

# Human ECT2 Is an Exchange Factor for Rho GTPases, Phosphorylated in G2/M Phases, and Involved in Cytokinesis

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**Abstract.** Animal cells divide into two daughter cells by the formation of an actomyosin-based contractile ring through a process called cytokinesis. Although many of the structural elements of cytokinesis have been identified, little is known about the signaling pathways and molecular mechanisms underlying this process. Here we show that the human *ECT2* is involved in the regulation of cytokinesis. *ECT2* catalyzes guanine nucleotide exchange on the small GTPases, RhoA, Rac1, and Cdc42. *ECT2* is phosphorylated during G2 and M phases, and phosphorylation is required for its exchange activity. Unlike other known guanine nucleotide exchange factors for Rho GTPases, *ECT2* exhibits nuclear localization in interphase, spreads through-

out the cytoplasm in prometaphase, and is condensed in the midbody during cytokinesis. Expression of an *ECT2* derivative, containing the NH<sub>2</sub>-terminal domain required for the midbody localization but lacking the COOH-terminal catalytic domain, strongly inhibits cytokinesis. Moreover, microinjection of affinity-purified anti-*ECT2* antibody into interphase cells also inhibits cytokinesis. These results suggest that *ECT2* is an important link between the cell cycle machinery and Rho signaling pathways involved in the regulation of cell division.

**Key words:** cell division • phosphorylation • nucleotide exchange • oncogene • microinjection

CELLULAR division into two daughter cells occurs through nuclear division and cytokinesis. In cytokinesis, formation of an actomyosin-based contractile ring separates the cytoplasm to two daughter cells. A small GTPase RhoA appears to be localized in the cleavage furrow during cytokinesis (Takaishi et al., 1995). In lower eukaryotes, such as sand dollar, *Dictyostelium*, and *Xenopus*, involvement of Rho GTPases in cell division has been reported (Kishi et al., 1993; Mabuchi et al., 1993; Larochelle, 1996; Drechsel et al., 1997). In *Saccharomyces cerevisiae*, cell division is achieved by budding, which is controlled by Cdc42 (Chant and Pringle, 1991). In contrast, in mammalian cells, where Rho GTPases also regulate cell surface cytoskeletal organization, the role of Rho GTPases in cell division is not well-documented.

The Rho family of small GTPases, represented by RhoA, Rac1, and Cdc42, act as molecular switches of diverse biological functions involving cytoplasmic actin remodeling (Hall, 1998). The GTP-bound form of Rho proteins is active, whereas the GDP-bound form is inactive. Activation of the Rho proteins is promoted by guanine nu-

cleotide exchange factors (GEFs),<sup>1</sup> which catalyze the replacement of bound guanosine diphosphate (GDP) by GTP. The GTP-bound form of Rho proteins can specifically interact with their effectors or targets and transmit signals to downstream molecules. Rho proteins are inactivated through the hydrolysis of bound GTP to GDP by intrinsic GTPase activity, assisted by GTPase activating proteins (GAPs). In fibroblasts, RhoA, Rac1, and Cdc42 regulate formation of actin stress fibers, membrane ruffling, and filopodia, respectively (Hall, 1998). The upstream stimuli that regulate these GTPases are largely unknown, although lysophosphatidic acid, PDGF, and Ras have been shown to regulate the cytoskeletal changes controlled by these GTPases (Hall, 1998).

We previously isolated the *ect2* oncogene in a search for mitogenic signal transducers in epithelial cells, where a murine keratinocyte expression cDNA library was introduced into fibroblasts to induce foci of morphologically transformed cells (Miki et al., 1991). Removal of the NH<sub>2</sub>-terminal half of *Ect2* strongly activates the transforming activity of *ect2*, and injection of *ect2* transfectants into nude mice efficiently induces tumor formation. The cen-

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1. **Abbreviations used in this paper:** BRCT, BRCA1 COOH-terminal repeat; DAPI, 4',6-diamidino-2'-phenylindole; DH, Dbl homology domain; ECT2-C, ECT2 COOH-terminal half; ECT2-F, full-length ECT2; ECT2-N, ECT2 NH<sub>2</sub>-terminal half; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein.

tral domain of Ect2 has sequence similarity with the *dbl* oncogene (Miki et al., 1993), which has been shown to catalyze guanine nucleotide exchange on the Rho family of small GTPases (Hart et al., 1991). Ect2 associates with a subset of the Rho family proteins: RhoA, RhoC, and Rac1 (Miki et al., 1993).

In this study, we show that human ECT2 catalyzes guanine nucleotide exchange on Rho proteins. ECT2 is phosphorylated in a G2/M phase-specific manner, and phosphorylation is required for the exchange activity of ECT2. In interphase cells, ECT2 is mainly localized in the nucleus. However, in mitotic cells, ECT2 is localized predominantly in the midzone, where the formation of cleavage furrow starts. We found that the inhibition of ECT2 by expression of a dominant negative mutant or microinjection of anti-ECT2 antibody specifically blocks the completion of cytokinesis, resulting in multinucleated cells.

## Materials and Methods

### Guanine Nucleotide Exchange Assays

The open reading frame of human ECT2 was introduced into the mammalian expression vector pCEV32F3 (Lorenzi, et al., 1999) to express a FLAG-ECT2 fusion protein. COS-7 cells were plated in 100-mm dishes and transfected with 10  $\mu$ g of plasmid DNA with Lipofectamine (GIBCO BRL). Transfected cells were cultured for 48 h, harvested, and lysed in 1 ml of cold lysis buffer (25 mM Hepes, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 20  $\mu$ g/ml each of leupeptin and aprotinin, and 100  $\mu$ g/ml PMSF). FLAG-ECT2 fusion proteins were immunoprecipitated from the lysates with 9  $\mu$ g/ml of anti-FLAG mAb (Sigma Chemical Co.) and protein G-Sepharose beads (Amersham Pharmacia Biotech). Guanine nucleotide exchange assays were performed essentially as described (Horii et al., 1994) using these immunoprecipitates. In brief, 3  $\mu$ g of GDP-loaded recombinant GTPases were incubated with 5  $\mu$ M [<sup>35</sup>S]GTP $\gamma$ S (0.25 mCi mmol<sup>-1</sup>) and 10  $\mu$ l of protein G beads suspension in 190  $\mu$ l of exchange buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.5 mM DTT, 100 mM NaCl, 0.5 mg/ml BSA). At the indicated times, 30  $\mu$ l of the reaction was removed and passed through nitrocellulose filters. Filters were washed and then counted in a liquid scintillation counter. For phosphatase treatment, immunoprecipitates were incubated with recombinant VHR protein (Ishibashi et al., 1992) or  $\lambda$  phosphatase (New England Biolabs) for 30 min at 30°C, and then used for exchange assays.

### Preparation of Anti-ECT2 Antibodies

GST-Ect2 fusion protein was expressed in *Escherichia coli* and used for immunizing rabbits. The NH<sub>2</sub>-terminal half (ECT2-N; amino acids 1–421) or Dbl homology domain (DH; amino acids 414–639) of human ECT2 was expressed as fusion proteins with thioredoxine and oligohistidine using the pET-32 vector (Novagen). Anti-ECT2 antibodies were prepared by passing antiserum through affinity columns coupled with the corresponding human ECT2 proteins using AminoLink Plus Immobilization Kit (Pierce). Anti-ECT2-DH recognized a single ECT2 protein of ~100 kD. Anti-ECT2-N also recognized the endogenous ECT2 protein, although some additional bands were weakly detected.

### Analysis of ECT2 Modification during Cell Cycle Progression

HeLa cells were grown in DMEM (GIBCO BRL) supplemented with 10% FCS in 7% CO<sub>2</sub> at 37°C. Cells were synchronized at the G1/S boundary by a thymidine-aphidicolin double block (Golsteyn et al., 1995). In brief, cells were incubated with 2 mM thymidine for 14 h, released from arrest, and then arrested at G1/S again with aphidicolin (1  $\mu$ g/ml) (Sigma Chemical Co.). Cells were then placed under normal growth conditions (time 0). After 6 h, nocodazole (final concentration 100 ng/ml) was added to arrest the cells at prometaphase. Mitotic cells were collected by mechanical shake-off and replated in normal growth medium. Following

trypsinization, samples were analyzed by flow cytometry using FACS® II instrument (Becton Dickinson). For phosphatase treatment, G1 or M phase arrested cells were lysed in TNE buffer (10 mM Tris-HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA pH 7.0, 10  $\mu$ g/ml aprotinin) and incubated with 1  $\mu$ g/ml of recombinant VHR protein for 30 min at 30°C. Samples were resolved by 6% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting using affinity-purified anti-ECT2 antibodies.

### Subcellular Localization of ECT2

To determine the localization of endogenous ECT2, HeLa cells were grown on coverslips, fixed for 10 min with 3.7% formaldehyde in PBS, permeabilized for 10 min with 0.2% Triton X-100 in PBS, and then incubated with 1% BSA in PBS for 10 min. For costaining of endogenous ECT2 and tubulin, coverslips were incubated with affinity-purified anti-ECT2-DH antibody and anti- $\beta$ -tubulin (TUB2.1; Sigma Chemical Co.) mAbs for 1 h at 4°C. After washing, samples were incubated with FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch, Inc.) for 1 h at room temperature. To express green fluorescent protein (GFP)-fused ECT2 proteins in U2OS cells, ECT2-N, ECT2-C, and ECT2-F were introduced into pEGFP-C1 (Clontech). After transfection, cells were fixed and proteins visualized by green fluorescence using a Zeiss Axiovert microscope equipped with a Photometrics digital camera and IPLab software (Signal Analytics Co.).

### Microinjection

HeLa cells were grown on poly-L-lysine-coated glass coverslips for 18–24 h until the cultures reached ~50% confluency. The coverslips were transferred into fresh medium containing 10 mM Hepes, pH 7.6, before injection. Antibodies were dialyzed and concentrated to 1 mg/ml using a Centricon-30 concentrator (Amicon). Rabbit IgG (Sigma Chemical Co.) was used as a control. Microinjection into the cytoplasm was performed using Eppendorf semiautomatic microinjection apparatus. Immediately after injection, coverslips were washed with PBS and incubated in growth medium for the indicated time period.

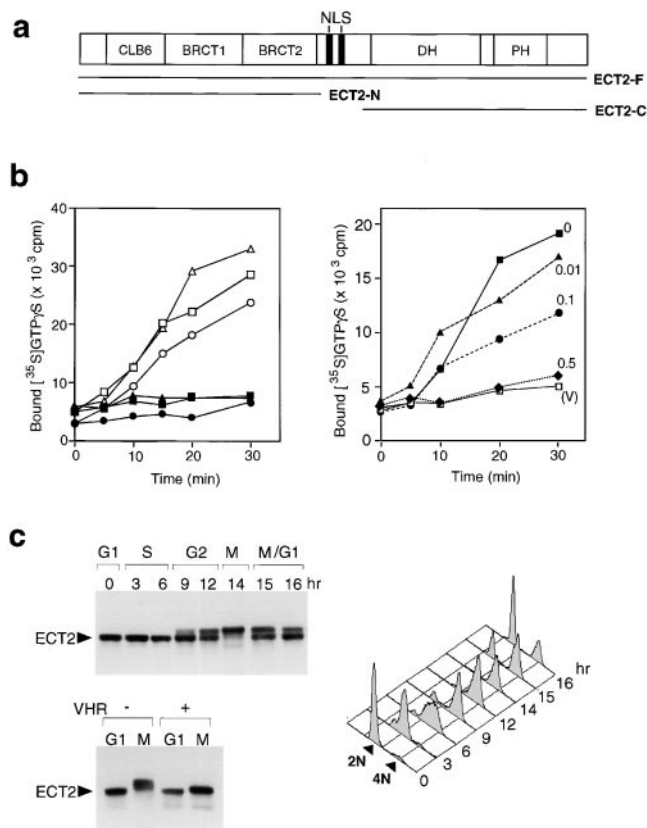
## Results

### ECT2 Is a GEF for RhoA, Rac1, and Cdc42

To examine the function of ECT2 as a Rho GEF, we expressed ECT2 as a FLAG epitope-tagged protein in COS cells immunoprecipitated with anti-FLAG mAb, and used immunoprecipitated protein for exchange assays. Full-length ECT2 efficiently stimulated nucleotide exchange on three representative members of the Rho GTPases, RhoA, Rac1, and Cdc42 in vitro, whereas similar immunoprecipitates from vector transfectants showed no significant activity (Fig. 1 b, left panel). In contrast to the activity on Rho GTPases, ECT2 did not exhibit significant exchange activity on two Ras family GTPases, H-Ras and Rap1A (data not shown).

### ECT2 Is Phosphorylated Specifically in G2 and M Phases of the Cell Cycle

The predicted ECT2 protein also contains a novel domain homologous to yeast S phase cyclin Clb6 (Schwob and Nasmyth, 1993; Stuart and Wittenberg, 1998), and two tandem repeats of the recently identified BRCA1 COOH-terminal repeat (BRCT) motif (Fig. 1 a) that is widespread throughout checkpoint and repair proteins (Saka et al., 1994; Bork et al., 1997; Zhang et al., 1998). Since these structural similarities suggest the possibility that ECT2 is involved in cell cycle control, we examined in detail the expression pattern of ECT2 during the cell cycle. HeLa cells were synchronized at the G1/S boundary, and the expres-



**Figure 1.** (a) Schematic representation of the human ECT2 protein. The human ECT2 cDNA clone was isolated from a B5/589 human epithelial cell cDNA library. The detailed structure will be published elsewhere. CLB6, a domain homologous to a yeast S phase cyclin CLB6. BRCT1 and BRCT2, BRCA1 COOH-terminal repeats. DH, Dbl-homology domain. PH, pleckstrin-homology domain. NLS, nuclear localization signals. The regions carried by ECT2-F, ECT2-N, and ECT2-C are shown. (b) Guanine nucleotide exchange activity of ECT2 on Rho GTPases. (Left panel) Exchange activity of immunoprecipitates from FLAG-ECT2-expressing cells (open symbols) or FLAG-expressing cells (filled symbols) on RhoA (circles), Rac1 (squares), or Cdc42 (triangles). Shown are representative results of at least three independent experiments. (Right panel) FLAG-ECT2 immunoprecipitates were treated with the indicated concentrations ( $\mu\text{g/ml}$ ) of VHR at  $30^\circ\text{C}$  for 1 h and then subjected to exchange assays on Rac1. V, immunoprecipitates from vector alone-transfectants. VHR-treated ECT2 immunoprecipitates did not show apparent degradation. (c) G2/M-specific phosphorylation of ECT2. HeLa cells were synchronized at G1/S boundary by thymidine/aphidicolin double block. ECT2 protein was detected with anti-ECT2 antibody in the cells as they progress through the cell cycle upon release from the drug arrest (upper left panel). Lysates of cells arrested at G1 phase by aphidicolin or at M phase by nocodazole were incubated with a protein phosphatase VHR, separated by SDS-PAGE, and analyzed for ECT2 (lower left panel). DNA contents of the cells in the above samples were analyzed by flow cytometry following the release from the G1 arrest (right panel). Positions of cells in G1 phase (2N) and G2/M phase (4N) are shown by arrowheads.

sion of ECT2 protein was examined by immunoblotting after the release from G1 arrest. Although we did not observe significant changes in the overall expression level of ECT2 protein during progression of the cell cycle, a shift

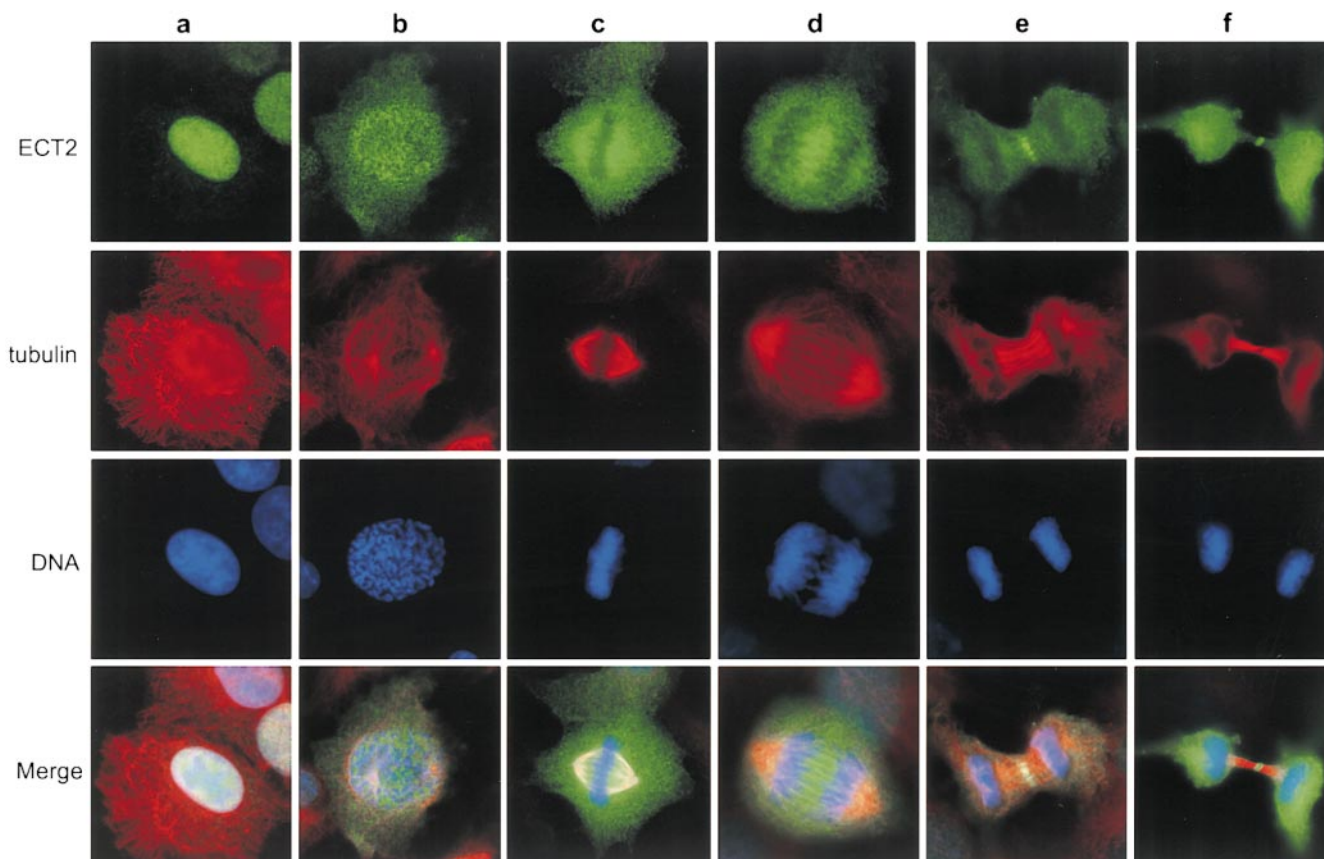
of the mobility of ECT2 was detected by anti-ECT2 antibody (Fig. 1 c, left panel). A slowly migrating protein appeared at 9 h as cells entered G2 phase. At 14 h, as cells entered M phase, the slowly migrating band predominated. The fraction of slowly migrating protein gradually decreased as cells entered G1 phase (Fig. 1 c). To determine whether the shift of mobility of ECT2 was due to protein phosphorylation, we treated M phase-arrested cells with the tyrosine/serine/threonine phosphatase VHR. No mobility shift was detected in M phase cells after VHR treatment (Fig. 1 c, left panel). Therefore, ECT2 appeared to be phosphorylated specifically at G2 phase, and this state was manifested through M phase.

### Phosphorylation Is Required for the Exchange Activity of ECT2

To further examine the effect of phosphorylation on the exchange activity of ECT2, we treated FLAG-ECT2 immunoprecipitates with various concentrations of VHR. As shown in the right panel of Fig. 1 b, the exchange activity of ECT2 was inhibited by VHR in a dose-dependent manner, and the incubation for 1 h with  $0.5 \mu\text{g/ml}$  of VHR decreased the activity of FLAG-ECT2 to background levels. Inhibition of exchange activity was also observed by treatment of FLAG-ECT2 with  $\lambda$  phosphatase, and this inhibitory effect was strongly prevented by a phosphatase inhibitor vanadate (data not shown). These results suggest that phosphorylation is required for the exchange activity of ECT2 in vitro.

### ECT2 Colocalizes with the Mitotic Spindle and Midbody in M Phase

Next, we examined the subcellular localization of endogenous ECT2 during cell cycle progression by immunofluorescent analysis of HeLa cells. Interestingly, in interphase cells ECT2 was located predominantly in the nucleus, where no expression of Rho family proteins has been reported (Fig. 2 a). After nuclear membrane breakdown in prometaphase, ECT2 spread into the cytoplasm (Fig. 2 b). During metaphase, ECT2 accumulated in the regions where the mitotic spindle is present (Fig. 2 c). Costaining of ECT2 and tubulin generated the yellow colocalization signal. As cells entered anaphase, ECT2 appeared to concentrate in the central region of the spindle (Fig. 2 d). In late anaphase and telophase, ECT2 was mainly located in the midzone, where the cleavage furrow is formed (Fig. 2 e). During cytokinesis, ECT2 accumulated at the midbody, a region in the middle of the bridge that connects the two daughter cells (Fig. 2 f). When the cells exited mitosis and the nuclear envelope reassembled, ECT2 translocated to the nucleus again (data not shown). To locate the region of ECT2 responsible for midbody localization, we transfected cells with plasmids containing the NH<sub>2</sub>-terminal half (ECT2-N), the COOH-terminal half (ECT2-C), or full-length (ECT2-F) ECT2 as GFP fusion proteins. GFP proteins were visualized 72 h after transfection (Fig. 3 A). Like endogenous ECT2, GFP-tagged ECT2-F was detected in the midbody of dividing cells. Although ECT2-N localized in the cytoplasm of interphase cells (data not shown), it accumulated in the midbody of dividing cells. In contrast, ECT2-C was detected in the cytoplasm of mitotic



**Figure 2.** Subcellular localization of ECT2 in interphase and mitotic cells. Endogenous ECT2 in HeLa cells was detected by affinity-purified anti-ECT2 antibodies (green). Tubulin (red) and DNA (blue) were also stained with anti- $\beta$ -tubulin antibody and 4',6-diamidino-2'-phenylindole (DAPI), respectively. Merged images are also shown at the bottom, where colocalization of ECT2 and tubulin results in yellow color. a, interphase; b, prometaphase; c, metaphase; d, anaphase; e, telophase; f, cytokinesis.

cells. These observations indicate that the NH<sub>2</sub>-terminal half of ECT2 is required for midbody localization. The nuclear localization of ECT2-F in interphase cells may be attributed to nuclear localization signals located in the central region of ECT2 (Fig. 1 a).

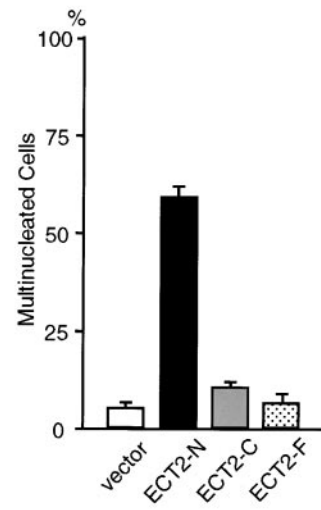
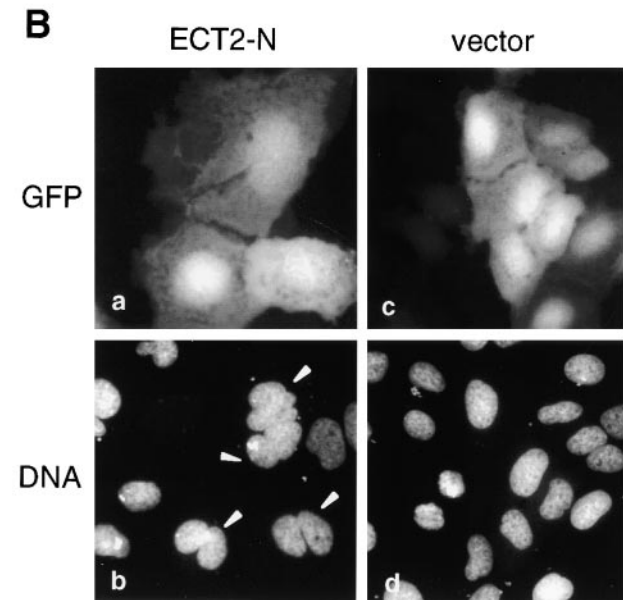
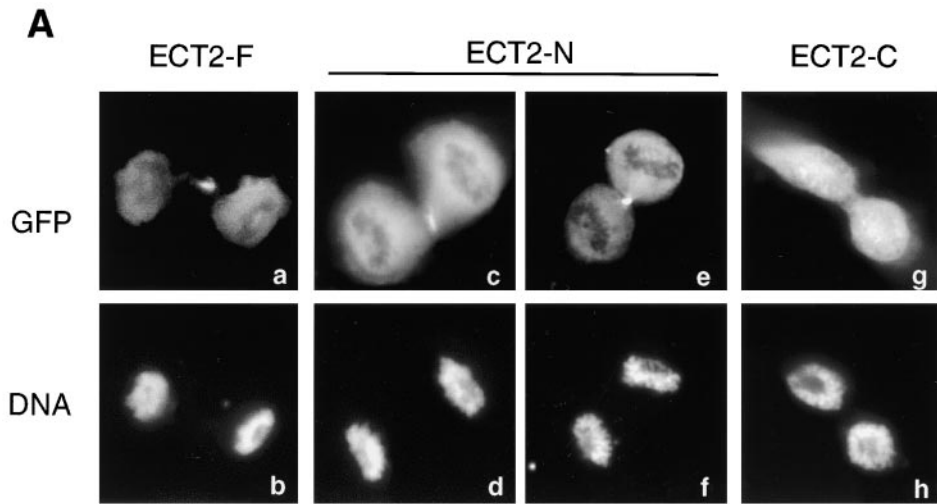
### **Expression of ECT2 NH<sub>2</sub>-Terminal Domain Inhibits Cytokinesis**

The dynamic change of subcellular localization and G<sub>2</sub>/M-specific modification of ECT2 suggest a possible role of ECT2 in cell division. If ECT2 controls cell division through the regulation of Rho GTPases, expression of ECT2 mutants containing the domain that is required for localization, but lacking the catalytic domain, may induce a dominant negative phenotype. Therefore, we closely examined the morphology of cells expressing ECT2-N. Surprisingly, ~60% of cells expressing ECT2-N became multinucleated, with most cells containing two nuclei 72 h after transfection (Fig. 3 B). Division of ECT2-N-expressing cells appeared to proceed to a stage just before the separation of two daughter cells with single nuclei (Fig. 3 A, panels c and e), suggesting that a very last step of cytokinesis was inhibited by ECT2-N expression. In contrast, cells expressing ECT2-F, ECT2-C, or GFP expression vec-

tor alone did not exhibit such phenotypes. Since most of the cells expressing ECT2-N underwent normal nuclear division (Fig. 3 A, panels d and f), it is unlikely that these multinucleated cells were generated as a consequence of aberrant nuclear division. These results suggest that ECT2-N can function as a dominant negative mutant to inhibit cytokinesis.

### **Microinjection of Anti-ECT2 Antibodies Specifically Inhibits Cytokinesis**

To further examine the involvement of ECT2 in cytokinesis, we inhibited ECT2 function by microinjection of affinity-purified anti-ECT2 antibodies into asynchronously growing HeLa cells (Fig. 4). Whereas cells injected with control IgG divided normally, ~60% of cells injected with anti-ECT2-DH, which recognizes the catalytic domain of ECT2, became larger with double nuclei 24 h after injection. Since 25% of the injected cells had not divided at this stage, ~80% of the cells were multinucleated upon completion of mitosis. After 48 h, 29% of anti-ECT2-DH-injected cells contained three or four nuclei. The presence of cells with more than two nuclei may indicate inhibition of multiple rounds of cytokinesis with anti-ECT2-DH. In contrast, very few control IgG-injected cells were multinucleated. To rule out the possibility that anti-ECT2 cross-



**Figure 3.** Effects of a dominant negative mutant of ECT2 on cytokinesis. (A) Subcellular localization of exogenously expressed ECT2 protein. The full-length (ECT2-F; panels a and b), NH<sub>2</sub>-terminal half (ECT2-N; panels c–f), or COOH-terminal half (ECT2-C; panels g and h) of ECT2 was transiently expressed in U2OS cells as a GFP-fused protein. GFP fusion proteins were detected by green fluorescence (panels a, c, e, and g). DNA was stained with DAPI (panels b, d, f, and h). (B) The NH<sub>2</sub>-terminal domain of ECT2 acts as a dominant negative mutant for cytokinesis. Cells were transfected with GFP-fused ECT2-F, ECT2-N, or ECT2-C, or GFP vector alone. GFP-expressing cells are visualized by green fluorescence (panels a and c). DNA was stained with DAPI (panels b and d). Arrowheads indicate multinucleated cells. Bars, 20  $\mu$ m. (Right panel) GFP-expressing multinucleated cells were scored under immunofluorescent microscopy 72 h after transfection. Data are average of three independent experiments.

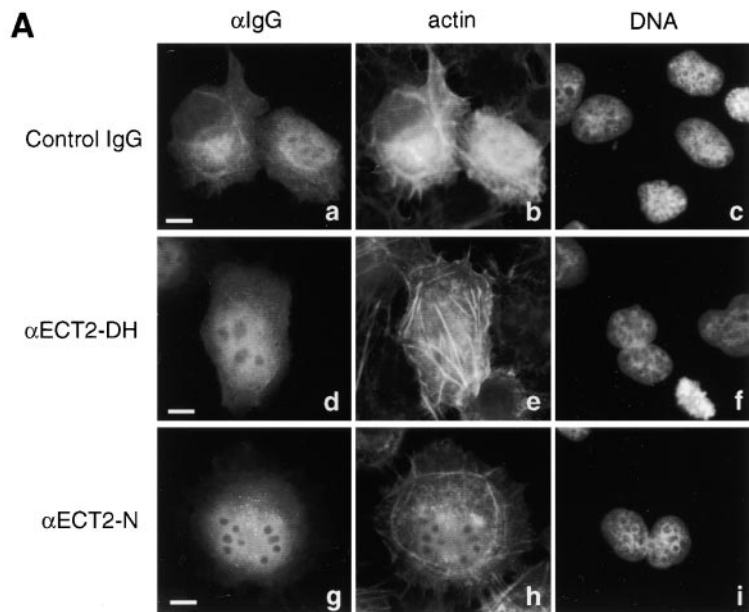
reacted with other molecules that regulate cytokinesis, we prepared a second affinity-purified antibody that specifically recognizes the NH<sub>2</sub>-terminal domain of ECT2. Microinjection of this antibody (anti-ECT2-N) also strongly inhibited cytokinesis (Fig. 3 A, panels g–i; Fig. 3 B). These results strongly suggest that ECT2 plays a critical role in cytokinesis.

### Discussion





The results from this study provide details about the biochemical and biological functions of the *ECT2* protooncogene product. We detected a mobility shift of ECT2 at G<sub>2</sub>/M phases. Treatment of the ECT2 immunoprecipitates with VHR dual specificity phosphatase resulted in shift back of the band to the normal size. This strongly suggests that ECT2 is modified by phosphorylation. We found that

dephosphorylation of ECT2 by phosphatases reduced the exchange activity *in vitro* in a dose-dependent manner. Therefore, ECT2 appears to be activated by phosphorylation, which occurs specifically in M phase. Since we could not detect phosphorylated ECT2 with antiphosphotyrosine antibodies (our unpublished results), ECT2 may be phosphorylated by serine/threonine kinases. Candidate protein kinases that can activate ECT2 may include Cdc2-regulated kinases and Cdc2 itself. The predicted amino acid sequence of ECT2 contains two consensus Cdc2 phosphorylation sites (amino acids 327–330 and 814–817). Further studies on ECT2 phosphorylation, including determination of phosphorylation sites and generation of phosphorylation-deficient mutants, will clarify the activation mechanisms of ECT2.

Rho proteins were found to regulate actin polymerization in studies on cytoskeletal organization of fibroblasts



**B** Effects of Antibody Injection on Cell Division

|   | Control IgG |          | αECT2-DH  |          | αECT2-N   |          |
|---|-------------|----------|-----------|----------|-----------|----------|
|   | 24 hr       | 48 hr    | 24 hr     | 48 hr    | 24 hr     | 48 hr    |
| No. of cells  | 180         | 57       | 277       | 66       | 245       | 63       |
|    | 45 (25%)    | 2 (3%)   | 69 (25%)  | 0 (0%)   | 60 (25%)  | 3 (5%)   |
|   | 135 (75%)   | 55 (97%) | 47 (17%)  | 24 (36%) | 37 (15%)  | 20 (31%) |
|  | 0 (0%)      | 0 (0%)   | 161 (58%) | 23 (35%) | 148 (60%) | 25 (40%) |
|  | 0 (0%)      | 0 (0%)   | 0 (0%)    | 19 (29%) | 0 (0%)    | 15 (24%) |

**Figure 4.** Inhibition of cytokinesis by microinjection of anti-ECT2 antibodies. (A) Affinity-purified anti-ECT2 antibodies specific to the NH<sub>2</sub>-terminal domain (αECT2-N), or the DH domain (αECT2-DH) were microinjected into unsynchronized cultures of HeLa cells. Injected cells were identified by immunostaining with anti-rabbit IgG antibody (panels a, d, and g). Cells were also stained for actin with phalloidine (panels b, e, and h) and for DNA with DAPI (panels c, f, and i) to determine the periphery of the cells and morphology of nuclei, respectively. Panels a–c show the morphology of cells injected with control antibody, where two daughter cells were observed. Microinjection of cells with αECT2-DH (panels d–f) or αECT2-N (panels g–i) resulted in single cells with two nuclei 24 h after injection. Longer incubation (48 h) also resulted in cells with more than three nuclei (data not shown). Bars, 10 μm. (B) Number of single interphase cells, normally divided cells, binucleated cells, and multinucleated cells are shown 24 or 48 h after microinjection of the designated antibodies. The last lane includes tri- and tetranucleated cells.

(Hall, 1998). During cytokinesis in animal cells, an actin-based contractile ring divides the cell into two daughter cells. We found that ECT2 catalyzes nucleotide exchange on Rho proteins and this activity appears to be dependent on G2/M phase-specific phosphorylation. ECT2 was released to the cytoplasm after the nuclear membrane breakdown and then accumulated in the midbody during cytokinesis. Since ECT2-N localized in the midbody, but ECT2-C did not, ECT2-N appears to determine the midbody localization of ECT2. We found that expression of ECT2-N strongly inhibited cytokinesis. Because ECT2-N localizes in the midbody but cannot catalyze nucleotide exchange, it appears to function as a dominant negative mutant. Moreover, microinjection of affinity-purified anti-ECT2 antibody also inhibited cytokinesis. Therefore, ECT2 might play an important role in cytokinesis. The nuclear localization of ECT2 may suggest another role of ECT2 in mitosis. This possibility is under investigation. However, the inhibition of cytokinesis with ECT2-N or anti-ECT2 might not be attributed to the inhibition of mitosis, because ECT2-N, which lacks nuclear localization signals, was expressed in the cytoplasm and anti-ECT2 was injected into the cytoplasm.

The presence of cell cycle regulator-related domains and G2/M phase-specific phosphorylation of ECT2 sug-

gest the regulation of ECT2 activity by cell cycle machinery. Therefore, ECT2 might be an important molecule linking cell cycle machinery to Rho signaling pathways involved in the regulation of cytokinesis. In *Xenopus*, multiple Rho proteins are involved in cytokinesis: whereas local activation of Rho is important for proper constriction of the contractile furrow, Cdc42 plays a role in furrow ingression (Drechsel et al., 1997). In *Drosophila*, cytokinesis is developmentally controlled during embryogenesis and is omitted during the initial nuclear division cycles. It starts in somatic cells with mitosis 14, but is blocked with mutations in the *pebble* gene (Lehner, 1992). Recently, it was reported that *pebble* encodes an ECT2-related protein, which can interact with *Drosophila* Rho1 GTPase (Prokopenko et al., 1999), suggesting that ECT2 is also involved in cytokinesis in *Drosophila*.

In mammalian cells, Rho GTPases also regulate cortical actin remodeling (Hall, 1998). Inhibition of RhoA in normal rat kidney cells revealed that Rho regulates the completion of cytokinesis possibly through modulating the mechanical strength of the cortex (O'Connell et al., 1999). Although the role of Rho in division of mammalian cells is not well-documented, recently it was reported that two Rho effectors, Rho-associated kinase and Citron kinase, play a role in cytokinesis (Goto et al., 1998; Madaule et al.,

1998). Further studies on ECT2, Rho GTPases, and their effectors should clarify the signal transduction pathways involved in the control of cell division.

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