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Local Inhibition of Cortical Rotation in *Xenopus* Eggs by an Anti-KRP Antibody

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The dorsal–ventral axis of amphibian embryos is specified by the "cortical rotation," a translocation of the egg cortex relative to the vegetal yolk mass. The mechanism of cortical rotation is not understood but is thought to involve an array of aligned, commonly oriented microtubules. We have demonstrated an essential requirement for kinesin-related proteins (KRPs) in the cortical rotation by microinjection beneath the vegetal cortex of an antipeptide antibody recognising multiple *Xenopus* egg KRPs. Time-lapse videomicroscopy revealed a striking local inhibition of the cortical rotation around the injection site, indicating that KRP-mediated translocation of the cortex is generated by forces acting across the vegetal subcortical region. Anti-tubulin immunofluorescence showed that the antibody disrupted both formation and maintenance of the aligned microtubule array. Direct examination of rhodamine-labelled microtubules by confocal microscopy showed that the anti-KRP antibody provoked striking three-dimensional flailing movement of the subcortical microtubules. In contrast, microtubules in antibody-free regions undulated only within the plane of the cortex, a significant population exhibiting little or no net movement. These findings suggest that KRPs have a critical role during cortical rotation in tethering microtubules to the cortex and that they may not contribute significantly to the translocation force as previously thought. **© 2000 Academic Press**

Key Words: **cortical rotation; cortex; microtubule; kinesin-related protein; egg; amphibian; endoplasmic reticulum; ER.**

INTRODUCTION

The cortical rotation is the cellular process that breaks the radial symmetry of the amphibian egg, specifying the orientation of the two embryonic body axes. The entire outer cortex of the fertilised egg rotates relative to the mass of inner cytoplasm by an angle of about 30° about an axis perpendicular to the primary animal–vegetal axis. As a result of this cortical rotation, "dorsal determinants," factors able to trigger the formation of the "organiser" region of the gastrula, are displaced from the vegetal pole region to a more equatorial position where they become activated (Gerhart *et al.*, 1989; Elinson and Holowacz, 1995; Chang *et al.*, 1999). The nature of these determinants is not known; however, they are thought likely to act in the Wnt signalling pathway (Marikawa *et al.*, 1997; Moon and Kimelman, 1998). 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 such as the notochord, central nervous system, and head.

The mechanism of the cortical rotation and of dorsal determinant translocation is not understood. A transient array of aligned vegetal subcortical microtubules that forms during the rotation period (Elinson and Rowning, 1988) is clearly implicated in both processes. The determinants could be transported along these microtubules by direct association with the moving cortex and/or by association with subcortical particles or vesicles that move rapidly in the same direction (Rowning *et al.*, 1997; Miller *et al.*, 1999). Such particles are probably transported by plus-end-directed microtubule-based motor proteins, the vast majority of the vegetal subcortical microtubules being oriented with their plus ends pointing in the direction of cortical displacement and determinant movement (Houliston and Elinson, 1991a). The cortical rotation movement is also thought to be generated by microtubule-based motor pro-

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teins rather than by the force of microtubule polymerisation, since polymerisation can be arrested during the rotation period without impairing the rotation (Houliston, 1994). To cause the rotation, plus-end-directed motor proteins attached to the vegetal cortex could transport the cortex along "tracks" of aligned subcortical microtubules anchored in the cytoplasm, and/or cytoplasmic minus-end motors could move against microtubules attached to the cortex (see Houliston and Elinson, 1992). The former possibility is favoured by the observation that many of the vegetal subcortical aligned microtubules arise by extension out of the deeper vegetal cytoplasm. Thus connections appear to be maintained between the cytoplasmic mass and the microtubule array during the rotation period (Houliston and Elinson, 1991b; Schroeder and Gard, 1992; Elinson and Palacek, 1993). Furthermore, studies of microtubule behaviour in live eggs have indicated that most of the vegetal subcortical microtubules translocate relative to the cortex during rotation (Houliston, 1994), with deeper ones possibly sliding against more peripheral ones (Larabell *et al.*, 1996). It should be noted, however, that individual microtubules were hard to distinguish in these previous studies and that there was some evidence for a subpopulation of microtubules that did not translocate with the vegetal yolk mass but rather remained attached to the cortex during rotation (Houliston, 1994).

The microtubule plus-end-directed motor, kinesin, together with a large number of other proteins related by extensive sequence similarity in the mechanochemical "motor" domain, form a superfamily of "kinesin-related proteins" (KRPs; Vale and Fletterick, 1997; Hirokawa, 1998; see also the kinesin home page, http://www.blocks.fhcrc.org/~kinesin). This superfamily includes all known plus-end-directed microtubule motors as well as some minus-end-directed motors. Many of the KRPs identified in *Xenopus* egg cytoplasm have been shown to participate in the formation and function of the mitotic spindle (Vernos *et al.*, 1993; Vernos and Karsenti, 1996; Walczak *et al.*, 1998). The overlapping and complementary roles of individual KRPs and of the minus-end-directed motor cytoplasmic dynein in these events have been dissected by analysis of spindle assembly in egg cytoplasmic extracts, in which individual KRPs can be removed or compromised, and by reduction of the concentrations of specific proteins in live eggs by depletion of the corresponding mRNAs from the oocyte (Sawin *et al.*, 1992a; Vernos *et al.*, 1995; Boleti *et al.*, 1996; Gaglio *et al.*, 1996; Heald *et al.*, 1996, 1997; Merdes *et al.*, 1996; Walczak *et al.*, 1996, 1997; Wood *et al.*, 1997). Multiple egg KRPs may also participate in the cortical rotation, perhaps contributing to microtubule stability, bundling, and/or alignment as well as possibly to the generation of force between the subcortical microtubules and the cortex. At least two KRPs, conventional kinesin (Houliston and Elinson, 1991a) and Eg5 (Houliston *et al.*, 1994; Chang *et al.*, 1996) have been shown to associate with the vegetal microtubule array during interphase of the first cell cycle.

To investigate whether and how KRPs contribute to the *Xenopus* egg cortical rotation we have microinjected antibodies recognising *Xenopus* egg KRPs beneath the vegetal cortex of fertilised eggs and analysed the effects on the cortical rotation and on the behaviour of subcortical microtubules. Our results show that KRPs acting across the entire vegetal subcortical region are required for the cortical rotation. More surprisingly they indicate that cortical KRPs probably act mainly to tether microtubules to the cortex rather than to drive the translocation of the cortex.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal anti-KRP peptide antibodies were generated by CovaLab (Lyon) against two conserved regions of the kinesin motor domain, LNLVDLAGSE ("LAGSE") and HIPYRESKLT ("HIPYR"), as described by Sawin *et al.* (1992b) and affinity purified on columns coupled with the corresponding peptide. Partially purified mouse monoclonal anti-sea urchin kinesin heavy chain antibodies SUK2 and SUK4 (Ingold *et al.*, 1988) were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa under Contract N01-HD-7-3263 from the NICHD. All antibodies for microinjection were exchanged into IB2 buffer (88 mM KCl, 10 mM Hepes, pH 7.6) and concentrated using Centricon filtration devices (30K cut-off). Ig concentrations were determined by comparing band intensities of Coomassie blue-stained SDS–PAGE gels.

For characterisation of antibodies by Western blot a preparation enriched in KRPs was prepared from *Xenopus* eggs by an adaptation of the method of Buster and Scholey (1991). Briefly, microtubules were polymerised by addition of 20 μM Taxol to a clarified egg cytoplasmic extract prepared by crushing eggs in the presence of PMEE buffer (0.1 M K · Pipes, pH 6.5, 2.5 mM $MgSO_4$, 0.1 mM EDTA, 5 mM EGTA, with 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 0.5 mM AEBSF, 1 mg ml^{-1} pTAME, 20 μg ml^{-1} benzamidine, 1 mM DTT, 1 mM sodium azide). ATP was then depleted using 10 units/ml hexokinase and 10 mM glucose for 30 min at 19°C. AMP · PNP (1 mM) was added for 20 min, and KRPs were eluted from pelleted microtubules using 10 mM MgATP and 0.5 mM GTP overnight at 4°C in the presence of 30 μM Taxol. SDS–PAGE and immunoblotting were performed according to standard methods using peroxidase-linked secondary antibodies and ECL detection.

Egg Handling and Microinjection

Female *Xenopus* (CNRS, Rennes) were stimulated to ovulate by injection with approx 700 IU HCG. Eggs squeezed into 80% Steinberg's solution (100% Steinberg's is 58 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 5 mM Tris–HCl, pH 7.4) were fertilised *in vitro* using crushed testis, dejellied using 2.5% cysteine (pH 8), and cultured in 20% Steinberg's solution. For microinjection, the perivitelline space was collapsed using 4% Ficoll in 80% Steinberg's solution. Diluted antibodies for microinjection were mixed vigorously just before use with 0.2-μm yellow-green carboxyl latex FluoSpheres (Molecular Probes) washed into IB2 buffer by centrifugation and resuspension. In peptide preabsorption experiments, diluted antibody was mixed with LAGSE peptide in a molar ratio of approx 1:25 for at least 18 h at 4°C, then centrifuged at top speed in an Eppendorf centrifuge to remove particulate aggregates before bead addition. Twenty to thirty nano-

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litres of each solution was injected into *Xenopus* eggs or embryos using a Drummond microinjection apparatus. To target the vegetal subcortical region of the egg, delivery in two or three small (7–10 nl) injections was found to be most effective. A normalised time scale is used to compare events in the first cell cycle: 0 NT is the time of insemination and 1 NT the time of first cleavage (Ziskind and Elinson, 1990).

In Vivo Observation and Imaging

To follow the cortical rotation, mitochondrial islands in the vegetal yolk mass were labelled using the lipophilic dye $DiOC_6(3)$ (Savage and Danilchik, 1993; Houliston, 1994). After removal of the fertilisation envelope, groups of five or six injected and uninjected eggs were mounted in observation chambers and images of each egg acquired every 15–20 s using a Zeiss Axiovert 100TV microscope equipped with a Ludl motorised stage and a cooled CCD camera (Princeton Instruments) driven by Metamorph software (Universal Imaging). Confocal imaging of live eggs was performed following subcortical microinjection of 20–30 nl rhodamine–tubulin (Cytoskeleton; diluted to 2–5 mg/ml in IB2) using a Picospritzer air pressure microinjection system. Images were collected using a MRC 600 Bio-Rad confocal imaging system mounted over a Zeiss LM35 inverted microscope equipped with a 100× Olympus objective with 1.6 numerical aperture. For display purposes, image sequences were manipulated using NIH Image software (available via http:/rsb.info.nih.gov/nih-image).

Original recordings can be viewed at our Web site: http://www.obs-vlfr.fr/xenope.htm.

Immunofluorescence

Eggs and embryos were fixed at −20°C in methanol or in methanol containing 3.7% formaldehyde and processed for immunofluorescence (Elinson and Rowning, 1988; Houliston, 1994; Houliston *et al.,* 1994) using rat monoclonal antibodies YL1/2 (anti-α-tubulin; gift from J. Kilmartin) and anti -BiP (gift from D. Bole) with Texas red-labelled secondary antibodies (Caltag) to label microtubules and ER, respectively (Houliston and Elinson, 1991a). Fluorescein-linked anti-rabbit Igs (Caltag) were used to detect anti-LAGSE and anti-HIPYR. Confocal images were acquired on a Leica confocal microscope (Houliston, 1994). Controls in which primary antibodies were omitted confirmed that no cross-reaction between antibodies occurred.

RESULTS

Local Inhibition of Cortical Rotation with an Anti-KRP Antibody

We raised rabbit polyclonal antibodies against peptides corresponding to two regions of the motor domain that are highly conserved amongst the kinesin superfamily, LAGSE and HIPYR, as described previously (Sawin *et al.*, 1992b; see Materials and Methods). The anti-LAGSE peptide was designed to be inhibitory. An equivalent anti-LAGSE antibody has been shown to block spindle elongation in a semi-*in vitro* system, while an anti-HIPYR had no appreciable effect (Hogan *et al.,* 1993) and so acts as a negative control. The profile of polypeptides recognised by the two antibodies on

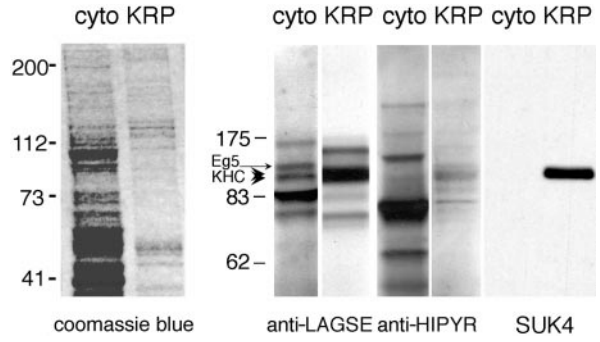

FIG. 1. *Xenopus* egg KRPs recognised by anti-LAGSE. Samples of *Xenopus* egg cytoplasmic proteins (cyto) and of a motor protein preparation enriched in certain KRPs (KRP—see Materials and Methods) were subjected to SDS–PAGE and then either stained with Coomassie blue to reveal total polypeptides or transferred electrophoretically to nitrocellulose for immunodetection with anti-LAGSE, anti-HIPYR, or the anti-kinesin heavy chain antibody SUK4 as indicated. The 130K band corresponding to Eg5 is indicated. Eg5 was detected by specific antibodies in egg cytoplasm and associates with microtubules (not shown), but was not released under the elution conditions used here.

Western blots (Fig. 1) corresponded closely to that described for the antibodies used in these previous studies (Sawin *et al.,* 1992b). The motor-protein fraction shown was prepared by AMP · PNP-enhanced binding and ATP release from Taxol-stabilised microtubules. Note that while enriched in many KRPs, including the kinesin heavy chain, such preparations are not expected to contain KRPs in proportion to their presence in the egg cytoplasm due to variations in binding properties. For instance, the abundant egg KRP, Eg5, was not fully eluted under the conditions used. On the basis of their apparent relative molecular weights, the entire range of peptides recognised by the antibody can be accounted for by known *Xenopus* egg KRPs.

We microinjected affinity-purified anti-LAGSE beneath the vegetal cortex of fertilised eggs and then monitored the cortical rotation by following the movement of mitochondrial islands relative to the vegetal cortex (Fig. 2; animated sequences available on our Web site). The mitochondrial islands are embedded in the vegetal yolk mass and displaced in a coordinated fashion when the egg surface is immobilised against the coverslip (Savage and Danilchik, 1993; Houliston, 1994; Figs. 2a and 2b). Using this method local perturbations insignificant for the overall cortical displacement can be revealed by time-lapse videomicroscopy. Co-injected fluorescent microspheres allowed the injected antibody to be located. Anti-LAGSE was found to provoke striking local inhibition of movement of mitochondrial islands relative to the cortex (Figs. 2d–2f and 2h). Effects varied from partial slowing of the movement to complete inhibition over patches of approximately 100–500 μm in diameter around the injection site. Clear inhibition was observed with an injected concentration of as little as 1

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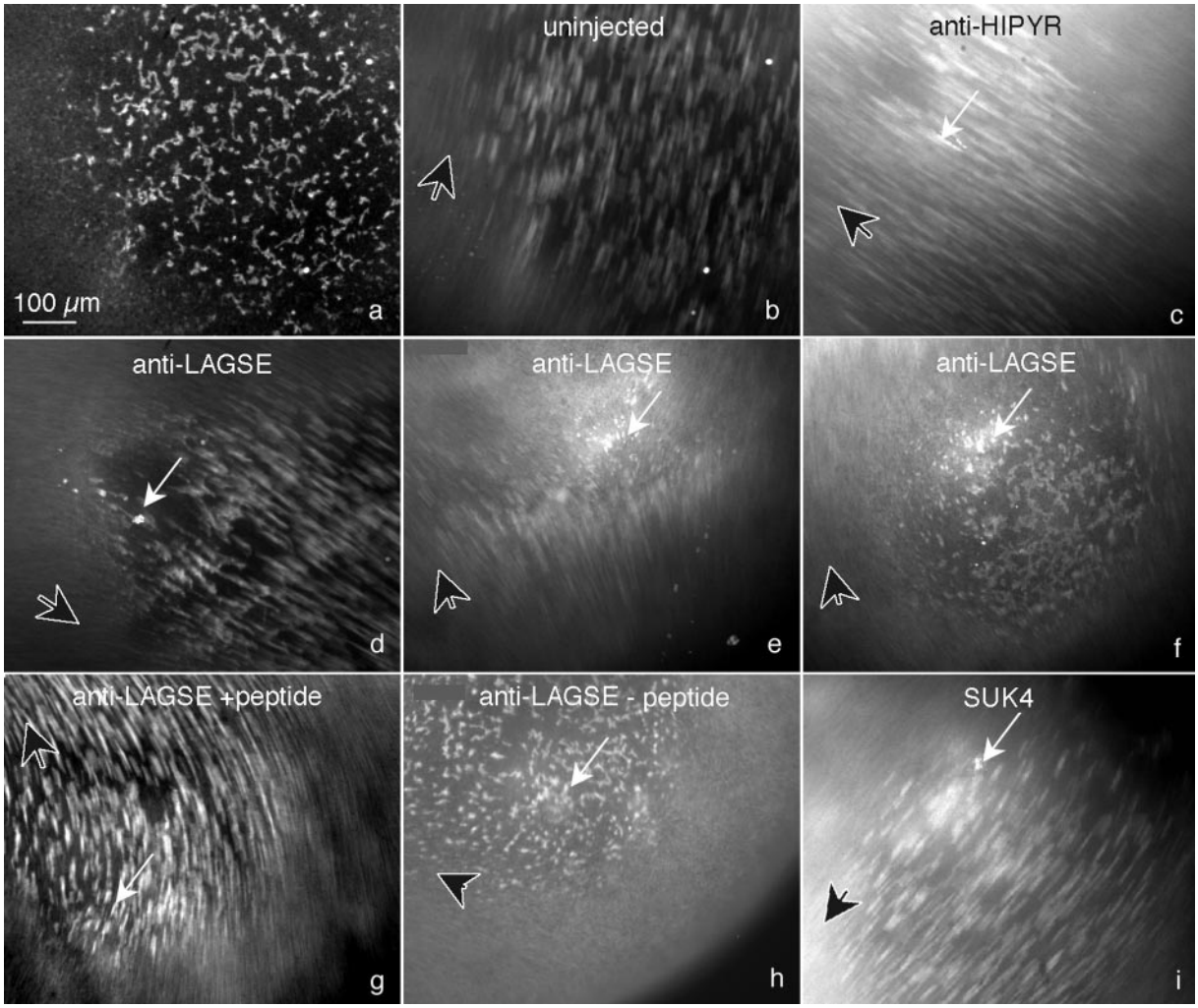

FIG. 2. Anti-LAGSE inhibits cortical rotation locally. (a) Image from a time-lapse video sequence showing $DiOC_6(3)$-labelled mitochondrial islands embedded in the vegetal cytoplasmic mass of a *Xenopus* egg. In all other images (b–i) the translocation of mitochondrial islands with respect to the immobilised vegetal cortex during cortical rotation is demonstrated by averaging 16–20 consecutive images. Black arrows have been added to show the direction of translocation. White arrows indicate the positions of fluorescent beads injected with the various antibody solutions beneath the vegetal cortex. (b) Translocation in the uninjected egg shown in (a). (c) Egg injected with 3 × 5 nl of 2.8 mg/ml anti-HIPYR at 0.5 normalised time (NT; see Materials and Methods). Translocation was barely perturbed. (d) Egg injected with 25 nl of 2 mg/ml anti-LAGSE at 0.5 NT. Mitochondrial island translocation was perturbed locally around the injection site, with "downstream" mitochondrial islands tearing as they moved away from the affected area. (e, f) Two eggs injected with 2 × 10 nl of 2 mg/ml anti-LAGSE at 0.6 NT showing extensive inhibition of translocation. Movement was completely blocked over most of the field shown in (f), while in (e) cytoplasm can be seen "piling up" behind the affected region. (g, h) Effect of pretreating anti-LAGSE with the LAGSE peptide. 2 × 10 nl of 2 mg/ml antibody was injected at 0.4 NT in both eggs. Incubation with a 25 molar excess of peptide substantially reversed the inhibitory effect of anti-LAGSE (g), whereas overnight incubation at 4°C did not reduce the effectiveness of the antibody alone (h). (i) Egg injected with 3 × 7 nl of 5 mg/ml SUK4 at 0.5 NT. Translocation was barely perturbed.

mg/ml. Inhibition was most effective when the antibody was introduced close to the cell surface in several small doses. Using this protocol, anti-LAGSE (2 mg/ml) was injected into 27 eggs (nine separate experiments). Complete local inhibition of cortical rotation was observed in 16 of the 27 eggs. In the remaining 11, the patches of antibody/bead mix visible at the surface were smaller, and the perturbations of cortical rotation were correspondingly less severe. Local inhibition of the cortical rotation following injection of anti-LAGSE did not depend on the time of injection relative to the onset of cortical rotation and the formation of the microtubule array (around 0.5 NT; Elinson

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and Rowning, 1988; Houliston and Elinson, 1991b), occurring whether the antibody was injected before (Figs. 2d and 2h) or after (Figs. 2e and 2f) this time.

Injection of the noninhibitory anti-HIPYR antibody at 2.8 mg/ml provoked only slight perturbations of the mitochondrial island displacement (in all 8 eggs in which beads were clearly visible at the surface from three experiments; Fig. 2c). As a further control for nonspecific effects of the injected anti-LAGSE solution, it was preincubated with excess LAGSE peptide. This substantially or completely rescued cortical rotation in 8 of 12 eggs from four experiments (Figs. 2g and 2h).

To address specifically the role of conventional kinesin in the cortical rotation, microinjection experiments were performed with SUK4 and SUK2, mouse monoclonal antibodies which recognise epitopes in the motor and stalk domains, respectively, of the sea urchin kinesin heavy chain (Ingold *et al.*, 1988). These both recognise the kinesin heavy chain specifically in *Xenopus* egg cytoplasm (Houliston and Elinson, 1991a; Fig. 1). SUK2 has no effect on kinesin function, whereas SUK4 inhibits kinesin-based microtubule motility *in vitro* (Ingold *et al.*, 1988) and blocks transport of exocytotic vesicles and lysosomes *in vivo* (Hollenbeck and Swanson, 1990; Scholey, 1998; Tuma *et al.*, 1998). In the six eggs from three experiments injected with 5 mg/ml SUK4 in which injected beads were visible at the egg surface, mitochondrial island displacement during cortical rotation was not disturbed (Fig. 2i). Similar results were obtained with 5 mg/ml SUK2 (six eggs in three experiments, not shown). These negative results should be treated with caution since we cannot prove that SUK4 effectively inhibits kinesin in the *Xenopus* egg cytoplasm. They suggest, however, that kinesin alone probably does not account for the KRP activity in the cortical region required to generate the cortical rotation.

Anti-LAGSE Blocks Cell Division

Microinjection of the anti-LAGSE antibody was found not only to block the cortical rotation but also to perturb mitosis and cleavage (Fig. 3) as reported for injection of anti-KRP antibodies into sea urchin eggs (Wright *et al.*, 1993). Injection into animal blastomeres of early embryos prevented the next or subsequent division so that by the blastula stage, cells containing the antibody were multinucleate with disorganised multipolar spindles forming during mitosis (Figs. 3c and 3d). Anti-HIPYR had no effect on mitosis or cleavage (Figs. 3e and 3f). This result supports the idea that anti-LAGSE but not anti-HIPYR effectively blocks KRP function since many KRPs are known to be involved in mitosis (Walczak *et al.*, 1998; see Introduction) and/or cytokinesis (Williams *et al.*, 1995; Adams *et al.*, 1997; Powers *et al.*, 1998; Raich *et al.*, 1998). Following injection beneath the vegetal cortex of the egg, the progression of the cleavage furrow was perturbed or arrested locally when large patches of anti-LAGSE lay in its path (Figs. 3a and 3b). This may be due to interference with

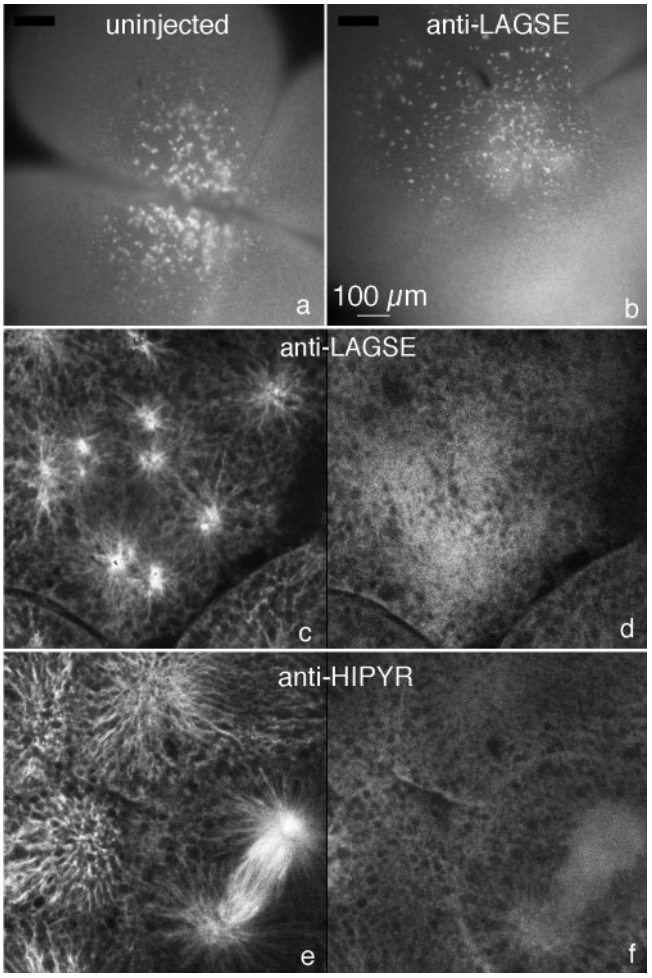

FIG. 3. Anti-LAGSE blocks cell division. (a, b) Vegetal subcortical injection of anti-LAGSE blocked cleavage furrow progression when the injected antibody was positioned appropriately. In the embryo shown in (b) both first and second furrows failed to complete, while an uninjected sibling (a) divided successfully to the four-cell stage. (c–f) Blastula stage *Xenopus* embryos which were injected at the eight-cell stage with anti-LAGSE (c, d) or anti HIPYR (e, f) (8–10 nl at 2 or 2.8 mg/ml, respectively, into single blastomeres). The progeny of the blastomeres injected with anti-LAGSE failed to divide while those injected with anti HIPYR continued to divide in parallel with uninjected blastomeres. Immunofluorescence with anti-tubulin antibodies (c, e) and anti-rabbit Ig antibodies to reveal the injected antibody (d, f) showed that division of nuclei and centrosomes in blastomeres containing anti-LAGSE antibody continued in a disorganised way without cleavage while blastomeres in sibling embryos containing anti-HIPYR showed normal interphase microtubule arrays and spindles.

a specialised microtubule array in amphibian eggs implicated in adding new membrane to the advancing cleavage furrow (Aimar, 1997; Danilchik *et al.*, 1998). As in the sea urchin egg (Wright *et al.*, 1993), neither SUK4 nor SUK2 affected cell division, except for very localised perturba-

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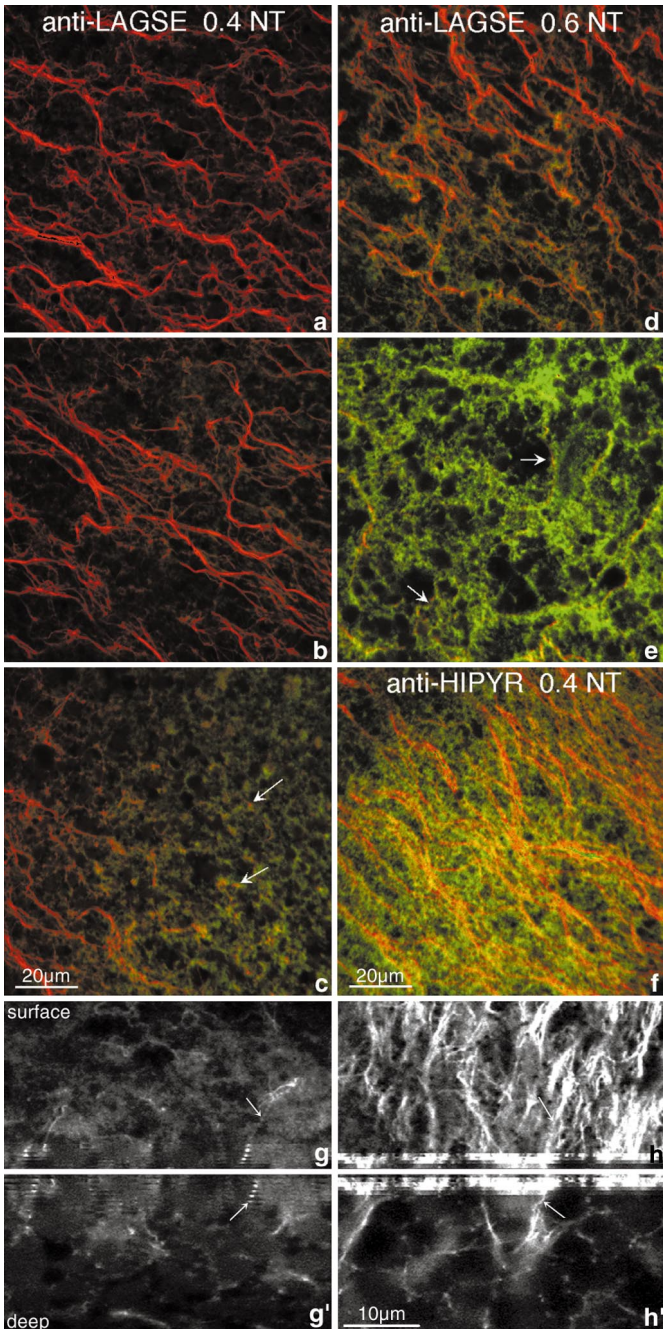

FIG. 4. Disruption of microtubule organisation by anti-LAGSE. Microtubule organisation in eggs injected with 2 × 10 nl of 2 mg/ml anti-LAGSE at 0.43 NT (a–c), of 2 mg/ml anti-LAGSE at 0.58 NT (d, e), of 2.8 mg/ml anti-HIPYR at 0.43 NT (f), and of 2 mg/ml anti-LAGSE at 0.55 NT (g) and in an uninjected egg (h). All eggs were fixed towards the end of the rotation period (0.7–0.8 NT). (a–f) Superimposed confocal images taken from the vegetal side of eggs processed for immunofluorescence with anti-tubulin antibodies (red) to show the distribution of microtubules and with anti-rabbit Ig antibodies (green) to reveal the location of the injected antibody. (a–c) Different vegetal regions of a single egg. Alignment of the subcortical microtubules was normal in regions lacking anti-LAGSE (a) but was progressively disrupted at increasing antibody concentrations (b, c). In areas where the antibody was most concentrated (c) only short microtubule segments were visible at the cortex (arrows). Anti-LAGSE also provoked disruption of microtubules in a dose-dependent manner when injected after the formation of the array (d, e). In contrast, anti-HIPYR had little or no appreciable effect on the organisation of the subcortical microtubules, whether injected before (f) or during (not shown) the cortical rotation. (g, h) Composite images constructed using the projection function in NIH Image software from stacks of six to eight images taken at 0.8-μm intervals downwards from the vegetal surface. For each egg, the stacks have been tilted by 40° with respect to a horizontal plane to show the upper (g, h) and lower (g′, h′) sides. Microtubules projecting from the deeper cytoplasm towards the surface (arrows) were clearly detectable in the presence of anti-LAGSE (g) as in uninjected eggs (h); however, these failed to turn and organise parallel to the cortex. This contributed to the severe disorganisation of the subcortical microtubule array provoked by the antibody. In contrast microtubules arriving at the surface in uninjected eggs (h, h′) turned to join bundles of aligned microtubules running parallel to the surface (for example at arrows).

tions of the cleavage furrow at the injection scar seen with both antibodies.

The immediate or delayed inhibition of cell division observed in eggs injected with anti-LAGSE in which the cortical rotation had been inhibited locally precluded analysis of their subsequent development.

Anti-LAGSE Disrupts Formation and Maintenance of the Microtubule Array

To determine whether the anti-LAGSE antibody interfered with the organisation of the vegetal subcortical microtubule array, double immunofluorescence was used to relate microtubule organisation to the presence of the injected antibody. When eggs injected beneath the vegetal cortex prior to the cortical rotation were fixed during the rotation period (Figs. 4a–4c), formation of the microtubule array was found to be severely disrupted. Microtubules and microtubule bundles lying parallel to the vegetal surface were less abundant in areas containing anti-LAGSE, and where present they were often misoriented (Fig. 4c). Eggs injected with anti-LAGSE after the onset of cortical rotation (Figs. 4d, 4e, and 4g) also showed disorganised microtubules in the presence of the antibody. Subcortical microtubules and microtubule bundles were clearly detectable but were severely misaligned. In contrast, anti-HIPYR (Fig. 4g) and SUK4 (not shown) had little effect on the organisation of the subcortical microtubules, irrespective of the time of injection.

Examination of microtubule organisation through the depth of the subcortical region in anti-LAGSE-injected eggs (Figs. 4g and 4g′) revealed that while the aligned microtubule array parallel to the surface was severely affected, microtubules projecting from the deeper cyto-

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plasm towards the surface were comparable to those in uninjected eggs (Figs. 4h and 4h′). In both the presence and the absence of the antibody, numerous microtubules could be detected perpendicular to the vegetal surface. Only in uninjected eggs, however, did these turn and extend to join the bundles lying parallel to the vegetal surface. These observations indicate that subcortically injected anti-LAGSE can both prevent the vegetal subcortical microtubule array from forming correctly and disrupt microtubule alignment once the array has formed. Microtubule alignment parallel to the cortex appears to be affected preferentially.

Anti-LAGSE Uncouples Microtubule–Cortex Interactions

Previous *in vivo* studies of microtubule behaviour during the cortical rotation (Houliston, 1994; Larabell *et al.*, 1996) had revealed generalised microtubule movement in the direction of yolk mass movement. These observations were considered to be evidence for microtubule translocation relative to the cortex and lent support to models for the cortical rotation in which plus-end-directed motors translocated the cortex with respect to subcortical microtubules (Houliston, 1994) and/or promoted microtubule–microtubule sliding (Larabell *et al.*, 1996). The much improved time-lapse recordings of microtubules in live eggs by confocal microscopy following microinjection of rhodamine-labelled tubulin in the current study (Fig. 5; recordings at http://www.obs-vlfr.fr/xenope.htm) significantly changed our appreciation of microtubule behaviour in the subcortical region. Although waves of movement rippling continually along the microtubules in the direction of cortical rotation do indeed give the impression of extensive microtubule translocation, we could identify many examples of microtubule segments undergoing little or no net translocation with respect to the cortex (Fig. 5a). Such microtubules were identified previously but thought to be a minority (Houliston, 1994). The relative lack of translocation of subcortical microtubules was emphasised at the edge of regions of microtubule disruption provoked by the injected anti-LAGSE antibody, where the cortical rotation was only partially inhibited and the ends of microtubule bundles were seen to oscillate laterally without net displacement (Fig. 5b).

In the centre of antibody-affected areas where translocation of yolk platelets (visible as unstained objects on the cytoplasmic side of the microtubules) was completely halted there was also clearly no microtubule displacement. The presence of injected antibody in affected regions was confirmed in some experiments by co-injection of Texas red-labelled 70-kDa dextran (not shown). Strikingly, microtubules in these regions were observed to exhibit vigorous uncoordinated flailing movements in three dimensions, with parts of the microtubule bundles moving in and out of the plane of focus (Fig. 5c). This contrasted with the directed rippling movements largely confined to the plane of the cortex in areas unaffected by the antibody. The disorganised microtubule flailing movements provoked by anti-LAGSE account for the severe perturbations in microtubule organisation observed by immunofluorescence of injected eggs (Fig. 4e).

Taken together our *in vivo* observations strongly suggest that in the undisturbed egg a significant subpopulation of microtubules is anchored to the cortex and that KRPs near the cortex act to tether or constrain microtubule movement. They also imply that motor molecules unaffected by the anti-LAGSE antibody promote microtubule movement in the subcortical region. We thus propose that the cortical rotation movement may not be generated by sliding between the microtubules and the cortex as previously thought, but that the force may be generated deeper in the egg by other motor molecules (see Discussion).

KRPs Colocalise with Cortical ER

The distribution of the proteins recognised by anti-LAGSE was examined by immunofluorescence on eggs fixed during the cortical rotation. Antigens recognised by anti-LAGSE were abundant, forming a dense carpet of anti-LAGSE staining at the level of the cortex (Figs. 6a and 6b). In subcortical regions their general distribution tended to coincide with that of the aligned microtubule bundles (Figs. 6c and 6d). This distribution was reminiscent of that of ER (Figs. 6e and 6f; Houliston and Elinson, 1991a). Double immunofluorescence using an anti-BiP antibody to reveal the distribution of ER showed that the punctate pattern of anti-LAGSE staining overlapped extensively with the ER network, both at the level of the cortex and associated with the microtubule bundles in the subcortical region. The coincidence of the punctate aggregates with parts of the ER network was emphasised by applying an edge-finding function to the anti-BiP image prior to merging of the confocal images (Figs. 6g and 6h).

These results indicate that a significant fraction of the KRP population in the vegetal subcortical and cortical regions associates with ER. The aggregates of anti-LAGSE staining on the ER may correspond to specialised sites of ER attachment to microtubules (Allan and Vale, 1994; Waterman-Storer *et al.*, 1995), which are thought to contain KRPs (Waterman-Storer and Salmon, 1998).

DISCUSSION

The experiments reported here provide the first strong evidence that the action of KRPs at the level of the vegetal cortex is essential for cortical rotation in amphibian eggs. More unexpectedly, they suggest that the main role of KRPs in the subcortical regions may not be in translocating the cortex but rather in promoting lateral interactions necessary to align subcortical microtubules and to tether them to the cortex. Whatever the function(s) of the KRPs at the vegetal cortex, it appears clear from our results that they are required locally across the

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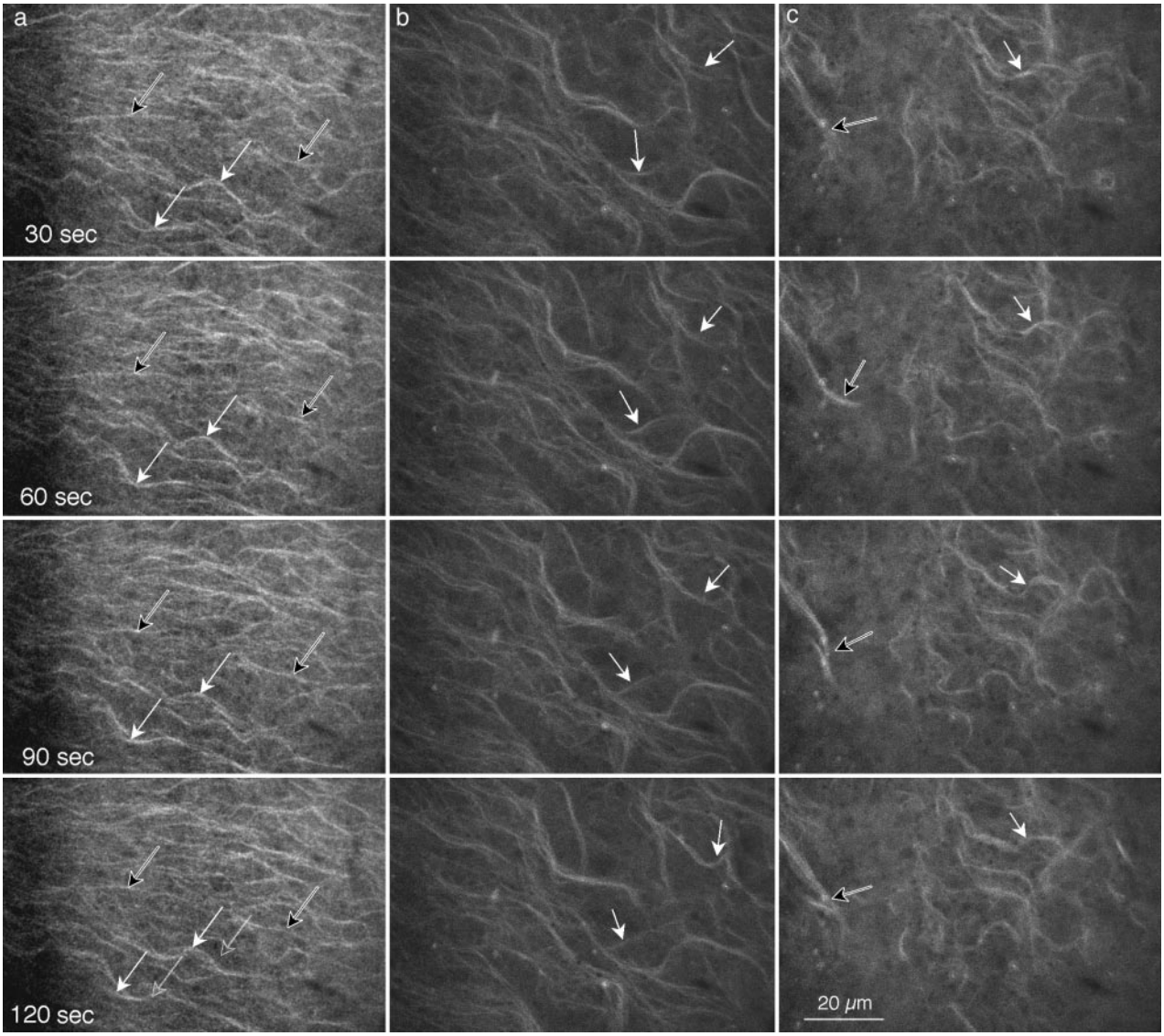

FIG. 5. Anti-LAGSE uncouples microtubule–cortex interactions. Confocal images from sequences of three regions taken from the vegetal side of a live prick-activated egg injected with 40 nl of 5 mg/ml rhodamine–tubulin 20 min after activation and 2 × 20 nl of 2 mg/ml anti-LAGSE 30 min later. Yolk platelets can be observed in the recording as unstained objects moving across the field, lying mainly in a deeper focal plane than the microtubules. (a) Region far from the antibody injection site. Coordinated translocation of vegetal yolk platelets occurred undisturbed from right to left relative to the immobilised cortex. The microtubules were well aligned and microtubule waves tended to move in the plane of the cortex in the same direction as the yolk (e.g., at white arrows: the initial position is also marked lightly on the final image). Many microtubule segments, however, showed little apparent movement (black arrow). (b) Region on the edge of the injected zone. On the right side of this field the speed of yolk platelet translocation was reduced and the alignment of microtubules disrupted, with exaggerated lateral waving occurring (arrows). (c) Region close to the antibody injection site where yolk platelet translocation was abolished. Microtubule behaviour was severely disrupted. No net microtubule displacement occurred within the field; however, uncoordinated flailing of microtubule bundles was observed both in the plane of the cortex and perpendicular to it (e.g., at arrows). The end of the bundle marked with a black arrow showed oscillating lateral movements but clearly did not move from the field of observation.

vegetal surface of the egg since inhibition of movement of the cytoplasm relative to the cortex by the anti-LAGSE antibody was restricted to the area receiving the antibody. This implies that the coherent movement of the vegetal yolk mass (Vincent *et al.*, 1986; Danilchik and Denegre, 1991) is the result of similar forces simultaneously pushing different parts of the vegetal cytoplasm across the cortex.

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Local KRP-Mediated Interactions Necessary for the Cortical Rotation

The *Xenopus* egg KRP Xklp1 is required both for correct spindle formation and for the microtubule-dependent process of germ plasm aggregation that occurs during the first few cell cycles (Vernos *et al.*, 1995; Robb *et al.*, 1996). Other KRPs present in the egg might likewise function during interphase as well as during mitosis, either by participating in specific events or by affecting microtubule bundling, stability, or organisation more generally. Inhibitory anti-KRP antibodies could thus perturb the cortical rotation directly or indirectly. Antibodies recognising the microtubule-associated protein XMAP230, implicated in microtubule organisation and stability, probably inhibit cortical rotation indirectly by preventing formation of the array (Cha and Gard, 1999). In the present study injections were targeted so as to restrict the antibody to patches at the vegetal cortex within which microtubule behaviour could be monitored. We were thereby able to confirm directly that cortical rotation could be halted by anti-LAGSE injection after 0.5 NT without destroying the subcortical microtubule bundles.

When the inhibitory antibody was introduced early in the cell cycle, the formation of the aligned subcortical microtubule array was severely affected. The substantial reduction in subcortical microtubule density in these early-injected eggs may in part reflect decreased microtubule stability in the absence of microtubule–cortex interactions (see below). The relative scarcity of microtubules beneath the vegetal cortex may in turn have contributed to the failure of cortical rotation. It is unlikely to be wholly responsible, however, since low doses of nocodazole can substantially reduce the density of the aligned subcortical microtubules without affecting the speed of rotation (Houliston, 1994). We speculate that the dramatic local block to cortical rotation we observed irrespective of injection time relates more to disruption of microtubule organisation than to reduction in microtubule numbers.

KRPs and Microtubule Tethering at the Cortex

The idea that KRPs act to tether microtubules to cortical structures during cortical rotation was suggested by our observations of microtubule behaviour in injected eggs. Under the influence of anti-LAGSE the subcortical microtubule bundles lost their alignment and their movements were much more exaggerated than in unaffected regions, no longer being confined to the plane of the cortex. KRPs are also thought to play tethering roles in other cellular processes. One example is at the kinetochore during mitosis, where the KRP CENP-E is implicated in maintaining connections to microtubule plus ends while allowing continuing microtubule dynamics (Lombillo *et al.*, 1995a; Hyman and Karsenti, 1996; Yao *et al.*, 1997). In cultured cells specialised ER domains containing KRPs appear similarly to maintain attachments to dynamic microtubule plus ends (Waterman-Storer and Salmon, 1998). In both these examples tethering affects microtubule dynamics, lowering transition frequencies between growth and shortening. Interference with KRPs mediating such tethering might destabilise microtubules (Lombillo *et al.*, 1995b) and thus explain the reduction in numbers of subcortical microtubules we observed in the presence of anti-LAGSE.

The identity of the KRP(s) mediating microtubule–cortex interactions and the nature of their association with the cortex remain to be established. Targeted injection of single or combined antibodies able to inhibit specifically known KRPs, combined with direct examination of the consequences for microtubule behaviour, may allow distinct roles for individual members of the kinesin superfamily to be distinguished. It remains possible, however, that cytoplasmic KRPs in the egg interact with cortical structures in a relatively nonspecific and redundant manner. Soluble plus-end-directed motors in the egg cytoplasm are able to associate promiscuously with injected beads, endogenous vesicles (Rowning *et al.*, 1997), and particles containing an overexpressed GFP–dishevelled fusion protein (Miller *et al.*, 1999). Likewise in crab and squid giant axons, a wide variety of negatively charged particles including carboxyl latex beads and actin filaments exhibit plus-end-directed transport upon microinjection (Adams and Bray, 1983; Terasaki *et al.*, 1995).

Potential cortical structures with which KRPs may associate include microfilaments, cytokeratin filaments, and ER (Klymkowsky *et al.*, 1987; Houliston and Elinson,

FIG. 6. KRPs colocalise with ER in the cortical region. (a–d) Double immunofluorescence of eggs fixed during the cortical rotation with anti-tubulin (a, c) and anti-LAGSE (b, d). Anti-LAGSE showed a carpet of superficial cortical staining (a, b) as well as a general codistribution with the more deeply bundled microtubules of the subcortical vegetal microtubule array (c, d). (e–h) Double immunofluorescence with anti-LAGSE and anti-BiP to reveal the ER. The distribution of ER at cortical (e) and subcortical (f) levels was similar to that of anti-LAGSE. Superimposition of images taken both levels (g, h) at high magnification, with the ER outlined in red by application of an edge-finding function to the anti-BiP image and anti-LAGSE in green, confirmed that the dots of anti-LAGSE overlay parts of the ER network (arrows).
FIG. 7. Possible mechanism for the cortical rotation. This diagram shows one possible mechanism which could account for our various observations. Cortically attached KRPs (blue) and cytoplasmic minus-end-directed microtubule motors (green) cooperate to produce the cortical rotation. Microtubules are indicated in red and the cortex in black. KRPs, perhaps associated with cortical ER, mediate lateral interactions between outward-extending microtubules and the cortex. The tethered subcortical microtubules are drawn further into alignment as the cortex moves (Vincent *et al.*, 1987; Ziskind and Elinson, 1990), with movement and alignment probably reinforcing each other (Gerhart *et al.*, 1989). In this model, minus-end-directed motors drive microtubules out from the inner cytoplasm and exert a force that displaces the cortex (green arrowheads).

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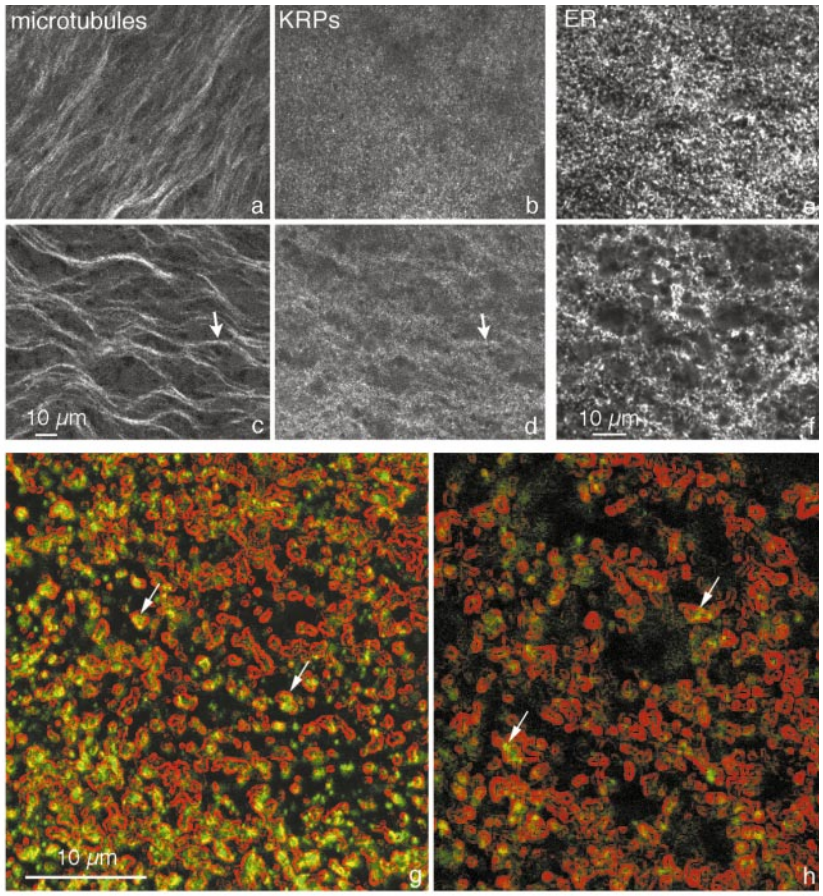

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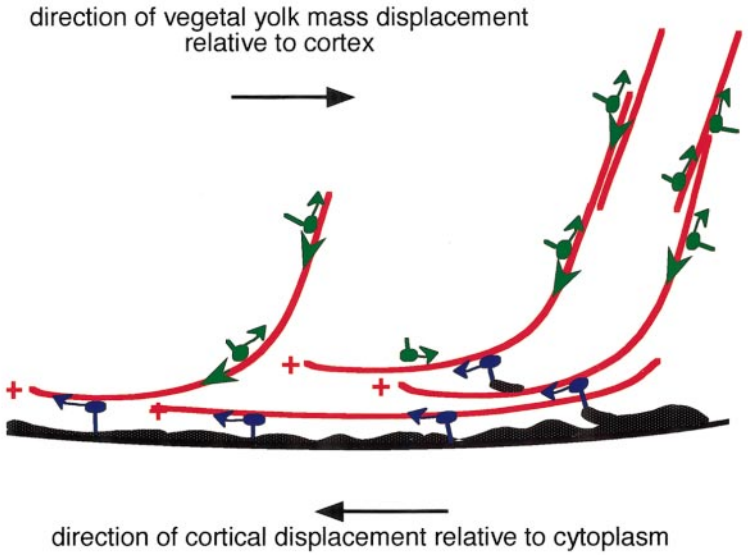

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1991a) as well as adjacent microtubules (Larabell *et al.*, 1996). ER is likely to provide an important interface between microtubules and the cortex during cortical rotation, with ER sheets and tubes closely apposed to the microtubules of the array (Houliston and Elinson, 1991a; Chang *et al.*, 1996). Furthermore kinesin associates with ER on isolated vegetal cortices (Houliston and Elinson, 1991a) and in the present study the abundant antigens recognised by anti-LAGSE were found to colocalise extensively with ER in the cortical region.

Force Production for the Cortical Rotation

Although we could not assess directly the contribution of KRPs to the translocation of the cortex relative to microtubules in our experiments because of the severe microtubule disorganisation provoked by the inhibitory antibody, a number of observations indicate that subcortical KRPs may not provide the main driving force for the cortical rotation. In particular the greatly improved discrimination of microtubules in live eggs allowed a substantial population of microtubules that showed little or no net displacement to be clearly identified, an observation difficult to reconcile with models in which microtubules are translocated across the cortex by plus-end-directed microtubule motors (Houliston, 1994). Furthermore lateral movement of subcortical microtubules along with atypical three-dimensional flailing was enhanced in the presence of anti-LAGSE, suggesting that other motor proteins and/or KRPs acting elsewhere in the egg contribute significantly to microtubule movement in the subcortical region.

The most obvious candidate for the generation of microtubule movement in the egg is the minus-end-directed motor cytoplasmic dynein. Dynein is a dominant microtubule motor in cytoplasmic extracts made from *Xenopus* eggs and early embryos (Verde *et al.*, 1991; Allan, 1995; Heald *et al.*, 1996; Merdes *et al.*, 1996; Niclas *et al.*, 1996; Lane and Allan, 1999). Cytoplasmic dynein distributed evenly in the egg, perhaps associating with cytoskeletal or ER networks, has been proposed to act along the lengths of the microtubules nucleated around the sperm centrosome in fertilised eggs and to push them out towards the cortex, thus accounting for the positioning of the sperm aster and associated pronuclei (Reinsch and Gönczy, 1998). Localisation of cytoplasmic dynein and the associated dynactin complex along astral microtubules has been reported in mitotic epithelial cells (Busson *et al.*, 1998), suggesting that an equivalent mechanism contributes to the positioning of mitotic spindles. Another related process, in which microtubules nucleated at the cell centre are transported outwards into the axons (Reinsch *et al.*, 1991; Baas, 1997) has been shown directly to depend on cytoplasmic dynein and dynactin in cultured neurones (Ahmad *et al.*, 1998). Microinjection of the inhibitory dynein intermediate chain antibody D70.1 (Gaglio *et al.*, 1996; Heald *et al.*, 1996; Reinsch and Karsenti, 1997; Walczak *et al.*, 1998) into *Xenopus* eggs had no detectable effect on the cortical rotation (our unpublished results); however, these negative results are inconclusive since local injections may not have an effect if dynein participates in the cortical rotation by exerting force along microtubules throughout the cytoplasm. It will be challenging to try and find other approaches to dissecting the role of dynein in this cortical rotation.

In summary, we have found that an antibody that interferes with KRP function blocks the cortical rotation locally and provokes unusual three-dimensional movements of subcortical microtubules. We have also revealed that a significant population of subcortical microtubules in undisturbed regions does not displace with respect to the cortex. These findings are difficult to reconcile with models in which KRPs transport the cortex along aligned subcortical microtubules. As a possible alternative mechanism compatible with currently available data (Fig. 7) we suggest that KRPs act at the cortex to promote lateral interactions between microtubules and the cortex necessary for the formation and maintenance of the aligned subcortical microtubule array. Cortically attached plus-end-directed KRPs may also contribute to the displacement of the cortex. Minus-end motors acting in the cytoplasm to push the microtubules towards the cortex may also make an important contribution to the cortical translocation. The arrival of microtubules at the vegetal cortex from the inner cytoplasm is initially biased on one side of the egg, corresponding to the side at which the sperm penetrated and the sperm aster formed (Houliston and Elinson, 1991b). This asymmetry in microtubule organisation ensures that cortical rotation occurs around a horizontal axis and that development of dorsoanterior structures is initiated correctly on one side of the embryo.

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