THE FUNCTION AND REGULATION OF MYOSIN-INTERACTING GUANINE

NUCLEOTIDE EXCHANGE FACTOR (MYOGEF) AND CENTROSOME/SPINDLE POLE

ASSOCIATED PROTEIN (CSPP) DURING MITOTIC PROGRESSION AND CYTOKINESIS

by

MICHAEL KWABENA ASIEDU

B.A. (Hons), University of Ghana, 1999

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

This dissertation describes the role of myosin-interacting guanine nucleotide exchange factor (MyoGEF) and centrosome/spindle pole associated protein (CSPP) in mitotic progression and cytokinesis. We have identified three mouse isoforms of CSPP, all of which interact and colocalize with MyoGEF to the central spindle in anaphase cells. The N-terminus of MyoGEF interacts with myosin whereas the C terminus interacts with the N-terminus of CSPP, forming a complex. The N-terminus of CSPP appears to be important for both localization and interaction with MyoGEF. CSPP plays a role in mitotic progression since its depletion by RNAi resulted in metaphase arrest. MyoGEF is required for completion of cytokinesis. Both MyoGEF and CSPP are phosphorylated by mitotic kinases including Plk1 and Aurora. Importantly, MyoGEF is phosphorylated at Thr-574 in mitosis by Polo-like kinase 1, and this phosphorylation is required for activation of RhoA. Thr-543 of MyoGEF is required for Plk1 binding in mitosis and phosphorylation of MyoGEF by Cdk1/cyclinB, possibly at Thr-543 may generate a Plk1 docking site, i.e., Cdk1 can phosphorylate MyoGEF at Thr-543, thereby allowing Plk1 to bind and phosphorylate MyoGEF at Thr-574. Finally, MyoGEF and CSPP are also phosphorylated by Aurora-B kinase in vitro. Taken together, we propose that Aurora-B may phosphorylate and recruit MyoGEF and CSPP to the central spindle, where phosphorylation of MyoGEF at Thr-543 promotes Polo kinase binding and additional phosphorylation of MyoGEF, leading to the activation of RhoA at the cleavage furrow.

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Dedication

This dissertation is dedicated to my parents, Katakyie and Sisi for being such wonderful parents, and to my beautiful daughter Janelle, for being the joy of my life.

CHAPTER 1 - INTRODUCTION

Overview of the cell division cycle

One of the critical decisions every dividing animal cell must make is whether to schedule another cell division cycle or exit into quiescent state. Likewise, cells in quiescent state must decide whether to persist in their quiescent state or abandon their non-proliferating state and re-enter the cell cycle. Most cells in an adult organism assume a quiescent state, whereas fewer cells during early development enter into quiescence (Malumbres and Barbacid, 2001).

The cell cycle is a complex process controlled by a complex network of regulatory proteins, which climaxes with the production of two identical daughter cells from one parent cell (Schaffer, 1998). The cell cycle, basically a cycle of duplication and division, is divided into four phases. Two of these phases are dedicated to the duplication of the entire genome (the synthetic or S phase) and to divide the cellular component into two daughter cells (mitosis or M phase). The preparatory phases or "gap periods" G1 and G2 are for synthesizing RNAs and proteins, and ensuring the completion of the S phase respectively. Non-dividing cells will exit the cell cycle and enter into quiescent or G0 state. The G1 and G0 phases are primarily controlled by growth factors. The restriction point or start point (in yeast) is the point at which the cells no longer respond to withdrawal of growth factors. The essence of the cell cycle is to accurately duplicate the DNA in the chromosomes and then segregate them accurately into two genetically identical daughter cells. Successful cell division requires tight regulation, which is achieved by ensuring the cell division follows a set of events in a specific order. The cell must possess a surveillance mechanism to sense and correct any mistakes and prevent mistakes from being transferred to the daughter cell. DNA replication is limited to only once per cycle (Tessema at al, 2004).

Regulation of the cell cycle

The cell cycle must be regulated to ensure that preceding events are completed before proceeding to the next. To ensure genomic integrity and orderly execution of cell-cycle events, mammalian cells have developed surveillance mechanism, comprised of regulatory pathways, referred to as checkpoint pathways. The checkpoint mechanism is responsible for ensuring high fidelity of chromosome segregation. In the event of DNA damage or errors in DNA replication or chromosome segregation, the checkpoint pathway is activated to induce cell cycle delay or arrest. The delay in the progression of the cell cycle, depending on the extent of the damage, allows time for either repair of the defect or the unstable cell is eliminated due to abortive mitosis. One of the important checkpoints is the regulatory site referred to as 'restriction' point. Under unfavorable conditions such as diminished size or absence of mitotic signaling, the cell may undergo apoptosis, terminal differentiation or exit into quiescence or G0 state. Two other well characterized checkpoints are the mitotic spindle checkpoint and the DNA damage checkpoint. A defective checkpoint pathway could lead to genomic instability and aneuploidy, resulting in congenital disorders as well as the transformation of normal cells into cancer cells (Tanaka et al, 2005; Elledge, 1996).

The main engine propelling the cell cycle forward is the action of cyclin dependent kinases (CDKs), a family of serine/threonine kinases, with their respective cyclin which are regulated in a cell cycle phase-specific manner. The CDKs are the catalytic subunits, and the cyclins are the regulatory units. As the names suggest, the CDK's association with the regulatory cyclins is a requirement for its kinase activity. The level of the cyclins independently fluctuates during the cell cycle. The master control system of the cell cycle, a complex of cyclin and CDKs phosphorylate various downstream targets to drive the cell through the cell cycle to activate DNA synthesis (in late G1 and S) and form mitosis-associated structural components (in late G2 and M). Progression through the transitions of the cell cycle is governed by the fluctuation in the levels of the cyclins, which is a consequence of their synthesis and concomitant degradation. Specific cyclins activate a specific group of CDKs. In mammalian cells, the G1 phase is characterized by elevated levels of the D cyclins (D1, D2, D3), which associate with CDK4 and CDK6 in a complex formation with the phosphorylation and activation of the CDKs. The activated CDK phosphorylates the retinoblastoma protein RB, a key regulator of G1 progression through the restriction point (Tanaka et al, 2005; Elledge, 1996).

The level of cyclin E increases as the cell progresses through late G1 phase, with the binding of cyclin E to CDK2 regulating the transition from G1 to S phase. Elevated levels of cyclin A are observed at late G1 phase through to the S phase. The synthesis of DNA at S phase requires the cyclinA/CDK2 complex. Cyclin A associates with CDK2 late in S phase. Incomplete DNA synthesis and DNA damage in the S phase are monitored by a G2 checkpoint, which delays progression into mitosis to allow DNA repair or abort the cell cycle (Malumbres and Barbacid , 2001; Nigg, 2001; Murray, 2004; Tessema et al, 2004; Johnson and Walker, 1999). The M phase is the most spectacular stage of the cell cycle culminating in the segregation of the duplicated genome (karyokinesis) and partitioning of the cytoplasm (cytokinesis) into two daughter cells.

M phase progression and regulation

The M phase is traditionally divided into six stages, which occur in a strict sequential order. The first five stages, prophase, prometaphase, metaphase, anaphase and telophase constitute mitosis while the sixth stage, which overlaps with mitosis, is referred to as cytokinesis. Precise execution of S phase events such as chromosomal DNA replication into two sister chromatids is essential for an error free separation of sister chromatids during mitosis. The M phase is associated with dynamic reorganization of the cell morphology, including nuclear envelope breakdown, disassembly and rearrangement of microtubule network into bipolar mitotic spindles apparatus, chromatin condensation, segregation of sister chromatids, assembly of the actomyosin contractile ring, and cell division (Nigg, 2001; Tessema et al, 2004).

Progression through M phase is primarily regulated by two interlinked post-translational modifications, namely protein phosphorylation and proteolysis or degradation. While phosphorylation by mitotic kinases (Figure 1-1) regulates the proteolytic machinery, several of these kinases are downregulated by proteolysis. Years of research has led to the identification of several mitotic kinases, prominent among them is the cyclin dependent kinases (CDK). The pioneer of the CDK family of cell-cycle regulators, CDK1, is the trailblazer of the club of mitotic kinases (Nigg, 2001; Morgan, 1997, Murray, 2004). The Cdks belong to a family of serine/threonine kinases that associate with cyclins to form active heterodynamic complexes (Malumbres, 1998)
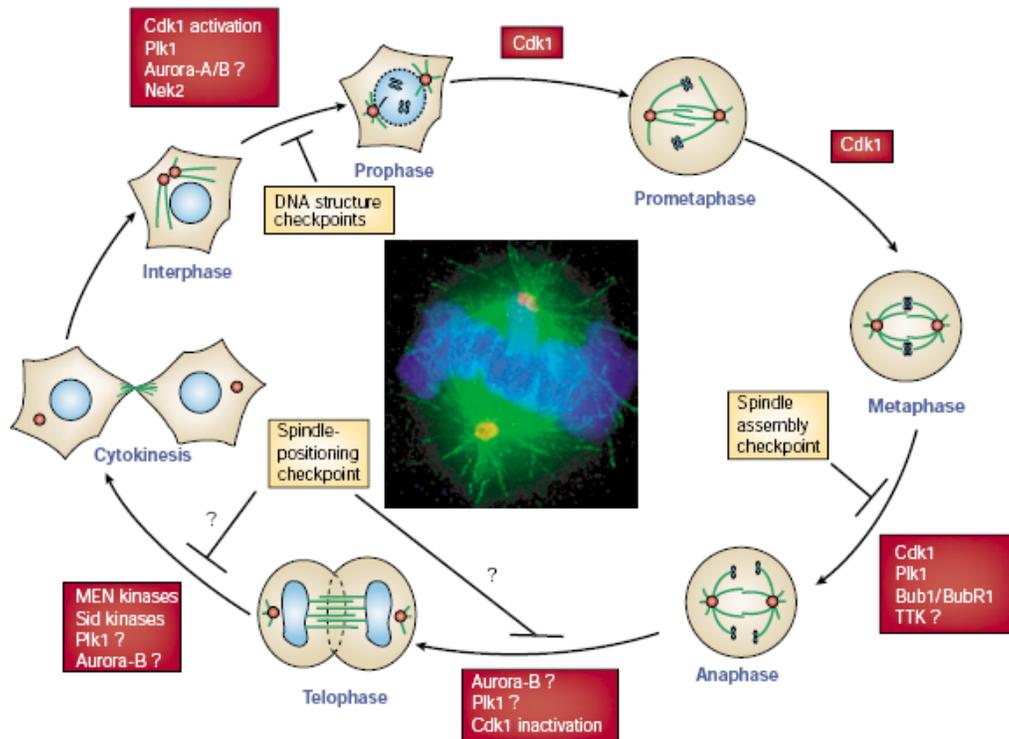
Figure 1-1. A schematic diagram of the regulation of cell division cycle by the principal mitotic kinases, emphasizing their multiple functions at different phases of the cell cycle. (Adapted with permission from Nature Review Molecular Cell Biology, 2, 21-32)

Entry into M phase is initiated by the activation of Cdk1/cyclin B holoenzyme, also known as the mitosis promoting factor (MPF). The MPF is activated through dephosphorylation by dual-specifity phosphatase CDC25C, which represents the rate-limiting step for entry into mitosis. CDK1 undergoes a conformational change upon cyclin B binding, resulting in an increase in its intrinsic activity. Furthermore, the T-loop also undergoes a conformational change leading to the opening up of the substrate-binding cleft. A 100-fold activation occurs by phosphorylation of threonine 161 on the T-loop of CDK1 by CDK-activating kinase (CAK). Thus, full activation and biological activity of the complex is achieved by both cyclin binding and T-loop phosphorylation (Nigg, 2001; Russo et al., 1996; Desai, et al., 1992; Jeffrey et al., 1995).

MPF, which phosphorylates other protein kinases as well as nuclear lamins and nuclear envelope proteins and condensins, is subject to regulation by phosphorylation on two inhibitory sites, threonine 14 and tyrosine 15, by Myt1 and Wee1 respectively, and by association with CDK inhibitors of the CIP/KIP class such as p21 and p27. Wee1 and Myt1 are CDK inhibitor kinases, whereas CIP is a Cdk-interacting protein and KIP, a kinase-inhibitor protein. The two inhibitory sites reside in the ATP binding site. (Lee and Yang, 2001; Nigg, 2001; Coleman and Dumphy, 1994; Nigg, 1996; Harper and Adams, 2001; Malumbres and Barbacid, 2001). Activation of Cdk1/cyclin B induces chromatin condensation, nuclear envelope breakdown, fragmentation, and reorganization of microtubules to form the mitotic spindle. The alignment of the sister chromatids on the metaphase plate and subsequent segregation to the spindle poles is dependent on the attachment of the spindle microtubules to the kinetochores of sister chromatids and the force generated. The kinetochore is a protein complex that mediates the attachment and movement of sister chromatids along the spindle microtubules. The mitotic spindle consists of microtubule associated proteins and a bipolar array of microtubules extending from one spindle pole to the other spindle pole, the chromosome and cortex. The mitotic spindles are required for segregation of daughter chromatids during cell mitosis (Tanaka et al, 2005; Nigg, 2001). CDK1/cyclinB complex is also regulated by the destruction of the cyclin regulatory partner to set the stage for mitotic exit and cytokinesis.

One other group of important mitotic kinases, which is receiving a lot of attention, is the polo and polo-like kinases (plks) named after the *Drosophila polo* gene. Polo is a Spanish word for pole. The founding member of this family of serine/threonine kinases, polo, was originally identified in mutants that fail to undergo mitosis in *Drosophila melanogaster*. The polo-like kinases are highly conserved in eukaryotes and play an essential role in regulating multiple cell cycle events (Sunkel and Glover, 1988, Hamanaka et al, 1995, Blagden and Glover, 2003, Barr et al 2004). Members of this family include one each from *D. melanogaster* (Polo), *Saccharomyces cerevisiae* (Cdc5), *Schizosaccharomyces pombe* (Plo1) and four in mammals namely, Plk1, Plk2 (Snk), Plk3 (Fnk/Prk), and Plk4 (Sak). Plk1 is the best- characterized member of the human Plks. Although Plk1 is expressed in the S, G2 and M phases of the cell cycle, its activity is low in S phases but increases in G2 and climaxes in M phase (Strebhardt and Ullrich, 2006, Golsteyn et al, 1994, Golsteyn et al, 1995). Plk1 exhibits dynamic localization and kinase activity during mitosis. It localizes to the centromere early in mitosis until late anaphase

and then relocates to the kinetochore as shown by the expression of recombinant Green Fluorescent Protein (GFP) fused to Pk1, suggesting a possible role in regulating chromosome and chromatin separation during anaphase (Arnaud et al, 1998). Plk1 then moves to the central spindle and the midbody region (Shirayama et al., 1998; Bahler et al, 1998; Logarinho and Sunkel, 1998; Moutinho-Santos et al, 1999; Song et al, 2000).

Polo kinases function in activation of Cdc2, centrosome maturation, bipolar spindle formation, chromosome segregation, regulation of anaphase-promoting complex (APC) and cytokinesis (Figure 2-2; Barr et al, 2004).
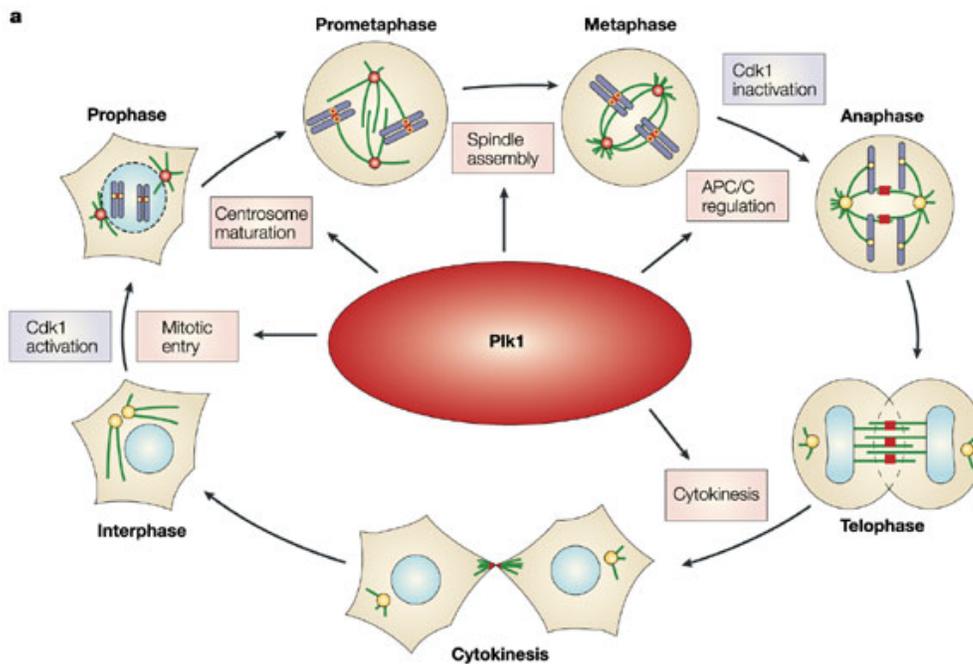
Figure 1-2. Schematic presentation of the cell cycle highlighting the multiple functions of Plk1. (Adapted with permission from Nature Review Molecular Cell Biology 5, 429-440)

The mammalian Plk1, which is believed to perform most of the function attributed to its orthologs Polo, Cdc5 and Plo1, is implicated in the activation of Cdk1/cyclin B at mitotic entry (Watanabe et al, 2004, Toyoshima-Morimoto et al, 2001, Toyoshima-Morimoto et al, 2002, Jackman et al, 2003), centrosome maturation (Lane and Nigg, 1996), formation of bipolar spindle (van Vugt et al, 2004 and Sumara et al, 2004), accumulation of spindle assembly

6

checkpoint proteins at kinetichores (Ahonen et al, 2005 and Kang et al 2006), activation of APC by phosphorylation and inactivation of APC inhibitor Emir1 (Hansen et al, 2004 and Moshe et al, 2004).

The Plks have a highly conserved amino terminal serine/threonine kinase domain and a much less conserved carboxy terminal polo-box domain. Plk1 interacts with the phosphospecific-binding module of its substrates, utilizing the polo-box domain. This interaction is believed to be responsible for Plk1 localization to its diverse structures during mitosis. The PBD recognizes an optimal sequence motif of Ser-[pSer/pThr]-[Pro/X], which suggests that the "priming" phosphorylation on docking proteins might be generated by Cdks, MAP kinases, other mitotic kinases or by Plk1 itself (Lin etal, 2000, Yarm, 2002, Kang et al, 2006, Elia et al, 2003a, Elia et al, 2003b and Neef et al, 2003).
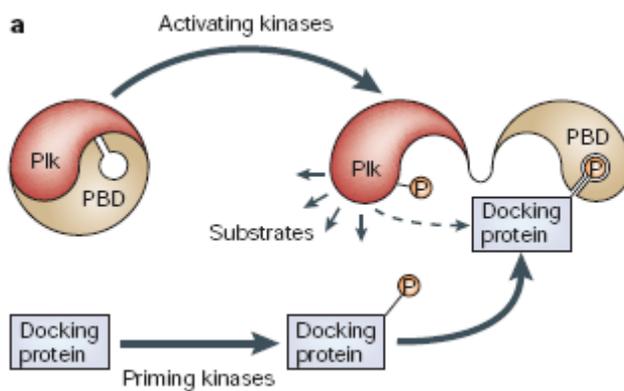
Figure 1-3. A model of Plk1 activation and polo-box domain-mediated targeting of Plk1 to its substrates.
Phosphorylation of Plk1 by activating kinases activates Plk1 releasing it from intramolecular inhibition and opening up the phospho-specific binding region in the PBD domain. (Adapted with permission from Nature Review Molecular Cell Biology 5, 429-440).

Another recently discovered serine/threonine family of mitotic kinases is the aurora, named after the aurora gene in *D. melanogaster* during a screen to identify mutants that showed defective spindle pole behaviour (Carmena and Earnshaw, 2003). Although the original gene was identified in *Drosophila*, the first such kinase, Ipl1 (for increase-in-ploidy 1) was isolated in *S. cerevisiae* from mutants with abnormal chromosome segregation (Sunkel and Glover, 1988,

Chan and Botstein, 1993). Whereas yeast have only one aurora, two paralogs (Aurora-A and –B) have been identified in both *Caenorhabditis elegans* and *D. melanogaster,* and the mammalian genome codes for three (aurora-A, -B and –C) (Giet and Prigent, 1999, Adams et al, 2001, Bischoff and Plowman, 1999 and Nigg, 2001). The auroras are key regulators of both the centrosome and nuclear cycles and regulate multiple events in mitosis such as centrosome duplication and condensation, bipolar mitotic spindle formation, kinetochore-microtubule interactions and chromosome alignment on the metaphase plate, spindle checkpoint monitoring and completion of cytokinesis (Carmena and Earnshaw, 2003; Keen and Taylor, 2004). The three mammalian aurora paralogs exhibit sequence similarity within the catalytic domain, with aurora A and B sharing 71% similarity, but they show significant diversity in the amino terminus (Carmena and Earnshaw, 2003). Aurora-A and -B show ubiquitous expression with the highest level of activity occurring late in G2- and M phases (Bischoff et al, 1998). Despite the level of similarity between the mammalian auroras, their localization and function are very different.

Aurora-A contains an amino-terminal centrosome-binding domain with localization to the duplicated centrosomes and spindle poles in mitosis. It is required for the assembly and stability of the bipolar spindle apparatus, particularly maturation and separation of centrosomes (Bischoff and Plowman, 1999; Dutertre et al 2002; Blagden and Glover 2003). Aurora-A interacts with, and its activity regulated by TPX2, a prominent component and a key player of mitotic spindle, regulated by small GTPase Ran (Kufer et al, 2002; Wittman et al, 2000). Depletion of Aurora-A by small interfering RNA (siRNA) delayed mitotic entry, and silencing TPX2 showed requirement for targeting the Aurora-A to the mitotic spindle microtubules but not centrosomes (Hirota et al, 2003; Kufer et al, 2002). On the other hand, overexpression of Aurora-A ablated the spindle checkpoint function and compromised cytokinesis (Anand et al, 2003 and Meraldi et al, 2002). Aurora-A is required for the recruitment of several components of the pericentriolar material (PCM) such as γ-tubulin, and it regulates the localization of microtubule dynamics factor, D-TACC (*Drosophila* transforming-acidic-coiled-coil protein). Aurora-A also associates with and phosphorylates Eg2, a spindle assembly protein (Giet et al, 1999 and Giet et al, 2002). Similar to most mitotic kinases, Aurora-A is regulated by both phosphorylation/dephosphorylation and proteolysis. Phosphorylation increases activity, whereas dephosphorylation during late mitosis/early G1 phase by protein phosphatase PPI and degradation by APC/C-related protein complex and Aurora-A-kinase-interacting protein (AIP)

downregulate Aurora-A activity (Francisco et al, 1994; Littlepage et al, 2002 and Walter et al, 2000).

Aurora-B exhibits dynamic localization during mitosis by concentrating along the centromere in prophase and then associating with the inner centromere at metaphase and finally relocating to the central spindle and cortex during anaphase. Aurora-B participates in a complex formation with inner centromere protein (INCENP) and survivin. Like Aurora-B, INCENP and survivin display dynamic localization to the centromere at metaphase and to the central spindle at anaphase. Proteins with such dynamic localization from the chromosome to the central spindle are referred to as chromosomal passenger proteins, CPP (Adams et al, 2000; Kaitna at al, 2000; Wheatley et al, 2004; Speliots et al, 2000). They are involved in regulating the functions of the chromosomes such as segregation and condensation (Katayama et al, 2003). The founding member of the chromosome passenger proteins is the yeast INCENP with reported function in chromosome segregation and cytokinesis, since it concentrates to the cortex (Adams et al, 2001). Survivin, the third member of the chromosomal passenger complex is an inhibitor of apoptosis protein and has also been implicated in mitotic progression and cytokinesis (Adams et al, 2001). INCENP and survivin are required for proper localization of Aurora-B during mitosis because overexpression of INCENP without the C terminus required for interaction with Aurora-B causes mislocalization of Aurora-B during mitosis (Adams et al 2000). A summary of the function of the Aurora-B/INCENP/Survivin complex includes (1) localizing Aurora-B to the centromere to phosphorylate Histone H3, (2) regulating the kinase activity of Aurora-B and (3) promote the relocalization of the CPP complex from the centromere to the central spindle (Bolton et al, 2002; Bishop et al, 2002; Speliotes et al, 2000).

Cytokinesis in eukaryotes

Cytokinesis, characterized by a series of dramatic cortical rearrangements, marks the final step of the cell division and culminates in the physical division of a cell into two independent daughter cells, each with full complement of nuclear and cytoplasmic content. The process begins at the onset of anaphase, closely following the separation of sister chromosomes

(Mishima et al, 2004). The precise temporal and spatial regulation of cytokinesis is to ensure high fidelity of cytokinesis in order to generate identical daughter cells and maintain genomic stability. Although the precise mechanism driving cytokinesis is not fully understood, the process can be divided into four steps, namely, (1) determination of cleavage plane or division site (2) formation of contractile ring (3) cleavage furrow assembly and ingression and (4) midbody formation and abscission (Glotzer 2001). Typically, a dividing cell forms an invagination or cleavage furrow immediately after anaphase onset. The cortical cytoplasm in close proximity to the site previously occupied by the chromosome ingresses inwards until it merges with central spindle microtubules forming the so-called midbody. The midbody is digested and the two daughter cells are separated from each other (Wolf et al, 1999).

Cleavage plane specification/furrow positioning

In animal cells, cytokinesis begins with specification of the division/cleavage site or the furrow position, usually at the cell equator and perpendicular to the axis of chromosome segregation, which ultimately becomes the site where the cleavage furrow is assembled. To ensure fidelity of the entire cell division process, chromosome segregation in mitosis directed by the mitotic spindle is spatially linked with cytokinesis such that the division site is specified after chromosome segregation and between the segregated chromosomes to guarantee that each daughter cell will have complete nuclear and cytoplasmic materials (Glover, 2001; Guertin et al, 2002; Wolf et al, 1999). The onset of cytokinesis and the specification of the division site are regulated both in time and space. The fact that cytokinesis closely follows mitotic exit suggest some coordinate control between the two events. It has been reported that the proteolytic degradation of cyclins resulting in inactivation of Cdk1 during mitotic exit may be required for the onset of cytokinesis (Wheatley et al, 1997) The spatial regulation of cytokinesis has been the subject of both intense research and debate regarding the role of different types of microtubules in specifying the cleavage plane (Glover, 2001). It is now agreed that the mitotic spindle determines the position of the furrow, however the nature of the component of the spindle responsible for inducing the cortex to furrow or the nature of the signal remains unsettled (D'Avino et al, 2005; D'Avino et al, 2006). The anaphase mitotic spindle is composed of different types of microtubules but it is not clear which type of types determines the positioning of the cleavage site. The following nomenclature is used to differentiate various types or

microtubules based solely on where they are found in the cell. Microtubules linking the centrosome to the kinetochore are referred to as kinetochore microtubules, whereas spindle midzone microtubules join two sister chromatids (Burges and Chang, 2005). Similarly, equatorial astral microtubules originate from the centrosome towards the equatorial cortex whiles the polar astral microtubules proceed from the centrosome towards the polar cortex (Burges and Chang, 2005).
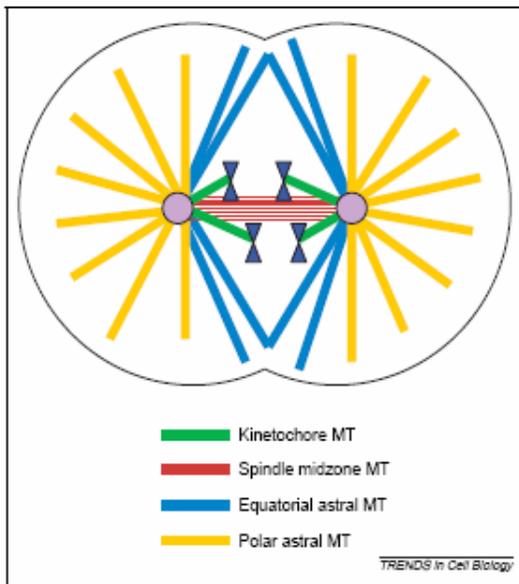
Figure 1-4. Different types of mitotic microtubules (MTs).
The MTs are classified based on their geometrical relationships. (Adapted with permission from Trends in Cell Biology, Vol 15, March 2005)

Initial evidence that the mitotic spindle induces cleavage furrow positioning and formation came from micromanipulation studies using echinoderm embryos and tissue culture cells (Rappaport, 1996; Glover, 1997). In these experiments, astral microtubules overlapping at the equator proved sufficient to induce cleavage formation at the equator even without intervening chromosomes or the presence of spindle midzone (Rappaport 1996). However, other experiments using mammalian and *Drosophila* cells, where the shape and size of the cells differ from the echinoderm embryos, provided conflicting results to the effect that interzonal microtubules (also called central spindle or spindle midzone microtubules), as opposed to the astral microtubules, are required for cytokinesis (Glover, 2004).

Evaluation of classical models

It has been known that crosstalk between the cortex and the microtubules determines cleavage plane specification. The question of how the spindle stimulates furrow formation has engaged the attention of researchers for a long time. Currently, three hypotheses or models of furrow induction have been proposed. They are the equatorial or astral stimulation model, central spindle model and the polar relaxation model.

| **Equitorial stimulation** | **Polar (astral) relaxation** | **Spindle mizone model** |
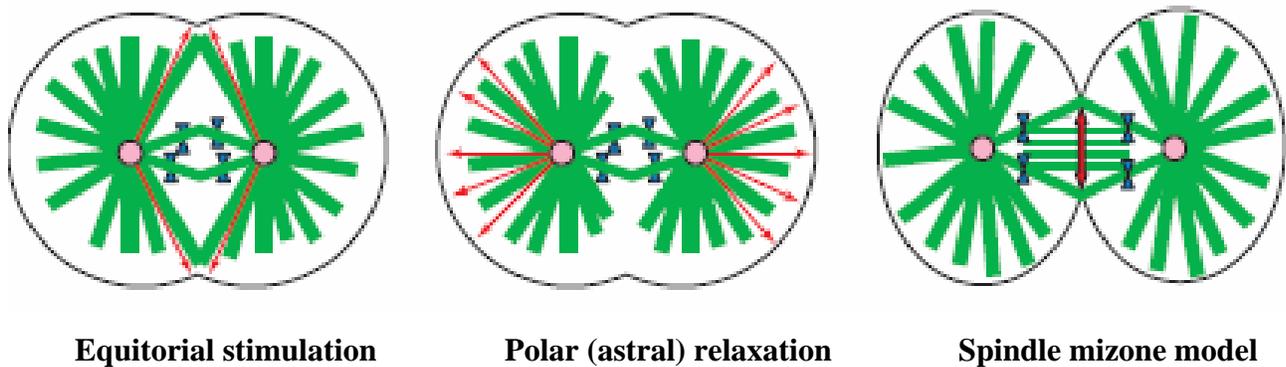
Figure 1-5. Different models for the role of MTs in induction of cleavage furrow.
Equitorial stimulation model. Astral microtubules from opposite pole overlapping at the equatorial cortex transmit positive signals to induce cleavage furrowing
Polar (astral) relaxation model. The tension at the polar cortex is relieved by the presence of polar astral MTs coupled with in increased tension at the equatorial cortex due to lack of equatorial MTs at this region.
Spindle midzone model. Movement of a complex of proteins from the central spindle MTs to the equatorial cortex stimulate furrow induction
(Adapted with permission from Trends in Cell Biology, Vol 15, March 2005)

The first hypothesis, the astral stimulation hypothesis, postulates that polar astral microtubules pass on a furrow-inducing signal to the cell cortex, where signals from two poles are somehow focused into a ring at the spindle equator. The basis of this hypothesis came from a variety of elegant experiments conducted by Rappaport and his group using invertebrate embryos which showed that an ectopic cleavage furrow can form between the asters of two separate

spindles in the same cell. Their results even showed that a cleavage furrow can be induced by a single microtubule aster in anaphase cells under certain conditions, arguing that the induction signal does not require interactions between microtubules from two poles. Other perturbation included cell shape deformation and removing parts of the central spindle. These resulted in premature and multiple cleavage furrows induced at several locations in the cell, suggesting that the cortex is globally competent to furrow, provided the appropriate signals are communicated. Application of pharmacological agents which depolymerized microtubules blocked cleavage induction by the spindle, demonstrating that molecular signals or factors may be transported by the microtubules and their associated proteins to initiate cleavage formation. Similar experiments using spherical echinoderm blastomeres showed that overlapping astral microtubules without intervening chromosomes or presence of spindle midzone is sufficient to specify the position of the cleavage plane (Rappaport R, 1985; Rappaport R, 1996).

The polar or astral relaxation hypothesis, on the other hand, suggests that astral microtubules interacting with the polar cortex generate negative signals that inhibit cleavage furrow formation. It states that the contraction of active actin–myosin bundles distributed throughout the cell cortex is inhibited near the spindle poles, resulting in a gradient of contractile activity that is highest at the midpoint between poles. Support for this model has come from experiments with embryos of *C. elegans*, and to some degree cultured mammalian cells, where defects in astral microtubule formation led to multiple ectopic cleavage furrows and widespread contractile activity, supporting the belief that the microtubules normally function to inhibit contraction at the cell cortex in these cell types.

The central spindle hypothesis, the most attractive of the hypotheses, proposes that the cleavage furrow is induced by a positive stimulus emanating from the central spindle, made up of anti-parallel microtubules and signaling molecules. Overwhelming evidence from many species backs this model (Cao and Wing 1996, Wheatley and Wang, 1996). Work in cultured mammalian cells in which physical blocks placed between the spindle midzone and the cortex prevented furrow formation. Disrupting the central spindle formation using genetic approaches or micromanipulation resulted in varying degress of cyokinetic defects (Dechant and Glotzer, 2003; Cao and Wing 1996; Eggert et al, 2006). Experiments that disturbed the central spindle formation blocked furrow formation in *Drosophila* (but not in *C. elegans*). Moreover, furrow formation occurred successfully in *Drosophila* meiotic cells or in cells without proper

centrosome function, suggesting that astral microtubules are dispensable in furrow formation (Glotzer, 2004). Furthermore, a number of proteins which localize to the central spindle are also required for cytokinesis. There is some evidence, however, that the central spindle may not be entirely responsible for furrow initiation and formation. When cells were depleted of PRC1, a central spindle protein, central spindle was disrupted but cleavage furrow proceeded normally (Mollinari et al 2005).

It appears that no single proposal could satisfactorily explain all the observations, and thus the positioning of the cleavage furrow is likely to be determined by some combination of these mechanisms and perhaps depending on the cell type, there will be variations in the importance of different mechanisms. In fact, a recent report in which asters were ablated with ultraviolet laser beam has provided evidence suggesting that furrow positioning is determined by two signals, first by astral microtubules followed by signals originated from the spindle midzone (Bringmann and Hyman, 2005). Furthermore, there is abundant evidence; at least in embryonic cells of *C. elegans*, for example, supporting the view that astral stimulation and central spindle mechanisms are both important. A clear understanding of these mechanisms is likely to arise from the complete identification and analysis of the signaling molecules that are involved in furrow positioning (Glotzer, 2004; Alsop and Zang, 2003; Bringmann and Hyman, 2005).

Molecular mechanism of cleavage formation

The nature of the signals that are delivered to the cortex by the microtubules of the mitotic spindle has also been the subject of intense study. The transition from metaphase to anaphase is marked with critical rearrangement of the mitotic spindle. These include shrinkage of the kinetochore microtubules to deliver sister chromatids to the spindle poles, sliding of other non-kinetochore microtubules leading to cell elongation, and bundling up of some non-kinetochore microtubules to form the central spindle. Microtubule-based motor proteins play a pivotal role in these rearrangements. In addition, proteins that localize to astral and/or central spindle microtubules, or to the equatorial cortex, are potential candidates for participation in the signal or its delivery system (Guertin et al, 2002).

Data from genetic studies have identified several conserved protein complexes that localize to the central spindle in cells in anaphase. One such protein complex is the chromosomal

passenger or the ABI complex, comprising INCENP, Aurora B kinase, Borealin and Survivin. At least some components of the passenger complex have been reported to be required for cytokinesis in both mammalian and *Drosophila* cells (Prigent et al, 2005; Terada et al, 1998; Adams et al, 2000).

Another complex that localizes to the central spindle and is required for cytokinesis is the centralspindlin which, in mammals, is made up of kinesin-related protein, MKLP1, and a Rho GTPase activating protein, MgcRacGAP. The orthologues of this complex in *D. melanogaster* and *C. elegans* are Pavorotti/RacGAP50C and Zen-4/Cyk-4 respectively. Other proteins that associate with the central spindle include motor proteins MKlp2, Kif4, mitotic kinase Plk1, structural protein PRC1, centrosome associated protein CSPP and guanine nucleotide exchange factors, pebble/Ect2 and MyoGEF (Kuriyama et al, 2002; Neef et al, 2003; Lee and Kim, 2004; Barr et al, 2004; Patzke et al, 2005; Wu et al, 2005). PRC1, a microtubule associated protein (MAP) with microtubule bundling activity interacts with Kif4, which limits its activity to the small region in the central spindle. Cdk1 has been reported to inhibit the microtubule bundling activity of both PRC1 and centralspindlin complex during metaphase. The use of small interfering RNA (siRNA) to silence PRC1 resulted in almost complete disruption of the central spindle. Similarly, RNAi depletion of ECT2 and MyoGEF results in multinucleation, a phenotype of cytokinesis failure (Prokopenko et al, 1999; Tatsumoto et al, 1999).

According to D'Avino et al (2000), a signal that will link the central spindle to the cortex and induce furrow formation must possess at least three properties. First, the signal must be necessary and sufficient to induce furrow initiation. Second, it must either be stimulated or reach the cortex before furrow initiation and thirdly, it must have regulatory control over the signaling pathways that modulate formation and ingression of the cleavage furrow (D'Avino et al, 2005). The centralspindlin appears to be an attractive candidate because it possesses all three properties. Both Pav and RcGAP50 are required for furrow formation and the expression of pav mutant lacking the motor activity leads to abnormal localization and concentration of the mutant protein as well as actin at the spindle pole, demonstrating that the centraspindlin recruits actin to the contractile ring. Secondly, in *Drosophila* embryos and S2 cells, both Pav and RacGAP50C accumulate at the equatorial cortex at anaphase (Adams et al, 1998; Goshima and Vale, 2003; Somers and Saint, 2003; Somma et al, 2002; Minestrini et al, 2003). Moreover, both MgcRacGAP and RacGAP50 inhibit Rac activity at the cleavage furrow in both mammals and

flies and RacGAP also interacts with RhoGEF resulting in Rho activation. The centralspindlin also regulates accumulation of RhoA and ECT2 at the equatorial cortex (Kamijo et al 2005; Zhao and Fang, 2005; Nishimura and Yonemura, 2006). These results suggest that RacGAP could regulate the signaling pathways that control cleavage formation and ingression. It could be deduced from the above that the MKLP1 of the centralspindlin complex could participate in transporting RacGAP along the microtubules to cell cortex and hence play a role in the signal delivery process. RacGAP will then promote cleavage furrow formation and actin myosin assembly. There is some coordination among the occupants of the central spindle, suggesting that the regulation of their function may involve an integrated approach. For example, interaction between MKLP1 and Aurora B orthologs in *C. elegans* has been reported in addition to data showing that INCENP regulates localization of the centralspindlin (Glotzer, 2004; Vagnarelli and Earnshaw 2004; Ruchaud et al, 2007, Terada et al, 2001).

Contractile ring assembly and cleavage furrow ingression

The signals that are received at the equatorial cortex involve proteins that concentrate at the cortex in a microtubule-dependent manner and are important for furrow formation. The contractile ring is formed at the site of division at the end of anaphase, resulting in a cleavage furrow that gradually constricts the parent cell into two daughter cells. The contractile ring is made up of a ring of proteins that positions beneath the cortex and envelops the cell equator. The components of the cleavage furrow include actin, myosion and other proteins that organized into the actomyosin contractile bundle. Myosin localizes to the cleavage furrow soon after actin. The interaction between myosin and actin generates the force that causes the ring to shrink and pulls the membrane inwards. Actin depolymerization and myosin perturbation led to delayed cytokinesis and abolished furrow ingression respectively, suggesting that the mechanical force driving the contractile ring ingression is provided by myosin (Alberts et al, 2002; Satterwhite and Pollard, 1992; Fujiwara and Pollard, 1976).

Type II myosin consists of two myosin heavy chains (MHCs), two essential light chains (ELCs) and two regulatory light chains (RLCs). All three subunits are required for myosin to function properly. Phosphorylation of RLC at Thr-18 and Ser-19 by myosin light-chain kinase has been shown to increase myosin activity. Although in vitro biochemical data supports the

functional relevance of RLC phosphorylation, two in vivo experiments using mutant RLCs containing nonphosphorytable myosin light chain kinase (MLCK) sites expressed in null backgrounds produced conflicting results in two different organisms. In *Dictyostelium*, phosphorylation at MLCK sites has no effect on RLC activity, whereas the opposite was observed in *Drosophila*. The apparent different could be attributed difference in the requirement of myosin function during cytokinesis in single cells compared to cells in tissue environment (Ostrow et al, 1994; Wolf et al, 1999).

Anilin, an essential cytokinesis protein, is a component of the contractile ring, and binds and bundles actin. Anilin is required for cyokinesis because microinjection of anilin antibodies resulted in a defect in cytokinesis. Although an anilin mutant forms a cleavage furrow, the contractile ring appears disorganized and cytokinesis is aborted at late stage (Oegema et al, 2000; Echard et al, 2004; Straight et al, 2005). Actin regulates the localization of anilin as shown by the absence of anilin at the cleavage furrow when cells were treated with latrunculin-A, an actin depolymerising agent. Anilin binds to myosin and regulates its contraction late in cytokinesis, but the interaction is regulated by phosphorylation of the myosin regulatory light chain (Straight et al, 2005).

A key regulator of the contractile ring in animal cells is the small GTPase RhoA. The Rho family of small GTPases has been implicated in the regulation of actin dynamics in response to both intracellular and external stimuli. Rho GTPases perform this function through interaction and regulation of a number of downstream targets such as Pak (p21-activated kinases), ROCK (Rho kinase), and formin-homology protein such as actin-modulating diaphanous-related proteins (Bokoch, 2003; Riento and Ridley, 2003; Evangelista et al, 2003). The Diaphenous formin-homology protein and members of the Rho-dependent kinases have been implicated as targets of Rho GTPase during cytokinesis (Tatsumoto et al, 1999; Kosako et al, 2000; Madaule et al, 1998).

Rho signaling pathway: regulators and effectors

The family of Rho GTPases belongs to the superfamily of Ras-related small GTPases found in eukaryotic cells. The subfamily is divided into groups based on sequence and functional similarities. They include Rho A, B, C, Rac 1, 2, 3 and two isoforms of Cdc42. The three best-studied members are RhoA, Rac1 and Cdc42 (Hall, 1998; Van Aelst and D'Souza-Schorey,

1997). Members of the family are highly conserved in eukaryotes and play a fundamental role in a variety of cellular processes such as cell polarity, cell movement and cell division. The effect of each subfamily on cytoskeleton architecture is distinct: RhoA and relatives RhoB and RhoC control cell adhesion and retraction by regulating the formation of actin stress fibers and focal adhesion, Rac1 and relatives Rac2 and Rac3 stimulate cell movement through the formation of lamellipodia, and Cdc42 and related proteins stimulate the formation of actin microspikes and filopodia for sensing extracellular cues (Ridley and Hall, 1992; Jaffe and Hall, 2005; Karnoub eta el, 2006). In addition to their role in the regulation of actin-myosin cytoskeleton, the Rho GTPases are also involved in cell growth, transcriptional regulation, development and G1 phase progression (Van Aelst and D'Souza-Schorey, 1997; Karnoub eta el, 2004).

The GTPases act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound state. This process is firmly regulated by Rho-specific guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Moon and Zeng, 2003; Wennerberg et al, 2005). The GEFs are the primary mediators of Rho GTPase stimulation but the regulation by GAPs and GDP-dissociation inhibitors (GDIs) are also contributory factors. Once activated, the Rho GTPase interacts with a plethora of diverse effectors to regulate a variety of cellular processes.

Activation of small GTPase RhoA spatially regulates an early step in cytokinesis. Inactivation of RhoA results in cytokinesis defects and furrow assembly inhibition. A guanine nucleotide exchange factor, pebble, and its human ortholog ECT2 have been identified as key activators of RhoA and required for cytokinesis. Both pebble and ECT2 localize to the cleavage furrow, and inactivation of RhoA or loss of pebble results in inability to recruit actin and failed furrow ingression (Prokopenko, et al 1999; Tatsumoto, 1999).

Several downstream effectors of RhoA have been identified and their roles in cytokinesis assessed. They include Rho kinase, Citron kinase and forming-homology proteins (Glotzer, 2001). RhoA promotes actin polymerization by interacting with proteins of the formin-homology protein family and stimulating their capacity to promote actin nucleation and growth. Myosin II, on the other hand, is directly regulated by RhoA, resulting in actin filament alignment and bundling. Formin proteins are not only required for cytokinesis in worms and flies but are targets of RhoA as well. They may play a role in early stage of cytokinesis, since loss of formin

prevented furrow formation. Mutation in either the diaphanous gene in flies or cyk-4 gene in worms resulted in cytokinesis defect (Castrillon & Wasserman, 1994; Chang et al, 1997; Evangelista et al 1997; Swan et al 1998; Figure 1-1). Formin-homology proteins are defined by the presence a tandem FH1 and FH2 domains at the C terminus. Their amino terminal region binds RhoA. The FH1 domain associates with actin-binding protein, profiling, through its proline-rich region. mDia has been reported to colocalize with RhoA and profilin and may regulate cellular events in cooperation with profilin. RhoA inhibition by C3 exoenzyme microinjection abolished the RhoA, mDia and profilin colocalization, suggesting a pathway of RhoA regulation of actin through mDia (Watanabe et al 1997; Carlier and Pantaloni, 1997; Schluter et al, 1997; Figure 1-1).
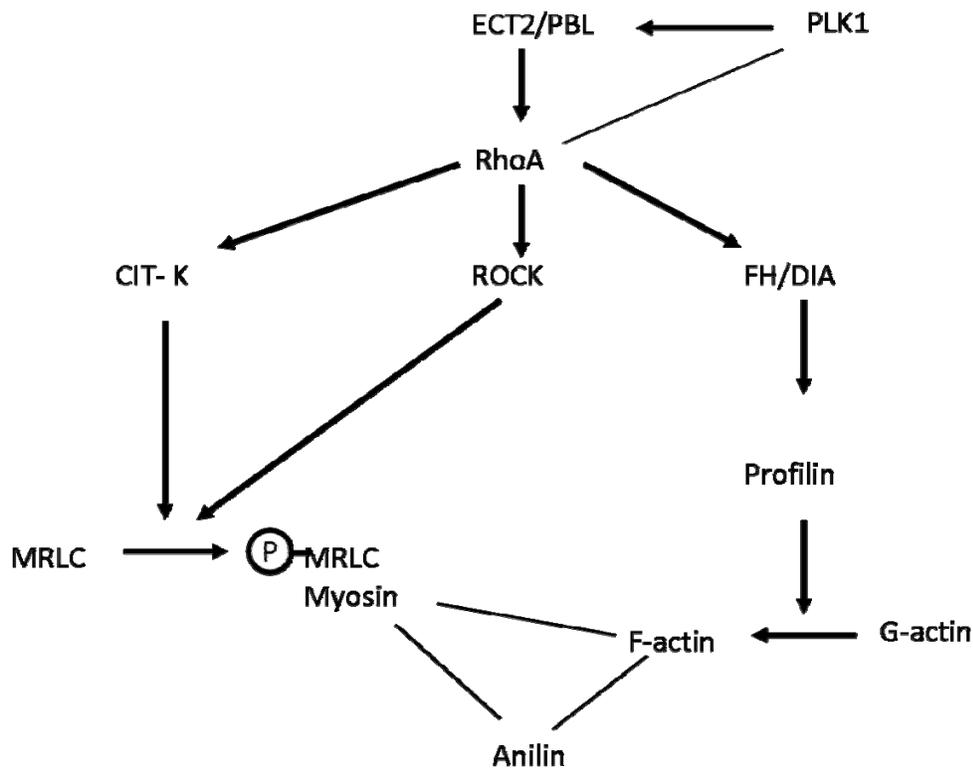

Figure 1-6. Signaling pathways regulating the cortical activity at the cleavage furrow during cytokinesis
Thick arrows denote activation whereas thin lines indicate interaction.

RhoA also associates with a variety of targets to increase myosin phosphorylation and promote the assembly and motor activity of myosin. RhoA associates with two kinases, ROCK and Citron, also with cytokinesis functions. Evidence from genetic and chemical studies suggested only a facilitating role for ROCK in cytokinesis, possibly due to the fact that the use of chemical agents and mutants may not completely abolish the function of ROCK, since it shows strong localization to the cleavage furrow. ROCK phosphorylates myosin light chains at least in vitro and the extent of myosin regulatory light chain (MRLC) phosphorylation in vivo is decreased by the use of chemical inhibitors of ROCK (Kosako et al, 1999; Kosako et al, 2000; Amano et al, 1996). Indeed RhoA promotes MRLC phosphorylation and therefore myosin activity using a two-prong approach: by associating with and activating ROCK and inhibiting MRLC dephosphorylation (Amano et al, 1996; Kawano et al, 1999). RhoA may also indirectly stabilize actin filaments and therefore activate contractile ring assembly through LIM-kinase, a substrate of ROCK. Activation of ROCK by RhoA then leads phosphorylation and activation of LIM-kinase, which then phosphorylates and inactivates cofilin thereby stabilizing actin filaments and inducing stress fibre formation (Sumi et al, 2001; Sumi et al 1999; Maekawa et al, 1999, Ohashi et al, 2000).

Another target of RhoA is Citron kinase. Citron kinase also phosphorylates MRLC but unlike ROCK, does not inhibit myosin phosphatase. However, the exact role of citron kinase during cytokinesis is not clear. Although expression of truncated genes in mammalian cells resulted in failed cytokinesis and abortive contractile ring contraction, citron knock-out mice exhibited tissue-specific cytokinesis defect during spermatogenesis (Yamashiro et al 2003; Madaule et al, 1998; Madaule, et al 2000; Di Cunto et al, 2000; Di Cunto et al, 2002). However, the ortholog of citron kinase in flies, Sticky, is required for cytokinesis in all tissues. The expression of its mutant or the use of RNAi results in incomplete cytokinesis with aberrant actin filaments, indicating a role in contractile ring assembly, as opposed to contraction (D'Avino et al, 2004, Echard et al, 2004; Naim et al, 2004; Shandala, et al 2004). It is interesting to note that, MRLC phosphorylation by either ROCK or Citron kinases have different effects. Whereas ROCK is involved in contraction, citron controls contractile ring assembly (D'Avino et al, 2005).

The role of Polo kinase in cytokinesis has been particularly difficult to study because of its earlier role in mitosis, and therefore knockdown of its gene results in prometaphase-like arrest. However, the availability small molecule inhibitors have made it possible to overcome

this hurdle. A recent report using ectopically expressed recombinant GFP-RhoA shows that RhoA colocalizes and interacts with Polo kinase 1 (Dai et al, 2007). It is not clear whether the interaction is mediated by other proteins such as ECT2, which is both a Polo kinase1 substrate and a RhoA activator. Cdc5, Polo kinase homolog in yeast has been found to activate RhoA, but it is not known whether the same is true in mammals or whether RhoA activates polo kinase instead (Dai et al, 2007; Niiya et al, 2006; Yoshida et al, 2006).

Guanine nucleotide exchange factors

Structure and function

Members of the Dbl family of guanine nucleotide factors (GEFs) are multifunctional proteins which activate Rho GTPases by acting as mediators of diverse intracellular signaling. They are defined by the presence of tandem Dbl-homology (DH) and pleckstrin-homology (PH) domains. The DH domain is responsible for catalyzing the GDP-GTP exchange reaction of small GTPases. The first mammalian RhoGEF, Dbl, was isolated from diffuse cell lymphoma (Dbl) as a transforming gene and has a region of 240 amino acids, belonging to the DH domain, showing significant sequence similarity to CDC24. CDC24 is an upstream activator of yeast CDC42, just as Dbl was later identified as an activator of human CDC42. It has been predicted, based on the data from the genome projects that there are at least 6 GEFs in yeast, 18 in worms, 23 in flies and about 69 in humans (Eva and Aaronson, 1985; Bender and Pringle, 1989; Venter et al, 2001, Rossman et al, 2005). The DH domain has been established as the domain responsible for the GEF activity, whereas the PH domain controls the localization of the GEF protein. The PH domain therefore modulates the localization of GEFs to the plasma membrane as well as association with actin cytoskeleton and has been reported to associate with phosphorylated phosphoinositides (PIPs) and proteins (Rebecchi and Scarlata, 1998; Lemmon and Ferguson, 2000). A second role of the PH domains is their ability to directly modulate the activity of the DH motif. For most GEFs, the DH-PH domain represents the minimum structure required for its transforming activity. The GEF activity of several of the known GEFs has been assessed based on their ability to exchange GTP for GDP on RhoA. The assay measures their ability to stimulate GDP dissociation, the addition of GTP in vitro or the cellular effect of their overexpression in vivo. Whereas some GEFs are specific for only one GTPase, for example,

Cdc24, Tiam1 and Lbc, others such as Dbl, Ect2, Ost and Bcr activates multiple Rho GTPases (Zheng, 2001).

The mechanism of guanine nucleotide exchange factor on Rho GTPase is thought to be a two-step process of GDP-dissociation and GTP-binding. The GEF first recognizes the GDP-bound Rho GTPase and induces the dissociation of the GDP to form a nucleotide-free binary GEF-Rho complex. GTP then binds to the complex resulting in the dissociation of GEF. The role of the GEF therefore is to (1) destabilize the GDP-Rho complex and (2) stabilize the intermediate nucleotide free complex (Rossman et al, 2005).

Mechanism of regulation

Each GEF is uniquely regulated by one or a combination of four possible regulatory mechanisms of activation and deactivation, but generally they involve removal of inhibitory sequences present in the molecule, promoting protein-protein interaction, control of intracellular localization and repression of the GEF activity.

The structure of most GEFs depicts the presence of regulatory domains that control activity through intramolecular interaction. Although the mechanism of removal of the autoinhibition has not been worked out, it is thought that it may involve phosphorylation or interaction with other proteins. The first mechanism involves an interaction between the DH and PH domains. An example is the PH domain of Dbl, Vav and Sos1, which by its interaction with PI-4,5-P_2 and PI-3,4,5-P_3 is believed to regulate the GEF activity by removing the constraint placed on the DH domain (Han et al, 1998, Nimnual et al, 1998, Russo et al. 2001; Das et al. 2000).

The second mechanism involves interaction of a regulatory domain with either the DH or PH domain of the GEF. Such interaction may inhibit the function of the DH or the PH domain by blocking access by the GTPase or modifying the cellular localization directed by the PH domain (Aghazadeh et al, 2000; Bi et al, 2001). An example is the N-terminus of Dbl, Vav, Asef, Tiam1, Ect2, and Net1, where N-terminal truncation mutants are constitutively active in vivo (Ron et al. 1989; Katzav et al. 1991; Miki et al. 1993; van Leeuwen et al. 1995; Chan et al. 1996; Kawasaki et al. 2000) or the C terminus of Lbc, where activation results from the removal of C terminus (Sterpetti et al. 1999). In the case of Vav, transient phosphorylation by Src and Syk tyrosine

kinases in response to extracellular stimuli results in increased activity, while constitutive activation occurs upon the removal of the first 66 amino acids from the N-terminus (Crespo et al. 1997; Han et al. 1997; Miranti et al. 1998; Katzav et al. 1991). The activity of ECT2 is also induced by Cdk phosphorylation during M phase progression (Tatsumoto et al 1999).

The third mechanism involves formation of oligomers between intermolecular DH domains. Examples include the regulation of RasGRF1, RasGRF2 and onco-Dbl (Anborgh et al 1999; Zhu et al, 2001). The fourth mode is through direct protein-protein interaction where the activities of the GEF is kept at the basal level by cellular factors (Zheng, 2001).

Another mechanism of regulating the spatio-temporal activation of GEFs via nuclear localization signals has been suggested for Rho-GEF, Ect2/pebble and Net1. In this scenario, the presence of two nuclear localization signals at the N-terminus functions to sequester the GEFs away from their targets until their function is needed. An example is Ect2/pebble, which is kept away in the nucleus but is released into the cytoplasm upon nuclear breakdown during mitosis to regulate cytokinesis (Prokopenko et al. 1999; Tatsumoto et al. 1999; Schmidt and Hall 2002).

Goal of my Research

The goal of this research was to determine the molecular mechanism of the role of MyoGEF in the regulation of cytokinesis. Our laboratory has previously identified a novel guanine nucleotide exchange factor (MyoGEF) which is required for cytokinesis. We have found that MyoGEF interacts with a centrosome/spindle pole associated protein (CSPP) using yeast two-hybrid system. We hypothesized that CSPP directs MyoGEF to the central spindle, where MyoGEF then regulates the activity of the small GTPase proteins and positions the myosin contractile ring. Specific aims of my research were to (1) characterize the interaction between MyoGEF and CSPP, as well as MyoGEF-myosin interaction *in vivo* and *in vitro* and determine the interacting domains, (2) determine the role of CSPP in cytokinesis using RNA interference and microscopy, (3) identify post-translational modifications of MyoGEF and upstream kinases, and correlate such modifications with the regulation of cytokinesis.

CHAPTER 2 - MYOGEF INTERACTS WITH MOUSE CENTROSOME/SPINDLE POLE-ASSOCIATED PROTEIN (CSPP) ISOFORMS TO REGULATE M PHASE PROGRESSION

Introduction

Rho GTPases including RhoA, Rac1 and Cdc42 play a role in cell morphogenesis by acting as regulators of actin-myosin cytoskeleton and inducing specific types of cell structure such as stress fibers, lamellipodia and filopodia (Burridge and Wennerberg, 2004; Ettienne-Manneville and Hall, 2002; Hall, 1998). The small GTPases act as molecular switches by cycling between inactive GDP-bound state and active GTP-bound forms. The activities of these GTPases are regulated mainly by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Mackay and Hall 1998). The GEFs catalyze the exchange of GDP to GTP, whereas the GAPs inactivate the GTPase by stimulating the intrinsic GTPase activity to generate GDP-bound form (Mackay and Hall, 1998).

Activated RhoA activates Rho-kinase which then phosphorylates myosin regulatory light chains as well as regulatory subunits of myosin phosphatase, resulting in an increase in myosin contractile activity (Kimura et al., 1996; Matsui et al., 1996). RhoA therefore regulates the organization of actin cytoskeleton and myosin activity at the cleavage furrow and contributes to contractile ring assembly and furrow ingression (Bement et al., 2005; Dean et al., 2005; Kamijo et al., 2005; Matsumura et al., 2001; Yoshizaki et al., 2004).

There is abundant evidence to suggest that the central spindle and GEFs/GAPs cooperate to spatially and temporally regulate the contractile ring formation (Burgess and Chang, 2005; Eggert et al., 2006; Glotzer, 2005). A *Drosophila* RhoGEF, Pebble, localizes to the cleavage furrow. Pebble, RacGAP50C (a GAP), and Pavarotti (a kinesin-like protein) associates to form a tri-molecular complex, whose proposed function is to position the myosin contractile ring to the site of furrowing during cytokinesis in *Drosophila* cells (Somers and Saint, 2003). In *Drosophila* cells, myosin has been reported to localize to the equatorial cortex first by

recruitment and then retention (Dean et al., 2005). Genes involved in the initial recruitment process are pebble, rho1, and rho kinase of the Rho pathway, whereas F-actin, the centralspindlin complex, formin (diaphanous), and profilin (chickadee) are responsible for myosin II retention at the cleavage furrow. The human homologue of pebble, ECT2, regulates the initiation of furrow ingression and the completion of cytokinesis in mammalian cells (Kamijo et al., 2005; Tatsumoto et al., 1999; Yuce et al., 2005; Zhao and Fang, 2005). MKLP1, a component of the centralspindlin promotes the localization of ECT2 at the central spindle, which in turn regulates cortical localization of RhoA and contractile ring assembly (Kamijo et al., 2005; Nishimura and Yonemura, 2006; Yuce et al., 2005). Phosphorylation of ECT2 by polo-like kinase 1 recruits ECT2 to the central spindle and induces the initiation of cytokinesis (Petronski et al., 2007). However, it has recently been shown that depletion of ECT2 did not affect RhoA activation at the cleavage furrow (Birkenfeld et al., 2007), suggesting that RhoA activation in cytokinetic cells is not regulated by only ECT2 but other GEFs may also contribute to the activation.

A family of proteins termed centrosome/spindle pole-associated proteins (CSPPs) has been reported to localize to the centrosome, spindle pole, and central spindle (Patzke et al., 2005; Patzke et al., 2006). Two human CSPPs isoforms, CSPP-S and CSPP-L, identified so far show differential cell cycle dependent regulation.   Ectopic expression of CSPP-S but not CSPP-L resulted in cell-cycle arrest in early G1 and mitotic phases with the formation of abnormal spindle such as monopolar and multipolar spindles. RNAi-mediated depletion of CSPPs in 293T cells resulted in cell cycle arrest at S-phase (Patzke et al., 2005; Patzke et al., 2006). Although CSPPs are predominantly concentrated at the spindle pole and central spindle, it is still unclear whether CSPPs play a role in the regulation of mitotic progression.

Wu et al recently reported the identification of a GEF, termed MyoGEF (myosin-interacting GEF), which localizes to the cleavage furrow during cytokinesis. MyoGEF binds to nonmuscle myosin II and activates the small GTPase protein RhoA. Disruption of MyoGEF by RNA interference (RNAi) results in the formation of multinucleated cells (Wu et al, 2006). The current study characterizes the functional interaction betweeen CSPPs and MyoGEF by in vitro and in vivo binding assays and demonstrates that the C-terminal region of MyoGEF interacts with the N-terminal region of CSPPs. Finally, time-lapse microscopy reveals that depletion of CSPPs by RNA interference (RNAi) leads to defects in spindle integrity as well as cell cycle arrest at metaphase. The interaction between CSPP and MyoGEF also facilitates MyoGEF-

myosin interaction. All together, the results suggest that myosin-MyoGEF-CSPPs interaction may play a role in regulating mitotic progression.

Material and Methods

Plasmid construction

The full length MyoGEF was cloned into pCS3+MT vector as previously described (**Wu** et. al., 2006). pDSRed-MyoGEF was generated by cloning the full-length MyoGEF cDNA into EcoRI/Sal sites of pDsRed-Monomer-C1 vector (Clontech). Four MyoGEF truncation mutants corresponding to amino acids 71-388, 71-565, 392-565 and 392-780 were cloned into pCMV-3Tag2B (Clontech) and pGEX-5X-1 (GE Healthcare) expression vectors using BamHI and XhoI sites. mCSPP-1, 2 and 3 were cloned into SalI/SalII site of pEGFP-C3 to generate pEGFP-C3-mCSPP-1, 2 and 3 constructs. To generate Myc-tagged mCSPP-1, the full length mCSPP-1 was amplified from GFP-C3-mCSPP-1 by PCR and subcloned into the Bgl2/XbaI site of CS3+MT vector. GFP-tagged CSPP was provided by Dr. Hans-Christian Aasheim. H2B-GFP plasmid was purchased fron Addgene (Addgene plasmid 11680) (Kanda et al, 1998).

Cell culture and transfection

HeLa Tet-ON cells (hereafter HeLa cells) (Clontech) were grown in DMEM supplemented with 10% fetal bovine serum. Cells were transiently transfected with the expression vectors or siRNA as described in each experiment using Lipofectamine 2000 (Invitrogen), according to the manufacturer instructions. HeLa cells were also cotransfected with Myc-MyoGEF and GFP-CSPP, and transfected with Myc-MyoGEF truncation mutants or GFP-tagged mCSPP-1, 2 and 3 by using Lipofectamine 2000 (Invitrogen). Plasmid transfected cells were analysed after 24 h of tranfection whereas cells transfected with siRNA were analysed 48-72 h after transfection.

Antibody generation

A polyclonal antibody, designated #4660, to detect mCSPPs proteins, was generated using the N-terminal 17 amino acids region of mCSPP-1 and mCSPP-3 as the antigenic epitope (Appendix A; amino acid residues shown in red). The antibody could detect mCSPP-1 and mCSPP-3 but not CSPP-L.

Protein expression and *in vitro* translation

GST-MyoGEF polypeptides were expressed in bacterial expression systems (*E. coli*) and purified from BL21 bacterial cells using glutathione agarose beads. BL21 bacterial cell pellets were homogenized by sonication using the Sonic dismembrator (Fisher Scientific) and lysed in 1X PBS containing 1% Triton X-100 for 1h at 4°C. The protein was purified using glutathione Sepharose 4B beads, eluted with 100 mM Tris-HCl (pH 8.0), 5 mM glutathione and dialyzed against 50 mM NaCl, 50mM Tris-HCl (pH 7.5).

Myc-tagged mCSPP-2 protein was synthesized by *in vitro* transcription coupled translation using the TNT SP6 quick coupled transcription/translation system (Promega) according to the instructions of the manufacturer. The *in vitro* transcription/translation product was subsequently used in GST pull-down assays with GST alone and GST-tagged MyoGEF truncation mutants.

Immunoprecipitation and GST pull-down assays

Transfected HeLa cells were lysed in Radioimmune precipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% deoxycholate, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM Na_2VO_4, 1 mM NaF with Protease inhibitor mixture) for 10 min on ice. Cell extracts were collected and precleared with protein A/G agarose beads. The precleared lysate was incubated with agarose-conjugated anti-Myc and anti-GFP antibody overnight at 4°C. After washing, the bound proteins were eluted with 2X SDS loading buffer. For GST pull-down experiments, GST-MyoGEF mutant proteins were immobilized with GSH-agarose beads for 1h on ice. After washing with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% Triton-X-100, 10% glycerol, 0.2 mM EDTA and 1 mM DTT), the GST or GST-MyoGEF mutants/GSH-agarose beads were incubated with *in vitro* translated CS3-mCSPP-2 protein overnight at 4°C. The beads were then washed, resuspended in 2X SDS loading buffer and bound proteins eluted.

Immunoblotting

Cell lysates, immunoprecipitates and pull-down proteins were separated on SDS-PAGE gels, transferred to an Immobilon-P transfer membrane (Millipore), blocked in 5% non-fat milk and incubated with mouse anti-Myc (9E10, 1:1000; Santa Cruz), rabbit anti-GFP (1:1000; Santa

Cruz) antibodies or rabbit anti-myosin IIA (1:5000;) polyclonal antibody as previously described (Philips et. al., 1995). The blots were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz) for 1 h at 23°C. After washing, the blots were visualized by SuperSignal West Pico Luminol/Enhancer solution (Pierce).

Immunofluorescence staining and time-lapse microscopy

HeLa cells grown on coverslips and transfected with plasmid encoding GFP-mCSPP-1, 2 and 3 were fixed in 4% paraformaldehyde for 12 min and permeabilized in 0.5% Triton X-100 for 10 min at 23°C. After blocking with 1% bovine serum albumin for 1hr at 23°C, actin filaments were stained with anti-beta-tubulin primary antibody (1:1000; Sigma) for 3 h and visualized by incubation with a secondary antibody, rhodamine –phalloidin (1:500; Molecular Probes) for 1 hr at 23°C. Cells were mounted in prolonged antifade reagent (Molecular Probes) and images taken with Nikon C-1 confocal microscope.

For live cell imaging, HeLa cells were grown in LabTek I chambered coverglasses (Nunc) and transfected with plasmid or siRNA as described. Before transferring to the microscope, the cell medium was changed to Leibovitz's L-15 Medium (ATCC) supplement with 10% fetal bovine serum. Images of dividing cells were acquired using a Leica DMI 6000 B microscope (Leica).

Results

Identification of mCSPP isoforms

The identification of a novel guanine nucleotide exchange factor, MyoGEF has been previously described (Wu et al, 2006). MyoGEF interacts with non-muscle myosin II, localizes to the cleavage furrow and is important for cytokinesis. MyoGEF was also found to interact with a 286-amino acid polypeptide belonging to the family of centrosome/spindle pole associated proteins in a two-hybrid screening using full-length MyoGEF as the bait (Appendix A: underlined amino acid sequence; Patzke et al, 2005; Patzke et al, 2006). Two isoforms of the human CSPP, designated CSPP and CSPP-L have been reported. CSPP-L differs from CSPP by having a 294 amino acid added to the N-terminus and an isertion of 51 amino acid between the first two coiled-coil domains (Patzke et al, 2005; Patzke et al, 2006). Three mouse CSPP isoforms, designated mCSPP-1, mCSPP-2 and mCSPP-3 were identified from the yeast two-hybrid screen and subsequent alignment with the mouse genomic DNA sequence. The corresponding amino acids of mCSPP-1, mCSPP-2 and mCSPP-3 are 1197, 1142 and 416 respectively (Figure 1A and Appendix A). mCSPP-3 appears to be a truncated form of mCSPP-1, since the entire amino acid sequence of mCSPP-3 except the 19 residues at the C terminus is similar to the N-terminus of mCSPP-1. The differences between mCSPP-1 and mCSPP-2 includes an insertion of 51 amino acids (amino acids 630-680) at the C terminus of mCSPP-1 and 8 amino acids (91-98) inserted at mCSPP-2 amino terminal region. Furthermore, amino acids 1-33 of mCSPP-1 and 1-21 of mCSPP-2 are not conserved Figure 1A; Appendix A).

MyoGEF interacts with mCSPPs

To confirm the interaction between myoGEF and mCSPP isoforms obtained from yeast two hybrid experiments, we tested whether MyoGEF will also interact with CSPPs in transfected HeLa cells. GFP-tagged mCSPP-1, 2 and 3 were each co-expressed in HeLa cells with Myc-tagged MyoGEF. The cell lysates were immunoprecipitated using agarose-conjugated anti-GFP or anti-Myc antibody to determine interaction between mCSPPs and myoGEF. When Myc-MyoGEF was immunoprecipitated with agarose conjugated anti-Myc antibody, GFP-mCSPP-1 was detected by anti-GFP antibody in the immune complex (Figure 2A). Similarly, Myc-MyoGEF was detected by anti-Myc antibody in the immune complex when GFP-mCSPP-2, GFP-mCSPP-3 and GFP-CSPP were immunoprecipitated with agarose conjugated anti-GFP

antibody (Figure 2B). This confirmed the interaction between MyoGEF and CSPP and also suggested that the N-terminal region of CSPPs may be important for the interaction with Myc-MyoGEF since the entire mCSPP-3 amino acid sequence except for 19 amino acids at its C terminus is identical to the N-terminus of mCSPP-1 (Figure 1A and Apendix 1).

To further define the region of interaction with mCSPP, four GST-tagged MyoGEF truncation mutants expressed in bacterial expression system were used in an *in vitro* binding experiment with *in vitro* translated Myc-tagged mCSPP-2. GST alone or GST-MyoGEF fragments were immobilized to the glutathione conjugated agarose and incubated with myc-mCSPP-2 to determine which MyoGEF could interact with Myc-mCSPP-2 *in vitro*. Immunoblot using anti-Myc antibody shows that the GST-tagged MyoGEF truncation mutant (amino acid 392-780) interacts with MyoGEF-mCSPP-2 interaction (Figure 2-4C). The rest of the GST-MyoGEF polypeptides did not show interaction with Myc-mCSPP-2 (top panel, compare lanes 1, 2, and 3 with 4), indicating that C-terminal region of MyoGEF corresponding to amino acids 565-780 in MyoGEF is required for interaction with mCSPPs.

mCSPP localizes to the spindle pole and central spindle during cytokinesis.

It has been reported that both CSPP-S and CSPP-L localized to the centrosome, spindle pole and central spindle (Patzke et al., 2005; Patzke et al., 2006). To determine whether the mCSPPs also localized to the same subcellular structures as the human homologs, cDNAs of the mCSPP isoforms were fused to the 3' end of GFP gene. HeLa cells were transiently transfected with GFP-tagged mCSPP-1, mCSPP-2 and mCSPP-3 for 24 h. The transfected HeLa cells were stained with anti-tubulin antibody to visualize both GFP-mCSPPs and microtubules by confocal microscopy. GFP-mCSPP-3 localized predominatly in the nucleus in interphase cells whereas GFP-mCSPP-1 showed diffuse distribution in both cytoplasm and nucleus (data not shown). GFP-mCSPP-2 on the other hand forms speckle-like structures which localized predominantly in the nucleus 18 h after transfection but re-distributed to both nucleus and cytoplasm after 18 h. However, consistent with the localization of its human homologs, Figure 3 shows that all GFP-mCSPP isoforms, GFP-mCSPP-1(panels a to f), GFP-mCSPP-2 (panels g to l) and GFP-mCSPP-3 (panels m to r) localized to the spindle pole and central spindle during cytokinesis, with the exception of GFP-mCSPP-1 which also showed diffuse distribution during cytokinesis. Similar to mCSPP interaction with MyoGEF, the N-terminal region of mCSPPs appears to be critical for

localization, as demonstrated by the localization of GFP-mCSPP-3 which almost constitutes the N-terminal region of the mCSPPs.

Strikingly, although human and mouse CSPP are conserved and CSPP-L and mCSPP-1 show 71% amino acid similarity, they localized differently in interphase cells. Unlike CSPP-S and CSPP-L, GFP-mCSPPs did not localized to the centrosome and microtubules in interphase cells suggesting that mouse and human CSPPs are regulated differently during interphase (Patzke et al 2005; Patzke et al 2006; data not shown). It has been reported that overexpression of both CSPP and CSPP-L in culture cells resulted in defect in spindle formation (Patzke et al, 2005; Patzke et al, 2006). Therefore to determine whether overexpression of mCSPP isoforms also induces cytokinetic defect, cell cycle progression of HeLa cells expressing GFP-mCSPP-1, GFP-mCSPP-2 and GFP-mCSPP-3 were monitored by time-lapse microscopy. HeLa cells expressing GFP-mCSPP-1 and GFP-mCSPP-3 (Movie S1) displayed normal cell cycle progression and localized to the spindle pole and central spindle. However, ~70% (29 of 40) of HeLa cells expressing RFP-mCSPP-2 and GFP-H$_2$B arrested at metaphase (Movie S2). Of the metaphase arrested cells, the spindles gradually shrank and concentrated to the poles. However GFP-mCSPP-2 concentrated at the spindle pole and central spindle in transfected HeLa cell that progressed beyond metaphase to complete cytokinesis.

MyoGEF colocalization with mCSPP during cytokinesis

As described above, GFP-CSPPs localized to the spindle pole and central spindle, whereas GFP-MyoGEF localized to the central spindle and cleavage furrow (Patzke et al, 2005; Patzke et al, 2006; Wu et al, 2006). To determine the relative localization of MyoGEF and CSPP during cytokinesis, MyoGEF and mCSPP-3 were fused to DsRed and GFP to produce RFP-MyoGEF and GFP-mCSPP-3 respectively. This made it possible to analyse the localization of both MyoGEF and mCSPP-3 in live HeLa cells by time-lapse microscopy. HeLa cells expressing both GFP-mCSPP-3 and RFP-MyoGEF were examined for colocalization at the central spindle. Figure 2-4 and Movie S3 shows a pattern of RFP-MyoGEF diffuse distribution in metaphase followed by gradual accumulation at the cleavage furrow and central spindle in anaphase cells. On the other hand, GFP-mCSPP-3 localized primarily to the spindle pole and central spindle. Notably, GFP-mCSPP-3 localized to the central spindle before the arrival of RFP-MyoGEF at the cleavage furrow zone and central spindle region, suggesting a functional

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role of the dynamic interaction between MyoGEF and CSPP that may regulate localization and/or function of MyoGEF at the cleavage furrow and central spindle.

RNAi-mediated depletion of CSPPs results in cell cycle arrest at metaphase

Patzke et al have reported that over-expression of human CSPPs led to the formation of monopolar and multipolar spindles during cytokinesis (Patzke et al, 2005; Patzke et al, 2006). However, flow cytometry analysis of RNAi-mediated depletion of CSPPs resulted in S-phase arrest, although the localization of CSPPs to the spindle pole and central spindle, suggested a role in regulating mitotic progression. It is possible that CSPP may have multiple functions and that the formation of speckle-like structures observed in some CSPP overexpressed cells which induced interphase arrest may suggest another function of CSPPs in interphase cells such as chromosome duplication. It is also plausible that that the S-phase arrest could have masked the role of CSPP in the regulation of mitotic progression that was missed by flow cytometry analysis. To assess the role of CSPPs in mitosis, cell cycle progression of siRNA mediated depletion of CSPP in HeLa cells expressing H2B-GFP, to allow visualization of chromosome movement, was monitored by time-lapse microscopy 72 h after siRNA treatment. Compared to control cells (Movie S4) which advanced to anaphase in about 30 minutes, approximately 30% (7/20) of the siRNA treated cells (Movie S5) arrested at metaphase after several hours of monitoring. Although the chromosomes aligned successfully at the metaphase plate in these cells, they failed to separate, possibly by activating the mitotic spindle checkpoint mechanism due to defects in chromosome attachment to the kinetochore (Figure 2-5A & B). Immunoblotting of lysates from HeLa cells treated with CSPP siRNA using CSPP-sepcific antibody also showed significant reduction of CSPP protein level (Figure 2-5C). These results clearly suggest that CSPP is important for progression through mitotic phase of the cell cycle.

CSPP and MyoGEF may regulate M phase progression via complex formation with myosin II

We have previously reported an interaction between MyoGEF and myosin II (Wu et. al., 2006), and as described above, Myc-mCSPP-2 also associates with the carboxy terminal region of MyoGEF (Figure 2-2C). To further define the region in MyoGEF responsible for myosin-MyoGEF interaction, four Myc-tagged MyoGEF polypeptides were expressed in HeLa cells.

Cell lysates were subjected to immunoprecipitation using anti-myc antibody. As shown in Figure (3-6A), myc-71-388 and myc-71-565 could immunoprecipitate myosin II (compare lanes 2 and 3 with lanes 4 and 5). To further understand the role of CSPP in MyoGEF-myosin interaction, HeLa cells were either transfected with plasmid encoding Myc-mCSPP-2 or Myc-MyoGEF alone or in combination with plasmid encoding GFP-MyoGEF or GFP-mCSPP-2. A Co-immunoprecipitation experiment showed that Myc-MyoGEF could precipitate more myosin in the presence of GFP-CSPP. This suggests that CSPP, by interacting with MyoGEF, could facilitate MyoGEF-myosin interaction and thereby regulate the function of MyoGEF. It would be interesting to find out the effect of CSPP depletion on the localization of MyoGEF. However, as described above, knockdown of CSPP arrested cells at metaphase. This made it difficult to analyse its effect on MyoGEF localization during cytokinesis. It is possible to circumvent this limitation with the use of small molecule inhibitors but unfortunately, chemical inhibitors of CSPP are not available at the present time.

Discussion

The identification and function of MyoGEF, which localizes to the cleavage furrow and regulates cytokinesis has recently been reported (Wu et al, 2006). The current study details the interaction between MyoGEF and CSPP, a spindle pole/centrosome-associated protein, during the cell cycle progression. The mouse CSPPs were identified in a yeast two-hybrid screen for MyoGEF interacting partners using full length MyoGEF as the bait (unpublished data). The mouse CSPPs, like their human homolog, localize to the spindle and central spindle during cytokinesis. Depletion of MyoGEF and CSPP leads to multinucleate formation and metaphase arrest respectively, suggesting a role of both proteins during mitotic progression. Minor differences were observed in the localization of the three mCSPP isoforms. Although they all localize to the spindle pole and central spindle, GFP-mCSPP-1 also shows diffuse distribution, whereas GFP-mCSPP-2 forms speckle-like structures in interphase cells. In terms of amino acid sequences, the difference between mCSPP-1 and mCSPP-2 is that the N-terminal 33-amino acids of mCSPP-1 and N-terminal 21-amino acids of mCSPP-2 are not conserved. In addition, they also differ by 51 amino-acids insertion in mCSPP-1. These differences appear to account for the differences in localization in both interphase and mitosis. Significantly, unlike the other two isoforms, overexpression of GFP-mCSPP-2 results in metaphase arrest with abnormal spindle formation and chromosome congression. This phenotype is consistent with the overexpression of human CSPP-S and CSPP-L including defects in spindle formation (Patzke et al 2005; Patzke et al 2006). Furthermore, siRNA depletion of CSPP resulted in metaphase arrest. Taken together, the results of overexpression and RNAi are consistent with each other and suggest an important role of CSPP in regulation mitotic progression.

The localization of CSPPs to the centrosome, spindle pole and central spindle is consistent with mitotic defects phenotypes observed from siRNA-mediated CSPP depletion in HeLa cells. The central spindle serves as a binding site for many proteins implicated in the regulation of cytokinesis, such as microtubule associated proteins (MAPs), microtubule motor proteins, kinases and chromosomal passenger proteins (Mollinari et al., 2002; Martineau-Thuillier et al., 1998; Zhu et al., 2005; Nigg, 2001; Barr et al., 2004; Ainsztein et al., 1998; Skoufias et al., 2000; Gassmann et al., 2004). Despite the well know function of kinetochores in sensing and regulating metaphase to anaphase transition, there is evidence to suggest that centrosomes may also play a role in this process (Maiato et al, 2004; Huang & Raff 1999). The

evidence came from studies in which the destruction of endogenous and GFP-tagged cyclin B in *Drosophila* cells began in the spindle poles and continued to the central spindle. Recent studies have implicated Fzy/Cdc20 and Fzr/Cdh1 as being responsible for the wave of Cyclin B destruction at the spindle and the cytoplasm respectively (Huang & Raff 1999; Raff et al, 2002). Since CSPPs also associate with centrosomes and microtubules, it is conceivable that they could play a role in mitotic progression.

The initiation and assembly of the cleavage plane is determined by astral microtubules via interaction with the components of the contractile ring, which therefore regulate the dynamics of the cleavage furrow in time and space (Bringmann and Hyman, 2005; Burgess and Chang, 2005; Canman et al., 2003; Eggert et al., 2006; Motegi et al., 2006). Recent studies have proposed a two-step process of positioning the cleavage furrow: a first signal is initiated by the astral microtubules followed by a second signal from the central spindle (Bringmann, 2005; Bringmann et al., 2007; Bringmann and Hyman, 2005). Meanwhile as described above, CSPPs interact with MyoGEF and localize to the spindle pole and central spindle, whereas MyoGEF concentrates at the cleavage furrow during anaphase, suggesting that interaction between MyoGEF and CSPPs could provide a link between the central spindle and the equatorial cortex and thereby regulate the assembly of the contractile ring. This hypothesis can be tested by assessing the effect of CSPP depletion on the localization of MyoGEF during cytokinesis, but unfortunately, silencing CSPP results in metaphase arrest. The metaphase arrest suggests an earlier role for CSPP in mitosis and does not allow analysis of its role in cytokinesis. This is similar to the difficulty researchers faced in trying to determine the role of polo-like kinase 1 during cytokinesis until chemical inhibitors were found to circumvent the problem. It also interesting to note that the spindle pole formation appeared normal at the start of metaphase but became disorganized after some time, indicating a possible role for maintenance of spindle microtubules.

It has also been shown that MyoGEF does not only interact and colocalize with myosin but also activates RhoA to regulate cytokinesis (Wu et al, 2006). Myosin is a component of the contractile ring and is important in the furrowing process. How does MyoGEF, CSPP and myosin cooperate to signal to the cleavage furrow and regulate the contractile ring? In *Drosophila*, a RhoGEF, pebble, regulates the organization of contractile ring by activating RhoA. A RhoGAP, RacGAP50C associates with a kinesin-like protein Pavarotti and regulates microtubule bundling. It has been hypothesized that the complex of RacGAP50C-Pavarotti migrates along the microtubules to the equatorial cortex to interact with Pepple to initiate

contractile ring formation. Similarly, MyoGEF associates with both myosin and CSPP through its N-terminus and C terminus respectively and furthermore, coexpression of CSPP enhances the interaction between MyoGEF and myosin. Is it therefore attractive to propose that the interaction between CSPP and MyoGEF facilitates the concentration of MyoGEF at the cleavage furrow to interact with myosin and activate RhoA thereby contributing to the initiation and positioning of cleavage furrow. It is also possible that the binding of CSPP to MyoGEF could induce a conformational change in MyoGEF, which will facilitate the interaction between MyoGEF and myosin. Human CSPPs contains several potential phophorylation sites including that of Polo and Aurora kinases and have been shown to be serine phosphorylated in vivo (Patzke et al, 2005). Polo kinase localizes to the spindle pole and centrosome and regulate centrosome maturation and and formation (Lane and Nigg, 1996; Sumara et al., 2004; van Vugt et al., 2004). Phosphorylation of ECT2, a cytokinesis regulator, has been shown to regulate RhoA localization and activity. Therefore identifying the mitotic kinases that phosphorylate MyoGEF and CSPP as well as understanding their functional interaction will provide further insight into the regulation of mitotic progression by CSPP.
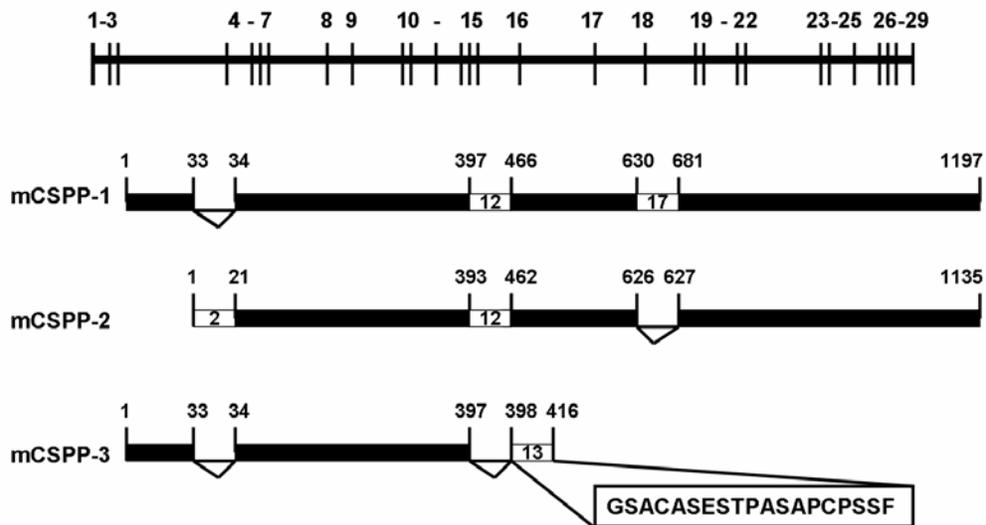
Figure 2-1. Gene structure of mCSPP and comparison of amino acid sequences of mCSPP-1, -2, and -3 isoforms.

(A) Schematic diagram of three isoforms of mCSPP proteins. The isoforms were form by exon skipping, alternative 3' splice site and a shift in reading frame of exon 13. The major differences among mCSPP-1, -2, and -3 are shown: a 51-amino acid insert in the mid-region of mCSPP-1; a 19 amino acids that are present in mCSPP-3, but not in mCSPP-1 and mCSPP-2. The number indicates the amino acids.
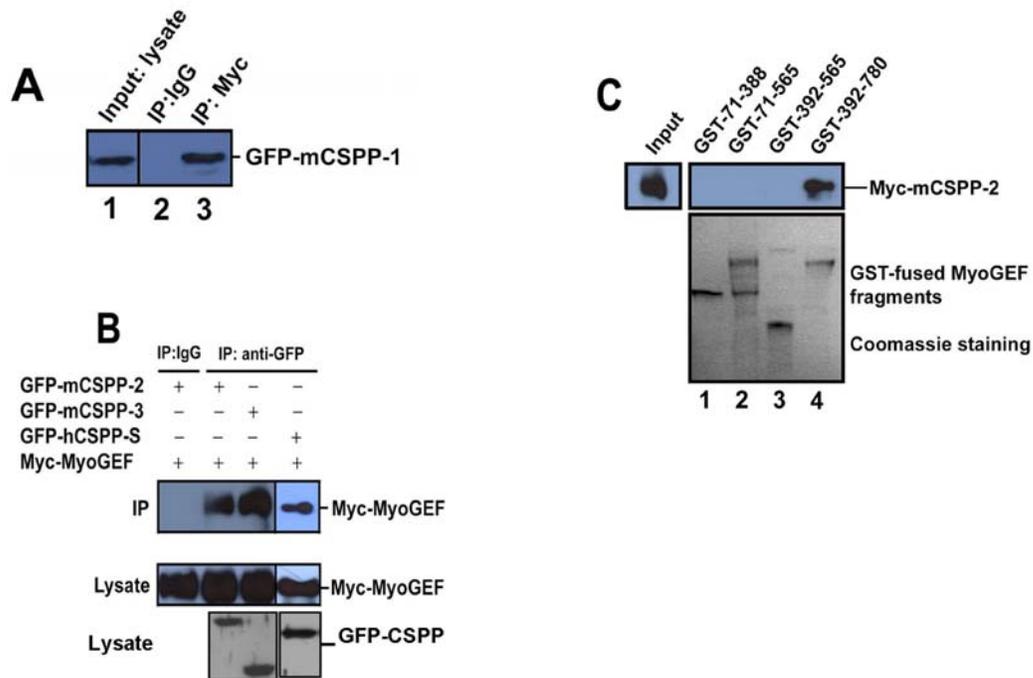
Figure 2-2. mCSPPs interact with MyoGEF.

 (A) Interaction between Myc-MyoGEF and GFP-mCSPP-1. HeLa cells were transfected with plasmids encoding Myc-MyoGEF and GFP-mCSPP-1. Anti-Myc-conjugated agarose was used to bring down GFP-mCSPP-1. (B) Interactions between Myc-MyoGEF and GFP-mCSPP-2, GFP-mCSPP-3, or GFP-hCSPP. A plasmid encoding Myc-MyoGEF was cotransfected into HeLa cells with plasmids encoding GFP-mCSPP-2, GFP-mCSPP-3, or GFP-CSPP. Anti-GFP-conjugated agarose was used to precipitate Myc-MyoGEF. (C) In vitro interaction between Myc-mCSPP-2 and GST-fused MyoGEF fragments. Immobolized GST-fused MyoGEF fragments were used to bring down the in vitro translated Myc-mCSPP-2. Note that only the C-terminal fragment (GST-392-780) can bring down Myc-mCSPP-2 (compare lane 4 with planes 1, 2, and 3).

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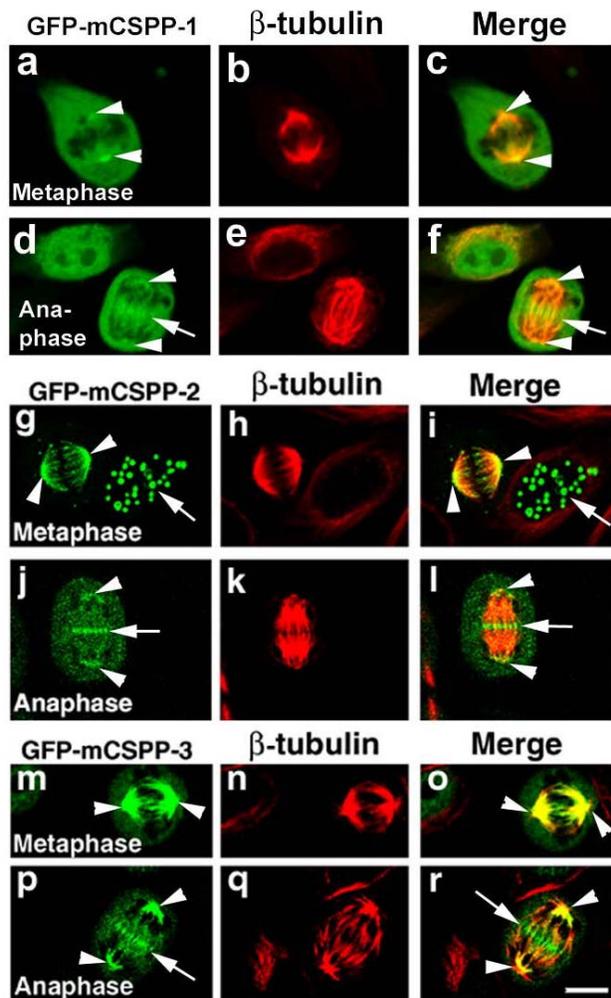
Figure 2-3. Localization of GFP-tagged mCSPP isoforms during cytokinesis.
Plasmids encoding GFP-mCSPP-1 (a-f), GFP-mCSPP-2 (g-l), and GFP-mCSPP-3 (m-r) were transfected into HeLa cells. 24 h after transfection, the transfected cells were fixed in 4% paraformaldehyde and stained with an antibody specific for beta-tubulin (red). Bar, 10 μm.
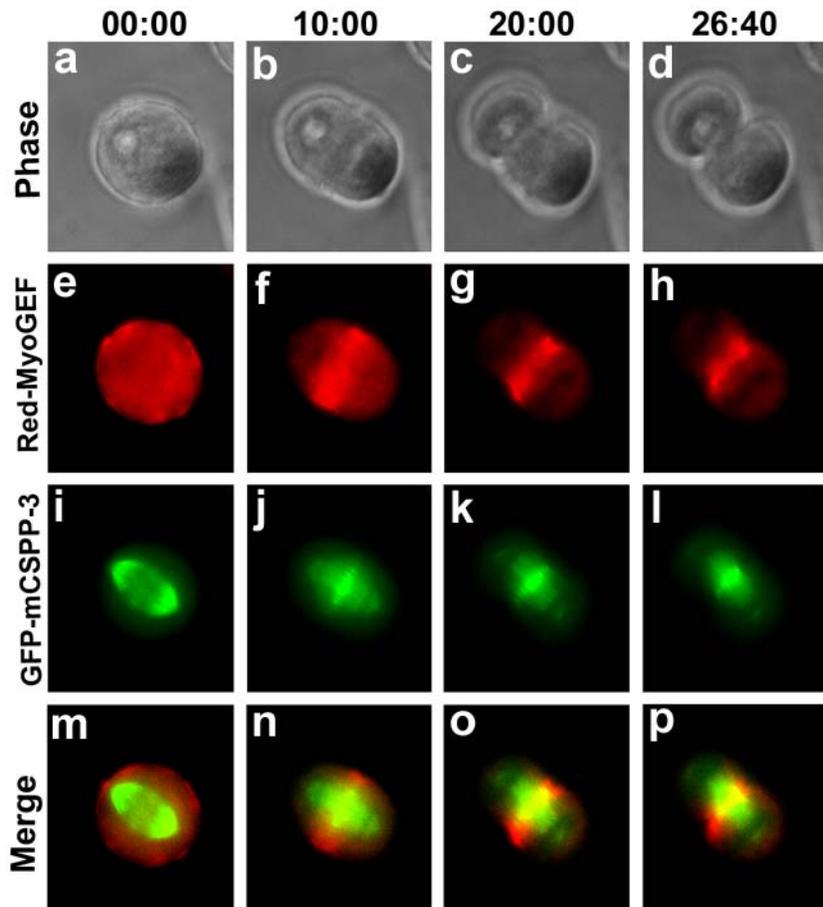
Figure 2-4.Colocalization of RFP-MyoGEF and GFP-mCSPP-3 at the spindle midzone during cytokinesis

 HeLa cells were transfected with plasmids encoding DsRed-MyoGEF (red; panels e-h) and GFP-mCSPP-2 (green; panels i-l). 24 h after transfection, the live transfected cells were monitored for the localization of DsRed-MyoGEF and GFP-mCSPP-2 during cytokinesis by using a Leica DMI 6000 B microscope (Leica). The number represents the time elapse in minutes:seconds.
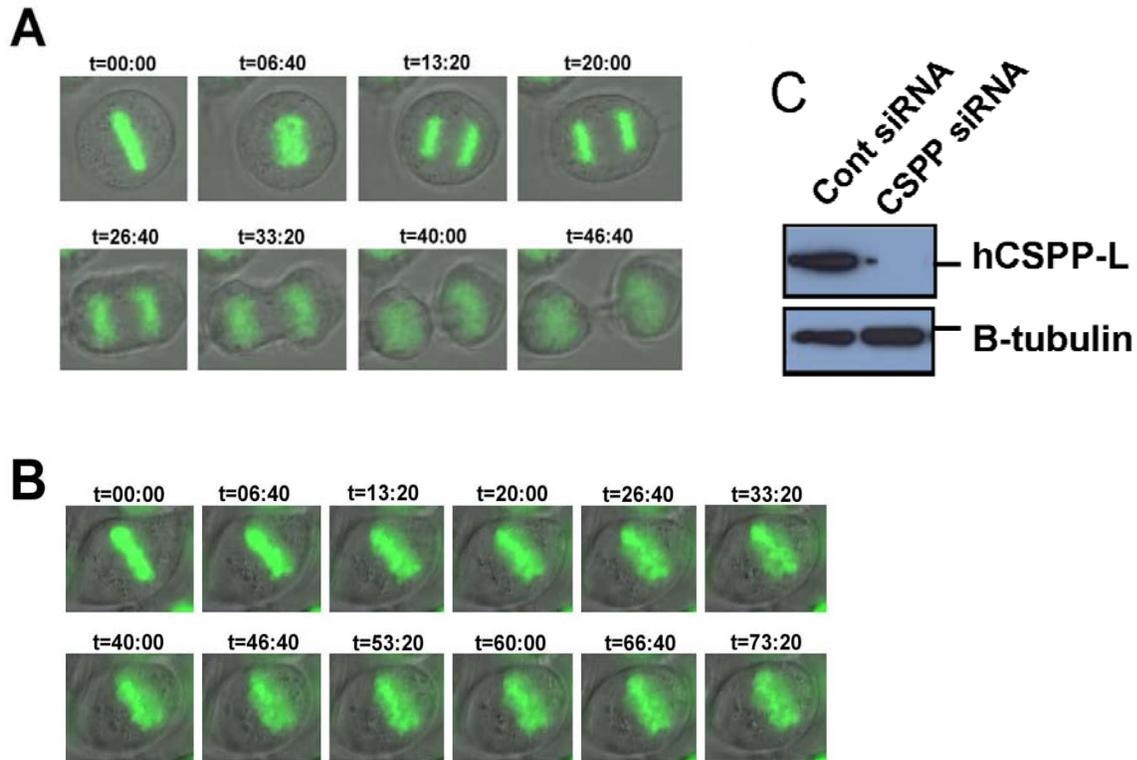
Figure 2-5. RNAi-mediated depletion of CSPP in HeLa cells leads cell cycle arrest at metaphase.

(A & B) HeLa cells were transfected with CSPP siRNA and a plasmid encoding H2B-GFP (green). 48-72h after transfection, the transfected cells were monitored by using a Leica DMI 6000 B microscope (Leica). The number represents the time elapse in minutes:seconds. (C) RNAi-mediated depletion of hCSPP-L in HeLa cells. HeLa cells were transfected with siRNA against CSPPs: control siRNA (lane 1) or CSPP siRNA (lane 2).
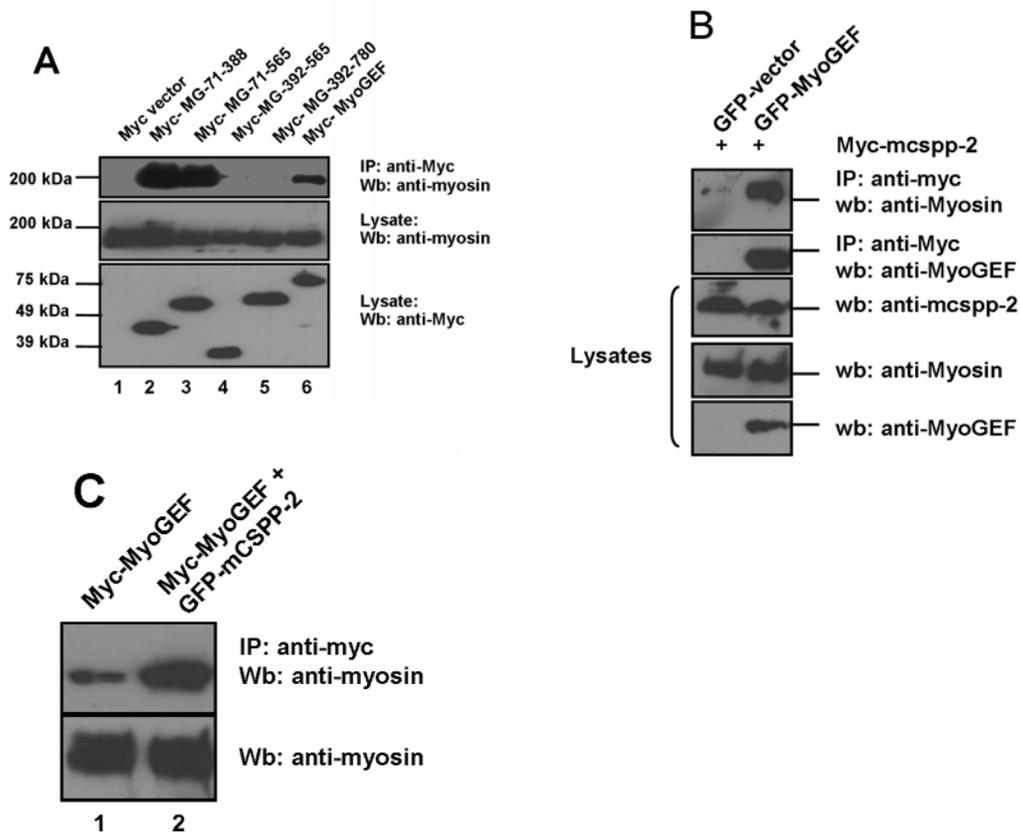
Figure 2-6. Myc-mCSPP-2, GFP-MyoGEF and myosin II form a molecular complex.
(A) Myosin II interacts with N-terminus of MyoGEF. HeLa cells were transfected with plasmid encoding Myc-tagged MyoGEF fragments and MyoGEF full length. 24 hours after transfection, cells lysates were subjected to immunoprecipitation followed by immunoblots with anti-Myc or anti-myosin II antibodies. (B, C) MyoGEF, CSPP and myosin II interact in vivo. HeLa cells were transfected with plasmid encoding Myc-mCSPP-2 or Myc-MyoGEF alone or in combinations with GFP-MyoGEF or GFP-mCSPP-2 plasmid. The transfected cells were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblots with anti-mCSPP, anti-MyoGEF or anti-myosin antibodies. Note that myosin was brought down by coexpression of mCSPP-2 and MyoGEF but not by mCSPP-2 alone.

CHAPTER 3 - PHOSPHORYLATION OF MYOGEF AT THR-574 STIMULATES ACTIVATION OF RHOA

Introduction

RhoA, a small GTPase protein, is a key regulator of the contractile ring in animal cells. RhoA exists either in an active GTP-bound form or inactive GDP bound form. Active GTP-bound RhoA interacts with multiple targets at the cleavage furrow, including proteins that regulate the organization and contraction of the actin–myosin ring (Mackay and Hall; Glover 2001).

RhoA–GTP induces actin filament formation by associating with formins and stimulating their ability to promote actin nucleation and growth. RhoA also interacts with multiple targets such as Rho-activated kinase, ROCK, which phosphorylates two activating sites on myosin regulatory light chain (MRLC), thereby stimulating the assembly and motility of myosin II. ROCK also phosphorylates the regulatory subunit of a myosin phosphatase, resulting in downregulation of the phosphatase activity and further enhancing MRLC phosphorylation (Kimura et al, 1996; Matsui et al, 1996; Matsumura et al 1998; Matsumura, 2005; Glover, 2001).

Upstream regulators of RhoA include guanine-nucleotide exchange factors (RhoGEF) which activates RhoA and GTPase-activating proteins (RhoGAP), which has the reverse effect. A mammalian RhoGEF, Ect2 (called Pebble in *Drosophila*) is required for cytokinesis and localized to the site of cleavage. Localization of Ect2 to the furrow, will ensure its activation by signals that determine the timing and position of cleavage furrow formation.

The central spindle is made up of a bundle of antiparallel microtubules that constitute the core of the central spindle. Other key components of the centralspindlin are MKLP-1 (also called Pavarotti in *Drosophila* and ZEN-4 in C. elegans), a plus-end-directed kinesin-6 motor and a RhoGAP, MycRacGAP (also called RacGAP50C in *Drosophila* and Cyk-4 in *C. elegans*). In mammals and *Drosophila*, Ect2/Pepple associates with a component of the centralspindlin MycRacGAP/RacGAP50, which formed the basis of the proposed model that centralspindlin travels to the cortex along the microtubules to regulate the assembly of the contractile ring via interaction with the RhoGEF (Yuce et al, 2005; Bement et al, 2005; Somers and Saint, 2003).

Other regulatory proteins that localize to the central spindle are passenger proteins, so called because they localize to the kinetochores during metaphase but then relocate to the central spindle at the onset of anaphase. An example is Aurora B, which is required for completion of cytokinesis partly by phosphorylating and thereby promoting the function of MKLP-1 (Guse et al, 2005).

Mitotic kinases, such as Cdk1, polo-like kinase 1 (Plk1), Aurora-A, and Aurora-B, have been found to regulate mitotic events such as microtubule reorganization, chromosome condensation, centrosome separation and nuclear envelope breakdown (Nigg et al., 1996; Nigg, 1998, Nigg, 2001; Severson et al., 2000; Hirota et al., 2003). Plk1 is required for normal mitotic phase progression by associating with mitotic spindles in metaphase but relocates to the midbody in cytokinesis (Nigg et al., 1996; Yuan et al., 2002). The polo binding domain (PBD) of Plk1 mediate the interaction of Plk1 with its substrates. The PBD interacts by recognizing phosphorylation at a specific phospho-Ser- or phospho-Thr-binding domain generated by proline-directed protein kinases, such as Cdk1 or Plk1 itself (Elia et al., 2003a;). ECT2 is phosphorylated by Cdk1 and Plk1 in vitro. The Cdk1 phosphorylation site in Ect2 is used to interact with Plk1 in a phospho-specific manner in M phase (Niiya et al, 2006).

MyoGEF, a novel guanine nucleotide factor involved in cytokinesis, interacts and colocalizes with myosin to the cleavage furrow during cytokinesis (Wu et al, 2006). CSPP localizes to the central spindle pole and spindle during cytokinesis. MyoGEF interacts with both human and mouse isoforms of CSPP and colocalizes to the central spindle. RNAi-mediated depletion of CSPP results in metaphase arrest. CSPP also facilitates the interaction between MyoGEF and mysion. However, it is not known how CSPP and MyoGEF are regulated during the cell cycle, specifically, whether MyoGEF and CSPP are regulated via phosphorylation by mitotic kinases and the function significance of such modifications.

In this study, we have analysed the functional regulation MyoGEF and CSPP by mitotic kinases including Cdk1, Plk1 and Aurora kinases. Plk1 phosphorylates MyoGEF in mitosis and cytokinesis, leading to activation of RhoA. Cdk1 phosphorylation 'primes' MyoGEF for interaction and phosphorylation by Plk1. We have also shown that MyoGEF is phosphorylated

by Aurora A and B kinases. CSPP is phosphorylated by Plk1 and Aurora-B kinases, and these modifications may regulate localization and activation of MyoGEF.

Materials and methods

Plasmid constructs

As described previously, the full length MyoGEF was cloned into pCS3+MT and pcDNA4/HisMax vectors to generate Myc-tagged MyoGEF and His-tagged MyoGEF (Wu et. al., 2006). Six MyoGEF truncation mutants corresponding to amino acids 71-388 (MG-1), 71-565 (MG-2), 392-565 (MG-3), 392-780 (MG-4), 559-790 (MG-5) and 479-780 (MG-6) were cloned into pCMV-3Tag2B (Stratagene) and pGEX-5X-1 (GE Healthcare) expression vectors using BamHI and XhoI sites. MyoGEF fragment 479-780 was cloned using EcoRI/XhoI sites. MyoGEF mutants (MyoGEF-T574A, MyoGEF-T574E, MyoGEF-T585A, MyoGEF-T620A, MG-4T574A, MG-6/ST543/4AA, MG-6SS696/7AA were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagene) and confirmed by DNA sequencing. To generate GFP-mCSPP-2, mCSPP-2 was cloned into SalI/SalII site of pEGFP-C3 vector. Myc-tagged mCSPP-2 was generated by amplifying the full length mCSPP-2 from GFP-C3-mCSPP-2 using PCR and subcloned into the Bgl2/XbaI site of CS3+MT vector. GST-CSPP-L fragments were subcloned using BamHI/XhoI sites into pGEX-5X-1 vector.

Cell culture and synchronization

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum. Cell cycle synchronization was performed by double thymidine block. Briefly, HeLa cells were cultured with 2 mM thymidine (Sigma) for 14 h, and then released into fresh medium for 8 h, and cultured again with thymidine-containing medium for 14 h, then released into fresh media to synchronize cells in different stages of the cell cycle. The cells were released from the interphase arrest at different times and collected at the same time to ensure equal transfection time and obtain cell at different stages of the cell cycle. In transfection experiments, the medium was changed to serum-free Opti-MEM (Invitrogen) before the second thymidine block. Cells were then transfected with expression vectors using lipofectamine (Invitrogen), according to the manufacturer's intructions and then and subjected to immunoprecipitation or GST pull-down assays.

Expression and purification of recombinant protein and *in vitro* translation

GST fused proteins such as MyoGEF polypeptides, CSPP polypeptides, RhoA, Rac1, Cdc42 and RBD were expressed in *Escherichia coli* as described above. The bacterial cell pellets were homogenized and lysed in 1X PBS containing 1% Triton X-100 for 1h at 4°C. Glutathione Sepharose 4B beads were used to purify the proteins and eluted with 100 mM Tris-HCl (pH 8.0), 5 mM glutathione and dialyzed against 50 mM NaCl, 50 mM Tris-HCl (pH 7.5). For GST-RBD purification, bacterial cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF, 1% Triton X-100 with protease inhibitor, and purified with glutathione Sepharose 4B bead.

Myc-tagged Polo kinase protein was expressed using *in vitro* using the TNT SP6 quick coupled transcription/translation system (Promega) according to the manufacturer's instructions. The recombinant proteins were then used in GST pull-down assays.

Immunoprecipitation, GST Pull-down assays and immunoblot

Transfected cells were lysed in Radioimmune precipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% deoxycholate, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM Na2V04, 1 mM NaF with Protease inhibitor mixture) for 10 min on ice. Cell lysates were precleared with protein A/G agarose beads and incubated with agarose-conjugated anti-Myc antibody. The bound proteins were eluted with 2X SDS loading buffer and subjected to immunoblot analysis. For GST pull-down experiments, GST-MyoGEF mutant proteins were immobilized with GSH-agarose beads for 1h on ice. After washing with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% Triton-X-100, 10% glycerol, 0.2 mM EDTA and 1 mM DTT), the GST or GST-MyoGEF mutants/GSH-agarose beads were incubated with *in vitro* translated CS3-mCSPP-2 protein overnight at 4°C. The beads were washed three times with lysis buffer, and bound proteins were eluted from the beads in SDS sample buffer. The eluted proteins were separated by SDS–PAGE and transferred to an Immobilon transfer membrane (Milipore). After blocking for 30 min at room temperature in 5% non-fat dry milk/PBS, the membrane was incubated with the primary antibodies for 3 h at room temperature or overnight at 4°C. The following antibodies were used; mouse anti-Myc (9E10, Santa Cruz), rabbit anti-phosphothreonine (Zymed), anti-RhoA and RhoC (Santa Cruz), anti-MyoGEF described in Wu

et al (2006) and anti-myosin as previously described in Philips et al, (1995). Bound primary antibodies were detected by using HRP-conjugated secondary antibody and visualized by the ECL System (Pierce).

In vitro kinase and phosphatase assays

For Polo kinase 1 assay, 5 μg of purified GST-fused MyoGEF and CSPP fragment were incubated with 0.5 μg recombinant His-Plk1 (Cell signaling) in kinase buffer (5 mMOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EGTA, 4 mM MgCl2, 0.05 mM DTT, 250 μM ATP) and 1 μCi of [γ32]ATP. For the Cdk1 kinase assay, 5 μg of GST-fused MyoGEF fragments were incubated with 0.5 μg of purified GST-fused recombinant Cdk1/cyclinB complex (Cell signaling) in kinase buffer (25 mM Tris–HCl pH 7.5, 10 mM MgCl2, 5 mM β-glycerophosphate, 0.1 mM Sodium Vanadate, 2 mM DTT, 200 μM ATP) and 1 μCi of [γ32]ATP. The reaction mixtures were incubated at 30°C for 30 minutes in 50 μl and resolved on SDS-PAGE gels. The gels were dried and subjected to autoradiography.

Dephosphorylation- After immunoprecipitation of Myc-MyoGEF with agarose- conjugated anti-myc antibody, MyoGEF immune complexes were divided in two equal portions, and each washed twice with complete lysis buffer, twice with lysis buffer without phosphatase inhibitors and finally once with the lamda phosphatase reaction buffer 1X (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij35). The two aliquots were incubated for 30 min at 30°C in presence or absence of 1 μl of lambda phosphatase (Biolabs) in a 50 μl. The reaction was stopped by washing the immunocomplexes twice in 1 ml of lysis buffer containing phosphatase inhibitors to neutralize and eliminate the lambda phosphatase, and then used in a GEF exchange assay.

In vitro guanine nucleotide exchange analysis

The GEF exchange assay was conducted using a fluorescence-based spectroscopic analysis, which measured the incorporation of a fluorescence analog of GTP, *N*-methylanthraniloyl (mant)-GTP onto small GTPase RhoA and RhoC. Briefly, lysates from HeLa cells expressing Myc-MyoGEF, Myc-MyoGEFT574A or from HeLa cells transfected with Plk1 siRNA and Myc-MyoGEF were immunoprecipitated using anti-Myc antibody and washed 4

times with lysis buffer. About 10 μg of GST-tagged RhoA and RhoC were equilibrated in exchange buffer containing 40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl$_2$, 1 mM dithiothreitol, 100 μg/ml BSA, and 400 nM mant-GTP (Cell signaling). After taking a few measurements of steady readings at 25°C using a Perkin-Elmer Life Sciences Victor 3, the anti-Myc immune complex acting as agonist or agarose-conjugated proteinA/G was added and relative fluorescence of incorporation of mant-GTP (excitation λ, 360 nm; emission λ, 440 nm) onto GST-RhoA and RhoC was monitored. The addition of protein A/G beads was to provide a negative control.

RhoA, RhoC and Rac1 Activation

GST-Rhotekin Rho binding domain (RBD) was purified from bacterial cells and immobilized with glutathione agarose (Sigma). HeLa cells were transfected with empty Myc vector, Myc-MyoGEF, Myc-MyoGEFT574A or Myc-MyoGEFT574E using Lipofectamine (Invitrogen) according to the manufacturer's instructions.   24 h after transfection, the cells were lysed in lysis buffer (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% Deoxycholate, 1% Triton X-100 and 0.5 mM MgCl$_2$ with protease inhibitors), centrifuged at 13,000 rpm for 6 minutes at 4°C. Cell lysates were incubated with 10 ug of the GST-RBD for 30 minutes at 4°C and washed 4 times with lysis buffer without SDS and deoxycholate. Bound proteins were eluted in sample buffer and analysed by immunoblot with mouse anti-RhoA antibody.

For the in vitro interaction between MyoGEF and small GTPase proteins, GST-tagged RhoA and Rac1 were immobilized on agarose beads and preloaded with GDP or, a non-hydrolyzable analogue of GTPγS at 30°C for 15 minutes while shaking. The reaction was stopped by the addition of MgCl$_2$ and washed. The GDP or GTPγS bound RhoA and Rac1 were incubated with ThioHis-MyoGEF and then analysed by Immunoblot using anti-MyoGEF antibody. For RhoC activation, purified His-tagged RhoC was preloaded with GDP and GTPγS and then incubated with MyoGEF fragment GST-71-565. The bound proteins were eluted and analyzed on a Immunoblot with anti-RhoC antibody (Santa Cruz).

Results

Cell cycle regulatory proteins are commonly regulated through phosphorylation and dephosphorylation by mitotic kinases. Several mitotic kinases such as Cdk1, Plk1 and Aurora-B kinase have been implicated in the regulation of cytokinesis. Ect2 is activated via phosphorylation first by Cdk1 and then by Plk1. Cdk1-mediated phosphorylation is a prerequisite for Plk1 binding to Ect2 (Niiya et al, 2006). Activated Ect2 or its orthologs, Pebble (*Drosophila*) and Let21 (*C. elegans*) catalyze the activation of RhoA, which then activates myosin at the cleavage furrow via interaction with two downstream targets, Rho-kinase and Citron-kinase. Rho-kinase directly phosphorylates myosin regulatory light chains (MRLC) and it also inhibits myosin phosphatase by phoshorylating its regulatory domain, thereby promoting myosin phosphorylation indirectly. Citron-kinase also stimulates myosin activity through phosphorylation. Whereas ROCK phosphorylation regulates contraction, Citron-kinase controls contractile ring assembly (Matsumura et al 1998; Matsumura, 2005; Glover, 2001; D'Avino et al, 2005).

Plk1 phosporylate MyoGEF at Thr-574 in vitro

To determine whether MyoGEF is phosphorylated by Plk1, four MyoGEF fragments GST-MG-71-388, GST-MG-71-565, GST-MG-392-565 and GST-MG-392-780 were used in an *in vitro* kinase assay with purified recombinant Plk1. The results showed that Plk1 phosphorylated fragment GST-MG-392-780 but not GST-MG-392-565, indicating that the phosphorylation site is located between amino acids 565 to 780 (Figure 3-1A). We then analysed the amino acid sequences of MyoGEF from 565 to 780 for possible phosphorylation sites using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/). Several potential phosphorylation sites were predicted. To identify the Plk1 phosphorylation site, we analysed the predicted sites for conformity with Plk1 consensus phosphorylation site given by [D/E-X-S/T-φ], where X is any amino acid and φ is a hydrophobic amino acid (Kang et al, 2006, Elia et al, 2003a, Elia et al, 2003b and Neef et al, 2003). Of all the potential phosphorylation sites, the region surrounding threonine-574 was the closest match to Plk1 recognition site. We, then, expressed a T574A mutant of GST-MG-392-780 and used it as substrate for the Plk1 in vitro kinase assay. Remarkably, the threonine to alanine mutation abolished the phosphorylation of GST-MG-392-780, indicating that Thr-574 is a Plk1 phosphorylation site (Figure 3-1A). To

determine whether Thr-574 is also phosphorylated in vivo, we transfected HeLa cells with plasmids encoding Myc-tagged full-length wild-type or mutated MyoGEF and then immunoprecipitated these Myc-tagged proteins from the transfected cells 24 h after transfection. Phosphorylation of wild-type or mutants was assessed by immunoblotting with anti-phosphothreonine antibody. As shown in Figure 3-1(B), Myc-MyoGEF (lane 1), Myc-MyoGEF-T585A (lane 3), and Myc-MyoGEF-T620A (lane 4) were threonine-phosphorylated in transfected HeLa cells. In contrast, threonine phosphorylation of Myc-MyoGEF-T574A was dramatically decreased (compare lane 2 with lanes 1, 3, and 4). These results confirmed that MyoGEF is phosphorylated at threonine 574 in vivo.

MyoGEF is phosphorylated at Thr-574 at metaphase and anaphase

We have shown that MyoGEF could be phosphorylated *in vivo* and *in vitro* at Thr-574. To understand the physiological significance of the Plk1 phosphorylation, we examined the timing of MyoGEF phosphorylation at Thr-574 in vivo. HeLa cells were transfected with plasmids encoding Myc-MyoGEF or Myc-MyoGEF-T574A and then synchronized at interphase and mitotic phase using the double thymidine block. Threonine phosphorylation of Myc-MyoGEF or Myc-MyoGEF-T574A was assessed from 0 h to16 h after thymidine block release. This was confirmed by immunofluorescence showing the percentage of cells in mitosis at the different times analysed. We observed threonine phosphorylation of Myc-MyoGEF from 8 h to 16 h after thymidine release (Figure 3-2A), corresponding to metaphase to G1 phases of the cell cycle. Importantly, threonine phosphorylation of the MyoGEF T574A mutant was delayed until G1 phase, suggesting that MyoGEF was phosphorylated at Thr-574 during metaphase and early cytokinesis and that threonine phosphorylation at other sites may be responsible for G1 phase phosphorylation (Figure 3-3B). MyoGEF interacts with both CSPP and myosin. Therefore, we asked whether Plk1 phosphorylation plays any role in regulating MyoGEF-CSPP or MyoGEF-myosin interaction. HeLa cells were transfected with Myc-mCSPP-2 plasmid and either empty GFP vector, or plasmids encoding GFP-MyoGEF or GFP-MyoGEFT574A. Twenty-fours hours after transfection, cell lysates were subjected to immunoprecipitation with agarose conjugated anti-Myc antibody, followed by immunoblotting with antibodies specific for myosin, MyoGEF and mCSPP-2 (Figure 3-3C). As shown in Figure 3-6(A), T574A mutant still interacted with CSPP or myosin II, suggesting that phosphorylation at Thr-574 may not regulate MyoGEF-CSPP

or MyoGEF-myosin interactions. Plk1 has been reported to play a role both in the activation of Ect2 and localization to the cleavage furrow (Tatsumoto et al, 1999; Petronczki et al, 2007).

Identification of Thr-574 in MyoGEF as a Plk1 docking site

The importance of the polo-binding domain, PBD, in the function of Plk1 is well established. The PBD mediates the interaction between Plk1 and its substrates by recognizing a consensus phosphospecific-binding module generated by proline-directed kinases including Cdk1 (Elia et al., 2003a). For example, phosphorylation of Ect2 by Plk1 depends on earlier phosphorylation by Cdk1 at the Plk1 docking site (Niiya et al, 2006). To determine whether Cdk1 phosphorylate MyoGEF, six MyoGEF polypeptides, including one with mutations at potential Cdk1 phosphorylation site, were subjected to *in vitro* kinase assays with Cdk1/cyclinB complex in vitro. As shown in Figure (3-3C), Cdk1 phosphorylates MG-479-780. The marginal decrease in the phosphorylation of fragment MG-6/T543A suggests that Thr-543 may be one of the many Cdk1 phosphorylation sites. To further determine whether the phosphorylation at Thr-543 is required for Plk binding to MyoGEF at mitosis, HeLa cells were cotransfected with plasmid encoding GFP-Plk1 and MycMG-4 or Myc-MG-4T543A and synchronized at interphase and mitosis using double thymidine block. Cell lysates were immunoprecipitated with agarose conjugated anti-Myc antibody. Immunoblot analysis with GFP antibody revealed an interaction between C terminus of MyoGEF and GFP-Plk1 at mitosis. Remarkably, GFP-Plk1 did not bind to a MyoGEF fragment that lacked the Plk1 docking site (MG-4-T543A) (compare lane 4 with lane 8). These results suggest that Cdk1 phosphorylates MyoGEF at Thr-543, generating a docking site for Plk1 to bind and phosphorylate MyoGEF.

MyoGEF interacts with RhoA, RhoC and Rac1

MyoGEF has been reported to activate RhoA (Wu et al, 2006). To confirm this, we tested whether MyoGEF interacts with RhoA, Rac1 or Cdc42. GST-tagged RhoA and Rac1 immobilized on agarose beads were preloaded with GDP or GTPγS, a non-hydrolyzable analogue of GTP. GDP- and GTPγS-bound GST-RhoA and Rac1 were incubated with ThioHis-MyoGEF and then analysed by immunoblotting using anti-MyoGEF antibody. For RhoC interaction, His-tagged RhoC was preloaded with GDP or GTPγS and then incubated with

MyoGEF fragment GST-71-565 that contained both DH and PH domains. The results showed that MyoGEF interacted with both GDP- and GTPγS-bound RhoA and RhoC but interacted with only GDP bound Rac1 (Figure 3-4 A & B).

Phosphorylation of MyoGEF is required for activation of RhoA and RhoC

We previously reported that RNAi-mediated depletion of MyoGEF led to a decrease in RhoA activity (Wu et al, 2006). To determine MyoGEF activity towards RhoA in vitro, we used a fluorescent based biochemical GEF exchange assay to measure the incorporation of a fluorescence analog of GTP, *N*-methylanthraniloyl (mant)-GTP onto small GTPase RhoA and RhoC in the presence or absence of immunoprecipitated Myc-MyoGEF. The assay exploits the different spectroscopic properties exhibited by Mant-GTP bound and unbound small GTPase. Lysates from HeLa cells expressing Myc-MyoGEF were immunoprecipitated with anti-Myc antibody. GST-tagged RhoA and RhoC were then equilibrated in GEF exchange buffer and spectroscopic readings taken for 5 minutes at 25 °C using a Perkin-Elmer Life Sciences Victor 3 fluorometer. The anti-Myc immune complex containing Myc-MyoGEF or Myc-MyoGEFT574A mutant was then added and the relative fluorescence units of mant-GTP incorporation onto GST-RhoA and RhoC was measured for 30 minutes every 30 seconds (excitation λ, 360 nm; emission λ, 440 nm). Protein A/G beads were used as negative control (Figure 3-5A & B). This in vitro assay showed that MyoGEF can activate both RhoA and RhoC.

We then asked whether phosphorylation is important for MyoGEF activity. Myc-MyoGEF immunoprecipitates were treated with or without lambda phosphatase and then used in a GEF exchange assay. Figure (3-5C, D) shows that dephosphorylation of MyoGEF significantly reduced the GEF activity of MyoGEF towards RhoA and RhoC, indicating that phosphorylation is important for MyoGEF activity.

Next we examined whether the effect of phosphorylation on RhoA activation observed above is due to Thr-574 phosphorylation by Plk1. Two different approaches were used to answer this question. HeLa cells were transfected with plasmid encoding Myc-tagged MyoGEF with or without Plk1 siRNA, or Myc-tagged MyoGEF T574A. Twenty-four hours after transfection or 48 h in the case of the siRNA treatment, the cell lysates were immunoprecipitated with agarose-conjugated anti-Myc antibody and the immunoprecipitates were used in labeled GTP incorporation as described above. A decrease in the rate of GTP incorporation assay, indicated

by relative fluorescence unit, suggested that depletion of Plk1 or Thr-574 mutation negatively affect activation of RhoA by MyoGEF (Figure 3-6A). To confirm these results, the rhotekin pull-down assay was performed using wild-type MyoGEF or mutant MyoGEF T574A. RhoA binds to and signals through rhotekin, a downstream target. The Rho-binding domain of rhotekin (RBD) specifically recognizes and binds to active GTP bound RhoA. Purified GST-RBD was immobilized by glutathione-conjugated agarose beads. The beads were then incubated with lysates from HeLa cells expressing Myc empty vector, Myc-MyoGEF, Myc-MyoGEFT574A or Myc-MyoGEFT574E. Immunoblot analysis using anti-RhoA antibody showed a decrease in the amount of GTP-RhoA in HeLa cells expressing Myc-MyoGEFT574A, suggesting that phosphorylation at Thr-574 is important for RhoA activation (Figure3-6B).

MyoGEF and CSPP are also phosphorylated by Aurora kinases

In addition to Plk1, the Aurora kinases, another family of serine/threonine kinases, also regulate multiple events in mitosis including centrosome duplication and condensation, kinetochore-microtubule interactions and chromosome alignment on the metaphase plate, bipolar mitotic spindle formation, spindle checkpoint monitoring and completion of cytokinesis (Carmena and Earnshaw, 2003; Keen and Taylor, 2004). Aurora-A localizes to duplicated centrosomes and spindle pole in mitosis, whereas Aurora-B, also called chromosome passenger protein, exhibits dynamic localization from centromere in prophase to inner centromere in metaphase to central spindle and cortex in anaphase (Bischoff and Plowman, 1999; Dutertre et al 2002; Blagden and Glover 2003). Depletion of Aurora-B kinase by RNAi altered the localization of Ect2 to the central spindle and midbody structures (Chalamalasetty et al, 2006). Since the Aurora kinases, like Polo kinases, are also important in the regulation of mitosis and cytokinesis, we asked whether Aurora-A and B also phosphorylate CSPP and MyoGEF. The same approaches for determining the phosphorylation of Plk1 was used. GST fused MyoGEF or CSPP fragments containing potential Aurora kinase phoshorylation sites were used in an in vitro kinase assay with Aurora-A and B-kinases. As shown in Figure 3-7A, Aurora-B kinase phosphorylated MG-392-780 and MG-588-790 (see Appendix B), indicating that the phosphorylation site is located at the C terminus of MyoGEF. Aurora-A, on the other hand, phosphorylated MG-71-388, MG-392-780, MG-558-790 (Appendix B) and to some extent, MG-392-565, indicating the presence of several Aurora-A phosphorylation sites within MyoGEF

(Figure 3-7B). CSPP was also found to be phosphorylated by Aurora-B. As shown in Figure 3-7C, CPL-254-366 and CPL-893-1001 but not CPL-110-176, CPL-470-535, CPL-893-981 and CPL-1041-1170 were phosphorylated by Aurora-B. CPL-893-981 contains one Aurora-B phosphorylation site (S963) whereas CPL-893-1001 contains two sites (S963, S986), suggesting that amino residue S986 is most likely the Aurora B phosphorylation site (Figure 3-7C). Site-directed mutagenesis will be performed to confirm that Aurora B can phosphorylate CSPP at S986. Phosphorylation by Aurora A was not tested.

Discussion

In this study, we report the identification of mitotic kinases as regulators of the activity and biological function of MyoGEF. We found that Plk1 interacts with MyoGEF and phoshorylates both MyoGEF and CSPP. Plk1 phosphorylates MyoGEF at threonine 574 in vivo and in vitro and the phosphorylation at threonine 574 is required for the activation of the small GTPase protein RhoA. The mutant MyoGEFT574A showed decreased activity towards RhoA. Consistent with this, depletion of Plk1 by RNAi also decreased MyoGEF-mediated RhoA activation.

We have shown that Plk1 phosphorylates MyoGEF at threonine 574. Plk1 localizes to the centrosome, kinetochores, and later to the midzone and regulates mitosis (Barr et al, 2004). In agreement with the notion that Plk1 functions in mitotic and cytokinetic phases, phosphorylation of MyoGEF at Thr-574 by Plk1 occurred during mitosis and early cytokinesis. This coincides with the expression of Plk1 during late G2 and M phases (Golsteyn et al, 1994, Barr et al, 2004). Importantly, phosphorylation in mitotic phase and cytokinesis was abolished when threonine 574 residue was mutated to alanine. However, phosphorylation of the mutant MyoGEF was also detected late in cytokinesis at telophase, most likely due to phosphorylation at a different site. Plk1 also phosphorylates CSPP, a centrosome/spindle pole associated protein found to interact with MyoGEF and localizes to the spindle pole and central spindle. As shown in figure 3-7D, Plk1 predominantly phosphorylates a CSPP fragment that contains a Plk1 phosphorylation site (serine 1156), suggesting that Plk1 may phosphorylate CSPP at S1156. It is known that the polo-binding domain of Plk1 (PBD) recognizes specific phospho-Ser or phospho-Thr motifs phosphorylated by proline-directed mitotic kinases such as Cdk1 (Elia et al, 2003). MyoGEF contains several Cdk1 consensus phosphorylation sites including threonine 543. In vitro kinase assay indicated that Cdk1 can phoshorylate MyoGEF. However, T543A mutation only led to a marginal decrease in phosphorylation signal on the autoradiograph. One possibility is that Cdk1 could also phosphorylate MyoGEF on other residues. Nonetheless, our results indicate that threonine 543 is important for Plk1 binding to MyoGEF in mitosis, because T543A mutant did not interact with MyoGEF in mitosis. It is tempting to speculate that the interaction between MyoGEF and Plk1 *in vivo* may require phosphorylation at threonine 543 by Cdk1 to generate a phospho-binding domain for Plk1 interaction. This mode of regulation is well documented for Plk1. Human Ect2, a critical GEF in cytokinesis, is also a substrate of both

Cdk1 and Plk1. Cdk1 phosphorylates threonine 412, which then serves as a consensus phosphospecific binding region for Plk1. Plk1 phosphorylation leads to an increase in accumulation of GTP-bound RhoA by stimulating Ect2 catalytic activity (Tatsumoto et al, 1999; Niiya et al, 2006). Plk1 phosphorylation also initiates cytokinesis by recruiting Ect2 to the central spindle to promote RhoA activation. We confirmed that MyoGEF can bind and activate RhoA. Furthermore, activation of RhoA by MyoGEF is also regulated by Plk1 phophorylation. Treatment of the immunoprecipitated Myc-MyoGEF with lambda phosphatase resulted in decreased RhoA activation, suggesting that Plk1 phosphorylation is critical for MyoGEF-mediated RhoA activation. Importantly, depletion of Plk1 by siRNA also decreased MyoGEF-mediated RhoA activation. Further, expression of Plk1 phosphorylation-deficient mutant T574A did not induce activation of RhoA. These findings suggest that Plk1 can regulate RhoA activation by phosphorylating MyoGEF.

In contrast, the phospho-mimic mutant T574E did stimulate RhoA activation. Although threonine to alanine mutation is believed to be structurally conserved, it may be argued that the inability of T574A mutant to induce RhoA activation is due to generation of nonfunctional mutant or major structural change as opposed to inability to phosphorylate. However, the structure of the T574 mutant may not have been critically altered based on the fact that residual RhoA activation was still detected and secondly, the mutant protein still localized to the cleavage furrow and central spindle during cytokinesis. This explanation also holds true for the inability of Plk1 to bind to the mutant T543A in mitosis.

CSPP is also phosphorylated by Plk1, most likely at Ser-1156. Localization of CSPP to the centrosome or spindle pole and central spindle is similar to that of Plk1 (Barr et al, 2004). siRNA-mediated depletion of CSPP leads to metaphase arrest, suggesting that CSPP regulates mitotic progression. HsCyk-4 or MycRacGAP is a component of the centralspindlin complex which localizes to the central spindle and regulates cytokinesis (Mishima et al, 2002). HsCyk-4 forms a complex with Ect2 and this link is believed to trigger cortical localization of Ect2 (Saint and Somers, 2003). Plk1 has been proposed to stimulate cytokinesis initiation by inducing a complex formation between Ect2 and HsCyk-4, which then mediate the localization of Ect2 to the central spindle (Petronczki et al, 2007). Similarly, as Plk1 phosphorylates both CSPP and MyoGEF, it is reasonable to speculate that Plk1 may regulate the activity of MyoGEF through CSPP-MyoGEF interaction. CSPP phosphorylation by Plk1 may regulate its interaction with

MyoGEF. This interaction would then recruit MyoGEF to the central spindle to to regulate cytokinesis. Determining the spatial regulation MyoGEF by Plk1 could provide the evidence to support this hypothesis.

The Aurora kinases also regulate mitosis and cytokinesis via their localization to duplicated centrosomes, spindle pole, centromere and central spindle and cortex during mitosis and cytokinesis (Bischoff and Plowman, 1999; Dutertre et al 2002; Blagden and Glover 2003). Aurora-B kinase modulates the localization of Ect2 to the central spindle and midbody structures. Although it is not known whether Aurora-B directly phosphorylates Ect2, the MKLP2-Aurora-B complex directly interacts with Ect2 and regulates the localization of Ect2 to the central spindle (Chalamalasetty et al, 2006). However, it has been reported that Aurora-B phosphorylates both components of the centralspindlin complex, MycRacGAP and MKLP1. We have shown that both MyoGEF and CSPP are directly phosphorylated by Aurora-B kinase in vitro. In addition, MyoGEF is also phosphorylated by Aurora-A.
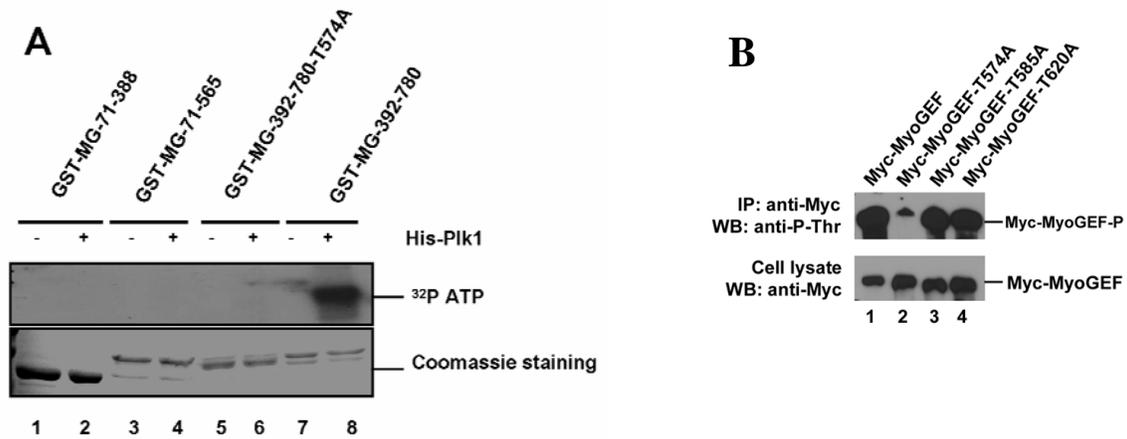
Figure 3-1 Plk1 phosphorylates MyoGEF at Thr-574

(A) Plk1 phosphorylates MyoGEF *in vitro*. Four GST-tagged MyoGEF polypeptides including a T574A mutant were incubated with or without purified Plk1 in the presence of $[\gamma^{32}ATP]$. The proteins were resolved on SDS-PAGE gel visualized by autoradiography or coomassie blue staining. (B) Phosphorylation of MyoGEF *in vivo*. HeLa cells were transfected with plasmids encoding Myc-MyoGEF, Myc-MyoGEF T54A, Myc-MyoGEFT 585A or Myc-MyoGEF T620A. Anti-Myc-conjugated agarose was used to precipitate Myc-tagged proteins from transfected cells, followed by immunoblot using anti-phosphothreonine antibody.
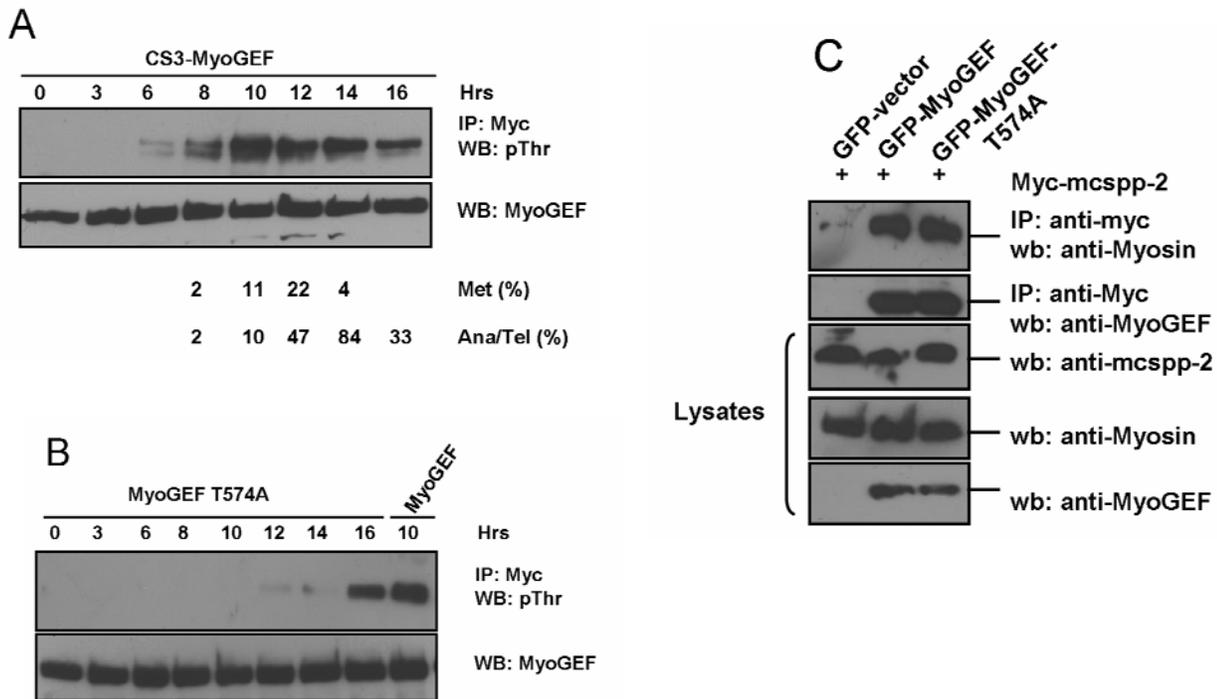
Figure 3-2. MyoGEF is phosphorylated at Thr-574 in mitosis and cytokinesis
(A) Phosphorylation of MyoGEF at mitosis and cytokinesis. HeLa cells expressing Myc-tagged
MyoGEF was synchronized at interphase by double-thymidine block and then released to
progress through the cell cycle. Threonine phosphorylation of MyoGEF at different stages of the
cell cycle was assessed by immunoprecipitation with anti-Myc-conjugated agarose followed by
immunoblotting with phosphothreonine-specific antibody. (B) Phosphorylation of
MyoGEFT574A in mitosis and cytokinesis. HeLa cells expressing Myc-MyoGEFT574A were
synchronized at mitosis and cytokinesis upon release from thymidine arrest. The synchronized
transfected cells were subjected to immunoprecipitation with anti-Myc-conjugated agarose. The
precipitated Myc-MyoGEFT574A proteins were immunoblotted with phosphothreonine-specific
antibody. (C) Threonine 574 mutation is not required for MyoGEF-CSPP interaction. Lysates
from HeLa cells coexpressing Myc-mCSPP-2 and either GFP vector, GFP-MyoGEF or GFP-
MyoGEF-T574A were immunoprecipitated with anti-myc conjugated agarose and analyzed by
immunoblotting using anti-myosin and anti-MyoGEF antibodies.
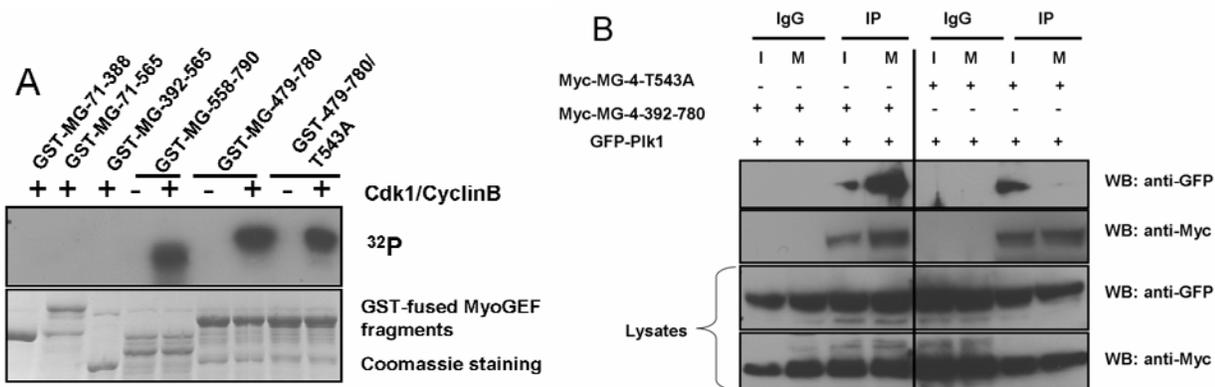
Figure 3-3. Phosphorylation of MyoGEF by Cdk1/CyclinB complex may mediate binding and phosphorylation by Plk1.

(A) Cdk1/CyclinB complex phosphorylates MyoGEF. Six purified GST-tagged MyoGEF polypeptides including a T543A mutant were incubated with purified recombinant Cdk1/cyclinB complex in the presence of [γ^{32}ATP] for an in vitro kinase assay. Phosphorylated proteins were visualized by autoradiography. Equal amounts of GST-MyoGEF polypeptides were verified by coomassie staining. (B) Threonine 543 of MyoGEF is required for Plk1 binding in mitosis. HeLa cells co-expressing GFP-Plk1 and Myc-392-780(MG-4) or Myc-MG4T543A were synchronized at interphase (I) and metaphase (M) by double thymodine block. Mitotic cells were obtained 12 h upon release from thymidine arrest. Myc-MG-4 and Myc-MG-4T543A were brought down by anti-myc-conjugated agarose followed by immunoblot with anti-GFP antibody. Note that T543A mutant did not interact with Plk1 at mitosis (compare lane 4 with lane 8).
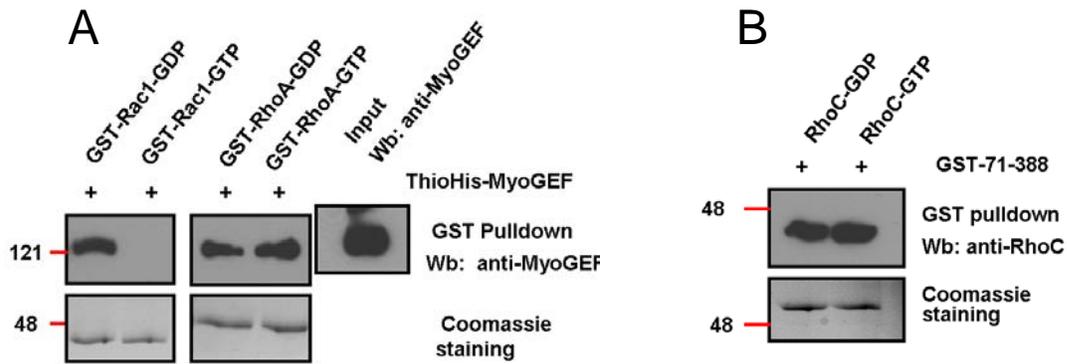
Figure 3-4. MyoGEF interacts with small GTPase proteins including RhoA
(A) MyoGEF interacts with GTP and GDP-bound forms of RhoA. GST-tagged RhoA and Rac1 were preloaded with GDP or GTγP and incubated with ThioHis-MyoGEF. GST pull-down proteins were immunoblotted with anti-MyoGEF antibody. (B) MyoGEF interacts with RhoC in vitro. His-tagged RhoC preloaded with GDP and GTPγS was incubated with MyoGEF fragment GST-71-565. GST pull-down proteins were separated on SDS-PAGE and detected with anti-RhoC antibody.
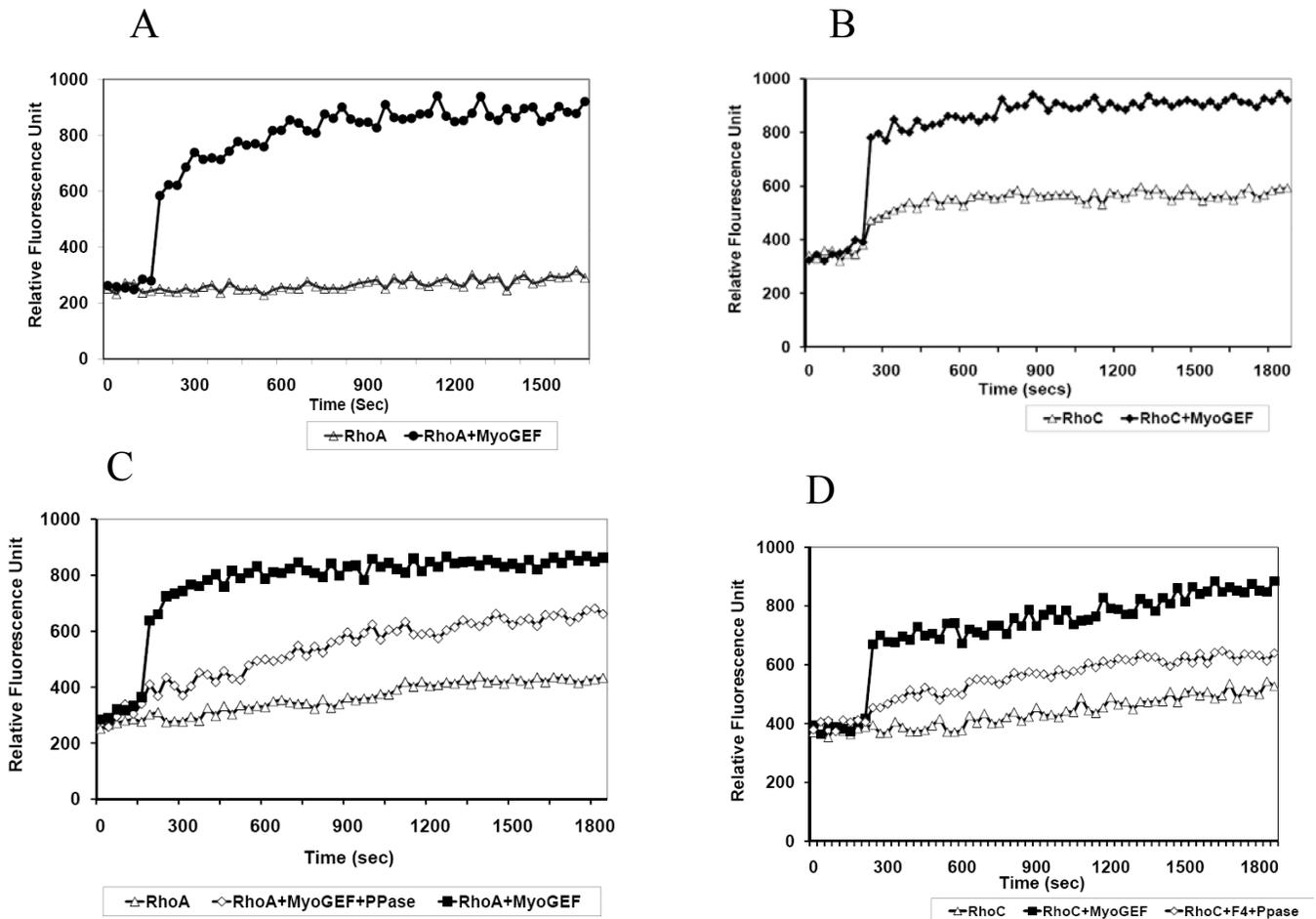
Figure 3-5. Phosphorylation of MyoGEF regulates activation of RhoA and RhoC.
(A & B) Activation of RhoA and RhoC by MyoGEF. The Myc-MyoGEF immunoprecipitate was incubated with mant-GTP and purified GST-tagged RhoA or RhoC, and the rate of nucleotide incorporation was measured over time. (C & D) Dephosphorylation of the immunoprecipitated Myc-MyoGEF decreased the incorporate of GTP onto RhoA and RhoC. Agarose conjugated anti-Myc immunoprecipitate treated with lambda phosphatase was incubated with mant-GTP and GST-RhoA or RhoC. The rate of mant-GTP incorporation onto RhoA and RhoC were indicated by relative fluorescence units.
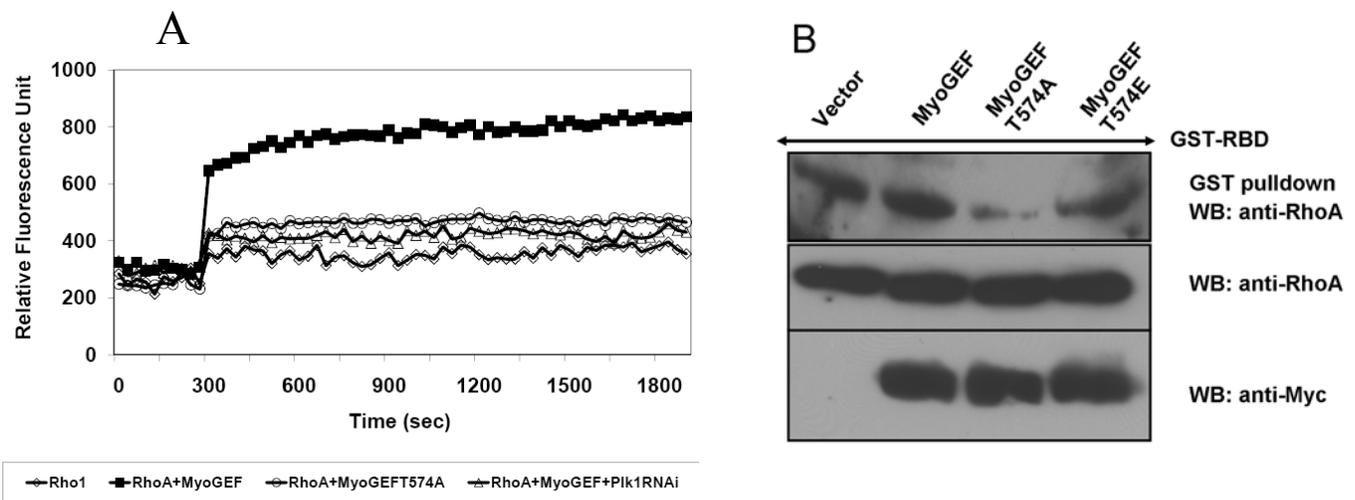
Figure 3-6. MyoGEF-mediated activation of RhoA is regulated by phosphorylation at Thr-574 by Plk1.

(A) Effect of Plk1 phosphorylation on MyoGEF activity. HeLa cells expressing Myc-MyoGEF were depleted of Plk1 by siRNA treatment. Alternatively, HeLa cells were transfected with a plasmid encoding Myc-MyoGEFT574A. Anti-myc conjugated agarose was used to precipitate Myc-MyoGEF and Myc-MyoGEFT574A. The precipitates were incubated with RhoA and mant-GTP to measure RhoA activation as described above. Note that Myc-MyoGEFT574A or Myc-MyoGEF from Plk1-depleted cells did not activate RhoA. (B) Phosphorylation of MyoGEF at Threonine 574 by Plk1 is required for activation of RhoA. GST-RBD of rhotekin was incubated with lysates from HeLa cells expressing either Myc empty vector, Myc-MyoGEF, Myc-MyoGEFT574A or Myc-MyoGEFT574E. The GST pull-down was immunoblotted using anti-RhoA antibody.
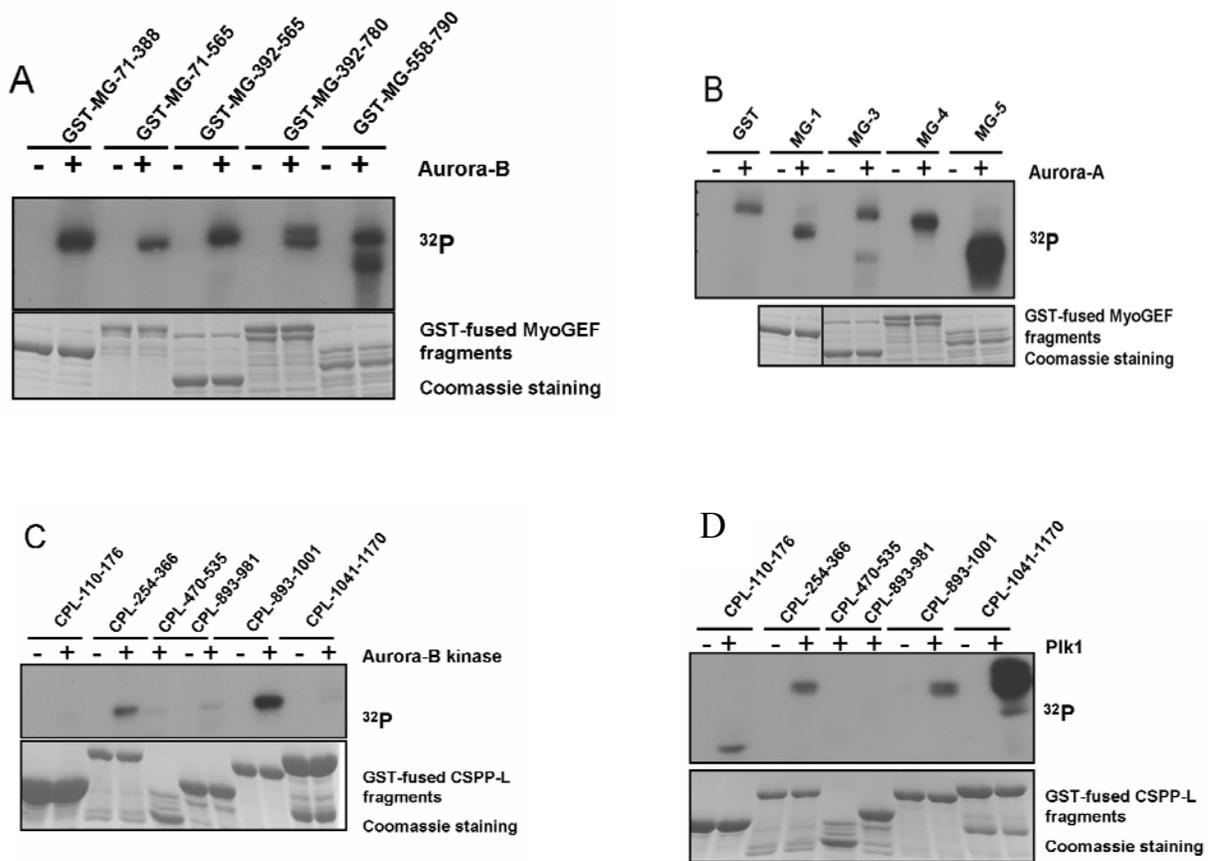
Figure 3-7. MyoGEF and CSPP are phosphorylated by Aurora A & B kinases.

(A & B) Five GST-fused MyoGEF fragments and GST only protein were used in *in vitro* kinase assay with Aurora-A or B kinase. Phosphorylated proteins were detected by autoradiography. (C & D) Six CSPP fragments were incubated with [γ^{32}ATP] and Aurora-B (C) or Plk1 (D). The proteins were separaeted on SDS-PAGE gel and visualized by autoradiography. Equal amounts of GST-MyoGEF and CSPP polypeptides were verified by coomassie staining.

CHAPTER 4 - SUMMARY

This dissertation characterizes the function and regulation of myosin-interacting guanine nucleotide exchange factor, MyoGEF, and censtrosome/spindle pole associated protein, CSPP. These proteins play an important role in the regulation of mitotic phase progression and cytokinesis.

Cytokinesis, the final step of cell division, is defined as the physical separation of a parent cell into two daughter cells with each having its full complement of the nuclear and cytplasmic components. It is a critical step in cell proliferation and therefore must be spatially and temporally regulated to ensure genomic stability from one cell generation to another (Glotzer, 2001). Cytokinesis is tightly coordinated with chromosome segregation and must occur only after chromosomes have faithfully segregated. The division plane must also be positioned between the segregated chromosomes. Failure to delay cytokinesis before completion of mitosis can result in multinucleated cells leading to aneuplody and polyploidy, which are contributing factors to uncontrolled cell proliferation and cancer formation (Guertin et al, 2002). Therefore coordination between mitosis and cytokinesis is critical for maintenance of genomic stability. In addition, the timing and the positioning of cytokinesis is also crucial for a successful cytokinesis. It is known that the central spindle determines the positioning and timing of the contractile ring. A key regulator of the formation and contraction of the contractile ring is the small GTPase protein RhoA. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) act as upstream regulators of small GTPases. The Rho GTPase pathway plays a critical role in signaling to the myosin contractile ring. However, the mode and nature of the signals that are transmitted to the contractile ring by Rho GTPase pathway is still not well understood.

The actin–myosin ring is a central component of the cytokinesis machinery, and its assembly marks a crucial step in the cytokinesis process. The interaction between myosin and actin generates the force required to drive the furrow ingression (Saterwhite and Pollard, 1992). A key component of the contractile ring is a nonmuscle myosin II which localizes to the cleavage furrow (Saterwhite and Pollard, 1992, Dean et al, 2005, Wu and Pollard 2005, Wei and Adelstein, 2000). In animal cells, in which the mechanism that initiate actin–myosin ring contraction is well understood, the contraction is regulated primarily by phosphorylation of the

myosin regulatory light chains (MRLC) on a pair of serine residues (serines 18 and 19 in vertebrate MRLC) close to the amino terminus of the MRLC. MRLC phosphorylation is not only required for the ATP-dependent motor activity but may also contribute to the assembly of myosin II into the bipolar filaments required for the formation actin–myosin bundles. The phosphorylation at these sites increases myosin II activity at the cleavage furrow at the end of mitosis (Matsui et al, 1996; Matsumura et al 1998; Matsumura, 2005; Glover, 2001).

The small GTPase proteins RhoA, Rac1 and Cdc42, function as key regulators of the assembly of actomyosin cytoskeleton (Burridge and Wennerberg, 2004). RhoA GTPases play a central role in the contractile ring assembly by activating the downstream pathway leading to actin polymerization and myosin activation. RhoA regulates the phosphorylation state of MRLC through interaction with multiple targets including the Rho-activated kinase (ROCK), Citron-kinase and mDia. RhoA may induce actin filament formation by interacting with mDia, a formin-homology containing protein and stimulate its ability to induce actin nucleation and growth (Matsumura et al 1998; Matsumura, 2005; Glover, 2001; Kato et al, 2001). RhoA activates both ROCK and Citron kinases. ROCK in turn activates myosin by phosphorylating two activating sites on MRLC and the regulatory subunit of myosin phosphatase, resulting in activation of myosin and inhibition of myosin phosphatase activity (Kimura et al, 1996; Jaffe and Hall, 2005).

The activity of RhoA GTPase is regulated by GEFs and GAPs. GEFs activate RhoA by catalyzing the exchange of GTP for GDP. GAPs, on other hand, inactivate RhoA by stimulating the intrinsic GTPase activity of RhoA to hydrolyse GTP to GDP. RhoA is activated locally at the cleavage furrow by GEFs. Activation of RhoA by GEFs and an increase myosin contractile activity are essential for the initiation and ingression of the cleavage furrow (Bement et al, 2005, Kamijo, et al, 2005). Since RhoA localizes to the cleavage furrow, it is reasonable that its activating GEFs will localize to the cleavage and/or central spindle. Ect2, a critical human GEF, localizes to the central spindle and is required for both initiation and completion of cytokinesis (Tatsumoto et al, 1999; Yuce at al, 2005; Zhao and Fang, 2005). Ect2 and the centralspindlin complex cooperate to promote RhoA accumulation at the equatorial cortex and cleavage furrow formation. The centralspindlin is made up of a GAP called HsCyk-4/MycRacGAP and a kinesin-like protein known as MKLP1. The *Drosophila* orthologs of Ect2, MgcRacGAP and MKLP1 are Pebble(Pbl), RacGAP50C and Pavarotti (Pav) in that order (Nishimura and Yonemura, 2005,

Mishima et al, 2002). It has been proposed that in *Drosophila*, the tricomplex of Pebble and RacGAP50C/Pavarotti links the contractile ring to the cortical microtubules at the cleavage site, where Pebble then regulates the organization of actin-myosin ring and Pav mediates microtubule bundling (Somers and Saint, 2003). The spatial and temporal regulation of Ect2 is modulated by several mitotic kinases. Cdk1 phosphorylates Ect2 to generate phosphospecific binding site for Plk1. Plk1 phoshorylation promote recruitment of Ect2 to the central spindle to initiate cytokinesis. Aurora-B and MKLP2 complex regulate Ect2 localization to the central spindle (Chalamalasetty et al, 2006). It is possible to find the existence of redundancy and tissues-specific mechanisms for the regulation of cytokinesis based on studies from different cell type or tissues (Piekny et al, 2005; Uyeda et al, 2004). For instance, cytokinesis was inhibited in HeLa cells expressing a dominant negative mutant of Ect2, but that phenotype was not observed in Rat1A cells (Yoshita et al, 2004). Furthermore, the fact that over 69 human GEFs have been predicted from the human genome suggests that additional GEFs may participate in independent mechanisms to regulate cytokinesis.

We recently reported the identification of a novel guanine nucleotide exchange factor, MyGEF, which interacts and colocalizes with myosin at the cleavage furrow. MyoGEF regulates cytokinesis via activation of RhoA. Inactivation of MyoGEF by RNAi resulted in the formation of multinucleated HeLa cells (Wu et al, 2006).

A recently identified family of centrosome/spindle pole associated protein (CSPP), localize to the centrosome, spindle pole and central spindle. The two human CSPPs isoforms, CSPP-S and CSPP-L so far identified, exhibited differential cell cycle dependent regulation. Ectopic expression of CSPP-L arrested cells in early G1 and mitotic phases with the formation monopolar and multipolar spindles. Inactivation of CSPP-S by RNAi resulted in cell cycle arrest at S-phase (Patzke et al., 2005; Patzke et al., 2006). Although CSPPs are predominantly concentrated at the spindle pole and central spindle, it is not known if they play any role in the regulation of mitotic progression.

The current study describes the interaction between MyoGEF and CSPP, during the cell cycle progression. Three mouse CSPP isoforms were identified in a yeast two-hybrid screen for MyoGEF interacting partners using full length MyoGEF as the bait (unpublish data). Similar to the localization exhibited by the humans homologs, the mouse CSPP isoforms localized to the spindle pole and central spindle during cytokinesis. Depletion of MyoGEF led to formation of

multinucleate cells whereas losts of CSPP resulted in metaphase arrest. These results suggest that MyoGEF and CSPP regulate mitotic progression. Structurally, there are minor differences between the isoforms. First of all, even though all the isoforms localize to the spindle pole and central spindle, GFP-mCSPP-1 also shows diffuse distribution whereas GFP-mCSPP-2 forms speckle-like structures in interphase cells. Secondly, the first 33 amino acid at the N-terminus of mCSPP-1 and the first 21 amino acids at the N-terminus of mCSPP-2 are not conserved. Thirdly, there is a 51 amino acids insertion after amino acid 629 in mCSPP-2. These differences appear to account for the disparity in localization between mCSPP isoforms in both interphase and mitosis. Significantly, unlike mCSPP-1 and 3, overexpression of GFP-mCSPP-2 results in metaphase arrest with abnormal spindle formation and chromosome congression. This phenotype is similar to those reported for overexpression of human CSPP-S and CSPP-L (Patzke et al 2005; Patzke et al 2006). Inhibition of CSPP by siRNA depletion resulted in metaphase arrest, which conflicts with an earlier report of S-phase arrest from CSPP depletion. Our results, from overexpression and RNAi inhibition, show a consistent phenotype and also correlate localization of CSPP with its function in the regulation mitotic progression. This is supported by the fact that, central spindle serves as a binding site for many proteins implicated in the regulation of cytokinesis, such as microtubule associated proteins (MAPs), microtubule motor proteins, kinases and chromosomal passenger proteins (Mollinari et al., 2002; Martineau-Thuillier et al., 1998; Zhu et al., 2005; Nigg, 2001; Barr et al., 2004; Ainsztein et al., 1998; Skoufias et al., 2000; Gassmann et al., 2004). Centrosomes have been reported to play a role regulating metaphase to anaphase transition (Maiato et al, 2004; Huang & Raff 1999). Therefore, it is reasonable that the localization of CSPPs to the centrosomes and microtubules could suggest a role in mitotic progression.

We have shown that the C terminus of MyoGEF interacts with N-terminus CSPP *in vitro* and in vivo and the N-terminus of MyoGEF also interacts with myosin, forming a complex. MyoGEF interact with myosin in the absence of CSPP, but the presence of CSPP facilitates the interaction. What then is the function of MyoGEF-CSPP interaction or MyoGEF-myosin interaction or CSPP-MyoGEF-Myosin interaction? It is known that initiation and assembly of the cleavage furrow is determined by astral microtubules via interaction with the components of the contractile ring. Astral microtubules therefore regulate the dynamics of the cleavage furrow in time and space (Bringmann and Hyman, 2005; Burgess and Chang, 2005; Canman et al., 2003;

Eggert et al., 2006; Motegi et al., 2006). It has recently been suggested that two consecutive signals initiate cleavage furrow positioning: a first signal initiated by the astral microtubules followed by a second signal from the central spindle (Bringmann, 2005; Bringmann et al., 2007; Bringmann and Hyman, 2005). Alternatively, mCSPP-2 interacts with MyoGEF and localizes to the spindle poles and central spindle before the onset of anaphase when MyoGEF accumulates at the cleavage furrow and later to the central spindle. Since CSPP-MyoGEF interaction precedes the start of mitosis it is conceivable that the interaction could recruit MyoGEF to travel along the spindle microtubules to the equatorial cortex to associate with myosin and activate RhoA, leading to cleavage furrow formation. This hypothesis suggests that interaction between MyoGEF and CSPPs could provide a link between the central spindle and the equatorial cortex and thereby regulate the assembly of the contractile ring. It would be interesting analyse the effect of CSPP depletion on the localization of MyoGEF during cytokinesis. The mitotic arrest induced by CSPP depletion suggests a role for CSPP in mitosis which could mask its role later in cytokinesis. CSPP may also play a role in the maintenance of spindle microtubules based on the observation that the spindle pole formation appeared normal at the start of metaphase but became disorganized after extended period of arrest.

If MyoGEF, CSPP and myosin form a complex to regulate mitotic progression and cytokinesis, what then is the mechanism involved the formation and functional regulation of the complex? One common mechanism of regulating complex formation and function of proteins involved in mitosis is phosphorylation by mitotic kinases including Cdk1, Polo kinase and the Aurora kinases. For example, ECT2 is phosphorylated by Cdk1 and the Plk1 resulting in recruitment to the central spindle and activation of RhoA (Tatsumoto et al, 1999; Petronczki et al, 2007). We identified several kinases that phosphorylate MyoGEF and CSPP at specific amino acid residues and regulate the interaction and function of CSPP and MyoGEF.

We have shown that Plk1 interacts and phosphorylate MyoGEF specifically at threonine 574 *in vivo* and *in vitro* and that the phosphorylation at threonine 574 is required for RhoA activation. Alternatively, the mutant MyoGEFT574A did not activate RhoA. Depletion of Plk1 by RNAi decreased MyoGEF-mediated activation of RhoA. We have identified Thr-543 in MyoGEF as the docking site for Plk1. Our results also show that Plk1 interacted with C terminus of MyoGEF strongly in mitosis but not in interphase. Substitution of Thr-543 to alanine abolished the Plk1-MyoGEF interaction. Since the Thr-543 is a consensus Cdk1 phosphorylation site, it is expected that Cdk1 first phosphorylate MyoGEF at Thr-543, which then serve as binding site for Plk1. Plk1 in turn phosphorylates Thr-574. Plk1 regulates mitosis by localizing

to the centrosome, kinetochores and later to the midzone (Bar et al, 2004). In agreement with Plk1 function we found that the Plk1 phosphorylation at Thr-574 occurred during mitosis and early cytokinesis, which coincides with the expression of Plk1 during late G2 and M phases (Golsteyn et al, 1994, Barr et al, 2004). Remarkably though, the phosphorylation during mitosis and cytokinesis was abolished when T574 was mutated to alanine, although some level of phosphorylation was detected late in cytokinesis at telophase, more likely due to a different kinase other than Plk1.

Ect2 is a also substrate of both Cdk1 and Plk1. Cdk1 phosphorylation of threonine 412 serves as a consensus phosphospecific binding region for Plk1. Plk1 phosphorylation of Ect2 leads to activation of Ect2 catalytic activity as well as an increase in accumulation of GTP-bound RhoA (Tatsumoto et al, 1999; Niiya et al, 2006). Plk1 phosphorylation also initiates cytokinesis by recruiting Ect2 to the central spindle to promote activation of RhoA. Consistent with the regulation of Ect2 by Plk1, we confirmed that MyoGEF could bind and activates RhoA. Furthermore, activation of RhoA by MyoGEF is also regulated by Plk1 phosphorylation. Dephosphorylation of MyoGEF by lambda phosphatase treatment, resulted in reduced RhoA activation, suggesting that Plk1 phosphorylation is critical for RhoA activation. It may be argued that mutation altered the structure of the protein and hence the reduction in activity or binding but the counter-argument is that threonine to alanine mutation is conserved structurally, coupled with the proper localization of the mutant and also some level of activation detected by the mutant.

We have shown that Plk1 can phosphorylate CSPP, most likely at Ser-1156. CSPP localization to the centrosome or spindle pole and central spindle and siRNA-mediated depletion resulting in metaphase arrest, suggest that CSPP may regulate mitotic progression. Plk1 has been reported to stimulate cytokinesis initiation by inducing a complex formation between Ect2 and HsCyk, which then mediates the localization of Ect2 to the central spindle (Petronczki et al, 2007). Since Plk1 phosphorylates both CSPP and MyoGEF, it is possible that Plk1 may regulate the activity of MyoGEF by phosphorylating CSPP. Plk1 phosphorylates CSPP which then interacts with and recruits MyoGEF to the central spindle.

The Aurora kinases also regulate mitosis and cytokinesis (Bischoff and Plowman, 1999; Dutertre et al 2002; Blagden and Glover 2003). Aurora-B kinase regulates Ect2 localization to the central spindle and midbody strucrures through interaction with MKLP2 (Chalamalasetty et

al, 2006). Interestingly, we have shown that both MyoGEF and CSPP are directly phosphorylated by Aurora-B kinase in vitro. In addition, MyoGEF is also phosphorylated by Aurora-A. Although the exact role of MyoGEF and CSPP phosphorylation by Aurora kinases is not known, this phoshorylation could provide basis for further research to unravel the function and regulation of CSPP and MyoGEF and also the mechanism of cytokinesis.

In summary, we have identified three mouse isoforms of CSPP and have characterized their interaction with MyoGEF as well as the trimolecular complex formation involving myosin. We have determined that CSPP is required for mitotic progression and that both CSPP and MyoGEF are phosphorylated by Cdk1, Plk1 and Aurora A and B kinases. Plk1 phosphorylates MyoGEF at Thr-574, and this modification is required for activation of RhoA and therefore cytokinesis. However, many questions, including the role of CSPP in the localization of MyoGEF and the role of Aurora kinase phosphorylation of MyoGEF and CSPP in MyoGEF-CSPP interaction and/or their their localization still needs to be addressed in order to fully understand the function and regulation of CSPP and MyoGEF in mitosis and cytokinesis (Figure 4-1).
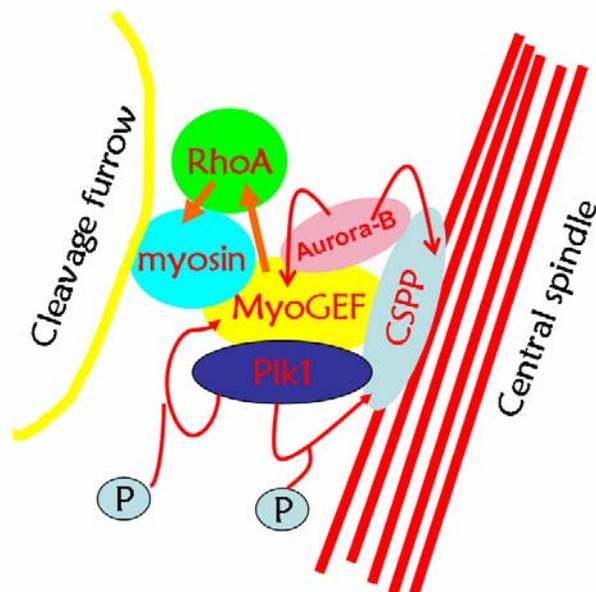

Figure 4-1. A model of interaction among MyoGEF, myosin and CSPP, and phosphorylation by mitotic kinases Plk1, Cdk1 and Aurora-B.

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Appendix A - Sequence comparison of CSPPs

```
hCSPP-L  MLFPLQVAAVTSSVRDDPLEHCVSPRTRARSPEICKMADNLDEFIEEQKARLAEDKAELESDPPYMEMKGKLSAK 75
hCSPP-S  --------------------------------------------------------------------------- 
mCSPP-2  ------------------------------------M--DLNCY-----PHLVAGHAH-----SYLIWRGKASEK 27
mCSPP-1  ------------------------------------ADSLDEFIEEQKAKLAKDKAELESDPPYMEMKGKASEK 39
mCSPP-3  ------------------------------------ADSLDEFIEEQKAKLAKDKAELESDPPYMEMKGKASEK 39

hCSPP-L  LSENSKILISMAKENIPPNS-QQTRGSLGIDYGLSLPLGEDYERKKHKLKEELRQDYRRYLTQ------------ 137
hCSPP-S  --------------------------------------------------------------------------- 
mCSPP-2  LSENSKILISMAKENIPPSSQQQPKGPLGIEYGLSLPLGEDYEQKKHKLKEELRQDYRRYLTQGITQAKRKKNFL 102
mCSPP-1  LSENSKILISMAKENIPPSSQQQPKGPLGIEYGLSLPLGEDYEQKKHKLKEELRQDYRRYLTQ--------KNFL 106
mCSPP-3  LSENSKILISMAKENIPPSSQQQPKGPLGIEYGLSLPLGEDYEQKKHKLKEELRQDYRRYLTQ--------KNFL 106

hCSPP-L  -------------------------ERLKLERNKEYNQFLRGKEESSEKFRQVEKSTEPKSQRNKKPIGQVKPDLTS 189
hCSPP-S  --------------------------------------------------------------------------- 
mCSPP-2  STGETDPSTLGVSLPIDERLSAKERLKLERNREYNQFLRGKAESTEKVRQVEKNIEPKSQRNKNPISQGKSDLPL 177
mCSPP-1  STGETDPSTLGVSLPIDERLSAKERLKLERNREYNQFLRGKAESTEKVRQVEKNIEPKSQRNKNPISQGKSDLPL 181
mCSPP-3  STGETDPSTLGVSLPIDERLSAKERLKLERNREYNQFLRGKAESTEKVRQVEKNIEPKSQRNKNPISQGKSDLPL 181

hCSPP-L  QIQTSCENSEGPRKDVLTPSEAYEELLNQRRLEEDRYRQLDDEIELRNRRIIKKANEEVGISNLKHQRFASKAGI 264
hCSPP-s  --------------------------------------------------------------------------- 
mCSPP-2  QIQTAYTHSEGPW-------------LS--RQEEGLYRQLDGEIELRSRRPLKQTKEEVGISGAEHPSLSGSAGV 237
mCSPP-1  QIQTAYTHSEGPW-------------LS--RQEEGLYRQLDGEIELRSRRPLKQTKEEVGISGAEHPSLSGSAGV 241
mCSPP-3  QIQTAYTHSEGPW-------------LS--RQEEGLYRQLDGEIELRSRRPLKQTKEEVGISGAEHPSLSGSAGV 241

hCSPP-L  PDRRFHRFNEDRVFDRRYHRPDQDPEVSEEMDERFRYESDFDRRLSRVYTNDRMHRNKRGNMPPMEHDGDVIEQS 339
hCSPP-S  ------------------------------MDERFRYESDFDRRLSRVYTNDRMHRNKRGNMPPMEHDGDVIEQS 45
mCSPP-2  PERRARRANGERVLDRQHCRADRDPGVSEDMDERFRFESDFDRRLLRVYTNGRP----HGSRRGYVDGDDVPEEP 308
mCSPP-1  PERRARRANGERVLDRQHCRADRDPGVSEDMDERFRFESDFDRRLLRVYTNGRP----HGSRRGYVDGDDVPEEP 312
mCSPP-3  PERRARRANGERVLDRQHCRADRDPGVSEDMDERFRFESDFDRRLLRVYTNGRP----HGSRRGYVDGDDVPEEP 312

hCSPP-L  NIRISSAENKSAPDNETSKSANQDTCSPFAGMLFGGEDRELIQRRKEKYRLELLEQMAEQQRNKRREKDLELRVA 414
hCSPP-S  NIRISSAENKSAPDNETSKSANQDTCSPFAGMLFGGEDRELIQRRKEKYRLELLEQMAEQQRNKRREKDLELRVA 120
mCSPP-2  NTQISAAENKSVHCNGPPRSADLDITSPFAGMLFGGEGRELTKRRKEKYRQELLEQIAEQQKKKRREKDLAFGVT 383
mCSPP-1  NTQISAAENKSVHCNGPPRSADLDITSPFAGMLFGGEDRELTKRRKEKYRQELLEQIAEQQKKKRREKDLAFGIT 387
mCSPP-3  NTQVSAAENKSVHCNGPPRSADLDITSPFAGMLFGGEDRELTKRRKEKYRQELLEQIAEQQKKKRREKDLAFGIT 387

hCSPP-L  ASGAQDPEKSPDRLKQFSVAPRHFEEMIPPERPRIAFQTPLPPLSAPSVPPIPSVHPVPSQNEDLRSGLSSALGE 489
hCSPP-S  ASGAQDPEKSPDRLKQFSVAPRHFEEMIPPERPRIAFQTPLPPLSAPSVPPIPSVHPVPSQNEDLRSGLSSALGE 195
mCSPP-2  TSGVQDPEKSPDRLKQSSLTPRHFEEM-PPERPRVAFQTPPPPFSAPSSPSVPPVHSAPSHNEDLHSGLGSTLGE 457
mCSPP-1  TSGVQDPEKSPDRLKQFSLTPRHFEEM-PPERPRVAFQTPPPPFSAPSSPSVPPVHSAPSHNEDLHSGLGSTLGE 461
mCSPP-3  TSGVQDPEKSGSACASESTPASAPCPSSF---------------------------------------------- 416

hCSPP-L  MVSPRIAPL----PPPPLLPPLATNYRTPYDDAYYFYGSRNTFDPSLAYYGSGMMGVQPAAYVSAPVTHQLAQPV 560
hCSPP-S  MVSPRIAPL----PPPPLLPPLATNYRTPYDDAYYFYGSRNTFDPSLAYYGSGMMGVQPAAYVSAPVTHQLAQPV 266
mCSPP-2  LAHPRVLPVPLNPPPPPLLAPPASNYRTPYDDAYYFYGARNTLDPNIVYYGSGMIGGQPAPHVSAPVTHQVAPPA 532
mCSPP-1  LAHPRVLPVPLNPPPPPLLAPPASNYRTPYDDAYYFYGARNTLDPNIVYYGSGMIGGQPAPHVSAPVTHQVAPPA 536
mCSPP-3  --------------------------------------------------------------------------- 

hCSPP-L  VNTVGQNELKITSDQVINSGLIFEDKPKPSKQSLQSYQEALQQQIREREERRKKEREEKEEYEAKLEAEMRTYNP 635
hCSPP-S  VNTVGQNELKITSDQVINSGLIFEDKPKPSKQSLQSYQEALQQQIREREERRKKEREEKEEYEAKLEAEMRTYNP 341
mCSPP-2  VNTVGQNEQKVLSDGLRNSGLVFEDKPKPSTQSLQSYQEALQEQIREREARRKKERLEKEEYEAKLEAEMRIYNP 607
mCSPP-1  VNTVGQNEQKVLSDGLRNSGLVFEDKPKPSTQSLQSYQEALQEQIREREARRKKERLEKEEYEAKLEAEMRIYNP 611
mCSPP-3  --------------------------------------------------------------------------- 

hCSPP-L  WGKGGGGAPLRDAKGNLITDLNRMHRQNIDAYHNPDARTYEDKRAVVSLDPNLATSNAENLEDAANKSSGHMQTQ 710
hCSPP-S  WGKGGGGAPLRDAKGNLI-----------------------------------------------------SHMQTQ 365
mCSPP-2  WGKGGGGAPLRDAKGNLI-----------------------------------------------------SPLQTQ 631
mCSPP-1  WGKGGGGAPLRDAKGNLITDLNRMHRQNIDAYHNPDARTYEDKRAVVSIDQNLATSNAENLEDSANKNSGPLQTQ 686
mCSPP-3  --------------------------------------------------------------------------- 
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hCSPP-L  SSPFARGNVFGEPPTELQIKQQELYKNFLRFQIEEKKQREEAERERLRIAEEKEERRLAEQRARIQQEYEEEQEK  785
hCSPP-S  SSPFARGNVFGEPPTELQIKQQELYKNFLRFQIEEKKQREEAERERLRIAEEKEERRLAEQRARIQQEYEEEQEK  440
mCSPP-2  SSPFARGNTFGEPLSELQIKQQELYKNFLRFQIEEKRQREEAEREKLRVAEEKEEKRLAEQRARIQQEYEEEQER  706

mCSPP-1  SSPFARGNTFGEPLSELQIKQQELYKNFLRFQIEEKRQREEAEREKLRVAEEKEEKRLAEQRARIQQEYEEEQER  761
mCSPP-3  --------------------------------------------------------------------------

hCSPP-L  KREKEEEQRLKNEEHIRLAEERQKEAERKKKEEEGKYNLQLQHYCERDNLIGEETKHMRQPSPIVPALQNKIASK  860
hCSPP-S  KREKEEEQRLKNEEHIRLAEERQKEAERKKKEEEGKYNLQLQHYCERDNLIGEETKHMRQPSPIVPALQNKIASK  515
mCSPP-2  RREKEEEQRLKNEELIRLAEERRKEAERKKKEEEEKHNLQLQHYYERENIIGDETKHLRQPSPVVPALQNKIASK  781
mCSPP-1  RREKEEEQRLKNEELIRLAEERRKEAERKKKEEEEKHNLQLQHYYERENIIGDETKHLRQPSPVVPALQNKIASK  836
mCSPP-4  --------------------------------------------------------------------------

hCSPP-L  LQRPPSVDSTIRSFIHESSMSRAQSPPVPARKNQLRAEEEKKNVIMELSEMRKQLRSEEKRLQERLLHMDSDDEI  935
hCSPP-S  LQRPPSVDSTIRSFIHESSMSRAQSPPVPARKNQLRAEEEKKNVIMELSEMRKQLRSEEKRLQERLLHMDSDDEI  590
mCSPP-2  LQRPPSVDTIISSFIHESSMSRAQSPPVPARKNQLRAEEEKKNVIMELSEMRKQLRSEERRLQGRLLHLDSDDEI  856
mCSPP-1  LQRPPSVDTIISSFIHESSMSRAQSPPVPARKNQLRAEEEKKNVIMELSEMRKQLRSEERRLQGRLLHLDSDDEI  911
mCSPP-3  --------------------------------------------------------------------------

hCSPP-L  PIRKKERNPMDIFDMARHRLQAPVRRQSPKGLDAATFQNVHDFNELKDRDSETRVDLKFMYLDPPRDHHTLEIQQ  1010
hCSPP-S  PIRKKERNPMDIFDMARHRLQAPVRRQSPKGLDAATFQNVHDFNELKDRDSETRVDLKFMYLDPPRDHHTLEIQQ  665
mCSPP-2  PMRKRERNPMDIFDMARHRVQAPVRRPSPKGLDATTFQNIHDFNELRERDSDTRVDLRLMYPDPPRDHHTLEIQQ  931
mCSPP-1  PMRKRERNPMDIFDMARHRVQAPVRRPSPKGLDATTFQNIHDFNELRERDSDTRVDLRLMYPDPPRDHHTLEIQQ  986
mCSPP-3  --------------------------------------------------------------------------

hCSPP-L  QALLREQQKRLNRIKMQEGAKVDLDAIPSAKVREQRMPRDDTSDFLKNSLLESDSAFIGAYGETYPAIEDDVLPP  1085
hCSPP-S  QALLREQQKRLNRIKMQEGAKVDLDAIPSAKVREQRMPRDDTSDFLKNSLLESDSAFIGAYGETYPAIEDDVLPP  740
mCSPP-2  QALLREQQKRLNRIKMRRDAGADLDTICTDNAQGRRMPRDDTNDFLKNSLLESDSAFIGAYGETYPVIEDNAFPP  1006
mCSPP-1  QALLREQQKRLNRIKMRRDAGADLDTICTDNAQGRRMPRDDTNDFLKNSLLESDSAFIGAYGETYPVIEDNAFPP  1061
mCSPP-3  --------------------------------------------------------------------------

hCSPP-L  PSQLPSARERRRNKWKGLDIDSSRPNVAPDGLSLKSISSVNVDELRVRNEERMRRLNEFHNKPINTDDESSLVDP  1160
hCSPP-S  PSQLPSARERRRNKWKGLDIDSSRPNVAPDGLSLKSISSVNVDELRVRNEERMRRLNEFHNKPINTDDESSLVDP  815
mCSPP-2  PSQLPSARERRRNKLKGLDFDSSRLHTPQDGLSLKSISSVNVDQVRMRNEDRMRRLTEQQKKPTNTDDEGSLVDP  1081
mCSPP-1  PSQLPSARERRRNKLKGLDFDSSRLHTPQDGLSLKSISSVNVDQVRMRNEDRMRRLTEQQKKPTNTDDEGSLVDP  1136
mCSPP-3  --------------------------------------------------------------------------

hCSPP-L  DDIMKHIGDDGSNSVATEPWLRPGTSETLKRFMAEQLNQEQQQIPGKPGTFTWQGLSTAHG                1221
hCSPP-S  DDIMKHIGDDGSNSVATEPWLRPGTSETLKRFMAEQLNQEQQQIPGKPGTFTWQGLSTAHG                876
mCSPP-2  DDIMRHLSDDGRNSAATEPWLRPGTSESLKRFMAEHLNEEQHKGPGKPGTFTWQGLSAAHA                1142
mCSPP-1  DDIMRHLSDDGRNSAATEPWLRPGTSESLKRFMAEHLNEEQHKGPGKPGTFTWQGLSAAHA                1197
mCSPP-3  ------------------------------------------------------------
```

Appendix B - Structure, essential residues and polypeptide of MyoGEF and CSPP