Novel Functions of Ect2 in Polar Lamellipodia Formation and Polarity Maintenance during “Contractile Ring-Independent” Cytokinesis in Adherent Cells

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Some mammalian cells are able to divide via both the classic contractile ring-dependent method (cytokinesis A) and a contractile ring-independent, adhesion-dependent method (cytokinesis B). Cytokinesis A is triggered by RhoA, which, in HeLa cells, is activated by the guanine nucleotide-exchange factor Ect2 localized at the central spindle and equatorial cortex. Here, we show that in HT1080 cells undergoing cytokinesis A, Ect2 does not localize in the equatorial cortex, though RhoA accumulates there. Moreover, Ect2 depletion resulted in only modest multinucleation of HT1080 cells, enabling us to establish cell lines in which Ect2 was constitutively depleted. Thus, RhoA is activated via an Ect2-independent pathway during cytokinesis A in HT1080 cells. During cytokinesis B, Ect2-depleted cells showed narrower accumulation of RhoA at the equatorial cortex, accompanied by compromised pole-to-equator polarity, formation of ectopic lamellipodia in regions where RhoA normally would be distributed, and delayed formation of polar lamellipodia. Furthermore, C3 exoenzyme inhibited equatorial RhoA activation and polar lamellipodia formation. Conversely, expression of dominant active Ect2 in interphase HT1080 cells enhanced RhoA activity and suppressed lamellipodia formation. These results suggest that equatorial Ect2 locally suppresses lamellipodia formation via RhoA activation, which indirectly contributes to restricting lamellipodia formation to polar regions during cytokinesis B.

INTRODUCTION

The final step in the process of cell division is cytokinesis, during which the cellular contents are divided and the two daughter cells are formed. Formation of the furrow that ultimately leads to separation of the two daughter cells is mediated in large part by RhoA (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993; Jantsch-Plunger *et al.*, 2000), one of the Rho-type small GTPases, which also include Rac1 and Cdc42; these mediators regulate actin dynamics during a variety of cellular events (reviewed by Etienne-Manneville and Hall, 2002). In the classic “purse string” model of cytokinesis, actomyosin-dependent contraction of the contractile ring drives cleavage of the equatorial region (reviewed by Glotzer, 2005). RhoA-dependent stimulation of actin polymerization through regulation of formins is crucial to formation of the contractile ring (Sagot *et al.*, 2002; Kovar *et al.*, 2003), while phosphorylation of myosin light chain by Rho-associated kinase (ROCK) leads to myosin activation and contraction of the ring (Amano *et al.*, 1996; Kimura *et al.*, 1996; Piekny and Mains, 2002; Matsumura, 2005).

Rho GTPases are activated by guanine nucleotide exchange factors (GEFs). Among the numerous GEFs that have been identified, Ect2 (Epithelial cell transforming protein 2) has been shown to play a key role in cytokinesis. Ect2 was originally identified as a transforming protein in an expres-

sion-cloning assay (Miki *et al.*, 1993). Its role in cytokinesis was first identified in studies of *Drosophila melanogaster*. The *Drosophila* and *Caenorhabditis elegans* orthologues of Ect2, Pebble and LET-21, respectively, have both been shown to be required for contractile ring formation and cytokinesis (Prokopenko *et al.*, 1999; Morita *et al.*, 2005). Although Ect2 appears to act as a GEF with all three Rho-type small GTPases in vitro, recent studies suggest that RhoA is the primary downstream target of Ect2 in vivo (Kimura *et al.*, 2000; Yuce *et al.*, 2005; Kamijo *et al.*, 2006; Nishimura and Yonemura, 2006; Birkenfeld *et al.*, 2007).

Interestingly, under appropriate conditions in some cell types, cytokinesis proceeds fairly normally without contractile ring activity. For example, when adhering to a substrate, myosin II-null cells of the cellular slime mold *Dictyostelium discoideum* are able to divide by making use of traction forces, which move the daughter cells away from one another (Neujahr *et al.*, 1997; Zang *et al.*, 1997; Nagasaki *et al.*, 2002). This process was named “attachment-assisted mitotic cleavage” (Neujahr *et al.*, 1997) or “cytokinesis B” to distinguish it from “cytokinesis A,” which refers to the adhesion-independent, contractile ring-dependent “classic” cytokinesis (Zang *et al.*, 1997; Nagasaki *et al.*, 2002). On highly adherent substrates, certain types of mammalian cells are also able to divide in an adhesion-dependent, contractile ring-independent manner when the activity of the contractile ring is blocked by the myosin II-specific inhibitor blebbistatin (Kanada *et al.*, 2005). In addition, Burton and Taylor (1997) reported a case of successful division of a fibroblast that was driven by traction forces after regression of the initial equatorial furrow under physiological culture condi-

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tions. This observation can be interpreted to mean that cytokinesis B is activated when cells fail to properly complete cytokinesis A and that mammalian cytokinesis B serves as a backup mechanism. In that case, adherent mammalian cells capable of cytokinesis B must have a mechanism that enables them to make use of cytokinesis A or B to ensure successful cell division.

In the present study, we explored the functions of Ect2 in mammalian cytokinesis A and B using HeLa cells, which rely on cytokinesis A for division, and HT1080 human fibrosarcoma cells, which are able to divide using cytokinesis B when cytokinesis A is inhibited (Kanada *et al.*, 2005). Our findings provide the first direct evidence that Ect2 is dispensable for cytokinesis in certain types of mammalian cells and that RhoA localized at the equatorial cortex is indirectly required for the maintenance of appropriate polar lamellipodia formation in mitotic cells undergoing cytokinesis B.

MATERIALS AND METHODS

Plasmid Construction and RNA Interference

The construct for the dominant active form of human Ect2 (Ect2-DA, amino acids 415-884) was created by PCR from a HT1080 cDNA library using specific oligonucleotides and iProof High-Fidelity DNA polymerase (Bio-Rad, Tokyo, Japan) and was cloned into pEGFP-C3 (Clontech, Tokyo, Japan) in which the EGFP cDNA was replaced with mCherry cDNA. The mCherry expression plasmid (Shaner *et al.*, 2004) was kindly provided by Dr. R. Y. Tsien (University of California, San Diego). All DNA constructs were confirmed by sequencing.

Depletion of Ect2 was achieved using the short hairpin RNA (shRNA) expression vector piMARK (Nagasaki *et al.*, 2007), which mediates expression of both shRNA and the blasticidin resistance (Bsr) protein fused with enhanced green fluorescent protein (EGFP), enabling both rapid elimination of untransfected cells and visual identification of knockdown cells. The targeting sequence, GCAGTTGATGACTTTAGAA, was designed using Dharmacon's design algorithm (Boulder, CO; <http://www.dharmacon.com/sidesign/default.aspx>), which was followed by the loop sequence 5'-ACGTGTGCTGCTGTC-CGT-3' and the sequence complementary to the target sequence. To confirm the depletion of Ect2, cell lysate was analyzed by Western blotting with anti-Ect2 (sc-1005, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution) and anti- β -actin (Clone AC-74, Sigma, Tokyo, Japan; 1:2000 dilution) antibodies. The secondary antibody was horseradish peroxidase-conjugated anti-mouse antibody (474-1806, KPL, Gaithersburg, MD; 1:3000 dilution). Chemiluminescence (Super Signal West Dura Extended Duration Substrate, Pierce, Rockford, IL) was detected using an Las-3000 imager (Fuji-film, Tokyo, Japan).

Cell Culture and Transfection

HT1080 cells (Rasheed *et al.*, 1974) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen, Tokyo, Japan). HeLa S3 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.

Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells transfected with piMARK were maintained in the presence of blasticidin S at a concentration of 10 μ g/ml for HeLa cells and 20 μ g/ml for HT1080 cells.

Observation of Live Cells

To assess cytokinesis B in HT1080 cells, untreated polystyrene dishes (Asahi Techno Glass, Tokyo, Japan) were coated with 0.01% collagen type I solution (IFP 9660, Research Institute for the Functional Peptides, Yamagata, Japan) overnight (Kanada *et al.*, 2005). The S-(–)-enantiomer of blebbistatin (Toronto Research Chemicals, Toronto, ONT, Canada) used to inhibit myosin II, and thus cytokinesis A, was dissolved at 10 mM in DMSO. CT04 (Cytoskeleton, Denver, CO), the cell permeable derivative of C3 exoenzyme, was dissolved at 200 μ g/ml in 50% glycerol. HT1080 cells were incubated in the presence of 5 μ g/ml CT04 for 2 h under the growth conditions before observation.

Cells were maintained at a constant temperature in stage incubators (Onpu-4; Taihei Denki, Kasama, Japan; or MI-IBC, Olympus, Tokyo, Japan) attached to inverted microscopes (IX50 or IX71; Olympus) and observed using a 20 $\times$ objective with or without phase-contrast optics for differential interference contrast. Images were captured using a CCD camera (ORCA-AG or ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan) controlled with the IPLab software (Solution Systems, Funabashi, Japan).

Immunofluorescence

Cells were fixed in either 10% trichloroacetic acid (TCA) for 15 min (for RhoA staining; Yonemura *et al.*, 2004) or 3% formaldehyde, 2% sucrose in phosphate-buffered saline (PBS) for 30 min (for Ect2 staining), washed twice with PBS, and treated with 0.1% Triton X-100 in PBS for 5 min.

To assess cytokinesis B, the hydrophilic surfaces of glass-bottomed dishes (Asahi Techno Glass) were treated with hexamethyldisilazane (Shin-Etsu Chemical, Tokyo, Japan) to increase its hydrophobicity before coating it with collagen as described above. Forty-five minutes after addition of blebbistatin (30 μ M), cells on collagen-coated glass-bottomed dishes were fixed with appropriate reagents.

Cells were stained with either anti-RhoA (sc-418, Santa Cruz Biotechnology; 1:200 dilution) or anti-Ect2 (sc-1005, Santa Cruz Biotechnology; 1:100 dilution) antibody, followed by a mixture of Alexa 546- or 488-conjugated anti-mouse-IgG or anti-rabbit-IgG (Invitrogen) and 1 μ g/ml Hoechst 33258 (Wako Pure Chemical, Osaka, Japan).

Immunostained cells were observed using an inverted microscope (IX-70, Olympus) equipped with a confocal laser scanning unit (CSU 10, Yokogawa, Tokyo, Japan) or epifluorescence optics.

Activation Assay for Rho GTPases

Relative levels of active RhoA and Rac1 were estimated using the method described by Ren and Schwartz (2000) and Benard and Bokoch (2002), respectively, with slight modifications. Recombinant GST-RBD and GST-PBD were prepared and conjugated with glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). HT1080 cells on collagen-coated culture dishes were transfected with mCherry or mCherry-Ect2-DA expression vectors. To assay RhoA activity, cells were lysed 24 h after transfection in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol supplemented with 1 mM dithiothreitol, 50 mM NaF, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 70 μ g/ml tosyl phenylalanylchloromethyl ketone, 75 μ g/ml *p*-toluenesulfonyl-L-arginine methyl ester, 2 μ g/ml aprotinin, and 160 μ g/ml benzamide. To assay Rac1 activity, cells on collagen-coated dishes were lysed 24 h after transfection in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, and 10% glycerol supplemented with 1 mM dithiothreitol, 50 mM NaF, 1 mM NaVO₄, and the protease inhibitors. Lysates were clarified by centrifugation and then incubated with GST-RBD or GST-PBD beads for 45 min at 4°C with gentle agitation. The beads were then spun down and washed five times with lysis buffer. Total lysate and pull-downs were analyzed by Western blotting using anti-RhoA (1:200 dilution) or anti-Rac1 (05-389, Upstate, Lake Placid, NY; 1:2500 dilution) antibody. The density of each band was determined by densitometric analysis of Western blots after quantitating chemiluminescence signals. The relative amount of GTP-RhoA or Rac1 in cells expressing mCherry-tagged Ect2-DA was calculated as follows: the density of GTP-RhoA or Rac1 band/the density of total RhoA or Rac1 band and were expressed as ratios to those in control cells expressing mCherry alone.

RESULTS

Ect2 Is Not Essential for HT1080 Cell Growth

We initially used immunofluorescence microscopy to examine the spatial relationship between RhoA and Ect2 during cytokinesis in HeLa and HT1080 cells. RhoA was immunostained in cells fixed with TCA, which has been known to preserve active RhoA (Yonemura *et al.*, 2004; Yuce *et al.*, 2005), whereas Ect2 was immunostained in cells fixed with formaldehyde. We confirmed the findings of an earlier study (Chalamalasetty *et al.*, 2006) showing that RhoA accumulated at the equatorial cortex during anaphase in both HeLa and HT1080 cells (Figure 1A, left panels). Ect2 localized at the cell cortex as well as the central spindle in HeLa cells but predominantly at the central spindle in HT1080 cells (Figure 1A, middle and right panels).

To study the function of Ect2 during cytokinesis in the two cell lines, we next depleted Ect2 using a novel shRNA vector, piMARK (Nagasaki *et al.*, 2007). Cells harboring piMARK and expressing shRNA were selected by culture in the presence of blasticidin S, and efficient depletion of Ect2 was confirmed by Western blot analysis carried out 48 and 72 h after transfection (Figure 1B). Inhibition of cytokinesis in Ect2-depleted cells was then evaluated morphologically. When assessed 72 h after transfection, Ect2 depletion re-

Figure 1. Localization of RhoA and Ect2 during cytokinesis and the effects of Ect2 depletion on cytokinesis in HeLa cells and HT1080 cells. (A) Confocal microscopic images of mitotic HeLa and HT1080 cells showing the distributions of RhoA (green), Ect2 (green), and DNA (blue); scale bars, 10 μ m. Magnified views of the Ect2 staining (dashed boxes) are provided on the right. (B) Depletion of Ect2 in HeLa and HT1080 cells was confirmed by immunoblotting using anti-Ect2 antibodies 48 and 72 h after transfection. β -actin was detected as a loading control. (C) Frequencies of multinucleate cells. In each of three independent experiments, 200 cells were examined. Values are shown as mean (%) $\pm$ SD. (D) Persistent depletion of Ect2 in a stably transfected HT1080 cell line was confirmed by immunoblotting (left) and by confocal immunofluorescence microscopy (right) using anti-Ect2 antibodies; scale bar, 10 μ m.

sulted in 59% of HeLa cells being multinucleate (Figure 1C), which was consistent with earlier studies (Tatsumoto *et al.*, 1999; Kimura *et al.*, 2000; Kim *et al.*, 2005). By contrast, only 18% of HT1080 cells were multinucleate. During extended culture, all of the Ect2-depleted HeLa cells became severely multinucleated and eventually died. On the other hand, with selection in the presence of blasticidin S, a large number of HT1080 cell colonies emerged in which expression of Ect2 was stably suppressed. This enabled us to establish nine cell lines in which the majority of cells were mononucleate, though expression of Ect2 was strongly suppressed. In the cell line chosen for further characterization, the expression of Ect2 was suppressed by greater than 90%, as determined by densitometric analysis of Western blots. Immunofluorescence staining of these cells revealed that the amount of Ect2 at the spindle structures was reduced to an undetectable level (Figure 1D). These unexpected results indicate that whereas Ect2 is essential in HeLa cells, it is dispensable for cytokinesis and growth in HT1080 cells.

Effects of Ect2 Depletion on the Progression of Cytokinesis A

We used time-lapse videomicroscopy to analyze the effects of Ect2 depletion on the progression of cytokinesis A in mitotic HT1080 cells. In control cells, smoothly curved, U-shaped furrows began to form in the lateral equatorial region 2.7 ± 0.5 min (average $\pm$ SD; N = 6) after the onset of anaphase (3 and 4 min after anaphase onset, Figure 2A). The furrows became V-shaped as the ingression grew deeper, and the ingression was complete in $\sim 6.3 \pm 1.9$ min. By contrast, Ect2-depleted cells formed lateral cleavage furrows that were V-shaped from the beginning (6 min after anaphase onset, Figure 2A). These cells took a longer time to form cleavage furrows (4.7 ± 0.5 min; N = 6, after anaphase onset), but showed rapid ingression that was completed in 2.8 ± 0.8 min once the furrow was formed (Figure 2, A and B, and Supplementary Movies 1 and 2). The accelerated ingression in Ect2-depleted cells suggests that one of the

Figure 2. Effects of Ect2 depletion on cytokinesis A. (A) Cytokinesis A in control and Ect2-depleted HT1080 cells. Numbers indicate the time in minutes after onset of anaphase; scale bars, 20 μm . (B) Changes in the widths of the furrows in control ($\blacklozenge$) and Ect2-depleted ($\blacksquare$) cells after anaphase onset. Values are shown as means $\pm$ SD ($n = 6$). (C) Confocal microscopic images of control and Ect2-depleted mitotic HT1080 cells showing the distributions of RhoA and DNA; scale bars, 10 μm .

functions of Ect2 present at the central spindle is temporal regulation of the ingression process through regulation of the behavior of the equatorial cortex via RhoA or some other factor(s).

O'Connell *et al.* (1999) reported that *Botulinum* C3 exoenzyme (C3) did not prevent furrow ingression in normal rat kidney (NRK) cells or 3T3 fibroblasts. In addition, a recent fluorescence resonance energy transfer analysis showed that entire process of cytokinesis in Rat1 cells does not involve

RhoA activation (Yoshizaki *et al.*, 2004). These earlier studies suggest the possibility that HT1080 cells also divide in a manner independent of RhoA activation at the equatorial cortex. To examine this possibility, the cell permeable derivative of C3 was loaded into HT1080 cells. The C3-loaded cells failed to accumulate RhoA at the equatorial cortex (Supplementary Figure 1A) and were unable to form equatorial furrows ($N = 6$; Supplementary Figure 1B and Movie 7). Furthermore, immunofluorescence microscopy of Ect2-depleted HT1080 cells revealed that RhoA accumulated at the equatorial cortex, though the region in which it accumulated was narrower than in control cells (Figure 2C). We thus conclude that active RhoA, presumably at the equatorial cortex, is necessary for cytokinesis of HT1080 cells, but that Ect2 is not the primary GEF mediating activation of RhoA at the equatorial cortex of HT1080 cells.

Effects of Ect2 Depletion on the Progression of Cytokinesis B

We then used time-lapse videomicroscopy to examine the effects of Ect2 depletion on blebbistatin-induced cytokinesis B in HT1080 cells. In the presence of 30 μM blebbistatin, 15/15 control mitotic cells on collagen-coated substrates rounded up slightly and then respread, extending polar lamellipodia immediately after the onset of anaphase. Thereafter, the lamellipodia grew and became large and fan-shaped around the two poles, accompanied by formation of tight equatorial furrows (Figure 3A and Supplementary Movie 3). Only two of the 15 cells that formed tight furrows in the presence of 30 μM blebbistatin completed the division to yield separate daughter cells within 2 h; in the remaining 13 pairs, thin cytoplasmic bridges connecting the well-separated daughter cells persisted. Six of those 13 pairs were observed for another 6 h, and four pairs completed the scission, whereas the remaining two pairs eventually fused back to form binucleate cells. Thus, the overall success rate of cytokinesis B of HT1080 cells on collagen coated surfaces

Figure 3. Effects of Ect2 depletion on cytokinesis B. (A) Cytokinesis B in control HT1080 cells on collagen coated substrates in the presence of 30 μM blebbistatin. (B–D) Three representative patterns of cytokinesis B in Ect2-depleted cells cultured as in A. See text for details. Numbers indicate the time in minutes after anaphase onset; scale bars, 20 μm . (E) Growth of the distance from pole to pole in control (diamonds) and Ect2-depleted (squares) daughter cells after anaphase onset in the presence of blebbistatin. Values are shown as means $\pm$ SEM ($n = 8$). The differences between the two cell lines are statistically significant ($p < 0.05$) at 4 and 8 min after anaphase onset (Mann-Whitney test).

in the presence of 30 μ M blebbistatin was estimated to be 71%, although all of them temporarily formed tight equatorial furrows.

In contrast to this, the 13 Ect2-depleted cells observed in detail exhibited three distinct types of morphological abnormalities during cytokinesis B induced by blebbistatin. Five cells were classified as “delayed” (Figure 3B and Supplementary Movie 4). This group showed a delay in respreading after the onset of anaphase; all other processes during cytokinesis B appeared normal. Six cells were classified as “delayed and uncoordinated” (Figure 3C and Supplementary Movie 5). This group was characterized by compromised pole-to-equator polarity in the presumptive daughter cells during the respreading process. They formed uncoordinated lamellipodia around the poles and ectopic lamellipodia near the equatorial region, as well as delay in respreading. Nevertheless, all of these cells eventually formed tight furrows in their equatorial regions (Figure 3C at 50 min). The remaining two cells were classified as “delayed and one-sided” (Figure 3D and Supplementary Movie 6). This group also showed a delay before respreading, after which one side of the cells began respreading before the other side started. Although one of these two cells did form a furrow briefly, it immediately regressed, leaving a binucleate cell. The other cell did not even form a slight furrow, again resulting in formation of a binucleate cell. Two of the 11 Ect2-depleted cells that formed tight furrows in the presence of 30 μ M blebbistatin completed the division; thin cytoplasmic bridges connecting the well-separated daughter cells in the remaining nine pairs persisted during the 2-h observation period. It thus appears that there is no significant difference in the success rate of cytokinesis B between control and Ect2-depleted cells.

To quantitate the efficiency of opposite migration polarity, we measured changes in distance from pole to pole during cytokinesis B in the control and Ect2-depleted cells (Figure 3E). The “one-sided” group was excluded from this analysis because expansion between the two poles was unobservable. We found that the rate in increase in the pole-to-pole distance in control cells was about twice that in Ect2-depleted cells. Taken together, the most striking differences between control and Ect2-depleted cells in the presence of blebbistatin are that, unlike control cells, Ect2-depleted cells fail to form polar lamellipodia immediately after the onset of anaphase, and to maintain appropriate lamellipodial activities throughout cytokinesis B.

Narrower Accumulation of RhoA at the Midzone Cortex in Ect2-depleted Cells Undergoing Cytokinesis B

Because Ect2 appeared necessary for the formation and maintenance of polar lamellipodia during cytokinesis B, we next used a combination of immunofluorescence and phase-contrast microscopy to study the relationship between the localization of RhoA and lamellipodia formation in the presence of blebbistatin (Figure 4A). TCA-fixed control cells with two large fan-shaped polar lamellipodia showed broad accumulation of RhoA over a large area of the equatorial cortex. Within this region, lamellipodia formation was strongly inhibited. On the other hand, Ect2-depleted cells of the “delayed and uncoordinated” type showed narrower accumulation of RhoA at the equatorial cortex, in a region only above the central midzone. To compare the levels of RhoA present in the equatorial region in control and Ect2-depleted cells, we calculated the ratios of the total fluorescence in the equatorial region over that in the region where the lamellipodia formed. The amount of RhoA present in the equatorial region of fixed cells was reduced to 45% of control

Figure 4. Effects of Ect2 depletion on RhoA localization during cytokinesis B. (A) Phase-contrast and conventional epifluorescence micrographs of control and Ect2-depleted HT1080 cells undergoing cytokinesis B on collagen-coated substrates in the presence of 30 μ M blebbistatin. The cells were stained by anti-RhoA antibodies. Dashed boxes ($20 \times 25 \mu$ m) represent regions in which relative RhoA levels were measured; scale bars, 20 μ m. (B) Levels of RhoA present in the equatorial region are expressed as ratios of the total fluorescence in the equatorial region over that in the region where lamellipodia were formed. RhoA accumulation is significantly reduced ($p < 0.01$) in the equatorial region of Ect2-depleted cells (Mann-Whitney test). Values are means $\pm$ SEM ($n = 14$ and 12 for control cells and Ect2-depleted cells, respectively).

in Ect2-depleted cells (Figure 4B). The diminished RhoA levels were accompanied by weaker inhibition of lamellipodia formation, which was restricted to a narrower region than in control cells, so that ectopic lamellipodia formed in regions where they normally would be inhibited. This result suggests that RhoA present at the equatorial cortex suppresses formation of lamellipodia there during cytokinesis B and that Ect2 is involved in the broad cortical accumulation of RhoA over the midzone.

Rho Activity Is Required for Distinctive Polarity Formation during Cytokinesis B in HT1080 Cells

C3 inhibited the formation of equatorial furrows in HT1080 cells on surfaces without collagen coating (Supplementary Figure 1B and Movie 7). Thus, in C3-loaded mitotic HT1080 cells, the RhoA activity was inhibited to the extent that the myosin II-dependent contractile activity of the cleavage furrow was almost completely abolished. This prompted us to ask whether these cells are able to carry out cytokinesis B in the absence of RhoA activity when placed on collagen-coated substrates, in a manner similar to blebbistatin-treated cells. The C3-loaded cells on collagen-coated substrates hardly formed polar lamellipodia after the onset of anaphase. At 27.8 ± 3.6 min (average $\pm$ SD; $N = 13$) after the onset of anaphase, these cells suddenly formed lamellipodia all along the cell periphery, resulting in formation of binucleate cells (Figure 5B and Supplementary Movie 8). It thus appears that the mechanism that regulates lamellipodia formation was dramatically switched from Rho-dependent to Rho-independent at about 28 min after the onset of anaphase of cytokinesis B. Furthermore, this result indicates that the Rho activity is required for the formation of distinctive polarity during cytokinesis B in HT1080 cells.

A Dominant Active Form of Ect2 Suppresses Formation of Lamellipodia through Enhancement of RhoA Activity

Ect2 reportedly increases the rate of guanine nucleotide exchange by all three Rho-type small GTPases in vitro (Tat-

Figure 5. Effects of the cell permeable derivative of C3 exoenzyme on cytokinesis B. (A) Cytokinesis B in an HT1080 cell on a collagen-coated substrate in the presence of 30 μ M blebbistatin. (B) Failed cytokinesis B in a C3-loaded cell on a collagen-coated substrate. Numbers indicate the time in minutes after onset of anaphase; scale bars, 20 μ m.

sumoto *et al.*, 1999). In addition, Westwick *et al.* (1998) suggested that an oncogenic form of mouse Ect2 induces both lamellipodia formation via Rac1 activation and stress fiber formation via RhoA activation in one type of endothelial cell. These two results suggest the possibility that Ect2 directly enhances Rac1 activity in the polar regions of HT1080 cells undergoing cytokinesis B. To determine whether Ect2 directly enhances Rac1 and/or RhoA activity in HT1080 cells, we examined the effects of a dominant active form of Ect2 (Ect2-DA; Saito *et al.*, 2004). For this purpose, Ect2-DA was fused to a monomeric red fluorescent protein, mCherry, which has been proven less toxic than other red fluorescent proteins (Shaner *et al.*, 2004) and was overexpressed in interphase HT1080 cells.

We first used confocal microscopy to confirm that Ect2-DA localized at the cell cortex of interphase HT1080 cells (Figure 6A). The majority of cells expressing mCherry-Ect2-DA did not spread on the poorly adherent glass-bottomed dishes, even after 6 h of culture, whereas the control cells expressing mCherry spread completely within this period. We next used time-lapse videomicroscopy to observe lamellipodia formation by cells plated on highly adherent collagen-coated surfaces. Control cells showed distinct motility (Figure 6B and Supplementary Movie 9), with lamellipodia at defined locations along the periphery. In cells expressing constitutively active mutant of Rac1 (V12-Rac1), a prominent lamellipodium was formed all along the periphery (Supplementary Figure 2C and Supplementary Movie 11). By contrast, these normal lamellipodia were not apparent in the cells expressing mCherry-Ect2-DA when observed with phase-contrast microscopy. Instead, dark structures along the cell periphery and a halo over the rim of the cells were observed (Figure 6B), indicating that the cell periphery was thick. In addition, these cells formed short-lived protrusions that were pulled back immediately, resulting in marked inhibition

Figure 6. Activation of RhoA and inhibition of lamellipodia formation by mCherry-tagged Ect2-DA. (A) HT1080 cells were transfected with plasmids encoding either mCherry-Ect2-DA or mCherry. Twenty-four hours later the cells were transferred to glass-bottomed dishes for confocal microscopic observation; scale bars, 20 μ m. (B) Behavior of interphase HT1080 cells expressing mCherry or mCherry-Ect2-DA on collagen-coated substrates. The expression of each protein was identified by red fluorescence (left). Arrows show a newly formed lamellipodium in an mCherry-expressing cell; numbers indicate elapsed time in minutes; scale bars, 20 μ m. (C) mCherry-expressing (–) or mCherry-Ect2-DA-expressing (+) cells on collagen-coated substrates were assayed for RhoA activity using a GST-Rhotekin pull-down assay and for Rac1 activity using a GST-PAK binding domain pull-down assay. Typical immunoblots and the results of quantitative analysis (means $\pm$ SD, $n = 3$ and 4 for RhoA and Rac1, respectively) are shown.

of cell migration (Figure 6B and Supplementary Movie 10). These results suggest that mCherry-Ect2-DA-expressing cells are incapable of forming stable, typical lamellipodia. Staining fixed mCherry-Ect2-DA-expressing cells with fluorescently labeled phalloidin and anti-vinculin antibody revealed the presence of dense actin fibers along the cell periphery, as previously reported by Westwick *et al.* (1998), and focal adhesions over the whole basal cell membrane (Supplementary Figure 2, A and B).

Cells expressing mCherry-Ect2-DA were able to grow at a reasonable rate in the presence of G418 for more than 1 mo, and the above-mentioned phenotype was maintained during this period (Supplementary Movie 12). This rules out the possibility that mCherry-Ect2-DA exerts a general toxic effect to the cells and that this caused the severe inhibition of lamellipodial formation and cell migration.

Finally, to determine the effects of mCherry-Ect2-DA on the activities of small GTPases, we used pull-down assays to assess the activities of RhoA and Rac1 in cells expressing mCherry alone or mCherry-Ect2-DA on collagen-coated surfaces. RhoA activity was enhanced by more than twofold in mCherry-Ect2-DA-expressing cells. In contrast, the expression of mCherry-Ect2-DA had a weak inhibitory effect on Rac1 (Figure 6C). Apparently, Ect2 predominantly regulates the activity of RhoA in HT1080 cells.

DISCUSSION

Different Roles of Ect2 during Different Phases of Cytokinesis A

We investigated the activities of the GEF Ect2 during cytokinesis in HeLa cells, which rely on cytokinesis A for division, and HT1080 cells, which are able to use both cytokinesis A and cytokinesis B. The results of immunofluorescence staining, as well as of Ect2 knockdown experiments, provided the first evidence strongly suggesting that Ect2 is dispensable and that a substitute GEF can mediate activation of RhoA to form a contractile ring in certain types of mammalian cells, including HT1080 cells. VAV3 (Fujikawa *et al.*, 2002), MyoGEF (Wu *et al.*, 2006), and GEF-H1 (Birkenfeld *et al.*, 2007) are other GEFs reportedly involved in activation of RhoA during cytokinesis. Thus, one of these GEFs might play a major role to form a contractile ring in HT1080 cells.

Recently, Chalamalasetty *et al.* (2006) showed that in HeLa cells an N-terminal fragment of Ect2 (N-Ect2) that acts as a dominant negative form of Ect2 (Tatsumoto *et al.*, 1999; Kimura *et al.*, 2000; Yoshizaki *et al.*, 2004) does not prevent furrow ingression, but does prevent abscission, whereas RNA interference (RNAi)-dependent Ect2 depletion does prevent furrow formation. They concluded that a physiological level of Ect2 is not required at the central spindle for cytokinesis in HeLa cells, based on the observation that N-Ect2 displaced endogenous Ect2 incompletely from the central spindle. However, N-Ect2 lacks the membrane localization domain and is unlikely to displace endogenous Ect2 on the cortex. We thus propose another explanation: that in HeLa cells overexpressing N-Ect2, cortical Ect2 is sufficient to mediate formation of cleavage furrows. Depletion of the microtubule bundling protein PRC1 disrupts the central spindle, resulting in dispersal of the centralspindlin complex (Mollinari *et al.*, 2005), but RhoA accumulates at the equatorial cortex, where it induces contractile ring formation and contraction (Nishimura and Yonemura, 2006). This result is consistent with the above view, if Ect2 was also localized in the equatorial cortex in the absence of central spindle.

Still, these PRC1-depleted cells fail to carry out abscission (Mollinari *et al.*, 2005; Nishimura and Yonemura, 2006), which is consistent with the observation made in HeLa cells that accumulation of Ect2 at the central spindle is necessary for abscission (Chalamalasetty *et al.*, 2006). Contrary to this model, the Ect2-depleted HT1080 cells efficiently completed division. Perhaps migration of the daughter cells in opposite directions is sufficient to sever the cytoplasmic bridge in a midbody-independent manner. Consistent with that idea, we observed that under physiological culture conditions the majority of Ect2-depleted cells retained long, thin cytoplasmic bridges connecting the daughter cells after they had respread and that migration of the daughter cells away from one another eventually broke this cytoplasmic bridge to complete cytokinesis. By contrast, most control HT1080 cells successfully completed the division without the oppositely oriented migration of the daughter cells (Kanada and Uyeda, unpublished observations).

Regulation of Lamellipodia Formation by Ect2 during Cytokinesis B

We found that Ect2-depleted HT1080 cells are slower to start respreading after the onset of anaphase during blebbistatin-induced cytokinesis B. They also fail to maintain the characteristic fan-shaped polar lamellipodia, resulting in formation of uncoordinated lamellipodia around the poles and ectopic lamellipodia near the equatorial regions. Specific

Rho GTPases are believed to regulate specific subsets of the actin cytoskeleton during cell migration, such that Rac promotes membrane protrusion at the leading edge, whereas Rho regulates contractility at the tail (Burrage and Wennerberg, 2004; Raftopoulou and Hall, 2004). That Ect2-depleted HT1080 cells show delayed lamellipodia formation during anaphase suggests that, unlike equatorial RhoA activation for contractile ring formation during cytokinesis A, RhoA activation for lamellipodia formation during cytokinesis B mainly relies on Ect2. Furthermore, Ect2 is required to maintain appropriate polarity after anaphase. We also found that Ect2-depleted cells show narrower accumulation of RhoA at the cortex encircling the midzone and that inhibition of lamellipodia formation was restricted to that location. On the other hand, control cells showed broader accumulation of RhoA over the entire equatorial cortex, and lamellipodia formation was strongly suppressed in this region. Furthermore, experiments using C3 demonstrated that Rho activity is essential for the formation of distinctive polarity during cytokinesis B in HT1080 cells. Given these findings, we propose the following model for RhoA-dependent lamellipodia formation around the poles of mitotic cells undergoing cytokinesis B (Figure 7). On entry into anaphase, RhoA begins to accumulate at the equatorial cortex to form distinctive polarity, which is dependent on partially redundant functions of Ect2 and the unidentified GEF. This contributes to recruitment of the elements involved in lamellipodia formation to the poles, so that lamellipodia are formed around

Figure 7. A model of Rho-dependent polar lamellipodia formation during cytokinesis B. Ect2 and an unidentified Rho GEF accumulate at the central spindle and the equatorial cortex to activate RhoA, respectively (top and middle in Control). The two GEFs then induce broad RhoA accumulation at the equatorial cortex, which contributes to recruitment of the elements involved in lamellipodia formation to the polar peripheries (bottom in Control). Ect2-depleted cells are unable to form the broad RhoA accumulation as in Control cells, so that ectopic lamellipodia form near the equatorial region (bottom in Ect2-depleted). In C3-loaded cells, equatorial RhoA accumulation is completely inhibited, so that lamellipodia form all around the cell periphery (bottom in C3-loaded). In addition to this, a basal level of active RhoA seems necessary for lamellipodia formation during the first half hour after the anaphase onset, but that aspect of RhoA activity is not included in this scheme.

both poles. Thereafter, equatorial RhoA contributes to prevention of lamellipodia formation in that region, thereby maintaining the appropriate polarity. In this model, RhoA acts indirectly to promote polar lamellipodia formation during cytokinesis B. This view is, however, inconsistent with the fact that the global inhibition of RhoA by C3 prevented lamellipodia formation during the first half hour after the anaphase onset. Therefore, there seems to be an abrupt, qualitative change in dependence of lamellipodia formation on RhoA activities at about half hour after the onset of anaphase during cytokinesis B. During this first period of cytokinesis B, a smaller amount of RhoA may directly contribute to polar lamellipodia formation. This view is consistent with the report that RhoA directly regulates membrane protrusion at the cell periphery (Fukata *et al.*, 1999; Palazzo *et al.*, 2001; Pertz *et al.*, 2006).

Finally, we reported that Ect2-DA severely inhibits normal lamellipodia formation and enhances RhoA activity. These findings indicate that Ect2 mainly regulates activation of RhoA in HT1080 cells, though it regulates all three Rho-type small GTPases in vitro. It has been reported that under certain circumstances reciprocal balance between Rho-type small GTPases determines cellular morphology and behavior (reviewed by Uyeda *et al.*, 2004). In our experiment, the activity of Rac1 was not dramatically suppressed by overexpression of Ect2-DA, though the activity of RhoA was strongly enhanced. We suggest that during cytokinesis active RhoA might regulate Rac1 effector proteins such as p21-activated kinase (PAK) independently of Rac1 activity. Although there is currently no evidence directly pointing to such signaling pathways, there is some evidence that one member of the small GTPase family may regulate the effector of another member. For example, down-regulation of Rac1 induces phosphorylation of myosin light-chain kinase, which in turn phosphorylates myosin II in the absence of RhoA activation (Mandato *et al.*, 2000; Yoshizaki *et al.*, 2004). By an analogy, active RhoA might regulate Rac1 effector molecules without suppressing Rac1 activity. According to this model, equatorial RhoA activated by Ect2 during cytokinesis B might suppress Rac1 effector, resulting in suppression of lamellipodia formation in the region.

If cytokinesis B is a backup mechanism activated upon failure of cytokinesis A, cells must have a novel mechanism to sense that cytokinesis A has failed. Continued study of the mechanisms underlying cytokinesis B will surely lead us to a more comprehensive understanding of how the multiple modes of cytokinesis are regulated in a cooperative manner.

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