Mechanism of Contractile Ring-independent Cytokinesis in Adherent Mammalian Cells

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ABSTRACT

Division of animal cells is believed to be mediated by the 'purse-string' mechanism, which entails furrowing of the equatorial region, driven by the interaction between actin and myosin II filaments within contractile rings. It has been thought that this 'purse-string' mechanism is the conserved method by which cells ranging from lower eukaryotes such as yeast and slime molds to mammalian cells divide. Nonetheless, myosin II-null *Dictyostelium* cells on substrates divide efficiently in a cell cycle-coupled manner. This process, termed cytokinesis B, appears to be driven by polar traction forces. This thesis describes the results of studies that were aimed at revealing that cytokinesis B mechanism is conserved in mammalian cells, and understanding the mechanism of cytokinesis B in mammalian cells.

Chapter I describes the background information of research on animal cytokinesis, and brief summary and significance of this study.

Chapter II describes the demonstration of cytokinesis B in mammalian cells (Kanada *et al.*, 2005). In this work, I found that, in the presence of 30 μM blebbistatin, which is sufficient to reduce the myosin II ATPase activity by >90% *in vitro* (Straight *et al.*, 2003; Kovacs *et al.*, 2004), normal rat kidney (NRK) cells adhering to fibronectin-coated surfaces formed equatorial furrows and divided in a manner strikingly similar to myosin II-null *Dictyostelium* cells. I next showed that such blebbistatin-resistant cytokinesis was absent under a poorly adherent condition and in subconfluent cultures where the advancement of the polar lamellipodia was disturbed by neighboring cells. These results indicate that certain adherent mammalian cells are able to form tight equatorial furrows and divide in the environment in which cell adhesion is facilitated and cells are able to advance the polar lamellipodia even when the activity of contractile rings is inhibited.

Chepter III describes the exploration of the mechanism of cytokinesis B in mammalian cells. The effects of Ect2 depletion or Rho inactivation on cytokinesis A and cytokinesis B were examined, respectively, using HT1080 human fibrosarcoma cells, which are also able to divide using cytokinesis B when cytokinesis A is inhibited (Kanada *et al.*, 2005). Studies of the effects on cytokinesis A provided the first evidence that Ect2 is dispensable for the activation of RhoA during cytokinesis A in HT1080 cells, suggesting that Ect2 is not the primary GEF mediating activation of

RhoA at the equatorial cortex during cytokinesis A in this cell line. In addition, the results obtained from the study of effects on cytokinesis B suggested that equatorial Ect2 locally suppresses lamellipodia formation via RhoA activation, which indirectly contributes to restricting lamellipodia formation to polar region during cytokinesis B (Kanada *et al*., 2008). This study indicated that Ect2 and RhoA are not only important for cytokinesis A, but also for the regulation of cell polarity during cytokinesis B, suggesting that cytokinesis A and cytokinesis B are regulated by the same common upstream regulators of the signaling pathway for cytokinesis derived from the mitotic spindle.

Finally, Chapter IV describes concluding remarks and future directions to address the many questions that remain. In addition, possible reasons why adherent mammalian cells have the two modes of cytokinesis are discussed from evolutionary and beneficial points of view.

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ABBREVIATIONS

BSR	Blasticidin S resistance gene
C3	*Botulinum* C3 exoenzyme
DMSO	Dimethylsulfoxide
Ect2	Epithelial cell transforming protein 2
Ect2-DA	Dominant active form of Ect2
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GEF	Guanine nucleotide exchange factor
GST	Glutathion S-transferase
INCENP	Inner centromere protein
MKLP1	Mitotic kinesin-like protein 1
MT	Microtubule
N-Ect2	N-terminal fragment of Ect2
NRK	Normal rat kidney
PAK	p21-activated kinase
PBD	p21 binding domain
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PRC1	Protein regulator of cytokinesis 1
PtK2	Male rat kangaroo kidney epithelial
RNAi	RNA interference
RBD	Rho binding domain of Rhotekin
ROCK	Rho-associated kinase
shRNA	Small hairpin RNA
TCA	Trichloroacetic acid
V12-Rac	Constitutively active mutant of Rac1

CHAPTER I

INTRODUCTION

During cytokinesis, cytoplasm of a cell is precisely partitioned into two so that each daughter cell receives a full complement of chromosomes and other essential organelles. Understanding the mechanism of cytokinesis is not only a central issue in cell biology (Glotzer, 1997), but also important in the context of human disease, since there is accumulating evidence suggesting that failure of cell division is closely related to tumorigenesis (Fujiwara *et al.*, 2005; Caldwell *et al.*, 2007; Roversi *et al.*, 2006).

Conceptually, cytokinesis in animal cells is comprised of a series of distinct steps. In anaphase, a cleavage plane is specified at the site where the cleavage furrow will form. A contractile ring containing actin and myosin II filaments assembles at this site, followed by ingression of the equatorial region. An intracellular bridge and a dense midbody structure are formed when the furrowing completes. Finally, scission completes the process, yielding two separated daughter cells.

Mechanism of cleavage plane determination

Since the early studies of Rappaport, it has become clear that mitotic apparatus plays key roles in cleavage furrow formation during animal cytokinesis (Rappaport, 1986). The mitotic apparatus contains three sets of microtubule (MT) organization: spindle MTs formed between two spindle poles, astral MTs emanating radially from spindle poles, and midzone MTs which are anti-parallel overlapping MTs formed between sister chromatids after chromosome segregation. In the classic view, astral MTs are primarily responsible for signaling of cytokinesis. Its main evidence came from the study by Rappaport (1961), who created sand dollar eggs having two sets of mitotic spindles and demonstrated that in addition to the two normal furrows formed perpendicular to the spindles, a furrow was also formed between astral arrays of MTs that are not connected by the spindle MTs. Since there were no spindle MT bundles or chromosomes between the unconnected astral arrays of MTs, this result was taken to indicate that it is the astral arrays of MTs that determine the cleavage position and induce furrows. However, there is now reason to question the generality of this model, since more recent experiments indicated that in some cells the midzone MTs appear to determine the position of the cleavage furrow. The possibility that midzone MTs are important for cytokinesis arose from attempts to reproduce some of the experiments on

which the original model had been based, but in cultured somatic cells rather than in a large embryo. For example, to determine whether two asters that are not connected by a spindle can induce formation of an ectopic furrow, the behavior of cells containing multiple spindles was examined using cultured mammalian cells (Wheatley and Wang, 1996; Rieder *et al.*, 1997; Eckley *et al.*, 1997). The formation of such furrows was somewhat variable and dependent on the geometric relationship between the spindles. Among these studies, Wheatley and Wang (1996) elaborately demonstrated that MT bundles formed between the asters are insufficient to induce furrows, and midzone MT bundles play a critical role in furrowing in normal rat kidney (NRK) cells. It thus appears indisputable that animal cells determine the positions of cleavage furrows via two pathways. In one case, furrows are formed between two astral arrays of MTs, while in the other they are formed near the midzone MT bundles. In large embryos, radially emanating astral MTs are required to transmit signals derived from the mitotic spindle to the distant equatorial cortex avoiding the dilution of them. In contrast, somatic cells primarily use the midzone MT bundle-dependent method, because mitotic spindles in these cells are relatively larger, so that signals derived from midzone appear to easily reach the equatorial cortex.

Recent studies of MT dynamics during cytokinesis established another, novel direction to explore regarding methods of furrow induction. These studies demonstrated that stabilized MTs induce equatorial ingression, while dynamic MTs inhibit cortical ingression in the polar regions during cytokinesis. For instance, in drug-induced monopolar Ptk2 cells, stable MTs extending from chromosomes to the cell cortex induce furrow formation, whereas interaction between dynamic astral MTs and the cortex does not (Canman *et al.*, 2003). In addition, artificially stabilized MTs were shown to induce cortical ingression during cytokinesis in Ptk1 cells (Shannon *et al.*, 2005). Some components of chromosome passenger complex, aurora B and inner centromere protein (INCENP), reportedly relocate from chromosomes to the spindle midzone after chromosome separation, and they function in stabilizing MTs in the spindle midzone (Adams *et al.*, 2001; Giet and Glover, 2001; Murata-Hori *et al.*, 2002; Wheatley *et al.*, 2001). Surprisingly, Aurora B kinase activity is also required for the maintenance of dynamic astral MTs, which prevents mis-positioning of the cleavage furrow (Miyauchi *et al.*, 2007). However, the exact molecular mechanism that regulates the dynamics of MTs during cytokinesis is still under investigation.

After cytokinesis of an adherent cell, the equator of the mitotic mother cell becomes the rears of the two daughter cells that migrate away from one another, and polar regions of the mother cell become the leading edges of the daughter cells (Burton and Taylor, 1997; Fukui and Inoue, 1991; Moores *et al*., 1996; Sanger, 1975). Both the polar regions of mitotic cells and the leading edges of interphase cells exhibit protrusive activities, while the equator and the rear regions are contractile. Thus, mitotic cells can be viewed as two polarized cells joined in a tail-to-tail manner (Fukui and Inoue, 1991). In this context, Waterman-Storer *et al*. (2000) reported that, during cell migration, MTs in the anterior regions of interphase cells are more dynamic than those in the posterior region. This suggests a functional similarity between each of the two sets of MTs present in mitotic cells and polarized interphase cells. For this reason, regulation of MT dynamics during cytokinesis is not only important for furrow induction, but also for the global regulation of cytoskeleton in mitotic cells.

Mechanism of contractile ring formation

Once the division plane is specified, the division machinery is assembled at this site. In the conventional model termed "purse string mechanism", a contractile ring comprised mainly of actin and myosin II filaments is assembled in the equatorial region (Mabuchi and Okuno, 1977; Cooper, 1987). Assembly of the contractile ring is mediated by GTP-bound active RhoA. RhoA is a member of the Rho-type small GTPases including RhoA, Rac1 and Cdc42 that regulate actin dynamics during a variety of cellular events (reviewed by Etienne-Manneville and Hall, 2002). The key RhoA effectors involved in cytokinesis are members of the formin family proteins, Rho-associated kinase (ROCK) and Citron kinase. Briefly, formin promotes the polymerization of actin necessary for formation of the contractile ring. ROCK regulates activation of myosin II, which is a crucial component of the contractile ring that generates the force necessary for contractile ring constriction. Finally, RhoA also activates Citron kinase, which is required in some cells for the late stage of cytokinesis after the furrow has ingressed to completion (reviewed by Piekny *et al.,* 2005).
Rho-type small GTPases are activated by guanine nucleotide exchange factors (GEFs). Among the numerous GEFs, the mitosis-specific GEF, Ect2, has been shown to play a key role in the activation of RhoA during cytokinesis in embryos of *Drosophila*

melanogaster and *Caenorhabditis elegans,* and HeLa cells (Prokopenko *et al.*, 1999; Morita *et al.*, 2005; Kimura *et al.*, 2000).

Myosin II-independent pathway of cell-cycle-coupled cytokinesis in *Dictyostelium* cells

Interestingly, while the purse string mechanism is the broadly accepted hypothesis, the essential role in cytokinesis of actin- and myosin II-dependent contraction of the equatorial cortex is now being questioned. Myosin II-null cells of the cellular slime mold *Dictyostelium discoideum* form cleavage furrows that are hardly distinguishable from those in wild-type cells when attached to a solid surface (Neujahr *et al.*, 1997). Formation of furrows in the absence of myosin II depends on the adhesiveness of the substrate surface. Non-adherent myosin II-null cells are unable to form an ingressing furrow (Zang *et al.*, 1997). This mode of cytokinesis is named "attachment assisted mitotic cleavage" (Neujahr *et al.*, 1997) or "cytokinesis B" to distinguish it from "cytokinesis A", which refers to the classic, adhesion-independent, contractile ring-dependent cytokinesis (Zang *et al.*, 1997; Nagasaki *et al.*, 2002). These studies revealed that *Dictyostelium* cells have two parallel pathways both of which lead to cell-cycle-coupled cytokinesis (Figure 1), and suggested the possibility that the purse string mechanism is insufficient to describe cytokinesis in animal cells.

Cytokinesis B in mammalian cells

Whether or not cytokinesis B mechanism identified in *Dictyostelium* is relevant to other organisms was an interesting open question. There seemed to be no doubt that the purse string mechanism is essential for cytokinesis of an embryo. These cells are not adherent to the substrates, and in that sense, the requirement of myosin II is reminiscent of the same requirement of *Dictyostelium* cells suspended in the medium (cytokinesis A). By contrast, since most cultured mammalian cells require adhesion to substrates for their lives (e. g., Ben-Ze'ev and Raz, 1981), the idea that cytokinesis in adherent mammalian cells occurs only through the purse string (cytokinesis A) mechanism is really questionable. In fact, mammalian cells exhibit a number of behaviors that are difficult to explain on the basis of cytokinesis A mechanism alone. For instance, when cytochalasin D, which inhibits actin polymerization, was applied locally to the equatorial region of mitotic NRK cells, the cleavage process was

accelerated rather than inhibited, whereas local application of the drug to the polar regions inhibited the furrowing process (O'Connell *et al.*, 2001). Microinjection of inhibitory antibodies against myosin II into epitheloid cells inhibited morphogenetic cell movement, but only delayed cytokinesis (Zurek, *et al.*, 1990). Overexpression of non-phosphorylatable myosin II regulatory light-chain in COS and NRK cells completely inhibited receptor capping, but only partially inhibited cytokinesis (Komatsu *et al.*, 2000). Those phenomena can be interpreted as suggesting that highly adherent mammalian cells are also capable of dividing in an adhesion-dependent, contractile-ring-independent manner. Furthermore, the phenotype of certain knockout mice also supports the notion that the purse string mechanism is insufficient to describe animal cytokinesis. For instance, although mammalian cells have three subtypes of non-muscle myosin II, myosin IIA, B and C, cardiomyocytes do not express non-muscle myosin IIA *in vivo*. However, cardiomyocytes of mice lacking the gene for non-muscle myosin IIB heavy chain show only moderate degrees of cytokinetic failure (Takeda *et al.*, 2003). Therefore, cytokinesis of these mutant cardiomyocytes should be due either to myosin IIC expressed at low levels or to the cytokinesis B mechanism.

Recently, specific inhibitor of non-muscle myosin II ATPase activity, blebbistatin, was identified and shown to inhibit cytokinesis in HeLa cells (Straight *et al.*, 2003). This finding was interpreted as supportive of the essential role of the contractile ring in cytokinesis of mammalian cells. However, since HeLa cells are poorly adherent, I suspected that highly adherent mammalian cells might respond differently to blebbistatin treatments. I thus initially analyzed the effects of blebbistatin on cytokinesis of NRK cells, which were previously shown to exhibit behaviors that are difficult to explain in the context of the purse-string mechanism (O'Connell *et al.*, 1999, 2001), to reexamine the importance of the purse-string mechanism (cytokinesis A) in cytokinesis of animal cells.

Summary and significance of this study

The following chapters detail the results of studies that were aimed at revealing that cytokinesis B mechanism is conserved in mammalian cells, and understanding the mechanism of cytokinesis B in mammalian cells. This thesis grew out of a long-standing hypothesis of my advisor, Dr. Taro Q.P. Uyeda and Dr. Akira Nagasaki

(Uyeda *et al*., 2004) that cytokinesis B mechanism may be conserved in adherent mammalian cells.

Since mammalian cells have three subtypes of non-muscle myosin II, depletion of all the subtypes via gene knockout or RNA interference (RNAi) were not practical strategies. I thus took the advantage of blebbistatin to inhibit contraction of contractile rings, which was one of the two key technical elements of this study. The second key technical element of this study was the choice of highly adherent cells rather than commonly used, poorly adherent HeLa cells, and the application of extracellular matrix proteins adsorbed on hydrophobic surfaces to facilitate adhesion of cells to the substrates. These were my original breakthroughs that were essential for the following experiments.

This thesis initially describes that, on highly adherent substrates, certain types of mammalian cells are able to divide in an adhesion-dependent, contractile ring-independent manner when contraction of the contractile rings is blocked by the myosin II-specific inhibitor, blebbistatin. Next, depletion of Ect2 (Epithelial cell transforming protein 2), a major guanine nucleotide-exchange factor (GEF) for Rho-type small GTPases during cytokinesis in HeLa cells, and inactivation of RhoA were performed in HT1080 cells to explore the mechanism of cytokinesis B in mammalian cells. This study revealed that equatorial Ect2 suppresses lamellipodia formation via RhoA activation in the equatorial region, which indirectly contributes to restricting lamellipodia formation to polar regions during cytokinesis B in HT1080 cells.

As described above, it has become evident that Rappaport's rule and the purse string mechanism (cytokinesis A) are insufficient to describe cytokinesis in animal cells. I believe that my findings described here contribute to revise the classic model of animal cytokinesis, and continuing advances in understanding of the mechanisms underlying cytokinesis B are necessary for the complete understanding of the mechanism of animal cytokinesis in animal cells.

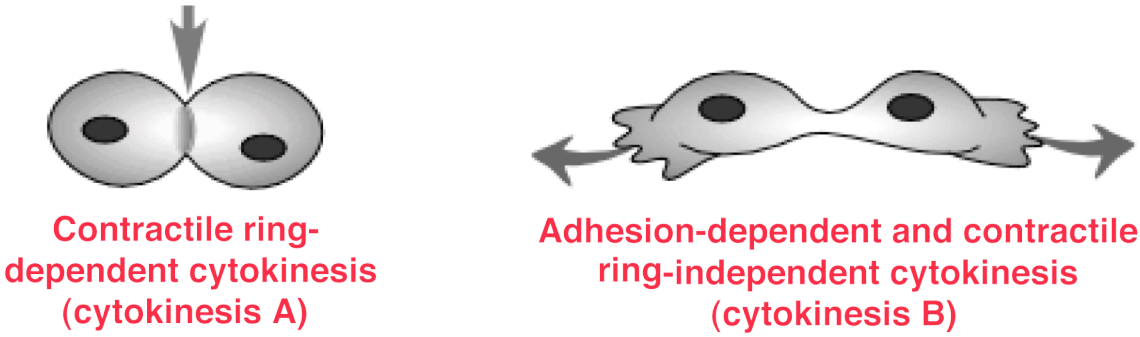

Figure 1. Two functionally redundant mechanisms leading to cell cycle-coupled division.

CHAPTER II

ADHESION-DEPENDENT AND CONTRACTILE RING-INDEPENDENT EQUATORIAL FURROWING DURING CYTOKINESIS IN MAMMALIAN CELLS

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ABSTRACT

Myosin II-dependent contraction of the contractile ring drives equatorial furrowing during cytokinesis in animal cells. Nonetheless, myosin II-null cells of the cellular slime mold *Dictyostelium* divide efficiently when adhering to substrates by making use of polar traction forces. Here, I show that in the presence of 30 μM blebbistatin, a potent myosin II inhibitor, normal rat kidney (NRK) cells adhering to fibronectin-coated surfaces formed equatorial furrows and divided in a manner strikingly similar to myosin II-null *Dictyostelium* cells. Such blebbistatin-resistant cytokinesis was absent in partially detached NRK cells and was disrupted in adherent cells if the advance of their polar lamellipodia was disturbed by neighboring cells. 40 μM Y-27632, which inhibits Rho-associated kinase, was similar to 30 μM blebbistatin in that it inhibited cytokinesis of partially detached NRK cells but only prolonged furrow ingression in attached cells. In the presence of 100 μM blebbistatin, most NRK cells that initiated anaphase formed tight furrows, although scission never occurred. Adherent HT1080 human fibrosarcoma cells also formed equatorial furrows efficiently in the presence of 100 μM blebbistatin. These results provide direct evidence for adhesion-dependent, contractile-ring-independent equatorial furrowing in mammalian cells and demonstrate the importance of substrate adhesion for cytokinesis.

Introduction

Following the onset of anaphase, animal cells assemble a contractile ring comprised of actin and myosin II filaments in their equatorial region, after which active sliding between the two filament systems contributes to the constriction of a furrow that eventually yields two daughter cells. It has been believed that this "purse-string" mechanism is the conserved method by which cells ranging from yeasts and slime molds to mammalian cells divide (Glotzer, 1997; Fishkind and Wang, 1995). When cultured on substrates, however, myosin II-null cells of the cellular slime mold *Dictyostelium discoideum* are able to divide efficiently in a manner clearly different from wild-type cells (Zang *et al*., 1997; Neujahr *et al*., 1997). Subsequent molecular genetic analyses revealed that *Dictyostelium* makes use of two parallel pathways leading to cell-cycle-coupled division, one involving the myosin II-dependent purse-string mechanism and another that depends on substrate adhesion and is independent of active contraction of the contractile ring (Nagasaki *et al*., 2002). The physical mechanism of the latter remains controversial (Gerisch and Weber, 2000; Robinson *et al*., 2002), but there is strong evidence to suggest that it is driven by traction forces that are generated around both poles and that move the two daughter cells away from one another (Nagasaki *et al*., 2002; Uyeda and Nagasaki, 2004). Consistent with this view, a recent modeling by Zhang and Robinson (2005) demonstrated that in elongated cells, such as adherent mitotic cells, a Laplace pressure resulting from material properties and the geometry of the cell generates force to help furrow ingression.

Mammalian cells also exhibit a number of behaviors that are difficult to explain on the basis of the purse-string mechanism (reviewed by Uyeda and Nagasaki, 2004). For instance, when cytochalasin D, which caps the plus ends of actin filaments and inhibits their polymerization, is applied locally to the equatorial region of mitotic normal rat kidney (NRK) cells, the cleavage process is accelerated rather than inhibited, whereas local application of the drug to the polar regions inhibits the furrowing process (O'Connell *et al*., 2001). Those phenomena can be interpreted as suggesting that highly adherent mammalian cells are also capable of dividing in an adhesion-dependent, contractile-ring-independent manner. Furthermore, it has been shown that myosin II is

not essential for cytokinesis and viability in certain strains of the budding yeast *Saccharomyces cerevisiae* (Watts *et al*., 1987; Bi *et al*., 1998).

These and other (Young *et al*., 1993; O'Connell *et al*., 1999; Takeda *et al*., 2003) observations paint a complex picture in which cytokinesis in animal and fungal cells cannot be entirely explained by the simple purse-string mechanism. Recently, blebbistatin, a potent and specific inhibitor of non-muscle myosin II ATPase activity, was identified and shown to inhibit cytokinesis in HeLa cells and cultured *Xenopus* tissue cells (Straight *et al*., 2003). This finding was interpreted as supportive of the essential role of the contractile ring in cytokinesis. But because myosin II may also be involved in post-mitotic spreading (Cramer and Mitchison, 1995), spindle formation (Rosenblatt *et al*., 2004), and maintenance of cell polarity during interphase (Straight *et al*., 2003; Pierini *et al*., 2003), it seems premature to conclude that blebbistatin disrupts cytokinesis by inhibiting contraction of the contractile ring. Moreover, cells other than HeLa or *Xenopus* tissue cells might respond differently to blebbistatin treatments.

Thus, to reexamine the importance of the purse-string mechanism in cytokinesis of animal cells, I analyzed the effects of blebbistatin on cytokinesis of NRK cells, which were previously shown to exhibit behaviors that are difficult to explain in the context of the purse-string mechanism (O'Connell *et al*., 1999, 2001). The results of this and related experiments that I report here establish experimentally that certain mammalian cells are able to form tight equatorial furrows in a traction force-dependent and contractile-ring-independent manner, under appropriate adhesion conditions.

MTERIAL AND METHODS

Culture and observation of cells

NRK cells (NRK-52E; Health Science Research Resources Bank, Tokyo, Japan; cell number IF050480) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). HT1080 cells (Rasheed *et al.*, 1974) were cultured in RPMI-1640 supplemented with 10% FBS. To facilitate cell adhesion, untreated polystyrene dishes (#1000-035X, Asahi Techno Glass Co., Tokyo, Japan) were coated with fibronectin (Yagai Research Center, Yamagata, Japan) at 50 μg/ml or 0.01% collagen type I solution (Wako Pure Chemical, Osaka, Japan) overnight. Trypsinized NRK cells were allowed to attach to the fibronectin matrix for 3 to 5 h before addition of blebbistatin or Y-27632. To assess cytokinesis in partially detached NRK cells, mitotic cells were obtained by treatment for 4 h with nocodazole (40 ng/ml), followed by wash-off and transfer to Easy Grip tissue culture dishes (#353001, Falcon) containing medium with or without blebbistatin or Y-27632. The S-(-)-enantiomer of blebbistatin was purchased from Toronto Research Chemicals (Toronto, Canada) and dissolved at 10 mM in DMSO. Y-27632 (MELK, Tokyo, Japan) was dissolved at 5 mM in water. Cells were maintained at constant temperature in a stage incubator (Onpu-4; Taiei Denki, Kasama, Japan) attached to an inverted microscope (IX50; Olympus, Tokyo, Japan) and observed using a 20X objective with phase-contrast optics. Images were captured with a CCD camera (C5985; Hamamatsu Photonics, Hamamatsu, Japan) and a time-lapse recording system (ARGAS-20; Hamamatsu Photonics). Using accumulation times of 1-2 s enabled me to reduce the intensity of illuminating light to minimize the phototoxic effects (Kolega, 2004).

Immunofluorescence

One hour after addition of blebbistatin (100 μM), cells on fibronectin-coated glass-bottom dishes (Mat-Tek Corp., Ashland, MA) were fixed for 15 min in 80 mM PIPES (pH 7.0), 1 mM $MgCl_2$, 10 mM EGTA, 0.2% Triton X-100, and 4% formaldehyde, and then blocked in PBS containing 0.1% Triton X-100 and 2% bovine serum albumin. Cells were stained with anti-α-tubulin (clone DM 1A, Sigma, St. Louis, MO; 1:1,000 dilution) or anti-myosin IIA (M8064, Sigma; 1:1,000 dilution) antibody, followed by a mixture of Alexa 488-conjugated anti-mouse-IgG or

anti-rabbit-IgG antibody (Invitrogen, Carlsbad, CA), 3.3 nM rhodamine-phalloidin (Sigma), and 1 μg/ml Hoechst 33258 (Wako Pure Chemical). Immunostained cells were observed using an inverted microscope (IX-70, Olympus) equipped with a confocal laser scanning unit (CSU 10; Yokogawa, Tokyo, Japan).

RESULTS

Effects of blebbistatin on cytokinesis in NRK cells adhering to fibronectin-coated surfaces

To evaluate the consequences of inhibiting myosin II-dependent contractile-ring activity in NRK cells, I first examined the effects of 30 μM blebbistatin, which is sufficient to reduce myosin II ATPase activity by >90% in vitro (Straight *et al.*, 2003; Kovacs *et al.*, 2004). Within 5 min after addition of the drug, interphase cells lost their single leading lamellipodia and distinct polarity, and instead exhibited repeated uncoordinated expansion and contraction of the cell periphery (unpublished observations).

Upon entry into mitosis, control NRK cells in sparse cultures treated with 0.3% dimethylsulfoxide (DMSO) alone withdrew their lamellipodia and became thicker and less adherent to the substrate, as suggested by the observation that these cells were easily dislodged by shaking the culture dishes. These cells then formed cleavage furrows in their equatorial regions, respread, and divided into two daughter cells via a process that was typically completed within 10 min after the onset of anaphase (Figure 1A, Table 1, and Supplemental Movie 1 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1). In contrast, in the presence of 30 μM blebbistatin plus 0.3% DMSO, mitotic NRK cells on fibronectin-coated substrates remained adherent, but initiated chromosome segregation. Subsequently, the cells respread and formed fan-shaped lamellipodia around both poles. The radial advance of the lamellipodia was accompanied by a furrowing of the equatorial region that progressed over a period of about 60 min (Figure 1B, Table 1, and Supplemental Movie 2 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1). This sequence of morphological changes is very different from that seen in the absence of blebbistatin but is strikingly similar to the division of myosin II-null *Dictyostelium* cells on substrates (Figure 1D) (Zang *et al.*, 1997; Neujahr *et al.*, 1997; Nagasaki *et al.*, 2002).

Interestingly, only seven of the 16 cells that formed tight furrows in the presence of 30 μM blebbistatin completed the division to yield separate daughter cells (Figure 1E); three started to form furrows, but one of the polar lamellipodia stopped migrating, after which the furrow slowly regressed, resulting in binucleate cells (Figure

1F). The oppositely oriented locomotion of daughter cells away from one another usually stopped within 2 h, and in the remaining six cells, thin cytoplasmic bridges connecting the daughter cells persisted during the 2-h observation period, even though cleavage appeared almost complete (Figure 1G). Two of those pairs subsequently fused back to form binucleate cells; the fate of the remaining four pairs was not determined.

Cytokinesis in sub-confluent NRK cultures in the presence of blebbistatin

In sub-confluent cultures treated with 30 μM blebbistatin, the polar lamellipodium of a mitotic cell typically encountered a neighboring cell before a tight equatorial furrow was formed, which stopped the advancement, and the cell failed to form a furrow or divide (Figure 2, B-D; Table 1; Supplemental Movie 3 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1). In contrast, all control cells divided successfully in sub-confluent cultures despite the lack of polar advancement (Figure 2, A, C, and D; Table 1). This suggests that for the cytokinesis in the presence of blebbistatin, it is necessary that lamellipodia are formed around both poles and that they migrate away from one another while front-to-rear polarity is continuously maintained in both daughter cells. It further suggests that this mode of cytokinesis is dependent on polar advancement and expansion of the mitotic cell, rather than on active contraction of the contractile ring.

Cytokinesis in partially detached NRK cells in the presence of blebbistatin

To rule out the possibility that cytokinesis in the presence of 30 μM blebbistatin was driven by residual myosin II activity, I sought conditions under which cytokinesis in NRK cells was clearly dependent on the activity of the contractile ring. For instance, in *Dictyostelium*, cytokinesis in cells in suspension requires active contraction of the contractile ring (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein *et al.*, 1989; Zang *et al.*, 1997). However, consistent with earlier reports on mammalian cells (Ben-Ze'ev and Raz, 1981; Winklbauer, 1986), I found that when mitotic NRK cells were transferred to agarose plates, to which they could not adhere, cytokinesis was completely blocked even in the absence of blebbistatin (unpublished observations). I then transferred mitotic NRK cells to fresh Easy Grip tissue culture dishes containing medium and asked whether these cells could divide

while they were still in the process of secreting extracellular matrix protein to establish adhesion. Time-lapse observation of individual cells demonstrated that successful cytokinesis under these conditions yields pairs of semicircular cells (7/14 cells: Figure 3A and Supplemental Movie 4 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1), whereas failure yields single, round, binucleate cells (7/14 cells). Within 10 min of replacement of the culture medium with a trypsin-EDTA solution, all of the semicircular cells detached from the substrate to become two separated spherical cells (Figure 3B), demonstrating that these pairs of semicircular cells have indeed completed division. I thus used these morphological features to distinguish between successful and unsuccessful cytokinesis in 1,000 cells (200 cells from five independent plates) surveyed 5-10 h after transfer to fresh dishes in the presence or absence of 30 μM blebbistatin. In the absence of blebbistatin, 60±14% (average ± SD) of mitotic NRK cells divided successfully, but in the presence of blebbistatin, 96±2% of cells failed to divide when in this partially detached condition. Time-lapse observation of 18 individual cells showed that no noticeable equatorial furrows or polar lamellipodia were formed in the presence of blebbistatin; those cells eventually respread as binucleate cells (Fig. 3C and Supplemental Movie 5 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1).

Thus, 30 μM blebbistatin was clearly sufficient to inhibit contractile ring-dependent cytokinesis, which confirms that the successful cytokinesis on fibronectin-coated surfaces in the presence of 30 μM blebbistatin was mostly independent of contractile-ring activity.

Effects of a high concentration of blebbistatin on cytokinesis in NRK cells

I also examined cytokinesis of NRK cells in the presence of 100 μM blebbistatin, which reduces myosin II ATPase by >95% in vitro (Straight *et al.*, 2003; Kovacs *et al.*, 2004). Rosenblatt *et al.* (2004) recently reported that myosin II is required for efficient karyokinesis in cells in which the nuclear envelope breaks down. In that regard, I also found in a preliminary experiment that the tighter inhibition of myosin II activity achieved with the higher dose of blebbistatin resulted in frequent (41%) failure of karyokinesis, halting the process prior to anaphase (Table 1). Therefore, to focus on the effects of 100 μM blebbistatin on the furrowing process, I

analyzed in a separate experiment only those cells that initiated anaphase normally. Of 15 such cells, 12 formed lamellipodia around both poles and formed tight furrows in their equatorial regions (Table 1). In the remaining three cells, a lamellipodium was formed all along the cell following karyokinesis, but no noticeable furrows were formed. In five of the 12 cells that formed furrows, lamellipodia were not formed simultaneously around each pole. This caused the furrows to be misplaced, forming between a binucleate fragment and an anucleate fragment.

None of the cells that formed furrows in the presence of 100 μM blebbistatin ultimately divided to two separate cells within the 2-h observation period. The majority (11/12) retained the furrow (Table 1), but the daughter cells gradually lost their front-to-rear polarity, and their lamellipodial movements became fragmented and uncoordinated. This led to fragmentation of the daughter cells into several pieces connected by thin cytoplasmic strands (Figure 4A and Supplemental Movie 6 at http://www.molbiolcell.org/cgi/content/full/E05-03-0233/DC1).

Immunofluorescence microscopy revealed that high concentrations of actin and myosin II filaments were present in the equatorial regions of cells treated with 100 μM blebbistatin (Figure 4, C and E), which was similar to control cells treated with DMSO alone (Figure 4, B and D). These cells also showed extensive arrays of astral microtubules behind the lamellipodia, and in the later phases of the separation of the daughter cells, the nuclear envelopes reformed and the microtubule cytoskeletons became more like those of interphase cells (Figure 4C).

Effects of Y-27632 on cytokinesis in NRK cells

Y-27632 inhibits Rho-associated kinase (ROCK) and thus disturbs actomyosin-ring contraction by inhibiting the phosphorylation of the light chain of myosin II (Kosako *et al.*, 2000; Glotzer, 2001; Yoshizaki *et al.*, 2004). I therefore examined its effects on cytokinesis in NRK cells. Under the conditions used to produce partial detachment (see above), 17/17 mitotic cells failed to divide and subsequently respread as binucleate cells in the presence of 40 μM Y-27632. Four of these cells showed a slight equatorial furrowing (Figure 5A), which eventually regressed, but the remaining 13 did not form detectable furrows. I next evaluated the effects of Y-27632 on cytokinesis in cells adhering to fibronectin-coated substrates. In this case, 9/9 mitotic cells divided successfully in the presence of 40 μM Y-27632,

albeit somewhat more slowly than in its absence (Figure 5B). These effects of Y-27632 are similar to, although weaker than, those of blebbistatin as expected.

Adhesion-dependent and contractile-ring-independent equatorial furrowing in HT1080 cells

It is important to know whether other mammalian cells are also capable of carrying out adhesion-dependent and contractile-ring-independent cytokinesis. In that regard, I tested the effects of blebbistatin on cytokinesis in highly adherent human HT1080 fibrosarcoma cells (Rasheed *et al.*, 1974) on substrates coated with collagen type I. Upon entry into mitosis, 7/7 control cells treated with 1% DMSO rounded up, formed cleavage furrows in their equatorial regions, respread, and successfully divided into two daughter cells (Figure 6A and Table 1). In contrast, in the presence of 30 μM blebbistatin, HT1080 cells rounded up slightly and then respread without forming equatorial furrows. The majority (10/11) subsequently formed lamellipodia around both poles and tight furrows in their equatorial regions (Figure 6B and Table 1), while one failed to form lamellipodia around both poles and a tight equatorial furrow. The cells that formed tight furrows lost their oppositely oriented polarity of the presumptive daughter cells immediately after the formation of cytoplasmic bridges connecting the daughter cells. Only one cell completed the division, and two cells fused back to form binucleate cells, within the 2-h observation period. In the remaining seven cells, the cytoplasmic bridges persisted for at least 2 h (Figure 6B and Table 1). Two of those pairs subsequently fused back to form binucleate cells, and one pair eventually completed the division; the fate of the remaining pairs was not determined.

I also examined cytokinesis of HT1080 cells that initiated anaphase in the presence of 100 μM blebbistatin. Like the cells treated with 30 μM blebbistatin, these cells first rounded up and then respread. Of 11 cells, seven subsequently formed lamellipodia around both poles and tight furrows in their equatorial regions (Figure 6C and Table 1), while four failed to form lamellipodia around both poles and a tight equatorial furrow. Daughter cells of two of the seven cells that formed tight furrows eventually lost distinct polarity and became fragmented, as was the case in NRK cells in 100 μM blebbistatin. In the remaining five cells, the daughter cells fused back to form binucleate cells within the 2-h observation period (Figure 6C and Table 1).

DISCUSSION

I have shown here that NRK and HT1080 cells are able to form tight furrows in an adhesion-dependent manner, even when myosin II activity is greatly suppressed by blebbistatin. Furthermore, in the presence of 30 μM blebbistatin, the majority of the daughter NRK cells maintained front-to-rear polarity after the furrow was formed, and the resultant migration away from one another played an important role in completing the separation of the two cells. These results are inconsistent with the view that active contraction of the contractile rings is essential for equatorial furrowing and cytokinesis of mammalian cells, and suggest the critical importance of substrate adhesion in cytokinesis of adherent animal cells.

Straight *et al*. (2003) observed that blebbistatin completely inhibits contraction of the contractile rings and cytokinesis in HeLa cells and *Xenopus* tissue culture cells. I was able to confirm a strong inhibitory effect of blebbistatin on cytokinesis of HeLa cells, which round up and mostly detach from substrates during cytokinesis (unpublished observations). Therefore, my data support the view of Straight *et al*. (2003) that active contraction of contractile rings is inhibited by blebbistatin, and hence requires the activity of myosin II. They are also consistent with the data of Rosenblatt *et al*. (2004) and Komatsu *et al*. (2000), who observed that efficient karyokinesis in mammalian cells requires myosin II. On the other hand, my findings differ from those of Cramer and Mitchison (1995), who observed that the respreading of male rat kangaroo kidney epithelial (PtK2) cells after karyokinesis was inhibited by butanedione monoxime and suggested that this process is myosin II dependent. I found that even in the presence of 100 μM blebbistatin, the majority of the NRK and HT1080 cells that successfully initiated anaphase respread and formed lamellipodia, which indicates that this process is not critically dependent on myosin II in these cells. A similar result was also obtained by Pelham and Wang (1999) using 3T3 cells.

In addition, I found that NRK and HT1080 daughter cells were unable to maintain large, fan-shaped lamellipodia in the presence of 100 μM blebbistatin; instead, the uncoordinated formation of leading edges resulted in fragmentation of the cells into smaller pieces connected by cytoplasmic strands, while the original furrow was maintained. I therefore suggest that either a trace amount of myosin II activity and/or

some other factor that is inhibited by a high concentration of blebbistatin is required for maintenance of the appropriate polarity of the daughter cells. Consistent with the former possibility, it has been demonstrated that filaments of myosin II accumulate at the bases of lamellipodia in PtK 1 and REF 52 cells (Ponti *et al.*, 2004; Verkhovsky *et al.*, 1995), and inhibition of myosin light chain kinase by ML-7 resulted in the loss of polarity in interphase neutrophils (Pierini *et al.*, 2003). On the other hand, Shu *et al.* (2005) recently reported that 100 μM blebbistatin inhibits motility of mutant *Dictyostleium* cells lacking myosin II, supporting the possibility that 100 μM blebbistatin interfered with myosin II-independent processes in NRK and HT1080 cells.

In summary, then, my results suggest that three aspects of cytokinesis in NRK cells require myosin II: karyokinesis, active contraction of the contractile rings, and maintenance of cell polarity after furrow formation. Moreover, comparison of the differential sensitivities to 30 and 100 μM blebbistatin suggests that a higher level of myosin II activity is required to maintain contraction of the contractile rings than is necessary for karyokinesis or appropriate cell polarization. Most importantly, however, equatorial furrow formation in appropriately adherent cells does not require myosin II in either NRK cells or HT1080 fibrosarcoma cells.

This adhesion-dependent, contractile-ring-independent mode of cytokinesis was first characterized in the cellular slime mold *Dictyostelium discoideum* (Neujahr *et al.*, 1997; Zang *et al.*, 1997; Nagasaki *et al.*, 2002). It was named "attachment-assisted mitotic cleavage" (Neujahr *et al.*, 1997) or "cytokinesis B" to distinguish it from "cytokinesis A", which refers to the classic, adhesion-independent, contractile-ring-dependent cytokinesis (Zang *et al.*, 1997; Nagasaki *et al.*, 2002). It has been suggested that cytokinesis B is a more primitive process than cytokinesis A (Gerish and Weber, 2000; Nagasaki *et al.*, 2002). Although there are a number of observations in the literature that can be interpreted as suggesting that mammalian cells also have the capacity for cytokinesis B (reviewed by Uyeda *et al.*, 2004), the present study provides the first direct demonstration that particular mammalian cell types are able to carry it out.

The phenotypes of certain mutant animals also support the possibility that cytokinesis B plays important roles in animal cells. For instance, knocking out the single non-muscle myosin II heavy chain gene in *Drosophila* is lethal, but developing larvae appear to die because of failure in morphogenetic cell-sheet movements, while

cytokinesis proceeds normally (Young *et al.*, 1993). It is possible that maternal material present in larvae provided sufficient amounts of myosin II to drive cytokinesis, but then cytokinesis must require less myosin II activity than cell-sheet movements. Furthermore, cardiomyocytes of mice lacking the gene for non-muscle myosin IIB heavy chain show only moderate degrees of cytokinetic failure (Takeda *et al.*, 2003). These cells do not express non-muscle myosin IIA, and therefore cytokinesis of these mutant cardiomyocytes should be due either to myosin IIC expressed at low levels or to the cytokinesis B mechanism.

It has been suggested that active Rho-dependent phosphorylation of myosin II by ROCK has a facilitating, but not an essential, role in cytokinesis, so that inhibition of ROCK by Y-27632 results in a delay but not blockage of cytokinesis (Kosako *et al.*, 2000; Piekny *et al.*, 2000; Yoshizaki *et al.*, 2004; reviewed by Glotzer, 2001). I confirmed that cytokinesis is slowed by Y-27632 in adherent NRK cells. However, in partially detached NRK cells, cytokinesis was completely inhibited by 40 μM Y-27632. It may be that NRK cells are somehow more sensitive to Y-27632 than other types of cells. Alternatively, cytokinesis B may contribute to the Y-27632-resistant, slower cytokinesis reported earlier.

Nonetheless, the physiological significance of cytokinesis B in mammalian cells is not clear at this point. I speculate that the conservation of the cytokinesis B mechanism in both cellular slime molds and mammalian cells suggests that cytokinesis B has some important role. Burton and Taylor (1997) reported division of a fibroblast that was driven by oppositely oriented traction forces following regression of the initial equatorial furrow. I also observed that mitotic NRK cells that failed to constrict a furrow in the absence of blebbistatin or Y-27632 respread and formed prominent lamellipodia that moved apart in opposite directions (unpublished observations). Therefore, there seems to be a correlation between the failure of cytokinesis A and the subsequent activation of the cytokinesis B mechanism, which suggests the possibility that cytokinesis B in mammalian cells may be a backup mechanism when cells fail to undergo cytokinesis A. Piel *et al.* (2001) reported that post-anaphase repositioning of the centriole to the midbody triggers the completion of cell division and facilitates severing of the midbody. Furthermore, in adherent and flattened L929 and 3T3 cells on fibronectin-coated substrates, post-anaphase centrioles did not reposition to the midbody and rather migrated toward the lamellipodia, and the cytoplasmic bridge was

severed only after migration of the daughter cells away from one another. This behavior of L929 and 3T3 cells on fibronectin-coated substrates is reminscent of NRK cells undergoing cytokinesis B, and it is interesting to speculate that the cytokinesis B mechanism is activated by failure of the reposition of the post-anaphase centriole to the midbody.

Finally, because astral microtubules are dynamic (Canman *et al.*, 2003) and the distal ends of dynamic microtubules in interphase cells are known to activate Rac1 (Waterman-Storer *et al.*, 1999), I speculate that cytokinesis B is dependent on polar Rac activation by astral microtubules. Furthermore, in certain types of cells, antagonistic relationships between the activities of Rac and Rho-type GTPase proteins have been demonstrated, suggesting that equatorial activation of Rho-type GTPase proteins might elicit polar Rac activation, or vice versa (reviewed by Uyeda *et al.*, 2004). In addition, activation of integrin by extracellular matrix also leads to activation of Rac1 (del Pozo *et al.*, 2004), and this may be how adhesion to a fibronectin-coated substrate contributes to efficient cytokinesis B in NRK cells. It thus appears that in order to fully understand the interdependent network of regulatory pathways leading to successful cytokinesis in animal cells and, more broadly, the origin and principles of cytokinesis, it will be necessary to understand the mechanism of cytokinesis B.

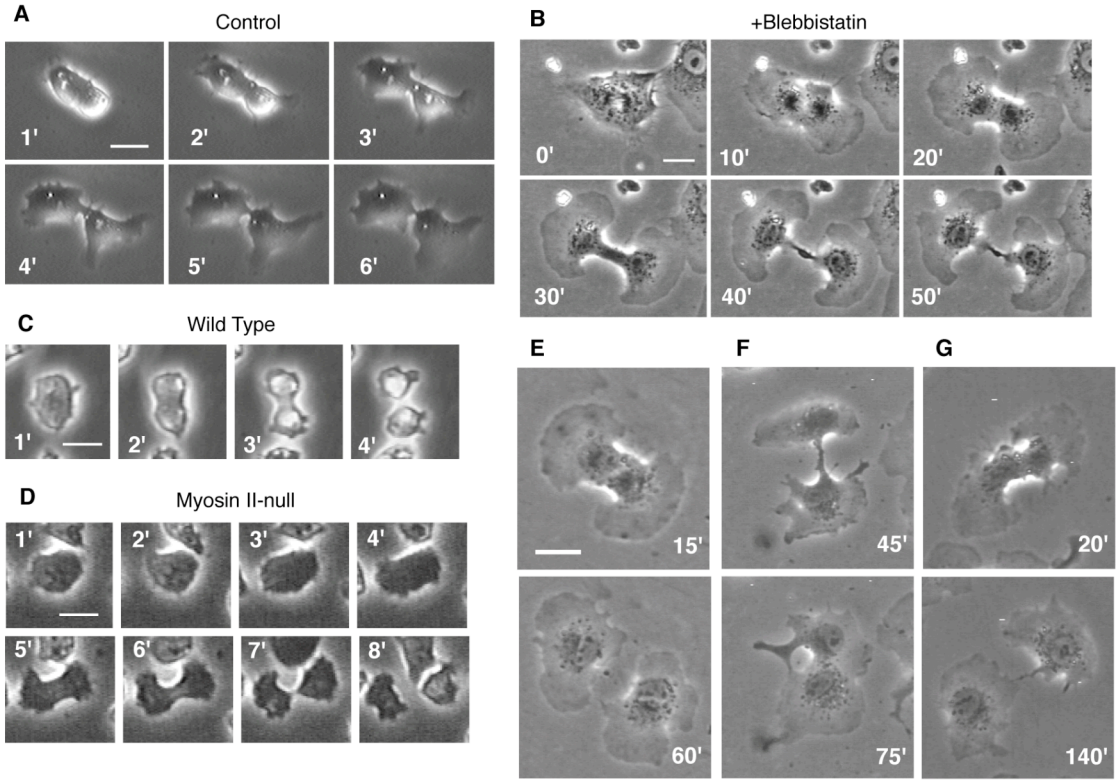

Figure 1. Cytokinesis in NRK and *Dictyostelium* cells and the effects of blebbistatin. (A, B) Cytokinesis in sparsely cultured NRK cells on fibronectin-coated substrates in the presence of 0.3% DMSO (A) or 30 μM blebbistatin and 0.3% DMSO (B). (C, D) Cytokinesis in wild-type (C) and myosin II-null (D) *Dictyostelium* cells (data modified from Nagasaki *et al*., 2002). (E, F, G) Three representative patterns of cytokinesis in NRK cells cultured as in B. See text for details. Numbers indicate time in minutes after onset of anaphase; bars indicate 20 μm (A, B, E-G) or 10 μm (C, D).

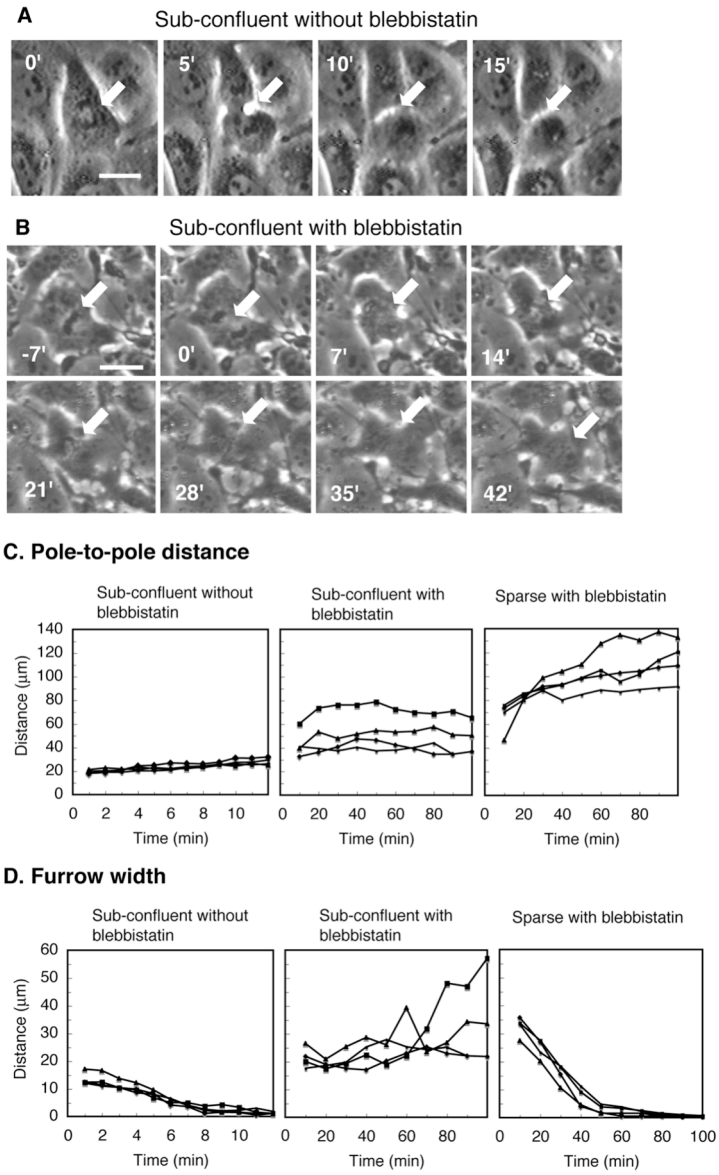

Figure 2. Failed cytokinesis in sub-confluent cultures of NRK cells on fibronectin-coated substrates in the presence of 30 μM blebbistatin. (A and B) Mitotic cells in the presence of 0.3% DMSO (A) or 30 μM blebbistatin and 0.3% DMSO (B). Arrows, the original position of the cell's equator; numbers, time in minutes after onset of anaphase; bars, 20 μm. (C and D) Changes in the distances between the poles of the daughter cells (C) and the widths of the furrows (D) in four individual mitotic cells in each of three groups: DMSO-treated cells in sub-confluent cultures (left), blebbistatin-treated cells in sub-confluent cultures (middle), and blebbistatin-treated cells in sparse cultures (right).

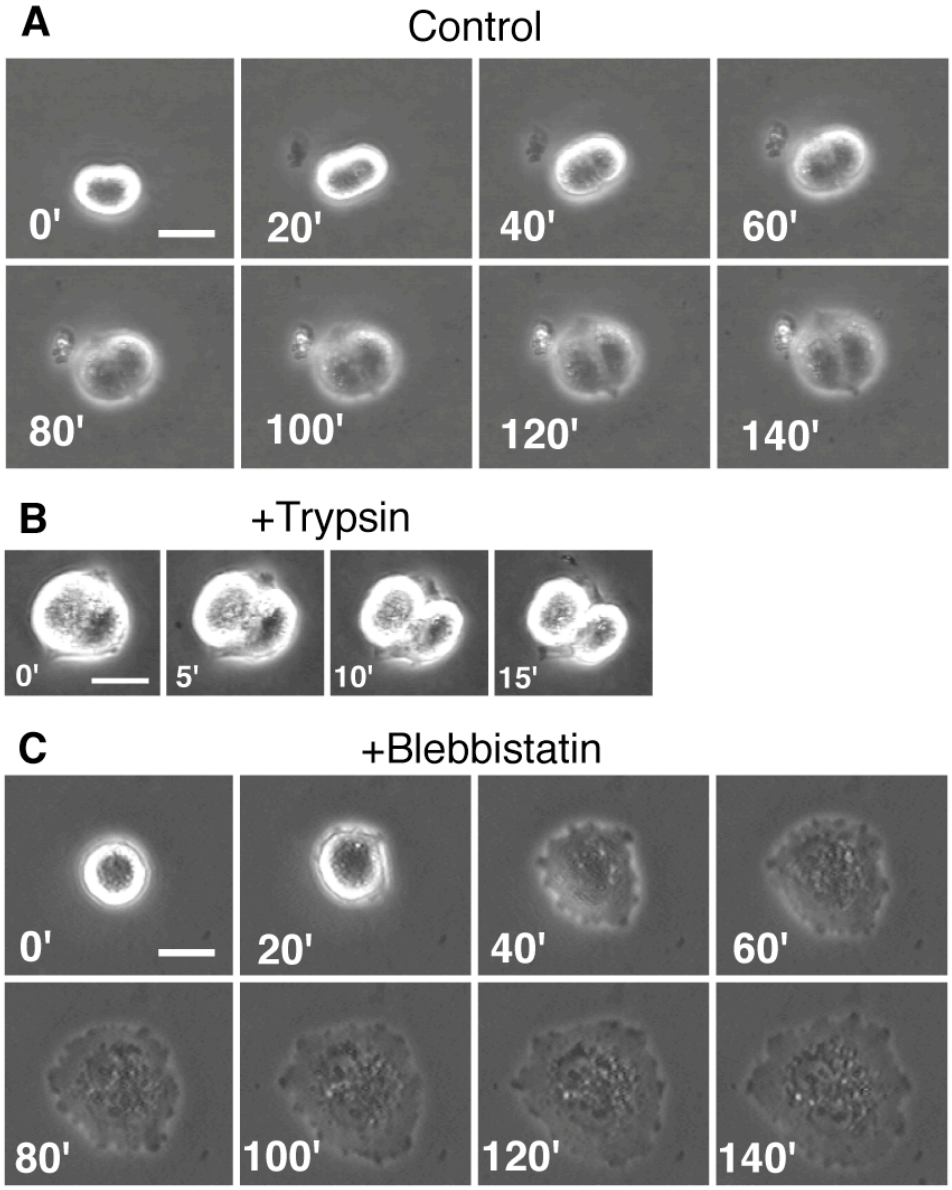

Figure 3. Effects of blebbistatin on cytokinesis in partially detached NRK cells. Mitotic cells were harvested and replated on fresh Easy Grip tissue culture dishes. (A) Successful cytokinesis of a control cell. (B) Successful cytokinesis in (A) was confirmed by the formation of two spherical cells after trypsinization. (C) Failed cytokinesis in the presence of 30 μM blebbistatin. Numbers, time in minutes after onset of anaphase (A and C) or after addition of trypsin (B); bars, 20 μm.

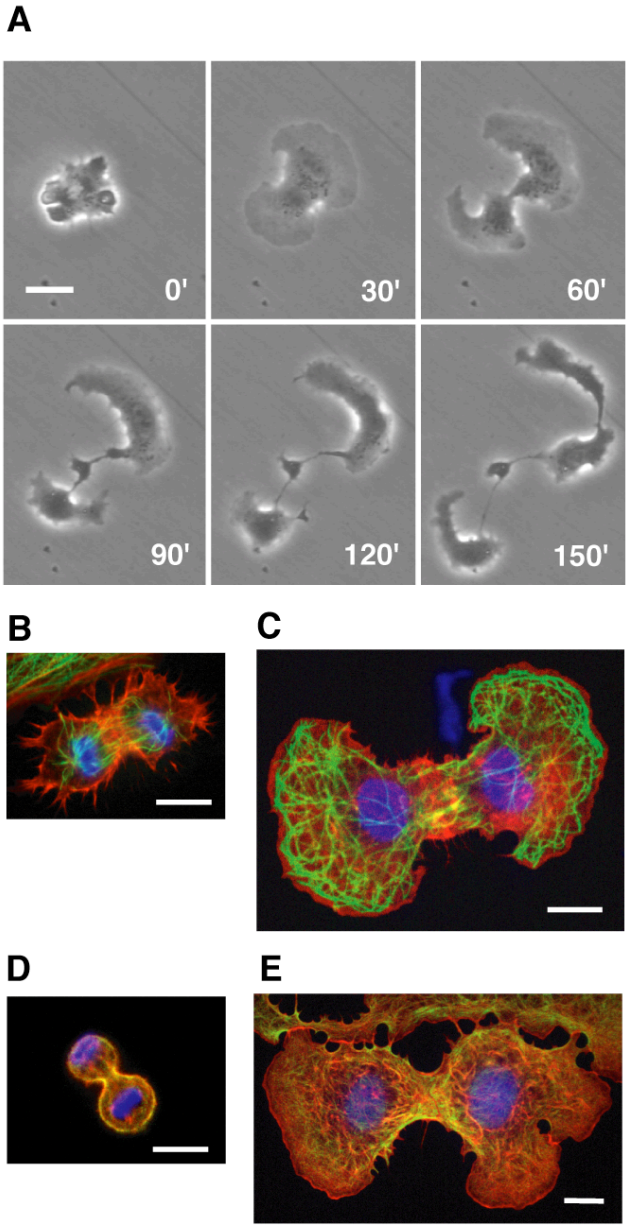

Figure 4. Failed cytokinesis in NRK cells on fibronectin-coated substrates in the presence of 100 μM blebbistatin. (A) Time-lapse microscopy of an abnormal division. Numbers indicate times in minutes after onset of anaphase. (B-E) Cytoskeletal organization of cells treated with 1% DMSO as a control (B and D) or 100 μM blebbistatin plus 1% DMSO (C and E) during cytokinesis. Cells were immunostained with anti-tubulin antibody (B and C) or anti-myosin-IIA antibody (D and E), followed by a mixture of Alexa 488-conjugated secondary antibodies (green), Hoechst 33258 for DNA (blue), and rhodamine-phalloidin for actin filaments (red). Bars, 20 (A) or 10 (B-E) μm.

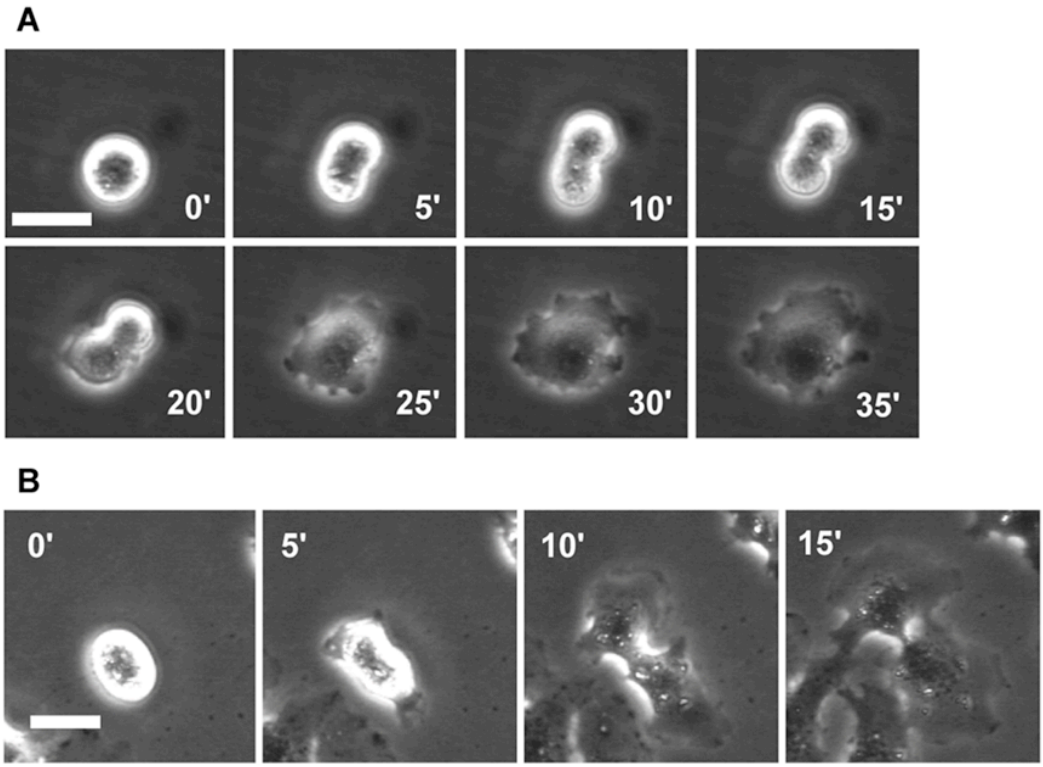

Figure 5. Effects of 40 μM Y-27632 on cytokinesis in NRK cells. (A) Failed cytokinesis in a partially detached cell. (B) Successful cytokinesis in a cell on a fibronectin-coated substrate. Numbers, time in minutes after onset of anaphase; bars, 20 μm.

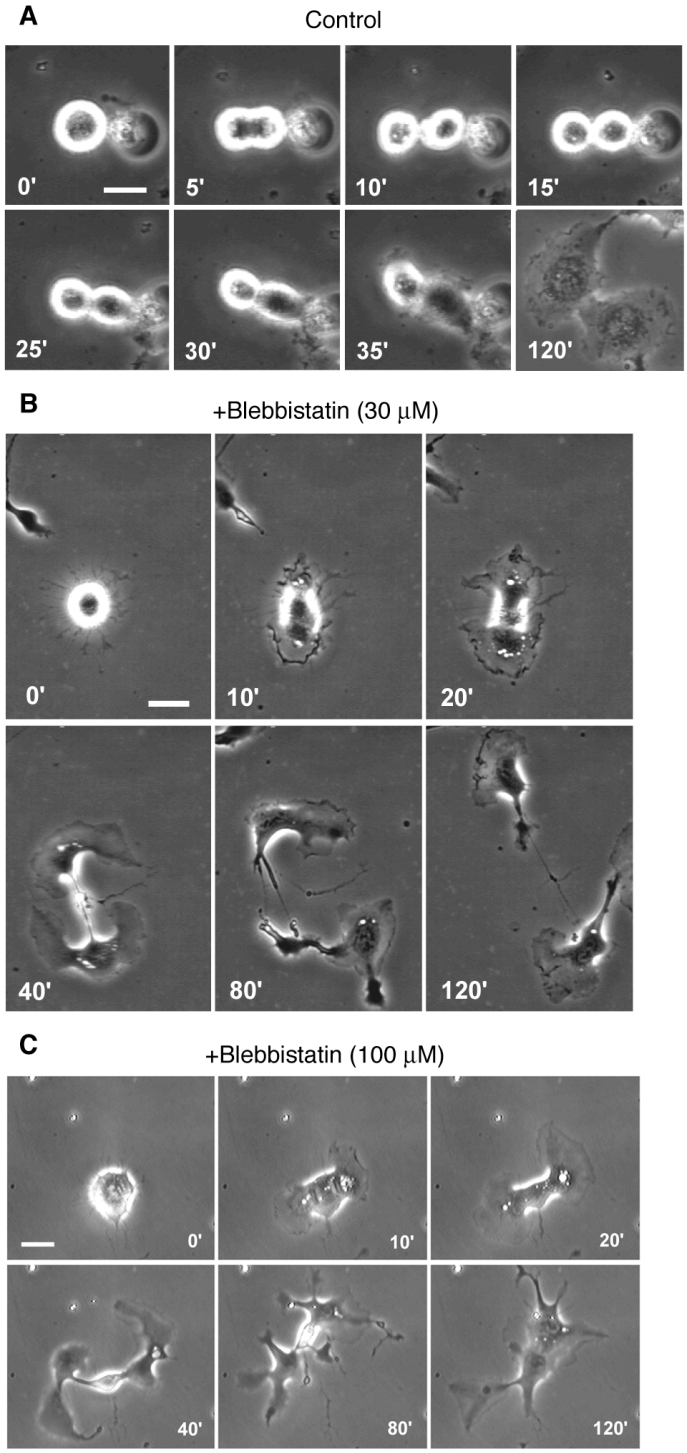

Figure 6. Cytokinesis in HT1080 cells on substrates coated with type I collagen in the presence of 1% DMSO (A), 30 μM blebbistatin plus 0.3% DMSO (B), or 100 μM blebbistatin plus 1% DMSO (C). Numbers, time in minutes after onset of anaphase; bars, 20 μm

Table 1. **Effects of blebbistatin on three steps of cytokinesis of adherent NRK and HT1080 cells**

Cell	Cell density	No. of cells examined	Blebbistatin (μM)	Karyokinesis (%)	Furrow formation* (%)	Scission# (%)	Regression# (%)	Bridged# (%)
NRK	Sparse	9	0	ND	100	100	0	0
NRK	Sparse	16	30	100	100	44	19	38
NRK	Sparse	15	100	59@	80	0	7	73
HT1080	Sparse	7	0	ND	100	100	0	0
HT1080	Sparse	11	30	100	91	9	18	64
HT1080	Sparse	11	100	ND	64	0	45	18
NRK	Sub-confluent	15	0	ND	100	100	0	0
NRK	Sub-confluent	10	30	ND	0	-	-	-

ND: not determined.

*Frequencies of furrow formation among cells that completed normal karyokinesis.

Fractions of cells among those that formed tight furrows that completed successful scission, regressed to form binucleate cells, or remained connected by cytoplasmic bridges.

@ This datum was obtained in an experiment involving 22 cells. The other data in this line are from a separate experiment involving 15 cells.

CHAPTER III

NOVEL FUNCTIONS OF ECT2 IN POLAR LAMELLIPODIA FORMATION AND POLARITY MAINTENANCE DURING "CONTRACTILE RING-INDEPENDENT" CYTOKINESIS IN ADHERENT CELLS

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ABSTRACT

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, I 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 me 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 the separation of the two daughter cells is regulated 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 in 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 expression-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 (Chapter II; 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 conditions. 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 coordinate cytokinesis A and B to ensure successful cell division.

In the present study, I 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 (Chapter II; Kanada *et al.*, 2005). My 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, USA), and was cloned into pEGFP-C3 (Clontech, USA) 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. All DNA constructs were confirmed by sequencing.

Depletion of Ect2 was achieved using the 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 (http://www.dharmacon.com/sidesign/default.aspx), which was followed by the loop sequence 5'-ACGTGTGCTGCTGTCCGT-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; 1: 200 dilution) and anti-β-actin (Clone AC-74, Sigma, USA; 1:2,000 dilution) antibodies. The secondary antibody was horseradish peroxidase-conjugated anti-mouse antibody (474-1806, KPL, USA; 1:3,000 dilution). Chemiluminescence (Super Signal West Dura Extended Duration Substrate, Pierce, USA) was detected using an Las-3000 imager (Fujifilm, 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 (Gibco, USA). HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% antibiotic-antimycotic.

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

Observation of Live Cells

To assess cytokinesis B in HT1080 cells, untreated polystyrene dishes (Asahi

Techno Glass Co., Japan) were coated with 0.01% collagen type I solution (IFP 9660, Research Institute for the Functional Peptides, Japan) overnight (Chapter II; Kanada *et al.*, 2005). The S-(-)-enantiomer of blebbistatin (Toronto Research Chemicals, Canada) used to inhibit myosin II, and thus cytokinesis A, was dissolved at 10 mM in DMSO. CT04 (Cytoskeleton Inc., USA), the cell permeable derivative of C3 exoenzyme, was dissolved at 200 μg/ml in 50 % glycerol. HT1080 cells were incubated in the presence of 5 μg/ml CT04 for 2 hours under the growth condition before observation.

Cells were maintained at a constant temperature in stage incubators (Onpu-4; Taiei Denki, Japan; or MI-IBC, Olympus, Japan) attached to inverted microscopes (IX50 or IX71; Olympus, Japan) and observed using a 20X 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, Japan) controlled with the IPLab software (Solution Systems, 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 Co., Japan) were treated with hexamethyldisilazane (Shin-Etsu Chemical, Japan) to increase its hydrophobicity before coating it with collagen as described above. Forty-five min after addition of blebbistatin (30 μM), 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, USA) and 1 μg/ml Hoechst 33258 (Wako Pure Chemical).

Immunostained cells were observed using an inverted microscope (IX-70, Olympus) equipped with a confocal laser scanning unit (CSU 10, Yokogawa, 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 (Amersham Biosciences, USA). 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 $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10% glycerol supplemented with 1 mM dithiothreitol, 50 mM NaF, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 70 μg/ml tosyl phenylalanylchloromethyl ketone, 75 μg/ml p-toluenesulfonyl-L-arginine methyl ester, 2 μg/ml aprotinin and 160 μg/ml benzamidine. To assay Rac1 activity, cells on collagen-coated dished were lysed 24 h after transfection in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100 and 10% glycerol supplemented with 1 mM dithiothreitol, 50 mM NaF, 1 mM $NaVO_4$ 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 5 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, USA; 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 by (the density of GTP-RhoA or Rac1 band) / (the density of total RhoA or Rac1 band) and expressed as ratios to those in control cells expressing mCherry alone.

RESULTS

Ect2 is not essential for HT1080 cell growth

I 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), while Ect2 was immunostained in cells fixed with formaldehyde. I 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, I 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 h 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 resulted 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 me 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

I used time-lapse video microscopy 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 ± 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 about 6.3 ± 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 2A, B and Supplemental Movie 1, 2 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). The accelerated ingression in Ect2-depleted cells suggests that one of the 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 (Figure 3A), and were unable to form equatorial furrows (N = 6; Figure 3B and Movie 7 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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). I 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

I then used time-lapse video microscopy 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 4A and Supplemental Movie

3 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 while 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 in the presence of 30 μM blebbistatin was estimated to be 71%, although all of them temporalily 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 4B and Supplemental Movie 4 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). This group showed a delay in respreading following the onset of anaphase; all other processes during cytokinesis B appeared normal. Six cells were classified as "delayed and uncoordinated" (Figure 4C and Supplemental Movie 5 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 4C at 50 min). The remaining two cells were classified as "delayed and one-sided" (Figure 4D and Supplemental Movie 6 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 μM 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, I measured changes in distance from pole to pole during cytokinesis B in the control and Ect2-depleted cells

(Figure 4E). The "one-sided" group was excluded from this analysis because expansion between the two poles was unobservable. I found that the rate of the 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 conrol cells, Ect2-depleted cells fail to form polar lamellipodia immediately following 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, I 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 5A). 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, I 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 in Ect2-depleted cells (Figure 5B). 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 (Figure 3B and Supplemental Movie 7 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 me 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 ± 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 6B and Supplemental movie 8 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). It thus appears that the mechanism that regulates lamellipodia formation was dramatically switched from Rho-dependent to Rho-independent at around 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* (Tatsumoto *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, I 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.

I first used confocal microscopy to confirm that Ect2-DA localized at the cell cortex of interphase HT1080 cells (Figure 7A). 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. I next used time-lapse video microscopy to observe lamellipodia formation by cells plated on highly adherent collagen-coated surfaces. Control cells showed distinct motility (Figure 7B and Supplemental Movie 9 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1) 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 (Figure 8C and Supplemental Movie 11 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 7B), indicating that the cell periphery was thick. In addition, these cells formed short-lived protrusions that were pulled back immediately, resulting in marked inhibition of cell migration (Figure 7B and Supplemental Movie 10 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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 (Figure 8, A and B).

Cells expressing mCherry-Ect2-DA were able to grow at a reasonable rate in the presence of G418 for more than one month, and the above-mentioned phenotype was maintained during this period (Supplemental Movie 12 at http://www.molbiolcell.org/cgi/content/full/E07-04-0370/DC1). 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, I 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 2 fold in mCherry-Ect2-DA expressing cells. In contrast, the expression of mCherry-Ect2-DA had a weak inhibitory effect on Rac1 (Figure 7C). Apparently, Ect2 predominantly regulates the activity of RhoA in HT1080 cells.

DISCUSSION

Different roles of Ect2 during different phases of cytokinesis A

I 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, while 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. I thus propose another explanation: 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 this idea, I 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 (unpublished observations).

Regulation of lamellipodia formation by Ect2 during cytokinesis B

I found that Ect2-depleted HT1080 cells are slow 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, while Rho regulates contractility at the tail (Burridge 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. I 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, I propose the following model for RhoA-dependent lamellipodia formation around the poles of mitotic cells undergoing cytokinesis B (Figure 9). Upon 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 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, I 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 my experiment, the activity of Rac1 was not dramatically suppressed by overexpression of Ect2-DA, though the activity of RhoA was strongly enhanced. I suggest that during cytokinesis active RhoA might regulate Rac1 effecter 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 effecter 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 effecter molecules without suppressing Rac1 activity. According to this model, equatorial RhoA activated by Ect2 during cytokinesis B might suppress Rac1 effecter, 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 mechanism to sense that cytokinesis A has failed. Continued study of the mechanisms underlying cytokinesis B will surely lead me to a more comprehensive understanding of how the multiple modes of cytokinesis are regulated in a cooperative manner.

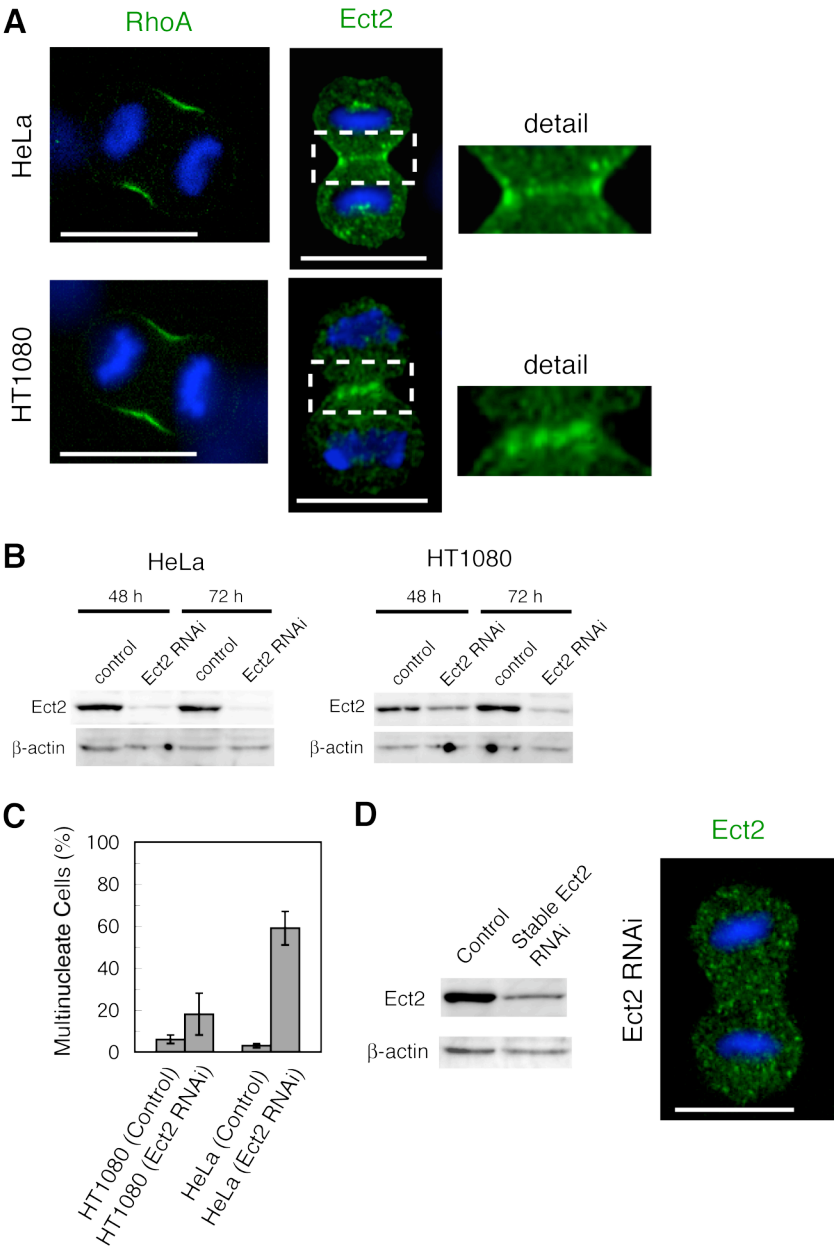

Figure 1. Localization of RhoA and Ect2 during cytokinesis and the effects of Ect2 depletion on cytokinesis in HeLa cells and HT1080 cells. (A) Confocal microscopic images of mitotic HeLa and HT1080 cells showing the distributions of RhoA (green), Ect2 (green) and DNA (blue); scale bars, 10 μm. Magnified 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 h 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 (%) ± 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 μm.

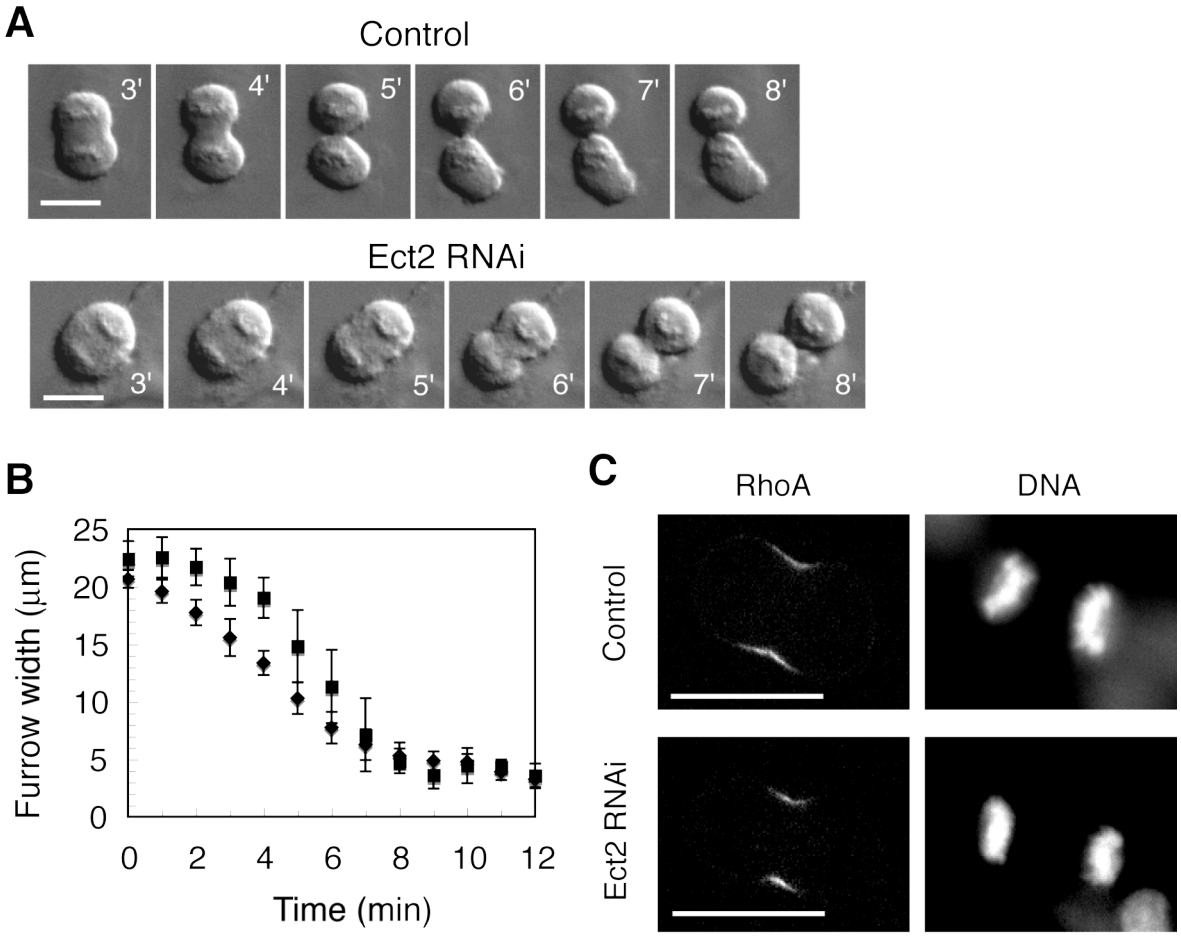

Figure 2. Effects of Ect2 depletion on cytokinesis A. (A) Cytokinesis A in control and Ect2-depleted HT1080 cells. Numbers indicate the time in minutes after onset of anaphase; scale bars, 20 μm. (B) Changes in the widths of the furrows in control (diamonds) and Ect2-depleted (squares) cells after anaphase onset. Values are shown as means ± 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.

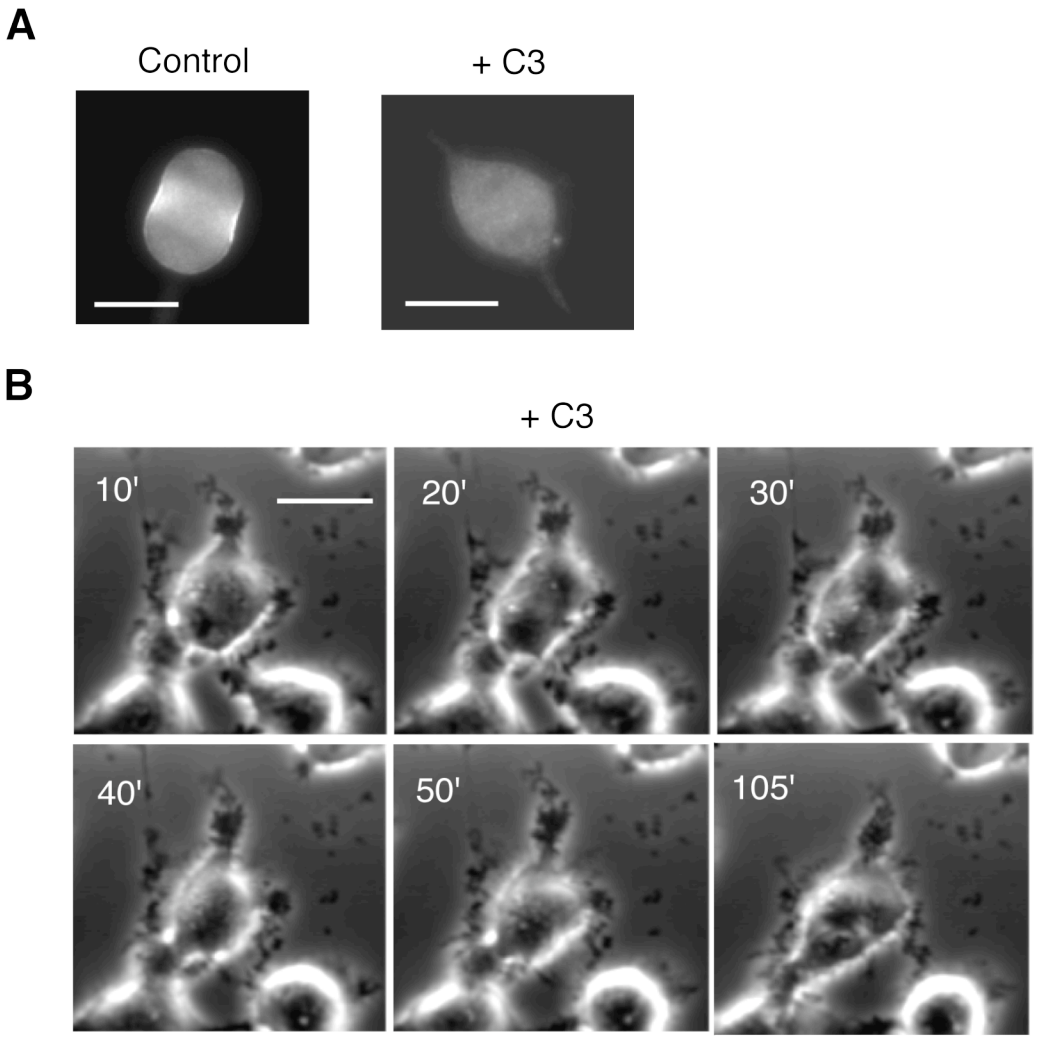

Figure 3. Effects of the cell permeable derivative of C3 exoenzyme on RhoA localization and cytokinesis. (A) Control and C3-loaded HT1080 cells were stained with anti-RhoA antibody after fixation with TCA; scale bars, 20 μm. (B) Failed cytokinesis in a C3-loaded HT1080 cell on a substrate without collagen coating. Numbers indicate the time in minutes after onset of anaphase; scale bar, 20 μm.

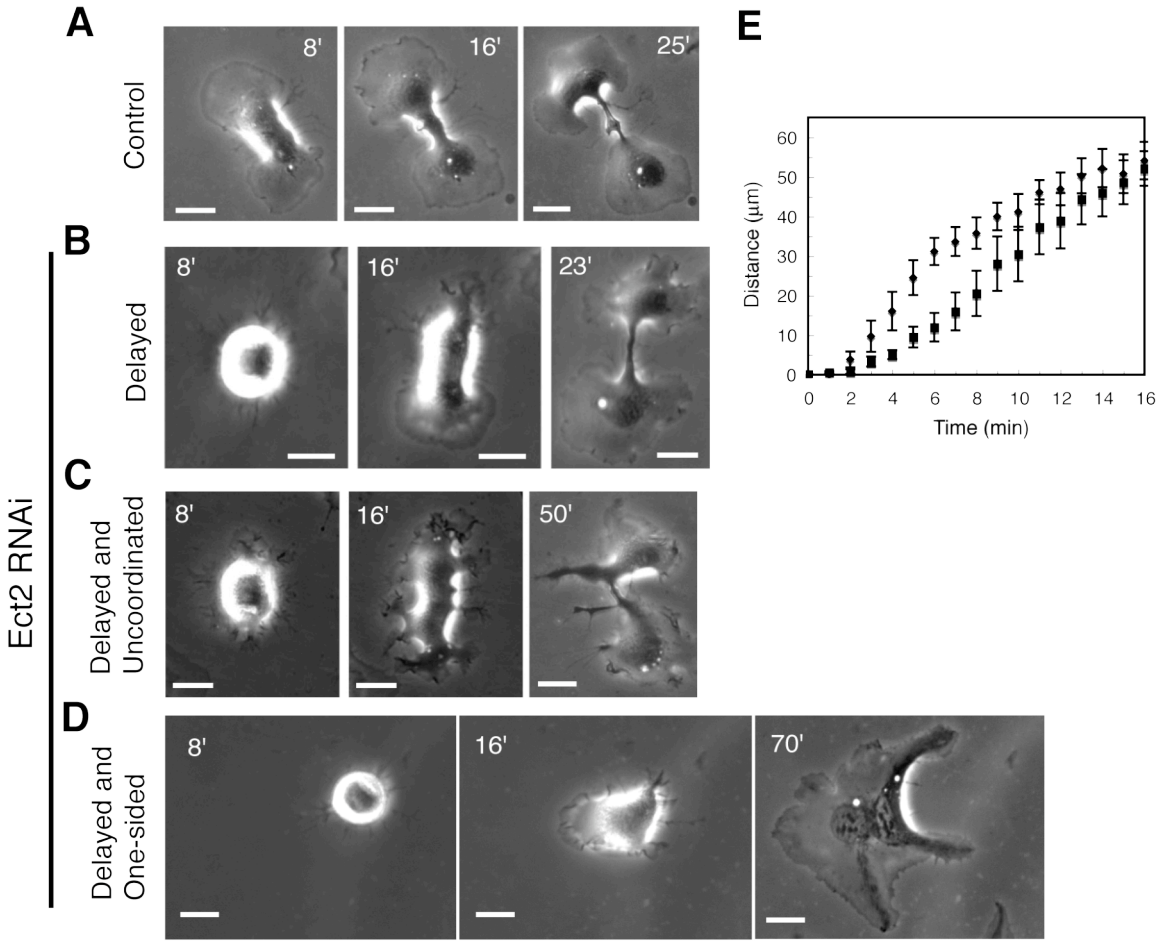

Figure 4. Effects of Ect2 depletion on cytokinesis B. (A) Cytokinesis B in control HT1080 cells on collagen coated substrates in the presence of 30 μM blebbistatin. (B-D) Three representative patterns of cytokinesis B in Ect2-depleted cells cultured as in A. See text for details. Numbers indicate the 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 ± SEM (n=8). The differences between the two cell lines are statistically significant ($P < 0.05$) at 4 min and 8 min after anaphase onset (Mann-Whitney test).

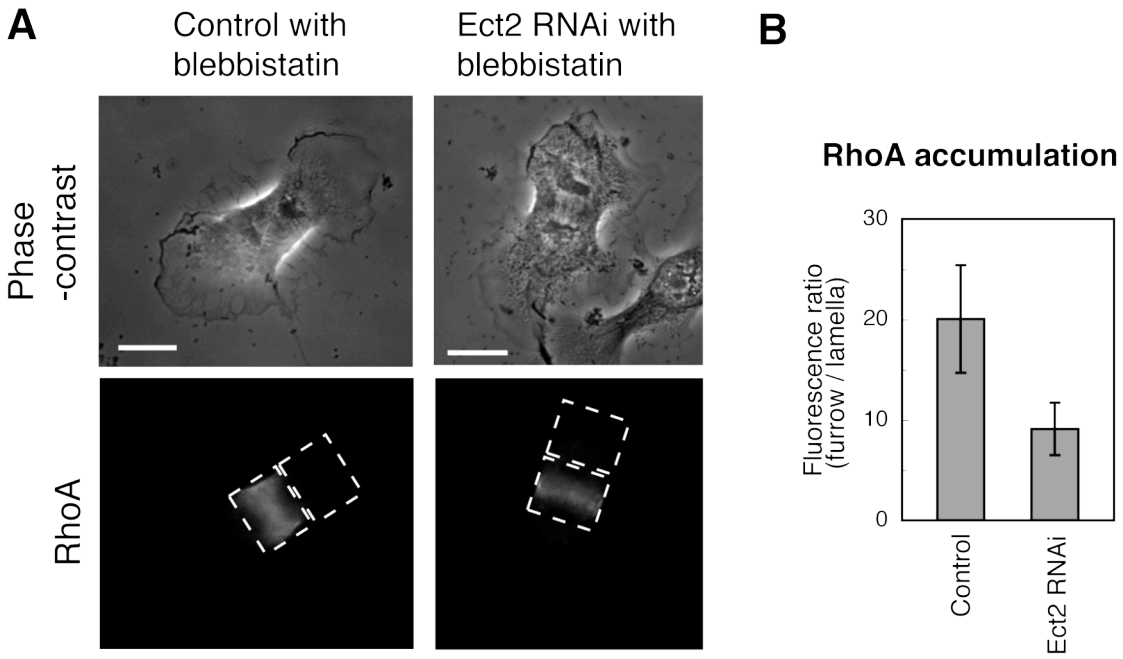

Figure 5. Effects of Ect2 depletion on RhoA localization during cytokinesis B. (A) Phase contrast and conventional epifluorescence micrographs of control and Ect2-depleted HT1080 cells undergoing cytokinesis B on collagen-coated substrates in the presence of 30 μM blebbistatin. The cells were stained by anti-RhoA antibodies. Dashed boxes (20 × 25 μm) represent regions in which relative RhoA levels were measured; scale bars, 20 μm. (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 ± SEM (n=14 and 12 for control cells and Ect2-depleted cells, respectively).

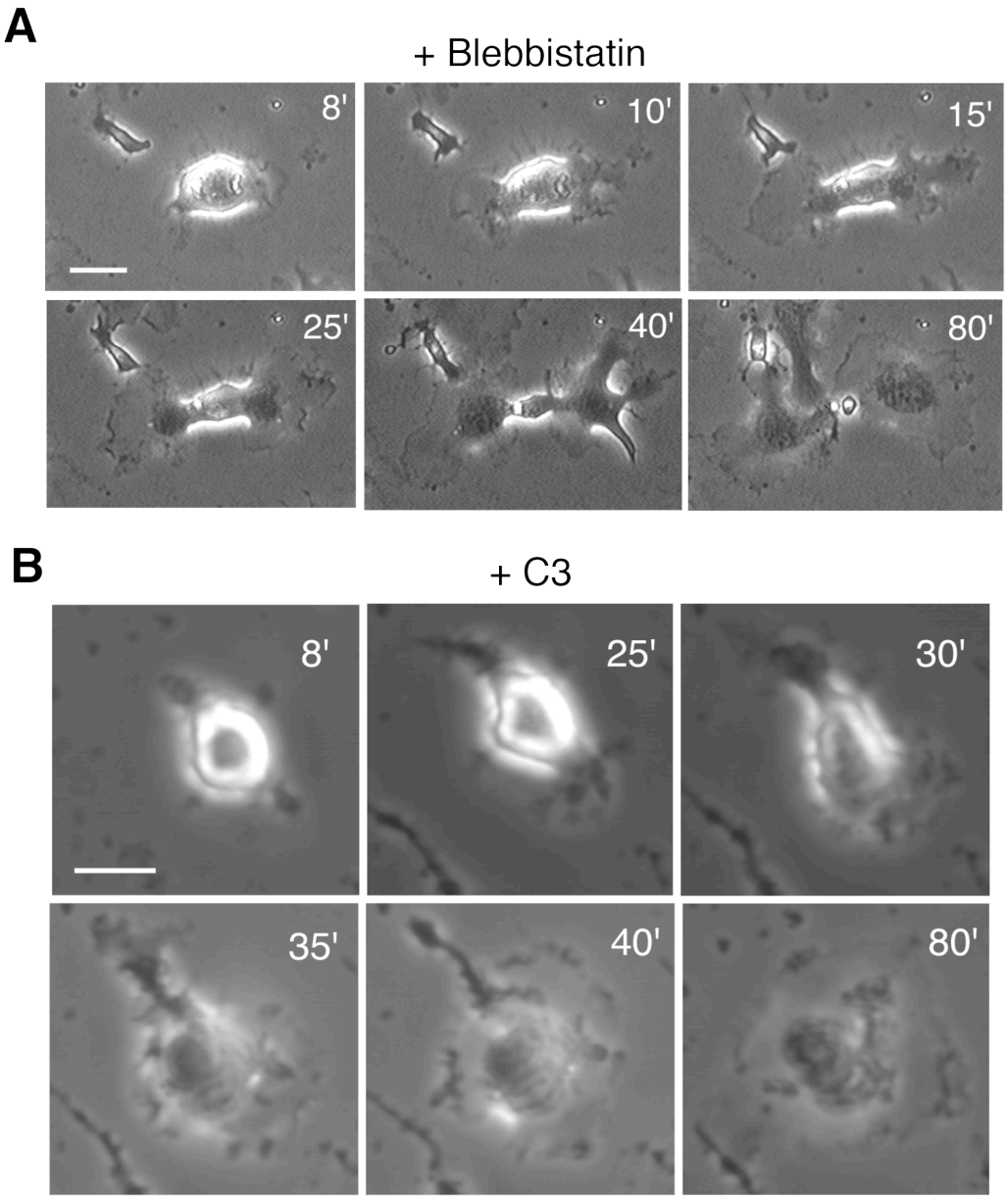

Figure 6. Effects of the cell permeable derivative of C3 exoenzyme on cytokinesis B. (A) Cytokinesis B in an HT1080 cell on a collagen-coated substrate in the presence of 30 μM blebbistatin. (B) Failed cytokinesis B in a C3-loaded cell on a collagen-coated substrate. Numbers indicate the time in minutes after onset of anaphase; scale bars, 20 μm.

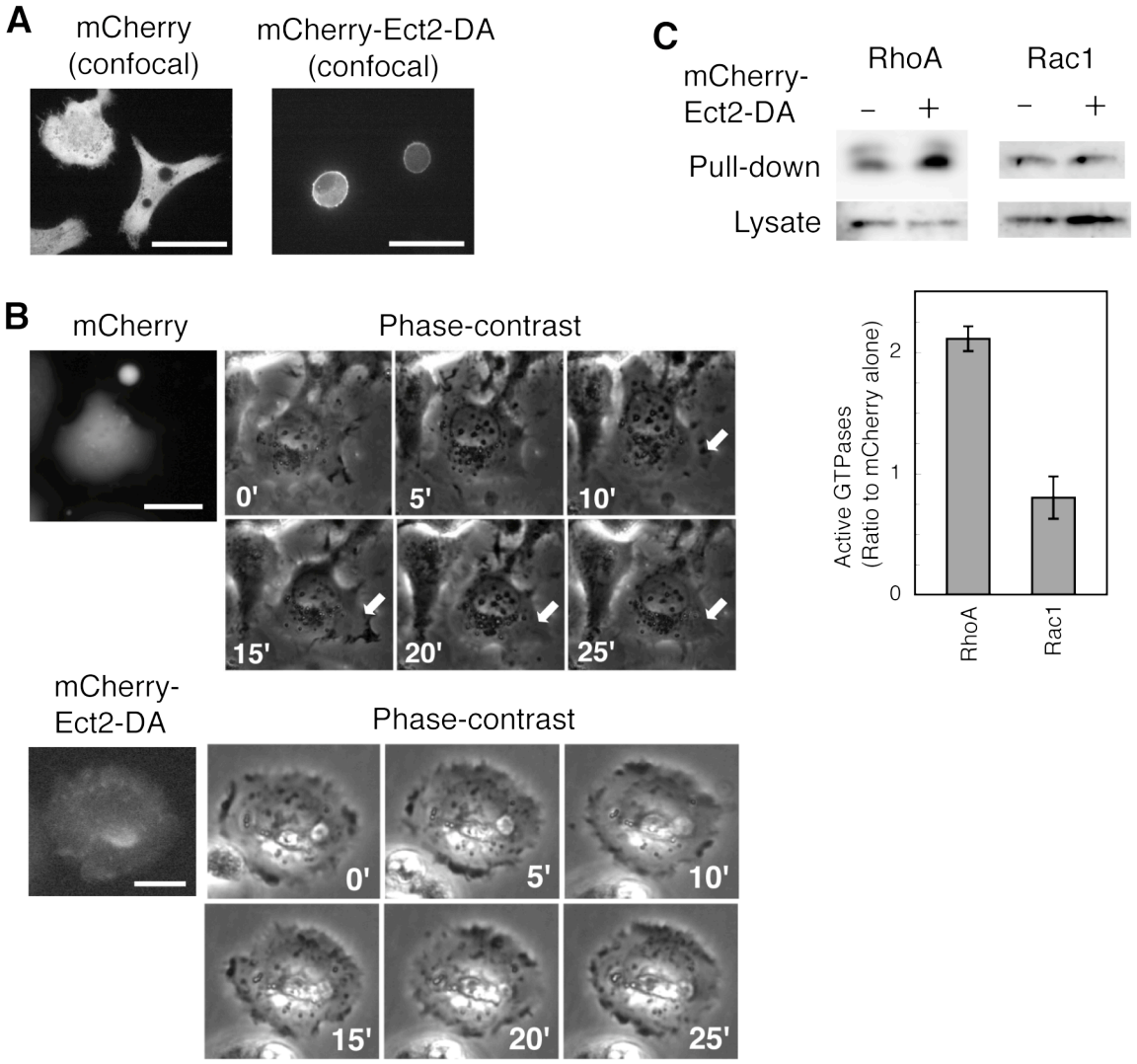

Figure 7. Activation of RhoA and inhibition of lamellipodia formation by mCherry-tagged Ect2-DA. (A) HT1080 cells were transfected with plasmids encoding either mCherry-Ect2-DA or mCherry. Twenty-four hours later the cells were transferred to glass-bottomed dishes for confocal microscopic observation; scale bars, 20 μm. (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 μm. (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 ± SD, n=3 and 4 for RhoA and Rac1, respectively) are shown.

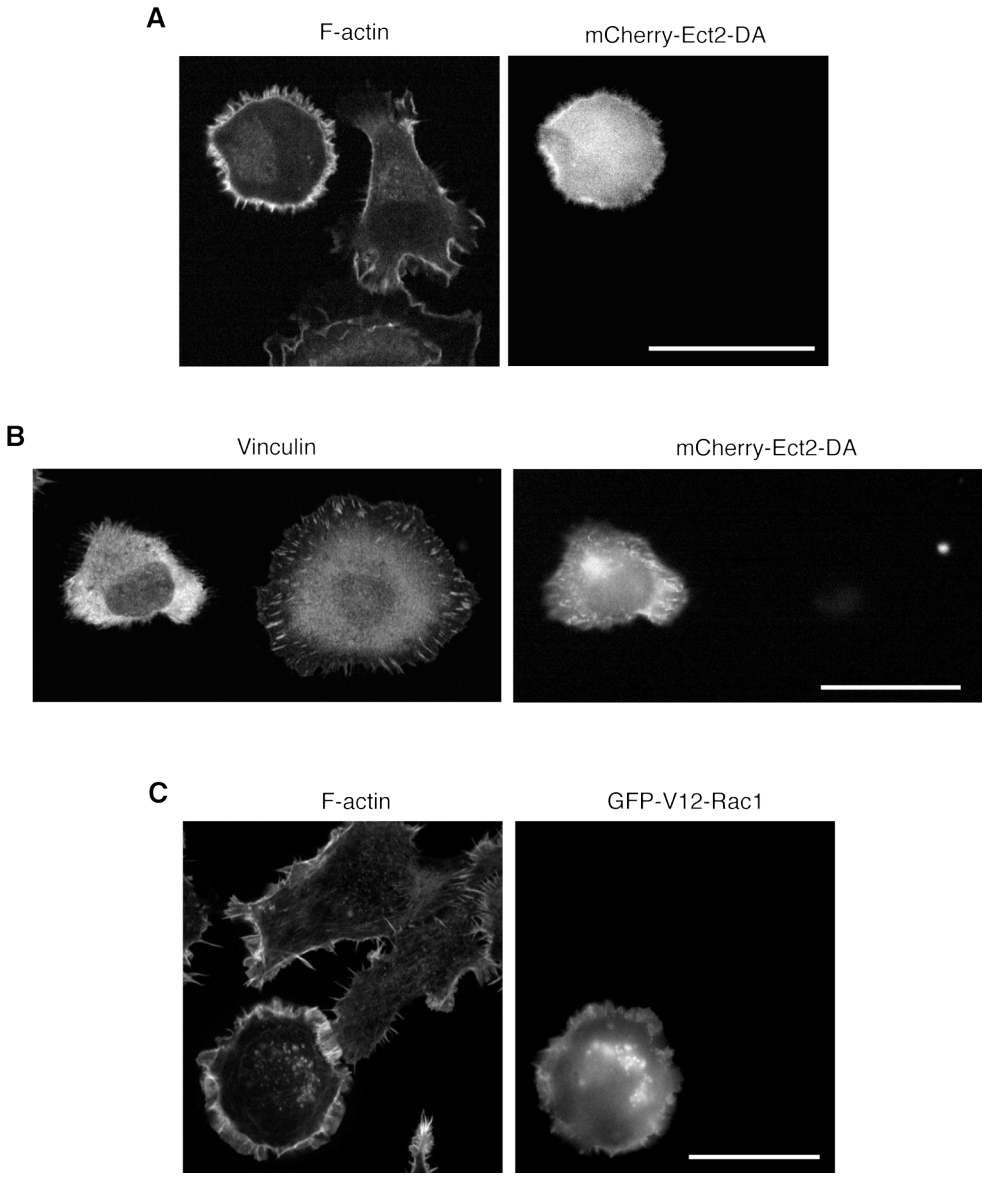

Figure 8. Actin and focal adhesion remodeling induced by overexpression of mCherry-Ect2-DA. (A, B) HT1080 cells were transfected with a plasmid encoding mCherry-Ect2-DA, and the actin cytoskeleton and the focal adhesions were visualized using FITC-labeled phalloidin (A, left) and anti-vinculin antibody (B, left; Clone hVIN-1, Sigma), respectively. Cells expressing mCherry-Ect2-DA was identified by mCherry fluorescence (right). (C) HT1080 cells were transfected with a plasmid encoding GFP-V12-Rac1 (unpublished). The actin cytoskeleton was visualized using rhodamine-labeled phalloidin (left). The expression of GFP-V12-Rac1 was confirmed by GFP fluorescence (right). Scale bars, 20 μm.

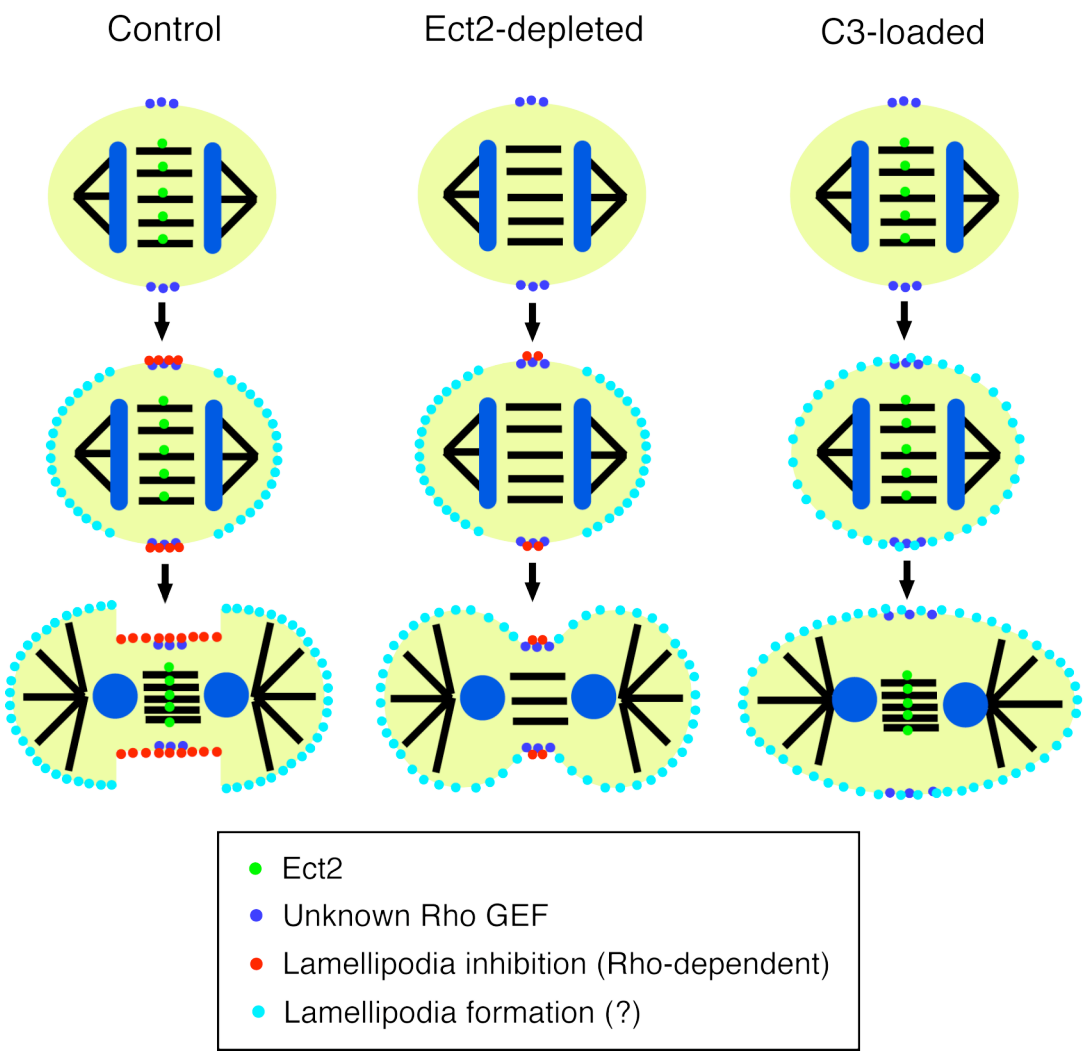

Figure 9. A model of Rho-dependent polar lamellipodia formation during cytokinesis B. Ect2 and an unidentified Rho GEF accumulate at the central spindle and the equatorial cortex to activate RhoA, respectively (top and middle in Control). The two GEFs then induce broad RhoA accumulation at the equatorial 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.

CHAPTER IV

CONCLUDING REMARKS AND FUTURE DIRECTIONS, POSSIBLE REASONS WHY ADHERENT MAMMALIAN CELLS HAVE TWO MODES OF CYTOKINESIS

Concluding remarks and future directions

Coordinated regulation of cytokinesis A and cytokinesis B

Through this study, I have shown that certain types of adherent mammalian cells have two modes of cell-cycle-coupled cytokinesis. These cells formed large fan-shaped lamellipodia around both poles when the contractile ring activity was inhibited, whereas the cells dividing by the contraction of the contractile rings did not form these characteristic polar lamellipodia. I thus speculate that contraction of contractile rings (cytokinesis A) and formation of polar lamellipodia (cytokinesis B) are regulated in a coordinated manner. In other words, cells appear to activate the cytokinesis B mechanism following the failure of the cytokinesis A mechanism. If this is the case, how do the cells sense that cytokinesis A mechanism has failed and consecutively activate the cytokinesis B mechanism? This is the most intriguing question that remains. Both cytokinesis A and cytokinesis B occur after chromosome separation. In addition, contractile rings during cytokinesis A are present in equatorial region, whereas lamellipodia is formed around both poles. Thus, there appears to be a negative spatio-temporal correlation between cytokinesis A and cytokinesis B, and there must be control systems that enable the two modes of cytokinesis to be coordinated. In this regard, in Chapter III (Kanada *et al.*, 2008), I have shown that equatorial Ect2 locally suppresses lamellipodia formation via RhoA activation, which indirectly contributes to restricting lamellipodia formation to polar regions during cytokinesis B. This finding indicates that Ect2 and RhoA are not only important for cytokinesis A, but also for the regulation of cell polarity during cytokinesis B. RhoA thus may be a key regulator that coordinates the two modes of cytokinesis in adherent mammalian cells.

One direction of research to fully understand the interdependent network of regulatory pathways between the two modes of cytokinesis is to identify all the key components involved in these processes. In this regard, the research of cytokinesis B mechanism in *Dictyostelium* as a molecular genetic model system has been very useful (Nagasaki et al., 2001), but recent progress in proteomics and RNAi technologies is enabling rapid and exhaustive identification of genes and proteins involved in cytokinesis in mammalian cells as well (reviewed by Friedman and Perrimon, 2004). By revealing the functions of these key components, I will be able to understand the principal biophysical and biochemical mechanisms underlying cytokinesis in adherent mammalian cells.

Correlation between cytokinesis A and cytokinesis B through regulation of MT dynamics

I have found a novel mechanism of the midzone MT stabilization to maintain midzone structure via RhoA activation, which ensures proper regulation of MT organization during cytokinesis (Kanada *et al.*, submitted). Based on these results, I hypothesize that the cells may change the dynamics of MTs through spatio-temporal regulation of RhoA activation when cytokinesis A has failed. MTs in the equatorial region are stabilized, while astral MTs are dynamic during cytokinesis (Canman et al., 2003; Shannon et al., 2005). In addition, stable MTs often correlate with the activation of Rho (Ishizaki et al., 2001; Palazzo et al., 2001; Ren et al., 1999), whereas dynamic MTs appear to correlate with the activation of Rac (Waterman-Storer et al., 1999). In my hypothesis, equatorial RhoA could modulate the global activity of Rho-type small GTPases during cytokinesis through the regulation of MT dynamics. To examine this interesting possibility, I should study the changes of MT dynamics and Rac activities in the polar regions when the contractile ring activity is locally inhibited, and analyze the correlation between the two. For this purpose, various live cell imaging techniques such as fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) are useful.

Significance of cytokinesis B mechanism *in vivo*

Cytokinesis B mechanism has not yet been demonstrated *in vivo*, but there are some pieces of evidence suggesting the existence. For instance, knocking out the single non-muscle myosin II heavy chain gene in *Drosophila* is lethal, but developing larvae appear to die because of failure in morphogenetic cell-sheet movements, while cytokinesis proceeds normally (Young *et al.*, 1993). It is possible that maternal material present in larvae provided sufficient amounts of myosin II to drive cytokinesis, but then cytokinesis must require less myosin II activity than cell-sheet movements. Furthermore, cardiomyocytes of mice lacking the gene for non-muscle myosin IIB heavy chain show only moderate degrees of cytokinetic failure (Takeda *et al.*, 2003). These cells do not express non-muscle myosin IIA, and therefore cytokinesis of these mutant cardiomyocytes should be due either to myosin IIC expressed at low levels or to the cytokinesis B mechanism. Before further studying the significance of cytokinesis B mechanism *in vivo*, I should first establish the cells that can divide using contractile ring-independent method *in vivo*. One approach for this purpose is to establish a cell line that can divide via only contractile ring-independent method *in vitro,* and then to examine whether they can form a

tumor under the skin of nude mouse. If the tumor formation is found, it means that the cells that cannot divide in a contractile ring-dependent manner can divide *in vivo*, suggesting the possibility that cytokinesis B mechanism is important *in vivo*.

Regarding the significance of cytokinesis B mechanism *in vivo*, there might be a concern that the cells in multicellular organisms are unable to undergo cytokinesis B because the advancement of polar lamellipodia should be inhibited by the surrounding cells, as was the case of blebbistatin-treated NRK cells in subconfluent cultures (Chapter II). To this concern, I can provide reasonable explanations. My experiments were performed in two-dimentional culture conditions, but the advancement of the polar lamellipodia may not be disturbed by surrounding cells *in vivo*, since the cells in three-dimentional environments are able to extend lamellipodia in any directions while avoiding encounters with the surrounding cells. Furthermore, I found that myosin II also localizes behind the polar lamellipodia during cytokinesis B, and the polar lamellipodia were fragmented when the activity of myosin II was inhibited almost completely by a high dose of blebbistatin (Chapter II). This finding suggests that myosin II is important for the integration of polar lamellipodia during cytokinesis B. I thus speculate that the cells that have defects in only contractile ring activity still retain higher activity of myosin II behind the polar lamellipodia, generating the power to push the surrounding cells away.

Possible reasons why adherent mammalian cells have the two modes of cytokinesis

From an evolutionary point of view, Mitchison (1995) reasoned that motility driven by polymerization of filaments such as actin filaments and microtubules evolved earlier than that driven by motor proteins such as myosin and kinesin. According to this theory, the cytokinesis B mechanism, which is driven by polymerization of actin filaments, is a more primitive pathway than the cytokinesis A mechanism, which requires contractile rings comprised of actin filaments and myosin motor proteins. This notion is consistent with the observation that acquisition of multiple modes of cytokinesis increases the efficiency of cell division. For instance, myosin II null *Dictyostelium* cells, which do not have the cytokinesis A pathway, are able to divide only on substrates. By contrast, wild type cells are able to divide efficiently in suspension as well, although they sometimes fail to divide (multinucleation rate ≈ 30%). Furthermore, the multinucleation rate of the wild type cells becomes nearly zero when they attach to the substrates, indicating that

cytokinesis B mechanism increases the efficiency of division.

From a beneficial point of view, the conservation of the cytokinesis B mechanism in both cellular slime molds and mammalian cells suggests that cytokinesis B has some important roles. Burton and Taylor (1997) reported division of a Swiss 3T3 fibroblast that was driven by oppositely oriented traction forces after regression of the initial equatorial furrow. Taken this report together with my speculation that the cells activate cytokinesis B mechanism following the failure of cytokinesis A mechanism, I hypothesize that cytokinesis B in mammalian cells may be a backup mechanism when cells fail to undergo cytokinesis A, which leads to successful cytokinesis.

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