Journal of Cellular Biochemistry 90:892–900 (2003)

Potential Roles of the Nucleotide Exchange Factor ECT2 and Cdc42 GTPase in Spindle Assembly in *Xenopus* Egg Cell-Free Extracts

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Abstract The ECT2 protooncogene encodes a guanine nucleotide exchange factor for the Rho family of small GTPases. ECT2 contains motifs of cell cycle regulators at its N-terminal domain. We previously showed that ECT2 plays a critical role in cytokinesis. Here, we report a potential role of XECT2, the *Xenopus* homologue of the human ECT2, in spindle assembly in cell-free *Xenopus* egg extracts. Cloned XECT2 cDNA encodes a 100 kDa protein closely related to human ECT2. XECT2 is specifically phosphorylated in M phase extracts. Affinity-purified anti-XECT2 antibody strongly inhibited mitosis in *Xenopus* cell-free extracts. Instead of bipolar spindles, where chromosomes are aligned at the metaphase plane in control extracts, the addition of anti-XECT2 resulted in the appearance of abnormal spindles including monopolar and multipolar spindles as well as bipolar spindles with misaligned chromosomes. In these in vitro synthesized spindle structures, XECT2 was found to tightly associate with mitotic spindles. The N-terminal half of XECT2 lacking the catalytic domain also strongly inhibited spindle assembly in vitro, resulting in the formation of mitotic spindles with a low density. Among the representative Rho GTPases, a dominant-negative form of Cdc42 strongly inhibited spindle assembly in vitro. These results suggest that the Rho family GTPase Cdc42 and its exchange factor XECT2 are critical regulators of spindle assembly in *Xenopus* egg extracts. J. Cell. Biochem. 90: 892–900, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: exchange factor; Cdc42; mitosis; oncogene; phosphorylation

Eukaryotic cells proceed through the cell division cycle in a strictly ordered fashion. Cells must precisely duplicate their chromosomal DNA during S phase, segregate the sister chromatids to opposite poles of the mitotic spindle during mitosis, assemble two nuclei, and then divide cell components into two daughter cells during cytokinesis. During mitosis, cytoskeletal components are dynamically reorganized to form fine architectures to divide genetic material correctly into daughter cells. These complicated transitions are coordinated through phosphorylation by cell cycle specific kinases in a temporally and spatially specific manner [Nigg, 1993; Glover et al., 1998]. It has been shown that a small GTPase Ran regulates spindle assembly in *Xenopus* cell-free extracts [Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Wilde et al., 2001] by altering microtubule dynamics and the balance of motor activities [Wilde et al., 2001].

Microtubules and actin represent dynamic cytoskeletal components that play essential roles during many cellular processes, such as cell motility and mitosis. In migrating cells, in response to numerous signals, a continuous but

Grant sponsor: Japan Society of Promotion of Sciences (fellowship to TT); Grant sponsor: Breast Cancer Think Tank Award (from National Institutes of Health).

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Received 26 August 2003; Accepted 25 September 2003

DOI 10.1002/jcb.10750

directed assembly of actin filaments results in protrusive filopodial or lamellipodial extensions [Waterman-Storer and Salmon, 1999]. Similarly, during cell division, interphase microtubules assemble into the bipolar spindle for chromosome segregation [Hyman and Karsenti, 1996]. Recently it was suggested that microtubules and actin are coordinated by the Rho family of small GTPases and their targets [Best et al., 1996; Cook et al., 1998; Gauthier-Rouviere et al., 1998; Ishizaki et al., 2001]. In addition to the regulation of cytoskeletal remodeling, Rho GTPases play crucial roles in diverse cellular activities such as membrane trafficking, transcriptional activation, and cell growth control [Van Aelst and D'Souza-Schorey, 1997]. Rho GTPases, represented by RhoA, Rac1, and Cdc42, are Ras-like small GTPases that function as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state. The activity of Rho GTPases is promoted by guanine nucleotide exchange factors, which catalyze the replacement of bound GDP by GTP [Cerione and Zheng, 1996]. The GTP-bound form of Rho GTPases can specifically interact with their effectors or targets and transmit signals to downstream molecules.

We previously isolated the ECT2 oncogene as a transforming cDNA from epithelial cells [Miki et al., 1991a, 1993] using a functional cloning system [Miki et al., 1991b]. ECT2 stimulates guanine nucleotide exchange on RhoA, Rac1, or Cdc42 in vitro [Tatsumoto et al., 1999]. ECT2 expression is induced in S phase and reaches the highest level in G2 and M phases [Sakata et al., 2000]. ECT2 is phosphorylated in G2 and M phases and the phosphorylation is required for the exchange activity of ECT2 [Tatsumoto et al., 1999]. ECT2 localizes to the nucleus in interphase and disperses to the entire cell after nuclear membrane breakdown. ECT2 is condensed on mitotic spindles during mitosis and then localized to the midbody during cytokinesis. Inhibition of ECT2 either with anti-ECT2 antibodies or a dominant negative form of ECT2 protein efficiently blocks cytokinesis [Tatsumoto et al., 1999].

In this report, we cloned the *Xenopus* homologue of ECT2 (XECT2), and showed that XECT2 is phosphorylated during mitosis and localized on the mitotic spindle. We also showed evidence that XECT2 and Cdc42 have potential roles in the formation of bipolar spindles in a *Xenopus* egg cell-free system.

MATERIALS AND METHODS

Isolation of XECT2 cDNA

A *Xenopus laevis* A6 kidney epithelial like cell cDNA library in λZAPII (1.8×10^6phages) was screened with human ECT2 cDNA (nucleotides, 1–1263) as probe. Prehybridization and hybridization were performed as described [Sagata et al., 1988] but at lower stringency (20% formamide). The hybridized filters were washed 50°C for 30 min with 2× SSC, 0.1% SDS, followed with 1× SSC, 0.1% SDS for 1 h.

Antibody Production

The coding sequences of amino-terminal *Xenopus* ECT2 (XECT2-N; amino acids, 1–390) were introduced into pET-32 (Novagen, Madison, WI) or pGEX4T (Pharmacia, Piscataway, NJ) vector, and expressed as oligohistidine or GST-fused proteins. Fusion proteins were purified according to the manufacturers' specifications. Antisera were obtained by immunizing rabbits with purified oligohistidine-fused XECT2-N. Antibodies were purified by passing through an affinity column containing GST-XECT2-N beads prepared by AminoLink Plus Immobilization Kit (Pierce, Rockford, IL). Purified proteins were dialyzed and concentrated in PBS using a Centricon-30 concentrator.

Preparation of *Xenopus* Egg Extracts and Sperm Nucleus

Mitotic extracts were prepared from unfertilized eggs of *Xenopus laevis* as described [Murray, 1991] except that a clarifying spin was performed for 60 min at 100,000*g* at 4°C. Interphase extracts were prepared from electrically activated eggs as described [Murray, 1991] except that cycloheximide (100 μg/ml) was added at the breakage step of eggs. Extracts were used immediately for the spindle assembly assay, or supplemented with 200 mM sucrose, and frozen in liquid nitrogen. Sperm nuclei were prepared as described [Murray, 1991] and stored at −80°C.

In vitro Spindle Assembly and Anaphase

Assembly of bipolar metaphase spindles and induction of anaphase were performed as described [Shamu and Murray, 1992]. Rhodamine-labeled tubulin (30 μg/ml, Molecular Probes, Eugene, OR) and sperm nuclei (100/μl) were added to freshly prepared mitotic extracts containing control non-immune rabbit IgG

(50 μg/ml, Sigma, St. Louis, MO) or affinity-purified anti-XECT2 antibody (50 μg/ml). After incubation at 20°C for 10 min, extracts were driven into interphase by the addition of $CaCl_2$ to a final concentration of 0.4 mM. The extracts were further incubated at 20°C for 80 min and a half volume of fresh extracts containing a proportional amount of the antibody was added to induce nuclear envelope breakdown and spindle assembly. Metaphase spindles were allowed to assemble for 80 min. To induce anaphase, a fraction of the metaphase extract was placed in new tubes and calcium was added to 0.4 mM. Samples were monitored for the progression of cell cycle under fluorescent microscopy, by means that 1.2 μl of extracts were spotted on a microscopic slide, 6 μl of fixation solution were dropped on top, and then squashed under a coverslip. To examine the effect of dominant negative Rho GTPases on spindle assembly, spindles were assembled in the same manner as described above in the extracts containing each recombinant protein (50 μg/ml).

Immunofluorescence Microscopy

For immunofluorescent analysis of extracts containing mitotic spindles, 20 μl of extracts were diluted with 1 ml of dilution buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 30% glycerol, 1% Triton X-100), and layered over 5 ml of cushion (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 40% glycerol) in a modified Corex tube containing a 12 mm round acid-washed and poly-L-lysine-coated coverslip at the bottom. The tubes were centrifuged for 15 min at 10,000 rpm in a Sorvall HB-4 rotor. The samples were fixed in methanol at −20°C for 5 min, transferred to PBS, and then incubated with affinity-purified anti-XECT2 antibody at 4°C for 1 h. After washing with PBS, they were incubated with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA) for 1 h at room temperature.

RESULTS

We used low stringency hybridization to screen a *Xenopus* cDNA library for the *Xenopus* homologue of human ECT2. Among the 11 positive clones identified, four contained 3.5 kb cDNA with a long open reading frame of 910 amino acids with a predicted molecular mass of 103 kDa. The amino-acid sequence was very similar to that of human ECT2 through the entire open reading frame, sharing 77% identity, except for an insertion of 31 amino acids at the amino-terminal region of the *Xenopus* clone (Fig. 1A,B). These high levels of sequence conservation suggest that this protein is the *Xenopus* homologue (XECT2) of human ECT2. XECT2 contains a Dbl homology (DH) domain followed by a pleckstrin homology (PH) domain in the central region. The combination of DH and PH domain is a common motif of guanine nucleotide exchange factors for the Rho GTPases [Horii et al., 1994; Cerione and Zheng, 1996]. The amino-terminal half of XECT2 contains a homology domain related to the fission yeast cell cycle regulator Cut5 [Saka and Yanagida, 1993]. The Cut5-related domain consists of two tandem repeats of the BRCA1 C-terminal (BRCT) motif, which is widespread in a number of molecules involved in cell cycle checkpoint and DNA repair [Bork et al., 1997]. The structure of each domain is well conserved between human and *Xenopus* ECT2, with the highest identity of 86% in their DH domains.

The amino-terminal domain of XECT2, which lacks similarity to other guanine nucleotide exchange factors for the Rho GTPases, was used to raise anti-XECT2 antibody. Affinity-purified anti-XECT2 antibody recognized a single band of approximately 100 kDa protein, which corresponded to the size predicted from the cDNA sequences, in interphase *Xenopus* egg extracts (Fig. 1C). In contrast, the size of the band detected by anti-XECT2 in mitotic extracts was slightly higher than that in interphase extracts. We have previously shown that human ECT2 is phosphorylated in a G2/M specific manner [Tatsumoto et al., 1999]. To test whether XECT2 is also phosphorylated in M phase, we treated the XECT2 immunoprecipitates with the dual-specificity phosphatase VHR. As shown in Figure 1D, VHR treatment converted the mitotic form of XECT2 to a form with a similar mobility of interphase XECT2. These results strongly suggest that XECT2 is phosphorylated specifically in M phase extracts.

To examine the role of XECT2 during mitosis, we reconstituted in vitro anaphase using *Xenopus* egg extracts (Fig. 2A). When buffer alone or non-immune rabbit IgG was added to the extract as control at the beginning of the assay, typical bipolar spindles with the chromosomes aligned at the metaphase plate were observed in 80% of all spindles counted. In contrast, when affinity-purified anti-XECT2 antibody

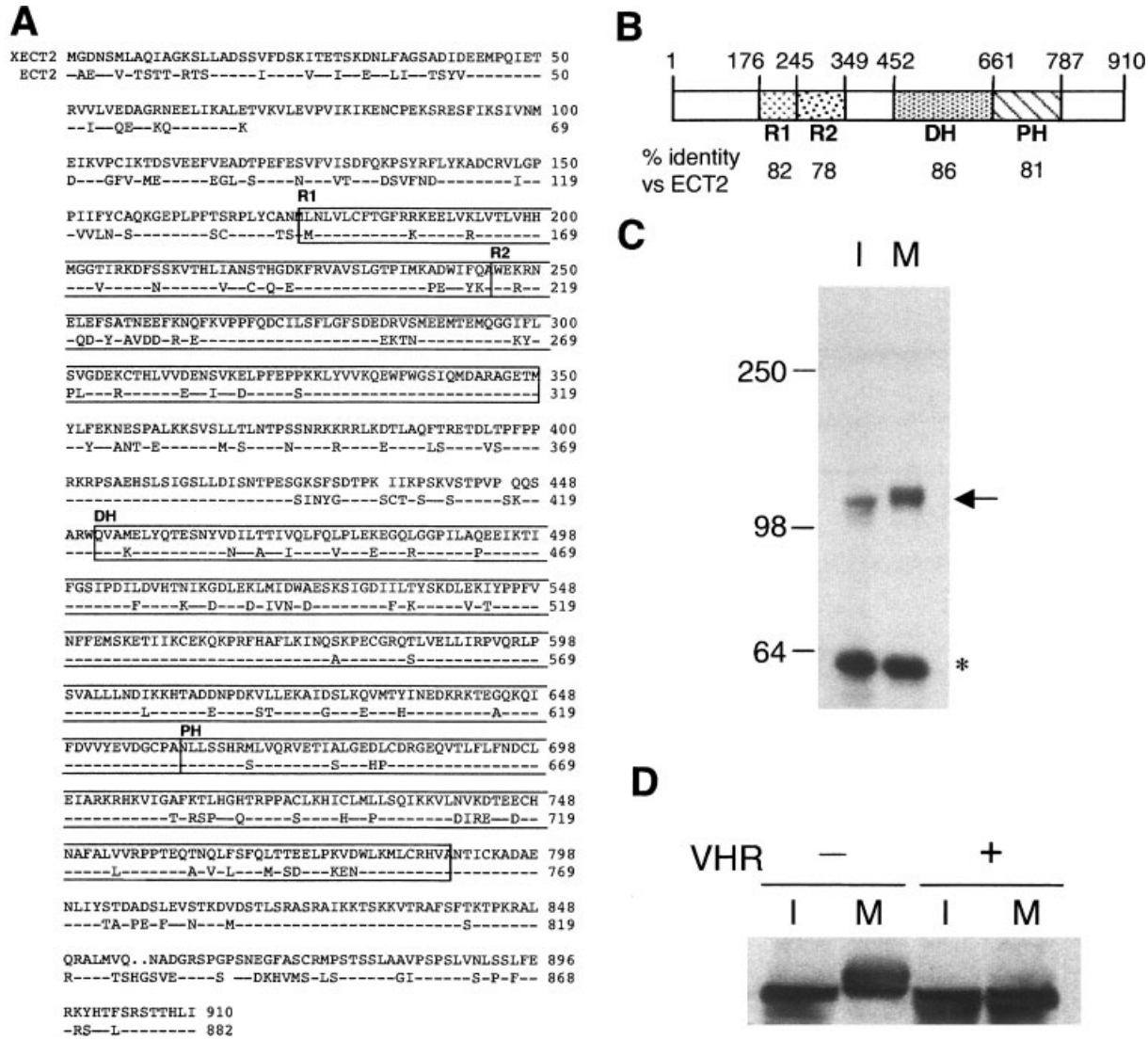

Fig. 1. Molecular cloning of *Xenopus* ECT2. **A**: An alignment of *Xenopus* ECT2 (XECT2) and human ECT2 protein sequences. cDNA sequence of *XECT2* was compiled from four overlapping cDNA clones. The human sequence is shown only where the amino acid differs from the *Xenopus* sequence. Identical residues are indicated by dashes. XECT2 contains an insertion of 31 amino acids (amino acids, 70–100) relative to ECT2. Following domains are boxed; R1, R2: BRCT repeats, Dbl homology (DH) domain, pleckstrin homology (PH) domain. **B**: Schematic representation of XECT2 protein predicted from the cDNA sequence. The percent identity of *Xenopus* and human ECT2 in each domain is shown. **C**: Identification of XECT2 protein. XECT2 was immunoprecipitated from mitotic (M) or interphase (I) extracts with anti-XECT2 antibody, resolved by 6% SDS–PAGE, and probed with anti-XECT2 antibody. An arrow and an asterisk indicate XECT2 and rabbit immunoglobulin heavy chain, respectively. It should be noted that the mobility of XECT2 in mitotic extract is slower than interphase extract. **D**: Phosphorylation of XECT2 in mitotic extracts. Mitotic (M) and interphase (I) extracts were incubated with (+) or without (−) VHR protein phosphatase, separated by SDS–PAGE, and analyzed for XECT2.

was added to the extracts, spindle assembly was drastically inhibited, in which approximately 90% of the spindles exhibited abnormal mitotic figures. These aberrant structures were apparent and became visible as early as the time point that corresponded to metaphase in control extracts. We frequently observed bipolar spindles with strongly reduced density of microtubules in the equatorial region. In this case, the chromosomes were attached to microtubules, however they did not align at the metaphase plate (Fig. 2A panel b). We also observed monopolar and multipolar structures. Monopolar structures consisted of microtubules radiated from single asters (Fig. 2A panel d), whereas multipolar structures contained microtubule arrays radiated from several asters, which were overlapped at the center without aligned

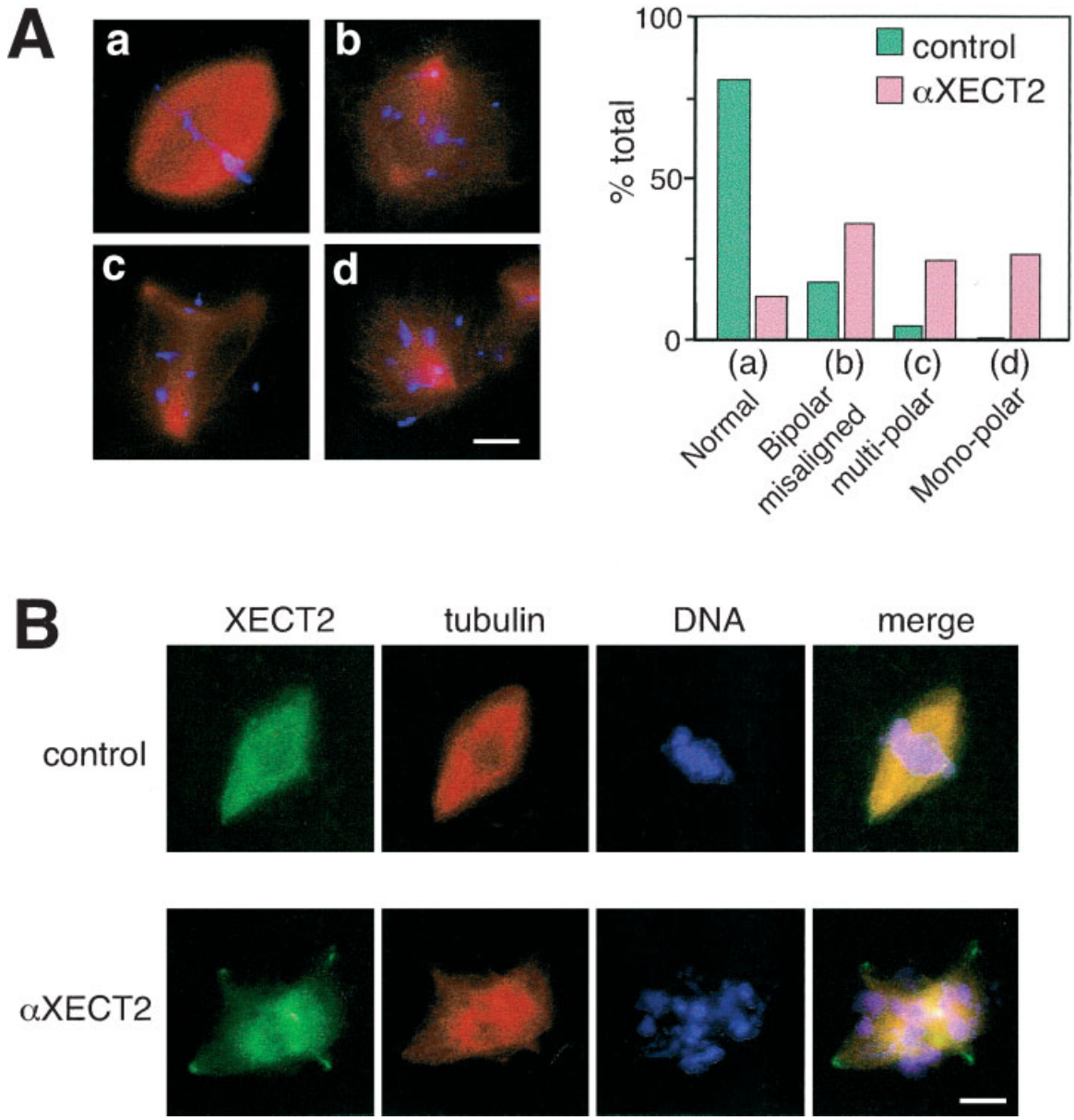

Fig. 2. Effects of anti-XECT2 antibody on spindle assembly in *Xenopus* egg extracts. **A**: Effects of affinity-purified anti-XECT2 on in vitro spindle assembly. **Left panel**: Representative structures of the mitotic spindles formed in the presence of rabbit IgG (**a**), or in the presence of anti-XECT2 antibody (**b–d**). Bipolar spindles with reduced density of microtubules in the equatorial region (b). Multipolar spindles (c). Monopolar spindles (d). Tubulin and chromosomes are seen in red and blue, respectively. Extracts containing sperm nuclei and antibody were cycled through interphase and arrested at the following metaphase. Bar, 1 μm. **Right panel**: Quantitation of structures formed in extracts containing 50 μg/ml nonimmune rabbit IgG (n = 159) or extracts containing 50 μg/ml anti-XECT2 antibody (n = 176). Spindle structures were scored 90 min after exit from interphase. Normal, bipolar spindles with chromosomes aligned at the metaphase plate; bipolar-misalign, bipolar spindles of a lower microtubule density with mis-aligned chromosomes; multi-polar, multipolar spindles; mono-polar, monopolar spindles. Data were accumulated from two independent experiments. **B**: Localization of XECT2 on mitotic spindles assembled in vitro. **Upper panels**: Mitotic spindles assembled in the presence of control IgG. Anti-XECT2 staining (green), rhodamine-tubulin (red), DAPI-stained chromatin (blue), and merged images are shown. **Lower panels**: Mitotic spindles assembled in the presence of anti-XECT2. Distribution of added anti-XECT2 antibody on assembled spindles is revealed by FITC-conjugated secondary antibody (green). Colocalization of ECT2 and tubulin generates yellow signals. Bar, 1 μm.

chromosomes (Fig. 2A panel c). These results suggest that XECT2 plays an important role in bipolar spindle formation.

We previously showed that ECT2 co-localizes to the mitotic spindle in HeLa cells, although it disperses over the entire cells after nuclear

membrane breakdown. To test whether XECT2 is tightly associated with the mitotic spindle in *Xenopus* egg extracts, we isolated the assembled spindles from control extracts and examined the localization of XECT2 using anti-XECT2. As shown in Figure 2B upper row panels, XECT2 co-localized with spindle microtubules assembled in vitro, suggesting that XECT2 is tightly associated with mitotic spindles. When anti-XECT2 was added to the extracts at the beginning of the in vitro anaphase reaction, abnormal spindle assembly was observed as described before. In this case, however, the added anti-XECT2 antibody was detected on microtubules with enrichment at spindle poles (Fig. 2B lower panels). This may suggest that XECT2 initially localized at spindle poles and the addition of anti-XECT2 to mitotic extracts inhibited the translocation of XECT2 along with microtubules, which might lead to the failure of normal bipolar spindle formation.

To further examine the possible involvement of XECT2 on spindle assembly, we used a dominant-negative form of XECT2. We previously found that the N-terminal half of human ECT2 (ECT2-N) efficiently inhibits cytokinesis in HeLa cells [Tatsumoto et al., 1999]. We expressed the N-terminal domain of XECT2 (XECT2-N), which corresponded to ECT2-N, as a maltose binding protein (MBP)-fused protein in *E. coli* and purified through an affinity column. As shown in Figure 3, MBP-XECT2-N, but not MBP alone, efficiently inhibited normal spindle assembly in *Xenopus* egg cell-free extracts. Similar to anti-XECT2, XECT2-N efficiently induced spindles with strongly reduced density of microtubules in the equatorial region. However, monopolar and multipolar spindles were not frequently observed. Although we observed approximately 10% of the spindles synthesized in the presence of XECT2-N appeared to lack apparent spindle poles (Fig. 3, unfocused), MBP alone also exhibited similar phenotype. These results suggest that XECT2 plays a critical role in spindle assembly.

Since ECT2 can catalyze guanine nucleotide exchange on RhoA, Rac1, and Cdc42 in vitro [Tatsumoto et al., 1999], we reasoned that ECT2 regulates spindle assembly through the activation of Rho GTPases. To test whether Rho

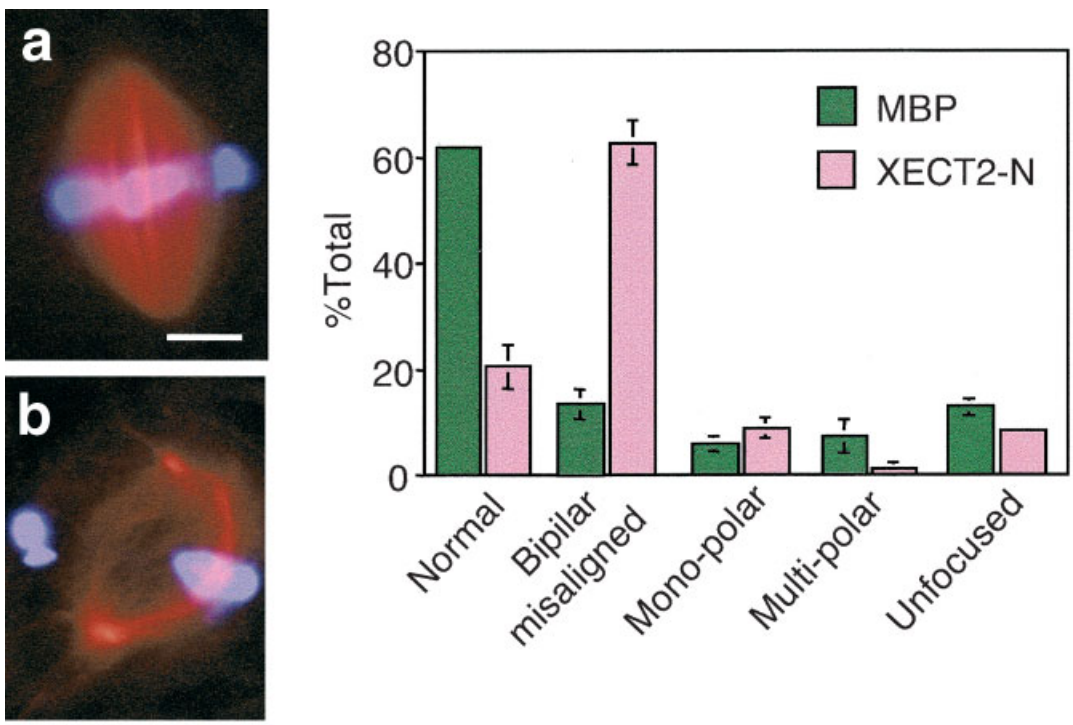

Fig. 3. Effects of XECT2-N on spindle assembly in *Xenopus* egg extracts. **Left panel**: Representative structures of the mitotic spindle formed in the presence of MBP alone (**a**), or MBP-XECT2-N at 1 μM (**b**). Bar, 1 μm. **Right panel**: Quantitation of structures formed in extracts containing maltose binding protein (MBP) alone or MBP-XECT2 fusion protein. Phenotypes are scored as in the legend to Figure 2. Unfocused, spindles lacking apparent spindle poles.

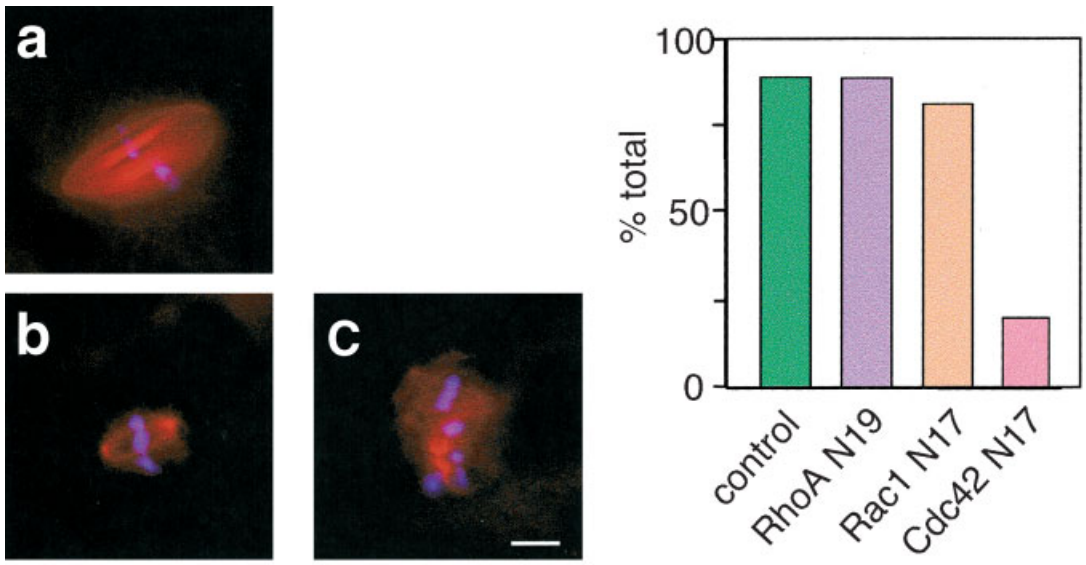

Fig. 4. Effects of dominant negative Rho GTPases on spindle assembly in *Xenopus* egg extracts. **Left panel**: Representative spindle morphologies obtained in extracts in the absence (**a**) or in the presence (**b–c**) of the dominant negative Cdc42. Bar, 1 μm. **Right panel**: Quantitation of structures formed in extracts containing 50 μg/ml each of GST (control), dominant negative RhoA (RhoA N19), Rac1 (Rac1 N17), or Cdc42 (Cdc42 N17). Phenotypes were scored 90 min after exit from interphase. Data were accumulated from two independent experiments.

GTPases are involved in spindle assembly, we utilized dominant negative mutants of the representative Rho GTPases. We observed that dominant negative Cdc42 (Cdc42 N17) markedly inhibited spindle assembly, whereas dominant negative RhoA (N19) or Rac1 (N17) as well as GST protein alone had little or no effect (Fig. 4). Although spindle assembly was potently inhibited in the presence of Cdc42 N17, bipolar spindle structures were still formed at a low frequency. In this case, however, the density of microtubules were markedly reduced and the spindle poles did not fully extend outward from chromosome mass (Fig. 4, compare panels a and b). In less frequent cases, collapsed microtubule arrays lacking poles were observed around chromosomes (Fig. 4 panel c). These results suggest that Cdc42 plays a critical role in spindle assembly in *Xenopus* egg cell extracts.

DISCUSSION

We have cloned the *Xenopus* homologue of ECT2, and found that its overall structure is very similar to human and mouse ECT2. Affinity-purified anti-XECT2 antibody strongly inhibited normal bipolar spindle assembly in *Xenopus* egg extracts. Most of the spindles formed in the presence of anti-XECT2 exhibited abnormal structures, including monopolar and multipolar spindles with scattered chromosomes. Even when bipolar spindles were formed, the microtubule density in the spindles, in particular in the equatorial region, was much lower than that found in bipolar spindles in control extracts. Thus, it appears that anti-XECT2 neutralized the ECT2 function and resulted in reduced spindle assembly. Alternatively, anti-XECT2 inactivated the XECT2 complex containing a factor(s) important for spindle assembly. As our anti-XECT2 did not efficiently deplete XECT2 from the extracts, we could not exclude the latter possibility.

We also found that XECT2-N efficiently inhibited normal spindle assembly in *Xenopus* cell-free extracts. These results suggest that XECT2 is a critical factor for spindle assembly in vitro. As XECT2-N lacks the catalytic domain, XECT2-N presumably associated with a site where XECT2 regulates spindle assembly, and thus prevented endogenous XECT2 to catalyze the reaction at the site. This phenotype was very similar to that observed by anti-XECT2 except for the absence of mono- and multi-polar spindles. The reason for this difference is not clear. A possible explanation is that anti-XECT2 can neutralize the function of

XECT2 at spindle poles, whereas XECT2-N cannot inhibit the function at this site.

Most of the spindles formed in the presence of anti-XECT2 or XECT2-N exhibited much lower microtubule density than those in control extracts. One possible explanation for these aberrant mitotic figures is that XECT2 may be required for the formation of the spindle midzone. In *Xenopus* egg extracts, bipolarity appears to come from interactions between antiparallel microtubules in the spindle midzone [Sawin and Endow, 1993], where plus-end motors cross-link antiparallel microtubules, eventually driving into two poles. Rho GTPases have been shown to interact with motor proteins, such as kinectin, a protein implicated in anchoring organelles to kinesin, and cytoplasmic dynein [Hotta et al., 1996]. XECT2 may regulate the midzone spindle through the stabilization of microtubules or interaction with motor proteins.

We have previously shown that microinjection of anti-ECT2 or expression of ECT2-N inhibits cytokinesis of HeLa or U2OS cells [Tatsumoto et al., 1999]. In these experiments, the occurrence of aberrant mitosis was not significantly higher than controls. It is possible that the control of spindle assembly by XECT2 is a *Xenopus* specific event. Alternatively, microinjection of anti-ECT2 or expression of ECT2-N might not be effective enough to inhibit spindle assembly in mammalian cells. In contrast to in vivo systems, higher concentrations of similar inhibitors could be used to efficiently inhibit normal spindle assembly in cell-free systems.

We demonstrated that dominant negative Cdc42 efficiently inhibited spindle formation in *Xenopus* egg extracts. Consistent with our findings, inactivation or activation of Cdc42 in mammalian cells also induces abnormal mitotic spindles (Yasuda and Narumiya, personal communication). It has also been reported that Cdc42 regulates the formation of the mitotic spindle organization center through the activation of the polarity determinant Par6 [Etienne-Manneville and Hall, 2001]. A constitutively active PAK, a Cdc42 effector protein kinase, induces abnormal mitotic spindles [Vadlamudi et al., 2000]. Cyk-4, a GAP for Cdc42 and Rac1, is also known to regulate central spindle formation [Jantsch-Plunger et al., 2000]. Therefore, it is possible that ECT2 controls spindle assembly through the regulation of Cdc42. Alternatively, Cdc42 may be regulated by a different exchange factor and XECT2 may regulate other GTPase(s) to control spindle assembly. There is evidence that microtubule dynamics is associated with Rho-mediated signal transduction pathways: Rac1 binds tubulin in vitro [Best et al., 1996]. Growth of microtubules is associated with an increase in cellular GTP-bound Rac1 [Waterman-Storer and Salmon, 1999]. RhoG localizes on microtubules and activates both Rac1 and Cdc42 in a microtubule dependent manner [Gauthier-Rouviere et al., 1998]. RhoA induces to stabilize a subset of microtubules that align with the direction of cell migration [Cook et al., 1998]. The Rho effector mDia regulates bundling of microtubules [Ishizaki et al., 2001; Palazzo et al., 2001]. XECT2 may function to induce spindle formation through these or unidentified Rho GTPases.

We have shown that inhibition of XECT2 or Cdc42 perturbs normal spindle assembly in *Xenopus* egg cell-free extracts. Our results suggest novel roles of XECT2 and Cdc42 in mitosis. Further studies on the role of Cdc42 and XECT2/ECT2 in the *Xenopus* cell-free system as well as in mammalian cells will clarify the molecular mechanisms of spindle assembly.

ACKNOWLEDGMENTS

We thank Dr. J.H. Pierce and Dr. D.R. Lowy for support; Dr. K. Fukazawa and Dr. G.F. Vande Woude for valuable suggestions; Dr. O. Peterson and Dr. D.P. Bottaro for materials. We also thank Dr. Shuh Narumiya for communicating their results prior to publication.

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