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Activation of myosin V–based motility and F-actin–dependent network formation of endoplasmic reticulum during mitosis

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It is widely believed that microtubule- and F-actin–based transport of cytoplasmic organelles and membrane fusion is down-regulated during mitosis. Here we show that during the transition of *Xenopus* egg extracts from interphase to metaphase myosin V–driven movement of small globular vesicles along F-actin is strongly inhibited. In contrast, the movement of ER and ER network formation on F-actin is up-regulated in metaphase extracts. Our data demonstrate that myosin V–driven motility of distinct organelles is differently controlled during the cell cycle and suggest an active role of F-actin in partitioning, positioning, and membrane fusion of the ER during cell division.

Introduction

It is a well-entrenched dogma that microtubule- and F-actin–based intracellular organelle transport is down-regulated during mitosis in order to ensure the accuracy of stochastic partitioning of the diverse organelles into daughter cells (Warren and Wickner, 1996). Unlike the Golgi apparatus, which undergoes fragmentation during mitosis, the highly interconnected ER does not undergo dramatical fragmentation and is maintained during mitosis (Warren, 1993). In mitosis, all microtubule-dependent movement of membranous organelles, including that of ER, is inhibited (Allan and Vale, 1991), presumably because microtubules and microtubule-associated motors are needed for spindle formation and chromosome segregation. We considered the possibility that during mitosis the F-actin–based system facilitates not only cleavage furrow formation but also the positioning and partitioning of the ER. That F-actin system can generally interact with ER is supported by the finding that the ER in neurons is transported on F-actin by myosin V (Tabb et al., 1998) and by the study showing that F-actin is involved in ER dynamics throughout the cell cycle in yeast (Prinz et al., 2000).

A convenient system to study cell cycle–dependent motility and network formation of ER is interphase-arrested extract (I extract)* and metaphase-arrested extract (M extract) from *Xenopus* frog eggs (Allan and Vale, 1991). We use this system here to investigate whether mitotic reorganization affects ER movement on F-actin and found that both myosin V–driven ER motility and F-actin–dependent ER network formation, a consequence of membrane movement and fusion, became activated in M extracts. To our knowledge, this is the first study demonstrating an up-regulation of both membrane organelle motility and fusion during mitosis.

Results and discussion

Our goal here was to study organelle motility along F-actin in I and M extracts from *Xenopus* frog eggs. For this we developed an in vitro procedure to generate stable three-dimensional F-actin networks attached to a coverslip surface under conditions where microtubules are absent. This was achieved by diluting *Xenopus* egg extracts with a glycine buffer containing nocodazole following incubation in the presence of rhodamine-phalloidin and an ATP-regenerating system. The network was stable and did not change in density for at least 60 min of observation (Fig. 1 A). The density and

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*Abbreviations used in this paper: CaMKII, calcium/calmodulim-dependent kinase II; CYCΔ, nondegradable cyclin B; I extract, interphase-arrested extract; M extract, metaphase-arrested extract; ECaSt/PDI, ER calcistorin/protein disulfide isomerase.

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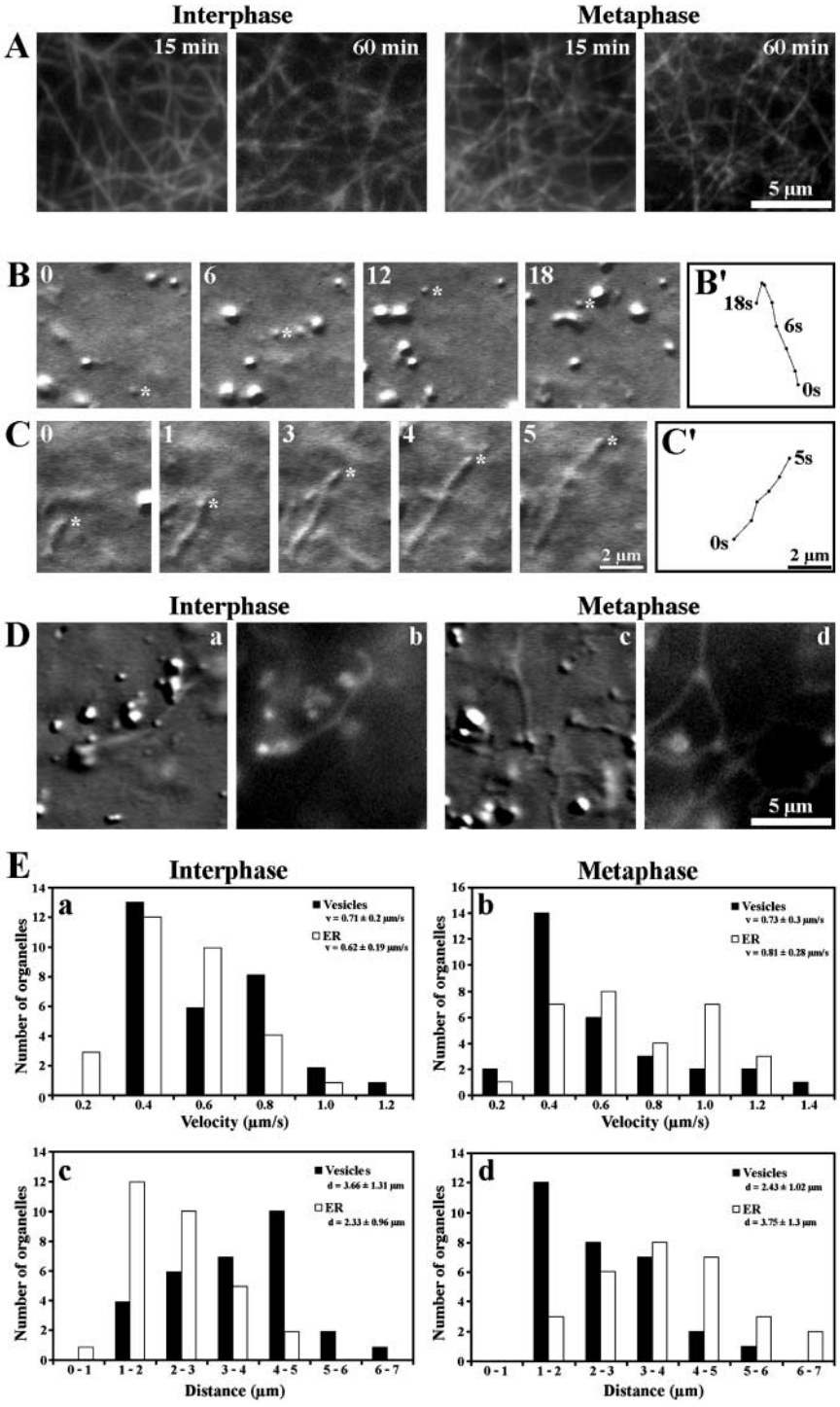

Figure 1. **Reconstitution of organelle motility on F-actin in extracts isolated from *Xenopus* eggs.** (A) Stability of an F-actin network in I (Interphase) and M (Metaphase) extracts visualized by fluorescence microscopy after 15 and 60 min of incubation with 0.5 μM rhodamine-phalloidin. (B) Movement of a globular vesicle on F-actin monitored by DIC microscopy. Selected frames of a video sequence covering 18 s are shown. (B′) Track diagram mapping the movement of the vesicle marked by an asterisk in B. (C) Movement of a tubular organelle (asterisk) on F-actin monitored by DIC microscopy. Selected frames of a video sequence covering 5 s are shown. (C′) Track diagram mapping the movement of the tubular organelle shown in C. The numbers in the top left corner of B and C indicate the time points of image acquisition. (D) Tubular organelles in I (Interphase) and M (Metaphase) extracts visualized by DIC microscopy (a and c) and by fluorescence microscopy after ER staining with $DiOC_6$ (b and d). (E) Motion analysis of organelle movement on F-actin. (a and b) Distribution of the velocities and average velocities (v) attained by 30 globular vesicles (Vesicles) in I extracts (a) and by 30 tubular organelles (ER) in meiosis II M extracts (b). (c and d) Distributions of the frequencies of run distances and average run distance (d) attained by 30 globular vesicles (Vesicles) in I extracts (c) and by 30 tubular organelles (ER) in meiosis II M extracts (d). See also videos 1 and 2 available at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1

stability of F-actin networks formed in M extracts were very similar to those observed in I extracts (Fig. 1 A).

In both I and meiosis II M extracts, the motility of two morphologically different types of organelles was detected, namely that of globular membrane vesicles (Fig. 1, B and B'; video 1 available at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1) and tubular membrane structures (Fig. 1, C and C'; video 2 available at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1). The latter observations were highly reminiscent of moving ER tubules observed previously in both *Xenopus* egg extracts (Allan and Vale, 1991) and squid axoplasm preparations (Tabb et al., 1998). To identify these membrane tubules, we first labeled I and M extracts with fluorescent dye $DiOC_6$, which preferentially labels ER (Jaffe and Terasaki, 1993), and C_6-NBD-Cer, which labels the Golgi apparatus (Martin et al., 1993). In both types of extracts, $DiOC_6$ labeled specifically the tubular structures suggesting their ER origin (Fig. 1 D). This was further confirmed by immunofluorescence microscopy using an antibody to an ER-resi-

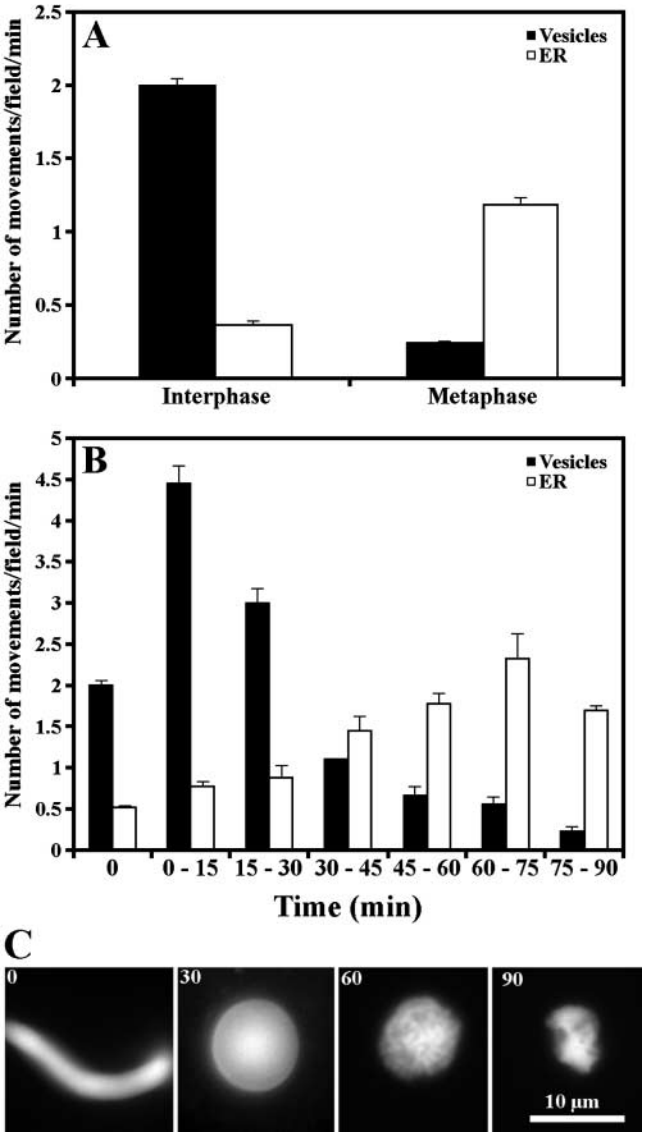

Figure 2. **Quantitative analysis of actin-dependent organelle movement.** (A) Motile activities of globular vesicles (Vesicles) and tubular organelles (ER) in I and meiosis II M extracts. (B) Motile activities of globular vesicles (Vesicles) and tubular organelles (ER) during transition from interphase to mitotic metaphase induced by CYCΔ. The values at the 0-min time point indicate the motile activity in the same I extract in the absence of CYCΔ. (C) Determination of cell cycle status during transition from interphase to mitotic metaphase induced by CYCΔ using morphology assay of added sperm nuclei.

dent protein, ER calcistorin/protein disulfide isomerase (EcaSt/PDI) (Lucero et al., 1994) (see below). No labeling of tubular membranes was found after treatment with C_6-NBD-Cer (unpublished data).

The motion analysis showed that the movement of all membranous organelles on F-actin was ATP dependent and unidirectional. The average velocity of moving globular vesicles was almost identical in both types of extracts (Fig. 1 E, a and b; Student's *t* test, $P = 0.94$). However, the velocity of moving ER tubules in M extracts was significantly higher (30%; Student's *t* test, $P = 4.6 \times 10^{-4}$) than in I extracts (Fig. 1 E, a and b). Greater differences were observed for the distribution of the measured distances of the moving organelles. The average distance moved by globular vesicles in the I extracts was 50% greater (Student's *t* test, $P = 2.2 \times 10^{-4}$) than in M extracts (Fig. 1 E, c and d). In contrast, the average distance moved by ER tubules in I extracts was 60% shorter (Student's *t* test, $P = 2.0 \times 10^{-5}$) than in M extracts (Fig. 1 E, c and d). We also performed a quantitative analysis of organelle movement on F-actin. We found that the motile activity (number of organelle movements per field per min) of globular vesicles was down-regulated approximately fivefold (Fig. 2 A). In contrast, the motile activity of the ER tubules was greatly up-regulated (about fourfold) in M extracts (Fig. 2 A). Together, these results demonstrate that ER movement and movement of globular vesicles on F-actin is differentially regulated throughout the cell cycle.

To confirm our finding that the movement of different organelles on F-actin is differently regulated by the cell cycle, we analyzed the F-actin–based motility in extracts during the transition from interphase to mitotic metaphase, the latter being induced by addition of nondegradable cyclin B (CYCΔ) to I extracts (Glotzer et al., 1991). During the first 15 min of incubation with CYCΔ, corresponding to the switch from interphase to mitosis, the motile activity of globular vesicles was significantly increased (Fig. 2 B). After 15 min of incubation, we detected a gradual inhibition of globular vesicle movement and a concomitant stimulation of ER movement (Fig. 2 B). The maximum rate of ER motile activity was observed at mitotic prophase (60–75 min of incubation, according morphology assay of added sperm chromatin; Fig. 2 C). At mitotic metaphase (75–90 min of incubation), the motile activity of globular vesicles and ER was almost identical to that detected in meiosis II M extracts (Fig. 2, A and B).

In M extracts, we detected not only activation of F-actin–dependent ER motility but also the formation of a reticular network (Fig. 3 A, a). This network formed after incubation of extract for 30 min was labeled with both the fluorescent dye $DiOC_6$ and an antibody to EcaSt/PDI (Fig. 3 A, b and e). Under the conditions used, we did not detect ER network formation in I extracts (Fig. 3 A, c, d, and f). Furthermore, ER network formation in M extracts was dynamic (video 3 available at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1) and strongly dependent on the presence of F-actin, since in the presence cytochalasin D, a drug preventing actin polymerization, no ER network was observed (Fig. 3 B). Collectively, our results argue that both F-actin–dependent ER network formation and ER tubule movement on F-actin are activated in M extracts. Notably, the microtubule-dependent ER network formation and movement (Allan and Vale, 1991) and the ER network formation by a controlled fusion reaction (Dreier and Rapoport, 2000) are inhibited in these M extracts.

We next asked what type of myosin might be responsible for ER transport on F-actin in *Xenopus* egg extracts. The average velocity (~0.7 μm/s) of organelle movement on F-actin we detected in extracts was similar to that seen with myosin V–dependent motility assays in vitro (Kuznetsov et al., 1992; Tabb et al., 1998). Furthermore, an equal amount of myosin V was found tightly associated with endogenous membrane organelles isolated from both I and M extracts

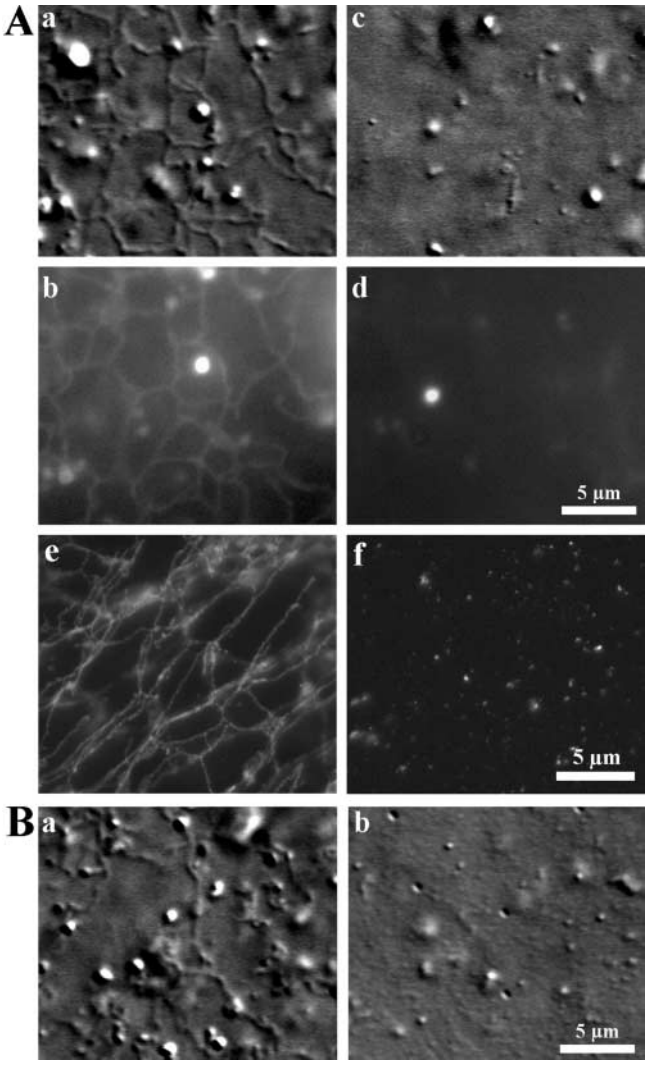

Figure 3. **F-actin–dependent ER network formation in M extracts.** (A) An extensive network of ER was visualized with both DIC microscopy (a) and fluorescence microscopy after staining with $DiOC_6$ (b) and immunofluorescence labeling with an antibody against ECaSt/PDI (e) in M extracts. No membrane network was detected in I extracts (c, d, and f). (B) An ER network was also observed in mitotic M extract obtained by incubation of I extracts with CYCΔ (a). An ER network did not form in the presence of 5 μM cytochalasin D (b). See also video 3 available at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1.

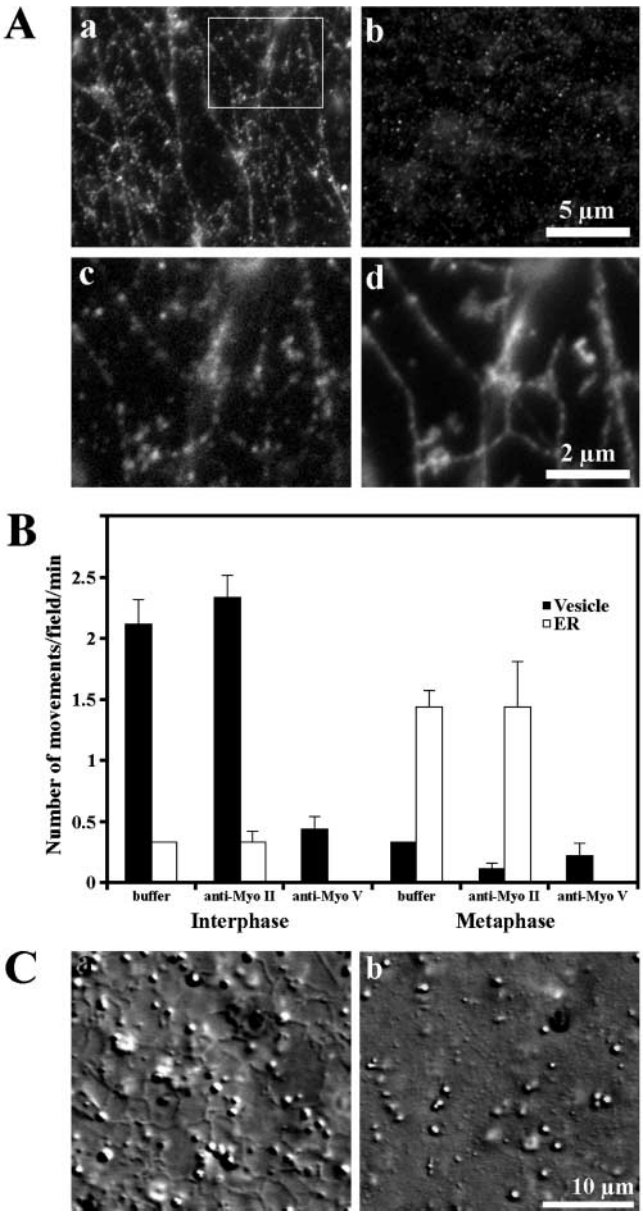

Figure 4. **Identification of myosin V as a motor responsible for organelle motility and ER network formation.** (A) The DIL2 antibody against myosin Va (a) but not a control antibody against myosin II (b) labeled the network in fixed preparations of M extract. The labeling of ER network with DIL2 antibody (c) and with an antibody against ECaSt/PDI (d) showed strong colocalization of myosin V immunosignal with ECaSt/PDI immunosignal. Panels c and d represent the region marked in panel a. (B) Inhibition of organelle motility on F-actin by an antibody against myosin Va. Motile activity in I and M extracts treated with DIL2 antibody (anti-Myo V) and a control antibody (anti-Myo II). (C) In M extracts ER network formation occurred in the presence of anti–myosin II (a) but not in the presence of DIL2 antibody (b).

(see Fig. 5 B, a; Rogers et al., 1999). Since the ER is the most abundant membrane organelle in egg extracts, these data make it unlikely that myosin V dissociated from the ER during mitosis and suggest that this myosin could be responsible for the ER transport we observe in M extracts.

To test directly whether myosin V is indeed still associated with ER in M extracts, we performed indirect immunofluorescence labeling of ER networks with a DIL2 antibody against mouse myosin Va, which strongly reacted with *Xenopus* myosin V as shown by immunoblotting (see Fig. 5 B). DIL2 antibody labeling revealed a pattern very similar to the ER network (Fig. 4 A, a). This pattern was not observed when a control antibody against myosin II was used (Fig. 4 A, b). Strikingly, the bulk of myosin V labeling colocalized with the immunosignal for EcaSt/PDI, the ER marker protein (Fig. 4 A, c and d). These data indicate that myosin V is associated with ER in M extracts.

To show directly that myosin V is involved in organelle transport, the extracts were treated with a DIL2 antibody and, as a control, the antibody against myosin II. Motile activity of all visible organelles on F-actin was inhibited in both types of extracts

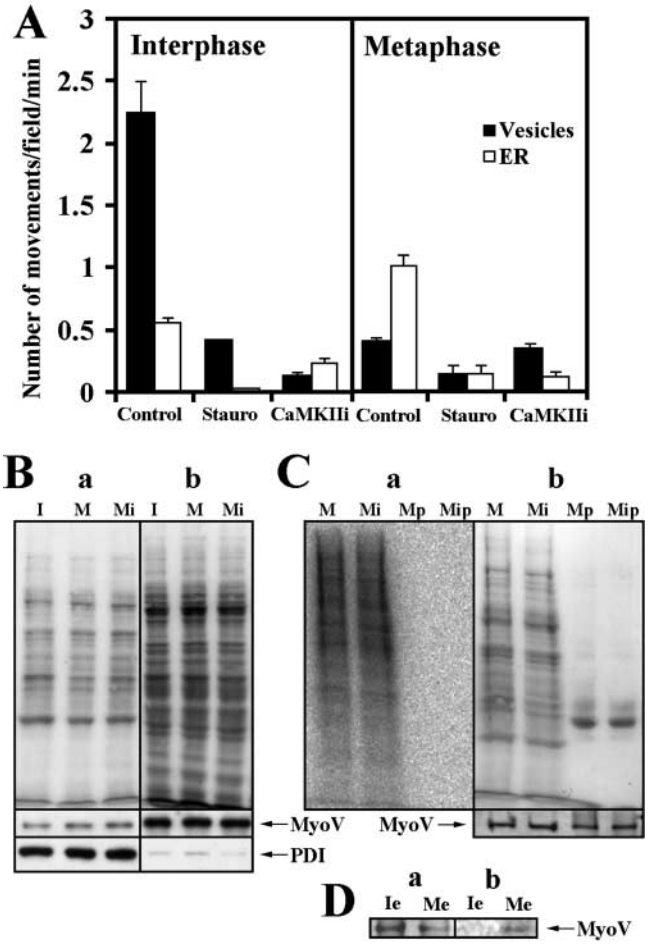

Figure 5. **Effect of protein kinase activity on myosin V–based organelle motility.** (A) 2 μM of staurosporine (Stauro) or 2 μM of CaMKII inhibitor (CaMKIIi) inhibited organelle motility in both I and meiosis II M extracts. The same effect was observed in mitotic M extract (data not depicted). (B) Immunoblotting of membrane fractions (a) and membrane free fractions (b) isolated from I extract (I), M extract (M), and M extract treated with CaMKII inhibitor (Mi) with DIL2 antibody (MyoV) and ER marker EcaSt/PDI (PDI). The equal amount of myosin V and EcaSt/PDI associated with membrane fractions. (Top) Ponceau S staining to demonstrate approximately equal protein load. (C) Phosphoimaging of membranes isolated from ^{32}P-labeled mitotic M extract (M) and ^{32}P-labeled mitotic M extract treated with CaMKII inhibitor (Mi) and myosin V immunoprecipitates (Mp and Mip) from the respective membrane fractions (a). (b) Ponceau S staining (top) and immunoblotting with DIL2 antibody (MyoV) (bottom) as in panel a to demonstrate approximately equal protein load and the presence of myosin V in membrane fractions and immunoprecipitates. (D) Immunoblotting (a) and phosphoimaging (b) of myosin V immnunoprecipitates from the nonfractionated I extract (Ie) and M extract (Me).

by >90% in the presence of DIL2 antibody (Fig. 4 B). However, the antibody against myosin II showed no effect on the motile activities in both types of extracts (Fig. 4 B). Moreover, the treatment of M extracts with the antibody against myosin Va but not against myosin II fully blocked the F-actin–dependent ER network formation (Fig. 4 C). Thus, these experiments are consistent with the notion that myosin V is the motor protein responsible for the F-actin–based movement of both globular vesicles and ER in I and M extracts and for F-actin–dependent ER network formation in M extracts.

Because myosin V is more strongly phosphorylated in metaphase compared with interphase (Rogers et al., 1999) and the phosphorylation of myosin V by calcium/calmodulin-dependent kinase II (CaMKII) resulted in the release of the motor from pigment granules (Karcher et al., 2001), we tested the involvement of protein phosphorylation in the movement of membrane organelles on F-actin. We first treated extracts with 2 μM staurosporine, a broad spectrum inhibitor of protein kinases (Ruegg and Burgess, 1989). This treatment blocked strongly both vesicle and ER motility in I and M extracts (Fig. 5 A). Surprisingly, the addition of 2 μM CaMKII (281–309) inhibitor peptide, which completely inhibits CaMKII activity in *Xenopus* egg extracts (Matsumoto and Maller, 2002), also blocked an activation of both ER movement in M extracts and vesicle movement in I extracts (Fig. 5 A).

To address whether motility inhibition reflects dissociation of myosin V from organelles, we isolated membrane fractions by flotation from M extracts preincubated with and without the CaMKII inhibitor. Quantitative analysis showed that this method yielded a distinct membrane fraction that contained ~80% of the total ER and ~5% of total myosin V (Fig. 5 B, a and b). We found that inhibition of CaMKII activity did not affect the amount of myosin V that copurified with the membrane fraction (Fig. 5 B, a). In addition, the CaMKII inhibitor did not change the level of protein phosphorylation in membrane fractions isolated from M extracts prelabeled with ^{32}P (Fig. 5 C, a). In contrast to the immunoprecipitation of myosin V from the nonfractionated ^{32}P-labeled M extract (Fig. 5 D), the immunoprecipitation of myosin V from membrane fractions isolated from ^{32}P-labeled M extract did not reveal any myosin V phosphorylation either in the absence or presence of the CaMKII inhibitor (Fig. 5 C, a and b). Thus, our results indicate that CaMKII activity is required for activation of myosin V–based movements of both ER in M extracts and of vesicles in I extracts. However, CaMKII activity alone is evidently not sufficient to mediate the regulatory mechanism responsible for controlling the myosin V–driven motility of different organelle types in the cell cycle. Since myosin V associated with membrane organelles does not undergo additional phosphorylation during mitosis, we suggest that CaMKII has additional targets of phosphorylation, which could, for example, modify the ATPase activity or processivity of myosin V during the cell cycle. These putative target proteins and the precise mechanisms regulating organelle motility on F-actin throughout the cell cycle remain to be determined. Since the number of myosin V isoforms in *Xenopus* is not yet known, we also cannot exclude the possibility that different isoforms undergo differential regulation relative to each other and to the cell cycle.

Our data provide strong evidence that ER membranes can move along F-actin and fuse during mitosis. The activation of myosin V–driven motility that we have observed could also explain the increase of ER motility seen during meiotic maturation of *Xenopus* oocytes (Kume et al., 1997) and the accumulation of ER in the F-actin–rich cortex of frog, mouse, and hamster eggs in vivo (Houliston and Elinson, 1991; Mehlmann et al., 1995; Shiraishi et al., 1995). It thus appears that the role of microtubules in moving the ER during interphase is taken over during mitosis by the actin system.

Materials and methods

Preparation of *Xenopus* egg extracts

I and M extracts were isolated from *Xenopus* eggs according to Murray (1991) with some modifications. I extracts were obtained by activation in the presence of 0.4 μg/ml ionophore A23187 for 5 min followed by washing in MMR/4 buffer containing 100 μg/ml cyclohexemide and incubation at RT for 30 min. The eggs were washed in ice-cold Tris-buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM sucrose). Protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin, 10 μg/ml TPCK) were added to the last washing step. After egg crushing by centrifugation, the cytoplasmic extract was removed and protease inhibitors and 60 μM nocodazole were added. Meiosis II M extracts were prepared without egg activation and cycloheximide addition, and the last washing step of the eggs was performed with Tris buffer containing 15 mM EGTA.

For some experiments, the transition from I to mitotic M extracts was induced by 1:0.75 (vol:vol) dilution of I extract in glycine buffer (300 mM glycine, 120 mM D-gluconic acid, 100 mM taurine, 20 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 30 μM nocodazole, pH 7.2) supplemented with 0.1 mg/ml bacterially expressed CYCΔ (Glotzer et al., 1991) and after incubation at RT in the presence of 0.5 μM rhodamine-phalloidin and ATP-regenerating system (1 mM ATP, 0.5 mg/ml phosphocreatine kinase, 10 mM creatine phosphate). The morphology of added sperm chromatin (Murray, 1991) and the histone H1 kinase assays were used to monitor the status of the cell cycle and the activity of $p34^{cdc2}$kinase (Felix et al., 1993).

In vitro motility assay and ER network formation

I or M extracts were diluted by 1:0.75 (vol:vol) in glycine buffer. Diluted extracts were supplemented with an ATP-regenerating system and 0.5 μM rhodamine-phalloidin and incubated for 15, 30, 45, and 60 min at RT. At each time point, the extracts were perfused into a microscope chamber. Organelle motility was monitored by video-enhanced contrast differential interference (DIC) microscopy, and the presence of rhodamine-phalloidin–labeled F-actin was confirmed by fluorescence microscopy. At each time point of incubation, three random fields (23 μm × 22 μm) were recorded for 3 min. The myosin-based motile activity was determined by counting moving organelles on tracks invisible with DIC microscopy and averaging the numbers obtained from 12 random fields after all time points of extract incubation. For organelle motility during transition from interphase to metaphase, the extract was examined by microscopy immediately after dilution, and continuous recording of 30 random fields for 3 min during 90 min of observation was performed.

ER network formation was monitored by both DIC microscopy and fluorescence microscopy after staining with 1 μg/ml $DiOC_6$ (Molecular Probes). To stain the Golgi apparatus, the extracts were incubated with 5 μM C_6-NBD-Cer (*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl D-*erythro*-sphingosine) (Molecular Probes) and examined by fluorescence microscopy. For immunofluorescence labeling of ER, 5–10 μl of extracts prepared as for DIC microscopy were placed on a poly-L-lysine–coated coverslip followed by incubation for 30 min at RT and then fixed by adding carefully 200 μl of 4% PFA with 4% sucrose in PBS. After fixation for 30 min, immunofluorescence labeling of the ER was performed as previously described (Kaether et al., 1997) using an mAb against rat ECaSt/PDI (StressGen Biotechnologies) and DIL2 polyclonal rabbit antibody against a GST fusion protein containing heavy chain residues 910–1,106 of mouse myosin-Va (Wu et al., 1997).

The effect of the following reagents on organelle motile activity and ER network formation was tested: a DIL2 antibody at 1:25 dilution, a polyclonal rabbit antibody raised against skeletal myosin II (M7648; Sigma-Aldrich) at 1:25 dilution, and 5 μM cytochalasin D (Sigma-Aldrich), 2 μM staurosporine (Calbiochem), and 2 μM CaMKII (281–309) inhibitor peptide (Calbiochem). For this, the diluted extracts were preincubated with the agents mentioned above for 30 min at RT.

Fractionation of extracts, ^{32}P labeling of membranes, and myosin V immunoprecipitation

Membrane fractions were prepared by flotation as described in Lane and Allan (1999) which was slightly modified. 300 μl of I extracts were diluted 1:0.75 (vol:vol) in glycine buffer containing an ATP-regenerating system. For obtaining the membrane fraction from mitotic M extracts, diluted I extracts were supplemented with CYCΔ. To study the effect of CaMKII activity on membrane protein phosphorylation, 2 μM CaMKII inhibitor and 50 μCi [γ-^{32}P]ATP (Amersham Biosciences) were added to diluted extracts. All samples were incubated for 60 min at RT and then were diluted once again with 2.75 ml of ice-cold glycine buffer containing 65% sucrose and 10 mM β-glycerophosphate. After centrifugation, the membrane fractions floated through 0.8 ml of glycine buffer with 50% sucrose were subjected to SDS-PAGE followed by Western blot and phosphoimaging. The immunoprecipitation of myosin V from the membrane fractions using affinity-purified DIL2 antibody was performed as described (Kromer et al., 1998).

Online supplemental material

Video 1 shows globular organelle movement on F-actin in I extract. The video illustrates the movement of globular organelles along F-actin observed with DIC microscopy and described in Fig. 1 B. Video 2 shows movement of tubular membrane structures (ER) on F-actin in meiosis II M extract. The video illustrates the movement of tubular membrane structures (ER) along F-actin observed with DIC microscopy and described in Fig. 1 C. Video 3 shows F-actin–dependent dynamics of ER network in meiosis II M extract. The video illustrates the movement along F-actin of tubular extensions of ER network observed with DIC microscopy and shown in Fig. 3 A. Videos 1–3 are available online at http://www.jcb.org/cgi/content/full/jcb.200204065/DC1.

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