha$ -mannopyranosyl- and  $\alpha$ -glucopyranosyl-containing glycoproteins and glycolipids and has previously been shown to recognize the ER, as well as other organelles, in tissue culture cells (Virtanen et al., 1980; Fuhrmann and Bereiter-Hahn, 1984).

As both the *trans*-Golgi network (TGN) and endosomes can form tubular networks in living cells (Cooper et al.,

1990; Hopkins et al., 1990), it was possible that such elements could form a distinct sub-set of the networks observed in vitro. Comparison of the networks labeled with anti-ER antibodies with the phase contrast image showed that virtually all the membrane networks contained ER proteins (data not shown). In contrast, fluorescein-conjugated wheat germ agglutinin, which recognizes terminal sialic acid and N-acetyl glucosaminyl residues of glycosyl side chains and has been widely used as a marker for the Golgi apparatus (Virtanen et al., 1980), did not label the tubular membrane networks, but instead gave strong staining of scattered globular structures (Fig. 1 *f*). Similarly, an antibody that recognizes *Xenopus*  $\beta$ -COP and which labels the Golgi apparatus of the *Xenopus* tissue culture cell line XTC (V. Allan and T. Kreis, unpublished results), did not label the *Xenopus* membrane networks (data not shown). None of the available antibodies to endosomal/lysosomal proteins tested so far cross-react with XTC cells, raising the possibility that an endosomal network might pass undetected. Wheat germ agglutinin, however, should label endocytic organelles in addition to the Golgi apparatus, but did not stain the membrane networks in vitro (Fig. 1 *f*). Taken together, these data suggest that the membrane networks formed in vitro from unfractionated *Xenopus* egg membranes are indeed ER.

Given that rough ER is characterized by the presence of ribosomes on its cytoplasmic surface, electron microscopy was used to investigate whether such recognizable features could be seen on the ER networks formed in vitro. As described in Materials and Methods, ER networks were formed on nitrocellulose-coated EM grids and then fixed and osmicated to preserve membrane structure. The networks could then be visualized by negative staining (data not shown) or by positive staining with uranyl acetate followed by rotary shadowing with a light coat of platinum/carbon (Fig. 2, *a* and *c*). Networks on EM grids were also freeze-dried without staining and then rotary shadowed (Fig. 2 *b*). Similar structural features were observed in all cases. A low magnification view (Fig. 2 *a*) reveals a network of lamellar and tubular membrane structures superimposed on a layer of microtubules attached to the nitrocellulose surface. Occasional close contacts were observed between microtubules and membrane tubule tips or projections (Fig. 2 *a*, arrowheads), but more often the membranes appeared to lie across the microtubules (Fig. 2, *b* and *c*). The surfaces of both lamellar and tubular regions of the membrane networks were dotted with small ( $\sim 20$ -nm-diam) particles, which were better revealed at higher magnification (arrows in Fig. 2, *b* and *c*), and which may correspond to individual ribosomes. In some cases, strings of these particles were observed on the membrane surface (Fig. 2, *b* and *c*, double arrows), and the particles often appeared to consist of two subunits. These strings of particles resemble free polysomes observed by EM (Miller et al., 1970), and so may provide clear examples of membrane-bound polysomes. The extracts are prepared in the presence of cycloheximide, so that such membrane-bound polysomes are arrested during protein synthesis and translocation into the ER, resulting in stable attachment of the polysome to the membrane. The presence of polysomes and ribosomes on the membrane networks provides further proof that the membrane tubules and networks seen moving by VE-DIC are RER.



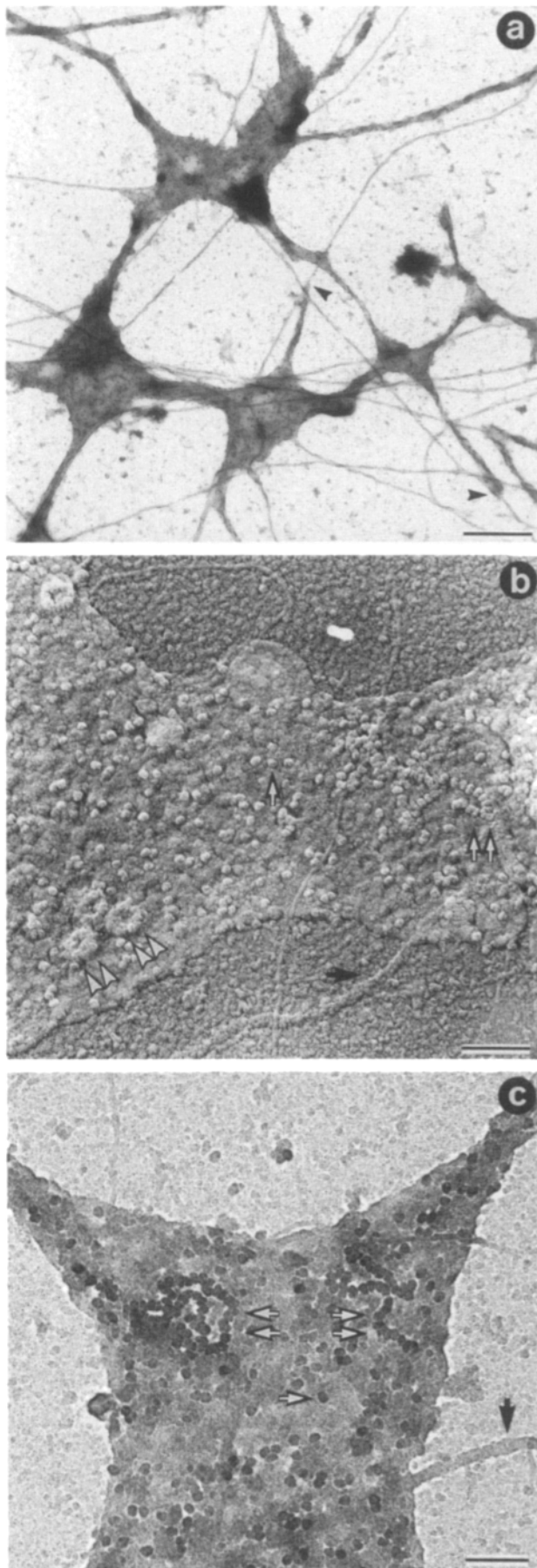
**Figure 1.** Membrane networks formed in vitro contain ER resident proteins. Interphase *Xenopus* egg cytosol and membrane fractions were incubated for 45 min to allow membrane networks to form, which were then fixed and processed for immunofluorescence as described in Materials and Methods. Coverslips were double labeled with mouse anti-PDI (1D3; *a*) and rabbit anti-tubulin (*b*), revealing an extensive tubular/lamellar PDI-containing network extended over a layer of microtubules. The membrane networks also contained the ER membrane protein TRAP $\alpha$  (*c*), but were not labeled by secondary antibodies alone (*d*). Double-labeling with fluorescently-conjugated lectins revealed that the membrane networks bound con A (*e*) but not wheat germ agglutinin (*f*). Bar, 5  $\mu$ m.

### ***Okadaic Acid Activates the Movement of ER Along Microtubules***

Cell cycle-dependent phosphorylation has previously been shown to regulate ER movement in vitro, as the presence of active mitotic p34<sup>cdc2</sup> kinase in *Xenopus* extracts inhibits the movement of ER membrane tubules and other membrane structures (Allan and Vale, 1991). In order to test whether protein phosphorylation might play a role in regulating ER movement during interphase (in the absence of active p34<sup>cdc2</sup>

kinase), phosphatase inhibitors were included in the motility assay. The presence of 2  $\mu$ M OA resulted in a marked increase in the extent of ER networks formed within a 15 min incubation, as did the addition of 2  $\mu$ M microcystin LR (Fig. 3) and calyculin A (data not shown). An inactive analogue of OA, NO, had no effect on ER network formation versus control, however (compare Fig. 3, *a* and *c*). Large, lamellar regions of ER were more common in control and NO-treated networks than after phosphatase inhibitor treatment. The



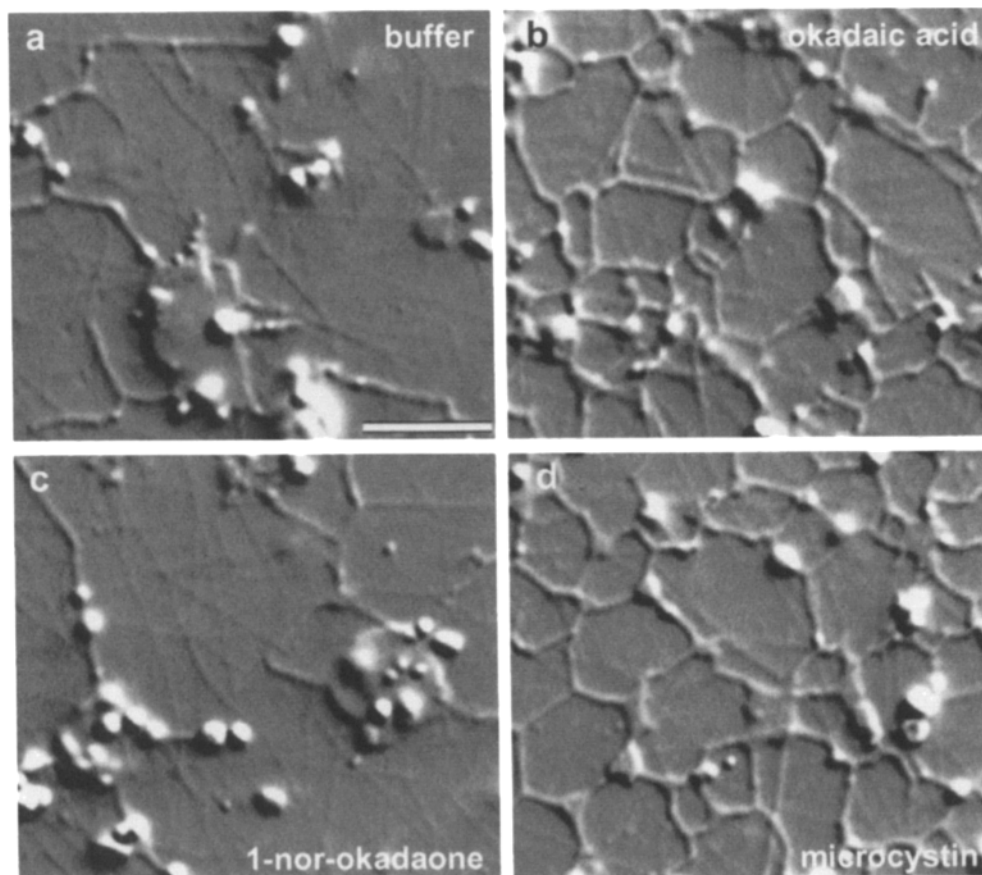


networks formed in the presence of OA and microcystin LR were similar in composition to control networks, however, as they possessed both PDI and TRAP $\alpha$ , as well as ribosomes (data not shown). Inhibition of phosphatases in interphase extracts therefore stimulates the formation of ER networks.

Observation of the ER networks by VE-DIC suggests that the phosphatase inhibition increased the amount of movement and hence the number of ER tubules which extended along microtubules. When two such moving tubules come into close contact, membrane fusion occurs, leading to the formation of a polygonal ER network (Allan and Vale, 1991). In order to establish whether ER tubule movement was indeed stimulated by OA, the number of moving tubules was counted and the total duration of tubule movement assessed both in the presence and absence of OA (see Materials and Methods). The amount of membrane present in these assays was reduced threefold compared to those shown in Fig. 3, to reduce the amount of movement to a level that could readily be quantitated (Table I). OA increased the number of moving ER tubules 21-fold, and the cumulative duration of ER movements was correspondingly increased by 16.3-fold compared to control. This increase in motility was directly correlated with the extent of ER network formed, as determined by counting the membrane tubule intersections in the polygonal ER network, which was likewise stimulated 16.3-fold by OA. As counting the three-way (or greater) junctions in the ER network was much more rapid than counting either total movements or duration of movement and still provided a similar estimate of activation, this measure was used in most subsequent experiments.

As ER membrane network formation clearly involves membrane fusion and dynamics, it seemed possible that a hydrophobic compound like OA was affecting the membrane directly, rather than acting via inhibition of phosphatases. However, this was ruled out by testing the effects of NO, which has a similar structure to OA but does not affect phosphatase activity. NO treatment did not stimulate ER network formation (Table I). In addition, the phosphatase inhibitors microcystin LR and calyculin A are structurally unrelated to OA, and yet cause a similar activation of ER movement (data not shown) and network formation (Fig. 3). Presuming that the stimulation of ER motility by inhibiting phosphatases is mediated by the phosphorylation of as yet unidentified com-

*Figure 2.* Ultrastructure of ER networks formed in vitro. ER networks extended on nitrocellulose-coated ER grids after a 20 min (a) or 40 min (b and c) incubation were fixed as described in Materials and Methods. Grids were either positively stained with uranyl acetate and lightly rotary shadowed with platinum/carbon (a and c), or freeze dried before rotary shadowing (b). Attachments can be seen between ER tubules tips and microtubules (black arrowheads) in a. Microtubules (black arrows) and thinner intermediate filaments commonly pass underneath the ER membranes. Globular particles (white arrows), which may be individual ribosomes, are seen on almost all membrane surfaces. Strings of these particles (double white arrows), which may be membrane-bound polysomes, are seen on some lamellar ER regions (b and c). Circular structures resembling nuclear pores (double white arrowheads) are found in both lamellar (b) and tubular (not shown) regions of the networks. Such structures may represent early stages in the formation of annulate lamellae. Bars: (a) 1  $\mu$ m; (b and c) 200 nm.



**Figure 3.** ER network formation is stimulated in the presence of phosphatase inhibitors. VE-DIC images of *Xenopus* egg cytosol and membranes which were incubated for 15 min in the absence of inhibitors (a), or the presence of 2  $\mu$ M OA (b), 2  $\mu$ M microcystin LR (d), or 2  $\mu$ M NO (c), which is an inactive analogue of OA. Both OA and microcystin LR treatments led to extensive ER network formation as compared with controls. Bar, 5  $\mu$ m.

ponents, then activation by OA should be blocked by a general kinase inhibitor such as 6-DMAP (Rime et al., 1989). Preincubation with 0.5 mM 6-DMAP for 5 min at room temperature did indeed prevent the OA-induced activation of ER movement, whereas 6-DMAP alone had no effect

(Table I). These results confirm that ER motility is stimulated by hyperphosphorylation.

Another possible explanation for the effects of phosphatase inhibitors is that they somehow cause a 20-fold increase in the amount of membrane present on the coverslip surface,

**Table I.** Quantitation of the Effect of OA on ER Tubule Movement and ER Network Extent

Addition to the motility assay	Total number of motile events*	Total duration of tubule movements*	Three-way junctions per field $\pm$ SD <sup>‡</sup>
s			
<b>Extract 1</b>			
Buffer alone	12	117.6	3.6 $\pm$ 2.3
5 $\mu$ M OA	252 (21.0) <sup>§</sup>	1913.2 (16.3)	58.8 $\pm$ 24.2 (16.3)
<b>Extract 2</b>			
Buffer alone	ND	ND	2.1 $\pm$ 2.7 <sup>  </sup> (1.0)
5 $\mu$ M NO	ND	ND	3.2 $\pm$ 5.1 <sup>  </sup> (1.5)
5 $\mu$ M OA	ND	ND	52.8 $\pm$ 27.9 <sup>  </sup> (25.1)
<b>Extract 3</b>			
Buffer alone	18 <sup>†</sup> (1.0)	ND	ND
0.5 mM 6-DMAP	19 <sup>†</sup> (1.06)	ND	ND
6-DMAP + OA	12 <sup>†</sup> (0.67)	ND	ND
2 $\mu$ M OA	480 <sup>†</sup> (26.7)	ND	ND

\* Each sample was incubated for 10 min at room temperature and then 10 fields were observed by VE-DIC and recorded for a total of 5 min. The number of moving ER tubules (motile events) and the total duration of their movements were then determined from tape.

<sup>‡</sup> 20 random fields from the same samples were recorded after 15-min incubation and the average number of three-way (or more) junctions in the membrane network per field was determined  $\pm$ SD.

<sup>§</sup> Numbers in brackets show relative increase over control values.

<sup>||</sup> The average number of three-way junctions per field  $\pm$ SD was determined from 20 random fields recorded from quadruplicate assays incubated for 15 min (therefore  $n = 80$  fields per condition).

<sup>†</sup> Samples were preincubated for 5 min at room temperature in the presence of buffer or 6-DMAP. OA was then added and motility was observed after 10 min. The number of motile events was determined during 5 min. Numbers shown are the averages from duplicate flow cells.

ND, not determined.

and so 20× more motor attachment sites would be available per field after a given time of incubation. Although simple observation (Fig. 3) did not support this hypothesis, it was nevertheless tested directly as follows. Control interphase ER networks were formed during a 30-min incubation and then control cytosol was flowed through to remove excess membrane, leaving just the ER networks attached to the microtubules on the glass surface. After a 5-min incubation, the amount of ER tubule movement was determined (Table II). Cytosol plus OA was then flowed through the same chamber, and the same parameter was assessed after a further 5-min incubation. It is clear that OA indeed promoted ER tubule movement (Table II) under conditions where the amount of membrane present remained constant, or decreased.

### Phosphatase Inhibition Regulates Membrane-associated Cytoplasmic Dynein Activity

The next question to be addressed was what parameters of ER movement were affected by phosphatase inhibition, as this information might provide clues to the mechanism of activation. For instance, was the direction of ER movement altered by OA treatment? We have previously shown that membrane tubule movement in *Xenopus* egg extracts occurred towards the minus ends of microtubules (Allan and Vale, 1991), and this has been confirmed in this study (Table III). As the ER tubule movement is also inhibited by 50 μM sodium orthovanadate (data not shown), it is likely that cytoplasmic dynein is the motor responsible for this movement. OA treatment had no effect either on the direction of ER movement (Table III) or on its vanadate sensitivity (data not shown), which suggests that the increase observed in membrane motility reflects an increase in cytoplasmic dynein activity.

In addition to investigating whether OA affected the direction of ER tubule movement, other parameters of motility were assessed. Since OA increased both the number of ER tubules moving and the cumulative time spent moving (Table I), the implication is that the duration of each individual tubule movement might not be affected. This is confirmed by the data presented in Fig. 4, which reveals that the range of times that each tubule spent moving before either stopping or being released from the microtubules was not obviously altered by phosphatase inhibition. The rate of ER movement was likewise unaffected by OA (Table III). Taken together, these results suggest that the motor-microtubule interaction

Table II. Effect of OA-treated Cytosol on ER Tubule Movement in Preexisting ER Networks

	Total number of ER tubule movements	
	Control cytosol	Cytosol + 2 μM OA
Flow cell 1	15	402 (26.8)*
Flow cell 2	16	367 (22.9)
Flow cell 3	13	285 (21.9)

ER networks were formed by incubating control cytosol and membranes for 30 min. Control cytosol was then flowed through the chamber, washing away excess membrane. After a 5-min incubation, movement of the network was observed by VE-DIC and recorded for 5 min. Cytosol + 2 μM OA was then flowed through the same chamber, incubated for 5 min, and observed and recorded for 5 min. The number of moving ER tubules were then determined from tape. \* Numbers in brackets show relative increase over control values.

Table III. Effects of Okadaic Acid on Motor Protein Activity

	0 μM OA	5 μM OA
% of ER tubules moving towards the minus end	100 (n = 61)	100 (n = 76)
Rate of ER tubule movement (μm/s ± SD)	1.65 ± 0.29 (n = 29)	1.62 ± 0.20 (n = 30)
No. of bead movements/mm of microtubule/min		
minus end-directed	11.6*	9.8‡
plus end-directed	0.5*	0.0‡

All measurements and quantitation was performed on membrane tubules or beads moving along microtubules nucleated from the plus ends of salt-washed sea urchin axonemes (see Materials and Methods). These microtubules were stationary on the coverslip surface.

\* 74 minus end- and three plus end-directed bead movements were observed in 954 s over 400 μm of microtubules.

‡ 34 minus end-directed movements were counted in 653 s over 319 μm of microtubules.

is not being altered. Instead, it may be the amount of active motor present on the membrane that is the critical factor. If this is true, then one important question is whether all cytoplasmic dynein (both membrane-associated and soluble) is affected in the same way, or whether the activation only influences the membrane-associated motor. To investigate

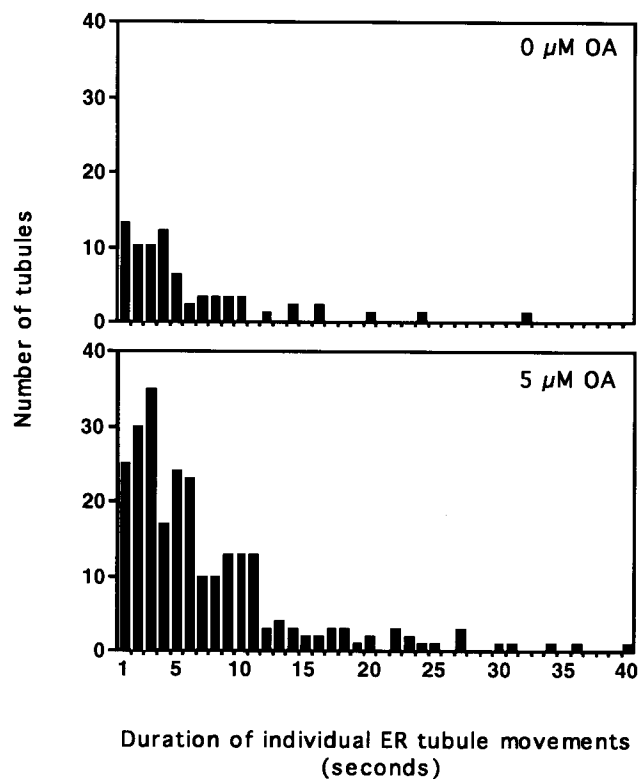


Figure 4. OA treatment increases the number of ER tubules moving with little effect on the duration of individual movements. Cytosol and membrane fractions were incubated for 10 min in the absence (a) and presence (b) of 5 μM OA, and were then observed by VE-DIC and recorded for 5 min. The duration of each ER tubule movement in one OA and three control samples was then determined from video tape. A total of 15 min of tape was then analyzed for a versus 5 min for b. The total duration and number of ER movements in the OA-treated sample and one of the controls, and the extent of ER network formed, are presented in Table I (extract 1).

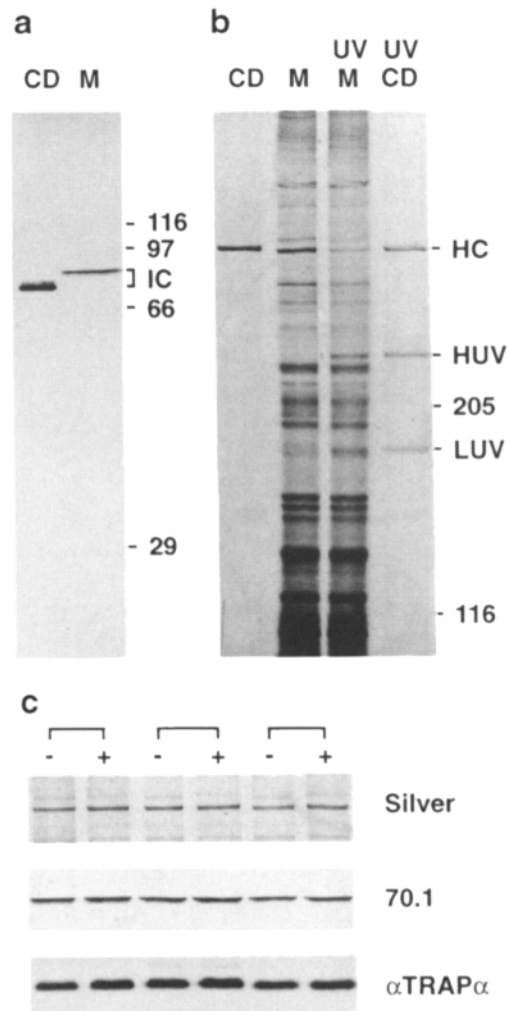


this, the amount of soluble motor protein activity was assessed by quantitating the movement of carboxylated latex beads, which adsorb soluble motor proteins onto their surface. Such an approach has previously been used to show that while membrane movement is greatly reduced in mitotic *Xenopus* extracts, the activity of soluble cytoplasmic dynein is not (Allan and Vale, 1991). Similarly, OA treatment of cytosol had no effect on the number of beads moving towards the minus ends of microtubules (Table III). Little plus end-directed bead movement was observed in either case (Table III). OA treatment therefore appears to be acting only on the membrane-associated cytoplasmic dynein, and not on the soluble pool of motor proteins.

### Membrane Association of Cytoplasmic Dynein Is Unaffected by OA

There are two possible mechanisms which could regulate cytoplasmic dynein-driven ER movement. Firstly, motor recruitment to the membrane could be stimulated by inhibition of phosphatases. Secondly, if the number of ER-bound dynein molecules is fixed, then the overall activity of the motors, or the proportion of active motors, could be increased by hyperphosphorylation. In order to distinguish between these two models, the amount of membrane-associated cytoplasmic dynein was assessed with, and without OA treatment. Because a considerable proportion of *Xenopus* egg cytoplasmic dynein is either soluble or associated with detergent-insoluble particulate material (data not shown), a membrane isolation protocol was designed to minimize cross-contamination by using flotation up through a sucrose gradient (see Materials and Methods). The membranes were isolated after only two centrifugation steps performed in the presence of ATP and, where appropriate, OA, to maintain the phosphorylation state of the membranes. The amount of cytoplasmic dynein intermediate chain was then assessed by immunoblotting using the monoclonal antibody 70.1, which recognizes a single band of approximately 83,000  $M_r$ , both in *Xenopus* motor fractions and crude extracts (data not shown) and in isolated membranes (Fig. 5 a), which is slightly larger than pig brain cytoplasmic dynein intermediate chain (74,000  $M_r$ ; running at 77,000 apparent  $M_r$  on these gels). Anti-TRAP $\alpha$  was used as an indicator of the relative amount of ER present in each membrane fraction. Immunoblots using a chromogenic substrate showed little qualitative change in the amount of cytoplasmic dynein intermediate chain in the membrane fraction after incubation with OA (Fig. 5 c). Similar results were obtained with 8 different interphase extracts. This result was confirmed for one extract using a chemiluminescent substrate for alkaline phosphatase, which showed that OA-treated membranes possessed 94.6% of control intermediate chain, after correction for the amount of membrane present.

Since it was possible that 70.1 recognized only a subset of *Xenopus* cytoplasmic dynein intermediate chains, the heavy chain of cytoplasmic dynein was followed directly using silver staining of 4% polyacrylamide-urea gels, which resolve the high molecular weight proteins very effectively. Putative cytoplasmic dynein heavy chain was identified in the membrane fraction by comigration with purified pig brain cytoplasmic dynein (Fig. 5 b). This band was cleaved by exposing the membranes to UV light in the presence of 200  $\mu$ M vana-



**Figure 5.** Membrane association of cytoplasmic dynein is unaffected by OA. The distribution of cytoplasmic dynein heavy and intermediate chains in membrane fractions was analyzed by silver staining and immunoblotting, respectively (see Materials and Methods). The mAb 70.1 recognized pig brain cytoplasmic dynein intermediate chain (a, lane CD; 74,000  $M_r$ , running here with an apparent  $M_r$  of 77,000) and a single band of  $\sim$ 83,000  $M_r$  in *Xenopus* egg membrane fractions (a, lane M; c, middle). Cytoplasmic dynein heavy chain was identified in *Xenopus* membrane fractions (b, lane M) by comigration with pig brain cytoplasmic dynein (b, lane CD), and by its susceptibility to UV-vanadate cleavage (b, lane UV-M and UV-CD) which gives rise to two fragments, HUV and LUV. When membranes were isolated following incubation in the absence (c, lanes marked -) or presence of 2  $\mu$ M OA (c, lanes marked +), little qualitative difference was seen in the amount of cytoplasmic dynein heavy chain (c, top, silver stain) or intermediate chain (c, middle, 70.1 antibody immunoblot). Three different extracts are shown,  $\pm$ OA treatment. Anti-TRAP $\alpha$  was used to reveal the relative amount of membrane present (c, bottom panel). The 70.1 and  $\alpha$ TRAP $\alpha$  panels are from the same gel lanes, obtained by cutting the immunoblot horizontally in the 60,000  $M_r$  region. Standard 10% SDS-polyacrylamide gels were used for immunoblotting (a and c, middle and bottom) and 4% urea-polyacrylamide gels were used for silver staining (b and c, top). Equal proportions of the isolated membrane fractions were loaded in each lane, with 10 $\times$  less membrane and 13 $\times$  less purified cytoplasmic dynein loaded for silver staining versus immunoblotting. The positions of  $M_r$  markers are shown (K) in a and b.

date and ATP (Fig. 5 b): a feature characteristic of the dyneins (Lee-Eiford et al., 1986). The amount of cytoplasmic dynein heavy chain in the membrane fraction was qualitatively unaffected by the presence of OA (Fig. 5 c), and densitometry revealed that the OA-treated band was 117% of the control ( $n = 8$  extracts,  $SD = 15\%$ ). At the loadings used, a twofold difference in the amount of cytoplasmic dynein present could be easily detected by densitometry (data not shown), even though the silver staining response is not strictly linear. Taken together, these results clearly show that the large activation of movement caused by inhibiting phosphatases can not be due to increased recruitment of cytoplasmic dynein to the membrane. Rather, it seems that phosphorylation modifies the activity of the membrane-associated motors themselves.

### PP1 Activity Correlates with the Amount of ER Movement

OA is a useful tool for identifying particular protein phosphatase activities as it is a more potent inhibitor of purified PP2A ( $IC_{50} = 0.1$  nM) than PP1 ( $IC_{50} = 10$  nM), whereas it has only a weak effect on PP2B ( $IC_{50} = 10$   $\mu$ M) and no effect on PP2C (Cohen et al., 1989). The activation of ER movement caused by OA is therefore unlikely to be due to inhibition of PP2C. In addition, since the extracts contain 5 mM EGTA but PP2B activity requires calcium, it is clear that PP2B is also not involved. The use of OA to distinguish between PP1 and PP2A is complicated, however, by the fact that the amount of OA required to inhibit each enzyme is dependent on the phosphatase concentration (Cohen et al., 1989). Accordingly, in concentrated *Xenopus* egg extracts the observed  $IC_{50}$  for PP2A and PP1 are 375 nM and 900 nM, respectively (Felix et al., 1990). Since the work described here uses extracts prepared by a different procedure and which are diluted with 2 vol of buffer before use, it was important to determine the sensitivity of PP1 and PP2A to OA in the motility assay in order to establish which phosphatase regulates ER movement. Under these conditions, PP2A had an  $IC_{50} \sim 70$  nM OA, assayed using phosphocasein, whereas PP1 had an  $IC_{50} \sim 250$  nM, assayed using the  $\beta$  subunit of phosphorylase kinase (Fig. 6). Half-maximal activation of ER movement was observed at  $\sim 420$  nM, and a comparison of the dose-response profiles (Fig. 6) strongly suggested that PP1 is responsible for controlling ER motility, rather than PP2A or the related PPX (Brewis et al., 1993). Similarly, when the extent of ER network formation versus OA concentration was determined using an extract from a different batch of eggs, half-maximal activation was observed at 400 nM. The activation of ER movement requires slightly higher OA concentrations compared to PP1 inhibition, presumably because the regulation of ER motility does not depend solely on the activity of PP1, but involves a balance of kinase activity relative to PP1 activity. Therefore, while the release of radioactive phosphate from phosphorylase kinase is a direct assay of PP1 activity, the stimulation of ER movement is an indirect consequence of PP1 inhibition. OA at 400 nM inhibits both PP2A and PP1, so it is possible, therefore, that both phosphatases are involved in regulating ER movement. Inhibiting PP2A alone is clearly insufficient for activation of motility, whereas inhibiting PP1 is an absolute requirement.

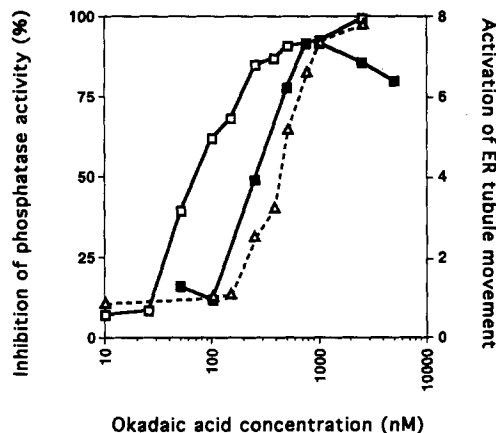


Figure 6. Comparison of the OA-sensitivity of PP1, PP2A and ER movement. The activity of PP2A (open squares) and PP1 (filled squares) in cytosol/membrane samples was determined in duplicate after a 10-min preincubation at room temperature in the presence of various concentrations of OA, as described in Materials and Methods. Phosphorylated casein and phosphorylase kinase  $\beta$  subunit were used as substrates for PP2A and PP1, respectively. The effect of OA concentration on the number of ER tubule movements was assessed by VE-DIC after a 10-min preincubation in a microscope flow cell. The movement in each sample was recorded for 5 min and is expressed as relative number of movements versus control (open triangles). The same batch of extract was used for all three assays. This extract, however, had a higher level of ER motility in the absence of OA compared to those shown in Tables I–III and Fig. 4, which results in a lower relative increase in movements occurring in the presence of OA.

### Discussion

The reconstitution of microtubule-based movement in vitro has proved a very valuable approach which has led to the identification and characterization of kinesin and cytoplasmic dynein (Vale et al., 1985; Paschal et al., 1987). This method has also been widely used to study the movement of membranous organelles along microtubules, including the formation of membrane networks (Dabora and Sheetz, 1988; Allan and Vale, 1991, 1994). As many organelles within the cell are able to form tubular membrane networks or tubules (Johnson et al., 1980; Swanson et al., 1987; Cooper et al., 1990; Hopkins et al., 1990; Lippincott-Schwartz et al., 1990), it is clearly important to identify the moving organelles present in an in vitro assay. Although previous studies have used the lipophilic dye, DiOC<sub>6</sub>(3), to label membrane networks formed in vitro (Dabora and Sheetz, 1988; Allan and Vale, 1991), this dye is unspecific, since it labels all membranous organelles in the cell (Terasaki and Reese, 1992). In this work, the membrane networks which form in interphase *Xenopus* egg extracts have been identified as ER because they contain the ER resident luminal protein PDI and the ER membrane protein TRAP $\alpha$ . This conclusion is confirmed by the presence of ribosomes and polysomes in the networks. In contrast, wheat germ agglutinin, which recognizes the sialated proteins present in the Golgi apparatus and endocytic pathway, does not label the networks. This lectin staining provides strong evidence that endosomes, lysosomes and the TGN do not form a subset of membrane networks. While it has not proved possible

to confirm this using antibodies to the TGN or endosomes/lysosomes because of lack of cross-reactivity, similar ER networks formed from rat liver membrane fractions (Allan and Vale, 1994) do contain ER resident proteins and do not label with antibodies to TGN or endosomal/lysosomal markers, or wheat germ agglutinin (V. Allan, unpublished data). These results suggest that the networks do not consist of a mixture of organelles which fuse artifactually in vitro. In conclusion, although the membrane fraction used in these experiments contains a mixture of all organelles, prepared under very mild conditions, the membrane networks formed in vitro derive only from the ER.

It has previously been suggested that the ER moves towards the plus ends of microtubules, based upon the observation that the ER collapses back around the nucleus after microtubule depolymerisation and then reextends out along microtubules to the cell periphery upon microtubule regrowth (Terasaki et al., 1986; Lee et al., 1989). This movement has been proposed to be driven by kinesin (e.g., Henson et al., 1992; Hollenbeck, 1989; Houliston and Elinson, 1991; Wright et al., 1991; Toyoshima et al., 1992). The ER in *Xenopus* egg extracts, however, moves exclusively towards the minus ends of microtubules (Allan and Vale, 1991; this study), using a motor that is sensitive to low concentrations of vanadate—properties typical of cytoplasmic dynein. It should be noted, however, that the experimental protocol of the studies performed in epithelial cells (Terasaki et al., 1986; Lee and Chen, 1988) was not designed to follow any movement of the ER towards the cell center. The question of whether tissue culture cell ER can move in both directions therefore remains open. Why should *Xenopus* egg ER move exclusively using cytoplasmic dynein? The egg extracts certainly possess active plus end-directed motors, because most vesicles move in that direction (Allan and Vale, 1991). The use of cytoplasmic dynein to drive ER movement may therefore reflect differences in sub-cellular organization required by a cell 1 mm in diameter, or may be required for an egg-specific function.

The movement of ER networks is regulated by phosphorylation in *Xenopus* extracts, being active in interphase and inhibited in metaphase (Allan and Vale, 1991). In addition, the results presented here reveal that interphase ER motility is greatly stimulated by inhibiting PP1, without affecting the direction of ER movement. While increased phosphorylation in interphase extracts stimulates cytoplasmic dynein-driven ER tubule movement and the extent of ER network formation, it is striking that in metaphase extracts phosphorylation by p34<sup>cdc2</sup> or a down-stream kinase leads to inhibition of ER movement (Allan and Vale, 1991). It might be expected that OA treatment of interphase extracts would lead to a pseudo-mitotic state, as has been observed in tissue culture cells (Yamashita et al., 1990; Lucocq et al., 1991; Vandr  and Wills, 1992), in which p34<sup>cdc2</sup> kinase is active and ER movement should be turned off. The interphase extracts used here, however, are made 15 min after release from metaphase arrest, when the levels of cyclins A and B should be very low, and further cyclin synthesis is inhibited by the presence of cycloheximide. Previous work has shown that when cyclin is absent, OA does not cause p34<sup>cdc2</sup> activation (Picard et al., 1989; Felix et al., 1990; Gliksman et al., 1992; Walker et al., 1992). The simplest model for the regulation of ER movement, therefore, is that p34<sup>cdc2</sup> (or a

down-stream kinase) turns off ER movement whereas in interphase, phosphorylation by a distinct, as yet unidentified, kinase results in activation of motility.

Exactly how this posttranslational control is exerted remains to be determined, but it is striking that the activity of membrane-associated cytoplasmic dynein is modulated in both metaphase and interphase extracts, while that of the soluble cytoplasmic dynein remains unaffected (Allan and Vale, 1991; this study). It is also clear that activating ER motility does not alter the speed of ER tubule movement, and that this activation occurs without any change in the amount of membrane-associated cytoplasmic dynein. Inhibiting PP1 in interphase extracts therefore results in specific activation of ER-associated cytoplasmic dynein without affecting motor recruitment to the membrane. In contrast, serum- or calcium-starvation of tissue culture cells leads to the loss of cytoplasmic dynein from endosome/lysosome membranes (Lin and Collins, 1993) and a reduction in motility (Hamm-Alvarez et al., 1993). This suggests that the mechanisms involved in turning off cytoplasmic dynein-driven movement may be different to those responsible for stimulating activity, as described here. In this context, it will be interesting to establish what happens to the level of cytoplasmic dynein during the inactivation of ER movement which occurs in metaphase (Allan and Vale, 1991). Cytoplasmic dynein itself may not be regulated directly by phosphorylation, however, since it was not phosphorylated in a cell cycle-dependent manner (Verde et al., 1991). It remains to be established whether dynactin (Gill et al., 1991; Holzbaaur et al., 1991; Schroer and Sheetz, 1991) is involved in controlling ER-associated cytoplasmic dynein activity, and whether it is the target of phosphorylation.

How does this regulation of ER movement compare with other examples of regulated organelle transport? Phosphorylation has also been shown to regulate the movement of organelles in a number of systems, especially in pigment cells of fish and amphibian skin. The phosphatase implicated in activating the minus end-directed pigment granule movement in melanophores is the calcium-dependent PP2B (Thaler and Haimo, 1990). This phosphatase is unlikely to be involved in the activation of ER movement described here, since the regulation occurs in the presence of EGTA and is maximally stimulated by 1-2  $\mu$ M OA (Fig. 6), much lower than the observed IC<sub>50</sub> for purified PP2B (10  $\mu$ M). OA does not inhibit PP2C significantly (Cohen et al., 1989), so the activation of ER movement is due to inhibiting PP1, PP2A, or a related enzyme such as PPX (Brewis et al., 1993). The OA dose-response curves (Fig. 6) strongly suggest that it is PP1 which is responsible for regulating ER movement. The possibility that both PP2A and PP1 need to be turned off in order to stimulate ER motility can not be ruled out, however. Serum-starved CV1 cells provide another example in which OA treatment activates organelle movement (Hamm-Alvarez et al., 1993), although the phosphatase involved has not been identified. This activation was manifested as an increase in the number of moving vesicles, coupled with an increase in their speed and duration of movement. In addition, OA treatment of chicken embryo fibroblast or T-helper hybridoma cell cytosols stimulated both soluble and lytic granule-associated kinesin activity twofold (McIlvain et al., 1994). In the *Xenopus* extracts, however, inhibiting PP1 had no obvious effect on vesicle movement (V. Allan, unpublished

results), and did not cause activation of either speed or duration of ER tubule movement (Table III, Fig. 4). Different control mechanisms may therefore exist for different classes of organelles and motors.

ER movement is clearly regulated by phosphorylation in vitro (Allan and Vale, 1991; this study), but is there any evidence for similar regulation in intact cells? One intriguing observation in living epithelial cells was that only a small proportion of cells possessed actively moving ER (Lee and Chen, 1988), raising the possibility that cells may activate ER movement at some point in the cell cycle, or when undergoing spreading or locomotion, for example. It has recently been observed that the synthesis of a PPI regulatory protein, inhibitor 2, oscillates with the cell cycle (Brautigan et al., 1990). The levels of inhibitor 2 are very high in telophase, after nuclear envelope re-formation (Brautigan et al., 1990), when p34<sup>cdc2</sup> activity should be low and when PPI is still mainly nuclear (Fernandez et al., 1992). It is tempting to speculate that these circumstances may result in very low cytoplasmic PPI activity as the cells enter G1—exactly the time at which the cells may need motile ER in order to reestablish their interphase network—and that this is reconstituted in vitro in the activated extracts described here. Whether the ER in vivo is indeed exposed to varying levels of PPI activity may be hard to assess, however, given the difficulties inherent in determining the activity of phosphatases towards endogenous substrates in concentrated extracts or intact cells (e.g., Walker et al., 1992; Clarke et al., 1993; Ferrigno et al., 1993), and the need to consider both the cell cycle-dependent redistribution of PPI (Fernandez et al., 1992) and its targeting to different compartments, including ER, via associated subunits (e.g., Hubbard et al., 1990). It is also possible that cells may regulate ER movement according to physiological requirements other than the cell cycle. For instance, ER motility might be inversely correlated with the level of protein synthesis. This idea is based on the observation that when pancreatic acinar carcinoma cells (AR42J) are stimulated to increase protein synthesis and secretion, their ER morphology changes from a tubular network extending throughout the cell to stacks of lamellar ER located near the nucleus (Rajasekaran et al., 1993). Such ER lamellae are more common in less motile samples in vitro (Fig. 3), so the formation of lamellar ER could be promoted by turning off ER movement. It seems likely that ER morphology, movement and membrane traffic will prove to be very closely linked in living cells. The ability to reconstitute the regulation of one of these aspects of ER behavior in vitro is a promising first step towards understanding such complex processes.

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