University of Alberta

Cell Cycle Regulation by the p42 Mitogen Activated Protein Kinase Pathway in Cycling *Xenopus* Egg Extracts

by

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List of Abbreviations

A	Alanine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAK	Cdk activating kinase
CC	Chromosome condensation
Cdc	Cell division cycle
Cdk	Cyclin dependent kinase
CSF	Cytostatic factor
Cyclin BΔ90	Cyclin B with the N-terminal 90 amino acids removed
DMSO	Dimethyl sulfoxide
E	Glutamic acid
EDTA	Ethylenediamine tetraacetic acid
EM	Enriched medium
ERK	Extracellular signal regulated kinase
g	Gravitational force
G1	Gap phase 1
G2	Gap phase 2
GVBD	Germinal vesicle breakdown
HCG	Human chorionic gonadotropin
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
Hr	Hour
IgG	Immunoglobulin G
IP	Immunoprecipitation
IU	International unit
kDa	Kilodalton
L	Leucine
LB	Luria-Bertani broth
MAPK	Mitogen activated protein kinase

MEK	MAPK/ERK Kinase
MEK(QP)	Constitutively-active MEK protein
MII	Metaphase II
Min	Minute
Mos	MAPK kinase kinase
MPF	Maturation (M phase) Promoting Factor
M-phase	Mitotic phase
MWt	Molecular weight
N	Asparagine
NEBD	Nuclear envelope breakdown
P	Proline
p42MAPK	42 kDa isoform of mitogen activated protein kinase
$p90^{Rsk}$	90 kDa Ribosomal S6 kinase
Plx1	Polo-like kinase
$pp90^{Rsk}$	Phosphorylated $p90^{Rsk}$
Q	Glutamine
R	Arginine
RPM	Revolutions per minute
S	Serine
S-phase	DNA synthesis phase
S287A	Serine mutated to an alanine on residue 287
SAC	Spindle assembly checkpoint
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec	Second
T	Threonine
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type
Y	Tyrosine

Chapter 1: Introduction

1.1 General Introduction to the Cell Cycle

The cell cycle consists of an ordered set of biochemical events that leads to the segregation of replicated chromosomes into two daughter cells. Cell cycle regulation is critical for the normal development of multicellular organisms. The standard cell cycle is typically divided into four stages. The first gap phase (G1), S phase (when DNA replication occurs), and the second gap phase (G2) together comprise interphase. Mitosis (M-phase) is the phase when nuclear division (chromosome segregation) and cytoplasmic division (cytokinesis) takes place, and is subdivided into prophase, metaphase, anaphase and telophase. G1 and G2 are the periods of growth during which the cell prepares for DNA synthesis and mitosis, respectively.

There are major monitoring systems that occur during G1, G2 and mitosis. These systems, called cell cycle checkpoints, are intracellularly-stimulated signaling pathways that monitor critical events during cell cycle progression such as DNA replication and chromosome segregation. If a defect is detected, the checkpoints are activated and the cell cycle is prevented from progressing; either the cellular damage is repaired or pathways are activated that lead to programmed cell death. There are several checkpoints that have been well-characterized which include the restriction point (Start in yeast), the DNA damage checkpoint, DNA replication checkpoint, and the spindle assembly checkpoint (SAC) (reviewed by Elledge, 1996). The DNA damage checkpoint and the SAC will be discussed further in this thesis.

1.2 Control of the Cell Cycle

The transition from one phase of the cell cycle to the next is controlled by a number of cellular proteins. The major regulatory proteins that control cell cycle progression are the cyclin dependent kinase (Cdk) complexes which are composed of a regulatory cyclin subunit and a catalytic cyclin-dependent kinase subunit. These serine/threonine protein kinases regulate progression through the cell cycle. Cyclins are regulatory subunits that are continuously synthesized and periodically degraded during the cell cycle to periodically activate Cdk (Evans *et al.*, 1983). To date, five of the nine Cdks that have

been identified are active during the cell cycle (reviewed by Vermeulen *et al.*, 2003). Depending on the stage of the cell cycle, different cyclins are required. The activation of the Cdk complexes induces the activation of downstream processes by selective phosphorylation of proteins (reviewed by Vermeulen *et al.*, 2003). The D type cyclins are G1 cyclins that bind to Cdk4 and Cdk6 and are essential for entry into G1. Cyclin E, another G1 cyclin, binds with Cdk2 to regulate the transition from G1 to S phase. Cyclin A and B are known as a mitotic cyclins because they form a complex with Cdk1 to facilitate entry into M-phase. Cyclin A also associates with Cdk2 and is important during S phase. Lastly, the complex of Cdk7 and cyclin H is known as Cdk activating kinase (CAK).

The complex of Cyclin B and Cdk1 (commonly referred to as Cdc2) known as maturation or M-phase promoting factor (MPF) will be the focus of my thesis. MPF is a universal regulator of the transition from G2 to M-phase of the cell cycle and MPF and the other Cyclin/Cdks are important regulators of cell cycle progression during meiosis and mitosis (reviewed by Fan and Sun, 2004). However, in addition to the Cdk complexes, there are other regulators that control the cell cycle. For example, the mitogen activated protein kinase (MAPK) pathway is known to be an important pathway involved in cell cycle control. The regulatory function of the MAPK cascade paralleling the activity of MPF in regulating oocyte maturation and early embryonic cell cycles will be discussed further.

1.3 The *Xenopus* Model System

Oocytes and eggs of the African clawed frog, *Xenopus laevis*, have been extensively used for the study of the cell cycle. There are many advantages to using the *Xenopus* model system. For example, oocytes or eggs can be obtained in large numbers year-round since *Xenopus* has the ability to spawn when induced with an injection of gonadotropic hormone. Large quantities of oocyte and egg extracts can be obtained easily for biochemical manipulation and analysis. Also, oocytes/eggs are large in size (approximately 1.3 mm in diameter) and resistant to mechanical damage, facilitating microinjection experiments (Murray, 1991). One can obtain large populations of oocytes and eggs that are naturally arrested at specific stages of the cell cycle. By artificial

means, such as applying hormones, calcium ionophores or stimulation by electrical shock, the oocytes and eggs can be parthenogenetically released from their natural arrest and undergo synchronous cell cycle progression.

The cell-free system using *Xenopus* extracts, originally developed by Lohka and Masui (1983) from amphibian eggs, have proven to be very useful for studying the cell cycle. A number of cell-free systems have been well-established and faithfully reproduce *in vitro*, most of the regulatory processes of the cell cycle as they would occur naturally *in vivo* (Lohka and Masui, 1983; Lohka and Maller, 1985; Murray and Kirschner, 1989; Shibuya *et al.*, 1992). Many studies have shown that the *Xenopus* cell-free systems are useful for studying M-phase because they are capable of reproducing nuclear envelope breakdown, chromosome condensation and assembling bipolar microtubule spindles (characteristics of M-phase) *in vitro* (Lohka and Maller, 1985; Murray and Kirschner, 1989). Since their establishment, these cell-free systems have provided a powerful way in which to study the cell cycle *in vitro,* and conduct experiments that would otherwise have been impossible to perform *in vivo*.

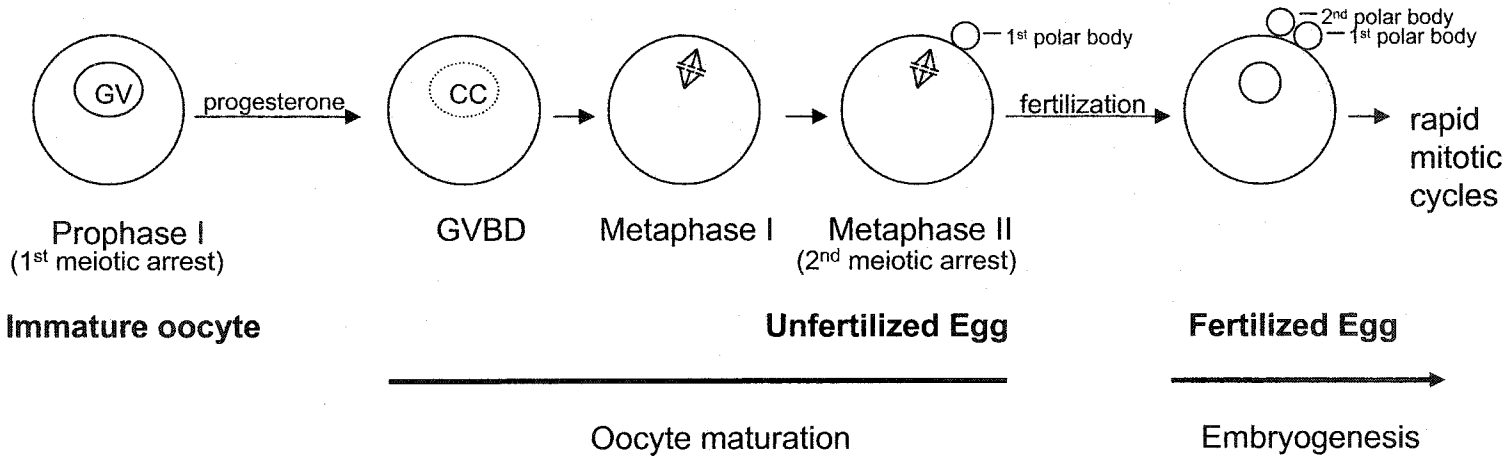
1.4 *Xenopus* Oocyte Maturation

In vertebrates, immature oocytes are transformed into fertilizable eggs by a process known as oocyte (meiotic) maturation in virtually all species (see Fig. 1.1). Immature oocytes are initially arrested in the prophase of the first meiosis (a specialized form of cell division that produces haploid gametes) (reviewed by Sagata, 1998). In *Xenopus*, meiotic maturation and ovulation are initiated when the ovary is stimulated by pituitary gonadotropins which cause follicle cells of the ovary to secrete progesterone. Following hormonal stimulation, the oocyte resumes meiosis I. The first externally visible sign of oocyte maturation is when the large nucleus of the oocyte called the germinal vesicle breaks down in a process called germinal vesicle breakdown (GVBD), synonymous with nuclear envelope breakdown (NEBD), which causes a white spot in the pigmented, animal hemisphere to form. Shortly after GVBD, chromosomes condensation (CC) occurs, the microtubules reorganize into a meiotic spindle and the oocyte completes the first meiotic division by emitting the first polar body (reviewed by Fan and Sun, 2004).

Figure 1.1 Schematic of *Xenopus* Oocyte Maturation

The steroid hormone progesterone causes the immature oocyte to be released from the first meiotic arrest in prophase and undergo oocyte maturation. The first externally visible sign of oocyte maturation is germinal vesicle breakdown (GVBD) followed by chromosome condensation (CC). The first meiotic division is completed on emission of the first polar body. The oocyte is arrested at metaphase II as a mature, fertilizable egg. Fertilization releases the egg from the second meiotic arrest. The second meiotic division is completed on emission of the second polar body and the fertilized egg enters a series of rapid, mitotic, embryonic cell divisions.

Figure 1.1 Schematic of *Xenopus* Oocyte Maturation



The oocyte then enters metaphase of the second meiotic division (meiosis II) and is arrested at this stage as a mature, fertilizable egg.

Fertilization releases the egg from this metaphase II arrest. The fertilized egg (zygote) emits the second polar body as meiosis is completed, and then enters a series of rapid, mitotic embryonic cell cycles. In *Xenopus*, cell cycles 2 to 12 of the embryonic cell cycles are rapid, synchronous 30-minute cycles consisting of solely of S and M-phases (Graham and Morgan, 1966). After 12 rounds of cleavage, gap phases are re-introduced into the cell cycle when transcription begins during the midblastula transition (MBT) (Newport and Kirschner, 1982). The cell cycles are further lengthened during gastrulation (cycles 13 to 15) (Howe *et al.*, 1995). Upon fertilization, the early embryonic cell cycles lack growth phases and most cell cycle checkpoint controls.

The *Xenopus laevis* embryonic system has been the choice of many laboratories for studying the cell cycle because of its many similarities to other organisms. Much of what has been learned using *Xenopus* has been used to increase our general understanding of cell cycle regulation.

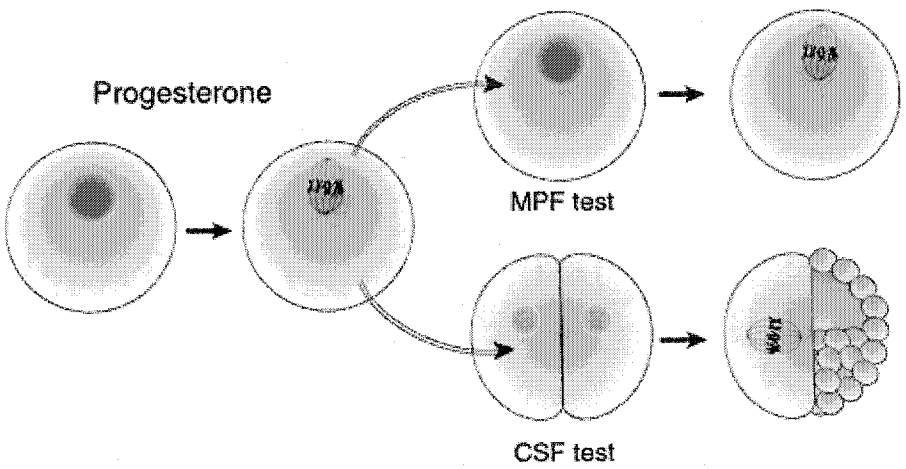
1.5 Cell Cycle Regulation by MPF in the *Xenopus* Early Embryonic System

In *Xenopus,* there are signal transduction pathways that are crucial for oocyte maturation in leading to the activation of maturation or M-phase promoting factor (MPF) and the p42MAPK (the 42 kDa isoform of mitogen activated protein kinase) pathway (reviewed by Tunquist and Maller, 2003). MPF, a complex of Cyclin B and Cdk1, was first discovered as a cytoplasmic factor that caused oocyte maturation (Masui and Markert, 1971). Using the oocytes of *Rana pipiens,* when cytoplasm from an oocyte that had undergone maturation in response to progesterone was transferred into an untreated, immature oocyte, the recipient oocyte was able to mature without hormone (progesterone) treatment (Fig. 1.2). This indicated that the oocyte treated with progesterone produced a factor in the cytoplasm that caused oocyte maturation, thus this factor was called maturation promoting factor (reviewed by Masui, 2001). MPF was biochemically purified from extracts of mature, unfertilized *Xenopus* eggs (Lohka *et al.*, 1988). Purified, active MPF consisted of two proteins, Cdk1 (p34 Cdc2 homolog) with

Figure 1.2 Identification of MPF (Maturation Promoting Factor) and Cytostatic Factor (CSF)

The transfer of cytoplasm from a mature egg into immature oocytes caused the immature oocyte to mature without hormone. Maturation promoting factor (MPF) was identified as the cytoplasmic factor that caused oocyte maturation. Also, transfer of the cytoplasm from a mature egg into a blastomere of a 2-cell embryo caused the injected blastomere to stop cleaving and arrest in metaphase of M-phase. The blastomere that was not injected continued to divide. This experiment demonstrated that the cytoplasmic activity called CSF could prevent zygotes from cleaving and cause a metaphase II arrest. (Figure from Masui, 2001)

Figure 1.2 Identification of Maturation Promoting Factor (MPF) and Cytostatic Factor (CSF)



an apparent mass of approximately 32 kDa (Gautier *et al.*, 1988) and Cyclin B with an apparent mass of 45 kDa (Gautier *et al.*, 1990). MPF (CyclinB/Cdk1) is now known to be a serine/threonine kinase that can phosphorylate the exogenous substrate histone H1 *in vitro*.

MPF later became known as M-phase promoting factor because it was found to be a universal regulator of entry into M-phase during both meiosis and mitosis in all organisms from yeast to man (Wasserman and Smith, 1978; reviewed by Yamashita *et al.*, 2000). MPF is first activated on resumption of meiosis I, inactivated between meiosis I and II, and reactivated at meiosis II. In fully-grown, immature *Xenopus* oocytes, MPF is present as a pre-formed, inactive complex of Cyclin B and Cdk1 (pre-MPF) (Fig. 1.3). Cdk activating kinase phosphorylates Cdk1 on threonine 161 which is necessary for activation. This phosphorylation induces a conformational change in Cdk1 and enhances the binding of cyclins (reviewed by Vermeulen *et al.*, 2003). However, Cdk1 remains inactive due to inhibitory phosphorylations of threonine 14 and tyrosine 15 residues of Cdk1 by Myt1, a dual-specificity kinase (Fig. 1.3) (Gautier *et al.*, 1991; Mueller *et al.*, 1995b). Also, *Xenopus* Wee1 kinase was found to be a major kinase for Tyr15 of Cdk1 (Mueller *et al.*, 1995a).

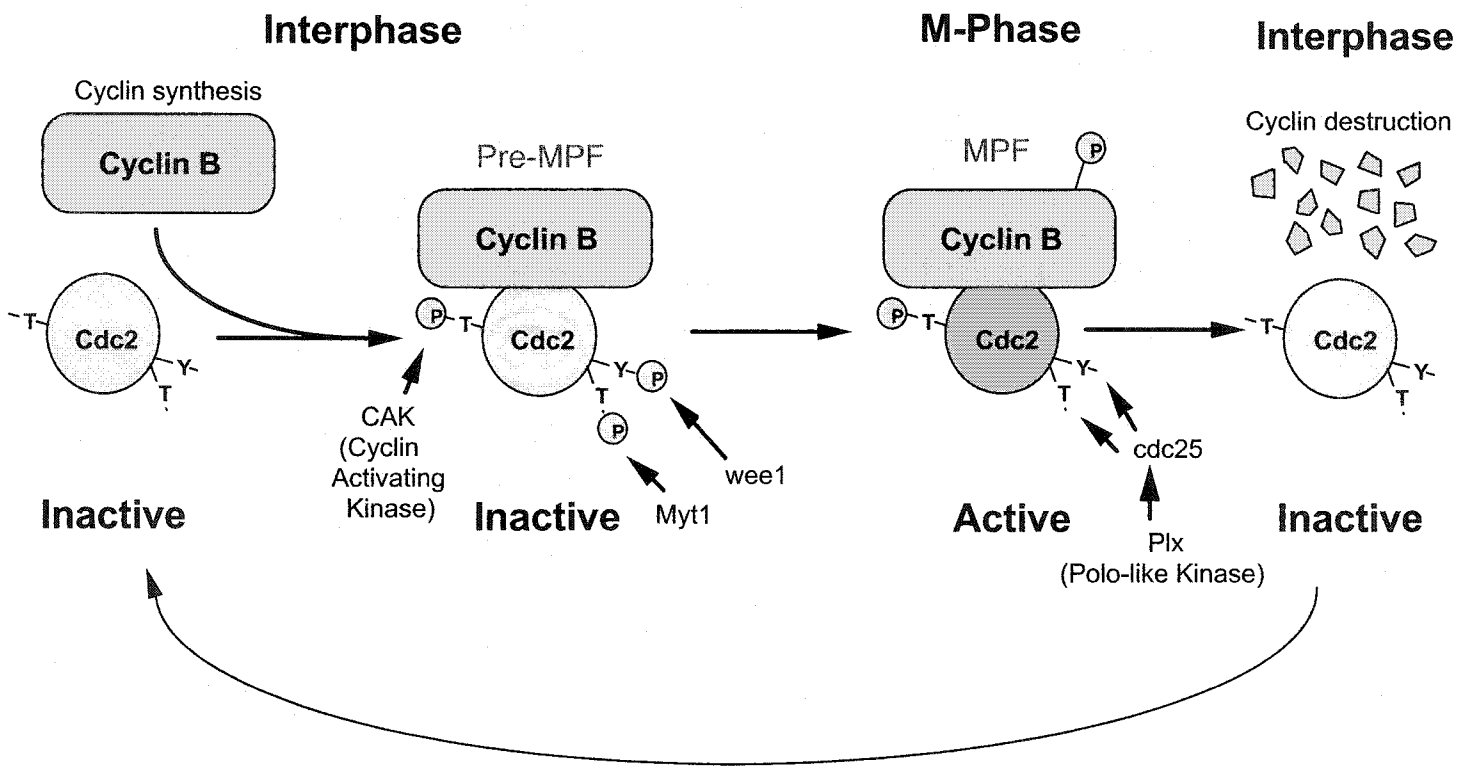
Progesterone also stimulates a pathway leading to the activation of Cdc25C, a dual-specificity phosphatase that removes the inhibitory phosphorylations from Cdk1 leading to the activation of MPF and entry into M-phase (Fig. 1.3) (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Lee et al., 1992; reviewed by Tunquist and Maller, 2003). There are three human homologues of Cdc25, Cdc25A, B, and C but only Cdc25B and Cdc25C can regulate entry into M-phase (reviewed by Nilsson and Hoffmann, 2000). During entry into M-phase in *Xenopus*, Cdc25C is hyperphosphorylated and active as a phosphatase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Kosako *et al.*, 1994). Cdc25C is best characterized for its function in catalyzing the removal of the inhibitory phosphorylations on Cdk1. It is activated by *Xenopus* polo-like kinase (Plx1) (Kumagai and Dunphy, 1996) when Plx1 phosphorylates Cdc25 on serine and threonine residues (Fig. 1.3) (Kumagai and Dunphy, 1996). When Plx1 activation is prevented by immunodepletion of Plx1, hyperphosphorylation of

Figure 1.3 Schematic of the Regulation of M-phase Promoting Factor (MPF) During the *Xenopus* Embryonic Cell Cycle

Cdc2 (Cdk1) exists as an inactive monomer in the cytoplasm. Cyclin B is synthesized and binds to Cdc2. However, inhibitory phosphorylations on threonine14 (Myt1) and tyrosine15 (Wee1) maintain MPF in an inactive state during S-phase, despite phosphorylation of threonine 161 by CAK (Cdk activating kinase). Plx1 activates Cdc25C, a dual-specificity phosphatase that removes the inhibitory phosphorylations from Cdk1. The action of Cdc25C activates MPF, driving the cell cycle from interphase into M-phase. Near the end of M-phase, Cyclin B is degraded and there is a rapid disappearance of MPF activity. Inactivation of MPF leads to the exit from M-phase, and the next cell cycle is initiated.

(Figure provided by Dr. Ellen Shibuya)

Figure 1.3 Schematic of the Regulation of M-phase Promoting Factor (MPF) During the *Xenopus* Embryonic Cell Cycle



Cdc25C and MPF activity is suppressed in *Xenopus* egg extracts (Abrieu *et al.*, 1998; reviewed by Nilsson and Hoffmann, 2000). In addition, Cyclin B/Cdk1 phosphorylates and activates Cdc25C in a positive feedback loop (Kumagai and Dunphy, 1992; reviewed by Nilsson and Hoffmann, 2000). Hence, the signaling pathway that activates Plx1 is required for the activation of MPF before GVBD because Plx1 is responsible for activating Cdc25C.

Upon fertilization and initiation of the early embryonic cell cycles, Cyclin B is fully degraded and MPF activity rapidly disappears. Endogenous Cyclin B is normally degraded by ubiquitin-mediated proteolysis. It has been shown that when the N-terminal 90 amino acids of sea urchin Cyclin B were removed (Cyclin BΔ90), Cyclin B Δ90 was able to bind to and activate Cdk1, but was not degraded at the end of mitosis (Murray *et al.*, 1989; reviewed by Irniger, 2002). In the presence of Cyclin BΔ90, M-phase was sustained with no decrease in MPF activity (Murray *et al.*, 1989). Thus, the degradation of Cyclin B (and all mitotic cyclins) is dependent on a conserved 9 amino acid motif, RXXLXXXXN (R, arginine; L, leucine; N, asparagine; X, any amino acid) in the N-terminal region of Cyclin B (Glotzer *et al.*, 1991) called the destruction box. This motif is required for cyclin proteolysis at the end of M-phase by the ubiquitin-mediated pathway. Following degradation of Cyclin B, the cell exits from M-phase, and once cyclin proteolysis is turned off, cyclins can accumulate and the next cell cycle is initiated (Fig. 1.3).

In summary, phosphorylations of Cdk1 by Myt1 and Wee1 keep Cyclin B/Cdk1 in an inactive state as pre-MPF (in the oocyte) or during S-phase (in the early embryonic cell cycles). Plx1 regulates entry into M-phase by hyperphosphorylating and activating Cdc25C phosphatase which then removes the inhibitory phosphorylations on Cdk1. The activation of MPF is tightly regulated to ensure the proper entry into M-phase.

1.6 Mitogen Activated Protein Kinase as a Regulator of M-phase Arrest

Mitogen activated protein kinases are a family of serine/threonine kinases that are distributed widely in eukaryotic cells and involved in regulating embryogenesis, immune-mediated inflammatory responses, cell cycle regulation, cell proliferation, cell differentiation, and apoptosis (reviewed by Pearson *et al.*, 2001; Hommes *et al.*, 2003).

MAPK has been studied in several different species including echinoderms, *Xenopus*, and mammals during oocyte maturation and embryonic development (Pelech *et al.*, 1988; Ferrell, Jr. *et al.*, 1991; Shibuya *et al.*, 1992; reviewed by Fan and Sun, 2004). There are three classes of members of the MAPK family, the extracellular signal-regulated kinases (ERK), c-jun terminal kinase/stress-activated protein kinases (JNK/SAPK) and p38 related kinases which have been well characterized (reviewed by Pearson *et al.*, 2001). All members of the MAPK family require dual phosphorylation of threonine and tyrosine residues in a TXY motif for full activity.

The extracellular signal-regulated kinases ERK1 and ERK2, also known as p44 and p42MAPK respectively, require phosphorylation on threonine 183 and tyrosine 185 in a TEY motif within its activation loop to become fully activated (reviewed by Tunquist and Maller, 2003). The p42MAPK signaling pathway is activated by numerous extracellular stimuli including growth factors, insulin, phorbol esters, and viral transformation (reviewed by Widmann *et al.*, 1999).

p42MAPK is phosphorylated and activated by the upstream kinase MEK, which itself is phosphorylated activated by Mos or Raf. Since Raf isoforms and Mos appear to phosphorylate only MEK1 and MEK2, they are exclusive to the p42 and p44 MAPK cascade (reviewed by Pearson *et al.*, 2001). In vertebrate oocytes, MEK is activated by phosphorylation by the serine/threonine protein kinase Mos. Mos is a germ cell-specific protein 39 kDa in size that was first identified in cells transformed by Moloney murine leukemia virus (Papkoff *et al.*, 1982). The mRNA for *c-mos* is stored in the oocytes, it is translated into protein shortly after stimulation of the oocytes with progesterone, leading to the activation of the p42MAPK pathway (Sagata *et al.*, 1988; reviewed by Tunquist and Maller, 2003). Downstream of p42MAPK is the serine/threonine kinase $p90^{Rsk}$ (ribosomal S6 kinase with a size of 90 kDa). $p90^{Rsk}$ is directly phosphorylated and activated by p42MAPK in oocytes and its activity is known to be regulated by serine/threonine phosphorylations (Blenis, 1993). From studies in *Xenopus* oocytes, evidence suggests that $p90^{Rsk}$ is the major effector of the p42MAPK pathway and functions in regulating the meiotic cell cycle progression and arrest of the mature egg (Bhatt and Ferrell, Jr., 1999; Gross *et al.*, 1999, 2000; reviewed by Fan and Sun, 2004).

p42MAPK will be emphasized and discussed further because it is expressed in

oocytes, well characterized, and plays a key regulatory role in *Xenopus* during oocyte maturation and the embryonic cell cycles (reviewed by Ferrell, Jr., 1999; Fan and Sun, 2004). In *Xenopus* oocytes, p42MAPK is activated by phosphorylation in response to extracellular signals or mitogens of the p42MAPK pathway reinitiating cell cycle reentry (reviewed by Ruderman, 1993). Activation of the p42MAPK pathway in *Xenopus* is important for mitosis since it maintains the metaphase II arrest of the mature egg and contributes to arrests in G2 and M phase. Much of the focus for the remainder of the thesis will be on the effect of p42MAPK during G2 and M-phase arrests in mitosis.

1.6.1 The Role of p42MAPK in Cytostatic Factor (CSF) Arrest

As previously mentioned, p42MAPK is required for mediating the metaphase II arrest of mature oocytes (reviewed by Sagata, 1997). Along with the discovery of MPF, Masui and Markert (1971) also identified an activity called cytostatic factor (CSF) which was responsible for inducing the metaphase II arrest. When the cytoplasm of a mature egg was transferred into one blastomere of a 2-cell embryo, the recipient blastomere stopped cleaving but the blastomere injected with cytoplasm from an immature oocyte continued to divide (Masui and Markert, 1971; reviewed by Masui, 2001). The inhibitory factor in the cytoplasm of mature eggs that prevented zygotes from cleaving disappeared upon fertilization and was termed CSF. CSF activity appears after MI with levels remaining high until a decrease upon fertilization. CSF, unlike MPF, does not appear again in cleaving zygotes (reviewed by Masui, 2001). Also, CSF activity is independent of the nucleus (Masui and Markert, 1971; reviewed by Masui, 2001).

Sagata *et al.* (1988) discovered that Mos had characteristics of CSF activity. When Mos anti-sense oligonucleotides were injected into progesterone-treated *Xenopus* oocytes, the oocytes did not undergo GVBD and oocyte maturation was inhibited (Sagata *et al.*, 1988). This group also demonstrated that expression of Mos in one blastomere of a two-celled embryo resulted in CSF arrest (Sagata *et al.*, 1989). Mos was identified as a MAPK kinase kinase that phosphorylates and activates MEK leading to the activation the p42MAPK pathway (Nebreda et al, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). The effect of Mos is most likely mediated by p42MAPK, since active p42MAPK

alone has CSF activity and Mos is unable to induce CSF arrest if MAPK kinase is inactivated (Haccard et al., 1993; Kosako et al., 1994).

Furthermore, pp90rsk is downstream of p42MAPK and has been shown to induce CSF arrest (Bhatt and Ferrell, 1999; Gross et al., 1999). When constitutively-active $pp90^{Rsk}$ was injected into one blastomere of a two-cell embryo, it caused CSF arrest without activating the endogenous MAPK pathway (Gross *et al.*, 1999). Also, when Rsk-2 (the more abundant isoform of $pp90^{Rsk}$) was immunodepleted from egg extracts, the extract did not undergo CSF arrest when Mos was added. Moreover, purified, wild-type Rsk-2 added back to the depleted extract was able to restore the CSF arrest in response to Mos (Bhatt and Ferrell, Jr., 1999).

CSF is now known to be an activity that is naturally present in unfertilized *Xenopus* eggs that appears during oocyte maturation and disappears after fertilization (reviewed by Masui, 2000). Mos protein was shown to be a part of CSF arrest since it activates the p42MAPK pathway. Also, the activation of $p90^{Rsk}$, which is downstream of p42MAPK, leads to CSF arrest. These studies demonstrate that activation of any component of the p42MAPK pathway upstream of $p90^{Rsk}$, or constitutively-active $p90^{Rsk}$ itself, can lead to CSF arrest.

1.6.2 p42MAPK in the Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint acts during mitosis or meiosis by delaying the onset of anaphase when a defect in spindle formation is detected. This checkpoint is activated when kinetochores of the sister chromatids are unattached or improperly attached to spindle microtubules and sister chromatid separation is prevented until all chromosomes are properly attached to the mitotic spindles.

Using *Xenopus* egg extracts, Minshull *et al.* (1994) discovered that p42MAPK played a role in the SAC. Normally, egg extracts do not activate cell cycle checkpoints unless the ratio of DNA to cytoplasm is substantially increased (Dasso and Newport, 1990; reviewed by Tunquist and Maller, 2003). For example, Dasso and Newport (1990) used a cell-free extract made from *Xenopus* eggs that oscillates between S and M-phase to show that mitosis is suppressed until the completion of DNA synthesis. In *Xenopus* eggs, the pathway that inhibits mitosis until DNA replication is completed is dependent on the

presence of a threshold concentration of unreplicated DNA (Dasso and Newport, 1990).

It has been observed that when cycling egg extracts were supplemented with greater than 9000 sperm nuclei/μl of extract, the addition of nocodazole (a microtubule depolymerizing agent) could activate the SAC (Minshull *et al.*, 1994). Addition of the dual specificity MAPK phosphatase MKP-1/CL100 to the egg extracts inactivated p42MAPK and the M-phase arrest due to the SAC was abolished (Minshull *et al.*, 1994). Furthermore, immunodepletion of endogenous p42MAPK from interphase extracts prevented the activation of the SAC, but the SAC could be restored by the addition of recombinant MAPK (Takenaka *et al.*, 1997; Wang *et al.*, 1997). In intact cells, MAPK has also been shown to regulate the SAC. When the *Xenopus* tadpole (XTC) cells were treated with nocodazole, the SAC was activated resulting in a metaphase arrest (Wang *et al.*, 1997). However, injection of MKP-1 into nocodazole-treated XTC cells abolished the metaphase arrest.

Many studies have established p42MAPK as a necessary component of the SAC in egg extracts as well as vertebrate cells. In *Xenopus* cycling egg extracts, the SAC can become activated when spindles are not attached properly to chromosomes causing an M-phase arrest as a result of activation of the p42MAPK pathway. The M-phase arrest induced by the SAC differs from CSF arrest in one important aspect, the SAC is not overcome by calcium, but the activation of p42MAPK is required for the establishment of both types of arrest.

1.6.3 The Role of p42MAPK in G2 and M-phase Arrests in *Xenopus*

Using demembranated sperm nuclei and egg extracts prepared by low speed centrifugation, Lohka and Masui (1983) first demonstrated that pronuclei could form from the sperm nuclei and undergo chromosome condensation in an *in vitro* system. Activated *Xenopus* eggs were later used to prepare a cell-free system in which sperm pronuclei formed in egg extracts underwent rounds of DNA replication and M-phase (nuclear envelope breakdown followed by chromosome condensation) (Lohka and Masui, 1984; Lohka and Maller, 1985). A few years later, Murray and Kirschner (1989) used a modified protocol to prepare a cell-free system (a cycling egg extract) that could undergo repeated cell cycles autonomously.

Since the initial establishment of the first cell-free system, numerous studies have used cell-free systems to study the role of p42MAPK in regulation of the cell cycle. In *Xenopus*, p42MAPK can regulate entry into and exit from M-phase (reviewed by Tunquist and Maller, 2003). During maturation, p42MAPK is activated by MEK and the level of phosphorylated p42MAPK remains high, causing an arrest at metaphase II. $pp90^{Rsk}$ is known to be necessary and sufficient for this CSF arrest (Bhatt and Ferrell, 1999).

However, in post-meiotic embryos and cycling egg extracts, the activation of p42MAPK has previously been shown to lead to an arrest of the early embryonic cell cycle in either G2 or M-phase depending on the timing of p42MAPK activation (Abrieu *et al.*, 1997b; Walter *et al.*, 1997; Bitangcol *et al.*, 1998; Murakami and Vande Woude, 1998). When p42MAPK is activated during entry into M-phase in cycling extracts, there is an arrest of the cell cycle at metaphase. In contrast, when p42MAPK is activated in cycling egg extracts during interphase prior to entry into M-phase, the cell cycle is arrested in G2, after DNA replication (Walter *et al.*, 1997; Bitangcol *et al.*, 1998). One way in which the p42MAPK pathway contributes towards G2 arrest is by phosphorylating and sustaining the activation of Wee1, the kinase that catalyzes the inhibitory phosphorylations of Cdk1, maintaining it in its inactive state (Murakami and Vande Woude, 1998; Walter *et al.*, 2000). In addition, G2 arrests have been observed by a number of groups as a result of phosphorylation of Cdc25C on a specific serine residue that maintains binding of 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998a; Duckworth *et al.*, 2002).

In *Xenopus*, activators of the p42MAPK pathway such as Mos has been shown to induce an M-phase arrest *in vitro* after addition to cycling egg extracts (Minshull *et al.*, 1994; Walter *et al.*, 1997; Bitangcol *et al.*, 1998; Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999; Chau and Shibuya, 1999). Studies by Chau and Shibuya (1998, 1999) have shown that p42MAPK activation on entry into M-phase can induce an M-phase arrest with stabilized Cyclin B and high levels of MPF activity. The M-phase arrest induced *in vitro* by the p42MAPK pathway is similar to the CSF arrest of unfertilized, mature eggs because it is characterized by the presence of metaphase spindles, high levels of Cdk1 kinase activity, and stabilized Cyclin B protein

(Minshull et al., 1994, Abrieu et al., 1996, Walter et al., 1997, Chau and Shibuya, 1998, 1999).

Another unusual type of M-phase arrest can be obtained when p42MAPK is activated slightly later during the entry into M-phase in *Xenopus* cycling egg extracts (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999). If p42MAPK is activated after the peak of MPF, the cell cycle does not exit from M-phase even though Cyclin B is degraded and MPF is inactivated (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999; Chau and Shibuya, 1999). In these experiments, activation of p42MAPK by Mos leads to an M-phase arrest with stabilized M-phase activities including chromosome condensation, spindle formation, and M-phase-specific phosphorylations (Chau and Shibuya, 1998, 1999). Surprisingly, however, the cell cycle does not exit from M-phase even though Cyclin B is degraded and MPF is inactivated (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999; Chau and Shibuya, 1999).

In summary, the activation of the p42MAPK pathway results in a different type of cell cycle arrest depending on the timing of its activation in a cycling egg extract. Activation of p42MAPK during interphase results in cell cycle arrest in G2, whereas p42MAPK activation during entry into M-phase causes an arrest of the cell cycle at M-phase. Furthermore, when p42MAPK is activated after the peak of MPF during M-phase, Cyclin B is degraded and MPF is inactivated, yet the cell cycle does not exit from M-phase and maintains M-phase activities. Therefore, the activation of the p42MAPK pathway has the ability to arrest the mitotic cell cycle during G2 and M-phase depending on the timing of p42MAPK activation.

1.7 Requirements for MPF and MAPK during M-phase

Normally, activation of MPF and Cyclin B degradation are thought to be necessary and sufficient for entry into and exit from M-phase, respectively. However, other kinases are also activated and required for the progression through M-phase. In *Xenopus*, the role of the p42MAPK pathway during M-phase is of particular interest since it is a key regulatory system that functions either in parallel or together with MPF during oocyte maturation (reviewed by Fan and Sun, 2004). *Xenopus* oocytes were used to discover

that the p42MAPK pathway is responsible for the CSF and metaphase II arrests of *Xenopus* oocytes (reviewed by Masui, 2000).

During the cell cycle, tyrosine dephosphorylation of Cdk1 in complex with Cyclin B leads to its activation that drives the cell cycle from interphase into M-phase. There is a generally held belief that the inactivation of MPF by the ubiquitin-mediated proteolysis of Cyclin B is essential and solely sufficient for exit from M-phase. However, as mentioned previously, there is evidence that after the cell cycle has entered M-phase, p42MAPK can stabilize M-phase activities and prevent the cell cycle from exiting from M-phase despite decreased MPF levels.

Shimada *et al.* (1998) demonstrated that Cyclin B/Cdk1 is essential for inducing chromosome condensation in *Xenopus* M-phase extracts, but alone, could not cause chromosome condensation (CC). When Cyclin B/Cdk1 was inhibited with butyrolactone I, a Cdk1 inhibitor, CC was abolished in M-phase extracts (Shimada *et al.*, 1998). However, when chromatin was incubated with active Cyclin B/Cdk1, CC was not induced indicating that CyclinB/Cdk1 was not sufficient to cause CC. Furthermore, CC and histone H1 phosphorylation occurred even when extracts were depleted of Cyclin B/Cdk1 activity using $p13^{Suc1}$ beads suggesting that these M-phase activities can be dissociated from Cyclin B/Cdk1 activity. Therefore, Cyclin B/Cdk1 activity may be activating downstream protein kinases such as p42MAPK rather than the direct phosphorylation of chromosomal proteins (Shimada *et al.*, 1998).

It has also been demonstrated that when p42MAPK was activated in cycling *Xenopus* egg extracts shortly after M-phase entry but too late to prevent Cyclin B degradation, Cyclin B dropped to undetectable levels and MPF was inactivated but the cell cycle was arrested in M-phase (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998). These Mos-treated, M-phase extracts had condensed chromosomes associated with microtubules and sustained M-phase specific phosphorylations even though they lacked Cdk1 activity (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998). In addition, similar M-phase arrested egg extracts that had p42MAPK activated and low levels of histone H1 kinase activity (MPF activity) only exited M-phase when the p42MAPK pathway was inhibited by the MEK-specific inhibitor PD98059 (Chau and Shibuya,

1999). These results suggest that even after the proteolysis of Cyclin B, M-phase can be maintained by p42MAPK.

These findings that show that M-phase is sustained by p42MAPK following Cyclin B degradation (MPF inactivation) are very surprising. Furthermore, these results suggest that p42MAPK activation may be acting independently of MPF to suppress exit from M-phase even after MPF has been inactivated potentially by activating downstream events. It is suspected that p42MAPK may be a good candidate for phosphorylating and regulating important M-phase substrates.

The mechanism by which p42MAPK is able to sustain an M-phase arrest after Cyclin B degradation has not been elucidated. However, since p42MAPK inactivation is required for exit from M-phase and is able to sustain M-phase activities after MPF inactivation, p42MAPK seems to be having additional effects that are independent of Cyclin B/Cdk1 activity.

1.8 The Role pp90^{Rsk} in G2 Arrest [MEK(QP)-G2 Arrest]

During G2 arrest induced by the constitutively active MEK, MEK(QP), in cycling egg extracts, activation of the p42MAPK pathway leads to the phosphorylation and activation of the 90 kDa ribosomal protein S6 kinase (p90^{Rsk}) (Bitangcol *et al.*, 1998). p90^{Rsk} is a serine/threonine kinase that is directly phosphorylated and activated by MAPK. There are two highly-conserved protein kinase domains present in p90^{Rsk}. The N-terminal domain is responsible for phosphorylating a variety of exogenous substrates *in vitro* containing an RXXS motif (Fisher and Blenis, 1996). This domain of p90^{Rsk} resembles the catalytic domains of p70^{S6K}, cAMP-dependent protein kinase A (PKA), and protein kinase C (Fisher and Blenis, 1996). The C-terminal domain is also catalytically active and involved in p90^{Rsk} autophosphorylation; it has been shown to cause the full activation of the N-terminal domain (Fisher and Blenis, 1996; Dalby *et al.*, 1998). Also, this domain has been shown to possess the site of interaction with MAPK (Fisher and Blenis, 1996). In *Xenopus* oocytes arrested in G2, unphosphorylated p90^{Rsk} is complexed with inactive p42MAPK. When these proteins are phosphorylated and activated during oocyte maturation, they dissociate (Hsiao *et al.*, 1994).

$p90^{Rsk}$ appears to be the mediator of MAPK-dependent CSF arrest in vertebrate unfertilized eggs (Gross *et al.*, 1999). However, in the early embryonic cell cycles, if $p90^{Rsk}$ is phosphorylated and activated during interphase, it can potentially act as a kinase for the cell cycle regulator Cdc25C phosphatase, which removes the inhibitory phosphorylations from Cdk1. Several sites in Cdc25C were found that have a consensus sequence for phosphorylation by $pp90^{Rsk}$, including serine 287 of Cdc25C. Other kinases including Chk1, protein kinase A, and calcium-calmodulin kinase II (CaMKII) have been identified that phosphorylate Cdc25C on serine 287 resulting in a G2 arrest of the cell cycle (Peng *et al.*, 1997; Kumagai *et al.*, 1998b; Duckworth *et al.*, 2002; Hutchins *et al.*, 2003). $pp90^{Rsk}$ does not phosphorylate the N-terminal domain of Cdc25C (Palmer *et al.*, 1998) but this domain is phosphorylated by Plx1 in *Xenopus* (Kumagai and Dunphy, 1996). Nevertheless, mitotic phosphorylation pattern of Cdc25C shows that there may be other sites that are not phosphorylated by either Cdk1 or Plx1 (Kumagai and Dunphy, 1996).

1.9 The Function of 14-3-3 Proteins

14-3-3 proteins are a family of conserved, acidic, dimeric proteins approximately 27 to 32 kDa in size that have diverse roles in both the positive and negative regulation of signal transduction pathways, cell cycle checkpoints, and apoptosis (Muslin *et al.*, 1996; reviewed by Fu *et al.*, 2000; Tzivion *et al.*, 2001). These proteins bind to specific sequence motifs in proteins that usually involve phosphoserine or phosphothreonine residues including the RSXpSXP (R, arginine; S, serine; pS, phosphoserine; P, proline; X, any amino acid) (Muslin *et al.*, 1996). The dimeric property of 14-3-3 proteins may bring together two proteins in a complex to facilitate regulation of signaling cascades. 14-3-3 proteins participate in a wide range of biological events and function in a variety of regulatory mechanisms by binding to phosphoserine in numerous partners (reviewed by Tzivion and Avruch, 2002).

Some signaling proteins phosphorylated on specific serine residues known to bind 14-3-3 proteins include protein kinase C, Raf, Cbl, phosphoinositide 3-kinase, BAD, and MEKK1 and 4 (reviewed by Tzivion *et al.*, 2001). More relevant to our studies, 14-3-3 proteins have been demonstrated by several groups to be important in mitotic regulation.

Some of the proteins involved in cell cycle control that associate with 14-3-3 proteins are Wee1, p53, Cdk1, Cdk2, and Cdc25C (reviewed by Tzivion *et al.*, 2001). Cdc25C phosphorylated on Ser287 in *Xenopus* and S216 in somatic cells has been shown to associate with 14-3-3 proteins approximately 30 kDa in size (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998b; Duckworth *et al.*, 2002). In yeast and humans, 14-3-3 proteins have been implicated in the DNA damage checkpoint (Peng *et al.*, 1997; Sanchez *et al.*, 1997). The association of 14-3-3 proteins with Cdc25C has been shown to be an essential component of the DNA damage checkpoint that arrest cells in G2 until the damaged DNA is repair. Also, in *Xenopus*, a mutant form of Cdc25C [Cdc25C(S287A), serine 287 was mutated to an alanine] was incapable of binding to 14-3-3 and overrode the DNA damage checkpoint caused by unreplicated and damaged DNA (Kumagai *et al.*, 1998b). The function and regulatory mechanism of the 14-3-3 proteins are diverse and versatile. Nevertheless, one very important function of 14-3-3 proteins appears to be regulating the G2 arrest of the cell cycle in response to the DNA damage checkpoint.

1.10 Proposal and Summary of Results

The p42MAPK pathway plays an important role in a G2 arrest and during M-phase to sustain NEBD/CC activities. My first study concentrated on the mechanism by which the activation of the p42MAPK by MEK(QP) during interphase caused a G2 arrest. The focus of my second project was to determine if p42MAPK activation after entry into M-phase could sustain nuclear envelope breakdown and chromosome condensation (NEBC/CC) activities of M-phase. *Xenopus* egg extracts were used to investigate the role of the p42MAPK pathway during a MEK(QP)-induced G2 arrest and during M-phase.

In the first part of my study, it was found that in MEK(QP)-G2 arrested egg extracts, S287 of Cdc25C was important in regulating G2 arrest. In an *in vitro* kinase assay, I showed that Cdc25C phosphatase is a target of active $pp90^{Rsk}$, and that activated $pp90^{Rsk}$ is able to phosphorylate Cdc25C(WT) on Ser287 suggesting that Cdc25C is a relevant target of $pp90^{Rsk}$ in egg extracts. Finally, recombinant 14-3-3ζ, was shown associate with Cdc25C(WT) in which Ser287 has been phosphorylated by $pp90^{Rsk}$ *in vitro*. These

results indicate that binding of 14-3-3ζ to $pp90^{Rsk}$ phosphorylated Cdc25C (on S287) is one way in which the MEK(QP)-induced activation of the p42MAPK pathway leads to G2 arrest of the cell cycle.

In the second part of my study, we hypothesized that nuclear envelope breakdown and chromosome condensation (NEBD/CC) activities, which are initially activated by MPF, may be sustained by activated p42MAPK independently of MPF activity. First, my results showed that when Cdk1 was inhibited by roscovitine in M-phase extracts, Cdc25C remained hyperphosphorylated and histone H1 phosphorylation was not abolished, which indicated the presence of activities with the ability to sustain activities of M-phase. Next, Cyclin BΔ90 protein was added to an interphase arrested extract and then the p42MAPK pathway was activated. When Cyclin BΔ90 was removed by immunodepletion and the supernatant was analyzed, there were NEBD/CC inducing activities only in extracts containing activated p42MAPK after the removal of Cyclin BΔ90. These preliminary results suggest that even upon removal of the MPF activity from an M-phase arrested egg extract, it is possible to have an extract with M-phase activities originally activated by MPF prior to its removal, and that p42MAPK may be able to sustain NEBD/CC activity without the presence of MPF activity.

Chapter 2: Materials and Methods

Table 2.1 List and Source of Chemicals and Reagents

Reagent	Supplier
Acetic acid, glacial	Fisher Scientific
Acrylamide (40% solution)	IBI (VWR)
[γ-^{32}P] Adenosine triphosphate	Amersham Biosciences
Adenosine trisphosphate (ATP)	Roche Diagnostics
Affi-Prep Protein A matrix	Biorad Laboratories Inc.
Agar	GibcoBRL (Invitrogen)
Ammonium persulphate (APS)	Sigma-Aldrich
Ampicillin Sodium	Novopharm
Amylose resin	New England Biolabs
Anti-Maltose binding protein (MBP) Antiserum	New England Biolabs
Anti-rabbit IgG (whole molecule)	Sigma-Aldrich
Aprotinin	Sigma-Aldrich
5-bromo-4-chloro-3-indolyl-1-phosphate(BCIP)	Promega
Bis-acrylamide	IBI (VWR)
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Brilliant blue R 250 (Coomassie)	Sigma
Bromophenol blue	BDH
Butanol	Fisher Scientific
Butyrolactone I	Biomol research laboratories
Calcium chloride	BDH
Calcium nitrate tetrahydrate	BDH
Calmidazolium	Sigma-Aldrich
Chymostatin	Sigma-Aldrich
Creatine kinase	Roche Diagnostics
Creatine phosphate	Roche Diagnostics
Cycloheximide	Sigma-Aldrich
L-Cysteine-HCl	Sigma-Aldrich
Cytochalasin B	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	BDH
Dithiothreitol (DTT)	Promega
EDTA (ethylenediamine-*N,N,N,N'*-tetraacetic acid)	BDH
EGTA (ethylene glycol-bis(2-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid)	Sigma-Aldrich
Ethanol, 95%	Fisher Scientific
Ethylene glycol-bis (succinic acid N-hydroxy-succinimide ester)	Sigma-Aldrich
D-Gluconic acid, hemimagnesium Salt	Sigma-Aldrich
D-Gluconic acid, potassium Salt	Sigma-Aldrich
Glucose	BDH
Glutathione	Sigma-Aldrich
Glycerol	EM Science

Glycine	Sigma-Aldrich
Goat serum	Gibco
HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)	Sigma-Aldrich
Histone H1	Boehringer Mannheim
Hoechst 33342	Sigma-Aldrich
Human chorionic gonadotropin (HCG)	Sigma-Aldrich
Hydrochloric acid	Fisher Scientific
isopropyl-β-D-thiogalactopyranoside (IPTG)	Rose Scientific
Leupeptin	Sigma-Aldrich
Magnesium chloride hexahydrate	EM Science
Magnesium sulfate heptahydrate	BDH
Maltose	Sigma-Aldrich
Mercaptoethanol	OmniPur (EM Science)
Methanol	BDH
NBT (nitro blue tetrazolium)	Promega
Nonidet P-40	EM Science
Nyosil M25	Nye Lubricants, Inc.
Ovalbumin	Sigma-Aldrich
Penicillin G sodium salt	Sigma-Aldrich
Pepstatin A	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
Polyethylene glycol	Sigma
Potassium Chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Potassium hydroxide	BDH
Pregnant mare serum gonadotropin (PMSG)	Sigma-Aldrich
Protein A Sepharose CL-4B	Pharmacia Biotech.
Protein kinase A inhibitor peptide	Sigma-Aldrich
Roscovitine	Calbiochem
Sepharose CL-4B	Sigma-Aldrich
Sodium azide	BDH
Sodium chloride	EM Science
Sodium dodecyl sulfate (SDS)	BDH
Sodium fluoride	Sigma-Aldrich
Sodium hydroxide	EM Science
Sodium orthovanadate	Sigma-Aldrich
Streptomycin sulfate	Sigma-Aldrich
Sucrose	ICN
Sulfathiazole	Sigma-Aldrich
TEMED (*N,N,N',N'*-tetramethylethylenediamine)	GibcoBRL (Life Technologies)
Tris(hydroxymethyl) aminomethane (Tris)	Invitrogen
Tryptone-peptone	Difco
Tween-20 (polyoxyethylene 20-sorbitan monolaurate)	Fisher Scientific
Versalube F-50	Andpak-EMA
Yeast extract	Difco

All solutions were made using deionized water that had been further purified using a Milli-Q (Millipore) cartridge purification system.

2.1 Preparation of *Xenopus* Egg Extracts

2.1.1 Inducing Ovulation of *Xenopus laevis*

Female *Xenopus laevis* frogs were primed for ovulation by injecting the frogs in the dorsal lymph sac with 100 I.U. of pregnant mare serum (PMS). One to three days later, the frogs were injected with 1000 I.U. of human chorionic gonadotropin (HCG). The frogs were transferred to individual tanks containing 0.1 M NaCl and allowed to ovulate for 12 to 13 hrs before harvesting the eggs. The spawned eggs and eggs gently squeezed from the frogs were collected.

2.1.2 Dejellying Eggs

Xenopus eggs were dejellied with gentle swirling in 2 g of L-cysteine hydrochloride dissolved in 100 ml of 0.16 M of NaOH solution with a pH between 7.80 to 7.82. The dejellied eggs were then washed at least 10 times with egg washing solution (0.1 M NaCl, 0.01 M HEPES-NaOH, pH 7.0). After washing, the eggs were transferred to agar coated glass petri dishes containing egg washing solution and examined under a dissection microscope to remove any activated or degenerated eggs.

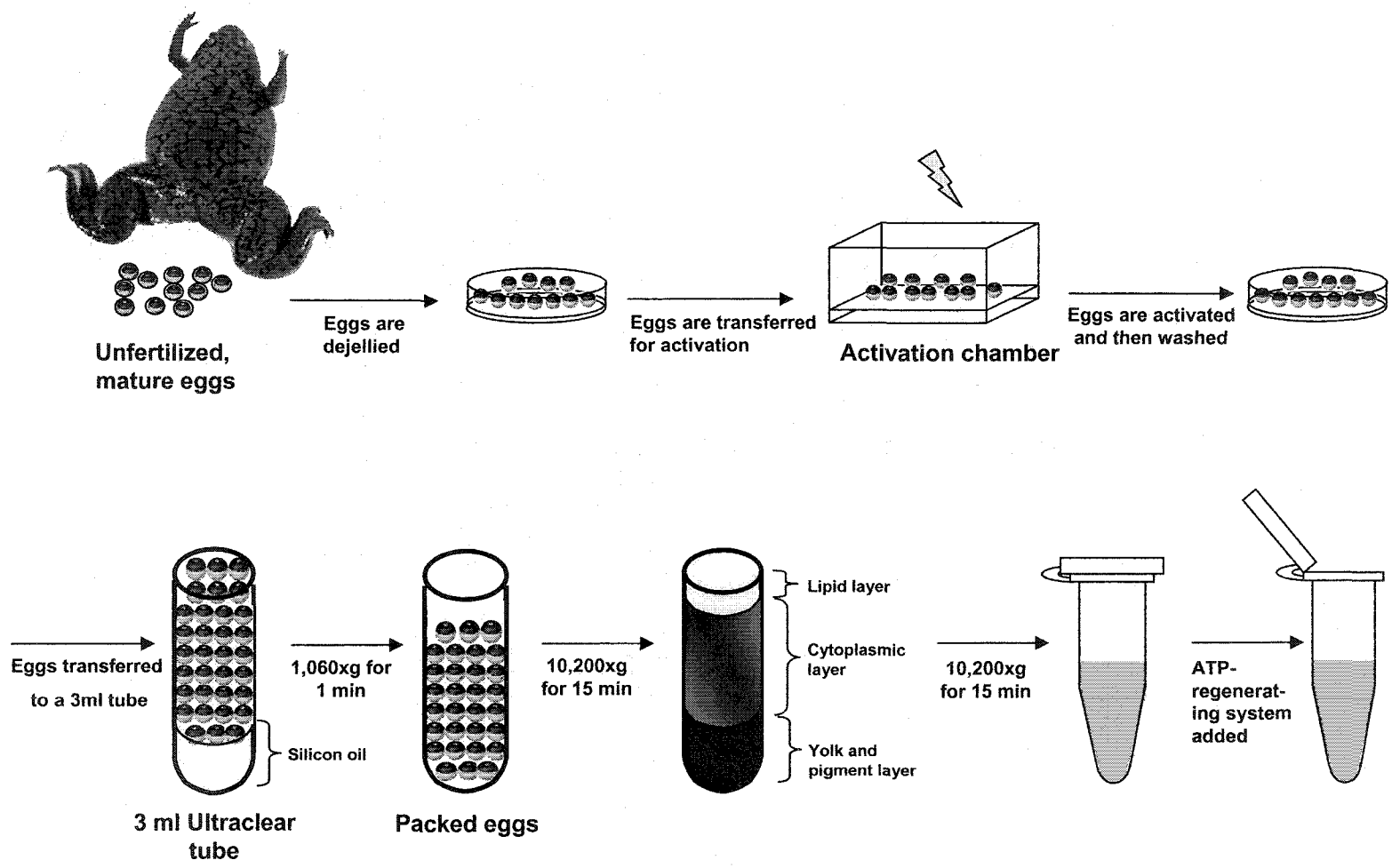
2.1.3 Cycling Egg Extracts

Cycling egg extracts that undergo repeated cell cycles were prepared from dejellied *Xenopus* eggs as described previously (Bitangcol *et al.*, 1998) (see schematic Fig. 2.1). Initially, 5 to 6 dejellied eggs were test activated in an activation chamber containing 20% Steinberg's solution (11.6 mM NaCl, 0.134 mM KCl, 0.08 mM $MgSO_4.7H_2O$, 0.68 mM $Ca(NO_3)_2.4H_2O$, 0.8 mM HCl, 0.006 mg penicillin G/ml solution, 0.01 mg streptomycin sulfate/ml solution, 0.02 mg sulfathiazole/ml solution, 0.92 mM Tris(hydroxyamino) methane, pH 7.4) using two, one-second electrical impulses (15 volts AC) separated by five seconds. The eggs were monitored for cortical contraction of the animal hemisphere which occurred 3 to 10 min post-activation. After confirming

Figure 2.1 Schematic for the Preparation of *Xenopus* Cycling Egg Extract

Xenopus cycling egg extracts are prepared by using unfertilized, mature eggs arrested at metaphase II of meiosis. The eggs are collected and dejellied. Next, the eggs are transferred to an activation chamber where they are released from the metaphase II arrest by electrical shock. Then, the activated eggs are washed with extraction buffer followed by packing and crushing by centrifugation. The cytoplasmic layer (crude extract) is subjected to centrifugation and cleared extract is removed, supplemented with ATP-regenerating system and used for cell-free assays.

Figure 2.1 Schematic for the Preparation of *Xenopus* Cycling Egg Extract



activation of the test eggs, the remaining eggs were activated in the same way. 10 to 12 min after activation, the eggs were washed three times with extraction buffer (EB: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, 10 mM HEPES-KOH, pH 7.7) and twice with EB supplemented with protease inhibitors (10 μg leupeptin/ml buffer, 10 μg chymostatin/ml buffer, 10 μg pepstatin/ml buffer).

After the washes, the eggs were transferred to 3.0 ml Ultraclear tubes (Beckman, Mississauga, Ontario, Canada) containing 0.6 ml silicon oil (Versalube F-50) overlaid with 0.6 ml EB (plus protease inhibitors and 100 μg cytochalasin B/ml of DMSO) which had been subjected to centrifugation at 1060xg for 1 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R centrifuge in order to sharpen the meniscus between the oil and buffer. About 17 to 18 min after activation, eggs were packed by centrifugation at 1060xg for 1 min at 4°C using the same centrifuge. The excess buffer and oil were aspirated then at 21 to 23 min post-activation, the packed eggs were crushed by centrifugation at 10,200xg for 15 min at 4°C using the same rotor. The cytoplasmic layer (extract) between the top lipid layer and the bottom yolk pellet was transferred to an ice-chilled 1.7 ml autoclaved microfuge tube using a wide bore blue pipetman tip. The extract was clarified by centrifugation once more at 10,200xg for 15 min at 4°C using the same centrifuge. The remaining lipid was aspirated and the pelleted residue was removed using a wide bore blue pipetman tip.

The final, cleared extract was removed and freshly-made ATP-regenerating system (40 mM ATP/Mg^{2+}, 0.4 M creatine phosphate, 2 mg creatine kinase/ml buffer) was immediately added to the extract (1 volume of ATP-regenerating system: 37 volumes of extract). These supplemented extracts were used immediately or kept on ice for up to 2 hours before use. Demembranated sperm nuclei (Vigers and Lohka, 1991) were added to a final concentration of approximately 100 to 500 sperm nuclei/μl reaction. MEK(QP)-G2 arrested egg extracts were prepared by incubating the cycling egg extracts with 0.06 mg MEK(QP) protein/ml reaction (Bitangcol *et al.*, 1998). Cdc25C proteins were assayed by addition to these cycling extracts.

2.1.4 CSF (M-phase Arrested) Unfertilized Egg Extracts

CSF extracts were prepared from unfertilized, *Xenopus* eggs as described previously with some modifications (Shibuya *et al.*, 1992). Dejellied unfertilized *Xenopus* eggs (arrested in the second meiotic metaphase) were washed three times with modified CSF extraction buffer (CSF-EB: 100 mM KCl, 5 mM $MgCl_2$, 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid (EGTA), 50 mM sucrose, 20 mM NaF, 10 mM HEPES-KOH, pH 7.7) and twice with CSF-EB supplemented with protease inhibitors (10 μg leupeptin/ml buffer, 10 μg chymostatin/ml buffer, 10 μg pepstatin/ml buffer). After the washes, CSF egg extracts were prepared in the same way as for the cycling egg extracts, except that extracts were supplemented with cytochalasin B to a final concentration of 50 μg cytochalasin B/ml extract after the first centrifugation. Also, depending on the batch of eggs used, an additional, clarifying centrifugation at 10,200xg for 15 min at 4°C using the same centrifuge was sometimes necessary.

2.1.5 Cycloheximide (Interphase Arrested) Egg Extracts

Cycloheximide (CHX) egg extracts arrested in interphase were prepared using unfertilized eggs that were dejellied and washed in the same way as for the cycling egg extract and prepared as described previously (Bitangcol *et al.*, 1998). Prior to activation, dejellied eggs were incubated in 20% Steinberg's solution containing 100 μg cycloheximide/ml solution for 10 to 15 min to inhibit protein synthesis. Then, eggs were activated using two electrical impulses (see section 2.1.3). Egg extracts were made following the procedure for cycling egg extracts, except all solutions contained 100 μg cycloheximide/ml solution, and activated eggs were crushed at 32 or 45 min post activation under the same conditions as for the cycling egg extract (see section 2.1.3). These interphase arrested, "CHX" egg extracts were used immediately or frozen in liquid nitrogen in aliquots of 50 μl and stored at -80°C for use in future experiments.

In certain experiments, interphase arrested egg extracts were prepared in the same way as for CHX egg extracts except that gluconate extraction buffer [GB: 10 mM gluconic acid (hemimagnesium salt), 10 mM gluconic acid (potassium salt), 20 mM HEPES-KOH, pH 7.5] was used. All incubations and washes prior to packing and crushing the eggs were done using GB containing 100 μg cycloheximide/ml GB.

2.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10%, 12.5%, or 15% polyacrylamide gels that were 0.75 mm thick (Anderson *et al.*, 1973). These gels differred from Laemmli gels (Laemmli, 1970) in that the proportion of acrylamide to bis-acrylamide was much higher. The proportion of acrylamide:bis-acrylamide for 10%, 12.5%, and 15% gels was 77:1, 120:1, and 174:1, respectively.

SDS-PAGE was done using a custom-made gel electrophoresis apparatus (8.0 cm x 14.5 cm "large" gels) or the Mini-Portean II Electrophoresis Cell (Biorad) (mini-gel). The resolving gels were prepared differently depending on the percentage of gel (see Table 2.2). SDS-PAGE was carried out using constant current. The highest current used for 10% and 12.5% gels was 22 mAmp and 25 mAmp for 15% gels.

2.2.1 Resolving Gel Preparation

The appropriate volumes of 40% acrylamide, 2% bis-acrylamide, 1.5 M Tris-HCl (pH 8.8) were added to water (see Table 2.2) in a small vacuum flask. 4.5 µl of 10% ammonium persulphate (APS)/ml gel mix was added to the gel mix and the mix was subjected to a vacuum for 5 min. Next, 0.45 µl TEMED/ml gel mix was added to the gel mix. Immediately, the gel was poured, overlaid with water-saturated butanol, and allowed to polymerize for 30 min to 1 hr at room temperature. After the gel was polymerized, the butanol was gently washed away with water, and the excess water was removed using a piece of filter paper.

2.2.2 Stack Gel Preparation

Approximately 2 ml of stack gel mix [5% acrylamide (v/v), 0.13% bis-acrylamide (v/v), 12.5% 1 M Tris-HCl (v/v), pH 6.8] was used for the large gels and 1 ml for the mini-gels. 10 µl 10% APS/ml gel mix and 1 µl TEMED/ml gel mix were added to the stack gel mix then poured on top of the polymerized resolving gel. Immediately, the comb was inserted into the stack gel and positioned so there was at least 0.5 cm between the resolving gel and the bottom of the wells of the comb. The stack gel was allowed to polymerize for 10 min before the comb was removed.

Table 2.2 Proportion of Reagents for Resolving Gels

Reagent	Gel Percentage		
	10% gel	12.5%	15%
40% Acrylamide	25% (v/v)	31.2% (v/v)	37.5% (v/v)
2% Bis-acrylamide	6.5% (v/v)	5.2% (v/v)	4.3% (v/v)
1.5 M Tris-HCl, pH 8.8	25% (v/v)	25% (v/v)	25% (v/v)

2.3 Expression of Recombinant Proteins in *E. coli*

2.3.1 Recombinant Cdc25C(S287A)

Recombinant *Xenopus* Cdc25C(S287A) with an N-terminal maltose-binding protein tag [MBP-Cdc25C(S287A)] was expressed in *Escherichia coli* DH10B cells and purified as previously described (Chau and Shibuya, 1999). 2 ml of a 3 ml overnight culture grown in Luria-Bertani medium (LB) [1% tryptone-peptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v)] containing 100 μg ampicillin/ml medium (from a stock solution of 100 mg ampicillin/ml water) and 200 μM glucose at 250 rpm at 37°C was subjected to centifugation at top speed for 5 min at room temperature using a swinging bucket rotor in a clinical centrifuge. The medium was aspirated and the pellet of cells was resuspended in 1 ml of fresh LB containing 50 μg ampicillin/ml LB. A 2 L flask containing 500 ml of LB with 100 μg ampicillin/ml LB and 200 μM glucose was inoculated with 200 μl of resuspended cells. The culture was grown for 5 to 6 hrs with shaking at 250 rpm at 37°C until the OD_{600} reached approximately 0.7.

Pre-induction samples were prepared by transferring 500 μl of bacterial culture from the flask to a 0.6 ml microfuge tube and subjecting the samples to centrifugation at 13,000 rpm for 1 min at room temperature using a fixed angle rotor in an Eppendorf 5410 centrifuge to pellet the cells. The medium was aspirated and 30 μl of 1X sample buffer [2% SDS (w/v), 10% glycerol (v/v), 0.01% bromophenol blue (w/v), 5% β-mercaptoethanol (v/v), 80 mM Tris-HCl, pH 6.8] (Laemmli, 1970) was added, the sample was vortexed and then boiled for 5 min. The bacterial culture was induced by adding isopropylthio-β-D-galactoside (IPTG) to 1 mM (from a stock of 1 M stored at -20°C) and further incubated with shaking at 250 rpm for 3 hours at room temperature. After 3 hours, a post-induction sample was prepared exactly as the pre-induction sample except

60 µl of 1X sample buffer was added before the sample was vortexed and boiled for 5 min.

3 hours after induction, the bacterial culture was collected in 250 ml centrifuge bottles. The bottles containing culture were weighed to balance and then subjected to centrifugation at 4000xg (5000 rpm) for 10 min at 4°C using a GSA rotor in a Sorvall centrifuge (rotor code 10). The supernatant was decanted and a graduated pipette was used to resuspend each pellet of cells in 10 ml of lysis buffer [100 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20 (v/v), 10 µg leupeptin/ml buffer, 10 µg chymostatin/ml buffer, 10 µg pepstatin/ml buffer, 2 µg aprotinin/ml buffer, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris-HCl, pH 8.0] on ice. The resuspended cells were pooled into a 50 ml polypropylene tube and frozen in liquid nitrogen.

At this time, the pre-induction and post-induction samples were analyzed by SDS-PAGE using a 10% mini-gel. The gel was stained with Coomassie stain [0.5% brilliant blue R 250 (w/v), 45% methanol (v/v), 10% glacial acetic acid (v/v)], destained [25% methanol (v/v), 7% glacial acetic acid (v/v)], and then dried.

The frozen cell suspension was subjected to two more rounds of freezing and thawing and then transferred to a 250 ml beaker. The cells were lysed by sonication on ice by using a broad tip probe (4 sets of 20 pulses at 6 output, 30% duty, time on hold, and 1 min intervals between each set for cooling). The sonicate was transferred to a screw-cap, round-bottomed, polypropylene tube and then subjected to centrifugation at 30,000xg for 20 min at 4°C using the SS-34 rotor in a Sorvall centrifuge. The supernatant was collected with a 10 ml graduated pipette and transferred to a 50 ml polypropylene tube. A pre-bead binding gel sample was prepared by mixing 1 volume of supernatant with 1 volume of 2X sample buffer. The pellet and supernatant were frozen in liquid nitrogen and stored at -80°C.

To verify protein expression and binding, 500 µl of thawed supernatant was mixed end-over-end with 10 µl of packed amylose beads (that had previously been washed with lysis buffer) for 1 hr at 4°C. The beads were pelleted at 10,200xg for 1 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R centrifuge and the supernatant was removed. Pre- and post-bead binding samples were prepared by mixing 10 µl of each supernatant with 10 µl of 2X sample buffer. The beads were washed twice with lysis

buffer and then the protein was eluted using 15 µl of 2X sample buffer. The supernatant and bead samples were analyzed by SDS-PAGE using a 10% mini-gel. Once protein expression and binding was verified, large scale protein purification was carried out using 10 µl packed amylose beads for every 500 µl of supernatant. The supernatant was bound to amylose beads (previously washed) for 1 hr at 4°C end-over-end in a 50 ml polypropylene tube. Pre- and post-bead binding samples were prepared in the same way as for the test binding experiment. The beads were pelleted by centrifugation at a speed setting of 5 for 2 min at room temperature using a fixed angle rotor in a clinical centrifuge. The supernatant was collected, the beads were resuspended in lysis buffer without protease inhibitors [100 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20 (v/v), 50 mM Tris-HCl, pH 8.0], and then transferred to a clean 15 ml falcon tube. Next, the beads were washed (each wash was 10 min at 4°C, end-over-end) with a series of solutions. The beads were washed twice with lysis buffer without protease inhibitors, twice with a second wash buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0), twice with a third wash buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0), and last, with a final wash buffer (100 mM NaCl, 20 mM HEPES-NaOH, pH 7.0).

The MBP-tagged Cdc25C(S287A) protein was eluted from the amylose resin using 2.5 volumes of elution buffer (100 mM NaCl, 10 mM maltose, 100 µM DTT, 20 mM HEPES-NaOH, pH 7.2) for 1 volume of packed, protein-bound amylose beads. Two rounds of elutions were done at room temperature for 10 min each, end-over-end. Then one elution was done using 20 mM maltose. The elution profiles of the eluate samples that were prepared by mixing 10 µl of eluate with 10 µl of 2X sample buffer, along with the pre-bead binding supernatant, post-bead binding supernatant, and BSA were analyzed by SDS-PAGE on a 10% mini-gel. The gel was stained, destained and dried.

The eluates that contained the MBP-tagged Cdc25C(S287A) protein were pooled together and dialyzed overnight at 4°C against 1L of dialysis buffer [100 mM KCl, 100 µM DTT, 10% glycerol (v/v), 20 mM HEPES-KOH, pH 7.5] using 3500 MWCO dialysis tubing (Fisher Scientific). The dialysis buffer was exchanged with fresh buffer after the first hour.

The proteins were concentrated using Ultrafree-MC protein concentrators (Millipore; 30,000 NMWL Filter Unit). Initially, the protein concentrators were washed twice with

dialysis buffer by centrifugation at 4,800xg at 4°C using a fixed angle rotor in an Eppendorf 5417R centrifuge. A pre-concentration sample of the protein was prepared by adding 6 µl of 2X sample buffer to 4 µl of dialyzed protein solution. The protein was concentrated by centrifugation at 4,800xg at 4°C using the same centrifuge with approximately 300 µl of protein solution added to the top chamber every 20 min for approximately 2 hrs. A pipetman was used to mix the protein solution every time more protein solution was added to the top chamber of the concentrator. Gel samples were prepared by adding 10 µl of 2X sample buffer to 10 µl of filtrate, and 18 µl of 2X sample buffer to 2 µl of concentrated protein solution. The samples were analyzed by SDS-PAGE on a 10% polyacrylamide gel with increasing concentrations of BSA. The gel was stained, destained and dried. The concentration of the protein was quantified by comparison with a standard graph of the BSA samples using the Image Gauge V3.0 software.

2.3.2 Recombinant Cyclin BΔ90

Recombinant sea urchin Cyclin BΔ90 [a non-degradable mutant missing the N-terminal 90 amino acids, (Murray *et al.*, 1989)], with an N-terminal maltose-binding protein tag (MBP-Cyclin BΔ90) was expressed in *E. coli* [BL21 (DE3) cells] and purified (Bitangcol et al., 1998). 10 ml of Enriched Medium (EM) [2% tryptone-peptone (w/v), 1% yeast extract (w/v), 0.5% NaCl (v/v), 0.2% glycerol, 50 mM KH_2PO_4, pH 7.2] containing 150 µg ampicillin/ml EM was inoculated with a single colony and grown with shaking at 250 rpm at 37°C overnight. The overnight culture was subjected to centifugation at top speed for 5 min at room temperature using a swinging bucket rotor in a clinical centrifuge. The EM was aspirated and the pellet of cells was resuspended in 10 ml of fresh EM containing 150 µg ampicillin/ml EM. 5 ml of resuspended cells were transferred to each of two 2 L flasks containing 490 ml of LB with 150 µg ampicillin/ml LB. The cultures were grown for 4 hrs with shaking at 250 rpm at 25°C until the OD_{600} reached approximately 0.450 to 0.600. Pre-induction samples were prepared by transferring 200 µl of bacterial culture from the flask to a 0.6 ml microfuge tube and subjecting the samples to centrifugation at 13,000 rpm for 1 min at room temperature using a fixed angle rotor in an Eppendorf 5410 centrifuge to pellet the cells. The medium

was aspirated and 20 µl of 1X sample buffer was added, the sample was vortexed and then boiled for 5 min. The bacterial culture was induced by adding isopropylthio-β-D-galactoside (IPTG) to 1 mM (from a stock of 0.5 M IPTG) and further incubated with shaking at 250 rpm for 3.5 hrs at 25°C. After incubation, a post-induction sample was prepared exactly as for the pre-induction sample, except 40 µl of 1X sample buffer was added. The bacterial culture was collected and cells were pelleted as for the MBP-Cdc25C protein (see section 2.3.1). The supernatant was decanted and each pellet of cells was resuspended in 5 ml of lysis buffer [150 mM NaCl, 10 mM EDTA, 0.05% Tween-20 (v/v), 10 µg leupeptin/ml buffer, 10 µg chymostatin/ml buffer, 10 µg pepstatin/ml buffer, 2 µg aprotinin/ml buffer, 1 mM PMSF, 50 mM Tris-HCl, pH 8.0] on ice. The resuspended cells were pooled into a 50 ml polypropylene tube, frozen in liquid nitrogen and then thawed.

At this time, the pre-induction and post-induction samples were analyzed by SDS-PAGE on a 12.5% mini-gel to verify expression of the protein. The gel was stained with Coomassie blue, destained, and then dried.

The cell suspensions were subjected to two more rounds of freezing and thawing, then the cells were lysed by sonication and the supernatant collected as described in section 2.3.1. A pre-bead binding gel sample was prepared by mixing 2 µl of supernatant with 8 µl 1X sample buffer. The pellet and supernatant were frozen in liquid nitrogen and stored at -80°C.

Approximately 20 ml of supernatant was bound to 4 ml of packed amylose resin (previously washed) overnight at 4°C end-over-end in a 50 ml polypropylene tube. A post-bead binding sample was prepared in the same way as for the pre-bead binding sample. The beads were pelleted by centrifugation at a speed setting of 5 for 2 min at room temperature using a swinging bucket rotor in a clinical centrifuge. 30 ml of lysis buffer without protease inhibitors [150 mM NaCl, 10 mM EDTA, 0.05% Tween-20 (v/v), 50 mM Tris-HCl, pH 8.0] was added to the beads and the beads were washed once end-over-end for 10 min at 4°C. The supernatant was collected, the beads were resuspended, and then transferred to a clean 15 ml falcon tube. Next, the beads were washed (each was for 10 min at 4°C, end-over-end) with a series of washes. The beads were washed again with the above lysis buffer without protease inhibitors, once with a second wash buffer

[500 mM NaCl, 0.05% Tween-20 (v/v), 50 mM Tris-HCl, pH 8.0], twice with a third wash buffer [100 mM NaCl, 0.05% Tween-20 (v/v), 50 mM Tris-HCl, pH 8.0], and three times with a final wash buffer (100 mM NaCl, 20 mM HEPES-NaOH, pH 8.0).

The MBP-Cyclin BΔ90 bound to amylose resin was transferred to a 2 ml screw-cap tube and eluted from the amylose resin using 1 volume of elution buffer (150 mM NaCl, 10 mM maltose, 50 mM Tris-HCl, pH 8.0) for 1 volume of packed protein bound amylose resin. Another elution was done using 20 mM maltose. Each elution took place at room temperature for 10 min each, end-over-end. The eluate samples were prepared by mixing 2 µl of eluate with 8 µl of 1X sample buffer, and were analyzed by SDS-PAGE on a 12.5% mini-gel along with the pre-bead binding supernatant, post-bead binding supernatant, and BSA. The gel was stained, destained and dried.

The eluates that contained the MBP-Cyclin BΔ90 protein were pooled together and dialyzed overnight at 4°C against 200 ml of dialysis buffer (100 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.7) using 3,500 MWCO dialysis tubing (Fisher Scientific). The dialysis buffer was exchanged with fresh buffer after the first hour. The protein was concentrated using the same dialysis buffer containing 10% polyethylene glycol (w/v) (MW 18 to 20,000) at 4°C.

Gel samples were prepared by adding 198 µl of 1X sample buffer to 2 µl of concentrated protein solution. The samples were analyzed by SDS-PAGE using a 12.5% polyacrylamide gel and quantified in the same way as for the MBP-Cdc25C(S287A) protein (see section 2.3.1).

2.3.3 Other Proteins

Wild-type recombinant *Xenopus* Cdc25C protein with an N-terminal maltose-binding protein tag [MBP-Cdc25C(WT)] had been prepared in the same way as MBP-Cdc25C(S287A) (Chau and Shibuya, 1999). Constitutively-active rat MEK(QP) with an N-terminal glutathione S-transferase (GST) protein tag had been expressed and purified as previously described (Bitangcol *et al.*, 1998). Human 14-3-3ζ protein with an N-terminal glutathione-S-transferase protein tag (GST-14-3-3ζ) was provided by Dr. Edmonds and Dr. Ostergaard (Arendt and Ostergaard, 2000).

2.4 Preparation of Cyclin dependent kinase 1 inhibitors

Roscovitine (Calbiochem) was prepared as a stock solution of 100 mM in DMSO and stored at -20°C. It was further diluted to 35 mM prior to experiments and added to reactions to a final concentration of 350 μM (1: 100 dilution). Butyrolactone I (Biomol) was prepared as a stock solution of 20 mM in DMSO and stored at -20°C. It was added to reactions to a final concentration of 200 μM (1: 100 dilution).

2.5 Cell Free Assays Using Egg Extracts

Unless stated otherwise, the reactions were prepared and the cell-free assays were carried out as previously described (Bitangcol *et al.*, 1998). Preparations of demembranated sperm nuclei (Bitangcol *et al.*, 1998) were thawed on ice, and nuclei were added to the reactions on ice to a final concentration of 200 to 500 sperm/μl reaction. The proteins and inhibitors [Cdc25C(WT), Cdc25C(S287A), Cyclin BΔ90, MEK(QP), roscovitine, and butyrolactone] were added to 5 to 10 % (v/v) of the reactions on ice. Once all of the additions to the reactions had been made on ice, the assays were started by transferring the reactions to a water bath where they were incubated at 21 to 23°C.

Histone H1 kinase (H1K), immunoblot and cytology samples were taken at intervals during incubation for analysis. The samples were prepared by removing 2 μl of reaction and mixing it with 20 μl of ice-chilled H1 kinase buffer (50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 10 mM NaF, 5 mM EGTA, 10 mM HEPES-KOH, pH 7.7). After thoroughly mixing, 17 μl of the mixture was added to 20 μl of 2X sample buffer (Laemmli, 1970) containing 10 mM NaF and 1 mM Na_3VO_4, and boiled for 5 min; the remaining 5 μl was immediately frozen in liquid nitrogen. For cytology samples, 2 μl of reaction was removed using a wide-mouth yellow pipetman tip and mixed gently with 10 μl of EGS fixative (see section 2.11). At the completion of the assay, H1K and immunoblot samples were stored at -80°C and the cytology samples were stored at 4°C.

2.5.1 Cell-Free Assay of MBP-Cyclin BΔ90

The reaction for Figure 3.10 was prepared by adding 0.7 µl of demembranated sperm nuclei (final concentration of 200 sperm/µl reaction from a stock of 14,000 sperm/µl buffer) to 49.3 µl of supplemented interphase arrested egg extract. The reaction was incubated for 60 min to allow decondensation of the sperm nuclei prior to the addition of MBP-Cyclin BΔ90. Once the nuclei were decondensed, 3 µl of soluble MBP-Cyclin BΔ90 (final concentration of 50 nM Cyclin BΔ90) was added to the reaction and H1 kinase, immunoblot, and cytology samples were taken at 10 min intervals.

2.5.2 Donor Reaction Preparation

The reactions for Figures 3.14 A and B were prepared by first allowing two, 1.7 ml microfuge tubes each containing 94 µl supplemented CHX extract to incubate at 23°C for 30 min to inhibit the activation of endogenous p42MAPK (see schematic Fig. 2.2). Next, 6.25 µl of soluble MBP-Cyclin BΔ90 protein (50 nM final concentration) was added to each tube. These reactions were placed in a water bath with a temperature of 23°C and tapped every 10 min. Samples were taken at 0, 30, and 45 min post-Cyclin addition. Next, 7.05 µl MEK(QP) or carrier buffer (7% of the total reaction volume) was added to the reactions. The reactions were further incubated at 21 to 23°C, and 0 min and 30 min after MEK(QP) protein addition samples were taken. Then, each of these reactions was divided into two equal aliquots and each aliquot was subjected to 3 rounds of immunodepletion (see section 2.7.4).

2.5.3 Recipient Reaction Preparation

Recipients reactions (Fig. 3.12 L) were prepared by initially allowing 48.2 µl of supplemented interphase arrested egg extract to incubate at 23°C for 30 min. Next, 1.8 µl of demembranated sperm nuclei (from a stock preparation of 14,000 sperm/µl buffer) were added to the extract to a final concentration of 500 sperm/µl reaction. The reactions were incubated for about 50 min to allow the sperm nuclei to form decondensed nuclei. The preparation and incubation of recipient reactions were timed to coincide with the time that the donor reactions would be ready for assay.

Figure 2.2 Schematic for the Preparation of a Cell-Free Assay for Nuclear Envelope Breakdown and Chromosome Condensation Activity

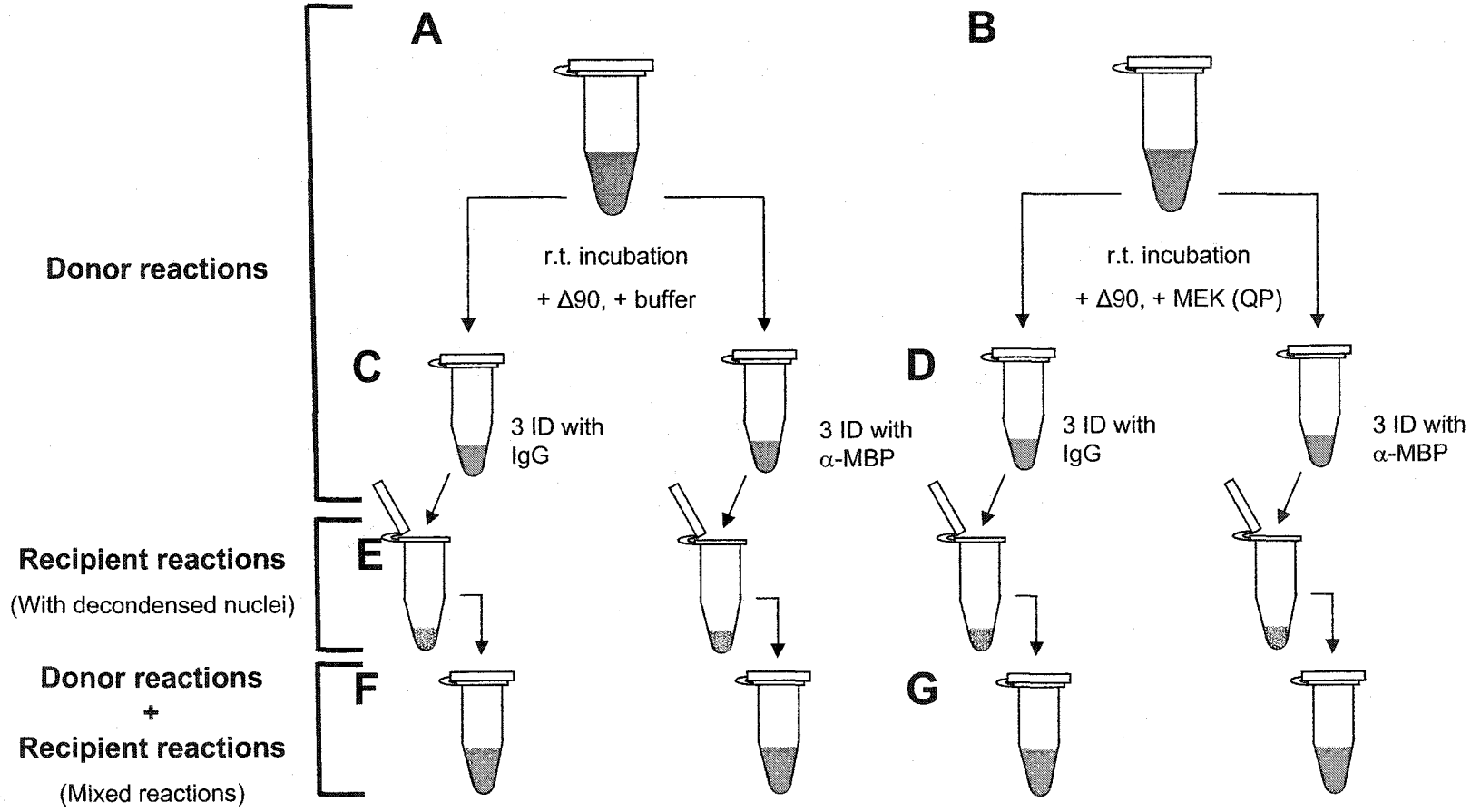
A,B) The donor reactions were prepared by first allowing two, 1.7 ml microfuge tubes each containing supplemented interphase arrested egg extract to incubate at room temperature for 30 min. Soluble MBP-Cyclin BΔ90 protein was added to each tube and the reactions were incubated at room temperature. 45 min after Cyclin BΔ90 addition, buffer (A) or MEK(QP) (B) was added to the reactions and the reactions were further incubated.

C,D) Each of the above reactions (A,B) was divided into two equal aliquots and each aliquot was subjected to 3 rounds of mock (IgG) or MBP-Cyclin BΔ90 (α-MBP) immunodepletions. After the third immunodepletion, 25 μl of immunodepleted supernatant from each donor reaction was collected.

E) The recipient reactions were prepared by adding demembranated sperm nuclei to an interphase arrested egg extract to a final concentration of 500 sperm/μl. The recipient extract was incubated for about 50 min to allow decondensation of the sperm nuclei. Before mixing with donor reactions, the recipient extract containing decondensed sperm nuclei was aliquotted into four tubes in 5 μl aliquots.

F,G) 20 μl of immunodepleted supernatant from each donor reaction was mixed with the 5 μl of recipient reaction containing decondensed sperm nuclei. The mixed reactions were assayed for NEBD/CC activity. H1 kinase, immunoblot, and cytology samples were taken.

Figure 2.2 Schematic for the Preparation of a Cell-Free Assay for Nuclear Envelope Breakdown and Chromosome Condensation Activity



2.5.4 Cell-Free Assay for Nuclear Envelope Breakdown and Chromosome Condensation Activity

A donor reactions were assayed for NEBD/CC activity after the three rounds of mock or MBP-Cyclin BΔ90 immunodepletions (see section 2.7.4). 25 µl of immunodepleted supernatant from each donor reaction was collected in clean 0.6 ml tubes (see schematic Fig. 2.2). 20 µl of donor immunodepleted supernatant was mixed with 5 µl of recipient CHX reaction containing 500 decondensed sperm nuclei/µl reaction. These mixed reactions were incubated in the water bath at 21 to 23°C, and H1 kinase, immunoblot, and cytology samples were taken from 0 to 90 min at 15 min intervals.

2.6 Antibodies and Immunoblotting

For the analysis of protein by immunoblotting, protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P filter, Millipore) using a Hoeffer blotting apparatus at 4°C for 2 hrs at 1 Amp per hour or overnight for a total amperage of 2 Amps. The blotting buffer was made according to a modification of standard methods (Harlow and Lane, 1988) [for Immobilon: 25 mM Tris, 192 mM glycine, 0.01% SDS (w/v), 16% methanol (v/v)]. Blocking was done in TBST [50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.1% Tween-20 (v/v)] containing 1.5% bovine serum albumin (w/v) and 0.02% sodium azide (w/v). The membranes were incubated in TBST containing antibodies, 10% goat normal serum (v/v), 1.5% BSA (w/v), and 0.02% sodium azide (w/v) either overnight or for 1 hr.

The primary antibodies used in the work described in this thesis were all rabbit polyclonal antibodies. The primary antibodies used were affinity-purified anti-Cdc25C (Bitangcol *et al.*, 1998), anti-ERK 837 (Boulton and Cobb, 1991), anti-Glutathione-S-Transferase (GST; Molecular Probes), anti-14-3-3 (Santa Cruz Biotechnology), anti-RSK1 (Santa Cruz Biotechnology), and anti-Maltose Binding Protein (MBP; New England Biolabs). Goat, anti-rabbit alkaline phosphatase-conjugated secondary antibodies (anti-rabbit; Cat. # A3687; Sigma) were used at 1:3000 dilutions for 45 min to 1 hr at room temperature to detect the primary antibodies. Immunoblots were developed in a reaction solution of 10 ml of alkaline phosphatase (AP) buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5), 33 µl of nitro blue tetrazolium (NBT) substrate, and

16.5 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to a modification of the manufacturer's instructions (Promega). The reaction was stopped in a solution of 20 mM Na_2EDTA (a 0.2 M Na_2EDTA disodium salt stock solution that was not neutralized and diluted 1:9 with Milli-Q).

2.7 Immunoprecipitation/Immunodepletion

2.7.1 Cdc25C Immunoprecipitation

Cdc25C was immunoprecipitated from immature oocyte extracts, unfertilized egg extracts, interphase egg extracts, and cycling egg extracts arrested in G2 by MEK(QP) (Bitangcol *et al.*, 1998). 50 µl of each extract was incubated with 0.5 µl of affinity-purified anti-Cdc25C antibodies on ice for 30 min. Then, each mixture of extract/antibody was mixed with 25 µl of protein A-Sepharose/Sepharose CL4B [1 volume protein A-Sepharose: 1 volume Sepharose CL4B (Pharmacia Biotech.) that had been washed in CSF-EB with 1 mM Na_3VO_4], and the resuspended resin was mixed by end-over-end rotation for 1 hr at 4°C. After pelleting, the resin was washed three times with CSF-EB with 0.1% Tween-20 (v/v) and three times with CSF-EB (see section 2.1.4). Proteins were eluted with sample buffer and analyzed by immunoblotting with anti-Cdc25C antibodies and anti-14-3-3 antibodies.

2.7.2 $pp90^{Rsk}$ Immunoprecipitation

$pp90^{Rsk}$ was immunoprecipitated either from a CSF egg extract (Fig. 3.5) or MEK(QP)-G2 arrested egg extract that was allowed to incubate for 30 min post MEK(QP) addition (unpublished data not shown). Anti-RSK1 antibodies (Santa Cruz Biotechnology, Inc.) were added to the egg extract and kept on ice for 1 hr. The extract containing the antibodies was then transferred to a fresh tube containing protein A-Sepharose resin and incubated further at 4°C for 30 min end-over-end. The beads were pelleted at 10,200xg at 4°C for 5 to 10 min and the lysate was removed. Beads were sequentially washed once in the above CSF-EB (see section 2.1.4) with 1% Tween-20 (v/v), once in CSF extraction buffer with 1% Tween-20 (v/v) and 0.5 M NaCl, twice with CSF extraction buffer without Tween-20, and a final wash with kinase buffer (1 mM β-

mercaptoethanol, 5 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.5). The final 50% slurry of $pp90^{Rsk}$-protein A Sepharose aliquots (suspended in 1 bead bed volume of kinase buffer; see section 2.8.2) was aliquotted into 0.6 ml microfuge tubes in 10 µl aliquots. These tubes were frozen in liquid nitrogen and stored at -80°C for future use in kinase assays.

2.7.3 Cdc25C Immunodepletion

Egg extracts were immunodepleted of Cdc25C using the same affinity-purified antibodies used for the immunoprecipitations (Fig. 3.1), except the antibodies were bound to protein A-Sepharose at a concentration of 2.0 µg antibody/µl beads, and the beads were washed several times with EB containing 1 µg BSA/ml EB and an ATP-regenerating system before addition to the egg extracts (Fig. 3.3). Anti-GST antibody-beads were used for the mock immunodepletions. Cycling egg extracts were incubated on ice for 45 min with either anti-Cdc25C antibody beads or anti-GST antibody beads (4 vol. egg extract: 1 vol. of antibody beads; mixing every few minutes). Antibody beads were pelleted by centrifugation at 10,200xg for 5 min and the depleted extract was used immediately for experiments. In some experiments, recombinant Cdc25C proteins (WT or S287A) were added to the depleted extracts at the same levels as that of endogenous Cdc25C in non-depleted egg extracts (Fig. 3.3).

2.7.4 Cyclin BΔ90 Immunodepletion

After taking the 30 min post-MEK(QP) addition samples from the donor reactions in the 1.7 ml tubes (see section 2.5.2), 2 aliquots of 42 µl from each of the donor reactions were transferred to 0.6 ml microfuge tubes containing either 10 µl of Affi-prep protein A-anti-rabbit IgG beads (mock immunodepletion) or Affi-prep protein A-anti-MBP beads (MBP-Cyclin BΔ90 immunodepletion). The extracts were subjected to 3 rounds of mock or MBP-Cyclin BΔ90 immunodepletion. Each round consisted of mixing the beads with the reaction for 10 min rounds at room temperature on a rocker. After 10 min of immunodepletion, the reactions were subjected to centrifugation at 10,200xg for 1 min at 21°C, and then the supernatants from each reaction were transferred to clean 0.6 ml tubes containing 10 µl of fresh Affi-prep protein A-anti-rabbit

IgG beads or Affi-prep protein A-anti-MBP beads for subsequent rounds of immunodepletion.

The immunodepletions for Figure 3.11 B were done in the same way except that an additional immunodepletion was done using 2 rounds of 30 min immunodepletions. The Affi-prep protein A beads were stored in cycling EB after the immunodepletions.

2.8 Kinase Assays

2.8.1 Histone H1 Phosphorylation Assay

Histone H1 phosphorylation assays were done as previously described (Bitangcol *et al.*, 1998). The H1 kinase samples from cell-free assays were thawed on ice and subjected to centrifugation at 13,000 rpm for 30 sec at room temperature using a fixed angle rotor in a Baxter centrifuge and then kept on ice. Next, 3 µl of kinase mixture (0.2 mg histone H1/ml kinase mixture, 0.5 µCi [γ-^{32}P]ATP/µl kinase mixture, 60 µM unlabeled ATP/Mg^{2+}, 20 µM protein kinase A inhibitor peptide (PKI), 100 µM calmidazolium, and 20 mM HEPES-NaOH, pH 7.5) was added to each tube and the reactions were immediately incubated at room temperature for 20 min. After 20 min, each reaction was quenched by adding 8 µl of 2X sample buffer and thoroughly mixing. The samples were analyzed by SDS-PAGE using a 15% polyacrylamide gel. The gel was stained, destained, dried and then processed for autoradiography. Analysis and quantification of ^{32}P incorporation was done using the phosphorimager.

2.8.2 Cdc25C and Inh2 Phosphorylation Assay

pp90^{Rsk} was immunoprecipitated from an M-phase arrested *Xenopus* egg extract or a MEK(QP)-G2 arrested egg extract using anti-RSK1 antibody and protein A Sepharose (see section 2.7.2). 5 µl aliquots of packed pp90^{Rsk}-protein A Sepharose were incubated with 0.1 mg recombinant MBP-Cdc25C(WT)/ml buffer or 0.1 mg MBP-Cdc25C(S287A)/ml buffer in 10 µl kinase buffer (10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 µM DTT, 60 µM unlabeled ATP, 20 mM HEPES-KOH, pH 7.5) and 5 µCi of [γ-^{32}P]ATP. The kinase reactions were carried out at 27°C for 1 hr with occasional mixing. For Figure 3.6, 5 µl aliquots of pp90^{Rsk}-protein A Sepharose were

incubated with recombinant MBP-Cdc25C(WT), MBP-Cdc25C(S287A), or rabbit Inhibitor-2 to a final concentration of 0.1 mg protein/ml buffer in 15 μl kinase buffer. The reactions for Figure 3.6 were quenched with 20 μl of 2X sample buffer, boiled for 3-5 min, analyzed by SDS-PAGE using a 15% gel and processed for autoradiography.

For binding experiments (see section 2.9), kinase reactions were carried out in the same way, but reactions were not quenched. Instead, the reactions were subjected to centrifugation to sediment the immunoprecipitates before the supernatant containing the substrate protein was removed and used for binding reactions.

2.9 Cdc25C and 14-3-3ζ Protein Binding Assay

The supernatants (from the unquenched kinase assays; see section 2.8.2) containing the phosphorylated MBP-Cdc25C(WT) or MBP-Cdc25C(S287A) were collected. The pelletted $pp90^{Rsk}$ immunoprecipitates were briefly washed with 10 μl of binding buffer (20 mM EDTA, 0.5% Tween-20, 20 mM NaF, 1 mM Na_3VO_4, 10 mM HEPES-KOH, pH 7.5) and the wash added to the previously collected supernatant. Either 0.25 μg of GST or 0.5 μg of GST-14-3-3ζ was added to the supernatant containing either phosphorylated Cdc25C(WT), nonphosphorylated Cdc25C(WT), phosphorylated Cdc25C(S287A) or nonphosphorylated Cdc25C(S287A). In addition, 0.5 mg ovalbumin/ml buffer and binding buffer was added to a final volume of 20 μl. The mixture of soluble proteins was allowed to incubate on ice for 1 hr. 18 μl of the each reaction containing the soluble protein mixture was then transferred to approximately 7 μl of packed amylose resin that had been previously washed with binding buffer. The protein mixture was mixed with the amylose resin end-over-end at 4°C for an additional 1 hr.

After 1 hr of binding the protein mixture to amylose resin, the beads were pelleted at 10,200xg for 1 min at 4°C, washed with four alternating washes (20 mM NaF, 1 mM Na_3VO_4, 10 mM HEPES-NaOH, pH 7.5) containing either 500 mM NaCl with 0.5% Tween (v/v) (wash buffer 1) or 150 mM NaCl with 0.5% Tween (v/v) (wash buffer 2) and twice with wash buffer 2 without Tween (wash buffer 3). After the washes, the proteins were eluted from the beads by adding 20 μl of 2X sample buffer containing 1 mM NaF and 0.1 mM Na_3VO_4 to the beads after aspirating the final wash buffer. The

samples were boiled for 3 to 5 min, analyzed by SDS-PAGE using a 12.5% gel and analyzed by autoradiography.

2.10 Autoradiography and Phosphorimager Analysis

Polyacrylamide gels stained with Coomassie blue, destained and dried were processed for autoradiography by exposure to Kodak X-OMAT AR films in Kodak X-ray exposure holders for 2 to 24 hrs at room temperature. The films were developed using the Kodak X-OMAT M35 processor.

Incorporation of ^{32}P was quantified by phosphorimager analysis. Dried gels were exposed to a phosphorimager plate for 1 to 2 hrs and then the plate was processed using the phosphorimager (Typhoon 8600). The scans were quantified using the Image Gauge V3.0 software program.

2.11 Cytology and Fluorescence Phase/Contrast Microscopy

The EGS fixative buffer was prepared by mixing stock buffer with ethylene glycol-bis (succinic acid N-hydroxy-succinimide ester) (EGS) in DMSO and Hoechst 33342. Briefly, in a 1.7 ml microfuge tube, EGS was dissolved in DMSO to 0.2 M, vortexed, and subjected to centrifugation at 13,000 rpm for 5 min at room temperature in a microfuge using a fixed angle rotor. The supernatant was transferred to a new tube. Four volumes of 0.2 M EGS was mixed with 96 volumes of fixative buffer [100 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol (v/v), 20 mM HEPES-NaOH, pH 7.5] and 2 μg Hoechst 33342/ml buffer (from a stock of 1 mg Hoechst/ml DMSO). The fixative was subjected to centrifugation again, and the supernatant was transferred to a new tube and shielded from light.

After fixation for at least 1 hr, samples were mounted on microscope slides (VWR) using an extra wide-mouth yellow pipetman tip. 8.5 μl of fixed sample was mounted on each slide, covered with a 22 mm square cover slip (VWR), and sealed with nail polish. The samples were viewed using a fluorescence/phase Axioskop microscope (Zeiss) under the 25X and 100X oil immersion objectives. Hoechst fluorescence images and phase contrast images were taken using a digital camera (Canon Powershot G2) attached to the microscope.

Chapter 3: Results

3.1 Activation of the p42MAPK Pathway During Interphase Leads to a G2 Arrest

3.1.1 14-3-3 Proteins Bind to Inactive Cdc25C in MEK(QP)-G2 Arrested Egg Extracts, but not to Active Cdc25C in M-phase Egg Extracts.

Recombinant MEK(QP) (a constitutively-active mutant, in which glutamine 56 is mutated to a proline) was previously shown to activate p42MAPK and cause a G2 arrest when added to a cycling egg extract during interphase (Bitangcol *et al.*, 1998). It is known that 14-3-3 proteins, a class of acidic, dimeric proteins, bind to Cdc25C during interphase, but not during M-phase (Kumagai *et al.*, 1998b). To determine whether 14-3-3 proteins were sustained in a complex with Cdc25C in the MEK(QP)-G2 arrested extracts (as previously found in oocyte and interphase extracts, Kumagai *et al.*, 1998b), Cdc25C was immunoprecipitated from extracts and subjected to immunoblotting with anti-Cdc25C and pan-specific anti-14-3-3 antibodies. It is clear that the majority of the Cdc25C immunoprecipitated from the MEK(QP)-G2 arrested extract is hypophosphorylated. 14-3-3 proteins were associated with Cdc25C in immunoprecipitates from MEK(QP)-G2 arrested extracts (Fig. 3.1). In these immunoprecipitates, however, it appears as though the smaller isoforms of 14-3-3 [with an apparent molecular mass of 28 kDa and a mass of approximately 27 kDa based on the predicted amino acid sequence (Kumagai *et al.*, 1998b)], probably corresponding to the ζ isoform of 14-3-3 (Kumagai *et al.*, 1998b), are the primary isoforms of 14-3-3 bound to the Cdc25C (Fig. 3.1). In immunoprecipitates from M-phase egg extracts, Cdc25C is active and hyperphosphorylated, but is not associated with 14-3-3 proteins.

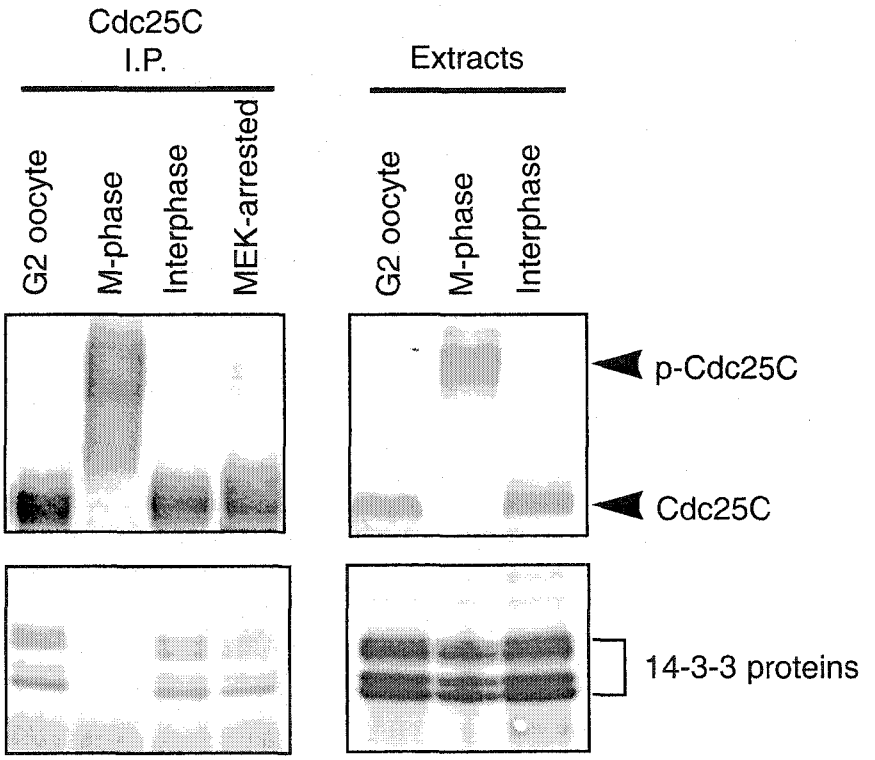
These results show that 14-3-3 protein binding to Cdc25C is sustained in the MEK(QP)-G2 arrested egg extracts coincident with the increase in tyrosine phosphorylated, inactive Cdk1 observed previously (Abrieu *et al.*, 1997a; Walter *et al.*, 1997; Bitangcol *et al.*, 1998).

Figure 3.1 14-3-3 Proteins Bind to Inactive Cdc25C in MEK(QP)-Interphase Arrested Egg Extracts but not to Active Cdc25C in M-phase Egg Extracts

Extracts made from immature oocytes (G2 oocyte), unfertilized eggs (M-phase), and cycling egg extracts in interphase, or MEK(QP) (MEK-arrested) egg extracts were immunoprecipitated using Cdc25C antibodies and then the extracts and immunoprecipitates were immunoblotted with anti-Cdc25C and pan-specific anti-14-3-3 antibodies.

The upper panels show the anti-Cdc25C immunoblot (p-Cdc25C indicates the hyperphosphorylated, active form of Cdc25C), and the lower panels show an anti-14-3-3 immunoblot. (Data provided by E. Shibuya).

Figure 3.1 14-3-3 Proteins Bind to Inactive Cdc25C in MEK(QP)-Interphase-Arrested Egg Extracts but not to Active Cdc25C in M-phase Egg Extracts



Data provided by Dr. E. Shibuya

3.1.2 Recombinant Cdc25C(S287A) Protein Accelerates the Cell Cycle in *Xenopus* Egg Extracts and Overcomes the G2 Arrest Induced by Activation of the p42MAPK Pathway

Recombinant wild type Cdc25C protein (WT) and a mutant Cdc25C protein in which S287 had been mutated to a nonphosphorylatable alanine (S287A) were expressed as fusion proteins with an N-terminal Maltose Binding Protein (MBP) epitope tag using *E. coli* (Chau and Shibuya, 1999). S287A had previously been found to overcome G2 cell cycle arrest in egg extracts induced by the DNA damage checkpoint (Kumagai *et al.*, 1998b; Chau and Shibuya, 1999). In this study, WT and S287A proteins were tested in cycling egg extracts and in extracts that had been arrested in G2 by MEK(QP). Addition of these proteins to cycling egg extracts showed that only the S287A protein accelerated cell cycles, in particular the second cell cycle (Fig. 3.2 A). Results from several experiments showed that S287A was able to drive entry into M-phase of the second cell cycle 15 to 20 min earlier than WT (Fig. 3.2 A). This difference in timing is consistent with previous results found for the effects of WT and S287A in overcoming cell cycle arrest in DNA damage checkpoint arrested extracts (Kumagai *et al.*, 1998b; Chau and Shibuya, 1999). These results suggest that phosphorylation of S287 of Cdc25C is necessary for preventing the premature activation of Cdk1 in normal cell cycles in egg extracts as well as inhibiting the activation of Cdk1 in extracts arrested in G2 by activation of the DNA damage checkpoint.

When proteins were added to MEK(QP)-G2 arrested egg extracts, S287A was able to overcome cell cycle arrest and drive these extracts into M-phase after about 100 min of incubation (Fig. 3.2 B). The same results were obtained with or without nuclei present in the egg extracts (unpublished data not shown). These results suggest that phosphorylation of S287 of Cdc25C is also of key importance in preventing activation of Cdk1 in MEK(QP)-G2 arrested egg extracts.

Figure 3.2 Cdc25C(S287A) Protein Accelerates the Cell Cycle in *Xenopus* Egg Extracts and Overrides the G2 Arrest Induced by p42MAPK

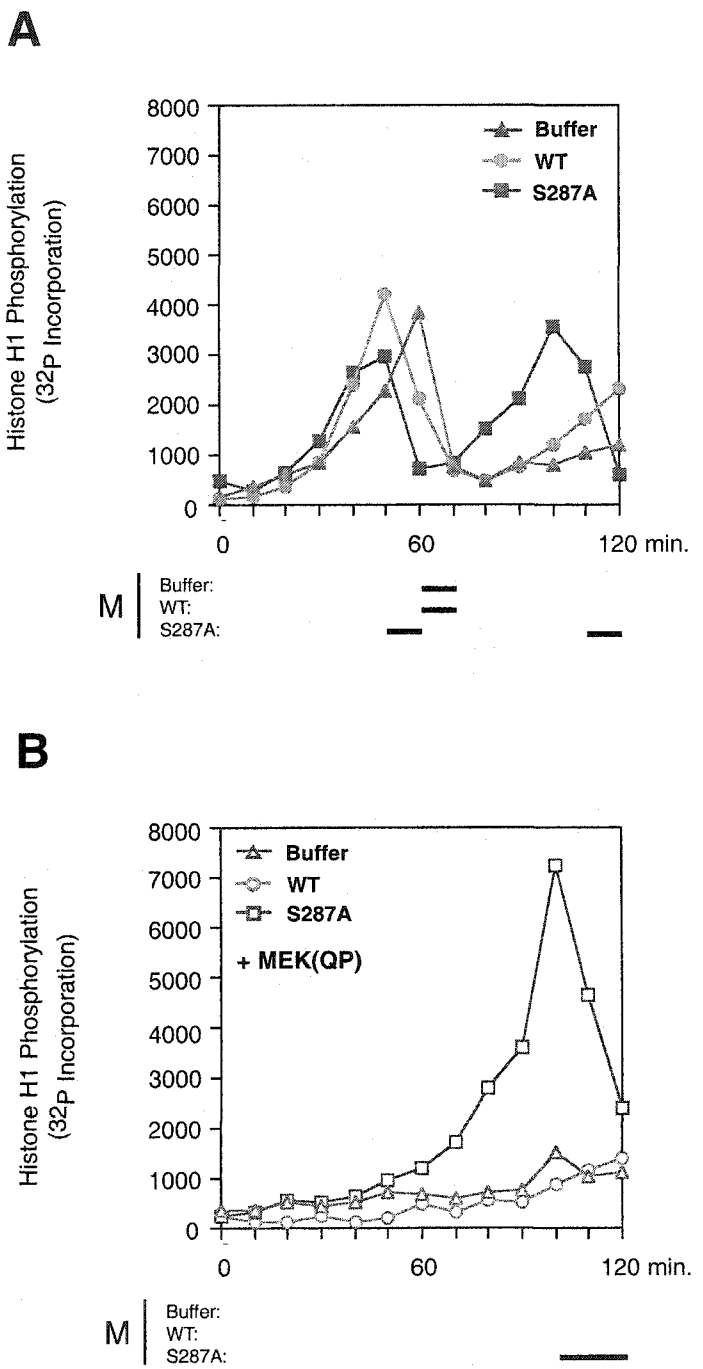
A) Cdc25C(S287A) Accelerates the Cell Cycle in *Xenopus* Egg Extracts

Recombinant MBP-Cdc25C(WT) or (S287A) fusion proteins were added to cycling egg extracts. Addition of S287A accelerates the cell cycle, particularly the second cell cycle. Bars beneath graphs indicate the samples in which M-phase nuclear morphology was observed [Nuclear Envelope Breakdown (NEBD), Chromosome Condensation (CC)].

B) Cdc25C(S287A) Drives MEK(QP)-G2 Arrested Egg Extracts into M-phase

Recombinant MBP-Cdc25C(WT or S287A) fusion proteins were added to cycling egg extracts with MEK(QP). S287A addition to MEK(QP)-G2 arrested extracts is able to override cell cycle arrest and cause entry into M-phase. Bars beneath graphs indicate the samples in which M-phase nuclear morphology was observed (NEBD, CC).

Figure 3.2 Cdc25C(S287A) Protein Accelerates the Cell Cycle in *Xenopus* Egg Extracts and Overrides the G2-Arrest Induced by p42MAPK



Date provided by A. Chau

3.1.3 Addition of Cdc25C(S287A) Protein to Cdc25C-Depleted Egg Extracts Containing Active p42MAPK Induces M-phase Earlier than Addition of Cdc25C(WT)

To eliminate the effects of endogenous Cdc25C in the MEK-induced G2 arrest, Cdc25C was immunodepleted from cycling egg extracts. WT and S287A Cdc25C were added back to these depleted egg extracts with MEK(QP). Mock-depleted extracts continued to cycle (Fig. 3.3 A), although slightly slower than non-depleted extracts (unpublished data not shown). However, as expected, Cdc25C depleted extracts did not cycle (Fig. 3.3 B). This result is consistent with the role of Cdc25C phosphatase as a necessary inducer of M-phase required for the dephosphorylation and activation of Cdk1 (Kumagai and Dunphy, 1992).

During the MEK(QP)-G2 arrest, Cdc25C appears to play a crucial role during the arrest. When WT and S287A were added back to extracts that had been arrested with MEK(QP), accelerated entry into M-phase was observed with S287A compared with WT, but the difference in timing of entry into M-phase was 30 to 40 min earlier, which was greater than in non-depleted extracts (Fig. 3.3 C,D). M-phase induced in depleted MEK(QP)-treated extracts by adding recombinant Cdc25C proteins was normal as judged by nuclear envelope breakdown, chromosome condensation, and microtubules organized into spindles (Fig. 3.3 E,F). The 30 to 40 min difference in timing of entry into M-phase establishes S287 as a key residue in Cdc25C that regulates the transition from a MEK(QP)-G2 arrest into M-phase.

3.1.4 *Xenopus* Cdc25C Has Consensus Sequences for $pp90^{Rsk}$ Phosphorylation and for 14-3-3 Binding

$pp90^{Rsk}$ is a serine/threonine kinase known to phosphorylate proteins such as the apoptotic protein BAD specifically on residue serine 112 within a 14-3-3 binding consensus sequence (Bonni *et al.*, 1999; Fang *et al.*, 1999; Shimamura *et al.*, 2000) . We were interested in determining whether or not the MEK(QP)-G2 arrest that we observed might be regulated by the activity of $pp90^{Rsk}$ on Cdc25C. $pp90^{Rsk}$ is a downstream, direct target of p42MAPK (Blenis, 1993) in the MAPK pathway that is able to phosphorylate specific serine residues. Hence, we examined the predicted amino acid sequence of

Figure 3.3 Addition of Cdc25C(S287A) Protein to Cdc25C-Depleted Egg Extracts Containing Active p42MAPK Induces M-phase Earlier than Addition of Cdc25C(WT)

A,B) Cdc25C was mock depleted using non-specific IgG (A) or immunodepleted (B) using a Cdc25C antibody from cycling egg extracts. Immunodepletion of Cdc25C prevents entry into M-phase as shown by the Cdc25C immunoblots (upper panels) and histone H1 phosphorylation (lower graphs). Bars beneath graphs indicate the samples in which M-phase nuclear morphology was observed (NEBD, CC). (Data provided by A. Chau).

C,D) MEK(QP) protein and either Cdc25C(WT) (C) or Cdc25C(S287A) (D) were added to extracts depleted of Cdc25C and samples were taken for analysis of MPF activity (H1 kinase activity) and cytology [bars beneath graphs indicate samples in which M-phase nuclear morphology was observed (NEBD, CC)]. The H1 kinase assays represented in C and D are from three different experiments with similarly colored graphs and cytology bars corresponding to the data from the same experiment in both panels. Since experiments were done at different times with radioisotope of different activities, levels of H1K phosphorylation are comparable only within each experiment. (Data provided by A. Chau).

E,F) Cytology samples taken at time points marked with an asterisk in (C) and (D). Dual exposure with Hoechst-stained DNA (blue) and phase/contrast. Nuclear envelope breakdown, chromosome condensation, and microtubules organized into spindles indicate M-phase, specifically metaphase. (Data provided by E. Shibuya).

Figure 3.3 Addition of Cdc25C(S287A) Protein to Cdc25C-Depleted Egg Extracts Containing Active p42MAPK Induces M-phase Earlier than Addition of Cdc25C(WT)

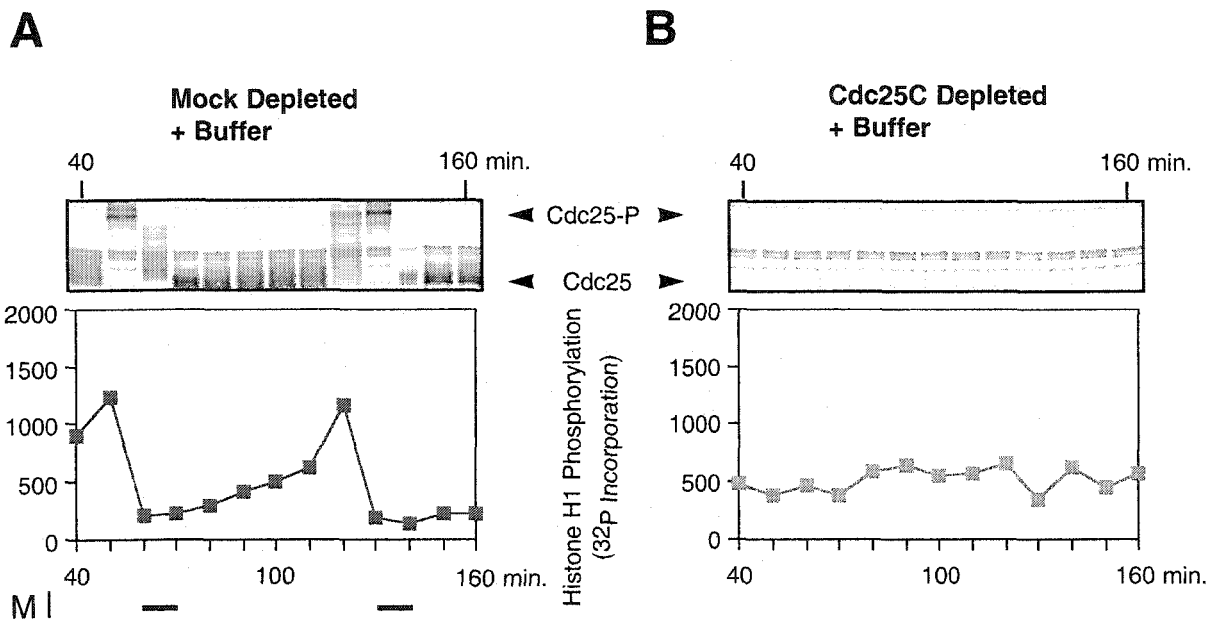
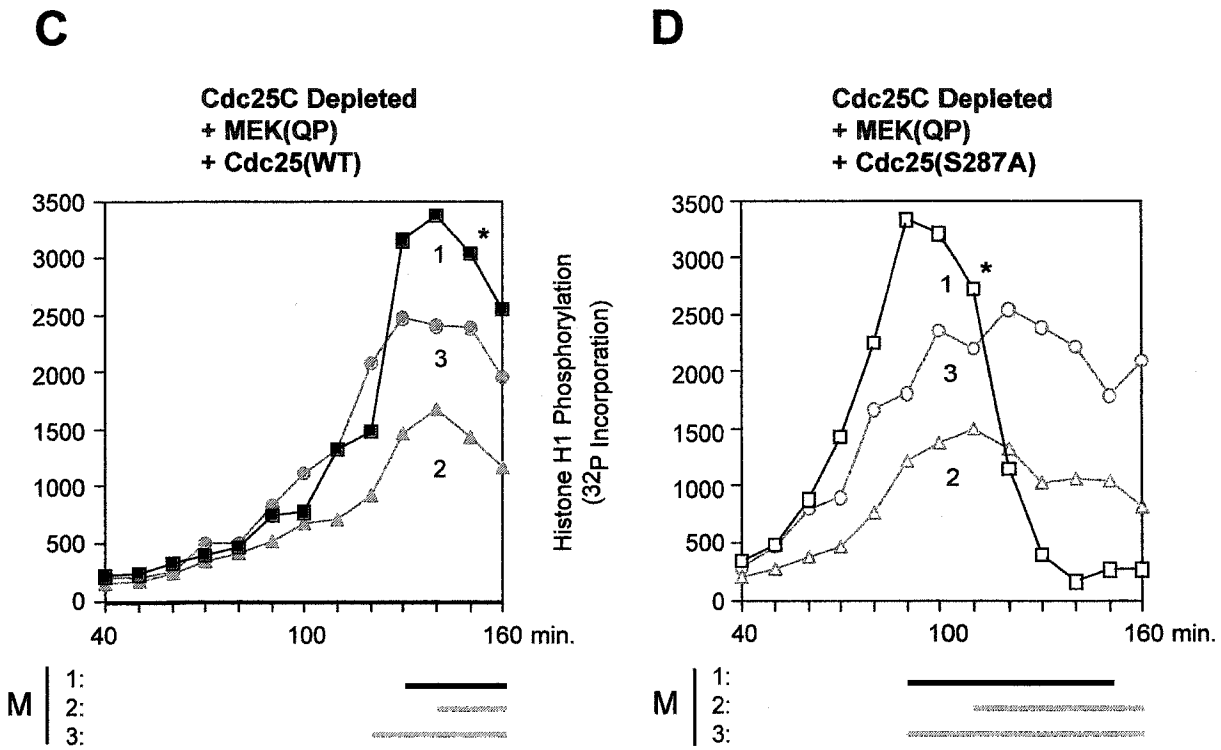
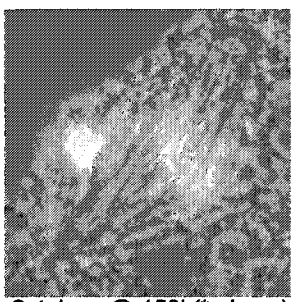
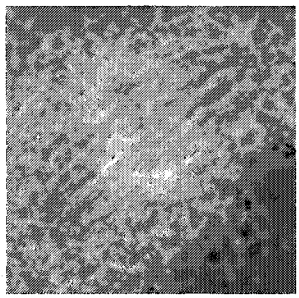


Figure 3.3




E

Cytology @ 150' (* above)


F

Cytology @ 110' (* above)

A-D: Data provided by A. Chau
E,F: Data provided by E. Shibuya

Cdc25C and identified potential sites able to be phosphorylated by $pp90^{Rsk}$ (Fig. 3.4). There are five consensus sequences for $pp90^{Rsk}$ phosphorylation (RXXS/T) in Cdc25C, including one that contained S287. Cdc25C phosphorylated on S287 in *Xenopus* and S216 in mammalian tissue culture cells has been shown to bind to 14-3-3 proteins, a family of acidic proteins approximately 30 kDa in size (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Duckworth *et al.*, 2002). Phosphorylation of Cdc25C at S287 mediates binding of 14-3-3 proteins since S287 resides within a consensus sequence (RXXpSXP) for 14-3-3 binding (Kumagai *et al.*, 1998b) (Fig. 3.4). The consensus sequences for $pp90^{Rsk}$ phosphorylation of Cdc25C suggested that it could be a potential target for $pp90^{Rsk}$ activated by the MEK(QP)-induced G2 arrest, and that phosphorylation of S287 may be the cause of 14-3-3 protein binding.

3.1.5 $pp90^{Rsk}$ Immunoprecipitated From an M-phase Arrested Egg Extract is Hyperphosphorylated

$pp90^{Rsk}$ was immunoprecipitated from an M-phase arrested egg extract (Fig. 3.5). In order to verify that the $pp90^{Rsk}$ immunoprecipitated from MII extracts was phosphorylated and in the active form, anti-RSK1 antibody was used to immunoprecipitate the protein. Immunoprecipitated protein bound to protein A-Sepharose beads, samples obtained from two different unfertilized egg extracts, and a CHX-induced interphase arrested egg extract were analyzed using anti-RSK1 antibody. The immunoprecipitated $p90^{Rsk}$ was present in the hyperphosphorylated form ($pp90^{Rsk}$), which exhibited a slower mobility on SDS-polyacrylamide gels (Fig. 3.5). The $pp90^{Rsk}$ eluted from the beads had the same mobility as the $pp90^{Rsk}$ detected in the MII extracts, when it is known to be fully active form. The $p90^{Rsk}$ from interphase extracts showed a faster mobility form (unphosphorylated form) of $p90^{Rsk}$ (Fig 3.5). The unphosphorylated form of $p90^{Rsk}$ is the inactive form normally present during interphase. These results confirmed that the $p90^{Rsk}$ immunoprecipitated from mature, unfertilized egg extracts is the phosphorylated form which suggests that it is active.

Figure 3.4 *Xenopus laevis* Cdc25C has Consensus Sequences for pp90^{Rsk} Phosphorylation and for 14-3-3 binding

Xenopus amino acid sequence was examined for Cdc25C (SWISS-PROT accession number: A42679; Kumagai and Dunphy, 1992. The underlining denotes the location of the pp90^{Rsk} phosphorylation consensus sites and the boldface indicates the binding site for 14-3-3 proteins.

Figure 3.4 *Xenopus laevis* Cdc25C has Consensus Sequences for pp90^{Rsk} Phosphorylation and for 14-3-3 Binding

Xenopus Laevis Cdc25C

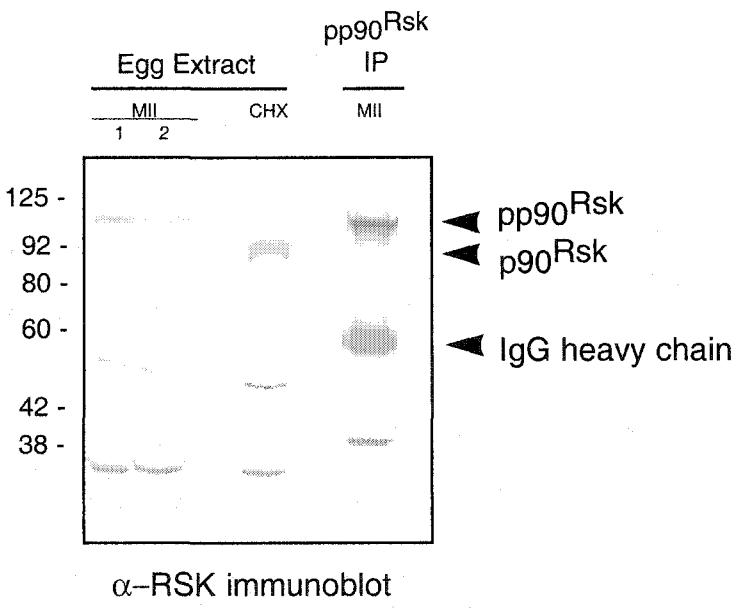
```
1   MAESHIMSSE APPKTNTGLN FRTNCRMVLN LLREKDCSVT FSPEQPLTPV TDLAVGFSNL
61  STFSGETPKR CLDLSNLGDE TAPLPTESPD RISSGKVESP KAQFVQFDGL FTPDLGWKAK
121 KCPRGNMNSV LPRLLCSTPS FKKTSGGQRS VSNKENEGEL FKSPNCKPVA LLLPQEVVDS
181 QFSPTPENKV DISLDEDCEM NILGSPISAD PPCLDGAHDD IKMQNLDGFA DFFSVDEEEM
241 ENPPGAVGNL SSSMAILLSG PLLNQDIEVS NVNNISLNRS RLYRSPSMPE KLDRPMLKRP
301 VRPLDSETPV RVKRRRSTSS SLQPQEENFQ PQRRGTSLKK TLSLCDVDIS TVLDEDCGHR
361 QLIGDFTKVY ALPTVTGRHQ DLRYITGETL AALIHGDFSS LVEKIFIIDC RYPYEYDGGH
421 IKGALNLHRQ EEVTDYFLKQ PLTPTMAQKR LIIIFHCEFS SERGPKMCRF LREEDRARNE
481 YPSLYYPELY LLKGGYKDFF PEYKELCEPQ SYCPMHHQDF REELLKFRTK CKTSVGDRKR
541 REQIARIMKL
```

SWISS-PROT accession number: A42679

Figure 3.5 $pp90^{Rsk}$ Immunoprecipitated from an M-phase Egg Extract is Hyperphosphorylated

$pp90^{Rsk}$ was immunoprecipitated (IP) from an M-phase arrested, unfertilized egg extract using an anti-RSK1 antibody and protein A-Sepharose beads. A sample of the immunoprecipitate was analyzed by SDS-PAGE and compared to two different MII extracts and a CHX (interphase arrested) extract using anti-RSK1.

Figure 3.5 pp90^{Rsk} Immunoprecipitated from an M-Phase Egg Extract is Hyperphosphorylated



3.1.6 Phosphorylation of MBP-Cdc25C by pp90^{Rsk}

After establishing Cdc25C as a potential regulator for the MEK(QP)-G2 arrest, we were interested in determining which kinase downstream of MEK was able to phosphorylate S287 of Cdc25C. Since S287 of Cdc25C was located within a consensus site for phosphorylation by pp90^{Rsk} (Blenis, 1993) that was also within a consensus site for 14-3-3 binding (Kumagai *et al.*, 1998b), we reasoned that the molecular mechanism by which 14-3-3 protein association was maintained in MEK(QP)-G2 arrested egg extracts was by phosphorylation of S287 by pp90^{Rsk}.

To test if pp90^{Rsk} could phosphorylate Cdc25C, pp90^{Rsk} was immunoprecipitated from an M-phase arrested extract or a cycling egg extract arrested in G2 by the addition of MEK(QP). pp90^{Rsk} was hyperphosphorylated and activated in both the M-phase and the MEK(QP)-G2 arrested egg extracts (Bitangcol *et al.*, 1998, Khandani, 2003; Fig 3.5). pp90^{Rsk} was tested for activity in an *in vitro* kinase assay using recombinant Cdc25C (WT or S287A) or inhibitor-2 (Inh2; rabbit) as the substrates. We verified that the pp90^{Rsk} immunoprecipitated from an unfertilized egg extract was active in this assay as shown by the phosphorylation of rabbit Inh2 (Fig. 3.6), a protein previously shown to be phosphorylated by pp90^{Rsk} (Wang *et al.*, 1995). The results from the kinase assay also showed that Cdc25C(WT) was substantially phosphorylated by pp90^{Rsk}, whereas phosphorylation of Cdc25C(S287A) was negligible, suggesting that S287 is the major phosphorylation site.

3.1.7 pp90^{Rsk} Immunoprecipitated from a MEK(QP)-G2 Arrested Egg Extract Phosphorylates S287 of Cdc25C Which Mediates the Binding of 14-3-3ζ

To determine if 14-3-3 proteins associate with Cdc25C in which S287 is specifically phosphorylated by pp90^{Rsk}, we immunoprecipitated pp90^{Rsk} from a MEK(QP)-G2 arrested egg extract (Fig. 3.7), which we had shown to be active. In *Xenopus* egg extracts, Cdc25C is stoichiometrically bound to the ε and ζ isoforms of 14-3-3 during interphase (Kumagai *et al.*, 1998b; Hutchins *et al.*, 2002). pp90^{Rsk} immunoprecipitates were used in an *in vitro* kinase assay with MBP-Cdc25C (WT and S287A) as substrates, and then these substrates (as well as proteins that had not been subjected to a kinase assay) were tested for their ability to bind to recombinant GST-14-3-3 fusion proteins.

Figure 3.6 pp90^{Rsk} Can Phosphorylate Cdc25C(WT) *in vitro*

pp90^{Rsk} was used to phosphorylate equal amounts of Cdc25C(WT), Cdc25C(S287A) and Inh2 (rabbit and *Xenopus*). Samples were analyzed by SDS-PAGE followed by processing for autoradiography.

A) The polyacrylamide gel stained with Coomassie blue confirms that equal amounts of MBP-Cdc25C(WT and S287A) proteins were used as substrates and analyzed by SDS-PAGE.

B) The autoradiogram of the gel in (A) shows that Cdc25C(WT) had incorporated substantial radiolabeled [^{32}P], whereas radiolabelling of Cdc25C(S287A) was negligible, indicating that pp90^{Rsk} specifically phosphorylated S287 of Cdc25C.

Figure 3.6 pp90^{Rsk} Can Phosphorylate Cdc25C(WT) *in vitro*

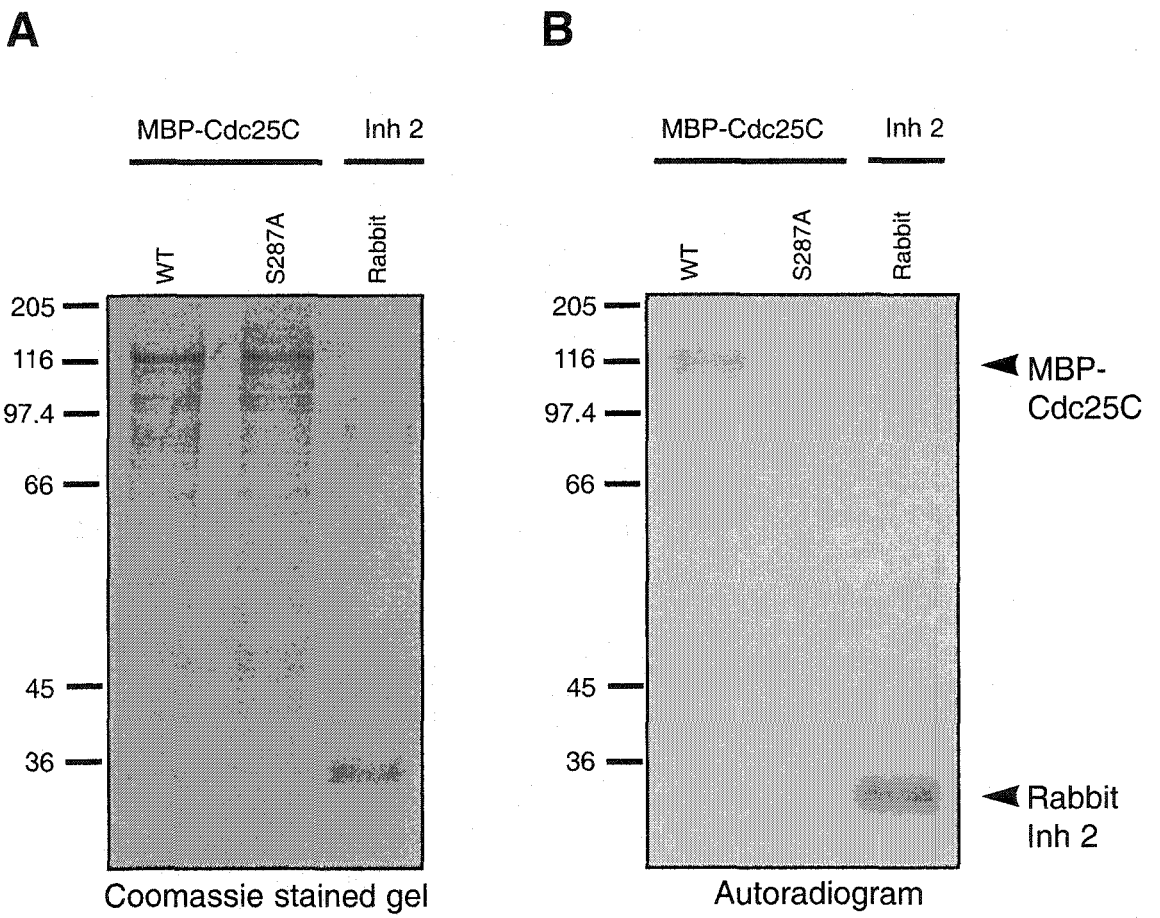
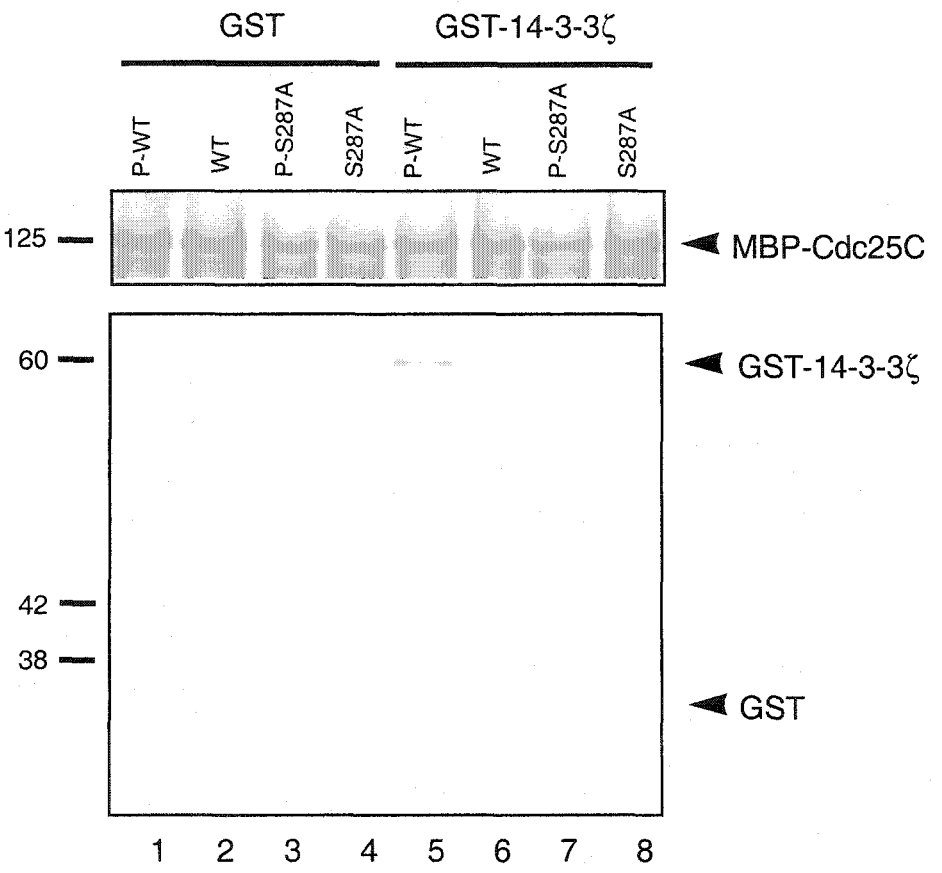


Figure 3.7 pp90^{Rsk} Immunoprecipitated from a MEK(QP)-G2 Arrested Egg Extract Phosphorylates Ser287 of Cdc25C Which Mediates the Binding of 14-3-3ζ

pp90^{Rsk} was immunoprecipitated from a cycling egg extract arrested in G2 by MEK(QP). Soluble, pp90^{Rsk} phosphorylated MBP-Cdc25C(WT) (lanes 1 and 5) and MBP-Cdc25C(S287A) (lanes 3 and 7) or nonphosphorylated MBP-Cdc25C(WT) (lanes 2 and 6) and MBP-Cdc25C(S287A) (lanes 4 and 8) proteins were incubated with GST or GST-14-3-3ζ. The protein complex was bound to amylose resin. The beads were then washed and bound proteins were eluted with 2X sample buffer and the samples were analyzed by immunoblotting with anti-MBP (upper) or anti-GST (lower) antibodies.

Figure 3.7 pp90^{Rsk} Immunoprecipitated from a MEK(QP)-G2 Arrested Egg Extract Phosphorylates S287 of Cdc25C Which Mediates the Binding of 14-3-3ζ



After the binding reactions, the MBP-Cdc25C proteins were retrieved from the reactions using amylose resin and the samples were analyzed by immunoblotting.

Using anti-Cdc25C antibodies, immunoblots showed that the amount of MBP-Cdc25C retrieved from the binding reactions by the amylose resin was approximately equal for both the phosphorylated and nonphosphorylated WT and S287A (Fig. 3.7). Using anti-GST antibodies, immunoblots showed the substantial presence of GST-14-3-3ζ only with the phosphorylated MBP-Cdc25C(WT). The anti-GST immunoblot showed negligible background binding of GST-14-3-3ζ to the nonphosphorylated MBP-Cdc25C(WT and S287A) and the phosphorylated MBP-Cdc25C(S287A) and no binding of the GST tag to any MBP-Cdc25C. These results indicated that 14-3-3ζ proteins bind with Cdc25C only when S287 of Cdc25C is phosphorylated by $pp90^{Rsk}$. They also suggested that one of the mechanisms that contributes to the MEK(QP)-induced G2 arrest of the cell cycle in egg extracts is the maintenance of 14-3-3 protein binding to Cdc25C by the sustained activation of the p42MAPK pathway.

3.2 Activation of the p42MAPK Pathway During M-Phase Sustains M-phase Inducing Activities (NEBD/CC) Without the Presence of MPF

3.2.1 Inhibition of Cdk1 by Addition of Roscovitine to an M-phase Arrested Egg Extract Does Not Lead to the Dephosphorylation of Cdc25C

In vivo and in egg extracts, the activation of p42MAPK has previously been shown to lead to an arrest of the early embryonic cell cycle in either G2 or M-phase (Abrieu *et al.*, 1997b; Walter *et al.*, 1997; Bitangcol *et al.*, 1998; Murakami and Vande Woude, 1998). Chau and Shibuya (1998, 1999) have shown that p42MAPK activation on entry into M-phase can induce an M-phase arrest with stabilized Cyclin B and high levels of MPF activity. If p42MAPK is activated after the peak of MPF, Cyclin B is degraded and MPF is inactivated, but unexpectedly, the cell cycle does not exit from M-phase (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999; Chau and Shibuya, 1999).

To determine if an M-phase extract could sustain M-phase activities when MPF was inhibited, a dose-response experiment using the Cdk1 inhibitor roscovitine was performed. Roscovitine was added to an M-phase arrested egg extract to concentrations of 0 to 250 μM and the reactions were assayed by immunoblotting and histone H1 phosphorylation assay. Cdc25C remained hyperphosphorylated at all concentrations of roscovitine tested (Fig. 3.8 A), and there appears to be some residual histone H1 activity even at the highest roscovitine concentration (Fig. 3.8 B,C). This suggested that either Cdk1 is not being completely inhibited by roscovitine, or there are other kinases activated in M-phase that are responsible for the histone H1 phosphorylation. Furthermore, the addition of up to 350 μM of roscovitine (Fig. 3.9 A) did not result in the dephosphorylation of Cdc25C. However, shortly after the addition of butyrolactone I (another Cdk1 inhibitor), both Cdc25C and p42MAPK became dephosphorylated (Fig. 3.9 A). These results show that roscovitine is able to inhibit Cdk1 activity (partial reduction of histone H1 phosphorylation) in a dose-dependent manner. However, since there are substantial levels of histone H1 activity remaining in the extract even after 350 μM of roscovitine is added, we determined if histone H1 phosphorylation was due to residual Cdk1 kinase activity uninhibited by roscovitine or the presence of other activities

Figure 3.8 Inhibition of Cdk1 by Addition of Roscovitine to an M-phase Arrested Egg Extract Does Not Lead to the Dephosphorylation of Cdc25C

A *Xenopus* M-phase arrested unfertilized egg extract was prepared (see Materials and Methods section 2.1.4). Increasing concentrations of the Cdk1 inhibitor roscovitine was added to 1.3% (v/v) of the final reaction. In addition, demembranated sperm nuclei were added to a final concentration of 400 sperm/μl reaction. At 15 min intervals, samples were taken for immunoblotting, histone H1 kinase assay, and cytology. Samples were analyzed by SDS-PAGE followed by immunoblotting.

A) Roscovitine was added to an M-phase extract to concentrations of 0 to 250 μM. The upper panel shows the anti-Cdc25C immunoblot (p-Cdc25C indicates the hyperphosphorylated form of Cdc25C), and the lower panel shows an anti-MAPK immunoblot (phosphorylated and activated p42MAPK is denoted by pp42MAPK). The bold line below the anti-MAPK immunoblot indicate samples in which M-phase nuclear morphology (NEBD, CC) was observed.

B) Samples were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and the gel was processed for autoradiography. The autoradiogram was from a 16.5 hrs exposure.

C) Phosphorimager quantitation of [^{32}P]-incorporation into histone H1 from gel in B, (expressed in arbitrary units).

Figure 3.8 Inhibition of Cdk1 by Addition of Roscovitine to an M-phase-Arrested Egg Extract Does Not Lead to the Dephosphorylation Cdc25C

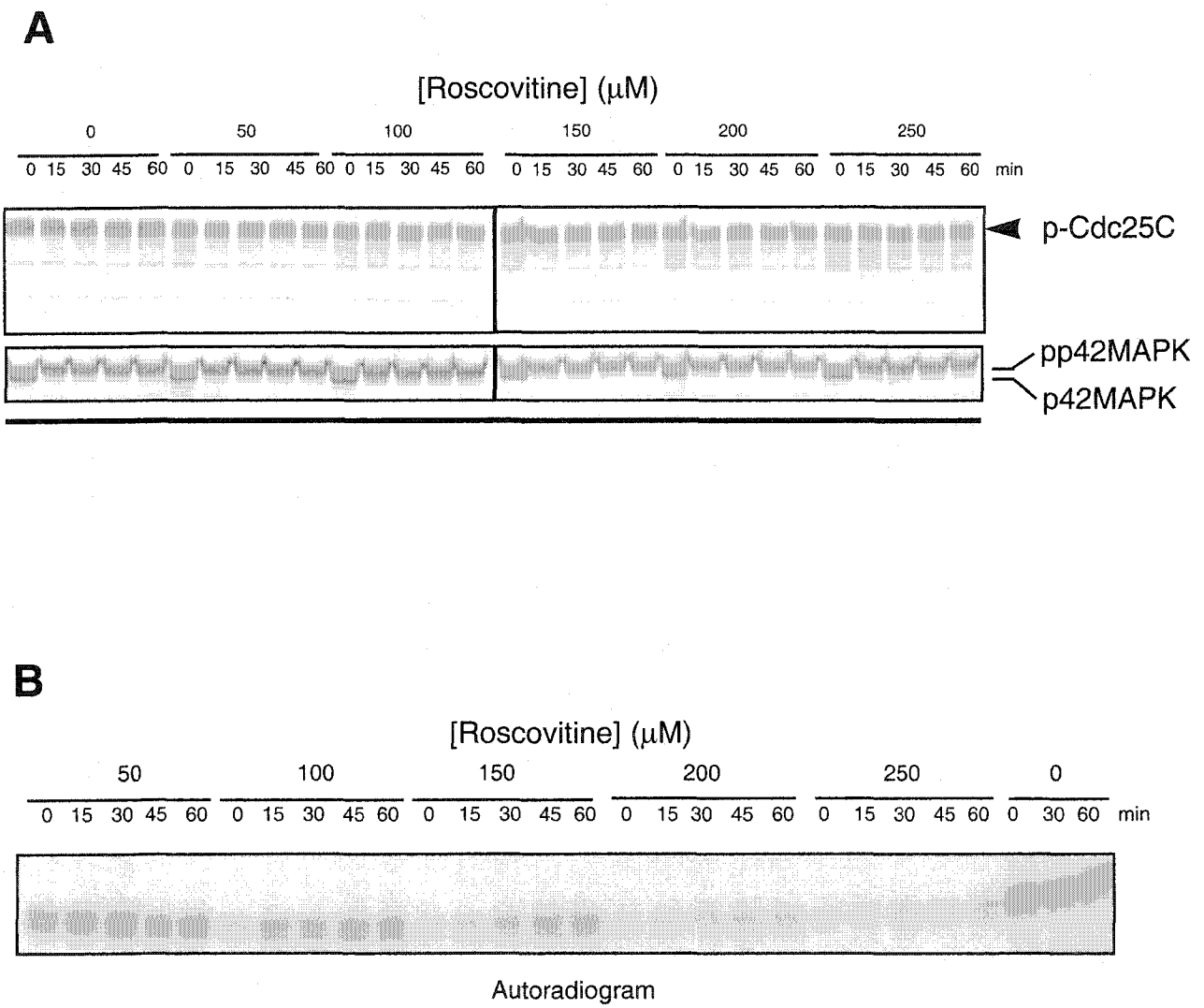


Figure 3.8

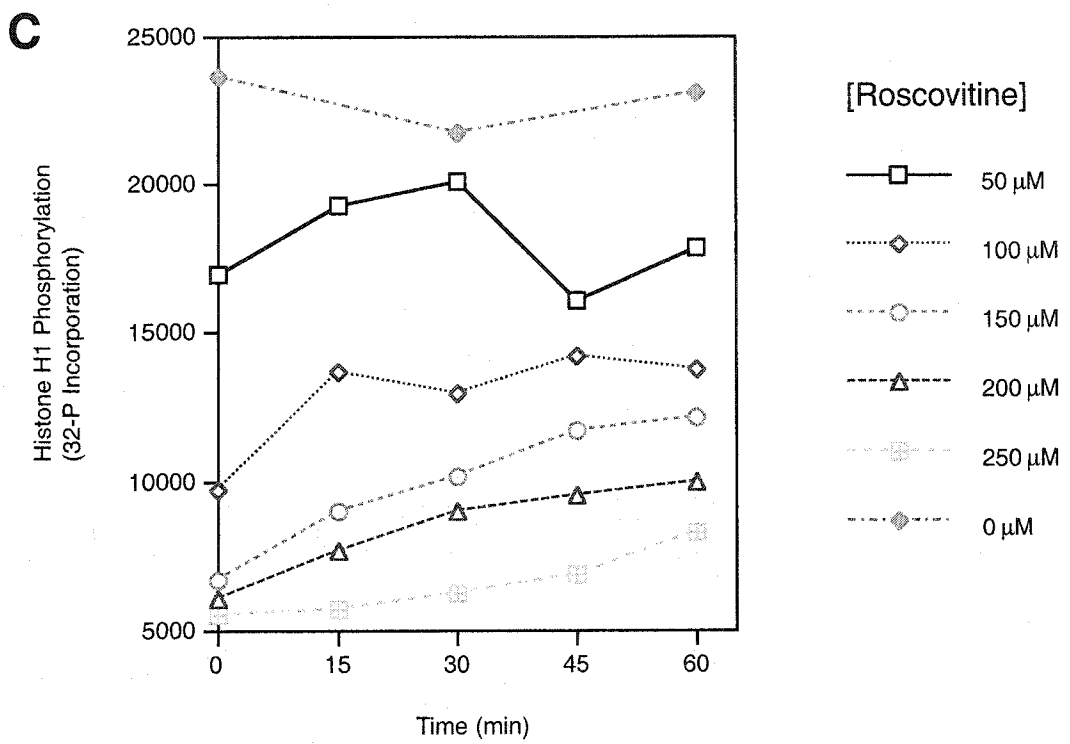


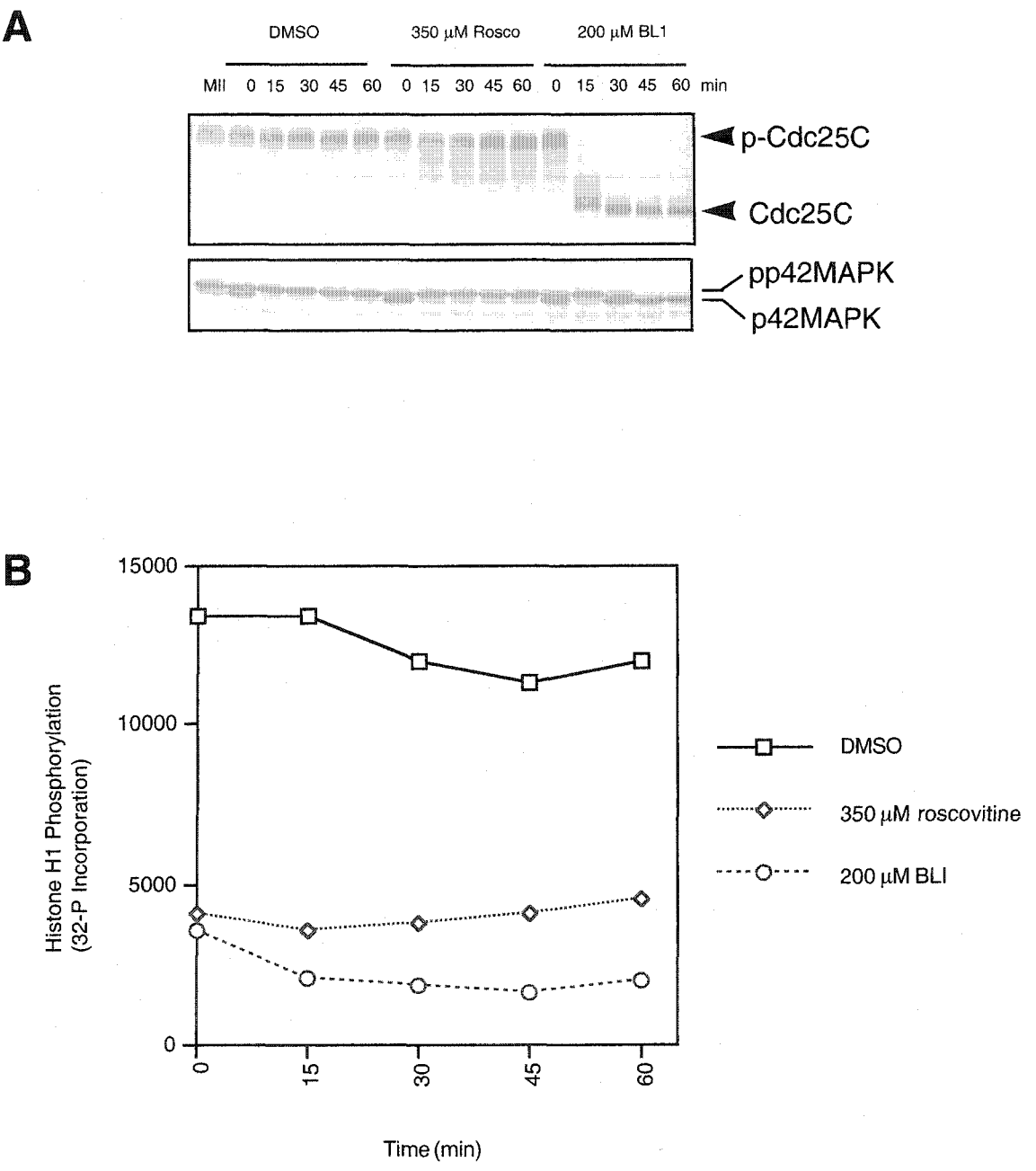
Figure 3.9 350 μM of Roscovitine Sustains Hyperphosphorylated Cdc25 and Phosphorylated p42MAPK

A *Xenopus* M-phase arrested unfertilized egg extract was prepared (see Materials and Methods). DMSO, 350 μM of roscovitine (rosco) or 200 μM butyrolactone I (BLI) was added to 1% (v/v) of the final reaction. In addition, demembranated sperm nuclei were added to a final concentration of 300 sperm/μl reaction. At 15 min intervals, samples were taken for immunoblotting and histone H1 kinase assay. Samples were analyzed by SDS-PAGE followed by immunoblotting.

A) 350 μM roscovitine or 200 μM BLI was added to an M-phase extract.
The upper panel shows the anti-Cdc25C immunoblot (p-Cdc25C indicates the hyperphosphorylated form of Cdc25C), and the lower panel shows an anti-MAPK immunoblot (activated p42MAPK is denoted by pp42MAPK).

B) Samples were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and the gel was processed for phosphorimager quantitation of [^{32}P]-incorporation into histone H1 (expressed in arbitrary units).

Figure 3.9 350 μM of Roscovitine Sustains Hyperphosphorylated Cdc25C and Phosphorylated p42MAPK



leading to the phosphorylation of histone H1.

3.2.2 The Addition of Cyclin BΔ90 to an Interphase Egg Extract Induces M-phase Without the Activation of p42MAPK

To determine if the p42MAPK pathway could sustain M-phase-inducing activities (NEBD/CC) we employed an alternative strategy by using a system in which *Xenopus* interphase arrested egg extracts were driven into M-phase by the addition of a nondegradable, truncated Cyclin B protein that was able to bind to and activate Cdk1. Interphase arrested egg extracts containing cycloheximide were used because they were devoid of endogenous mitotic cyclins, and could not synthesize any more. By using recombinant MBP-Cyclin BΔ90 (a mutant, non-degradable form of Cyclin B, with the N-terminal 90 amino acids removed, Murray *et al.*, 1989), an interphase arrested egg extracts could be driven into M-phase and arrested, with or without activation of the p42MAPK pathway. Then, the MBP-Cyclin BΔ90/Cdk1 complexes could be physically removed from the extract to determine the ability of p42MAPK in maintaining M-phase-inducing activities.

Soluble MBP-Cyclin BΔ90 was added to a final concentration of 50 nM to an interphase arrested egg extract containing decondensed sperm nuclei (see section 2.1.5), and samples were taken at 10 min intervals during incubation at 21 to 23°C. 20 min after the addition of MBP-Cyclin BΔ90, Cdc25C became hyperphosphorylated, but only the unphosphorylated form of p42MAPK was detected (Fig. 3.10 A). By 30 min of incubation, samples examined by fluorescence/phase-contrast microscopy showed NEBD and chromosome condensation of the sperm nuclei (Fig. 3.10 C). There was no nuclear envelope present and the chromosomes appeared to be condensed (Fig. 3.10 C). The results of the autoradiogram showed increasing histone H1 phosphorylation after MBP-Cyclin BΔ90 addition (Fig. 3.10 B). These results show that 50 nM of MBP-Cyclin BΔ90 is able to induce M-phase by 20 to 30 min, without the activation of p42MAPK. Addition of MBP-Cyclin BΔ90 alone is able to drive an egg extract into M-phase resulting in hyperphosphorylated Cdc25C. Under these experimental conditions, p42MAPK was not activated (unphosphorylated), as has been shown previously (Bitangcol *et al.*, 1998).

Figure 3.10 The Addition of Cyclin BΔ90 to an Interphase Egg Extract Induces M-phase Without the Activation of p42MAPK

50 nM of soluble recombinant MBP-Cyclin BΔ90 was added to an interphase arrested egg extract prepared using gluconate extraction buffer containing 100 μg/ml cycloheximide (see Materials and Methods section 2.5.1) and containing decondensed sperm nuclei. At 10 min intervals, samples were taken for immunoblotting, histone H1 kinase assay, and cytology. Samples were analyzed by SDS-PAGE followed by immunoblotting.

A) The top panel shows the anti-Cdc25C immunoblot (p-Cdc25C indicates the hyperphosphorylated form of Cdc25C), the middle panel shows an anti-MAPK immunoblot, the bottom panel shows the anti-MBP immunoblot. The bold line below the anti-MBP immunoblot indicate samples in which M-phase nuclear morphology (NEBD, CC) was observed.

B) Samples were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and the gel was processed for autoradiography. The autoradiogram was from an 11 hrs exposure.

C) The cytology samples were observed by fluorescence and phase/contrast microscopy. The upper row of images depicts sperm nuclei stained with the DNA-binding dye Hoechst, and the bottom row of images are the corresponding phase/contrast images.

Figure 3.10 The Addition of Cyclin BΔ90 to an Interphase Egg Extract Induces M-phase Without the Activation of p42MAPK

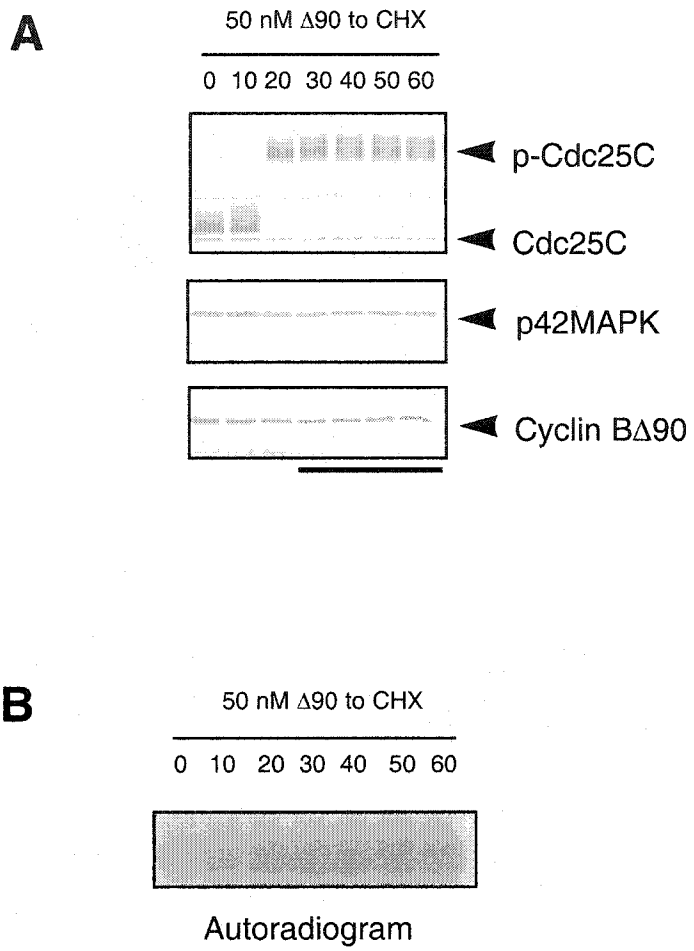
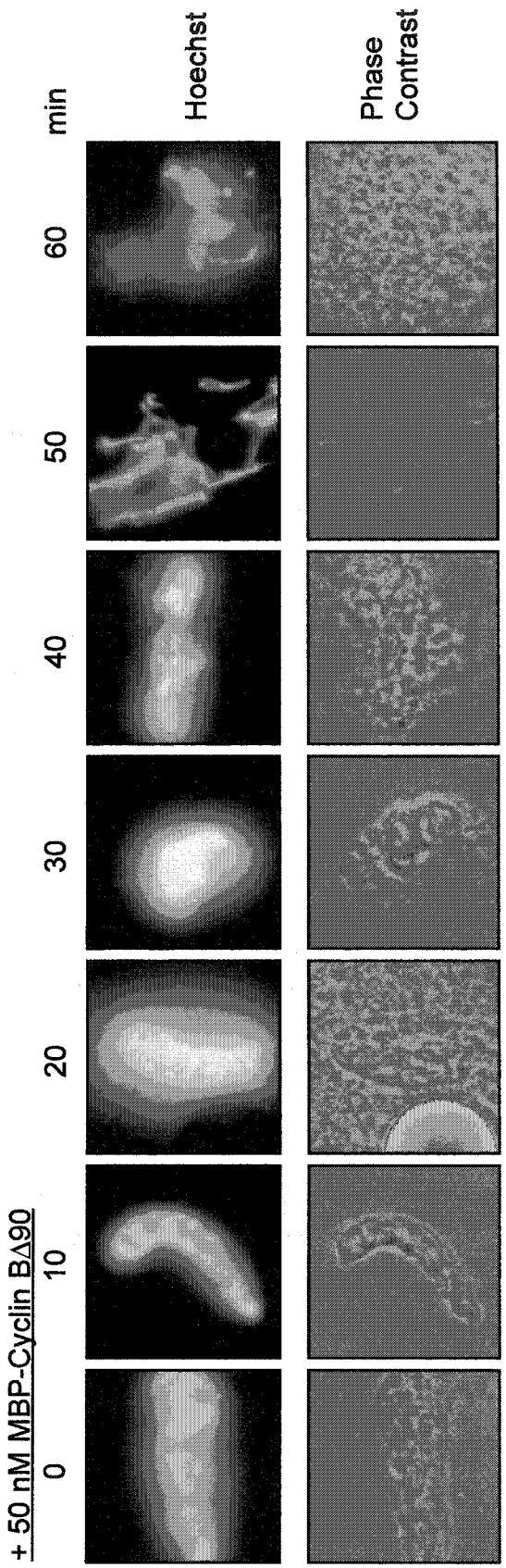


Figure 3.10



3.2.3 Immunodepletion of MBP-Cyclin BΔ90 from Egg Extracts

From the above results, it was shown that the addition of MBP-Cyclin BΔ90 to an interphase arrested egg extract was able bind to and activate Cdk1, driving the extract into M-phase and inducing interphase nuclei to undergo NEBD and CC. It is well documented that the activation of MPF is required for entry into M-phase of the cell cycle, but there is evidence that once M-phase has been achieved, MPF may not be necessary to sustain M-phase conditions (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999; Chau and Shibuya, 1999). When Mos(WT) was added to cycling *Xenopus* egg extracts leading to the activation of p42MAPK too late to prevent Cyclin B degradation, these extracts still arrested in M-phase with condensed chromosomes, microtubules and sustained M-phase-specific phosphorylations (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998). We hypothesized that once the egg extracts were driven into M-phase, p42MAPK activation could not only maintain M-phase conditions in the absence of Cyclin B/Cdk1 (MPF activity), but could also stabilize M-phase inducing activities such as those that induced NEBD/CC. This would indicate that Cyclin B/Cdk1 is necessary for entry into M-phase and the appearance of M-phase inducing activities, but that p42MAPK could sustain these activities after MPF had been activated, and even after MPF was inactivated.

In order to examine the role of p42MAPK activation in M-phase in the absence of Cyclin B/Cdk1, a protocol was established in which MBP-Cyclin BΔ90 could be removed from the egg extract by immunodepletion after it had induced an M-phase arrest. An interphase arrested egg extract was prepared with gluconate extraction buffer (see section 2.1.5) and soluble MBP-Cyclin BΔ90 was added to 50 nM to activate Cdk1 and drive the extract into M-phase. 60 min after protein addition, the extract was immunodepleted of MBP-Cyclin BΔ90 using anti-MBP bound to Affi-prep protein A beads. The extract was subjected to either three 10 min sets of immunodepletions (Fig. 3.11 A) or two 30 min sets of immunodepletions (Fig. 3.11 B). In these extracts, Cdc25C became hyperphosphorylated 30 min after MBP-Cyclin BΔ90 protein addition. Even after centrifugation and removal of the MBP-Cyclin BΔ90 from the extract, Cdc25C remained hyperphosphorylated in the supernatant. Also, p42MAPK became phosphorylated and activated since there was no room temperature incubation prior to the

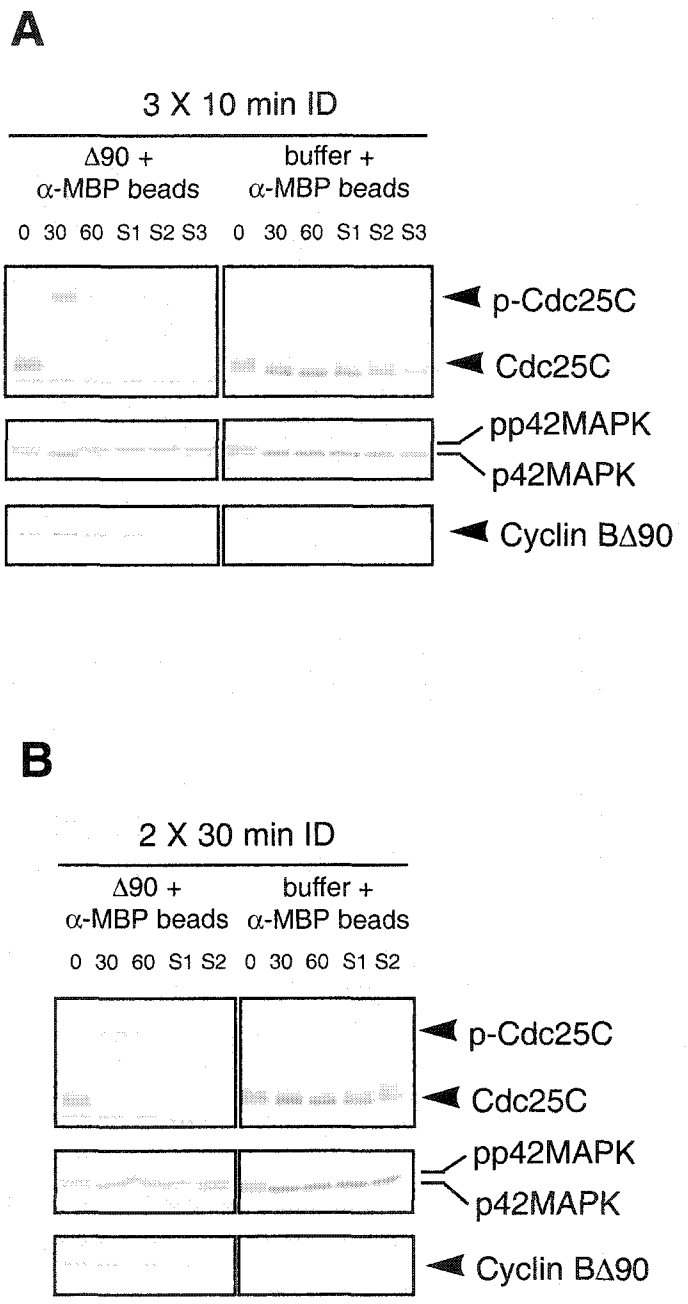
Figure 3.11 Immunodepletion of MBP-Cyclin BΔ90 from Egg Extracts

An interphase arrested egg extract was prepared using gluconate extraction buffer containing 100 μg/ml cycloheximide (see Materials and Methods section 2.1.5). Soluble MBP-CyclinΔ90 protein (50 nM) or buffer was added to the egg extract and allowed to incubate at 23°C for 60 min. Samples were taken from the reaction at 0, 30, and 60 min to verify activation of Cdk1 before immunodepletion. MBP-Cyclin Δ90 was immunodepleted from the egg extract using Affi-prep protein A-anti-MBP beads (see Materials and Methods section 2.7.3). The supernatants after each immunodepletion were analyzed by SDS-PAGE followed by immunoblotting. The top panel shows the anti-Cdc25C immunoblot, the middle panel shows an anti-MAPK immunoblot, and the bottom panel shows the anti-MBP immunoblot.

A) The results from 3 sets of 10 min immunodepletions. S1, S2 and S3 indicate the supernatants from the first, second, and third immunodepletions, respectively.

B) The results from 2 sets of 30 min immunodepletions. S1, S2 indicate the supernatants from the first and second immunodepletions, respectively.

Figure 3.11 Immunodepletion of MBP-Cyclin BΔ90 from Egg Extracts



activation of Cdk1 kinase (Fig. 3.11 A,B). From the anti-MBP immunoblot, it was clear that after the third round of 10 min immunodepletion (Fig. 3.11 A) and the second round of 30 min immunodepletion (Fig. 3.11 B), MBP-Cyclin BΔ90 was not detected. Since three 10 min sets of immunodepletions took less time than two sets of immunodepletions, the former was more appropriate to be used as a protocol for the cell-free assays.

These results established an appropriate protocol for immunodepletion of MBP-Cyclin BΔ90 from an egg extract in M-phase. By immunodepleting MBP-Cyclin BΔ90/Cdk1 from an egg extract after it had been driven from interphase to M-phase, the activities of an M-phase extract devoid of Cyclin B/Cdk1 could be tested.

3.2.4 p42MAPK is Able to Sustain Nuclear Envelope Breakdown and Chromosome Condensation (NEBD/CC) Inducing Activities in an M-phase Arrested Egg Extract After Removal of MPF

After establishing an immunodepletion protocol that enabled a way to study the activities of an M-phase extract without Cyclin B/Cdk1 activity, we wanted to determine whether or not p42MAPK could sustain M-phase inducing activities after an egg extract had entered M-phase without the presence of MPF activity. To test this notion, we devised an assay that would allow us to determine if an egg extract had the ability to induce NEBD and CC in interphase nuclei.

An interphase arrested egg extract was driven into M-phase by the addition of MBP-Cyclin BΔ90 (as in Fig. 3.10). After the addition of either buffer or MEK(QP), the extracts were either mock depleted or immunodepleted of MBP-Cyclin BΔ90/Cdk1. These "donor extracts" were then added to "recipient extracts" containing decondensed sperm nuclei to assay for the presence of NEBD/CC activity in the "donor extracts".

3.2.4.1 Donor Reaction Preparation

First, two replicate donor reactions containing supplemented interphase arrested egg extracts (without added sperm nuclei) were incubated for 30 min at room temperature to ensure that endogenous p42MAPK was not subsequently activated (Fig. 3.12 A,B; schematic Fig 2.2 A,B). Then, soluble MBP-Cyclin BΔ90 was added to each reaction to a concentration of 50 nM. By 30 min after MBP-Cyclin BΔ90 addition, Cdc25C had

Figure 3.12 p42MAPK is Able to Sustain M-phase Inducing Activities (NEBD/CC) in An M-phase Arrested Egg Extract After the Removal of MPF

An interphase arrested egg extract was prepared using cycling egg extract extraction buffer containing 100 μg/ml cycloheximide (see Materials and Methods section 2.1.5). Soluble MBP-Cyclin BΔ90 protein (50 nM) was added to 2 replicate cell-free reactions (without added sperm nuclei) and allowed to incubate at 21 to 23°C for 45 min (A and B; see schematic figure 2.2). Next, buffer (A) was added to one reaction and MEK(QP) (B) was added to the other reaction, and the reactions were incubated for an additional 30 min.

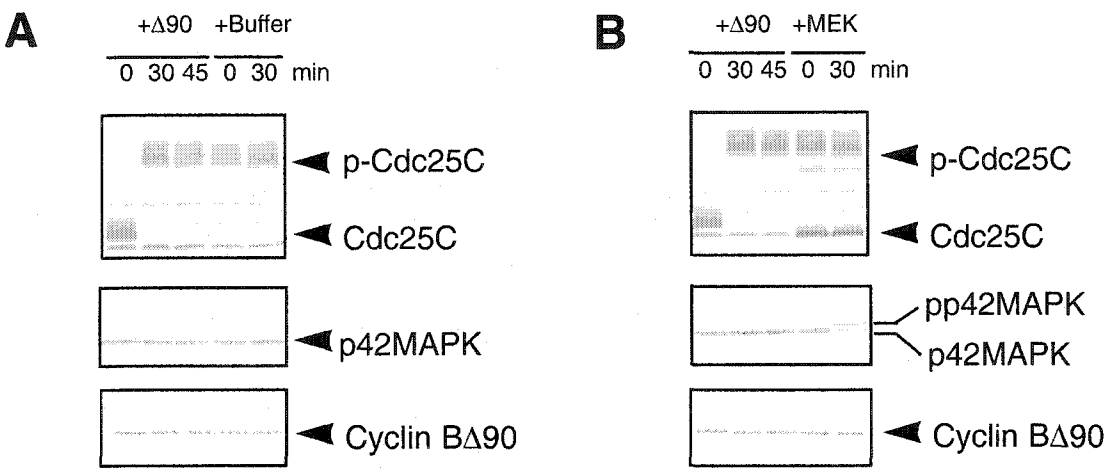
A) Addition of MBP-Cyclin BΔ90 protein to the interphase arrested egg extract (+Δ90) followed by the addition of buffer (+Buffer).

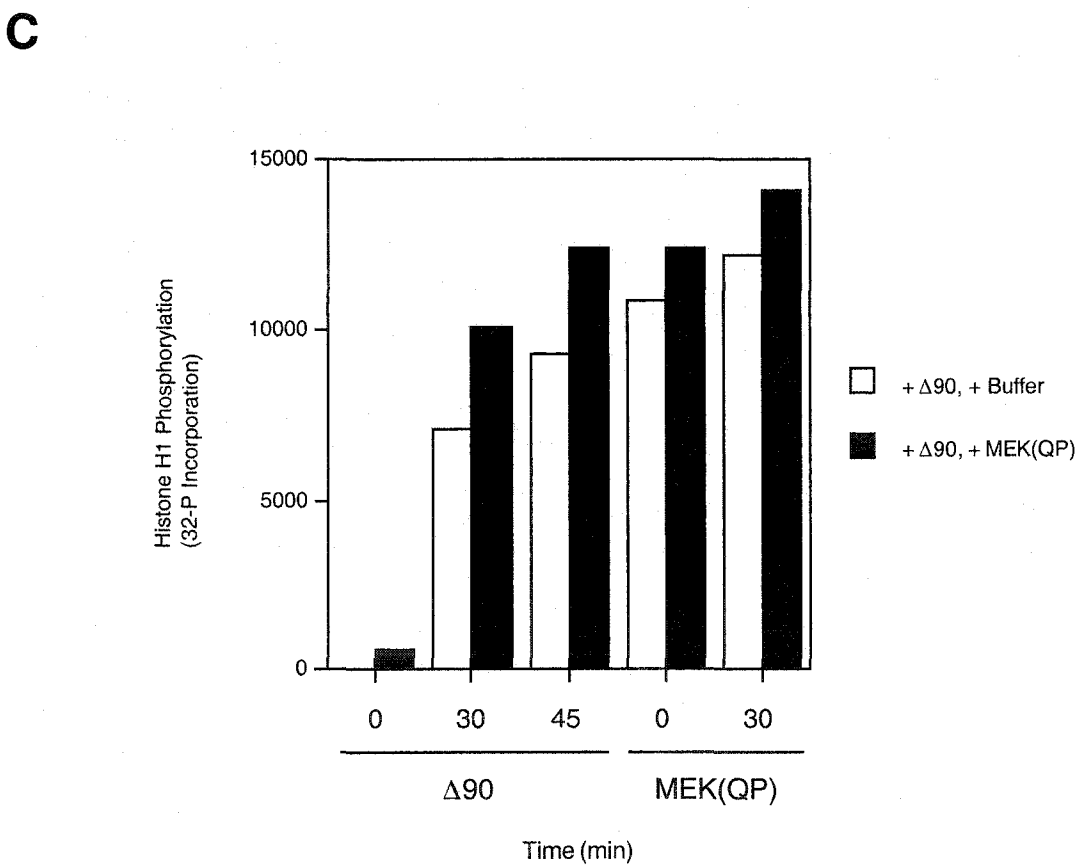
B) Addition of MBP-Cyclin BΔ90 protein to the interphase arrested egg extract (+Δ90) followed by the addition of MEK(QP) (+MEK).

C) Samples corresponding to the above reactions in (A) and (B) were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and followed by quantitation of [^{32}P]-incorporation into histone H1 (expressed in arbitrary units normalized to interphase egg extract values).

(A) and (B) were each divided into two equal volume reactions. One of the aliquotted reactions of A and B was subjected to 3 x 10 min rounds of immunodepletion using Affi-prep protein A-anti-rabbit IgG beads (control), and the other was immunodepleted using Affi-prep protein A-anti-MBP beads (to remove MBP-Cyclin BΔ90) (see Materials and Methods section 2.7.4 and figure 2.2). The depleted supernatant (donor extract) was then assayed by addition to recipient interphase arrested egg extracts which contained decondensed sperm nuclei . 4 volumes of donor extract

Figure 3.12 p42 MAPK is Able to Sustain M Phase-Inducing Activities (NEBD/CC) in M-Phase Arrested Egg Extract after the Removal of MPF





were mixed with 1 volume of recipient interphase arrested egg extract containing decondensed sperm nuclei (500 sperm nuclei per μl), and the mixed reactions were incubated at 21 to 23°C. Samples of the mixed reactions were taken at 45 min intervals for 90 min for immunoblot and histone H1 kinase activity. The cytology samples were taken at 15 min intervals for 90 min. The bold lines below the anti-MBP immunoblots indicate samples in which M-phase nuclear morphology (NEBD, CC) was observed.

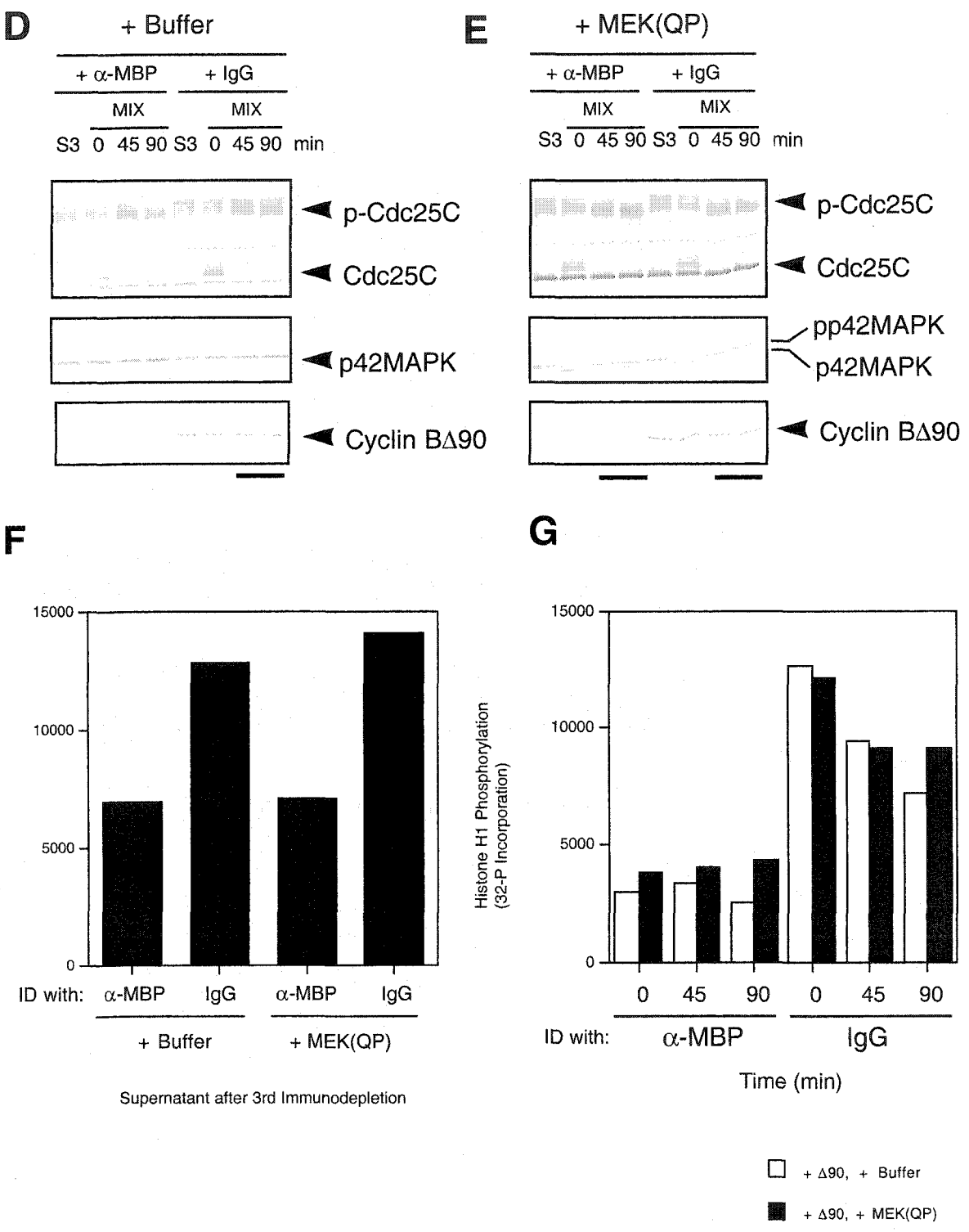
D) Results of mixing donor extract (S3) from reactions (A) (+ Buffer) that had been immunodepleted using Affi-prep-protein A-anti-rabbit IgG beads (IgG) or Affi-prep-protein A-anti-MBP-beads (α-MBP) with recipient egg extract containing decondensed sperm nuclei. S3 represents the supernatant of the donor extract after the third immunodepletion. MIX refers to the mixture of donor extract supernatant (4 vol.) with recipient extract (1 vol.).

E) Results of mixing donor extract (S3) from reactions (B) (+ MEK) that had been immunodepleted using Affi-prep-protein A-anti-rabbit IgG beads (IgG) or Affi-prep-protein A-anti-MBP-beads (α-MBP) with recipient egg extract containing decondensed sperm nuclei.

F) Supernatant samples after the third imunodepletion (S3) for the above reactions in (D) and (E) were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and followed by quantitation of [^{32}P]-incorporation into histone H1 (expressed in arbitrary units normalized to interphase egg extract values).

G) MIX samples for the above reactions in (D) and (E) were assayed for Cdk1 kinase activity in a histone H1 phosphorylation assay using [γ-^{32}P]ATP and histone H1 as a substrate. The samples from the phosphorylation assay were analyzed by SDS-PAGE and followed by quantitation of [^{32}P]-incorporation into histone H1 (expressed in arbitrary units normalized to interphase egg extract values).

Figure 3.12



The cytology samples from the mixed reactions corresponding to the data in Fig. C and D were observed by fluorescence and phase/contrast microscopy. In each figure, the upper row of images depicts sperm nuclei stained with the DNA-binding dye Hoechst, and the bottom row of images are the corresponding phase/contrast images.

Donor extract:

H) Extract from (A) immunodepleted using Affi-prep-protein A-anti-rabbit IgG beads (IgG) (mock immunodepletion).

I) Extract from (A) immunodepleted using Affi-prep protein A-anti-MBP beads (Δ90 immunodepletion).

J) Extract from (B) immunodepleted using Affi-prep-protein A-anti-rabbit IgG beads (IgG) (mock immunodepletion).

K) Extract from (B) immunodepleted using Affi-prep-protein A-anti-MBP beads (Δ90 immunodepletion).

Recipient extract:

L) Interphase arrested egg extract was incubated at room temperature for 30 min followed by the addition of demembranated sperm nuclei to a final concentration of 500 sperm/μl. Samples were taken at 0, 30, and 50 min after sperm addition. This recipient egg extract was added to the donor extract after 60 min of incubation.

Figure 3.12

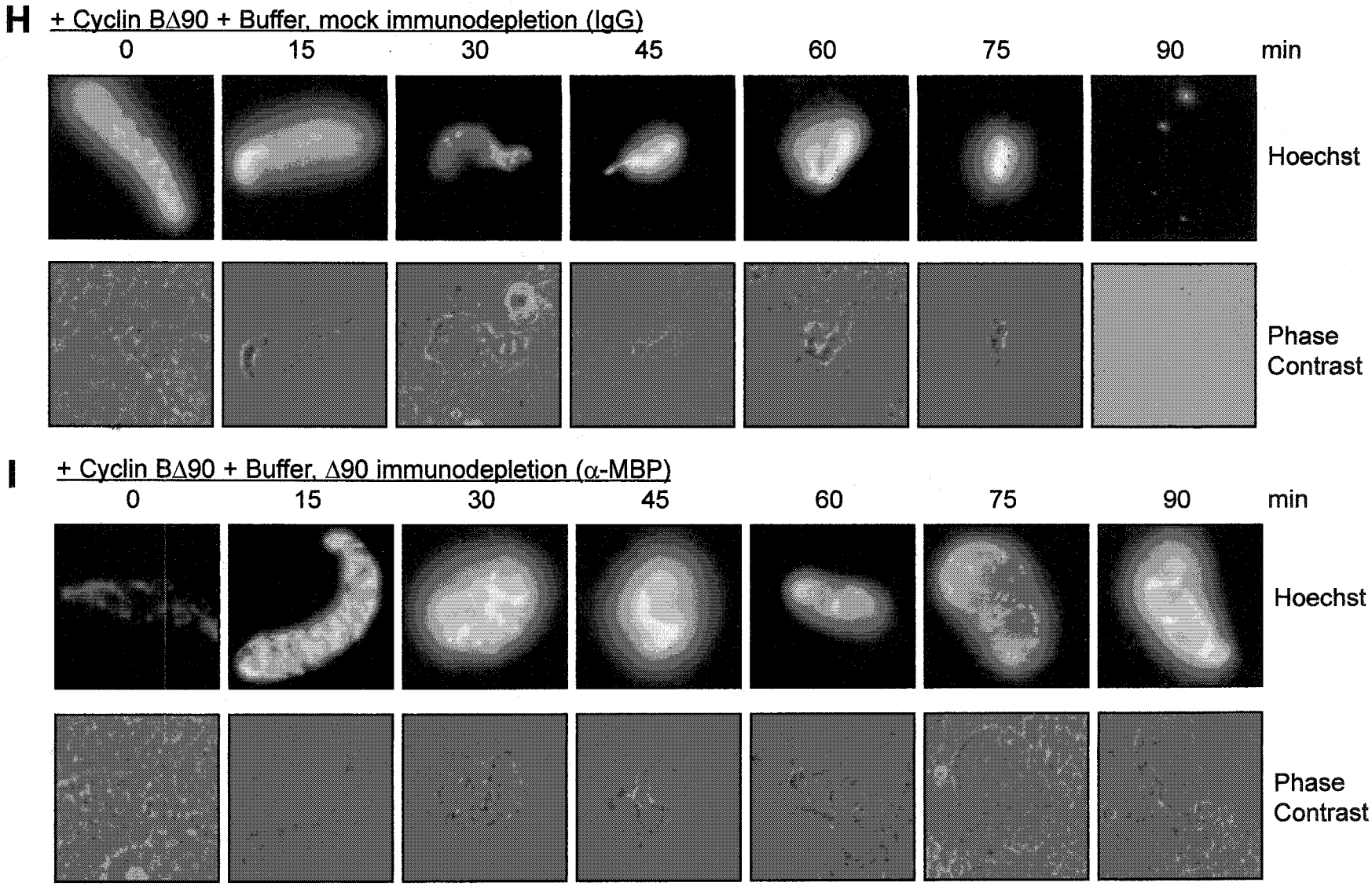


Figure 3.12

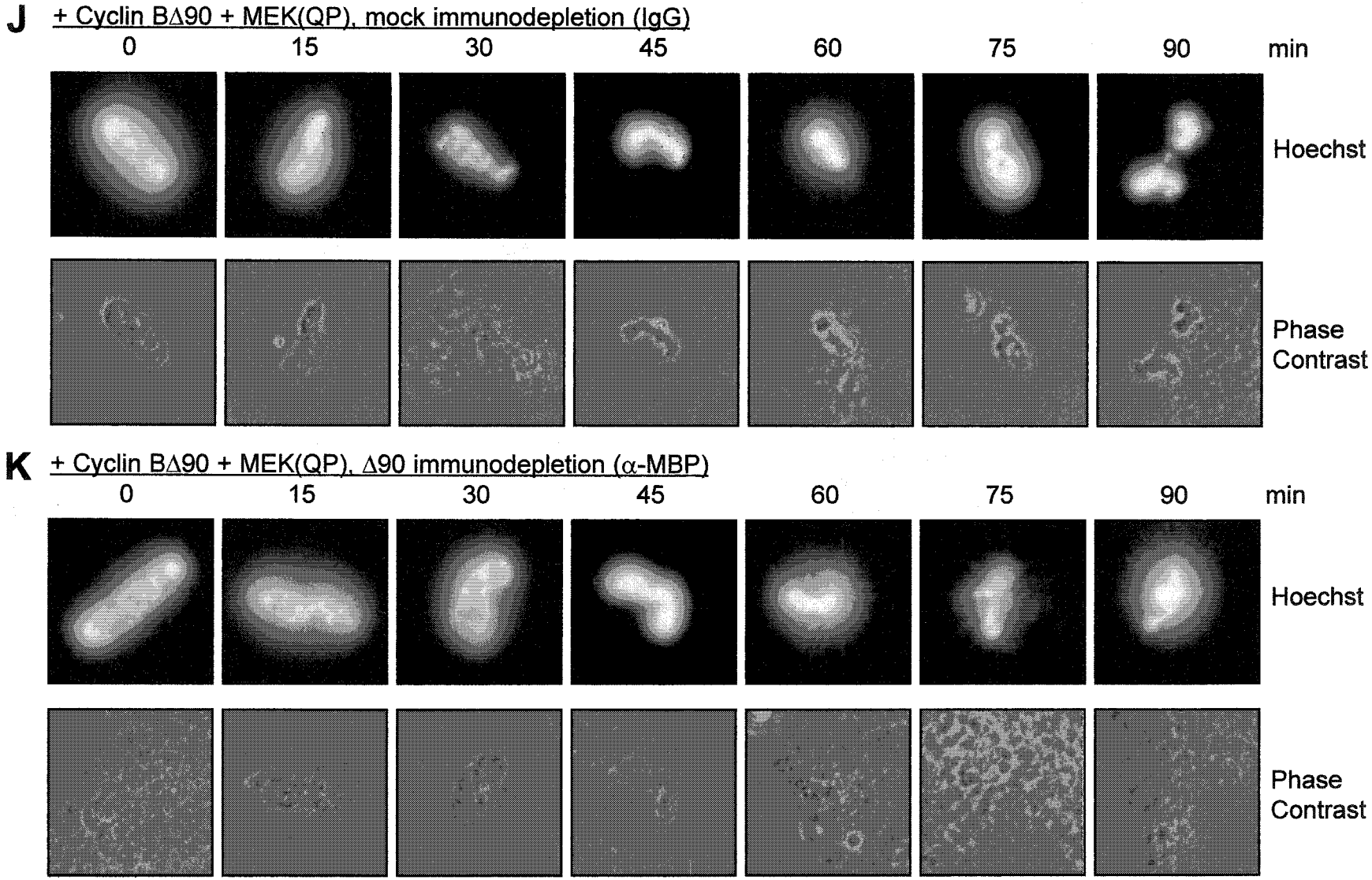
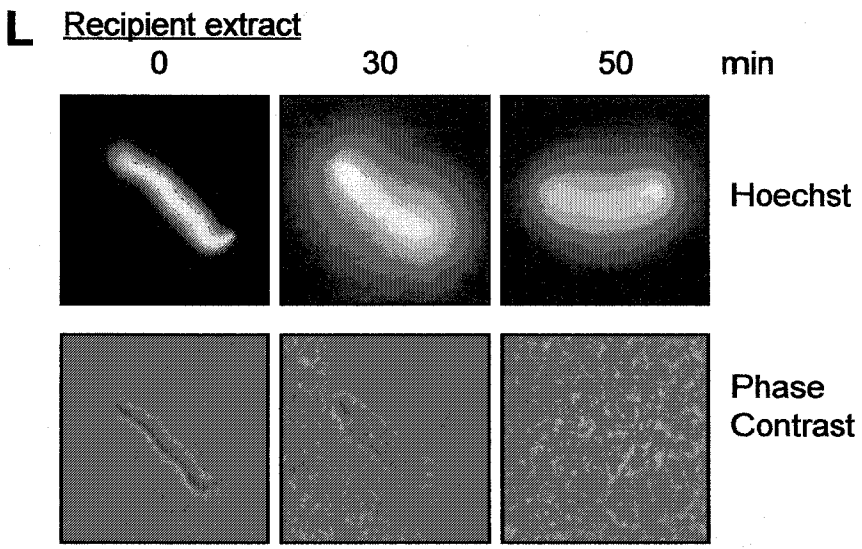


Figure 3.12



become hyperphosphorylated (Fig. 3.12 A,B). Also, p42MAPK remained inactive as shown by the presence of only the unphosphorylated, faster mobility form (Fig. 3.12 A,B). After 45 min of incubation, buffer was added to one reaction, and MEK(QP) was added to the other. 30 min later, p42MAPK was phosphorylated and activated in the reaction containing MEK(QP) (Fig. 3.12 B). Results from the histone H1 kinase assay showed a higher level of H1 phosphorylation for the reaction which received MEK(QP) even before the addition of the protein (Fig. 3.12 C). The reason for this difference is unknown since the two reactions were treated identically. However, after immunodepletion of Cyclin BΔ90/Cdk1 from the reactions with the inactive or activated p42MAPK, the histone H1 kinase activity levels of the supernatants from these donor reactions were very similar, as were the mixed donor/recipient reactions (Fig. 3.12 F,G).

3.2.4.2 Recipient Reaction Preparation

To assay the donor reactions for NEBD/CC inducing activity, recipient reactions were prepared by adding demembranated sperm nuclei to supplemented interphase arrested egg extract to a final concentration of 500 sperm/μl reaction. The recipient reactions were incubated at 21 to 23°C for 50 min, at which time nuclei with intact nuclear envelopes and decondensed chromatin had formed (Fig. 3.12 L).

3.2.4.3 Immunodepletion of MBP-Cyclin BΔ90/Cdk1 from Donor Reactions

30 min after buffer or MEK(QP) had been added to the donor reactions (Fig. 3.12 A,B), each donor reaction was divided in two: one aliquot was mock immunodepleted, and the other was immunodepleted of MBP-Cyclin BΔ90/Cdk1 using 3 rounds of immunodepletions. After the third round of immunodepletion, the donor reaction supernatants (S3) were collected and analyzed. The immunodepletions were considered successful since there was negligible MBP-Cyclin BΔ90 detected in the donor reactions immunodepleted with Affi-prep protein A-anti-MBP (Fig. 3.12 D,E). In comparison, MBP-Cyclin BΔ90 at a final concentration of 10 nM could be detected (unpublished data not shown). Also, Cdc25C in the donor reactions remained hyperphosphorylated, even after MBP-Cyclin BΔ90/Cdk1 had been removed. Moreover, p42MAPK remained

phosphorylated (the slower gel mobility form) after immunodepletion in both of the donor reactions containing MEK(QP) (Fig. 3.12 E).

The histone H1 phosphorylation assay showed high levels of histone H1 kinase activity in the donor reactions that had been mock immunodepleted with anti-rabbit IgG antibody and much lower levels for the donor reactions immunodepleted of MBP-Cyclin BΔ90/Cdk1 (Fig. 3.12 F,G). The levels of histone H1 kinase activity in the donor reactions immunodepleted of MBP-Cyclin BΔ90/Cdk1 were approximately half of peak levels (Fig. 3.12 F). Interestingly, even after the bulk of MBP-Cyclin BΔ90/Cdk1 was immunodepleted from the donor reactions, histone H1 kinase activity of the supernatants did not return to interphase level. Although the residual MBP-Cyclin BΔ90 detected in the immunodepleted donor reactions by immunoblot may account for some of the histone H1 kinase activity seen previously (Fig. 3.8 C, Fig. 3.9 B), it is possible that other histone H1 kinases that were activated on entry into M-phase remain in the supernatants after the immunodepletions.

Therefore, these results suggest that the histone H1 kinase activity of the immunodepleted donor reactions (S3) is due to residual Cdk1 kinase activity and/or other activated kinases that are able to phosphorylate histone H1 that remain in the supernatant.

3.2.4.4 NEBD and CC Inducing Activities That are Activated in M-phase are Sustained by p42MAPK in the Absence Cyclin B/Cdk1

The immunodepleted donor reactions (S3s) were added to the recipient reactions at a ratio of 4:1 (v/v) and mixed gently. After the S3s were added to the recipient reactions, the donor Cdc25C remained hyperphosphorylated in all four reactions (Fig. 3.12 D,E) regardless of whether MBP-Cyclin BΔ90/Cdk1 had been immunodepleted or not. Immediately after mixing the S3s with the recipient reactions, both the unphosphorylated (recipient) and hyperphosphorylated (donor) forms of Cdc25C were present (Fig. 3.12 D,E). However, after the mixture was allowed to incubate, only the hyperphosphorylated form of Cdc25C was present at 45 and 90 min (Fig. 3.12 D,E).

When p42MAPK was not activated in the donor reaction prior to the mock depletion with IgG, the nuclei in the mixed reaction underwent NEBD and CC (Fig. 3.12 H), indicating the presence of NEBD/CC inducing activity in the donor reaction. This result

is not surprising, since immunodepletion with IgG does not remove MBP-Cyclin BΔ90/Cdk1 from the donor reaction and levels of histone H1 kinase activity remain high (Fig. 3.12 F). In contrast, when the p42MAPK pathway was not activated in the donor reaction prior to the immunodepletion of MBP-Cyclin BΔ90/Cdk1, the nuclei in the mixed reaction did not undergo NEBD and CC, indicating the absence of NEBD/CC inducing activity (Fig. 3.12 I). The chromatin in these nuclei remained decondensed and the nuclear envelope remained intact (Fig. 3.12 I). Moreover, the level of histone H1 kinase activity in the donor reaction was substantially lower than in the mock depleted donor reaction, which is consistent with the negligible amount of MBP-Cyclin BΔ90 remaining after immunodepletion (Fig. 3.12 F).

Upon mixing the mock or MBP-Cyclin BΔ90 immunodepleted donor S3s containing MEK(QP) with recipient reactions, both phosphorylated and unphosphorylated p42MAPK were initially detected, and p42MAPK phosphorylation increased during the incubation (Fig. 3.12 E). Similar to the results in Figure 3.12 H, when p42MAPK was activated by MEK(QP) in the donor reaction prior to mock depletion with IgG, the nuclei in the mixed reactions underwent NEBD and CC by 45 min after the donor extract was mixed with the recipient extract (Fig. 3.12 J). Since the MBP-Cyclin BΔ90/Cdk1 had not been depleted from this reaction, levels of histone H1 kinase activity were high in the donor reaction (Fig. 3.12 F). Surprisingly, however, when p42MAPK was activated by MEK(QP) in the donor reaction prior to immunodepletion of MBP-Cyclin BΔ90/Cdk1, the nuclei in the mixed reaction underwent NEBD and CC (Fig. 3.12 K) despite that fact that levels of histone H1 kinase activity were low in both the donor and mixed reactions (Fig. 3.12 F,G). The levels of histone H1 kinase activity in the donor extracts immunodepleted of MBP-Cyclin BΔ90/Cdk1 were virtually identical, yet only the donor extract in which p42MAPK had been activated was able to induce NEBD and CC.

The results from this experiment indicate that the NEBD and CC-inducing activities that are activated in M-phase can be sustained in the absence Cyclin B/Cdk1 only if p42MAPK has been activated. Also, theses results suggest that the residual histone H1 phosphorylation may be due to other activities present in the extract after it has been driven into M-phase.

Chapter 4: Discussion

4.1 Activation of the p42MAPK Pathway During Interphase Leads to a G2 Arrest

In the first part of my study, I examined the molecular mechanism by which G2 arrest is induced by the sustained activation of the p42MAPK pathway in *Xenopus* cycling egg extracts. Previously, it was shown that during the MEK(QP)-induced G2 arrest, the tyrosine-phosphorylated, inactive form of Cdk1 accumulated, Cdc25C failed to become hyperphosphorylated, and the extract was inhibited from entering M-phase (Bitangcol *et al.*, 1998). These data suggested that the p42MAPK pathway could be inhibiting Cdc25C, preventing the dephosphorylation and activation of Cdk1 and entry into M-phase.

Here, we showed that the binding of 14-3-3 proteins to Cdc25C was sustained in egg extracts arrested in G2 by MEK(QP). Also, we showed that a mutant recombinant *Xenopus* Cdc25C protein (S287A), that is unable to bind 14-3-3 proteins, can accelerate the cell cycles in egg extracts, causing a premature entry into M-phase that was most prominent in the second cycle. More importantly, S287A was able to overcome the G2 arrest induced by MEK(QP) and cause entry into M-phase. The S287A mutant is more potent than WT in driving MEK(QP)-G2 arrested egg extracts into M-phase in extracts depleted of endogenous Cdc25C. Finally, we showed that $pp90^{Rsk}$ is able to phosphorylate S287 of Cdc25C *in vitro,* and that this phosphorylation leads to the binding of 14-3-3 proteins. We have provided a possible molecular mechanism for a G2 arrest that is caused by the activation of the p42MAPK pathway.

4.1.1 Cdc25C is Associated With 14-3-3 Proteins in Egg Extracts Arrested in G2 by Activation of the p42MAPK Pathway

From experiments that had been done previously by others, it had been found that the Cdc25C immunoprecipitated from the MEK(QP)-G2 arrested egg extracts was associated with 14-3-3 proteins, similar to the Cdc25C immunoprecipitated from interphase arrested egg extracts and G2 arrested oocyte extracts (Fig. 3.1). Compared with Cdc25C retrieved from oocyte extracts and interphase arrested egg extracts, there appeared to be less of the larger isoforms (likely the ε isoform with a predicted molecular

mass of 28 kDa) but similar amounts of the smaller isoforms (likely the ζ isoform with a predicted molecular mass of 27 kDa) present in the MEK(QP)-G2 arrested extract (Fig. 3.1). Using *Xenopus* egg extracts, Kumagai *et al.* (1998b) found that the ε and ζ isoforms of 14-3-3 were bound to S287-phosphorylated Cdc25C during interphase, and that the ζ isoforms of *Xenopus* 14-3-3 were about 28 kDa in mass. Therefore, the ζ isoform of 14-3-3 was used in our binding studies, since it was known bind to *Xenopus* Cdc25C (Kumagai *et al.*, 1998b) and it appeared that the ζ isoform is the 14-3-3 protein that is predominantly associated with the Cdc25C immunoprecipitated from a MEK(QP)-G2 arrested egg extract.

The association of 14-3-3 with Cdc25C has been demonstrated by previous groups (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998b; Duckworth *et al.*, 2002). In these studies, 14-3-3 binding to Cdc25C provided a mechanism that contributed towards a G2 cell cycle arrest induced by the DNA damage cell cycle checkpoint. Peng *et al.* (1997) demonstrated that Chk1, a serine/threonine kinase activated during this checkpoint, can directly phosphorylate human Cdc25C phosphatase on an important serine 216 residue (serine 287 is the homologous site in *Xenopus* Cdc25C). Phosphorylation on serine 216 of Cdc25C led to the binding of 14-3-3 proteins and mutating serine 216 to an alanine (S216A) abrogated the binding of 14-3-3 to Cdc25C (Peng *et al.*, 1997). The binding of 14-3-3 to Cdc25C was suggested to act as a checkpoint control and overexpression of the mutant S216A abrogated the G2 checkpoint arrest.

4.1.2 S287A Accelerates the Cell Cycle and Overcomes the G2 Arrest Induced by Activation of the p42MAPK Pathway in *Xenopus* Cycling Egg Extracts

Chau and Shibuya (1999) have previously shown that recombinant Cdc25C proteins (WT and S287A) were capable of overcoming G2 arrest in egg extracts that had been induced by the DNA damage checkpoint (Chau and Shibuya, 1999). On addition of either Cdc25C(WT or S287A) recombinant proteins to cycling egg extracts, we found that only the S287A was able to accelerate the cell cycle significantly. Since this acceleration appears to occur in cycling egg extracts even in the absence of nuclei, these results are consistent with previous work in *Xenopus* which showed that the association

of Cdc25C with 14-3-3 proteins has a function that is independent of the nucleus. (Peng *et al.*, 1997; Kumagai *et al.*, 1998b).

When Cdc25C proteins were added to egg extracts that had been arrested in G2 by constitutively-active MEK(QP), S287A overcame the arrest and drove the extract into M-phase (Fig. 3.2 B). Furthermore, in egg extracts that had been immunodepleted of Cdc25C, followed by MEK(QP) addition, S287A was consistently able to drive the cell cycle into M-phase much sooner than WT (30 to 40 min) (Fig. 3.3 A). The nuclear morphology in these extracts that had been driven into M-phase showed condensed chromosomes aligned on a mitotic spindle. These data, along with the fact that 14-3-3 proteins remained bound to Cdc25C in MEK(QP)-G2 arrested egg extracts, suggested that the phosphorylation of S287 is maintained in these extracts and that phosphorylation of this site is necessary for the maintenance of G2 cell cycle arrest.

4.1.3 G2 Cell Cycle Arrest Induced by Activation of the p42MAPK Pathway is Not Mediated by Chk1

Initially, the effect of p42MAPK on Chk1 as a pathway leading to the MEK(QP)-G2 arrest was tested previously by others in the lab. Chk1 protein kinase can phosphorylate Cdc25C (Peng *et al.*, 1997; Sanchez *et al.*, 1997) and the phosphorylation of Cdc25C appears to inhibit Cdc25C activity when 14-3-3 proteins bind (Peng *et al.*, 1997). Chk1 functions in the DNA damage checkpoint in egg extracts by phosphorylating Cdc25C on serine 287 which serves as a binding site for 14-3-3 proteins (Kumagai *et al.*, 1998a). However, in using antibodies to the yeast and human homologues of Chk1, no specific reactivity (no phosphorylated, activated Chk1 detected) in the MEK(QP)-G2 arrest was detected. Furthermore, the addition of a yeast Chk1 protein to MEK(QP)-G2 arrested extracts did not lead to a phosphorylation or mobility shift in polyacrylamide gels (unpublished results). Therefore, these data suggested that the MEK(QP)-G2 arrest was not induced by activating Chk1, a known kinase of Cdc25C.

4.1.4 G2 Cell Cycle Arrest Induced by Activation of the p42MAPK Pathway is Mediated by pp90^{Rsk} Phosphorylation of Cdc25C

From apoptotic studies, it has been shown that pp90^{Rsk} can act as an anti-apoptotic kinase that is able to phosphorylate the pro-apoptotic protein BAD on specific serine residues (Bonni *et al.*, 1999; Tan *et al.*, 1999; Shimamura *et al.*, 2000). BAD normally interferes with the anti-apoptotic function of Bcl-2 and Bcl-X_L in the mitochondria. However, the pro-apoptotic activity of BAD is inhibited when 14-3-3 proteins bind to BAD in the cytoplasm of cells (Zha *et al.*, 1996). 14-3-3 proteins have been shown to bind to BAD only when serine 112 or 136, residues that lie within a consensus sequence known for 14-3-3 protein association, are specifically phosphorylated. It has been shown that serine 112 of BAD is phosphorylated by pp90^{Rsk} (Bonni *et al.*, 1999; Fang *et al.*, 1999; Shimamura *et al.*, 2000) or by mitochondria anchored PKA (Harada *et al.*, 1999). In addition, serine 136 of BAD is phosphorylated by Akt which is associated with the translocation of BAD from the mitochondria to the cytoplasm (Pastorino *et al.*, 1999).

Our results show that pp90^{Rsk} phosphorylates S287 of Cdc25C contributing towards G2 arrest. pp90^{Rsk} is hyperphosphorylated and activated during the MEK(QP)-G2 arrest (Bitangcol *et al.* 1998, Fig. 3.5), and the critical S287 of Cdc25C that must be phosphorylated for the association of Cdc25C with 14-3-3 proteins lies within a consensus site for phosphorylation by pp90^{Rsk} (Fig. 3.4). After we obtained our data, Duckworth *et al.* (2002) demonstrated that PKA acts as an upstream kinase for S287 of Cdc25C, and interestingly, there is a similarity between the N-terminal kinase domain of pp90^{Rsk} and PKA (Fisher and Blenis, 1996). Walter *et al.* (2000) have provided evidence for one mechanism that contributes towards the inhibition of Cdk1, the phosphorylation of Wee1 by p42MAPK. Here, we suggest that the p42MAPK pathway has an additional role since the kinase pp90^{Rsk} can phosphorylate Cdc25C on S287 leading to binding of 14-3-3 proteins. The phosphorylation and inactivation of Cdc25C complements the sustaining of Wee1 activity by p42MAPK (Murakami and Vande Woude, 1998; Walter *et al.*, 2000), since they have opposing effects on Cdk1.

4.1.5 14-3-3 Proteins May be Maintaining Cdc25C in an Inactive State

It is still unclear whether or not the phosphorylation of Cdc25C has an effect on its activity, but there is evidence that 14-3-3 protein binding to Cdc25C may promote the export of Cdc25C (Lopez-Girona *et al.*, 1999) from the nucleus or prevent its entry into the nucleus (Yang *et al.*, 1999). Hyperphosphorylated Cdc25C is able to dephosphorylate and activate Cdk1 when it is present in the nucleus. It appears that the regulation of the G2/M transition may be controlled by specifically localizing the Cdc25C phosphatase to either the cytoplasm or the nucleus. In fission yeast, when 14-3-3 is prevented from binding to Cdc25C, Cdc25C is able to accumulate in the nucleus (Zeng and Piwnica-Worms, 1999). Several groups have provided evidence indicating that 14-3-3 plays a role in the nuclear exclusion of Cdc25C.

There are two models that have been proposed to explain how 14-3-3 binding regulates the nuclear exclusion of Cdc25C. In a study done by Lopez-Girona *et al.* (1999), the 14-3-3 protein Rad24 in fission yeast was reported to contain a nuclear export sequence (NES) and it was suggested that Rad24 acts as an attachable NES which regulates the nuclear export of Cdc25C. The authors proposed that a dimer of Rad24 was able to interact with both Cdc25C and the nuclear export receptor CRM1 which was thought to facilitate nuclear export. However, it is unlikely that the residues within Rad24 serve to regulate the nuclear export of Cdc25C by functioning as an attachable NES since some of the residues comprising the NES of Rad24 are directly involved in substrate binding and others participate in intramolecular interactions (Wang *et al.*, 1998; Rittinger *et al.*, 1999).

Another model suggested by several groups predicts that 14-3-3 inhibits the nuclear import of Cdc25C by interfering with a nuclear localization sequence (NLS) (Kumagai and Dunphy, 1999; Yang *et al.*, 1999). Mutation of *Xenopus* serine 287 and the equivalent human serine 216 to an alanine in Cdc25C was shown to completely abrogate 14-3-3 binding (Kumagai *et al.*, 1998b). The 14-3-3 dimer may bind to the RXXpSXP motif and mask the adjacent NLS thus prevent nuclear import of Cdc25C.

In a recent study in *Xenopus*, Hutchins *et al.* (2002) proposed an alternative hypothesis that is more consistent with our results. 14-3-3 binding to S287-phosphorylated Cdc25C may induce an interphase arrest by maintaining this inhibitory

phosphorylation of Cdc25C. Thus, rather than the maintenance of the cytoplasmic localization of Cdc25C being responsible for the MEK(QP)-G2 arrest that we observe, S287-phosphorylated Cdc25C may be inactive both in the cytoplasm and the nucleus thus resulting in a G2 arrest. These results are consistent with those of Oe *et al.* (2001) using *Xenopus* in which the phosphorylation of S287 of Cdc25C appears to negatively affect its activity as a phosphatase. Our results would be consistent with this latter notion, since the S287A protein overcomes the MEK(QP)-induced G2 arrest regardless of whether or not nuclei are added to the egg extract.

4.1.6 Possible Physiological Significance of the G2 Cell Cycle Arrest Induced by Activation of the p42MAPK Pathway in Egg Extracts

The physiological significance of the G2 cell cycle arrest obtained by activation of the p42MAPK signaling pathway remains unclear. However, the p42MAPK induced G2 arrest may be important in an as yet undiscovered cell cycle checkpoint (Bitangcol *et al.*, 1998). In addition, there is evidence that the G2 arrest that we have observed in egg extracts may be representative of a cell cycle delay/arrest potentially induced by extracellular signals such as fibroblast growth factor (FGF). This effect on the cell cycle may be important for differentiation in the later development of embryos or in the growth of mammalian cells.

The findings from this study suggest that the p42MAPK pathway may have a newly-identified role that normally occurs later in the development in *Xenopus*. The G2 arrest caused by the activation of p42MAPK that we have observed may be important later in the differentiation of tissues of the developing embryo. The notion of egg extracts being used to study cell cycle events that normally occur later in development of embryos has been well-established with studies of the spindle-assembly checkpoint (Minshull *et al.*, 1994; Takenaka *et al.*, 1997), and the DNA damage checkpoint (Kumagai *et al.*, 1998a; Chau and Shibuya, 1999; Guo *et al.*, 2000).

During the early embryonic cell cycles of *Xenopus laevis*, cycles 2-12 consist solely of S and M-phases and are 30 minutes in length, without any intervening G1 and G2 phases (Graham and Morgan, 1966; Miake-Lye *et al.*, 1983) and transcription does not occur (Newport and Kirschner, 1982; Miake-Lye *et al.*, 1983). During the mid-

blastula transition (MBT), which occurs at cell cycle 12, the cell cycles lengthen as gap phases are re-introduced (Newport and Kirschner, 1982). The G2 arrest that we have observed using MEK(QP) in egg extracts may be a growth arrest of general physiological importance later in development of animals that can be induced by growth factors such as FGF.

Fibroblast growth factor (FGF) plays an important role during the development of embryos of various organisms. Activation of the FGFR by ligand binding stimulates many downstream signaling cascades including the p42MAPK pathway (Kim and Nishida, 2001). For example, p42MAPK plays a role in the early signaling events of FGF-mediated mesoderm induction in *Xenopus* (Hartley *et al.*, 1994). The phosphorylation of Cdc25C by $pp90^{Rsk}$ may contribute to the growth arrest that occurs during embryonic patterning. Interestingly, it has also been shown that 14-3-3 proteins are required for FGF-mediated mesoderm induction during early development of *Xenopus* (Wu and Muslin, 2002).

Another possible physiological significance of the p42MAPK-induced G2 arrest is suggested from studies using mammalian cells. In HeLa cells, EGF and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) have been shown to prevent entry into M-phase from G2 by inhibiting the activation of Cdk1 (Barth *et al.*, 1996). Since the p42MAPK signaling pathway is known to be activated by EGF and phorbol esters (Cobb *et al.*, 1991), the $pp90^{Rsk}$ phosphorylation of Cdc25C may be contributing towards the arrest in these cells. Furthermore, there was a reduction of Cdc25C activity in synchronized cell cultures when EGF and TPA were present (Barth *et al.*, 1996). In chondrocytes, it has recently been found that both the ERK1/2 (p44/p42 MAPK) and p38 MAPK pathways are involved in FGF-mediated growth arrest (Raucci *et al.*, 2004). In these cells, both FGF and nerve growth factor (NGF) (but not EGF) inhibited growth, which was accompanied by a sustained activation of ERK1/2 (p44/p42 MAPK) and p38 MAPK (Raucci *et al.*, 2004). Moreover, the growth arrest in chondrocytes induced by FGF-activated signaling pathways may be necessary for coordinating the onset of differentiation (Dailey *et al.*, 2003). The sustained activation of the MAPK pathways in these cells may be required to delay or inhibit growth, and parallels the sustained post-S-phase arrest that we have observed in egg extracts.

Future directions for this study would include investigating the mechanism by which $pp90^{Rsk}$ phosphorylated-Cdc25C (on S287) maintains the G2 arrest. The effect on Cdc25C does not depend on the presence of a nucleus, thus the effects of 14-3-3 binding to Cdc25C probably occur in the cytoplasm. After Cdc25C is phosphorylated on S287, it would be interesting to determine the exact function of 14-3-3 binding and how it prevents entry into M-phase. It would be interesting to determine if phosphorylation of Cdc25C on S287 prevents the hyperphosphorylation and activation of Cdc25C by the upstream kinase Plx1. Lastly, the mechanisms of action are sometimes different between mammals, amphibians, and yeast. Although 14-3-3 bound Cdc25C may be contributing to a nuclear exclusion of Cdc25C in yeast and mammalian cells (Kumagai *et al.*, 1999; Lopez-Girona *et al.*, 1999), 14-3-3 bound Cdc25C in *Xenopus* may not be able to interact with Plx1 since preventing the hyperphosphorylation and activation of Cdc25C by Plx1 may be the purpose of 14-3-3 binding to Cdc25C.

4.2 Activation of the p42MAPK Pathway During M-phase Sustains M-phase Activities (NEBD/CC) Without the Presence of MPF

In the second part of my study, the role of p42MAPK during M-phase arrest was examined. In *Xenopus* egg extracts, it has been shown that the activation of p42MAPK after the peak of MPF activity can lead to an M-phase arrest even after the inactivation of MPF activity and Cyclin B degradation (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999). These extracts are sustained in M-phase with the presence of M-phase-specific phosphorylations and condensed chromosomes (Chau and Shibuya, 1999).

We hypothesized that after entry into M-phase, the activation of the p42MAPK pathway could sustain M-phase activities, such as those that induce NEBD and CC, even after MPF inactivation. Various studies and model systems have provided contradictory results concerning the role of p42MAPK and MPF during M-phase (reviewed by Yamashita *et al.*, 2000). Therefore, the requirement for MPF after entry into M-phase was investigated.

In this study, we have shown that the Cdk1 inhibitor roscovitine was able to inhibit some histone H1 phosphorylation in an M-phase arrested egg extract while maintaining

Cdc25C in a hyperphosphorylated state. Also, addition of MBP-Cyclin BΔ90 to an interphase arrested egg extract was able to induce M-phase activities without activating p42MAPK. An immunodepletion protocol consisting of 3 rounds of 10 min immunodepletions was used to immunodeplete MBP-Cyclin BΔ90 after an egg extract had been driven into M-phase. Using the immunodepletion protocol, it was shown that an interphase egg extract driven into M-phase upon addition of MBP-Cyclin BΔ90 could sustain M-phase activities (NEBD/CC) even after the removal of MBP-Cyclin BΔ90/Cdk1, but only if p42MAPK was activated. These results suggest that the p42MAPK pathway can sustain M-phase activities, specifically, those that induce NEBD/CC even in the absence of MPF activity.

4.2.1 p42MAPK May be Sustaining the Hyperphosphorylated State of Cdc25C When MPF Activity is Inhibited by Roscovitine

In order to test if p42MAPK is able to sustain NEBD/CC without MPF activity, we attempted to inhibit MPF activity by using Cdk1-specific inhibitors. Two selective inhibitors of Cdk1 that compete with the ATP-binding domain, roscovitine and butyrolactone I (BLI), were added to M-phase arrested egg extracts to inhibit Cdk1 kinase activity.

Previously, Shimada *et al.* (1998) showed that in the presence of BLI, chromosome condensation of remodeled sperm chromatin did not occur in unfertilized egg extracts and chromosome condensation could not be induced by adding active Cyclin B/Cdk1, implying that another protein kinase other than Cyclin B/Cdk1 was responsible for the induction of CC. In my experiments, 200 μM of BLI was not able to sustain Cdc25C in a hyperphosphorylated state (Fig. 3.9 A). The reactions containing BLI, when compared to the reactions containing roscovitine, had histone H1 phosphorylation levels that were lower and more comparable to interphase levels. Butyrolactone I is known to inhibit other kinases at higher concentrations. At a concentration of 94 μM, BLI is known to inhibit p42MAPK in cells (Kitagawa *et al.*, 1993). In calyculin A (inhibitor of type 1 and type 2A phosphatases) treated sea urchin eggs, it has been shown that 250 μM BLI did not affect chromosome condensation (Tosuji *et al.*, 2003). Also, in extracts obtained from metaphase II arrested bovine eggs, 100 μM of BLI did not inhibit

p42MAPK activity (Kubelka *et al.*, 2000). In contrast, in bovine oocytes, GVBD was almost completely inhibited and Cdk1 and p42MAPK failed to become activated with 100 μM BLI (Kubelka *et al.*, 2000). It appears that depending on the system used, the effects induced by varying concentrations of BLI differ. Nevertheless, 200 μM BLI is most likely inhibiting p42MAPK since p42MAPK was dephosphorylated and inactivated (Fig. 3.9 A). We turned to using roscovitine because it was likely that BLI may be inhibiting p42MAPK in *Xenopus* egg extracts treated with 200 μM of BLI.

Roscovitine functions by acting as a competitive inhibitor of ATP, the same mechanism of action as BLI, but it is more selective for Cdk1. In previous work, roscovitine is known to inhibit p42MAPK when used in concentrations in the micromolar range (inhibitory concentration to inhibit 50% of the protein activity, IC_{50} = 14 μM for p42MAPK while the IC_{50} for Cdk1 is 650 nM; De Azevedo *et al.*, 1997). However, our results suggest that in M-phase arrested egg extracts, higher concentrations of Cdk1 inhibitors are required to inhibit MPF activity. Concentrations of roscovitine up to 350 μM (25-fold greater than the specified IC_{50} for even p42MAPK) was able to maintain hyperphosphorylated Cdc25C and phosphorylated, active p42MAPK in M-phase arrested egg extracts (Fig. 3.9 A). Higher concentrations of Cdk inhibitors may be required for M-phase arrested egg extracts because Cdk1 is most active during M-phase. Also, roscovitine and buytrolactone I function by acting as competitive inhibitors of ATP. Since the *Xenopus* egg extracts are supplemented with ATP, higher concentrations of Cdk inhibitors may be required.

The results showed that when 350 μM of roscovitine was used to inhibit Cdk1, most MPF activity was inhibited as shown by the very low levels of histone H1 phosphorylation (Fig. 3.9 B). However, Cdc25C remained hyperphosphorylated and p42MAPK was activated (Fig. 3.9 A) indicating that the extract still had characteristics of M-phase. As expected, the histone H1 phosphorylation assay indicated a dose-dependence effect. As the concentration of roscovitine was increased, more Cdk1 kinase activity was inhibited resulting in less histone H1 phosphorylation. The Cdk1 kinase activity for the reactions containing 150 μM to 250 μM showed a gradual increase in histone H1 phosphorylation (Fig. 3.8 B,C). It appears that either roscovitine is losing activity over time or endogenous Cyclin B is being synthesized and contributing to

histone H1 phosphorylation. Since cycloheximide was not added to the egg extract, it is possible that protein was being synthesized. Moreover, it has been shown that p42MAPK can stimulate Cyclin B translation in *Xenopus* (Howard *et al.*, 1999). Also, $p90^{Rsk}$ has been shown to downregulate the inhibitory activity of Myt1 on Cdk1 (Palmer *et al.*, 1998) thus maintaining the active state of Cdk1. Because p42MAPK is phosphorylated in the M-phase arrested egg extract containing roscovitine, it may be active and promoting the synthesis of Cyclin B and the activation of Cdk1 kinase activity thus causing the gradual increase in MPF activity after initial inhibition by roscovitine. Therefore, for later experiments, egg extracts containing cycloheximide were used.

Although BLI at 200 μM appeared to lead to the dephosphorylation of p42MAPK and Cdc25C in the *Xenopus* egg extracts (Fig. 3.9 A), p42MAPK was not inactivated and Cdc25C remained hyperphosphorylated when 350 μM of roscovitine was used (Fig. 3.9 A). Unexpectedly, when 350 μM of roscovitine was used, the histone H1 phosphorylation data showed inhibition of Cdk1 kinase activity but some residual histone H1 phosphorylation. It was expected that 350 μM of roscovitine would be sufficient to inhibit all Cdk1 kinase activity since this was the highest concentration that could be used before p42MAPK became inactivated in egg extracts.

There are two possible reasons why the histone H1 phosphorylation assay may be showing residual histone H1 phosphorylation. The most obvious reason may be that Cdk1 activity can increase slowly because the inhibitors function in a competitive manner. However, 350 μM appears to be near the concentration able to inhibit p42MAPK thus it was expected that 350 μM of roscovitine would be in the range to maintain an inhibition of virtually all Cdk1 activity. Second, there may be other kinases in the egg extract that can phosphorylate histone H1. For example, in a study by Kuang *et al.* (1991), when *Xenopus* egg extract was fractionated by gel filtration, two prominent peaks of H1 kinase activity were detected. One peak of 150 kDa co-purified with Cdk1 protein but the other peak with an apparent molecular mass of 600 kDa was identified as a novel M-phase-specific H1 kinase (Kuang *et al.*, 1991). In addition, only the 600 kDa kinase was detected by the mitosis-specific monoclonal antibody, MPM-2.

Kinases including Cdk2, Cdk5, p44MAPK and p42MAPK are inhibited at higher concentrations of roscovitine. When histone H1 phosphorylation levels are down to

interphase levels, both Cdc25C and p42MAPK were always dephosphorylated. There may be a threshold level of M-phase activities induced by MPF on entry into M-phase that sustain activities such as Cdc25C hyperphosphorylation and M-phase activities (NEBD and CC). Since 350 μM of roscovitine did not result in the dephosphorylation of p42MAPK, further studies would be required in order to determine whether residual MPF activity, the activity of p42MAPK or other downstream activities was sustaining hyperphosphorylated Cdc25C and M-phase arrest.

4.2.2 MBP-Cyclin BΔ90 can be Immunodepleted from an Egg Extract but Residual Histone H1 Phosphorylation may be Caused by Unidentified Kinases

Since residual histone H1 phosphorylation was observed when inhibitors of Cdk1 were used to eliminate MPF activity, an immunodepletion protocol which used 3 sets of 10 min immunodepletions was established to physically remove MPF activity from an egg extract. The 3 sets of immunodepletions for 10 min each was used rather than the 2 sets of immunodepletions carried out for 30 min in order to minimize the time for the immunodepletions to prevent loss of activities.

The anti-MBP immunoblot shows that by the third round of immunodepletion MBP-Cyclin BΔ90 was not detected. From the anti-Cdc25C immunoblot, it was observed that Cdc25C remained hyperphosphorylated and even after MBP-Cyclin BΔ90 had been immunodepleted (Fig. 3.11 A,B). Endogenous p42MAPK was activated 60 min after the addition of MBP-Cyclin BΔ90 (Fig. 3.11 A,B) because the room temperature incubation prior to protein addition was omitted. Although the MBP-Cyclin BΔ90 is not detected in the supernatant after the immunodepletion (anti-MBP blots, Fig. 3.11 A,B), the supernatants were not assayed for the histone H1 kinase activity.

The histone H1 phosphorylation assay showed residual kinase activity in the supernatant samples after Cyclin BΔ90 had been immunodepleted (Fig. 3.12 D,E), similar to the results obtained after Cdk1 inhibition with roscovitine (Fig. 3.8 C, 3.9 B). Also, there seems to be some residual MBP-Cyclin BΔ90 remaining (although in near undetectable amounts) in the supernatant (Fig. 3.12 D,E). One could argue that the residual MBP-CyclinΔ90 present in the supernatant was causing the extract to remain in M-phase. However, this amount of Cyclin BΔ90 was not enough to sustain NEBD/CC-

inducing activities (Fig. 3.12 I). Clearly, sperm nuclei remained decondensed with intact nuclear envelopes when the donor extract (immunodepleted of Cyclin BΔ90/Cdk1 and p42MAPK inactive) was added to a recipient CHX extract containing decondensed sperm nuclei (Fig. 3.12 I).

I suspect that the bulk of the histone H1 kinase activity detected after immunodepletion of Cyclin BΔ90/Cdk1 or inhibition of Cdk1 by roscovitine may not be due to residual Cdk1 kinase activity. During the immunodepletion of MBP-Cyclin BΔ90/Cdk1, other kinases such as the 600 kDa kinase mentioned earlier may be phosphorylating histone H1. In addition to the 600 kDa kinase, in interphase arrested *Xenopus* egg extracts, the addition of microcystin activated a kinase that can lead to the phosphorylation of Cdc25C and induced NEBD and CC in the absence of MPF activity (Izumi and Maller, 1995). Although the kinase responsible for activating Cdc25C is likely Plx1 (Kumagai and Dunphy, 1996; Qian *et al.*, 1998), p42MAPK appears to be necessary to sustain M-phase activities and may be working in combination with other kinases to sustain M-phase arrest.

4.2.3 p42MAPK is Able to Sustain M-phase Activities (NEBD/CC) Without MPF, Once an Egg Extract has Entered M-phase

Activation of p42MAPK in cycling *Xenopus* egg extracts has been shown to cause the cell cycle to arrest at G2 and M-phase depending on the timing of activation (Abrieu *et al.*, 1997b; Walter *et al.*, 1997; Bitangcol *et al.*, 1998). Bitangcol *et al.* (1998) clearly demonstrated that when MEK(QP) was added to a cycling egg extract 40 min within the start of incubation, the cell cycle arrested at metaphase with hyperphosphorylated Cdc25 and metaphase spindles. Also, Chau and Shibuya (1998) showed that the activation of p42MAPK by Mos in a cycling *Xenopus* egg extract after the peak of MPF activitiy, led to Cyclin B degradation, but the extract remained arrested in M-phase with condensed chromosomes, mitotic arrays of microtubules and sustained presence of phosphoproteins that were detected by the MPM-2 antibody (Chau and Shibuya, 1998). Similar results were found by Guadagno and Ferrell, Jr. (1998) who also showed that without Cdk1 activity, Mos-treated *Xenopus* extract remained in mitosis since chromosomes remained condensed.

In our experiments, using a constitutively-active activator of p42MAPK, MEK(QP), we showed that when MBP-Cyclin BΔ90 was added to an interphase arrested egg extract to drive the extract into M-phase and then removed by immunodepletion, the supernatant lacking MBP-Cyclin BΔ90/Cdk1 retained NEBD/CC activity in the reaction in which p42MAPK had been activated (Fig. 3.12K). These results showed that activated p42MAPK was able to maintain M-phase activities induced by the activation of MPF (Fig. 3.12K), which is consistent with the results obtained previously with Mos (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998). Furthermore, these results suggested that p42MAPK was not only able to maintain the M-phase activities that would normally keep nuclei in M-phase morphology (NEBD/CC), but in addition, the donor extract, immunodepleted of Cyclin BΔ90/Cdk1 and containing activated p42MAPK could cause decondensed sperm nuclei in a recipient extract to undergo NEBD/CC (Fig. 3.12 K), implying that p42MAPK could sustain NEBD/CC inducing activities normally attributable to Cyclin B/Cdk1. These results suggest that NEBD/CC activity develops as a consequence of MPF, but is not MPF itself, and that p42MAPK can stabilize these activities.

Our study complements and extends the results showing that M-phase arrest can be sustained even after Cyclin B/Cdk1 kinase is inactivated when p42MAPK is activated in *Xenopus* cycling egg extracts (Chau and Shibuya, 1998; Guadagno and Ferrell, Jr., 1998; Bhatt and Ferrell, Jr., 1999). Moreover, since MEK(QP) was used to activate the p42MAPK pathway, these results show that no other pathways that are independent of the p42MAPK pathway are involved (i.e. the Plx1 pathway). These results support the idea that the p42MAPK pathway maintains M-phase arrest by sustaining the NEBD/CC inducing activities of M-phase independently of MPF activity.

Although p42MAPK was shown to sustain NEBD and CC without the presence of MPF, as a future study, one could verify and identify the proteins that are necessary for NEBD/CC inducing activities. It has been suspected that there are unidentified proteins (reviewed by Karaiskou *et al.*, 2001). Kinases other than MPF have been demonstrated to be activated before GVBD during oocyte maturation (Cicirelli *et al.*, 1988), one of these kinases being p42MAPK (reviewed by Ruderman, 1993). These unidentified proteins might may also be activated or sustained by p42MAPK and work in unison with

MPF to sustain M-phase inducing activities. Also, the activities responsible for maintaining Cdc25C in a hyperphosphorylated state and the sustained M-phase phosphorylations recognized by the MPM-2 antibody without the presence of Cdk1 kinase activity would have to be further characterized. I would have to repeat my experiments and confirm that MPM-2 reactive phosphoproteins are present in the donor extract after immunodepleting MBP-Cyclin BΔ90/Cdk1. Also, there are two consensus sequences for p42MAPK phosphorylation in Cdc25C that are not recognized by the MPM-2 antibody (Shibuya, unpublished). It would be interesting to determine if the sustained hyperphosphorylation and activation of Cdc25C is dependent on the activity of p42MAPK after initial activation by Cyclin B/Cdk1 kinase activity. Further studies would be needed to determine what activities are required for these processes.

4.2.4 Summary

In summary, we have provided a molecular mechanism for a G2 arrest revealed in egg extracts that is mediated by $pp90^{Rsk}$. We showed that $pp90^{Rsk}$ is able to directly phosphorylate S287 of Cdc25C *in vitro,* and that this phosphorylation leads to the binding of 14-3-3 proteins. This G2 arrest may represent the general effects of activation of the p42MAPK kinase pathway that have been observed during later development in embryos or in the differentiation of cells which may be a growth arrest of physiological importance.

We have provided evidence that the activation p42MAPK can sustain M-phase activities. Although MPF is necessary for entry into M-phase and the appearance of M-phase characteristics such as the hyperphosphorylation of Cdc25, initial phosphorylation of histone H1, and the appearance of MPM-2 reactive phosphorylations, these phosphorylations can be sustained by p42MAPK. Furthermore, NEBD/CC inducing activities that are normally thought to be activated and dependent on Cyclin B/Cdk1 can be maintained by p42MAPK without the presence of Cyclin B/Cdk1.

Chapter 5: References

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