

Complementary roles for dynein and kinesins in the *Xenopus* egg cortical rotation[☆]

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Abstract

Aligned vegetal subcortical microtubules in fertilized *Xenopus* eggs mediate the “cortical rotation”, a translocation of the vegetal cortex and of dorsalizing factors toward the egg equator. Kinesin-related protein (KRP) function is essential for the cortical rotation, and dynein has been implicated indirectly; however, the role of neither microtubule motor protein family is understood. We examined the consequence of inhibiting dynein–dynactin-based transport by microinjection of excess dynamitin beneath the vegetal egg surface. Dynamitin introduced before the cortical rotation prevented formation of the subcortical array, blocking microtubule incorporation from deeper regions. In contrast, dynamitin injected after the microtubule array was fully established did not block cortical translocation, unlike inhibitory-KRP antibodies. During an early phase of cortical rotation, when microtubules showed a distinctive wavy organization, dynamitin disrupted microtubule alignment and perturbed cortical movement. These findings indicate that dynein is required for formation and early maintenance of the vegetal microtubule array, while KRPs are largely responsible for displacing the cortex once the microtubule tracks are established. Consistent with this model for the cortical rotation, photobleach analysis revealed both microtubules that translocated with the vegetal cytoplasm relative to the cortex, and ones that moved with the cortex relative to the cytoplasm.

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Introduction

The orientation of the embryonic dorsoventral axis in amphibians is specified by the cortical rotation, a translocation of the entire cortex with respect to the underlying cytoplasm. The cortical rotation is responsible for transporting dorsalizing factors from a region around the vegetal pole to a more equatorial position where they trigger the formation of Spemann’s organizer and initiate the development of dorsoanterior structures (Elinson and Holowacz, 1995; Weaver et al., 2003; Kageura, 1997; Miller et al., 1999; Vincent and Gerhart, 1987; Vincent et al., 1986). The cortical rotation is mediated by a spectacular array of aligned microtubules beneath the vegetal cortex (Elinson and Rowning, 1988). These microtubules align with their plus ends

mostly pointing in the direction of cortical translocation (Houliston and Elinson, 1991a). Formation of the aligned microtubule array coincides with the start of the cortical rotation (around 0.5 NT, where 0 NT is the time of fertilization and 1 NT the time of first cleavage; Elinson and Rowning, 1988; Vincent and Gerhart, 1987; Vincent et al., 1986). It begins on the future ventral side where the sperm enters, and progresses rapidly toward the future dorsal side (Houliston and Elinson, 1991b). Microtubules nucleated in inner regions, in particular by the sperm-derived centriole, extend through the yolky cytoplasm and contribute to the formation of the array at the vegetal cortex (Elinson and Palacek, 1993; Houliston and Elinson, 1991b; Schroeder and Gard, 1992). The cortical rotation stops at around 0.9 NT when the microtubules depolymerize under the influence of M-phase promoting factor (Marrari et al., 2003).

Both the formation of the vegetal subcortical microtubule array and the displacement of the cortex likely depend on the activity of microtubule-based motor proteins. These proteins fall into two families, kinesin-related proteins

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(KRPs), most of which move their cargos to microtubule plus ends, and cytoplasmic dynein, which is minus-end directed. The formation of the array has been shown to depend on KRP-mediated microtubule–cortex interactions, by localized microinjection of an antibody that inhibits their common “motor” domain (Marrari et al., 2000). It also may require dynein activity, which has been proposed on the basis of indirect evidence to bring microtubules nucleated in central egg regions to form the array beneath the vegetal cortex (Marrari et al., 2000, 2003). Dynein-dependent movement of this type has been described in *Xenopus* eggs cytoplasmic extracts as well as in fibroblasts and neurons (Abal et al., 2002; Ahmad et al., 1998; Smith et al., 2000; Waterman-Storer et al., 2000). The translocation of the cortex that occurs once the array has formed could conceivably function via plus end-directed motor molecules (which would transport the cortex along the microtubule tracks) and/or minus end-directed motors (which would translocate cytoplasmic elements along cortically anchored microtubules; see Houliston and Elinson, 1992). Both KRPs and dynein have been shown to be active in the vegetal region at this time (Marrari et al., 2000, 2003); however, existing data are not sufficient to distinguish their roles. The three previous studies in which fluorescently labeled microtubules were followed during the cortical rotation suggested that most microtubules displace relative to the cortex, favoring a plus end KRP-based model, but could not discount the presence of cortically attached microtubules because of the lack of reference points on the microtubules (Houliston, 1994; Larabell et al., 1996; Marrari et al., 2000). Introduction of inhibitory anti-KRP antibodies during the cortical rotation disrupts subcortical microtubule alignment, thus cannot resolve whether KRP motor activity is responsible for actively displacing the cortex.

To test directly the role of dynein in the formation of the subcortical microtubule array and to understand the contribution of dynein and KRPs to the translocation of the cortex, we disrupted dynein function by microinjection beneath the vegetal cortex of p50/dynamitin, a component of the dynactin complex. This complex links dynein to its cargo (Ahmad et al., 1998; Echeverri et al., 1996; Januschke et al., 2002; Muresan et al., 2001; Suomalainen et al., 1999) and enhances the processivity of the motor (King and Schroer, 2000). Excess dynamitin has been used to inhibit dynein-dependant processes by disrupting the dynactin complex in various cell types and in *Xenopus* egg extracts (Echeverri et al., 1996; see Wittmann and Hyman, 1999). We preferred dynamitin to the widely used inhibitory antidynein intermediate chain antibody D70.1, because our *in vitro* studies showed that this IgM requires prolonged preincubation to fully inhibit dynein function (Marrari et al., 2003), likely accounting for its variable effects *in vivo* (Marrari et al., 2000). The effects of dynamitin injection during the cortical rotation were compared with those of inhibitory anti-KRP antibodies. In a

second approach, we used photobleaching of fluorescein-labeled microtubules to discriminate cortically anchored from translocating microtubules during the cortical rotation. The results of these studies have allowed us to propose an integrated model for the cortical rotation in which the two families of microtubule motor proteins have distinct, essential roles.

Materials and methods

Egg handling and microinjection

Female *Xenopus* (CNRS, Rennes) were stimulated to ovulate by injection with approximately 500 UI HCG. Eggs squeezed into 80% Steinberg's solution (Marrari et al., 2000) were fertilized *in vitro* using crushed testes, dejellied using 2.5% cysteine (pH 8), and cultured in 20% Steinberg's solution. For microinjection, the perivitelline space was collapsed using 3% Ficoll in 20% Steinberg's solution. Dynamitin was mixed with 70 kDa lysine fixable tetramethylrhodamine dextran (Molecular Probes), to give a final solution of 3 mg/ml dynamitin and 9 mg/ml dextran in IBH buffer (50 mM HEPES, 0.1 mM MgCl₂, pH 6.9). BSA (5 mg/ml) in IBH was used for control injections. Injections (2 × 10 nl) were made into the vegetal subcortical region of the egg using a Drummond microinjection apparatus. Inhibitory rabbit polyclonal anti-KRP antibody (anti-LAGSE) was generated against conserved regions of the kinesin motor domain (Sawin et al., 1992), and purified and injected at 2 mg/ml as described previously (Marrari et al., 2000).

Events during the first cell cycle were timed using a normalized time scale: 0 NT is the time of insemination and 1 NT the time of first cleavage. In some time-lapse experiments, the visible surface contraction wave that coincides with the end of the cortical rotation, and crosses the vegetal cortex at around 0.9 NT (Marrari et al., 2003; Pérez-Mongiovi et al., 1998), was used to normalize the time.

In vivo observation and imaging

To follow the cortical rotation, mitochondrial islands in the vegetal yolk mass were labeled using the lipophilic dye DiOC₆(3) as previously described (Marrari et al., 2000, 2003). After manual removal of the fertilization envelope, groups of five or six injected and uninjected eggs were mounted in observation chambers and images of each egg acquired every 20 s using a Zeiss Axiovert 100 TV microscope equipped with a Ludl motorized stage and a cooled CCD camera (Princeton Instruments) driven by Metamorph software (Universal Imaging Corporation).

Imaging of microtubules in live eggs was performed using a 40× oil objective on a Leica SP2 confocal microscope equipped with argon and green neon lasers. Fluorescein-tubulin (2 × 10⁻¹⁵ nl, Cytoskeleton) was injected

beneath the vegetal cortex at 0.4–0.5 NT. Photobleaching was achieved by intense laser scanning of a central square using a zoom of 16 and line pass of 16 with about 35% maximum power of the 488 nm laser line. We did not analyze eggs that showed any disturbance of microtubules or of cytoplasmic yolk platelets (visible as unstained ovals) around the bleached zone. Images were acquired every 5 s immediately following bleaching.

To aid the detection of microtubules moving relative to the cytoplasm, sequences of confocal images were realigned to keep the position of the cytoplasm constant by using a macrocommand developed on Metamorph software (Universal Imaging Corporation). Yolk platelets detectable as negatively stained ovals were used as markers of cytoplasmic position. Following the subtraction of background levels of fluorescence in the cytoplasm, entry of fluorescent microtubule segments into the bleached zone was followed by measuring pixel intensity in regions of interest positioned along the microtubule bundles (Figs. 4B, C).

All movies are available on our web site at http://biodev.obs-vlfr.fr/recherche/houliston/Cortical_rotation/Marrari2004.html.

Immunofluorescence

Eggs were fixed at -20°C in methanol containing 1% formaldehyde and processed for immunofluorescence as described previously (Chang et al., 1996), using rat monoclonal antitubulin antibody YL 1/2 (Oxford Biotechnology Ltd 2000) and a FITC-labeled anti-rat Ig antibody (Jackson ImmunoResearch) to label microtubules. Confocal images were acquired on the Leica confocal microscope described above.

Production and purification of dynamitin

Mammalian p50 dynamitin was expressed from a pET-14b plasmid obtained from Dr. Richard Vallee (Echeverri et al., 1996) in BL21DE3PlysS *E. coli* at 20°C overnight in the presence of 0.4 mM IPTG. Bacterial lysates obtained by sonication and treatment with lysozyme and DNase I for 20 min at room temperature were clarified by centrifugation at $30\,000 \times g$ at 4°C . A prepurified fraction was obtained by adding 20% ammonium sulfate at 4°C (Wittmann and Hyman, 1999). Ammonium sulfate was exchanged for Binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM PMSF) using 30 K cut-off “Ultrafree” centrifugal filter units (Millipore). The soluble fraction was added to His-bind Resin (Novagen) loaded with nickel. After extensive washing, dynamitin was eluted with 6 volume of 0.5 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9 (see Novagen, pET System Manual 3rd edition), dialyzed against IBH, and concentrated to 15 mg/ml. Production and purification of dynamitin were verified by polyacrylamide gel electrophoresis.

Results

Formation of the vegetal microtubule array is dynein–dynactin-dependent

Direct evidence that dynein function is required for the formation of the array of aligned vegetal subcortical microtubules, and thus for the initiation of cortical rotation, was obtained by injection of dynamitin protein beneath the vegetal surface before the onset of cortical rotation (Fig. 1). Anti-tubulin immunofluorescence and confocal microscopy of eggs fixed at the time of injection confirmed the absence of vegetal subcortical microtubules at the time of injection (data not shown). In 12 dynamitin-injected eggs fixed 15 min after injection, analysis of the pattern of the microtubule array revealed severe perturbations in the organization of the array in zones of about 150 μm in diameter around the injection site (as visualized with co-injected 70 kDa rhodamine dextran). In other areas, and in control eggs fixed in parallel, extensive arrays of aligned microtubules had formed. In 10 of the 12 eggs examined, in the dynamitin-containing zone, the number of microtubules was strongly reduced at the level of the array (Figs. 1A, B), but at deeper levels was increased compared with surrounding regions (Figs. 1A', B', movie 1). Thus, the presence of dynamitin during array formation resulted in an inward shift in the distribution of microtubules, indicating that dynein–dynactin usually acts to move microtubules into the vegetal subcortical layer. We cannot discount that reduction in subcortical microtubule density close to the egg surface in the presence of dynamitin was partly due to microtubule depolymerization, although this would not explain the concomitant microtubule accumulation at deeper levels. Note that dynein inhibition in other systems has not been reported to provoke depolymerization (Echeverri et al., 1996; Januschke et al., 2002; Koonce et al., 1999; Rusan et al., 2002; Waterman-Storer et al., 2000). The effect of dynein–dynactin disruption on the formation of the vegetal microtubule array was distinct from that resulting from KRP inhibition, in which microtubules reached the vegetal cortex but remained oriented perpendicular to the surface (Marrari et al., 2000). Taken together, these results support the idea that KRP and dynein motors have different functions essential for the formation of the vegetal subcortical microtubule array, dynein being required to bring microtubules out from inner regions to the vegetal cortex, and KRPs to tether microtubules in the cortical/subcortical region, favoring their alignment parallel to the egg surface.

As expected, the absence of an organized microtubule array following dynamitin injection early in the cell cycle prevented cortical rotation locally. Cortical rotation was monitored by following the movement of mitochondrial islands relative to the vegetal cortex by time-lapse. The mitochondrial islands are embedded in the vegetal yolk mass and displace in a coordinated fashion when the egg surface is immobilized against the coverslip (Marrari et al.,

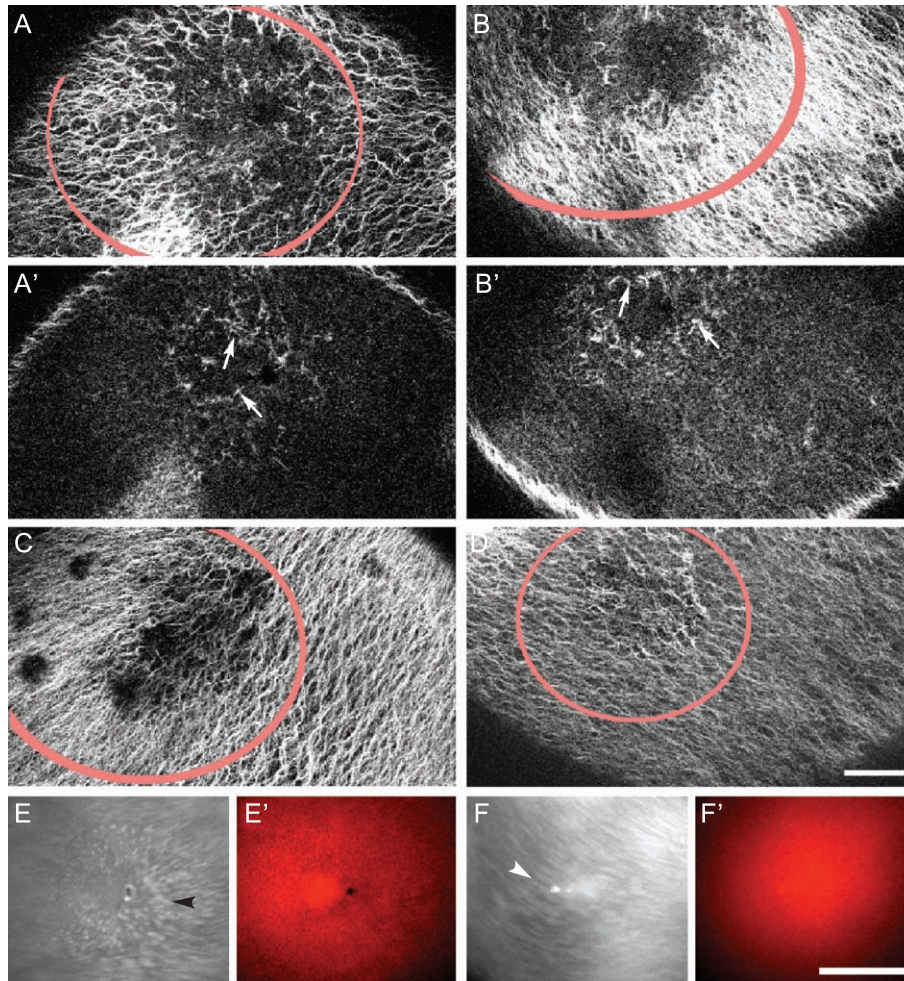


Fig. 1. Early dynamitin injection prevents microtubule array formation and cortical rotation. (A, B) Injection of dynamitin before the formation of the array (at 0.48 NT) resulted in severe reduction in the number of vegetal subcortical microtubules present 15 min later, as revealed by anti-tubulin immunofluorescence. The few microtubules detected at the egg surface around the injection site were not aligned. Accumulated wavy microtubules were detected at a depth of 16 μm within the affected zone (A', B') but not in surrounding regions. (C, D) Eggs injected with BSA at 0.5 NT also showed some reduction in the number of microtubules in the subcortical region 15 min later; however, the subcortical microtubules clearly retained their aligned organization. Rhodamine dextran (70 kDa) was co-injected in all experiments to determine the location of the injected solutions (regions outlined in pink). (E) Inhibition of cortical rotation following dynamitin injection illustrated by averaging 20 consecutive images from time-lapse video sequences of mitochondrial islands embedded in the vegetal cytoplasmic mass, which move with respect to the immobilized cortex in unaffected areas producing streaks on the combined images. Arrows indicate the direction of cytoplasmic translocation. Translocation of the mitochondrial islands was blocked locally in an egg injected with dynamitin (E) at 0.48 NT such that the islands can be distinguished individually in the affected area, but form streaks indicating movement in surrounding parts of the fields shown. BSA injection did not inhibit the cortical rotation (F). E' and F' are corresponding images of co-injected 70 kDa rhodamine dextran marking the location of the injected solutions. Scale bars: 100 μm . Movies can be viewed at http://biodev.obs-vlfr.fr/recherche/houliston/Cortical_rotation/Marrari2004.html.

2000; Pérez-Mongiovi et al., 1998; Savage and Danilchik, 1993). All movies are available on our web site at http://biodev.obs-vlfr.fr/recherche/houliston/Cortical_rotation/Marrari2004.html. Complete inhibition of movement occurred in a zone of about 100- μm diameter around the injection site in 7/7 injected eggs where a clear cortical rotation was detectable in surrounding areas (Fig. 1E, movie 2). In eggs injected with an equivalent volume of BSA at the same concentration and fixed 15 min later, the array was somewhat thinner than in controls (Figs. 1C, D), presumably due to local dilution of tubulin by the injected protein, but the microtubules present were correctly aligned and concentrated in the subcortical layer (see also Cha and Gard,

1999; Cha et al., 1998; Marrari et al., 2000). BSA injections had no effect on cortical rotation (6/6 eggs filmed; Fig. 1F, movie 3). Note that only eggs containing equivalent sized patches of rhodamine dextran were scored in BSA and dynamitin injection experiments.

KRPs but not dynein are required for cortical translocation

Having established that dynein–dynactin is required for the formation of the vegetal subcortical microtubule array, we next tested its requirement in the translocation of the cortex. In these experiments, dynamitin was injected after 0.5 NT, after the vegetal microtubule array had formed. We

confirmed by immunofluorescence that aligned microtubules fully covered the vegetal hemisphere in eggs fixed at the time of injection (not shown). Inhibition of the cortical rotation was observed in some of the injected eggs, but the extent of inhibition varied, some exhibiting complete inhibition similar to that resulting from injection before the cortical rotation (Fig. 2C; movie 4; index of inhibition = 3), while others showed slowing down of the cortical rotation (Fig. 2B; movie 5; 7; index of inhibition = 2), and others showed no effect (Fig. 2A; movie 6; index of inhibition = 1). The variation in inhibition did not relate to small differences in the quantity of injected dynamitin/dextran detectable beneath the vegetal cortex in these experiments (color coded in the graph Fig. 2D), but to the time of injection. Most injections performed between 0.54 and 0.64 NT completely inhibited the cortical rotation, injection at about 0.65 NT gave variable results, the speed of cortical rotation often being reduced, and late injection (after 0.68 NT) failed to affect the cortical rotation (Fig. 2D). Note that the delay

between injection of dynamitin and recording was 10–15 min irrespective of injection time. The lack of cortical rotation inhibition following late dynamitin injection contrasts sharply with the response to injection of the KRP inhibitory antibody anti-LAGSE. We previously noted that injection of anti-LAGSE during the cortical rotation blocked the displacement of mitochondrial islands locally (Marrari et al., 2000), but did not compare the effects of injections at different times within this period. In the light of our dynamitin injection results, we reanalyzed the recordings of anti-LAGSE-injected eggs and found that anti-LAGSE injections as late as 0.72 NT completely inhibited movement (Fig. 2D). These analyzes indicate that while dynein is not required for cortical translocation during the latter stages of cortical rotation, KRP function is essential during the whole process. The important implication is that the cortical rotation can occur independently of dynein, and thus that motors of the KRP family are responsible for the translocation of the cortex. It is also important to note that the

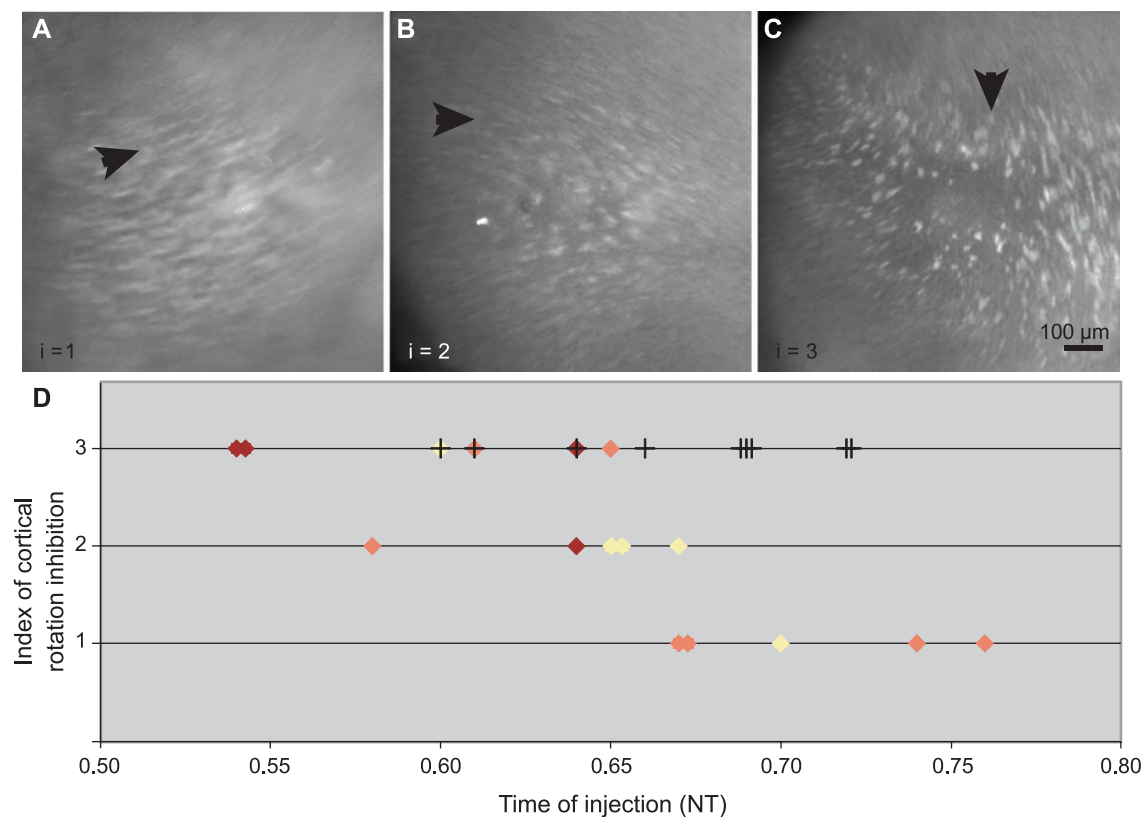


Fig. 2. Cortical rotation becomes dynamitin insensitive but remains dependent on KRPs. (A–C) Different degrees of cortical rotation inhibition demonstrated in three eggs injected with dynamitin after 0.5 NT. The translocation of cytoplasmic mitochondrial islands during the cortical rotation is demonstrated by averaging the first 13 images of each sequence (as in Figs. 1E, F). Arrowheads indicate the direction of cytoplasmic translocation. (A) Egg injected at 0.74 NT. Translocation was not perturbed (index of inhibition = 1). (B) Egg injected at 0.64 NT. Translocation slowed down locally (index of inhibition = 2). (C) Egg injected at 0.6 NT. Translocation was completely inhibited around the site of injection (index of inhibition = 3). (D) Comparison of the inhibitory effects of dynamitin (colored diamonds) and anti-LAGSE (+) on cortical rotation as a function of injection time. Inhibition by dynamitin was reduced following injection at later times, whereas anti-LAGSE completely blocked the cortical rotation irrespective of injection time. The delay between injection and observation was similar (10–15 min) for each egg scored. The degree of inhibition following dynamitin injection was not related to minor experimental variation in the targeting or diffusion of the injected solution to the subcortical region: red symbols indicate eggs with the largest detectable subcortical zones of injected dextran, orange indicates medium amounts, and yellow indicates smaller amounts.

failure of dynamitin, unlike anti-KRP antibodies, to inhibit the cortical rotation when injected late confirms that dynamitin does not interfere with essential KRP-mediated processes in the *Xenopus* egg, as it does in some cells (Deacon et al., 2003).

The progressive loss of dynein dependency of the cortical rotation described above was found to correlate with reduced disruption by dynamitin of microtubule organization (compare the microtubule disruption of the egg shown in Fig. 3B injected at 0.6 NT with that in Fig. 3C, injected at 0.7 NT). Visual assessment of the microtubule organization on confocal images revealed that the alignment of microtubules in the subcortical region was clearly disrupted in 5/6 eggs (Fig. 3B) following dynamitin injection early during the cortical rotation. In contrast, the vegetal subcortical microtubules remained aligned in 3/4 eggs (Fig. 3C) following injection after 0.68 NT. This is consistent with the lack of inhibition of cortical rotation. The maintenance of microtubule alignment and of cortical translocation in the presence of dynamitin injected late during cortical rotation was a further indication that the effects of early injection were not due to nonspecific toxicity. These results indicate that dynein activity is required to conserve the parallel pattern of the newly formed array of microtubules during the first half of the cortical rotation but not at later times.

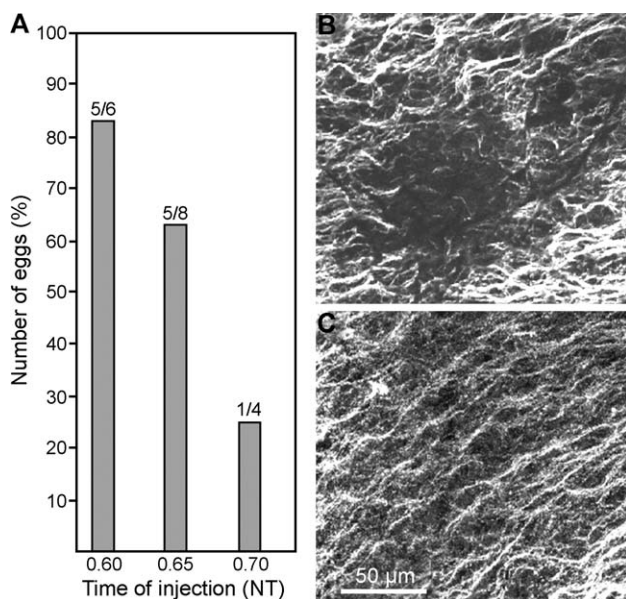


Fig. 3. Dynamitin disrupts the vegetal array during the first phase of cortical rotation. (A) Progressive decrease in the percentage of eggs exhibiting disruption of vegetal microtubule alignment as assessed following antitubulin immunofluorescence (example in B) as a function of injection time. Numbers of eggs scored are provided above each bar. Considerable variation in the effect on cortical rotation was observed following injections around 0.65 NT. (B) Example of egg injected at 0.60 NT and fixed 15 min later in which the alignment of subcortical microtubules was lost. (C) Example of egg injected at 0.7 NT and fixed 15 min later in which microtubules remained aligned.

Translocating and nontranslocating microtubules coexist in the vegetal array

The dynactin and anti-LAGSE injection experiments, along with analysis of microtubule movement on isolated cortices (Marrari et al., 2003), indicate that both dynein and KRPs are active in the region of the vegetal cortex during cortical rotation. Taken together, they show that dynein activity is crucial during an early phase of cortical rotation, while KRPs are required for cortex–microtubule interactions, being sufficient to translocate the cortex once the microtubule array is fully established. If both plus and minus end motors do indeed function during the cortical rotation, both translocating and nontranslocating microtubules should be detectable in the subcortical array during cortical rotation. Previous imaging of vegetal subcortical microtubules in live eggs has suggested that most microtubules displace relative to the cortex but were unable to unequivocally identify translocating and nontranslocating microtubules due to a lack of reference points on microtubules (see Introduction). We thus used the confocal microscope to photobleach subcortical microtubules labeled with injected fluorescein-tubulin. Fluorescently labeled microtubules in the center of a randomly chosen field beneath the vegetal egg surface were bleached by intense laser scanning. A coordinated displacement of the microtubule bundles in the subcortical region with the cytoplasm relative to the immobilized cortex was revealed by the uniform translocation of the bleached portions of the labeled microtubules, showing the same direction and speed as the surrounding yolk platelets (5/5 eggs examined: movie 7, see arrows in Fig. 4A). Thus, we can confirm that many of the vegetal subcortical microtubules displace relative to the cortex. This finding is consistent with the KRP-dependent translocation of the cortex proposed from our inhibitory injection studies (see above).

To aid the detection of microtubules that displaced with the cortex relative to the cytoplasm, we realigned the images of each sequence with respect to the position of cytoplasmic yolk platelets (see Materials and methods), which displace coordinately during the cortical rotation (Larabell et al., 1996). In four of the five eggs examined, unbleached microtubule segments were observed to enter the bleached zone immediately following bleaching (movie 8, arrow in Fig. 4B). In the fifth egg, no microtubules were appropriately positioned with respect to the bleach zone to analyze. Since bleaching does not affect microtubule integrity, this entry of microtubules into the bleach zone implies that some microtubules within the bundles displace relative to the general population described above. Comparison of the fluorescence intensity in adjacent segments along the microtubule bundle within the bleach zone confirmed that recovery of fluorescence was due to displacement of the bleached portions of some of the microtubules within the bundle, rather than to uniform fluorescence incorporation along the length of the microtubules (Fig. 4C). We verified that microtubules in deeper regions under the plane of

observation were bleached to preclude the possibility that the recovery of fluorescence was due to the “surfacing” of deeper microtubules (data not shown). It is unlikely that the fluorescence recovery was due to polymerization on plus ends since there is no reason to believe that plus ends would consistently lie immediately upstream of the bleach zone. We can thus conclude that some of the aligned subcortical

microtubules continue to displace relative to the surrounding cytoplasm during the cortical rotation, as predicted if cytoplasmic dynein acts upon them.

To summarize, the use of photobleaching has allowed the identification of populations of microtubules within the vegetal array that displace both with the cortex and with the cytoplasm. This finding does not conclusively demonstrate that either motor family is responsible for driving the cortical rotation since it remains possible that one of the microtubule sub-populations is displaced actively while the other is carried passively with either cortex or cytoplasm. Nevertheless, it reinforces the hypothesis that both plus end- and minus end-directed motors contribute to the cortical rotation.

Progressive change in microtubule organization during the cortical rotation

The requirement for dynein to maintain the vegetal subcortical microtubule array during early but not late periods of the cortical rotation implies that the organization of the array evolves progressively. We undertook a detailed immunofluorescence analysis of eggs fixed at successive times and were able to show that the array of microtubules did indeed change as the cortical rotation progressed. During the first half of the cortical rotation, the array consisted mainly of wavy cables covering most of the vegetal surface (Figs. 5A, C), while at later times, the wavy organization became less marked and patches of straighter “fine-combed” microtubules became apparent (Figs. 5B, D). This suggests that the microtubules recently arrived at the cortex have a wavy appearance while those residing there for longer straighten out. This conclusion is reinforced by the observation that in many eggs, particularly those fixed after 0.7 NT, the array was clearly asymmetric, with the wavy cables predominating on one side of the egg and the straighter microtubules on the other side (Fig. 5B). Examination of the relationship of these

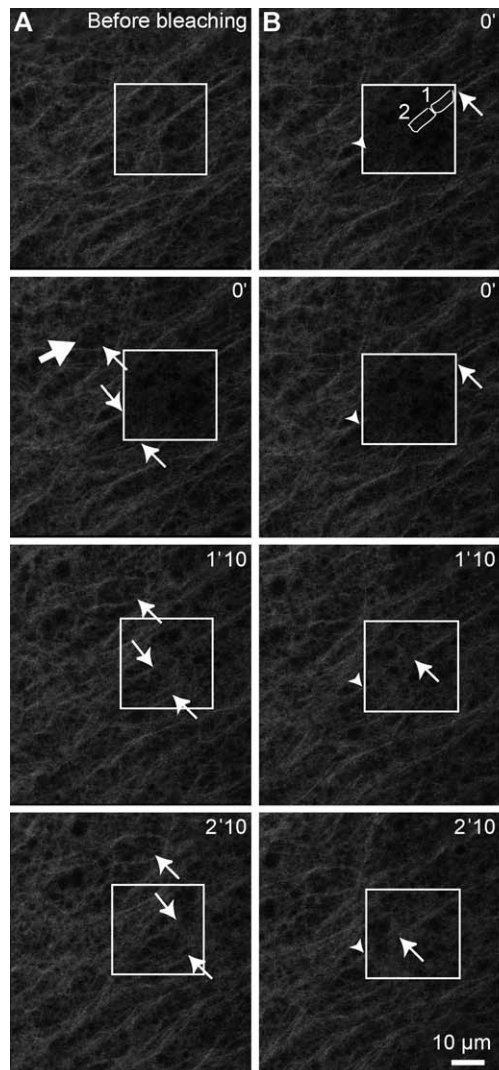


Fig. 4. Two microtubule populations in the vegetal array. Analysis of the movement of vegetal subcortical microtubules, labeled by incorporation of fluorescein-tubulin. Photobleaching (zone indicated by squares) was used to determine the nature of the microtubule movement in eggs mounted between coverslip and slide. Movies can be viewed at http://biodev.obs-vlfr.fr/recherche/houliston/Cortical_rotation/Marrari2004.html. (A) Images taken from a sequence of images acquired every 5 s following bleaching, showing that most microtubule bundles (arrows) translocate with the cytoplasm relative to the vegetal egg cortex, immobilized against the coverslip. The large arrow shows the displacement of cytoplasmic yolk platelets, in the same direction as the microtubule bundles. The first image shows the field before bleaching. Times after photobleaching are indicated. (B) Images from the same sequence realigned with respect to cytoplasmic yolk platelets. Displacement of some microtubules within bundles relative to the cytoplasm is revealed by their entry into the bleached square (e.g., at arrow). Other microtubules remained stationary in the cytoplasm (arrowhead). (C) Comparison of the rise in fluorescence intensity following bleaching in two adjacent zones (“1” and “2”) shown in B, expressed as % of unbleached levels, confirming that fluorescence recovery is not uniform along the microtubule bundle.

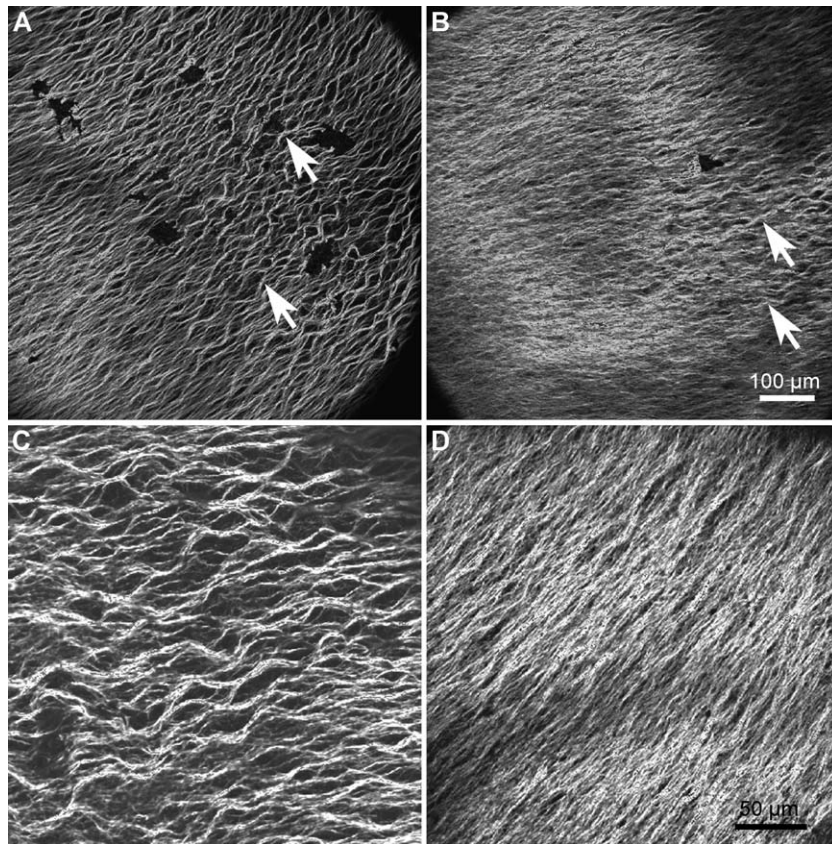


Fig. 5. Changes in subcortical microtubule organization during cortical rotation. Anti-tubulin immunofluorescence revealed that the vegetal subcortical microtubules developed a progressively straighter and less bundled appearance during the cortical rotation period, as seen in these eggs from the same batch and fixed at 0.60 NT (A) and 0.70 NT (B). General views show the wavy pattern of microtubules covering all the vegetal cortex (A) and straightened microtubules of the vegetal array (B). Microtubule bundles on the sperm entry side of the egg (D: egg fixed at 0.70 NT) were often straighter than those on the future dorsal side (C: egg fixed at 0.60 NT; also regions indicated by arrows in A and B).

patterns with the darkly pigmented sperm entry site in the animal half revealed that the straighter microtubules consistently lay on the sperm entry (presumptive ventral) side of the egg, where the vegetal subcortical microtubule array first forms as a result of sperm aster expansion (Houliston and Elinson, 1991b). We hypothesize that the wavy appearance of microtubules during the early, dynein-dependent period of the cortical rotation is a consequence of the outward-pushing action of dynein on microtubules being impeded by KRP-mediated interactions in the subcortical region.

Discussion

The data presented here have revealed that dynein plays an essential role in the *Xenopus* egg cortical rotation, responsible for the translocation of the vegetal cortex and of dorsalizing factors. They indicate that dynein–dynactin is required both to bring microtubules to the cortex from inner regions and to maintain the aligned subcortical microtubule array during an early phase of cortical rotation. Since inhibition of the other family of microtubule motors, the

KRPs, also causes disorganization of the aligned microtubules (Marrari et al., 2000), it appears that formation of the array is dependent on the cooperative action of KRPs and dynein. In addition, since inhibitory anti-KRP antibodies but not dynamitin injection prevented the cortical rotation when injected after 0.65 NT, we can conclude that KRP motors are responsible for translocation of the cortex once the microtubule array has formed, and not merely tethering them in the subcortical region (Marrari et al., 2000).

We can now propose an integrated model for the mechanism of cortical rotation, in which dynein and KRPs have complementary actions during different phases (Fig. 6): During the first half of the cell cycle, microtubules are projected toward the cortex under the influence of cytoplasmic dynein (Fig. 6A). When they arrive at the vegetal cortex, their plus end segments associate with cortically attached KRPs (Fig. 6B). Then begins the early phase of the cortical rotation (Fig. 6C), during which the combined action of cytoplasmic dynein pushing the microtubules outward and cortical KRPs moving to the microtubule plus ends conserves the alignment of the array. In the final phase of the rotation period (Fig. 6D), dynein activity is no longer

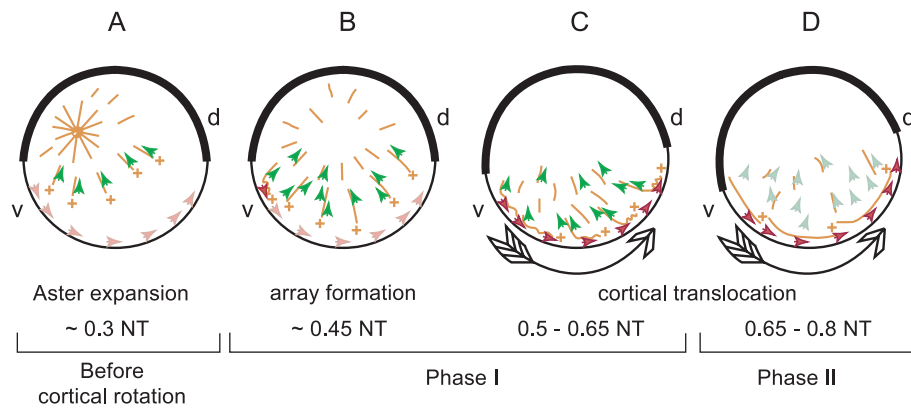


Fig. 6. Roles of microtubule motors during successive phases of the cortical rotation. We propose that during the first half of the cell cycle, microtubules (orange) are projected toward the cortex under the influence of cytoplasmic dynein (green arrows; A). When they arrive at the vegetal cortex, initially on the future ventral side of the egg (v) where the sperm aster is located, their plus end segments associate with cortically attached KRPs (pink/red arrows; B). During an initial phase of the cortical rotation (C), aligned microtubules become established across the entire vegetal subcortical region. They have a wavy appearance, possibly because interaction with subcortical elements impedes the progress of the outward-moving microtubules. During this phase, the cortical rotation is dependent both on KRPs, which mediate microtubule–cortex interactions, and dynein, which we propose acts to maintain sufficiently long microtubules in the subcortical region. In the final phase of the rotation period (D), dynein activity is no longer required to maintain the alignment of the subcortical microtubules. KRPs attached directly or indirectly to the cortex carry it toward microtubule plus ends. The motor molecules are represented in pale colors when they are not contributing to the cortical rotation, and dark colors when they are.

required to maintain the alignment of the subcortical microtubules, and KRPs continue to translocate the cortex on the established microtubule array.

Dynein-dependent establishment and maintenance of the vegetal microtubule array

Our results suggest that dynein-dependent outward transport of microtubules from inner region is essential for the proper formation of the subcortical array of aligned microtubules required for the cortical rotation (Figs. 6A, B). This proposed role of dynein is comparable with its described function in other cell types. Dynein function has been shown to push microtubules out from the centrosome to the cell periphery in many cell types, notably fibroblasts and neurons (Abal et al., 2002; Ahmad et al., 1998). In *Xenopus* egg extracts, dynein can function similarly to displace microtubules from the center of microtubule asters with their plus ends ahead (Marrari et al., 2003; Waterman-Storer et al., 2000). Dynein is clearly also active in the vegetal region in vivo since subcortical microtubule bundles undergo vigorous flailing movements when KRP function is inhibited (Marrari et al., 2000). These microtubules are nucleated around the sperm centriole early in the cell cycle, and can also polymerize independently of it in other cytoplasmic regions after 0.5 NT (Elinson and Palacek, 1993; Houlston and Elinson, 1991b). Our results suggest that microtubules nucleated deep within the cytoplasm are prevented from accumulating at the cortex when dynein function is blocked and support the idea that dynein functions to transport microtubules to the cell cortex from deeper regions of the cytoplasm to the vegetal subcortical region, thus playing a critical role in the formation of the aligned microtubule array (Figs. 6A, B).

An additional role for dynein during an early phase of the cortical rotation was revealed by the injection of dynamitin shortly after the microtubule array had formed. In these cases, dynamitin blocked or slowed the cortical rotation and caused disorganization of the microtubule array. We found that during this early period, the subcortical microtubules had a distinctive wavy appearance, perhaps because the progress of microtubules pushed outward by dynein is initially impeded by their interaction with the cortex causing them to buckle (Fig. 6C). We propose that dynein inhibition blocks or slows the cortical rotation during this early phase because dynein is required to maintain sufficiently long microtubule segments in the subcortical region to allow sustained translocation of the cortex by KRPs. The disruption of the organization of the array is presumably due to KRPs in the subcortical region acting on the ends of microtubules projecting from the cytoplasm (Fig. 6C).

KRPs provide the driving force for the cortical rotation

It had been established previously that KRPs are essential for maintaining microtubule organization during the cortical rotation, but their role in translocation remained in doubt (Marrari et al., 2000). Given the possibility, confirmed in this study, that some subcortical microtubules were cortically anchored, we previously proposed that dynein pushing microtubules out from inner regions might contribute significant force to displace the cortex during cortical rotation (Marrari et al., 2000, 2003). We have now essentially rule out this possibility, at least during the second phase of cortical rotation. Continuation of cortical rotation in the presence of dynamitin during this phase strongly implies that KRPs are responsible for displacing the cortex, although we cannot rule out the possibility that residual or

distinct dyactin-independent dynein activity is sufficient to assure cortical rotation during this phase. Our photobleaching analysis showed that most microtubule bundles in the subcortical region translocate relative to the cortex, supporting the idea that KRP activity is responsible for displacing the cortex toward the plus ends of microtubules.

The subcortical microtubule tracks can apparently be maintained during the late phases of cortical rotation independently of dynein action (Fig. 6D). Continuing microtubule polymerization in the vegetal subcortical region during cortical rotation (Houliston, 1994) probably favors the establishment of such tracks. The microtubules of the array may also be stabilized by interaction with other subcortical elements such as cyokeratin filaments, which progressively form a co-aligned network during the cortical rotation, and ER, which accumulates around the microtubules of the array (Clarke and Allan, 2003; Houliston and Elinson, 1991a; Klymkowsky et al., 1987).

Microtubule–cortex interactions mediated by motor molecules

In the model we propose for the cortical rotation, dynein acts throughout the cytoplasm while KRPs are attached directly or indirectly to the cortex. Consistent with this model, KRPs remain stably attached to isolated cortices whereas dynein can be released from them by washing (Marrari et al., 2003). Microtubules have been shown to interact with the cortex in many cell types, resulting in the displacement of internal structures such as nuclei and spindles, as well as of a specialized cytoplasmic domain in ascidian eggs known as myoplasm (Roegiers et al., 1999). In some examples such as *Caenorhabditis* embryos, budding yeast and mammalian epithelial cells, dynein associated with specific regions of the cortex has been implicated in microtubule–cortex interactions by pulling on astral microtubules (see Dujardin and Vallee, 2002). There are not, however any reported cases of cortically localized KRPs that mediate cortex–microtubule interactions. This may, at first sight, make the proposed role of KRPs during the *Xenopus* egg cortical rotation seem exceptional. It should be noted, however, that the “subcortical” region over which KRPs are thought to interact with microtubules is much thicker (5–10 μm) than the cortex of other cells (<0.1 μm). This complex region is packed with cellular organelles and cytoskeletal elements including cyokeratin filaments and a complex three-dimensional network of ER that envelopes the microtubules of the array (Houliston and Elinson, 1991a; Klymkowsky, 1995). If such elements are connected to the outer cortical layer of the egg, KRP-mediated interactions between them and the microtubules would indirectly cause the cortex to move. ER is a possible candidate for this role as KRPs localize to both subcortical and cortical ER networks in frog eggs (Houliston and Elinson, 1991a; Marrari et al., 2000), while in other cells, ER has been shown to move to microtubule plus ends using kinesin (e.g.,

Vale and Hotani, 1988; Waterman-Storer and Salmon, 1998). KRP-mediated interactions with cellular elements such as ER may also explain the movements of cytoplasmic regions in parallel with the formation of aligned microtubule arrays beneath the egg surface in ascidian eggs (Sawada and Schatten, 1988) and in sea urchin eggs in which microtubule polymerization is forced (Schroeder and Battaglia, 1985). They may also underlie the peripheral alignment of microtubules in cells in which dynein function has been disturbed, and the spiraling of the aster-centrosome complex in dynein-inhibited cells (Koonce et al., 1999; Smith et al., 2000).

Finally, it is interesting to note that determination of the future dorsal side in *Drosophila* oocytes also depends on microtubule-based processes involving both KRPs and dynein. Motors of both families participate in the relocation of the nucleus to the future dorsoanterior corner of the oocyte and thus in the establishment of an organized microtubule array (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003). Thus, as in amphibian eggs, dynein and KRPs cooperate in *Drosophila* oocytes to initiate the establishment of embryonic axes.

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