

# Analysis of microtubule movement on isolated *Xenopus* egg cortices provides evidence that the cortical rotation involves dynein as well as Kinesin Related Proteins and is regulated by local microtubule polymerisation

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Received for publication 15 July 2002, revised 16 January 2003, accepted 20 January 2003

## Abstract

In amphibians, the cortical rotation, a translocation of the egg cortex relative to the cytoplasm, specifies the dorsoventral axis. The cortical rotation involves an array of subcortical microtubules whose alignment is mediated by Kinesin-related proteins (KRPs), and stops as M-phase promoting factor (MPF) activation propagates across the egg. To dissect the role of different motor proteins in the cortical rotation and to analyse their regulation, we have developed an open cell assay system involving reactivation of microtubule movement on isolated cortices. Microtubule movements were dependent on ATP and consisted mainly of wriggling and flailing without net displacement, consistent with a tethering of microtubules to the cortex. Reactivated movements were inhibited by anti-KRP and anti-dynein antibodies perfused together but not separately, the KRP antibody alone becoming fixed to the cortex. Neither antibody could inhibit movement in the presence of MPF, indicating that arrest of the cortical rotation is not due to MPF-dependent inhibition of motor molecules. In contrast, D<sub>2</sub>O treatment of live eggs to protect microtubules from progressive depolymerisation prolonged the cortical rotation. We conclude that the cortical rotation probably involves cytoplasmic dynein as well as cortical KRPs and terminates as a result of local MPF-dependent microtubule depolymerisation.

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*Keywords:* Molecular motors; Maturation/M-phase promoting factor (MPF); *Xenopus* egg extracts

## Introduction

The cortical rotation is the cellular process that breaks the radial symmetry of the amphibian egg, specifying the orientation of the embryonic dorsoventral axis. Cortical rotation translocates the entire cortex with respect to the underlying cytoplasm and transports dorsal “determinants,” factors able to trigger the formation of Spemann’s organiser, from the vegetal pole to an equatorial position, where they become active (Vincent et al., 1986; Elinson and Holowacz, 1995; Miller et al., 1999). If the cortical rotation is blocked

experimentally, the dorsal determinants remain at the vegetal pole, no organiser forms, and the embryo develops without dorsoanterior structures.

The cortical rotation is a microtubule-dependent process involving a spectacular array of microtubules aligned beneath the vegetal cortex (Manes et al., 1978; Vincent et al., 1987; Elinson and Rowning, 1988). It starts at the time that the vegetal microtubule array forms, roughly halfway through the cell cycle. Microtubules nucleated in inner regions, in particular by the “sperm aster,” arrive at the vegetal cortex and align parallel to the surface with their plus ends pointing in the direction of cortical translocation (Houliston and Elinson, 1991a, b). The cortical rotation stops at mitosis, at the precise time that a wave of M-phase promoting factor (MPF) activation accompanied by a visible “Surface Contraction Wave” (SCW-1) crosses the egg

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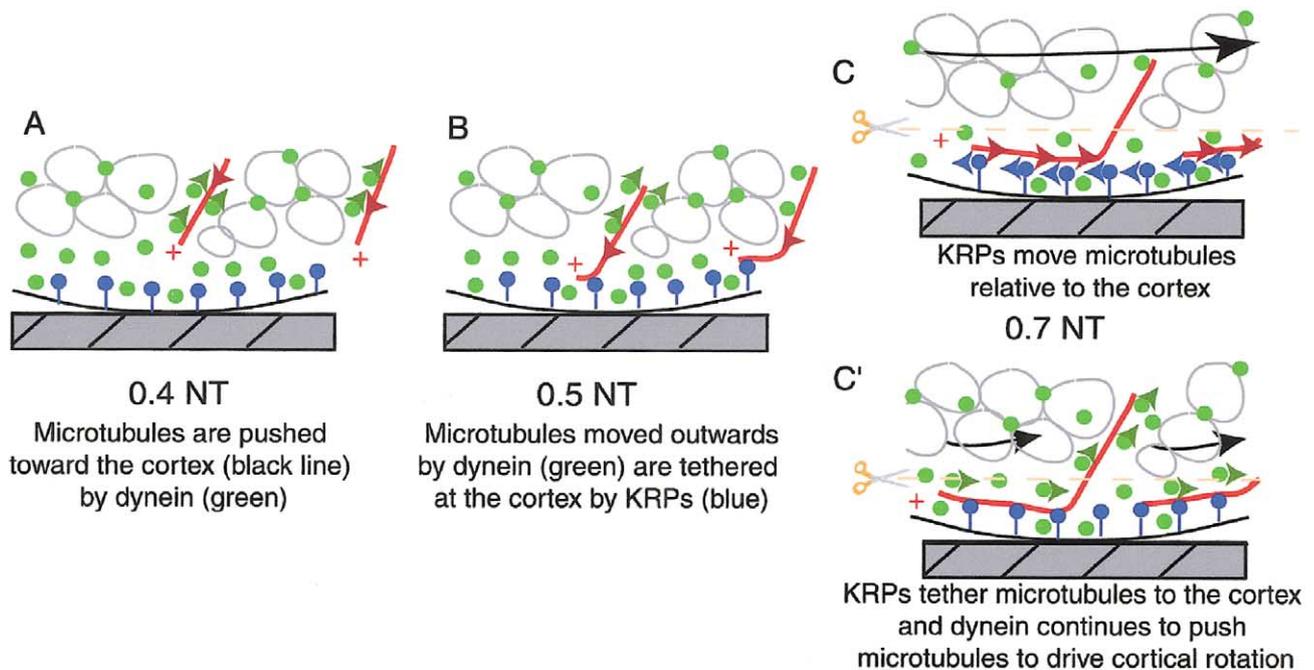


Fig. 1. Alternative models for the mechanism of cortical rotation. The vegetal region of an egg immobilised against a coverslip (grey) is shown. In this configuration, the large yolk platelets (white) embedded in the vegetal cytoplasm displace coordinately during cortical rotation (black arrows in C and C'), while the cortex remains stationary. Orange dashed lines in (C) and (C') indicate the portion of the cell removed with isolated cortices. Normalised times during the first cell cycle are indicated (NT, where 1NT is first cleavage). During the first half of the cell cycle (A), microtubules (red) nucleated in inner regions are displaced towards the vegetal cortex by cytoplasmic dynein (green). When they reach the vegetal cortex (B), they become anchored by KRPs (blue). One possibility to explain cortical rotation is that the cortically attached KRPs displace microtubules relative to the cortex by their action towards microtubule plus ends (C). Alternatively, the main force responsible for the cortical rotation could be provided by cytoplasmic dynein continuing to act between cytoplasmic elements and the microtubules, while KRP-based motility is insignificant and essentially plays a tethering role (C') (see text for more detailed explanations).

(Pérez-Mongiovi et al., 1998, 2000). Both the end of the cortical rotation and the SCW are clearly consequences of progressive activation of Cdc2, the kinase component of MPF. They may involve phosphorylation of distinct target molecules since depletion of the maternal RNA coding for the Kinesin-related protein (KRP) Xklp1 inhibits SCW1 but not the cortical rotation (Quaas and Wylie, 2002).

The mechanism of cortical rotation and its regulation by MPF remain to be understood. KRPs are known to play an essential role, since antibodies which interfere with interactions between KRP motor domains and microtubules block the cortical rotation locally when microinjected beneath the vegetal cortex (Marrari et al., 2000). These antibodies cause microtubules to detach from the cortex, indicating that KRP–microtubule interactions tether microtubules to the cortex in a similar way that the CENP-E motor tethers kinetochores to the plus ends of spindle microtubules (Lombillo et al., 1995). Current data do not allow us to decide to what extent KRP motor activity contributes directly to translocation of the cortex. One extreme possibility is that displacement of the cortex towards microtubule plus ends by cortically attached KRPs is solely responsible for the cortical rotation movement (see Fig. 1C; note that the experimental situation is shown in which the cortex is immobilised and displacement of the underlying cytoplasm is observed). Alternatively, given the predominant action of

cytoplasmic dynein relative to plus end motors in *Xenopus* egg cytoplasm (Allan and Vale, 1991; Lane and Allan, 1999), dynein could make a major contribution to the cortical rotation movement, with KRP-derived force at the cortex being insignificant (Fig. 1C'). Studies from a number of cell types support the possibility that dynein is active throughout the cell and so could drive microtubule displacement locally at all levels of the egg. In neuronal cells, for instance, dynein is required for the transport of microtubules into axons, where it is thought to act at the cortical level (Ahmad et al., 1998; Dillman et al., 1996). Strikingly, dynein also supports displacement of microtubules relative to structural components of low-speed “cytoplasmic” extracts of *Xenopus* eggs (Waterman-Storer et al., 2000). Microtubules detached from the sperm aster, as well as those nucleated independently in central regions of the egg (Houliston and Elinson, 1991b), could be moved similarly by dynein acting throughout the vegetal hemisphere to reach the vegetal cortex and eventually push the cortex relative to the cytoplasm (Fig. 1A, B, and C'). We know that formation of the vegetal subcortical microtubule array and cortical rotation can be driven uniquely by vegetal components, since they can occur in vegetal egg fragments as well as in eggs lacking nuclei and the sperm aster (Vincent et al., 1987; Houliston and Elinson 1991b; Elinson and Palacek, 1993).

The extreme hypotheses concerning the relative contributions of KRPs and dynein to the cortical rotation cartooned in Fig. 1 lead to different predictions concerning microtubule behaviour in the vegetal region. If KRPs drive the cortical rotation, microtubules should translocate with the vegetal cytoplasm relative to the cortex. If dynein provides the most driving force for the cortical rotation, microtubule translocation with respect to the cortex should be absent or weak, while vegetal cytoplasmic elements such as yolk platelets should displace relative to the tethered microtubules. The available studies of microtubule behaviour in live eggs have not allowed these possibilities to be clearly distinguished. Although the overall impression is of extensive microtubule movement in the direction of vegetal cytoplasm displacement (Houliston, 1994; Larabell et al., 1996), undulations of all microtubules make it difficult to unambiguously identify translocation with respect to the cortex (Houliston, 1994; Larabell et al., 1996; Marrari et al., 2000). Attempts to test the role of dynein *in vivo* by injection of inhibitory antibodies have also proven inconclusive (Marrari et al., 2000), probably because of the dominant and widespread action of cytoplasmic dynein in the egg (Allan and Vale, 1991; Lane and Allan, 1999).

We have developed an open cell preparation to analyse the mechanism of cortical rotation based on the reactivation of microtubule movement on isolated cortices. Cortical pieces isolated manually from amphibian eggs retain the main elements suspected to participate in the cortical rotation (Houliston and Elinson, 1991a; see Fig. 1C and C'). They consist of a thin "true" cortex ( $<0.5 \mu\text{m}$ ) corresponding to the outermost layer of the cell, comprising the plasma membrane, underlying actin and cortical ER, as well as a deeper, much thicker subcortical layer (5–10  $\mu\text{m}$ ). The subcortical layer contains the bulk of the vegetal array of microtubules and a three-dimensional network of associated ER (Houliston and Elinson, 1991a). We have been able to reactivate movement of microtubules on isolated *Xenopus* egg cortices by perfusion of ATP. Use of inhibitors combined with interphase or M-phase extracts has enabled us to analyse the contribution of KRPs and cytoplasmic dynein to microtubule movement at the vegetal cortex and to show that activity of both types of motor at the cortex persist through mitosis. This latter observation prompted us to conduct *in vivo* experiments and detailed confocal studies of microtubule organisation at the end of the cortical rotation, which indicated that cortical rotation is arrested not by MPF-dependant inhibition of molecular motors but as a result of MPF-induced microtubule depolymerisation.

## Materials and methods

### *Inhibitory agents and antibodies*

Most reagents, including ATP, AMP-PNP, sodium orthovanadate, and D70.1, a monoclonal antibody which

recognises an intermediate chain of cytoplasmic dynein (Walczak et al., 1998; Waterman-Storer et al., 2000), were obtained from SIGMA. To prepare a working stock of sodium orthovanadate, a 10 mM solution was adjusted to pH 10.2, boiled briefly, cooled, readjusted to pH 10.2, and boiled again. The final pH was 9.9 and the concentration 8.3 mM. Intermediate dilutions were prepared in ELB buffer (100 mM K-acetate, 3 mM Mg-acetate, 5 mM EGTA, 10 mM Hepes, 250 mM Sucrose, pH 7.6; Waterman-Storer et al., 2000) used to prepare extracts. The original buffer of commercially obtained D70.1 was exchanged for IB2 (10 mM K.Hepes, 88 mM KCl, pH 7.7) by using a centrifugal filter (Millipore; 30 KD cut-off) to give a 33-mg/ml stock. Inhibitory ("anti-LAGSE") and noninhibitory ("anti-HIPYR") rabbit polyclonal anti-KRPs antibodies were generated against conserved regions of the kinesin motor domain as described by Sawin et al. (1992) and purified as described previously (Marrari et al., 2000), giving a 5-mg/ml stock.

### *Preparation and observation of isolated cortices* (Figure 2)

Fertilisation and culture of embryos was as described previously (Marrari et al., 2000). To allow approximate comparison of cell cycle events despite variation in the length of the cell cycle, Normalised Time (NT) was used, where 0 NT was the time of insemination and 1 NT was the time when the furrow can be detected in roughly 50% of control eggs.

Approximately 10 nl of rhodamine-tubulin at 1 or 2 mg/ml in IBH buffer (50 mM Hepes, 0.1 mM  $\text{MgCl}_2$ , pH 6.9) prepared from twice-cycled pig brain tubulin as previously described (Houliston et al., 1993) was injected between 0.4 and 0.5 NT at two sites just beneath the vegetal cortex in fertilised and dejellied eggs to allow its incorporation into cortical microtubules during their polymerisation (Houliston, 1994). Vegetal cortices were peeled manually during the time of cortical rotation (0.5–0.8 NT) in AV buffer (100 mM K-acetate, 3 mM Mg-acetate, 5 mM EGTA, 10 mM Hepes, 50 mM Sucrose, pH 7.6; Allan and Vale, 1991) supplemented with 4  $\mu\text{M}$  taxol to stabilise microtubules, and protease inhibitors (10  $\mu\text{g/ml}$  leupeptin, 5  $\mu\text{g/ml}$  pepstatin, 10  $\mu\text{g/ml}$  aprotinin, 0.2 mM AEBSF; all from Sigma). Isolated cortices were attached and flattened onto paraplax-coated coverslips in a drop of PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM  $\text{MgCl}_2$ , pH 6.9; Schliwa et al., 1981). A glass slide with runners of paraplax-soaked lens-cleaning tissue (Whatman) was then lowered onto the coverslip, and the perfusion chamber was sealed with VALAB (vaseline, lanolin, bees' wax, 1:1:1). To preserve rhodamine fluorescence, we perfused an anti-bleach solution (10 mM glucose, 10  $\mu\text{g/ml}$  glucose-oxidase, 10  $\mu\text{g/ml}$  catalase, 1 mM DTT in AV/protease inhibitors/taxol; Blocker et al., 1996) and kept cortices in chambers in humid boxes at 18°C until use. Extracts used for perfusion

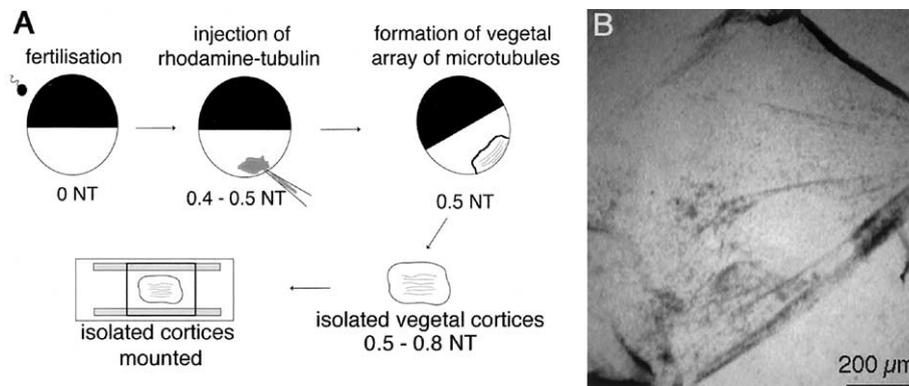


Fig. 2. The isolated cortex assay. (A) Rhodamine–tubulin was injected into fertilised eggs just before the formation of the microtubule array (between 0.4 and 0.5 NT). Pieces of cortex were isolated during the time of cortical rotation (0.5–0.8 NT) and mounted in perfusion chambers. Movements of rhodamine-labelled microtubules on isolated cortices were visualised by time-lapse fluorescent microscopy. (B) An isolated cortex mounted in a perfusion chamber observed by transmitted light microscopy.

were diluted in the same solution supplemented with an ATP regeneration system (1 mM Mg-ATP, 10 mM creatinine-phosphokinase, 80  $\mu\text{g/ml}$  creatine-phosphate; Blocker et al., 1996). Movement of microtubules on isolated cortices was recorded by time-lapse microscopy: Images were taken every 10 or 15 s with a LHESA SIT camera using a Zeiss Axiophot fluorescence microscope with automatic shutters run using “Metaview” software (Universal Imaging Corporation).

#### Preparation of cytoplasmic extracts

Interphase and metaphase extracts were prepared according to Allan (1993). Briefly, unfertilised and unactivated eggs were collected in 110 mM NaCl in tap water. For perfusion extracts, eggs were washed four times with XB buffer (100 mM KCl, 100  $\mu\text{M}$   $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, 50 mM Sucrose, pH 7.6) and two times with XB buffer/protease inhibitors (as above) containing 5 mM EGTA and 1 mM  $\text{MgCl}_2$ . For aster experiments, eggs were washed in ELB buffer and crushed by centrifugation at 9000g for 10 min. The cytoplasmic phase was clarified by a second centrifugation following addition of 100  $\mu\text{g/ml}$  cytochalasin B. To prepare interphase extracts from metaphase extracts, 1/50 volume of “conversion mix” (20 mM  $\text{CaCl}_2$ , 100 mM KCl, 1 mM  $\text{MgCl}_2$ ) and 100  $\mu\text{g/ml}$  cycloheximide was added. After 30 min of incubation at room temperature, EGTA was added to a final concentration of 0.4 mM to chelate calcium. Extracts were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

#### Preparation of sperm nuclei

*Xenopus* testes were crushed in about 3 ml of SUNAP (250 mM sucrose, 75 mM NaCl, 150  $\mu\text{M}$  spermine, 500  $\mu\text{M}$  spermidine, 20 mM HEPES, pH 7) at  $4^\circ\text{C}$  by using a Potter homogeniser (Hutchison et al., 1987). Plasma membranes of  $10^7$  sperm/ml were removed by using 400  $\mu\text{g/ml}$  lyso-

lethacin in SUNAP for 2 min at room temperature. Sperm nuclei were resuspended in 0.3% BSA/30% glycerol in SUNAP to obtain a concentration of  $10^4$  nuclei/ $\mu\text{l}$ , frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

#### Aster dispersion assay

A protocol to assess dynein-based aster dispersion was adapted from Waterman-Storer et al. (2000). A total of 8  $\mu\text{l}$  of converted extract supplemented with 1  $\mu\text{l}$  of rhodamine-tubulin at 1 mg/ml was mixed with approximately 100 nuclei in 1  $\mu\text{l}$ , and 0.5  $\mu\text{l}$  of the agent tested. D70.1 antibody was added at different concentrations before the addition of sperm nuclei and preincubated at  $4^\circ\text{C}$  for 30 min. The mix was incubated at  $18^\circ\text{C}$  for 16 min to allow the nucleation and dispersion of microtubules from nucleus-associated centrosomes, then fixed by addition of 10  $\mu\text{l}$  of 1% formaldehyde/40 mM Pipes/0.5 mM  $\text{MgCl}_2$ /0.5 mM EGTA/50% glycerol, pH 6.9. Two drops of 4  $\mu\text{l}$  from each tube, duplicated for each condition, were analysed. About 10 sperm nuclei per drop were chosen randomly, and asters associated with them were recorded with the Zeiss microscope and SIT camera described above. Camera settings were kept constant for each experiment. The number of saturated pixels representing the bright central area of the asters was counted for each recorded image by using “NIH Image” software (available via <http://rsb.info.nih.gov/nih-image>). The significance of differences between groups was tested by using Student’s *t* test at the 5% level.

#### Immunofluorescence of isolated cortices and whole eggs

Unfixed peeled cortices were incubated in extracts containing antibodies for 30 min with D70.1 or 15 min with anti-LAGSE, then washed twice with AV buffer. Cortices and eggs were fixed in methanol containing 1% formaldehyde at  $-20^\circ\text{C}$  and processed for immunofluorescence (Elinson and Rowning, 1988; Houliston, 1994; Houliston et

al., 1994) by using rat monoclonal antibody YL1/2 (anti- $\alpha$ -tubulin; Sera-Lab) with Texas Red-labelled anti-rat Ig (Jackson ImmunoResearch) to label microtubules, and fluorescein-linked anti-rabbit Igs or anti-mouse Igs (Jackson ImmunoResearch) to reveal anti-LAGSE or D70.1 associated with egg cortices. Confocal images were acquired on the Leica confocal microscope described previously (Houliston et al., 1994) or using a SP2 Leica microsystem. Controls in which primary antibodies were omitted confirmed that no cross-reaction between antibodies occurred.

#### *In vivo observations and D<sub>2</sub>O treatment*

To follow cortical rotation and SCWs by time-lapse microscopy, mitochondrial islands were labelled by using the lipophilic dye DiOC<sub>6</sub>(3) at 2.5  $\mu$ g/ml in H<sub>2</sub>O for 3 min (Savage and Danilchik, 1993; Pérez-Mongiovi et al., 2000). Eggs were transferred to 50% D<sub>2</sub>O/ 4% ficoll in 20% Steinberg starting about 5 min after the beginning of the cortical rotation. Fertilisation envelopes were removed 5 min later. Approximately 10 min after the beginning of cortical rotation, groups of five or six eggs, including three treated eggs, were mounted between slide and coverslip with a silicone rubber spacer. Five minutes later, cortical rotation and SCWs were recorded by time-lapse microscopy: Images were acquired every 15 s by using an inverted Zeiss microscope equipped with a motorised stage and CCD camera driven by “metamorph” software as described (Marrari et al., 2000). Movies were analysed with “NIH Image” software.

#### *Electrophoresis and Western blots*

To monitor cell cycle progression in perfused extracts and in D<sub>2</sub>O-treated eggs, the degree of Cdc25 phosphorylation was monitored by Western blotting. Standard histone H1 kinase assays to assess MPF activity of perfused extracts were not feasible due to variable dilution of extracts during perfusions. A total of 10  $\mu$ l of perfused extracts diluted at 1/8 was loaded on 10% acrylamide/bisacrylamide 28:8 gels. For comparison of the migration of Cdc25C isoforms, extracts with known active or inactive MPF were included. Migration and electrophoretic transfer were as described previously (Pérez-Mongiovi et al., 2000). For D<sub>2</sub>O experiments, two treated and two untreated eggs were monitored at successive times. Eggs were crushed in MR buffer (160 mM  $\beta$ -glycerophosphate, 40 mM EGTA, 30 mM MgCl<sub>2</sub>, 4 mM DTT, pH 7.5) supplemented by protease and phosphatase inhibitors (150  $\mu$ M NaF, 3 mM orthovanadate, 333  $\mu$ M AEBSF, 15.5  $\mu$ g/ml leupeptin, 15.5  $\mu$ g/ml Aprotinin, 16  $\mu$ g/ml pepstatin). Supernatants were diluted in MR containing inhibitors then heated with SDS sample buffer. To detect Cdc25C isoforms, a rabbit polyclonal anti-*Xenopus* Cdc25C at 1/200 (gift of E. Shibuya, Edmonton, Canada) was incubated with the blot for 1 h at room temperature and revealed by using a horseradish peroxidase-linked goat anti-

rabbit Ig (Zymed) by Enhanced Chemo-Luminescence. The intensity of bands corresponding to different phosphorylated Cdc25 isoforms was quantified by using NIH Image software.

## **Results**

### *Microtubule movement on isolated cortices promoted by ATP and egg extracts*

Fertilised *Xenopus* eggs were injected with rhodamine tubulin to label the array of cortical microtubules. Pieces of vegetal cortex were isolated manually during the period of cortical rotation and stored in an acetate/sucrose buffer that supports microtubule motor and ER movement (Allan and Vale, 1991). Taxol was included in all solutions to stabilise microtubules and prevent microtubule depolymerisation during experimental treatments. Movements of microtubules on isolated cortices perfused with various ATP-containing solutions (see below) were recorded by using time-lapse low light level microscopy (see Material and methods; Fig. 2).

Reactivated microtubule movement most commonly consisted of oscillations, wriggling, or flailing of microtubules in small groups or bundles on the cortex (Fig. 3A/movies 1, 2, and 3: all movies available at <http://biodev.obs-vlfr.fr/recherche/houliston>), sometimes accompanied by buckling of microtubule bundles (Fig. 3B/movies 4, 5, and 6). Although extensive areas of aligned microtubules were also detected on most cortices, microtubule movement in such areas could not be clearly distinguished. Translocations of microtubules for short distances relative to the cortex were observed very rarely, and sustained translocation was never recorded. We interpret the microtubule wriggling as resulting from the local action of motor proteins attached to cortical and subcortical components on parts of microtubules. Other parts of the same microtubules may be immobilised by association with other structures (including other microtubules) or subject to motor activity pushing in an opposing direction. It is probably not appropriate to interpret directly the types of microtubule movement observed in isolates in terms of the behaviour of microtubules during cortical rotation *in vivo*, given the overall the disturbances in organisation. Nevertheless, behaviour of microtubules on isolated cortices is compatible with their behaviour during the cortical rotation in whole eggs: *In vivo* recordings have revealed the presence of microtubule segments immobilised with respect to the cortex as well as waves of microtubule movement in the subcortical region (Larabell et al., 1996; Houliston, 1994; Marrari et al., 2000). The extent to which microtubule translocation occurs *in vivo* is unclear (see Introduction). The lack of translocation of microtubules on isolated cortices is compatible with a mechanism in which KRPs act mainly to tether microtu-

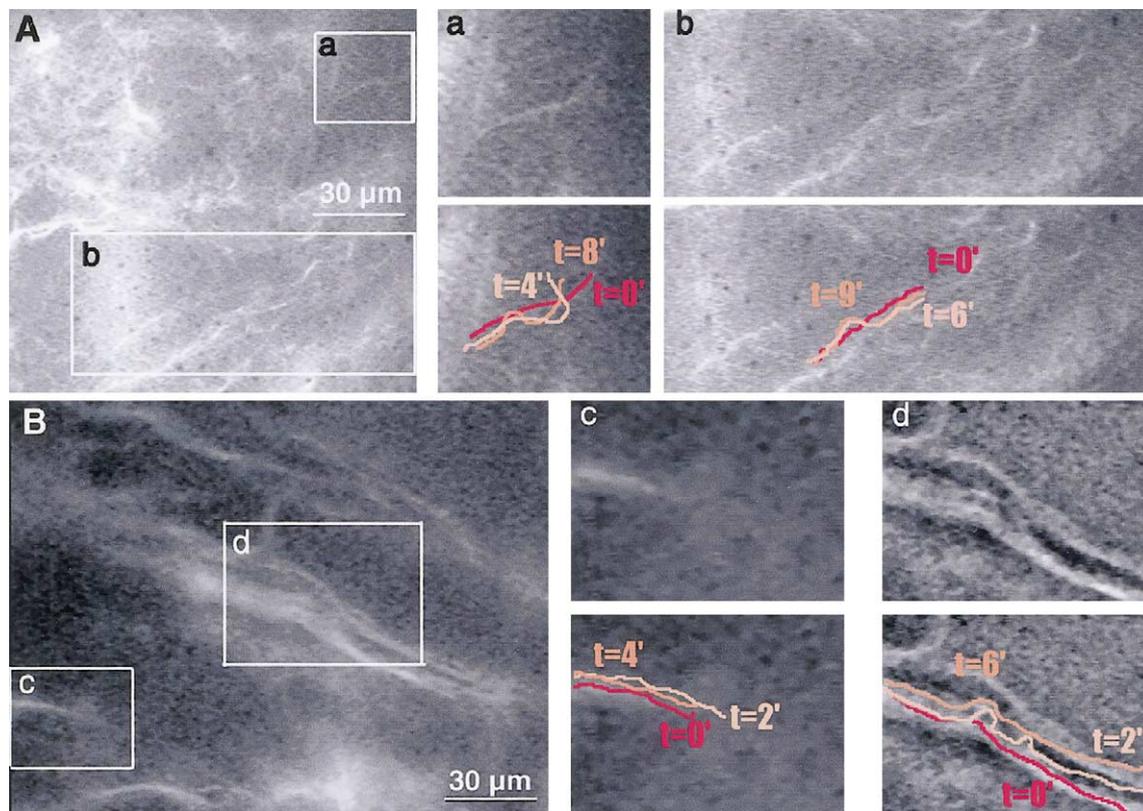


Fig. 3. Examples of microtubule movements on isolated cortices. Isolated cortices were perfused with buffer supplemented by an interphase extract diluted 1/4 and an ATP regenerating system. Initial images from sequences of 30 images acquired every 10 s are shown. The complete recordings are available (movies 1–6). In most cases, microtubules (A) wriggled or flailed relative to the cortex. In (B), microtubule movement provoked buckling of a microtubule bundle. For each sequence, two enlarged regions of the observed field are also shown (a,b/c,d), and the positions of certain microtubules traced from three representative images of the sequence superimposed on the same regions to illustrate the type of movement observed. Note that these do not come from consecutive frames or represent a single beating movement.

bules to the cortex and contributes only weakly to the force driving the cortical rotation (Marrari et al., 2000; Fig. 1C').

Perfusion of 1 mM  $Mg^{2+}$ /ATP in buffer was sufficient to reactivate microtubule wriggling persisting for several minutes on some isolated cortices. By inclusion of AMP-PNP, a nonhydrolysable ATP analogue, during peeling and mounting of cortices, microtubule wriggling could be reactivated more consistently and lasted longer following displacement of AMP-PNP with an excess of ATP (Vale and Hotani, 1988). The fact that microtubule movement on isolated cortices could be reactivated using only  $Mg^{2+}$ /ATP shows that components required to support microtubule-based movement and presumably functioning during cortical rotation are retained during manipulation of the isolates. The balance of contributions from cortical and cytoplasmic motors to microtubule movement may, however, be shifted compared with the *in vivo* situation, since the preparations contain cortical and subcortical structures but lack connections to deeper cytoplasmic regions.

The variability and limited duration of microtubule movement in these initial experiments, probably due to progressive loss of cytoplasmic factors during the experiment, did not allow comparison of different experimental

conditions. To prolong microtubule movement further, we supplemented the assay solution with diluted interphase cytoplasmic extracts of *Xenopus* eggs (see Materials and methods) to replenish soluble factors. Such extracts have been shown to support the displacement of organelles along microtubules as well as the translocation of microtubules on slides (Allan and Vale, 1991; Allan, 1993; Niclas et al., 1996). In the presence of ATP and egg extracts diluted up to six times, microtubule wriggling could persist for as long as 1 or 2 h, presumably because motors and/or their regulators were replenished by the extract. In subsequent experiments to test the effect of inhibitors, we standardly used interphase extracts diluted 1/4. The nature of the movement observed, and the variable proportion of microtubules wriggling in the fields under observation, precluded quantification of the movement. Two measures were taken to ensure objective comparison between conditions. Firstly, the same cortices were perfused with control solutions and test agents to allow direct comparison. Secondly, we scored movement in the presence of the test agents in a binary fashion, as equivalent to, or clearly reduced with respect to, movement in the corresponding control conditions. To ensure that any observed loss of activity was not due to damage to the cortex

Table 1  
Effect of inhibitors on microtubule movement on isolated cortices

Perfused agent <sup>a</sup>	Concentration	Number of cortices showing reduced microtubule movement <sup>b</sup>
AMP-PNP	5 mM	12/14
Sodium orthovanadate	25 $\mu$ M	0/6
	250 $\mu$ M	6/6
Cytochalasin B	10 $\mu$ g/ml	0/2
Cytochalasin D	10 $\mu$ g/ml	0/2
	10 $\mu$ g/ml (with 3 h of preincubation)	0/4

<sup>a</sup> Each perfusion contained interphase extract diluted at 1/4 supplemented with an ATP regenerating system.

<sup>b</sup> Movement was scored as “equivalent” or “clearly reduced” with respect to a control perfusion on the same isolated cortex.

during the course of the experiment, all cortices were finally washed and reperfused with control extracts. Only data from preparations on which movement of microtubules could be reactivated in these conditions, albeit less strongly than by initial control perfusions, were considered in our analyses.

#### *Motor proteins mediate microtubule movement on isolated cortices*

Since microtubule movement could be reactivated on isolated cortices using ATP, it seemed likely that molecular motors attached to cortices were involved. The involvement of ATP-dependant molecular motors was tested with drugs known to inhibit motor activity. AMP-PNP (5 mM) inhibits KRPs (Andrews et al., 1993; Muresan et al., 1996; Walczak et al., 1997) and probably other mechanochemical ATPases, including dynein. Perfusion of 5 mM AMP-PNP diluted in 1/4 interphase extracts clearly reduced microtubule movement on the isolated cortices (Table 1; movies 7–12). Note that general shifting of the cortex itself and perturbations provoked by the flow of perfusing solutions are responsible for movements in some recordings, which must be carefully distinguished from ATP-dependent wriggling and flailing. We also tested sodium orthovanadate, an inhibitor of ATPases frequently used to inhibit dynein activity. Concentrations of orthovanadate used in published studies to inhibit dynein in *Xenopus* egg extracts range from 1 to 100  $\mu$ M

(Verde et al., 1991; Heald et al., 1996; Blocker et al., 1997; Reinsch and Karsenti, 1997; Lane and Allan, 1999). At higher concentrations of orthovanadate (250  $\mu$ M), other ATP-dependent molecular motors, including KRPs, are considered to be inhibited (Blocker et al., 1997). Microtubule wriggling or translocations were clearly reduced in cortices perfused with 250  $\mu$ M orthovanadate (Table 1). Cortices perfused with 25  $\mu$ M orthovanadate continued to exhibit microtubule movement equivalent to controls; however, this cannot be interpreted as evidence for dynein independence since subsequent studies (see below) showed that dynein was not completely inhibited in our extracts by 25  $\mu$ M orthovanadate.

To test the involvement of the microfilament network in the reactivated microtubule movement, we treated cortices with 10  $\mu$ g/ml cytochalasin B or D, drugs known to disrupt actin filaments. No reduction of the microtubule movement was detected, even when cortices were preincubated for 3 h with cytochalasin D before perfusion of ATP and extracts (Table 1).

These inhibitor studies suggest strongly that ATP-dependent microtubule motors are responsible for the microtubule movement recorded on isolated cortices, and indicate that the cortical microfilament network does not actively participate. These characteristics correspond to those of the cortical rotation in vivo (Manes et al., 1978; Vincent et al., 1987).

#### *Both dynein and KRPs can support microtubule movement on isolated cortices*

To determine which motors participate in microtubule movements on isolated cortices, KRP and dynein motors were blocked with selective antibodies. KRPs were inhibited by using an antibody raised against a peptide corresponding to a motor domain sequence conserved amongst KRPs (“anti-LAGSE”; Hogan et al., 1993; Sawin et al., 1992). Perfusion of cortices with extracts containing anti-LAGSE or a noninhibitory anti-KRP antibody (anti-HIPYR) supported microtubule movement (Table 2; movie 13). Likewise in vivo, vigorous microtubule movement continues in the presence of anti-LAGSE, despite the local arrest of the cortical rotation (Marrari et al., 2000). These results indicate that KRP action is not solely responsible for

Table 2  
Effect of inhibitory antibodies on isolated cortices

Perfused antibodies <sup>a</sup>	Concentration and condition	Number of cortices showing reduced microtubule movement <sup>a</sup>
anti-LAGSE (anti-KRP motor domain)	0.5 mg/ml (15 min of incubation on perfused cortices)	0/4
D70.1 (anti-dynein intermediate chain)	1 mg/ml (30 min of preincubation in extract)	0/6
D70.1 and anti-LAGSE	1 mg/ml and 0.5 mg/ml	4/4
D70.1 and anti-LAGSE in undiluted extract	1 mg/ml and 0.5 mg/ml	2/2

<sup>a</sup> Perfusion conditions and scoring as in Table 1.

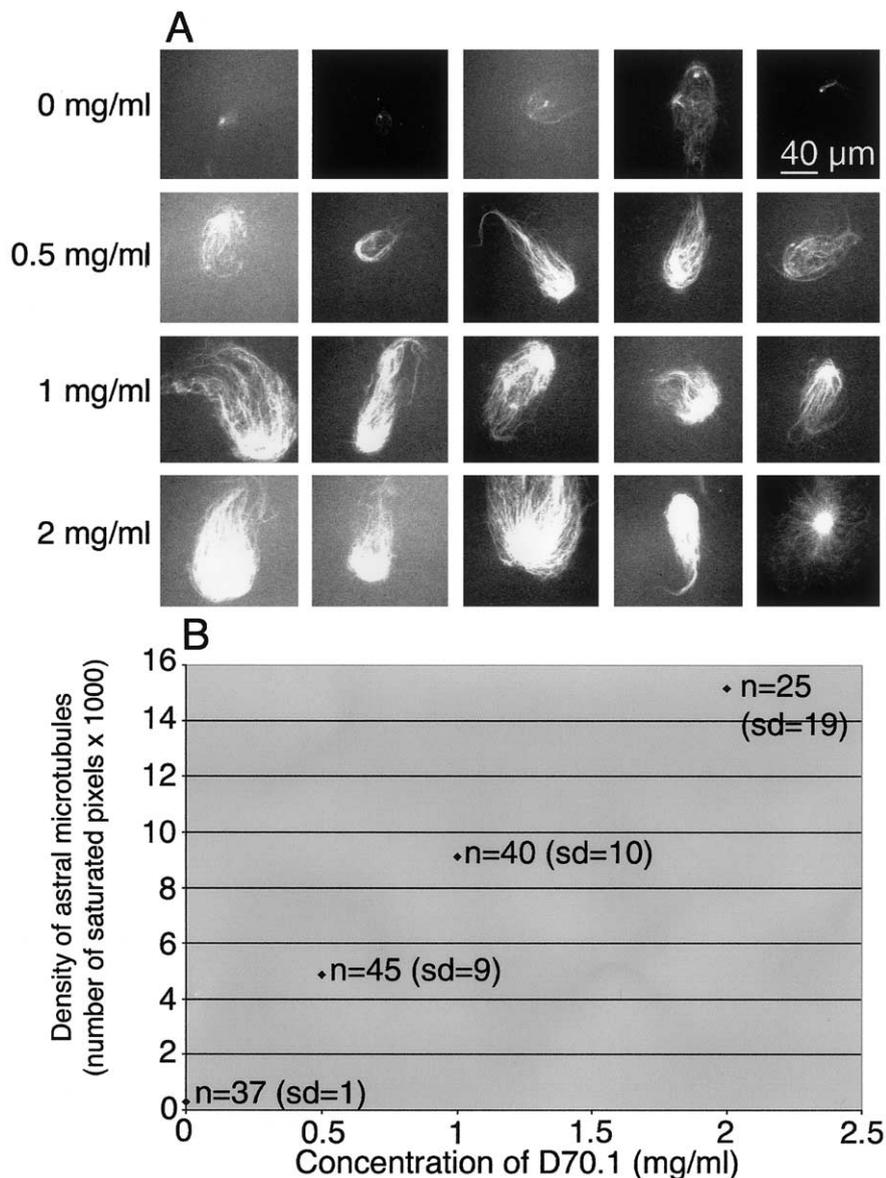


Fig. 4. Aster dispersion assay to test dynein inhibitors. (A) Images of fixed asters recorded with identical acquisition parameters were obtained in the presence of D70.1 at different concentrations, following 30 min preincubation at 4°C. The bright zone in the centre of the asters became bigger as the concentration of antibody was increased to 1 or 2 mg/ml. (B) The mean number of saturated pixels counted from  $n$  asters for each condition showed a significant difference in the presence of 0, 0.5, and 1 mg/ml of D70.1 but not between 1 and 2 mg/ml (Student's  $t$  test, 5% level).

generating the microtubule movement on isolated cortices, but do not rule out a role for KRPs in conjunction with other motor molecules.

To inhibit dynein, we tested two potential inhibitors, sodium orthovanadate (see above) and an antibody recognising an intermediate chain of dynein, D70.1. This antibody has been used to inhibit dynein activity in *Xenopus* egg extracts (Heald et al., 1996; Walczak et al., 1998); however, as with orthovanadate, published studies used a wide variety of concentrations in different experimental conditions. We tested the efficiency of inhibition of dynein motor activity by D70.1 and orthovanadate in our hands using an assay based on the expansion of microtubule asters nucleated by the centrosomes of sperm nuclei in *Xenopus*

extracts (see above; Waterman-Storer et al., 2000). Asters that form around the centrosomes of demembrated sperm nuclei disperse rapidly in untreated extracts as cytoplasmic dynein translocates the microtubules outwards relative to unidentified structures (Waterman-Storer et al., 2000). In the presence of dynein inhibitors, microtubules accumulate around the centrosome and form dense asters. We tested potential dynein inhibitors by incubating them in undiluted interphase extracts supplemented with rhodamine tubulin and centrioles linked to sperm nuclei (see Materials and methods). The number of saturated pixels in rhodamine-tubulin images around nuclei recorded under identical conditions (Fig. 4A) was used as a semiquantitative estimate of dynein activity (Fig. 4B). Initial trials established that inhi-

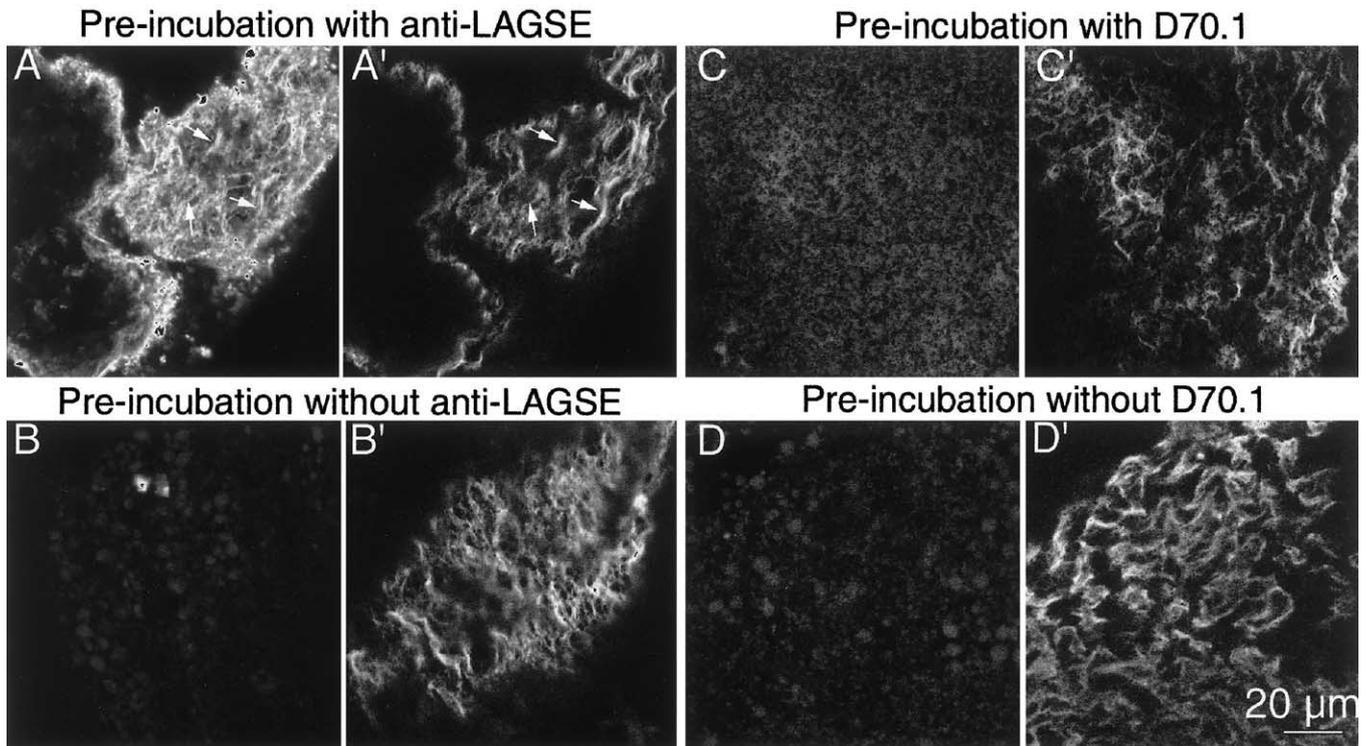


Fig. 5. Anti-LAGSE but not D70.1 attaches irreversibly to isolated cortices. Detection of anti-LAGSE (A, B) and D70.1 (C, D) using fluorescent secondary antibodies on isolated cortices incubated with these antibodies (A, C) or mock-incubated (B, D) and then washed with AV buffer before fixation. (A'–D') Anti-tubulin immunofluorescence of the same cortices. Anti-LAGSE attached irreversibly to isolated cortices and was found to remain colocalised with microtubule bundles (arrows) as well as distributed more generally over the cortex (A;  $n = 7$ ). In contrast, only a weak diffuse D70.1 distribution was observed on cortices incubated with D70.1 after washing (C;  $n = 9$ ).

tion by D70.1 was only achieved if the antibody was preincubated with the extracts for 30 min at 4°C (3 trials without incubation, 4 trials with incubation; data not shown). This requirement for preincubation may explain in part the variability in D70.1 effects reported in previous studies. A concentration-dependent effect of D70.1 was observed. Asters nucleated in the extracts incubated with 0.5 and 1 mg/ml of D70.1 were significantly different from each other and from controls ( $t$  test using data from 4 trials; Fig. 4A and B), whereas the effect of D70.1 at 1 and 2 mg/ml was not significantly different (2 trials; Fig. 4A and B). We also found that asters that formed in extracts incubated with 25  $\mu$ M orthovanadate were less dense than in 1 mg/ml D70.1, indicating that 25  $\mu$ M orthovanadate does not fully inhibit dynein activity (not shown).

The aster dispersion trials established that dynein was efficiently inhibited by using 1 mg/ml D70.1 following preincubation for 30 min. Cortices were thus perfused with extracts preincubated with D70.1 for 30 min at 4°C to block dynein present in the isolates. Equivalent microtubule wriggling persisted in the presence of D70.1 or of a control IgM (Table 2). Thus, dynein is not exclusively responsible for generating the observed movements. The movement observed in the presence of D70.1 is probably generated by KRPs. KRP motor action is essential for the cortical rotation as shown by the injection of anti-LAGSE in vivo. KRPs

could provoke microtubule movements on isolated cortices in the absence of dynein activity, irrespective of whether they are largely responsible for translocation of the cortex relative to microtubules in vivo (Fig. 1C), or whether their motor function is normally subordinate to that of dynein (Fig. 1C'),

When the inhibitory antibodies to both KRPs and dynein were perfused together, microtubule movement was clearly reduced compared with control conditions on the same cortices (Table 2, movie 14). The relative inhibition of microtubule movement in the combined presence of anti-LAGSE and D70.1 was not due to nonspecific effects of high antibody concentrations since the noninhibitory anti-KRP antibody anti-HIPYR (Marrari et al., 2000) combined with D70.1 in identical experimental conditions did not inhibit movement (data not shown). We conclude that the movement of microtubules on isolated cortices is generated by the combined action of KRPs and cytoplasmic dynein.

#### *Irreversible binding of anti-LAGSE to cortices*

We could reactivate movement of microtubules on cortices treated with a combination of anti-LAGSE and D70.1 by washing and reperfusion of extracts without antibody. In contrast, we were not able to restore microtubule movement on such cortices with extracts containing D70.1. This result

Table 3  
Microtubule movement on isolated cortices perfused with metaphase extracts supplemented with different antibodies

Perfusion <sup>a</sup>	Concentration and condition	Number of cortices showing reduced microtubule movement <sup>b</sup>
Metaphase extract		0/4
Metaphase extract + D70.1	1 mg/ml (30 min of preincubation in extract)	0/4
Metaphase extract + anti-LAGSE	0.5 mg/ml (15 min of incubation on perfused cortices)	0/3

<sup>a</sup> Each perfusion contained undiluted metaphase extract and ATP.

<sup>b</sup> Microtubule movement scored with respect to undiluted interphase extract.

could be explained by irreversible binding of anti-LAGSE to cortical KRPs. To investigate this possibility, cortices were incubated with extracts containing either of the two antibodies, washed with buffer in conditions equivalent to those used in the motility assay, then fixed and labelled with fluorescent secondary antibodies (Fig. 5). Perfused anti-LAGSE remained attached on isolated cortices, colocalising with some microtubules (Fig. 5A and A'), whereas D70.1 was barely detectable after washing (Fig. 5C and C'). These results indicate that the anti-KRP antibody attaches irreversibly to the cortex and that KRPs in the extract are not able to restore movement. In contrast, D70.1 is readily lost from the cortex by washing, possibly because cytoplasmic dynein is not strongly attached to cortical structures, and can be restored from fresh extracts.

Taken together our experiments with anti-KRP and anti-dynein antibodies indicate that microtubule movement reactivated on isolated cortices is due to the action of both KRPs and dynein, such that motors of either family can support microtubule movement on its own. The KRPs involved appear to be tightly attached to the cortex, while dynein forms weaker associations with cortical/subcortical structures.

#### *MPF activity does not affect the microtubule movement on isolated cortices*

The halt of cortical rotation coincides with the passage of a Surface Contraction Wave (SCW1), an animal–vegetal wave of local relaxation dependent in part on microtubules. SCW1 mirrors a wave of MPF activity which propagates across the egg (Rankin and Kirschner, 1997; Pérez-Mongiovi et al., 1998). Localised injection of MPF provokes an ectopic reorganisation wave and stops the cortical rotation (Pérez-Mongiovi et al., 2000). MPF activity is thus likely responsible for bringing an end to the cortical rotation.

We used the isolated cortex assay to test the effect of MPF activity on the molecular motors functioning at the vegetal cortex during cortical rotation. It is known that the activity of dynein and of certain KRPs can be modulated by MPF phosphorylation (Addinall et al., 2001; Allan and Vale, 1991; Blangy et al., 1995; Niclas et al., 1996; Sawin and Mitchison, 1995; Wittmann et al., 1998). Cortices were perfused with undiluted M-phase extracts prepared from unactivated *Xenopus* eggs, in which MPF activity is high

(Allan, 1993). We confirmed that metaphase extracts maintained a high MPF activity during the experiments by monitoring the phosphorylated isoforms of Cdc25C, an activator and substrate of MPF (Fig. 6, Pérez-Mongiovi et al., 2000). Microtubule movement was observed on isolated cortices perfused with metaphase extracts as with interphase extracts (Table 3), indicating that at least one of the motors was insensitive to MPF. To test the influence of MPF separately on the dynein or KRPs responsible for microtubule movement, we perfused metaphase extracts incubated with either D70.1 or anti-LAGSE. Microtubule movement was equivalent to controls in both cases (Table 3). Inclusion of both antibodies in metaphase extracts was not technically feasible, since the final dilution reduced MPF activity. Nevertheless, we were able to show that the combination of these antibodies added to undiluted interphase extracts was able to reduce microtubule movement on isolated cortices (Table 2).

These observations indicate that MPF activity does not inhibit either KRP or dynein activity at the vegetal cortex. This implies that MPF does not block the cortical rotation by inhibiting microtubule motors.

#### *Cortical rotation can be maintained during M-phase by stabilising microtubules*

If MPF does not inhibit microtubule movement driven by KRPs or dynein at the cortex, how could it stop the cortical rotation *in vivo*? One possibility is that MPF acts via destabilisation of microtubules. This was initially discounted as an explanation for the end of the cortical rotation since aligned subcortical microtubules in *Xenopus* eggs are detectable through the mitotic period, although they have been

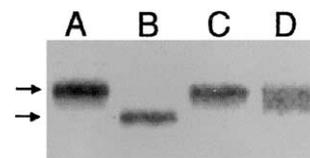


Fig. 6. MPF activity is maintained in perfused extracts. Western blot using anti-Cdc25C to monitor MPF activity in perfused extracts. Phosphorylated Cdc25C isoforms (upper arrow) in a control metaphase extract (A) and dephosphorylated isoforms (lower arrow) in an interphase extract (B) are shown for comparison. Before perfusion and incubation, metaphase extracts contained maximally phosphorylated isoforms (C). After perfusion and incubation, only a little dephosphorylation had occurred (D).

reported to disassemble gradually between 0.8 and 1.0 NT (Elinson and Rowning, 1988). Furthermore, if the cell cycle of *Xenopus* eggs is arrested in interphase, the vegetal cortical microtubule array persists longer, an effect that can be reversed by MPF (Schroeder and Gard, 1992). Our *in vitro* results prompted us to reexamine the possibility that MPF provoked a depolymerisation of certain vegetal microtubules, which would consequently stop the cortical rotation. Note that this possibility was not addressed in the isolated cortex experiments, in which microtubules were stabilised by using taxol.

We used deuterium oxide ( $D_2O$ ) to stabilise microtubules of the array during cortical rotation (Fig. 7). Although  $D_2O$  does not selectively affect microtubules, it readily penetrates the plasma membrane and so can be applied rapidly to the entire vegetal array. Microinjection of taxol, a more specific but non-cell-permeable microtubule stabilising agent, was attempted but provoked inhomogeneous movements centered around the injection site.  $D_2O$  treatment was initiated after cortical rotation was underway. Earlier treatment blocks the translocation of the cortex, probably because of an anarchical polymerisation of microtubules (Scharf et al., 1989). We confirmed that  $D_2O$  treatment stabilised the microtubule array by fixing eggs at later times and labelling microtubules by anti-tubulin immunofluorescence (Fig. 7C). In eggs fixed at 1.03 NT, much of the microtubule array was depolymerised in untreated eggs, although some aligned microtubules were still detectable over at least part of the vegetal cortex. In contrast,  $D_2O$ -treated eggs showed clear aligned microtubule arrays across the vegetal cortex at this time. As cortical rotation depends on the cell cycle, which may itself be affected by microtubule polymerisation (Pérez-Mongiovi et al., 2000), we also confirmed that  $D_2O$  treatment did not perturb the time course of MPF activity by monitoring the phosphorylation of Cdc25C (Fig. 7B).

The timing of the end of cortical rotation was compared in  $D_2O$  and untreated eggs by time-lapse microscopy of vegetal mitochondrial islands labelled with a vital dye (Savage and Danilchik, 1993). In untreated eggs, cortical rotation stopped at around 0.95 NT as SCW 1 passed (Fig. 7A; Pérez-Mongiovi et al., 1998). In  $D_2O$ -treated eggs, cortical rotation was prolonged until around 1.03 NT, and SCW-1 was not detected (Fig. 7A). This represents a delay in cortical rotation arrest of 5–15 min. Arrest of cortical rotation in  $D_2O$ -treated eggs occurred at approximately the time of a second SCW observable in control eggs. This “SCW-2” coincides with progressive MPF inactivation across the egg, is immediately followed by the cleavage furrow, and is thought to reflect modification of the cortical actomyosin network (Pérez-Mongiovi et al., 1998). Arrest of the cortical rotation in  $D_2O$ -treated eggs at this time is presumably a consequence of cleavage-associated cortical modifications.

Taken together, our *in vitro* results and the  $D_2O$  experiments suggested that cortical rotation is stopped by a partial and localised depolymerisation of the array of microtubules

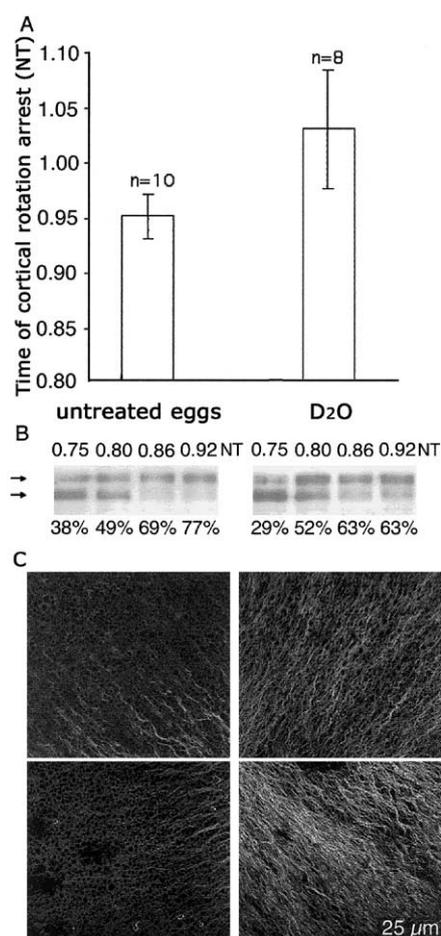


Fig. 7.  $D_2O$  treatment stabilises vegetal microtubules and prolongs the cortical rotation. (A) Comparison of the time of the arrest of the cortical rotation in untreated eggs and in eggs treated with  $D_2O$  starting during the cortical rotation, derived from time-lapse sequences of the displacement of mitochondrial islands.  $n$  is the number of eggs analysed. In untreated eggs, the cortical rotation stopped as SCW1 crossed the vegetal pole at around 0.95 NT. The timing of the cortical rotation arrest in eggs treated with  $D_2O$  was significantly delayed compared with untreated eggs (Student's  $t$  test, 5% level). (B) The time course of MPF activation, monitored by following the phosphorylation of Cdc25C in untreated (left) and  $D_2O$ -treated (right) eggs (annotation as in Fig. 6) was similar, as indicated by following the percentage of the intensity of the active upper band compared to the inactive lower band at successive times. (C) Examples of anti-tubulin immunofluorescence of vegetal cortical regions of  $D_2O$ -treated and untreated eggs fixed at 1.03 NT. In the absence of  $D_2O$ , the array of microtubules had clearly depolymerised over a large part of the egg vegetal surface (left), whereas  $D_2O$  stabilised microtubules across the entire vegetal surface (right).

provoked by the wave of MPF activation. To further investigate this possibility, untreated eggs were fixed at successive times between 0.90 and 1.05 NT, and cortical microtubules were examined in detail by immunofluorescence and confocal microscopy (Fig. 8). At 0.90 NT, a strong array of aligned microtubules covered the entire surface of the eggs (Fig. 8A). In contrast, eggs fixed around 0.96 NT, corresponding to the time of the passage of SCW-1 through the vegetal cortex (Fig. 7A, Pérez-Mongiovi et al., 1998), displayed a front of depolymerisation of microtubules (Fig.

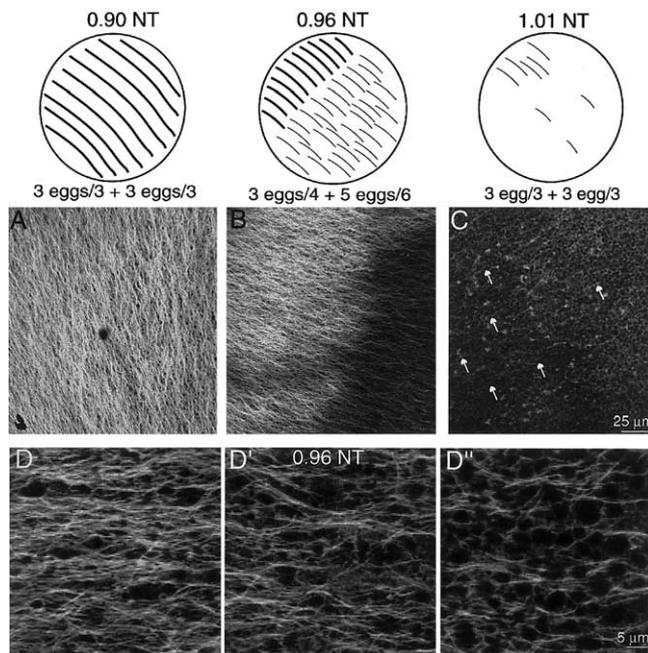


Fig. 8. A front of depolymerisation coincides with the arrest of cortical rotation. Confocal images of fertilised eggs fixed at successive times and processed for immunofluorescence detection of microtubules (combined data from two experiments). In all six eggs fixed at 0.90 NT (A), an array of microtubules covered the entire surface of the vegetal cortex as cartooned above. In most (8/10) eggs fixed around 0.97 NT (B), the time of the passage of the SCW1, a clear front of depolymerisation was detected. (D, D' and D'') Images of different regions of a single egg fixed at this time, spanning the front. At approximately 1.01 NT (C), the population of microtubules was dramatically reduced in all six eggs, a density gradient still being detectable across the vegetal surface.

8B, D–D''). On one side of this front, the pattern of the microtubule array was similar to that at 0.90 NT, while on the other side, the population of microtubules was clearly diminished (Fig. 8D–D''). In eggs fixed around 1.01 NT, the density of microtubules was generally low with a gradient in density still detectable across the vegetal cortex (Fig. 8C).

The striking front of microtubule depolymerisation detected at the time SCW1 passes through the vegetal hemisphere, together with *in vitro* data and the results of our D<sub>2</sub>O experiments, provide strong evidence that MPF activation does not inhibit the motor activity required for the cortical rotation but provokes a partial depolymerisation of microtubules, which brings this movement to a halt.

## Discussion

Experiments reported here indicate that the analysis of microtubule movement reactivated on isolated cortices can provide valuable insight into the mechanism and the regulation of the cortical rotation. Using this open system, we have demonstrated that dynein as well as KRPs can promote microtubule movement in the cortical/subcortical region, supporting the idea of cooperation between both types of

motors in the cortical rotation. Unexpectedly, our analyses also revealed that neither KRP nor dynein activity at the cortex was sensitive to elevated MPF activity. This conclusion was confirmed by *in vivo* experiments in which the cortical rotation was prolonged when microtubules were stabilised through mitosis. These data, along with the novel demonstration of a front of depolymerisation of vegetal microtubules prior to cleavage, strongly suggest that the timing of cortical rotation is controlled by cell cycle-dependent modulation of microtubule dynamics rather than of motor activity.

### *An open system to study cortical rotation*

Studies on whole eggs have revealed the basic features of the mechanism of cortical rotation (Elinson and Rowling, 1988; Marrari et al., 2000; Vincent et al., 1987); however, they have been limited by the difficulty of introducing specific inhibitory agents at precise cellular sites. On the other hand, *in vitro* analyses in *Xenopus* egg cytoplasmic extracts have provided a wealth of information concerning the activity and regulation of motor proteins (Allan and Vale, 1991; Heald et al., 1996; Niclas et al., 1996), but are not suited to address their localised regulation within the cell. For instance, in *Xenopus* egg extracts, ER is transported exclusively by cytoplasmic dynein but not by ER-associated KRPs (Allan and Vale, 1991; Lane and Allan, 1999), whereas *in vivo* studies indicate that KRPs associated with cortical ER are active (Marrari et al., 2000). In order to study locally at the cortex the activity of KRPs and dynein and their regulation by MPF, we have developed an open system in which microtubule movement on isolated vegetal cortices is reactivated in the presence of ATP and prolonged by supplementation with egg cytoplasmic extracts. In a similar way, cleavage furrow progression has been reactivated by using sea urchin zygote cortices (Walker et al., 1994) and calcium uptake and release by cortical ER studied on isolated sea urchin egg cortices (Terasaki and Sardet, 1991).

As for any *in vitro* motility system, it is impossible to be sure that the microtubule movement reactivated on isolated cortices faithfully reproduces the *in vivo* situation. We could nevertheless verify that the properties of this system we have studied were consistent with the available *in vivo* data: *In vivo*, the cortical rotation requires ATP and is driven by molecular motors rather than microtubule polymerisation (Vincent et al., 1987; Houliston, 1994), but is not perturbed by treatment with cytochalasin (Manes et al., 1978; Vincent et al., 1987). We found that microtubule movement on isolated cortices is likewise ATP-dependent and is blocked by inhibitors of mechanochemical ATPases, while being insensitive to cytochalasin. *In vivo*, inhibitory anti-KRP antibodies uncouple cortex–microtubule interactions but do not suppress microtubule movement. Likewise, on isolated cortices, movement persists in the presence of these antibodies. The fact that microtubules do not detach

from cortical isolates treated with anti-LAGSE can probably be explained by the presence in these preparations of elements of the thick subcortical layer, between which the microtubules are embedded. The lack of microtubule translocation on isolated cortices may at first seem incompatible with the overall impression of movement relative to the cortex *in vivo* (Houliston, 1994; Larabell et al., 1996); however, much of this movement may be due to waves of movement travelling along attached microtubules. Microtubule translocation has not been clearly demonstrated close to the cortex *in vivo*; indeed, nontranslocating microtubules have been identified at this level (Houliston, 1994; Marrari et al., 2000).

*In vitro* evidence supports roles for dynein as well as KRPs in the cortical rotation

Various lines of evidence from our studies on isolated cortices reinforce the hypothesis that dynein and KRPs cooperate during the cortical rotation (see Introduction; Fig. 1C'). The first concerns the nature of the microtubule movement reactivated on isolated cortices. Most microtubules oscillated or wriggled on the cortices, while translocations were observed rarely and covered only very short distances. This would be expected if the distal portions of subcortical microtubules are tethered to the cortex (Fig. 1C'), but not if cortically attached KRPs alone produce the force responsible for the cortical rotation and displace microtubules extensively relative to the cortex (Fig. 1C). Secondly, we demonstrated that dynein as well as KRPs contribute to the reactivated microtubule movement. Note that the persistence of microtubule movement in the presence of dynein inhibitors is entirely compatible with the hypothesis outlined in Fig. 1C', in which dynein is mainly responsible for cortical translocation. Since KRP motor function is essential for its function in the cortical rotation, KRPs are likely to provoke microtubule movements in the absence of dynein activity, even if their motor function is normally subordinate to that of dynein. Furthermore, the absence of deeper cytoplasmic regions from which the many of the subcortical microtubules originate may not only affect important organisational properties of the subcortical zone, but shift the balance of motor activity towards KRPs by reducing the extent of the zone over which dynein acts.

During expansion of the sperm aster during the first half of the cell cycle (Reinsch and Gönczy, 1998) and during cortical rotation (Marrari et al., 2000), dynein has been proposed to move microtubules relative to a wide-spread intracellular "matrix" distributed throughout the egg, including cortical and subcortical regions. This matrix potentially comprises multiple structures known to interact with dynein, including Golgi membranes (Fath et al., 1997; Roghi and Allan, 1999), other microtubules (Larabell et al., 1996), intermediate filaments (Shah et al., 2000), and microfilaments (Waterman-Storer et al., 2000). The association of dynein with such structures is mediated by the dynactin

complex (Blocker et al., 1997; Skop and White, 1998; Steffen et al., 1997; Wittmann et al., 2000) and/or involves direct interaction with phospholipids (Lacey and Haimo, 1994). The interaction of dynein with certain of its supports is inhibited by MPF; for instance, in *Xenopus* egg extracts, MPF phosphorylation of a dynein light intermediate chain dissociates dynein and dynactin from ER (Addinall et al., 2001; Allan and Vale, 1991; Blangy et al., 1995; Liao et al., 1994; Niclas et al., 1996; Sawin and Mitchison, 1995). The motor activity of dynein is not, however, inhibited in mitosis; indeed, it actively participates in spindle formation by targeting of NUMA, Xklp2, and Eg5 to spindle poles (Gaglio et al., 1996; Wittmann et al., 1998, 2000; Kapoor and Mitchison, 2001). Dynein activity in our isolated cortex assay was not inhibited by MPF, suggesting that it attaches to structures other than ER. Furthermore, dynein activity could be restored to isolated cortices perfused with anti-LAGSE and D70.1 from extracts following washing, indicating that there is a ready exchange between soluble and structure-bound pools of dynein during both interphase and mitosis.

Support for an anchoring role of KRPs tightly linked to the cortex is provided by the observation that, unlike D70.1, anti-LAGSE antibody incubated with isolated cortices remained attached following washing. Immunofluorescence studies in fixed eggs and isolated cortices have indicated that KRPs, including conventional kinesin associate with cortical ER (Houliston and Elinson, 1991a; Marrari et al., 2000); however, the identity of the cortical KRPs and their mode of attachment to the cortex remain to be determined. Candidate KRPs include conventional kinesin and kinesin II, both of which attach to membranous organelles and function during both interphase and mitosis (Scholey, 1996). The abundant KRP Eg5 also associates with the aligned vegetal microtubules during interphase (Houliston et al., 1994), despite the fact that its mitotic functions are activated by MPF (Blangy et al., 1995; Sawin and Mitchison, 1995; Blangy et al., 1997). Ultrastructural studies did not, however, reveal links between Eg5 and any other cortical structure (Chang et al., 1996). Another candidate, Xklp1, appears to be not essential for cortical rotation since Xklp1 depletion by injection of antisense oligonucleotides into the oocyte has no effect on the cortical rotation (Quaas and Wylie, 2002). Redundancy between multiple KRPs, including Xklp1, in the cortical rotation cannot be ruled out. Use of antibodies specifically inhibiting different KRPs in conjunction with D70.1 on isolated cortices will enable the activity of candidate KRPs at the cortex to be tested singly and in combination.

*MPF-induced microtubule depolymerisation halts the cortical rotation*

There is strong evidence that cortical rotation is arrested by a wave of propagated MPF activity which drives cortical changes manifest as SCW1 (Rankin and Kirschner, 1997;

Pérez-Mongiovi et al., 1998). Cortical rotation can be prolonged in vegetal fragments of eggs (Vincent et al., 1987) in which MPF activation does not occur (Pérez-Mongiovi et al., 2000), while injections of MPF can provoke ectopic SCW1-like waves which arrest cortical rotation (Pérez-Mongiovi et al., 2000). We initially favoured the possibility that an inhibition of molecular motors was responsible for the arrest of cortical rotation. In the isolated cortex assay, however, MPF activity did not inhibit detectable KRP or dynein activity. We were thus prompted to reexamine the hypothesis that the loss of microtubules from the array under the influence of MPF may halt the cortical rotation. The disassembly of the aligned vegetal microtubules depends on MPF activation (Schroeder and Gard, 1992), and their disappearance is delayed in vegetal fragments (Elinson and Palacek, 1993). We have now been able to detect a clear front of depolymerisation of microtubules correlating in time with the passage of the MPF wave through the vegetal cortex. We have also been able to show that cortical rotation can continue during mitosis when microtubules are experimentally stabilised, confirming that the motor molecules responsible are not sensitive to MPF.

Microtubules can be stabilised by a variety of cellular factors, including microtubule-associated proteins (MAPs). Three MAPs are known in *Xenopus* eggs: XMAP 215, XMAP230, and XMAP310 (Andersen et al., 1994; Andersen and Karsenti, 1997; Vasquez et al., 1999). These can all stabilise microtubules in vitro, XMAP 215 and XMAP 230 activity being modulated by MPF-dependent phosphorylation (Andersen et al., 1994; Shiina et al., 1992; Vasquez et al., 1999). Microtubule-severing enzymes are also activated by MPF (Shiina et al., 1992). XMAP 230 colocalises with microtubules until 0.95 NT (Cha and Gard, 1999), when the SCW1 stops the cortical rotation (Pérez-Mongiovi et al., 1998), while injections of anti-XMAP 230 antibodies destabilise the array (Cha and Gard, 1999), suggesting that the cortical rotation stops when XMAP 230 is phosphorylated by MPF. We suggest that partial depolymerisation of microtubules accompanying SCW1 provoked by phosphorylation of MAPs, such as XMAP230, occurs progressively across the cortex. This local microtubule depolymerisation uncouples the KRP-mediated anchoring of microtubules to the cortex and thus halts the cortical rotation.

A combination of in vitro and in vivo experiments has consolidated the evidence that dynein as well as KRPs are involved in cortical rotation and has allowed us to study the regulation of this spectacular developmental process by MPF. There is a tight correlation between the beginning of cortical rotation and the arrival of microtubules at the vegetal cortex (Elinson and Rowning, 1988; Schroeder and Gard, 1992), as well as between the end of the cortical rotation and microtubule depolymerisation (this study). Thus, the arrival and maintained contact between microtubule plus ends and the vegetal cortex essentially controls the timing of the cortical rotation, the motors required being

available and active throughout the cell cycle. We hope that the study of microtubule movement on isolated *Xenopus* egg cortices will allow further insight into the characterisation of the KRPs involved in the mechanism of cortical rotation. This in vitro assay and understanding of the cortical rotation may also be useful for the identification of the dorsal determinants attached to the cortex or subcortical structures and translocated by the cortical rotation.

## Acknowledgments

We thank Vicky Arrowsmith for assistance with cortex experiments, Mohamed Khmla for help with image processing, our research colleagues for many useful discussions and corrections of the manuscript, and Sébastien Motreuil for frog care. We are grateful to Ellen Shibuya (Edmonton) for generously supplying anti-Cdc25C serum. Research was funded by the CNRS, MRT (ACI), by ARC Grant 5893 and a HFSP network.

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