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B-Raf is an essential component of the mitotic machinery critical for activation of MAPK signaling during mitosis in Xenopus egg extracts

Sergiy I. Borysov

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B-Raf is an Essential Component of the Mitotic Machinery Critical for Activation of
MAPK Signaling During Mitosis in *Xenopus* Egg Extracts

by

Sergiy I. Borysov

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
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Jerry Wu, Ph.D.

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Keywords: MEK kinase, extracellular signal regulated kinase (ERK), Cdk1/cyclin B,
Cdc2/cyclin B, M-phase, signal transduction, cell cycle, phosphorylation

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<td>Alanine</td>
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<tr>
<td>AP-2</td>
<td>Adaptor protein 2</td>
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<tr>
<td>Apacid</td>
<td>ATP binding protein associated with cell differentiation</td>
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<td>APC</td>
<td>Anaphase-promoting complex</td>
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<tr>
<td>APKAP</td>
<td>Alanine-Proline-Lysine-Alanine-Proline</td>
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<td>AS</td>
<td>Ammonium sulfate</td>
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<tr>
<td>ASK</td>
<td>Apoptosis signal-regulated kinase</td>
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<td>ATR</td>
<td>Ataxia telangiectasia related</td>
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<td>N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea</td>
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<td>Bcl-2 homology 3</td>
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<td>Bim</td>
<td>Bel2-interacting mediator of cell death</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Bub</td>
<td>Budding uninhibited by benzimidazole</td>
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<td>CAS</td>
<td>Cellular apoptosis susceptibility</td>
</tr>
<tr>
<td>CCt8</td>
<td>Chaperonin containing theta</td>
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<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
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<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
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<td>CENP-E</td>
<td>Centromere-associated protein E</td>
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<td>Cn2</td>
<td>Cytosolic non-specific peptidase</td>
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<td>CNK</td>
<td>Connector enhancer of KSR</td>
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<td>CRD</td>
<td>Cysteine rich domain</td>
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<td>CSE1</td>
<td>Chromosome segregation gene 1</td>
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<td>CSF</td>
<td>Cytostatic factor</td>
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<td>Diethylaminoethyl</td>
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<td>DTT</td>
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<td>EB</td>
<td>Extract buffer</td>
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<td>ELB</td>
<td>Egg lysis buffer</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>EGTA</td>
<td>Ethylene glycol bis(aminoethylether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FF</td>
<td>Fast flow</td>
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<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<td>Glu</td>
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<td>Grb2</td>
<td>Growth factor receptor bound protein 2</td>
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<td>Glutathione S-transferase</td>
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<td>HP</td>
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<td>High Performance Liquid Chromatography</td>
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<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
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<td>Immunoprecipitate</td>
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<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>KSR</td>
<td>Kinase suppressor of Ras</td>
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<td>Mad</td>
<td>Mitotic arrest-deficient</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<td>Mixed lineage kinase</td>
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<td>Modified Ringers solution</td>
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<td>Maturation-promoting factor</td>
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<td>Nucleoporin</td>
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<td>PA</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>Protein phosphatase</td>
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<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>PXS*P</td>
<td>Proline-any amino acid-phospho-Serine-Proline</td>
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<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
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<tr>
<td>SNAP</td>
<td>SNAP adaptor protein</td>
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<td>Serum and glucocorticoid inducible kinase</td>
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<td>Soc-2</td>
<td>Suppressor of clear homolog</td>
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<td>Serine-Proline-Lysine-Threonine-Proline</td>
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<td>S*PXK/L</td>
<td>Phospho-Serine-Proline-any amino acid-Lysine or Leucine</td>
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<td>TAK</td>
<td>TGF-β-activated protein kinase</td>
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<td>TEY</td>
<td>Threonine-Glutamic acid-Tyrosine</td>
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<td>Wild-type</td>
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<tr>
<td>XB</td>
<td><em>Xenopus</em> buffer</td>
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B-Raf is an Essential Component of the Mitotic Machinery Critical for Activation of MAPK Signaling During Mitosis in *Xenopus* Egg Extracts

Sergiy I. Borysov

ABSTRACT

Activation of the MAPK cascade during mitosis is critical for spindle assembly and normal mitotic progression. The underlying regulatory mechanisms that control activation of the MEK/MAPK cascade during mitosis are poorly understood. The goal of my dissertation research is to identify the MEK kinase responsible for activation of the MAPK cascade during mitosis and to elucidate the biochemical mechanisms that regulate its activity. In the described herein work I purified and characterized the MEK kinase activity present in M-phase arrested *Xenopus* egg extracts. I demonstrate that B-Raf is the critical MEK kinase required for activation of the MAPK pathway at mitosis. Consistent with this, I show that B-Raf is activated in an M-phase dependent manner. Further, I provide data linking Cdk1/cyclin B to mitotic activation of B-Raf. Cdk1/cyclin
B associates with and phosphorylates B-Raf in M-phase arrested extracts and directly targets *Xenopus* B-Raf *in vitro* at a conserved Ser-144 residue. Phosphorylation at Ser-144 is critical for M-phase dependent activation of B-Raf and for B-Raf’s ability to trigger activation of the MAPK cascade at mitosis. Finally, I demonstrate that mitotic B-Raf undergoes feedback phosphorylation by MAPK at its conserved C-terminal SPKTP motif. Mutation of both phosphorylation sites within the SPKTP sequence to alanines increases activity of mitotic B-Raf. Further, inhibition or over-activation of MAPK during mitosis enhances or diminishes B-Raf activity, respectively. These results indicate that MAPK-mediated feedback phosphorylation negatively regulates B-Raf activity. Additionally, I show that active mitotic B-Raf exists in large multi-protein complex(s). By utilizing a proteomics approach I identify a set of proteins, which potentially associate with B-Raf at M-phase. Future studies are necessary to elucidate the involvement of these proteins in regulating B-Raf mitotic functions. In summary, my dissertation studies demonstrate that B-Raf activates MAPK signaling at mitosis and undergoes an M-phase dependent regulation. I propose that B-Raf has important functions at mitosis that contributes to its overall role in promoting cell proliferation.
Chapter One

General Introduction

General understanding of mitosis

The purpose of the cell cycle is to produce two daughter cells identical to the mother cell. This is achieved through a temporal coordination and execution of the cell cycle events. During G1-S-G2 phases of the cell cycle a cell commits to and prepares for cell division, whereas at M-phase the cell division is finally executed (Murray, 1992). M-phase is traditionally divided into two stages: mitosis (segregation of the chromosomes) and cytokinesis (division of the cytoplasm) (Satterwhite and Pollard, 1992). Therefore, mitosis is the culminating stage of the cell cycle, when the duplicated genome of the mother cell becomes redistributed into two daughter cells.

Mitosis is traditionally described as a series of intracellular morphological changes involving dynamic rearrangements of chromatin and cytoskeleton structures (Karsenti, 1991; McIntosh and Koonce, 1989; Wadsworth, 1993). The first signs of chromatin condensation within an intact nucleus mark the initial progression toward mitosis, recognized as prophase (Khodjakov and Rieder, 1999; Swedlow and Hirano, 2003). During prophase, a pair of centrosomes (Fukasawa, 2002), duplicated in S-phase,
undergoes separation defining the poles of the forming mitotic spindle (Raff and Glover, 1989). Recruitment of additional gamma-tubulin ring complexes into centrosomal matrix (Khodjakov and Rieder, 1999) provokes a dramatic increase in microtubule nucleation activity necessary for dynamic spindle assembly (Kuriyama and Borisy, 1981). The following nuclear envelope breakdown represents the first irreversible point of mitosis, prometaphase (Fields and Thompson, 1995; Rieder and Khodjakov, 1997). The dismantling of the nuclear envelope allows physical interaction between growing tubulin fibers of the mitotic spindle and the kinetochores of the chromosomes. Pushing and pulling forces of the kinetochore microtubules emitting from opposite poles and bound to the same chromosomal kinetochores position chromosomes halfway between the spindle poles, along the equator of a cell, called a metaphase plate (Sonoda et al., 2001; Tanaka et al., 2000). Until this stage of mitosis, sister chromatids are held together by cohesin (Nasmyth, 2001) selectively enriched in the neighborhood of the centromeres (Waizenegger et al., 2000). The metaphase alignment and proper attachment of chromosomes to the mitotic spindle triggers an abrupt and synchronous cleavage of the centromeric cohesin complexes (Hauf et al., 2001; Uhlmann et al., 2000). This provokes the sudden separation of sister chromatids and represents the second irreversible stage of mitosis, anaphase. Following the cleavage, chromosomes are separated first due to shortening of the kinetochore microtubules (anaphase A) (Mitchison and Salmon, 1992) and later due to growing of the polar microtubules and elongation of the whole spindle (anaphase B) (Aist et al., 1991). Mitosis is finished at the telophase when the kinetochore microtubules disappear (Wilson et al., 1994), a new nuclear envelope reforms around
each set of adjacent decondensing chromosomes (Gerace and Blobel, 1980; Newport, 1987) and the nucleoli are reformed (Dimario, 2004). The following cytokinesis is temporally and spatially coordinated to the completion of mitosis (Satterwhite and Pollard, 1992). The midbody, a microtubule-based structure assembled at telophase around the original spindle equator, directs proper cleavage of the cytoplasm during cytokinesis (Otegui et al., 2005).

An intricate biochemical network governs orderly execution and precision of mitotic events. Protein phosphorylation (Nigg, 2001) and dephosphorylation (Kumagai and Dunphy, 1996), protein-protein association (Hardwick, 2005; Kramer et al., 2000; Morgan, 1995; Musacchio and Hardwick, 2002; Visintin et al., 1997) and protein degradation (Bashir and Pagano, 2004; Holloway et al., 1993; Sudo et al., 2004) are the main mechanisms of the mitotic regulatory machinery.

Proper order and completion of the mitotic stages are accomplished by incorporating positive and negative feedback loops into the regulation of mitosis and creating two “points of no return” in mitotic progression (Murray, 1992; Murray and Kirschner, 1989b). The first “point of no return” overlaps with the prophase-prometaphase transition and is associated with a robust activation of Cdk1/cyclin B, the master regulator of mitotic entry (Dunphy et al., 1988). The irreversibility of Cdk1/cyclin B activation is ensured by the positive feedback loop existing between Cdk1/cyclin B and its upstream activator, Cdc25C phosphatase (Strausfeld et al., 1994), which removes the inhibitory phosphorylations from Thr-14 and Tyr-15 residues (Atherton-Fessler et al., 1993; Kumagai and Dunphy, 1996). This positive regulatory
circuit ensures an abrupt and robust accumulation of active Cdk1/cyclin B and enables Cdk1/cyclin B activation and progression toward mitosis to be independent from any further upstream inputs.

Cdk1/cyclin B activation sets up conditions for the next irreversible point in mitosis, activation of the anaphase-promoting complex (APC), a mitosis-specific ubiquitin-ligase, which targets mitotic proteins for degradation promoting exit from mitosis (Castro et al., 2005). Indeed, activation of Cdk1/cyclin B via direct and indirect mechanisms triggers formation of the mitotic spindle and its association with chromosomal kinetochores. Completion of this spindle-chromosome assembly during metaphase leads to activation of APC, which in turn ubiquitinates and targets securin and cyclin B for degradation. Securin proteolysis promotes the disjoining of sister chromatids and represents the main condition for anaphase initiation (Holloway et al., 1993). Degradation of cyclin B leads to inactivation of Cdk1/cyclin B complexes (Wheatley et al., 1997). Thus, activation of Cdk1/cyclin B triggers a series of events, which eventually leads to elimination of Cdk1/cyclin B signaling.

The spindle assembly checkpoint ensures that progression toward anaphase does not happen prematurely. It is thought that throughout spindle formation and establishment of kinetochore-microtubule interactions, the status of the kinetochore-microtubule attachments can be sensed and reflected in the levels of soluble “wait-anaphase” signaling complexes (Rieder and Salmon, 1998; Wassmann and Benezra, 2001), the presence of which delays activation of APC. Indeed, unattached kinetochores or low-tension kinetochores undergo phosphorylation and act as sites where spindle
checkpoint Mad2-Cdc20 protein complexes are assembled and released to abrogate APC activation (Chan and Yen, 2003; Hardwick, 2005; Yu, 2002).

In conclusion, mitosis is the final stage of the cell cycle, when segregation of replicated chromosomes occurs. To accommodate separation of the duplicated genomic DNA, the chromatin undergoes condensation and the cytoskeleton transforms into the mitotic spindle. These processes of mitosis are tightly regulated by mitotic biochemical machinery, which ensures proper timing, completeness and fidelity of mitotic stages.

**Protein kinases in regulation of mitosis**

Protein phosphorylation comprises one of the major modes of mitotic regulation (Nigg, 2001). Many kinases have been implicated in mitosis. These include Cdk1/cyclin B, members of Polo-like and Aurora kinase families, NIMA, mitotic checkpoint kinases (Bub1, BubR1 and Mps1), MAPK and others.

Cdk1/cyclin B is the main kinase involved in the regulation of mitotic onset (Arion et al., 1988; Gautier et al., 1988; Labbe et al., 1989; Lohka et al., 1988; Masui and Markert, 1971; Nurse, 1975). The contemporary dogma of the cell cycle regulation represents cyclin-dependent kinase activities, which fluctuate throughout the cell cycle, as a regulatory core of the cell cycle (Morgan, 1995). The identification of Cdk1/cyclin B as an initiator of M-phase and a prototype of Cdk/cyclin complexes was a discovery of Nobel Prize caliber (Paul Nurse, Leland Harwell and Tim Hunt, Nobel Prize in Medicine, 2001).
Cdk1/cyclin B initiates and establishes mitosis via phosphorylation of multiple substrates. For instance, active Cdk1/cyclin B directly phosphorylates lamins to induce dismantling of the nuclear envelope (Peter et al., 1991), condensins to contribute to further chromosome condensation (Kimura et al., 1998), and microtubule-associated proteins and kinesin-related motor proteins to affect centrosome separation and mitotic spindle assembly, respectively (Blangy et al., 1995). Besides this, Cdk1/cyclin B directly regulates APC and other components of the mitotic regulatory network (Patra and Dunphy, 1998; Zachariae et al., 1998). Inactivation of Cdk1/cyclin B occurs during the anaphase transition and is required for proper mitotic exit (Noton and Diffley, 2000). It is achieved through an APC-directed degradation of cyclin B, a regulatory partner of Cdk1 (Glotzer et al., 1991; Wheatley et al., 1997).

Polo-like kinases are a new emerging class of mitotic regulators. Originally, Polo-like kinases were identified in *Drosophila* (Sunkel and Glover, 1988) and yeast (Kitada et al., 1993) genomes and linked to mitotic regulation. There are at least three Polo-like kinase members in vertebrates (Lowery et al., 2005), one of which, Polo-like kinase 1 (Plk1), has been implicated in mitosis. It has been shown that Plk1 localizes to the mitotic apparatus (Arnaud et al., 1998; Golsteyn et al., 1995; Wianny et al., 1998), undergoes an M-phase dependent regulation (Charles et al., 1998; Golsteyn et al., 1995; Hamanaka et al., 1995) and has multifaceted roles during M-phase. It has been proposed that Plk1 triggers the Cdc25C-Cdk1/cyclin B positive feedback loop during the mitotic entry (Nakajima et al., 2003; Roshak et al., 2000; Toyoshima-Morimoto et al., 2002), controls mitotic spindle formation and functions (Ahonen et al., 2005; Casenghi et al.,
The family of Aurora kinases is another important group of mitotic regulators. Aurora kinases were originally identified in *Drosophila* as being involved in the regulation of centrosome functions during mitosis (Glover et al., 1995) and in budding yeasts as being involved in coordination of mitosis (Chan and Botstein, 1993). The metazoan genome encodes at least three Aurora kinases, Aurora A, B and C (Andrews et al., 2003). Thus far only Auroras A and B are directly linked to mitosis.

Auroras A and B differ from each other in timing of activation, intracellular localizations and functions during mitosis. Aurora A protein levels and activity peak at G2/M, whereas Aurora B is up-regulated and activated during late mitosis after Cdk1/cyclin B inactivation (Bischoff et al., 1998). Both kinases undergo proteasome-mediated degradation at the end of mitosis/G1 (Shu et al., 2003; Taguchi et al., 2002). Aurora A localizes to the centrosomes throughout mitosis (Stenoien et al., 2003) and is implicated mainly in centrosomal functions (Andrews et al., 2003; Giet et al., 1999). Additionally, Aurora A may be an important component of the M-phase onset signaling (Katayama et al., 2004; Krystyniak et al., 2006).

Aurora B localizes to the kinetochore regions from prophase until the metaphase-anaphase transition and to the midzone and midbody during telophase and cytokinesis, respectively (Andrews et al., 2003). It is thought that Aurora B’s association with INCENP (inner centromere protein) and survivin (Adams et al., 2000; Yasui et al., 2004)
is important for its roles in spindle formation (Moore and Wordeman, 2004) and the mitotic spindle checkpoint (Ditchfield et al., 2003; Hauf et al., 2003).

Genetic studies in the filamentous fungus *Aspergillus nidulans* identified a new mitotic kinase, NIMA (*never in mitosis A*) (Osmani et al., 1988). Functional studies characterized NIMA as one of the key regulators of mitotic onset (Osmani et al., 1991; Osmani et al., 1988; Osmani and Ye, 1996). However, the closest mammalian homologue of fungal NIMA, Nek2 (Fry, 2002), does not display a global effect on M-phase progression. It was shown that Nek2 is a centrosomal resident protein involved in centrosome separation (Fry et al., 1998a; Fry et al., 1998b).

Another important group of kinases involved in the regulation of mitosis is a set of kinases directly implicated in the generation of spindle checkpoint signaling. This includes kinases of Mad/Bub families (namely, Bub1 and BubR1/Mad3) and Mps1 kinase. It is proposed that Bub1 (Roberts et al., 1994) and BubR1 (Taylor et al., 1998) facilitate kinetochore recruitment of other members of the Mad and Bub families (Chen, 2002; Sharp-Baker and Chen, 2001) and participate in their rearrangements leading to the formation of spindle checkpoint protein complexes, which inhibit APC and postpone anaphase onset (Brady and Hardwick, 2000; Sudakin et al., 2001). Checkpoint kinase Mps1 (Weiss and Winey, 1996), similar to Bub1 and BubR1, facilitates kinetocore localization of the Mad proteins (Abrieu et al., 2001). However, unlike Bub1 and BubR1, Mps1 has not been shown to be a component of the “wait anaphase” complexes (Hoyt, 2001).
In conclusion, many kinases are involved in the regulation of mitosis. Cdk1/cyclin B is the major among them. Its activation and inactivation mark two of the most important transitional points in mitotic progression, commitment and exit from mitosis, and ensure the irreversibility of the process. The involvement of many other kinases in the mitotic regulatory network reflects the necessity of multilayered and branched control over the fidelity and integrity of cell division.

**Three-kinase module of the MAPK pathway**

The MAPK cascade is one of the most well characterized kinase pathways implicated in cellular signaling. It governs cellular responses to a variety of stimuli including cytokines, growth factors and hormones, and conditions, such as cellular stresses, cell adhesion and others (Widmann et al., 1999). The MAPK signaling module consists of three kinases that sequentially phosphorylate and activate one another. The up-stream kinase, MAPK kinase kinase (MEK kinase), phosphorylates and activates MAPK kinase (MEK), which in turn phosphorylates and activates MAPK (Fig. 1A) (English et al., 1999; Widmann et al., 1999). The MAPK cascade is unique for eukaryotes and conserved from yeast to humans (English et al., 1999; Widmann et al., 1999).

Mammalian cells express at least twenty members of the MAPK family (Pearson et al., 2001b). The most studied among them are classified into four distinct subfamilies (Widmann et al., 1999). These include extracellular signal-regulated kinases 1 and 2
Figure 1. Regulation of MAPK signaling by MEK kinases
A. General representation of the MEKK/MEK/MAPK signaling module. B. MEK kinase network in the regulation of MAPK pathway functions
(ERK1/2), Jun amino-terminal kinases 1, 2 and 3 (JNK 1/2/3), p38 proteins alpha, beta, gamma and delta (p38α/β/γ/δ) and extracellular signal-regulated kinase 5 (ERK5) (Widmann et al., 1999). Each MAPK subfamily is distinctly regulated and carries out distinct biological functions (Fig. 1B). ERK1/2 are predominantly implicated in the regulation of cell proliferation, growth, differentiation and survival. JNK and p38 are stress-response MAPKs, involved in apoptosis, stress responses to different cellular conditions or agents as well as cellular differentiation and some immune functions (Widmann et al., 1999). ERK5 is proposed to regulate cell survival and proliferation (Dong et al., 2001; Kato et al., 1998; Pearson et al., 2001a).

To ensure the integrity of intracellular processes, activation of a given MAPK cascade within a certain biological context must be precisely regulated (Pearson et al., 2001b; Widmann et al., 1999). Several biochemical mechanisms within a MAPK module ensure the specificity of MAPK signaling. First of all, the preferential phosphorylation of a certain set of intracellular targets by a given MAPK is conditioned by the intrinsic structural properties of a particular MAPK subfamily and its substrates. Despite the fact that all MAPKs phosphorylate their substrates at a similar consensus Ser/Thr-Pro sequence (Davis, 1993), substrate recognition by particular MAPKs varies depending upon the availability of appropriate docking sites (Fantz et al., 2001; Gonzalez et al., 1991; Gupta et al., 1996; Tanoue and Nishida, 2003).

The specificity of MAPK signaling is controlled as well on the level of MEK. Very specific combinations of MEK and MAPK are formed within the MAPK modules. MEK 1/2 activates ERK1/2, MEK 4/7 activates JNKs, MEK 3/6 activates p38s and
MEK5 activates ERK5 (Fig. 1B) (Pearson et al., 2001b; Widmann et al., 1999). This remarkable feature of the MAPK cascade organization prohibits cross-signaling on the level of MAPK kinases, thereby contributing to selective activation of a particular MAPK. It is well established that MAPK needs to be phosphorylated at both tyrosine and threonine residues within the TEY activation loop for its full activation (Payne et al., 1991). This phosphorylation is exerted by MEKs, dual specific kinases, which phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues (Nakielny et al., 1992; Zheng and Guan, 1993). Interestingly, this dual phosphorylation of MAPK is exerted in two phases (Ferrell and Bhatt, 1997) and, thus, serves as a threshold mechanism in activation of MAPK signaling (Ferrell, 1999).

The specificity of MAPK signaling regulation on the level of MEK kinases appears to be much more complex. There is a vast variety of MEK kinases, which are defined as enzymes that catalyze transfer of phosphate from ATP to hydroxyl side-chains of serine and threonine residues within MEK’s activation segment, inducing their activation (Zheng and Guan, 1994). Cumulative evidence demonstrates that some MEK kinases can activate more than one MEK as well as a certain MEK/MAPK cascade can respond to more than one MEK kinase (Fanger et al., 1997; Widmann et al., 1999; Xia et al., 2000; Yujiri et al., 1998) (Fig. 1B). Therefore, it is thought that MEK kinases create a signaling network that allows diversity of signaling inputs to trigger specific MAPK pathways (Widmann et al., 1999).

In summary, the MAPK pathway represents an evolutionary conserved signaling module implicated in the regulation of a variety of cellular processes. The core module
of the MAPK pathway consists of three kinases (MEK kinase, MEK and MAPK) that are sequentially activated via phosphorylation. The MAPK family is divided into four subfamilies, which are distinctly regulated and serve distinctive functions. Several intramodular layers of biochemical regulation ensure signaling specificity of MAPK pathways. These include selectivity of the MAPK-substrate reaction, activation of MAPK by a specific MEK and the capacity of the MAPK pathways to be linked to diversity of up-stream signals via a variety of MEK kinases.

**ERK1/2 cascade and mitosis**

ERK1/2 signaling has traditionally been implicated in the regulation of cell cycle initiation and G1/S transition following mitogenic stimulation (Kolch, 2000; Pearson et al., 2001b). However, evidence from different experimental systems demonstrates that the ERK1/2 pathway is an important regulator of the later stage of the cell cycle, mitosis. It is proposed that the ERK1/2 cascade regulates G2/M transition, formation of the mitotic spindle, the mitotic spindle checkpoint, exit from mitosis, Golgi fragmentation and the duration of mitosis (Fig. 2).

The first evidence implicating ERK1/2 in M-phase came from studies in *Xenopus* egg extracts. It has been shown that activation of the *Xenopus* homologue of ERK2, p42 MAPK, occurs during M-phase of the cell cycle (Gotoh et al., 1991a; Gotoh et al., 1991b; Minshull et al., 1994; Takenaka et al., 1997). Later works have demonstrated that the
Figure 2. ERK1/2 functions and localization during mitosis
ERK activity is necessary for proper spindle formation, metaphase/anaphase transition and overall mitotic timing. Localization of active (phosphorylated) MAPK to mitotic structures is shown schematically in red. Note that localization of monophospho-Y-ERK to Golgi is not shown.
ERK1/2 cascade is activated as well during mitosis in somatic cells (Harding et al., 2003; Roberts et al., 2002; Shapiro et al., 1998; Willard and Crouch, 2001; Zecevic et al., 1998).

Functional studies in somatic cell systems provided evidence that ERK1/2 signaling is important for proper transition to mitosis. Three different experimental settings have been used to study mitotic entry under the MEK/ERK1/2 loss-of-function conditions. Specifically, it was shown that expression of dominant-negative MEK (Wright et al., 1999), treatment of synchronized cell populations with PD98059 or U0126, MEK specific inhibitors, (Roberts et al., 2002; Wright et al., 1999) and depletion of MEK1/2 or ERK1/2 from synchronized cells by RNA interference (RNAi) technique (Liu et al., 2004) induced a G2/M arrest (Liu et al., 2004; Roberts et al., 2002; Wright et al., 1999), delayed and reduced activation of Cdk1/cyclin B (Liu et al., 2004; Wright et al., 1999), decreased nuclear translocation of cyclin B (Roberts et al., 2002), decreased reactivity with MPM-2 antibodies that recognize non-specific mitotic phosphoproteins (Wright et al., 1999) and reduced phosphorylation of histone H3 (Liu et al., 2004; Roberts et al., 2002). Thus, it was proposed that MEK/ERK signaling is important for Cdk1 activation and mitotic initiation.

Contrary to this, studies in Xenopus egg extracts showed that activation of Cdk1/cyclin B is not affected in p42 MAPK depleted extracts (Guadagno and Ferrell, 1998; Takenaka et al., 1997). Furthermore, constitutive activation of p42 MAPK in Xenopus egg extracts prior to mitosis inhibits Cdk1/cyclin B and delays M-phase onset (Bitangcol et al., 1998; Walter et al., 1997). Additionally, it was demonstrated that p42
MAPK can directly phosphorylate and activate Wee1, a kinase that inactivates Cdk1/cyclin B (Walter et al., 2000). Thus, involvement of ERK1/2 in regulation of Cdk1/cyclin B activity and mitotic onset remains disputable.

Early works have characterized ERK1/2 as a potential regulator of cytoskeleton functions. It was shown that active ERK1/2 phosphorylates components of the cytoskeleton (Hoshi et al., 1992; Ray and Sturgill, 1987; Verlhac et al., 1993) and largely associates with microtubule cytoskeleton in vivo (Reszka et al., 1995). Functional studies demonstrated that an increase in microtubule dynamics, typical for M-phase, depends on the presence of active ERK1/2. Specifically, it was shown that introduction of active p42 MAPK from M-phase to S-phase Xenopus egg extracts dramatically increases tubulin de-polymerization (Gotoh et al., 1991b). Consistently, the abrogation of p42 MAPK signaling in M-phase extracts via inhibition of MEK, immunodepletion of MEK or p42 MAPK dramatically decreases tubulin de-polymerization (Guadagno and Ferrell, 1998; Horne and Guadagno, 2003).

Fluorescent studies further supported implication of ERK signaling in the regulation of the mitotic apparatus. In 3T3 and Ptk1 cells, it was shown that active forms of ERK1/2 and MEK localize to the kinetochores from early prometaphase through mid-anaphase, to the spindle and spindle poles throughout mitosis, and the midbody during anaphase (Shapiro et al., 1998; Willard and Crouch, 2001; Zecevic et al., 1998) (Fig. 2). A similar pattern of intracellular p42 MAPK distribution was observed on mitotic spindles formed in Xenopus egg extracts (Horne and Guadagno, 2003) as well as in fertilized sea urchin eggs (Zhang et al., 2005).
The involvement of ERK1/2 signaling in formation of the mitotic spindle was directly shown by loss-of-function studies. Specifically, it was demonstrated that immunodepleting of p42 MAPK or blocking its activation during mitosis with a MEK inhibitor, U0126, induces abnormal spindle formations in mitotic *Xenopus* egg extracts and compromises stability of already formed spindles (Horne and Guadagno, 2003). Further, abrogation of mitotic spindle formation was observed as well in somatic cells pre-treated with U0126 at the late G2 (Horne and Guadagno, 2003). Recently, an altered formation of mitotic spindles and chromosome attachment was reported as well in sea urchin fertilized eggs treated with dominant-negative MEK 1/2 or U0126 (Zhang et al., 2005).

It was suggested that one of the substrates through which p42 MAPK may exert its spindle formation function is a kinetochore motor protein CENP-E (Zecevic et al., 1998). CENP-E is essential for bi-polar attachment of chromosome to microtubules (Yao et al., 1997). During mitosis p42 MAPK directly associates with and phosphorylates CENP-E at sites important for its association with microtubules (Zecevic et al., 1998).

More than a decade ago it was shown that an injection of thiophosphorylated ERK1/2 (Haccard et al., 1995) or c-Mos (Sagata et al., 1989) into *Xenopus* embryos induces metaphase arrest. Further, the addition of constitutively active MEK (Takenaka et al., 1997) or c-Mos (Chau and Shibuya, 1998; Guadagno and Ferrell, 1998) into mitotic *Xenopus* egg extracts maintains an M-phase-like state even following cyclin B degradation and inactivation of Cdk1/cyclin B. Consistently, blocking mitotic p42 MAPK activation by MEK immunodepletion or MEK inhibition shortens the duration of
M-phase in cycling *Xenopus* egg extracts (Guadagno and Ferrell, 1998). Thus, ERK1/2 signaling can induce a state similar to mitotic arrest and control the timing of mitosis.

*Xenopus* egg extracts have been utilized to address the involvement of p42 MAPK in the mitotic checkpoint. Two different experimental settings were used to mimic the mitotic spindle arrest. First, *Xenopus* egg extracts were supplemented with high concentrations of sperm nuclei and treated with nocodazole (Minshull et al., 1994). Second, UV-irradiated nuclei were added to extracts, which then were driven into mitosis by the addition of constitutively active Cdc25C phosphatase (Chau and Shibuya, 1999). These manipulations arrested extracts in an M-phase like state with high levels of Cdk1/cyclin B and ERK1/2 activities. Importantly, in both experimental systems p42 MAPK activity was critical for the maintenance of the mitotic arrest, which was confirmed by MAPK immunoprecipitation, treatment with a MAPK specific phosphatase (MKP1) (Minshull et al., 1994) and treatment with a MEK inhibitor, PD98059 (Chau and Shibuya, 1999).

Another study, which linked ERK1/2 to mitotic arrest functions, was a genetics analysis of *Drosophila*’s rolled/MAPK gene (Inoue and Glover, 1998), which showed that loss-of-function mutations of this gene abrogate the mitotic arrest upon treatment with colchicine (an inhibitor of microtubule polymerization).

It was proposed that ERK1/2 may exert its spindle checkpoint function via regulation of Cdc20, an activator of APC (Visintin et al., 1997) and a component of the anaphase-inhibitory protein complexes (Chan and Yen, 2003; Hardwick, 2005; Yu, 2002). It was shown that during mitotic arrest in *Xenopus* egg extracts ERK1/2 directly
phosphorylates Cdc20 at Thr-64 and Thr-68 residues (Chung and Chen, 2003). A Cdc20 mutant non-phosphorylatable at these (and two other) phosphorylation sites malfunctions during induction of the mitotic arrest: it does not form complexes with BubR1 and Mad2 and causes a decline in Cdk1 activity under the mitotic spindle checkpoint conditions. The same phenotype was observed when ERK1/2 activity was blocked with MEK inhibitors, U0126 or PD98059, which further links these Cdc20 functions to ERK1/2 signaling (Chung and Chen, 2003).

Regulation of Golgi fragmentation is another function of ERK signaling during mitosis. It was shown that phosphorylated MEK associates with membrane fractions in mitotic cells (Harding et al., 2003), localizes to Golgi during prophase (Colanzi et al., 2003) and is necessary for Golgi disassembly (Acharya et al., 1998). Further, a Golgi reassembly stacking protein of 55 kDa (GRASP55) was reported to be one possible *in vivo* target of the MEK/ERK pathway during mitosis (Jesch et al., 2001). Interestingly, further studies revealed that a non-traditional ERK cascade regulates Golgi fragmentation during mitosis. First, it was shown that MEK1, associated with membranes during mitosis, undergoes a Cdk1/cyclin B dependent cleavage (Harding et al., 2003), which blocks its interaction with full-length ERK (Harding et al., 2003). Subsequent studies showed that a novel alternatively spliced variant of ERK1, ERK1c, is involved in the regulation of Golgi fragmentation during mitosis (Aebersold et al., 2004; Shaul and Seger, 2006). ERK1c is regulated in an M-phase dependent manner, undergoes a preferential mono-tyrosine phosphorylation within the TEY activation loop and localizes to Golgi during early stages of mitosis (Shaul and Seger, 2006). Two speculations can be
made based on these studies. First, the mechanisms of ERK1/2 pathway activation during mitosis may be different from those during cell cycle entry. Second, activation of different functional pools of mitotic ERK may be regulated differentially.

In summary, ERK1/2 signaling displays a variety of roles in mitotic regulation. It is involved in the control of the G2/M transition, spindle formation, the mitotic checkpoint as well as Golgi fragmentation. Therefore, disruption of ERK1/2 functions during mitosis can impair the mitotic regulation at different levels and compromise the fidelity and/or order of mitotic events. Indeed, the constitutive activation of ERK1/2 signaling in v-mos or v-ras infected 3T3 fibroblasts provokes genomic instability via disregulation of mitosis (Fukasawa and Vande Woude, 1997; Saavedra et al., 1999). Further, it is proposed that the high incidence of cancer in patients with hereditary tyrosinemia type I (Weinberg et al., 1976) and hepatitis B (Margolis et al., 1991) might be a result of genomic instability caused by non-controlled ERK1/2 activation at mitosis (Jorquera and Tanguay, 2001; Yun et al., 2004).

Therefore, deciphering functions and regulation of the ERK1/2 pathway during mitosis is important for generation of a comprehensive vision of its role throughout the cell cycle. It will also contribute to understanding biochemical mechanisms underlying pathologies, which is necessary for future development of efficient medical diagnostics and treatment approaches.
Regulation of ERK1/2 signaling by MEK kinases

MEK kinases represent a key regulatory level in the MAPK module, where many different stimuli can converge to allow specific activation of a certain MEK/MAPK pathway (Fanger et al., 1997; Widmann et al., 1999; Xia et al., 2000; Yujiri et al., 1998, 2000). Consistent with this, activation of the MEK/ERK1/2 pathway under different cellular circumstances is regulated by different MEK kinases. Indeed, the ERK1/2 signaling during cell cycle entry and meiosis is activated by Raf-1 and c-Mos, respectively.

The cell cycle initiation is triggered by engagement of extracellular growth receptors and their oligomerization (Yarden and Schlessinger, 1987). This, in turn, stimulates conversion of a small GTP-ase protein Ras from an inactive GDP-bound to an active GTP-bound form (Kolch, 2000; Pearson et al., 2001b). Ras-GTP mediates membrane translocation and activation of Raf-1, a classical MEK kinase that triggers the MEK/ERK/1/2 cascade during the G0-G1-S transition. Activated MAPK directly phosphorylates cytoplasmic and cytoskeletal substrates or translocates into the nucleus to modulate transcription factors, which together promote progression into S-phase (Kolch, 2000; Pearson et al., 2001b).

Mechanism of ERK activation and its functions are strikingly different during meiosis. It is well established that hormonal stimulation triggers oocyte maturation, at least partially, via induction of synthesis of c-Mos, a MEK kinase that activates the MEK/ERK pathway during meiosis (Castro et al., 2001). With slight variations among
species, activated ERK during meiosis is involved in activation of MPF (Cdk1/cyclin B), maintenance of MPF activity between MI and MII of meiosis and, finally, in induction of the CSF (cytostatic factor) arrest of mature oocytes (Castro et al., 2001). Thus, activation of the ERK pathway by different MEK kinases may condition specificity of its cellular functions.

The underlying biochemical mechanisms that control activation of the MEK/ERK cascade during mitosis are poorly understood. It is unclear whether Raf-1 is involved in the regulation of the ERK pathway during mitosis. It was shown that Raf-1 undergoes hyperphosphorylation and activation in cells arrested at mitosis with nocodazole treatment (Laird et al., 1995; Pathan et al., 1996; Ziogas et al., 1998). Interestingly, this phosphorylation and activation of Raf-1 is thought to be directly regulated by Src and occur in a Ras-independent (Ziogas et al., 1998), but Rac/Cdc42/Pak-dependent manner (Zang et al., 2001). However, activation of Raf-1 at mitosis does not correlate with activation of the MEK/ERK cascade (Laird et al., 1999; Ziogas et al., 1998). Furthermore, none of the published works provide conclusive evidence for mitotic activation of Raf-1 in naturally cycling somatic cells. As well, it has not been clearly demonstrated that Raf-1 in mitotic cells is linked to some of MAPK’s mitotic functions. A study by Yue and Ferrell detected small amounts of germ-cell specific MEK kinase, c-Mos, in *Xenopus* egg extracts and provided evidence that c-Mos was important for activating the p42 MAPK pathway at mitosis (Yue and Ferrell, 2004). However, earlier studies show that c-Mos is efficiently degraded following egg fertilization (Nishizawa et al., 1993; Watanabe et al., 1991; Watanabe et al., 1989) and its expression is repressed in
somatic tissues (Xu and Cooper, 1995; Zinkel et al., 1992). Therefore, the involvement of Raf-1 and c-Mos in mitotic MAPK signaling is speculative and perhaps another MEK kinase is required for activation of the MAPK pathway at mitosis.

In conclusion, a variety of MEK kinases allows activation of the MAPK pathways by different stimuli. It is established that Raf-1 triggers activation of ERK1/2 signaling following mitogenic stimulation, whereas c-Mos activates the p42 MAPK cascade during meiosis. Circumstantial evidence indicates that neither Raf-1 nor c-Mos are involved in the regulation of the ERK cascade during mitosis. Thus, the identity of the mitotic MEK kinase remains elusive.

**Functions and structural organization of Raf family members**

Rafs are serine/threonine kinases, which are broadly implicated in regulation of many basic cellular processes, such as cell growth, proliferation, survival, differentiation and migration (Chong et al., 2001; Chong et al., 2003; Pearson et al., 2001b). In mammals, the family of Raf kinases is comprised of three members, Raf-1 (Kan et al., 1984; Rapp et al., 1983), A-Raf (Huleihel et al., 1986) and B-Raf (Calogeraki et al., 1993; Eychene et al., 1992), which are characterized by a substantial degree of homology and some overlapping functions.

Biochemical analyses demonstrate that the vast majority of intracellular Raf functions are exerted via MAPK signaling. Raf regulates cell growth and proliferation through MEK/ERK dependent promotion of the cell cycle progression (Roovers and
Assoian, 2000). It is demonstrated that at the G0/G1 border of the cell cycle the Raf/MEK/ERK pathway is involved in down-regulation of Cdk inhibitor p27, transcriptional up-regulation of cyclin D and other events that favor activation of the cyclin-dependent cell cycle machinery (Kerkhoff and Rapp, 1998). The cell survival functions of Rafs are exerted via the MEK/ERK-mediated activation of anti-apoptotic kinase Akt (von Gise et al., 2001), transcriptional repression of pro-apoptotic BH3-only members of the Bcl2 family (for instance, Bim) (Weston et al., 2003) and up-regulation of IAPs (Wiese et al., 2001). The longevity of the intracellular ERK signaling conditioned by kinetics of Raf activation determines the proliferation/differentiation decision of a cell (Brummer et al., 2002; York et al., 1998). It is proposed that prolonged ERK activation, directed by B-Raf, induces cell differentiation, whereas transient ERK activation via Raf-1 causes their proliferation (York et al., 1998). Raf family members as well are implicated in the regulation of cytoskeleton rearrangements necessary for cell migration, which is critical for embryogenesis, wound healing, angiogenesis, immune functions and tumor metastasis (Ehrenreiter et al., 2005; Pritchard et al., 2004).

Analysis of Raf functions on an organismal level demonstrated that members of the Raf family are critical for life. Indeed, A-Raf knockout mice display intestinal and neurological abnormalities, which can lead to postnatal death or survival with severe neurological defects (Wojnowski et al., 1998). Complete Raf-1 knockout mice display serious embryonic aberrations: growth retardation, placenta anomalies and under-development of the liver due to massive apoptosis of hepatocytes. These mice die at midgestation (Mikula et al., 2001). B-Raf-deficient mouse embryos are characterized by
massive apoptosis, causing aberrant vasculature development and vascular hemorrhage. These mice as well die at midgestation (Wojnowski et al., 2000; Wojnowski et al., 1997).

All three members of the Raf kinase family, Raf-1, B-Raf, and A-Raf, share a common architecture of domain organization. They all contain three large conserved regions: CR1, CR2, and CR3, and are functionally divided into the N-terminal regulatory domain and the C-terminal catalytic domain (Fig. 3) (Daum et al., 1994; Morrison and Cutler, 1997). CR1 and CR2 conserved regions are located in the N-terminal regulatory portion of Raf protein, whereas CR3 houses the C-terminal kinase domain. The N-terminal CR1 and CR2 conserved regions within the regulatory domain harbor protein binding and regulatory phosphorylation sites. Specifically, CR1 contains a Ras binding domain (RBD) and a cysteine rich domain (CRD). These sites are critical for Raf interaction with up-stream regulators: small GTP-ases, such as Ras and Rap1 (Chuang et al., 1994) (Fig. 3). The CR2 region contains a phosphorylation site, which is involved in negative regulation of Raf activity (Guan et al., 2000; Morrison et al., 1993; Zimmermann and Moelling, 1999) and represents a 14-3-3 binding phospho-epitope (Michaud et al., 1995) (Fig. 3). The kinase domain within the C-terminal conserved CR3 portion of Raf protein harbors two important phosphorylation regulatory sites: the N-region (Fabian et al., 1993; King et al., 1998; Mason et al., 1999) and activation loop (Chong et al., 2001; Zhang and Guan, 2000) (Fig. 3). Analogous to other kinases, it is thought that the phosphorylation within the activation loop of Raf induces conformational changes, which favor interaction of the ATP binding site with ATP (Huse and Kuriyan, 2002; Johnson and Lewis, 2001). Interestingly, unlike other Raf family members,
Figure 3. Domain organization of Raf family members
All members of the Raf family possess three conserved regions – CR1 and CR2, which comprise an N-terminal regulatory domain, and CR3, which comprises the catalytic domain. Note that B-Raf, unlike other members of the family, contains as well a unique N-terminal domain (shown in shaded box).
B-Raf contains a unique N-terminal domain, which may be involved in B-Raf specific regulation or functions (Fig. 3) (Kalmes et al., 1998).

In conclusion, Raf kinases are broadly implicated in a variety of cellular processes and are critical for proper organismal development and function. Members of the Raf kinase family share a significant sequence homology and a similar functional domain organization, with some exceptions for B-Raf.

**Regulation of B-Raf activity by phosphorylation and protein-protein interactions**

It is traditionally assumed that all members of Raf family are regulated by similar mechanisms. Indeed, all three Rafs have the same basic functional organization: N-terminal regulatory domain and the N-region and activation loop in the C-terminal kinase domain. It is believed that in an inactive state the N-terminal regulatory domain of Raf directly binds to the C-terminal kinase domain, blocking its accessibility (Heidecker et al., 1990). Thus, activation of Raf can be interpreted as “opening” of Raf’s tertiary structure by disrupting association between the N-terminal and C-terminal domains (Kolch, 2000). This is achieved by a series of coordinated regulatory phosphorylation and protein-protein association events. It is proposed that active GTP-bound Ras directly binds to the RBD and CRD within CR1 region (Fig. 3) (Chuang et al., 1994; Morrison and Cutler, 1997; Nassar et al., 1995; Okada et al., 1999; Vojtek et al., 1993) and provokes Raf’s translocation to the plasma membrane and removal of the inhibitory dephosphorylation from the CR2 (Jaumot and Hancock, 2001; Kubicek et al., 2002;
Mitsuhashi et al., 2003). These predispose Raf for positive regulatory phosphorylations at the catalytic domain, which finally induce the open active conformation. Despite the over-all similarities in regulation of Raf-1 and B-Raf activation, the accumulated evidence indicates that regulatory mechanisms involved in activation of Raf-1 and B-Raf differ in many important aspects. First, Raf-1 and B-Raf require a different “dose” of the regulatory phosphorylation to achieve full activation. Indeed, following Ras-mediated stimulation, activation of Raf-1 occurs as a two-set phosphorylation (Marais et al., 1997). This includes Ras and Src dependent phosphorylation of Ser-338 and Tyr-341 residues in the N-region (Diaz et al., 1997; Fabian et al., 1993; Marais et al., 1995; Mason et al., 1999) and Ras dependent phosphorylation of Thr-491 and Ser-494 residues in the activation loop (Fig. 3) (Chong et al., 2003). In contrast to this, B-Raf does not undergo regulation in the N-region: its Ser-445 residue is constitutively phosphorylated (Mason et al., 1999) and the residue corresponding to Raf-1’s Tyr-341 is a phospho-mimicking Asp-448 residue (Fig. 4). Hence, Ras-mediated activation of B-Raf requires only phosphorylation at Thr-599 and Ser-602 residues within the activation loop (Fig. 4) (Zhang and Guan, 2000). It is proposed that the constitutive presence of the negative charge in the N-region of B-Raf inhibits intramolecular association between the regulatory and catalytic domains (Tran et al., 2005), which explains the higher basal level activity of B-Raf compared to Raf-1 (Marais et al., 1997).
Figure 4. Phosphorylation in regulation of B-Raf activity

Activity of Human B-Raf is regulated both positively and negatively via phosphorylation. Positive regulation by phosphorylation includes: 1) constitutive phosphorylation at the N-region (constitutively phosphorylated Serine-445 and phosphorylation-mimicking Aspartic acid-448); and 2) Ras-dependent phosphorylation at the activation loop (Threonine-599 and Serine-602 residues). Both regulatory units are located within the C-terminal CR3 (catalytic) domain of B-Raf (shown in yellow). Phosphorylation at Serine-364, Serine-428 and Threonine-439 residues negatively regulates B-Raf and is executed by Akt. B-Raf is also phosphorylated by ERK at the C-terminal Serine-750 and Threonine-753 residues, but the effect of this phosphorylation on B-Raf activity is unknown.
Both B-Raf and Raf-1 undergo an inhibitory phosphorylation by Akt. However, Akt targets B-Raf at three residues (Ser-364, Ser-428 and Thr-439) (Chong et al., 2001; Guan et al., 2000) (Fig. 4), whereas Raf-1 at only one site (Zimmermann and Moelling, 1999). Interestingly, one of Akt’s phosphorylation sites in B-Raf, the Ser-364 residue, can as well be phosphorylated by SGK, a serum and glucocorticoid inducible kinase, which demonstrates homology to Akt (Zhang et al., 2001).

Recent work demonstrated that human B-Raf undergoes a feedback phosphorylation by ERK at Ser-750 and Thr-753 residues unique to B-Rafs C-terminal (Brummer et al., 2003) (Fig. 4). The effect of this phosphorylation on B-Raf activity was not determined. However, it is suggested that phosphorylation of B-Raf’s C-terminus may negatively affect B-Raf functions, such as cell differentiation (Brummer et al., 2003; Rushworth et al., 2006).

B-Raf activity is regulated as well on the level of protein-protein interactions. Several B-Raf’s interactors have been identified (Fig. 5), which can be classified in two groups. The first group comprises associations with the signaling and structural components of the MAPK module. These include B-Raf’s direct associations with its upstream activators, small GTP-ases, and down-stream target, MEK, association of B-Raf with other components of MAPK cascade via scaffolding proteins and formation of B-Raf/Raf-1 heterodimers. The second group of B-Raf’s protein partners includes non-enzymatic proteins outside of the MAPK cascade module, such as 14-3-3 protein and chaperone protein HSP90.
As mentioned above, B-Raf and Raf-1 directly associate with GTP-ases Ras and Rap1 (Berruti, 2000; Houslay and Kolch, 2000; Papin et al., 1996; Vossler et al., 1997; Yamamori et al., 1995; York et al., 1998). Interestingly, B-Raf and Raf-1 differ in their response to up-stream regulation by Rap1. Notably, Rap1 activates B-Raf and B-Raf-dependent activation of MAPK, but inhibits the Raf-1/MEK/ERK cascade (Bos et al., 2001; Houslay and Kolch, 2000; York et al., 1998).

B-Raf directly associates with MEK (Papin et al., 1996; Papin et al., 1995) and is characterized by a higher affinity to it than Raf-1 (Papin et al., 1998), which can explain the fact that B-Raf is a much stronger activator of MEK, compared to Raf-1 or A-Raf.
(Mason et al., 1999; Zhang and Guan, 2000). Interestingly, B-Raf undergoes a unique mode of additional regulation, alternative splicing (Barnier et al., 1995), which can modulate affinity of different B-Raf isoforms toward MEK (Papin et al., 1998).

The concept of integration of all components the MAPK signaling pathway within one functional unit was developed through studying mating and osmosensor pathways in budding yeast (Faux and Scott, 1996). Metazoan scaffolding proteins were identified by genetic screens in *Drosophila* and *C. elegans*. They include KSR (kinase suppressor of Ras), Soc-2 (suppressor of clear homolog) and CNK (connector enhancer of KSR). All of these proteins serve a structural framework for efficient interaction of Raf, MEK and ERK. To accomplish this they have to fulfill three criteria: 1) accommodate simultaneous interaction with all components of the MAPK module (Cacace et al., 1999; Stewart et al., 1999); 2) facilitate activation within the module (Li et al., 2000; Roy et al., 2002) and 3) may not possess any kinase activity (Cacace et al., 1999; Morrison, 2001; Stewart et al., 1999; Yu et al., 1998). It is assumed that B-Raf signaling, like Raf-1’s, is facilitated by scaffolding proteins.

Early work showed that a forced Raf oligomerization induces Ras dependent Raf activation (Luo et al., 1996). Furthermore, B-Raf was purified as a component of a Ras-dependent activator of Raf-1 (Mizutani et al., 1998). Thus, mutual association and regulation among Raf members was suggested. A more recent study directly showed that endogenous Raf-1 and B-Raf form heterodimers in response to Ras stimulation (Weber et al., 2001). Biological significance of this interaction is not fully understood. However, it
was shown that B-Raf/Raf-1 heterodimers possess a higher activity than corresponding monodimers (Rushworth et al., 2006).

14-3-3 protein is important for regulation of B-Raf activity (Kolch, 2000). Phosphorylation of B-Raf at Ser-364 residue creates a 14-3-3 binding motif, which analogously to Raf-1, may be necessary for locking B-Raf in an inactive conformation (Morrison and Cutler, 1997). Additionally, it is proposed that 14-3-3 protein contributes to activation of B-Raf signaling at least by facilitating B-Raf heterodimerization with Raf-1 (Garnett et al., 2005; Rushworth et al., 2006).

B-Raf has been shown to associate with heat-shock protein 90 (HSP90) (Grammatikakis et al., 1999; Jaiswal et al., 1996). It is accepted that association of HSP90 with B-Raf maintains B-Raf’s proper folding and functionality (Cissel and Beaven, 2000; Schulte et al., 1995).

In summary, regulation of B-Raf activation and function is exerted via controlled coordination of B-Raf phosphorylation and its association with regulatory, structural and effector proteins. Similar to other members of the Raf kinase family, activation of B-Raf involves unfolding of its inactive conformation. However, B-Raf stands apart due to several unique regulatory features. Unlike Raf-1, B-Raf is constitutively phosphorylated within the regulatory N-region, can respond differentially to an up-stream signaling by GTP-ases and can undergo alternative splicing, which may regulate B-Raf’s affinity toward MEK. B-Raf’s association with other proteins significantly contributes to regulation of its activity and functions. The most well studied interactions include: association with Ras and 14-3-3, formation of Raf/MEK/ERK signalosomes via
scaffolding proteins, B-Raf/Raf-1 heterodimerization, and stabilization of B-Raf molecules by association with chaperone HSP90.

**B-Raf as an oncogene**

Members of Raf kinase family were identified as proto-oncogenes (Jansen et al., 1984; Sutrave et al., 1984). However, extensive studies of Raf-1 mutations did not provide strong evidence for Raf-1 being involved in tumorogenesis. This situation was drastically changed in 2002 when B-Raf was claimed to be a potential cause of a significant proportion of human cancers (Davies et al., 2002). This key study, which performed a wide-range genomic screening of human tumors, demonstrated that B-Raf is mutated in 8% of all human tumors, including nearly 70% of melanomas, 14% of ovarian cancers, 14% of liver cancers and 12% of colorectal tumors (Davies et al., 2002). All of the detected B-Raf mutations were within its kinase domain. Interestingly, the overwhelming proportion of these mutations was represented by a single amino acid substitution: Val-600-Glu (B-Raf\textsuperscript{V600E}). It accounted for 80% of all mutations and for more than 90% mutations in melanomas (Davies et al., 2002). As described in the previous section, phosphorylation of the residues Thr-599 and Ser-602 flanking the mutated site is critical for full activation of B-Raf (Zhang and Guan, 2000). Therefore, it is not surprising that the phospho-mimicking Val-600-Glu mutation in the activation loop dramatically increases B-Raf’s activity (Davies et al., 2002). B-Raf\textsuperscript{V600E} induces transformation of NIH3T3 cells and cultured melanocytes via over-activation of the
MEK/ERK pathway (Davies et al., 2002; Wellbrock et al., 2004) and tumorigenicity in nude mice (Wellbrock et al., 2004). Further, treatment of melanoma cell lines harboring B-Raf$^{V600E}$ with Raf specific inhibitor BAY 43-9006 induces cell cycle arrest or apoptosis (Sharma et al., 2005).

Since the B-Raf$^{V600E}$ mutant displays all of major features of an oncogene and is expressed in a substantial percentage of melanomas, extensive studies focused on elucidating how it may be involved in the oncogenicity of melanoma. The accumulated evidence suggests that mutation of B-Raf occurs during the early benign stages of melanocyte dysplasia (nevi formation) and the B-Raf mutation must be combined with other genetic abnormalities to become malignant. Indeed, B-Raf$^{V600E}$ mutation was detected in more than 80% of nevi, which can remain quiescent for decades without progressing into malignancy (Michaloglou et al., 2005). Expression of B-Raf$^{V600E}$ mutant in human melanocytes resulted in their senescence-like arrest (Michaloglou et al., 2005) or in formation of non-malignant nevi in transgenic animals (Patton et al., 2005). Importantly, transgenic zebrafish carrying the oncogenic B-Raf on a p53 deficient background progressively developed invasive melanomas (Patton et al., 2005).

In conclusion, B-Raf has recently been recognized as a cellular oncogene. Its most frequently mutated version, B-Raf$^{V600E}$, is expressed in a large proportion of human cancers, ranking the highest in melanomas. This B-Raf mutant displays all the hallmarks of a classical oncogene: it possesses a greatly elevated kinase activity, promotes constitutive non-controllable ERK signaling, induces transformation of cultured cell lines and is required for survival of these tumors. It is proposed that B-Raf mutation occurs at
the early stages of cellular dysplasia and can provoke tumorigenesis in combination with other mutations. Identification of B-Raf as a critical oncogene highlights the importance of research focused on the elucidation of B-Raf regulatory mechanisms and B-Raf functions.

**Studying MAPK signaling in the cell-free system of *Xenopus* egg extracts**

Since the introduction of amphibian egg extracts as a research tool (Lohka and Masui, 1983), they have been shown to faithfully mimic many aspects of the cellular cell cycle and have been used to study a variety of cell cycle regulated processes. These include DNA replication (Arias and Walter, 2004; Blow and Laskey, 1986; Tutter and Walter, 2006), nucleus formation (Chan and Forbes, 2006; Hutchison et al., 1988; Lohka, 1998), vesicle fusion (Tuomikoski et al., 1989), cytoskeleton rearrangements (Belmont et al., 1990; Mandato et al., 2001), mitotic spindle formation and function (Desai et al., 1999; Horne and Guadagno, 2003; Maresca and Heald, 2006; Tremethick, 1999), apoptosis (Deming and Kornbluth, 2006) and oocyte maturation (Crane and Ruderman, 2006; Ohsumi et al., 2006).

The main advantage of the cell-free system of *Xenopus* egg extracts is that it permits many technical manipulations not feasible in tissue culture cells. This greatly increases the range of biochemical approaches which can be used for cell cycle analysis. Indeed, egg extracts are homogenous and synchronized for cell cycle progression, which is not the case for proliferating tissue culture cells. Importantly, extracts are much more
suitable for biochemical fractionation, immunoprecipitation and immunodepletion, and morphological analysis of sub-cellular structures.

The cell-free system of *Xenopus* egg extracts is a great experimental model for studying MAPK signaling. It has been repeatedly shown that the MAPK cascade is activated throughout meiosis in *Xenopus* oocytes (Abrieu et al., 2001; Gotoh et al., 1995; Gotoh et al., 1991a; Gotoh et al., 1991b; Kosako et al., 1994) and during M-phase of the subsequent embryonic cycles (Guadagno and Ferrell, 1998; Hartley et al., 1994; Takenaka et al., 1997). This implies that extracts prepared from *Xenopus* oocytes at different maturation stages or after fertilization can recapitulate MAPK signaling under different biological contexts, meiosis or mitosis respectively.

Several standard protocols have been developed in order to prepare *Xenopus* egg extracts that represent MAPK signaling during different stages of meiosis. Specifically, extracts prepared from immature oocytes arrested in prophase I of meiosis are used for studying MAPK regulation and functions during the G2/M transition and meiosis (Crane and Ruderman, 2006). Extract preparations derived from fully developed CSF (cytostatic factor) arrested *Xenopus* oocytes (Posada et al., 1993; Sagata et al., 1989) are a valuable research tool for studying MAPK signaling involved in meiotic metaphase arrest (Fig. 6) (Lohka and Maller, 1985; Nebreda and Hunt, 1993).

Extracts prepared from activated *Xenopus* eggs are routinely used for recapitulating the early embryonic cell cycles of S- and M-phases. The transition of a *Xenopus* oocyte to the first mitotic cell cycle is triggered during fertilization by a transient increase in intracellular Ca^{2+} concentration (Cuthbertson and Cobbold, 1985;
Lorca et al., 1993). To reproduce this biochemical trigger, mature *Xenopus* oocytes are parthenogenetically activated *in vitro* by a brief treatment with Ca\(^{2+}\) ionophore (Fig. 6) (Chen et al., 1998) or by subjecting them to a quick electro-shock (Murray, 1991). Since extracts prepared from these eggs recapitulate embryonic cell cycling, which does not include G1 and G2 phases, activation of MAPK in cycling extracts is restricted to M-phase. Thus, mitotic MAPK activation does not overlap with MAPK activation at the G1/S, as it occurs in somatic cells. Therefore, the cycling *Xenopus* egg extracts is an ideal biochemical model to study regulation of the MAPK cascade at M-phase.

The synchronous embryonic cell cycling during the early stages of *Xenopus* embryogenesis as well as in *Xenopus* egg cycling extracts is conditioned primarily by oscillation of mitotic cyclin B levels (Murray and Kirschner, 1989a). Since *Xenopus* egg extracts are very amenable to biochemical manipulations, it is relatively easy to arrest them in S- or M-phase. Specifically, preparation of extracts from parthenogenetically activated oocytes in the presence of cycloheximide blocks synthesis of cyclin B and arrests them in S-phase (Fig. 6). Supplementing of these extracts with recombinant non-degradable cyclin B (which lacks a ubiquitination D-box) (Glotzer et al., 1991) permanently arrests them in M-phase (Fig. 6). This approach is routinely used to prepare unlimited quantities of experimental material synchronously arrested at the S- or M-phases of the cell cycle.
Mature CSF-arrested oocytes (arrested in the second metaphase of meiosis) are collected and directly processed into CSF extracts or parthenogenetically activated with Ca\textsuperscript{2+} ionophore A23087 prior to processing into S-, M-phase arrested or cycling extracts.

**Figure 6. Cell-free system of Xenopus egg extracts**
In summary, the cell-free system of *Xenopus* egg extracts is a powerful experimental model, which surpasses tissue culture cells by the scope of feasible biochemical manipulations. The system is based on the natural ability of *Xenopus* oocytes to autonomously sustain the cell cycling processes. Many different aspects of cell cycling and signal transduction mechanisms can be addressed by using *Xenopus* egg extracts. Particularly, the cell-free system of *Xenopus* egg extracts is an optimal biochemical model to dissect functions and regulation of the MAPK signaling during mitosis.
The MAPK cascade is an important regulator of the cell cycle (Widmann et al., 1999). It is evident that MAPK signaling is implicated in control of at least two stages of the somatic cell cycle, namely, G1/S transition (Roovers and Assoian, 2000; Widmann et al., 1999) and mitosis (Gotoh et al., 1991b; Guadagno and Ferrell, 1998; Harding et al., 2003; Minshull et al., 1994; Takenaka et al., 1997; Willard and Crouch, 2001; Zecevic et al., 1998). Regulatory mechanisms and functions of the MAPK pathway during cell cycle initiation are well understood and represent a classical dogma of molecular biology (Roovers and Assoian, 2000; Widmann et al., 1999). In contrast, roles for MAPK and its activation during mitosis are much less defined. Nevertheless, data from different experimental systems have revealed involvement of MAPK signaling in the regulation of a variety of mitotic functions: mitotic onset (Liu et al., 2004; Wright et al., 1999), formation of the mitotic spindle (Horne and Guadagno, 2003; Zhang et al., 2005), mitotic metaphase arrest (Chau and Shibuya, 1999; Minshull et al., 1994), Golgi fragmentation (Aebersold et al., 2004; Shaul and Seger, 2006) and the duration of mitosis (Guadagno and Ferrell, 1998; Roberts et al., 2002). Therefore, to obtain a comprehensive understanding of how MAPK signaling is integrated into the cell cycle regulatory network, it is critical to decipher the mechanism of MAPK activation during mitosis. Furthermore, understanding how MAPK is controlled during mitosis may shed light on the biochemical mechanisms behind some pathological states, such as cancer. Indeed, the MAPK pathway is constitutively activated in over 30% of human cancers (Hoshino et
al., 1999). Based on the fact that MAPK plays a variety of roles throughout mitosis, it is plausible to suggest that disregulation of mitotic functions of the MAPK pathway may cause genomic instability and provoke tumorogenesis.

The activation of the MAPK cascade under certain biological contexts is conditioned by specific activation of particular MEK kinases (Fanger et al., 1997; Widmann et al., 1999). Therefore, I hypothesize that a specific MEK kinase is activated in an M-phase dependent manner and activates the MEK/MAPK pathway at mitosis. To address this hypothesis I am going to use M-phase arrested *Xenopus* egg extracts to purify and identify mitotic MEK kinase activity. Subsequently, I will utilize biochemical approaches to study how this MEK kinase is regulated during mitosis. The results of my dissertation studies will determine the mechanism of regulating the activation of the MAPK cascade during mitosis and expand our understanding of how MAPK signaling is implicated in overall cell cycle regulation.
Chapter Two

Identification of B-Raf as an M-phase MEK Kinase

Introduction

In order to decipher the regulatory mechanisms involved in activating the MEK/MAPK pathway at mitosis, the M-phase MEK kinase must be identified. To achieve this, I utilized biochemical approaches in order to purify and directly identify the mitotic MEK kinase. This research strategy requires an appropriate experimental model that fulfills several requirements. First, it should fairly represent the biochemistry of mitotic activation of the MAPK cascade. Secondly, this experimental system should be amendable for biochemical manipulations. And thirdly, the experimental material should be available in quantities suitable for extensive biochemical purification. The cell-free system of *Xenopus* egg extracts surpasses the tissue culture cells in all of the above-mentioned requirements and represents an ideal biochemical experimental model for studying mitotic MAPK signaling. Therefore, my biochemical studies were performed by using the cell-free system of *Xenopus* egg extracts.

For this part of my studies, *Xenopus* egg extracts that are arrested in M-phase were utilized. To prepare these extracts, mature (CSF-arrested) *Xenopus* oocytes were
collected and parthenogenetically activated in vitro by a brief incubation with calcium ionophore (Chen et al., 1998). The activated eggs were processed into extracts and permanently arrested in M-phase by the addition of non-degradable recombinant cyclin B (Glotzer et al., 1991).

First, I ascertained that my Xenopus extracts preparations represent an M-phase specific activation of the MAPK cascade. Specifically, it was checked whether they are void of meiotic MEK kinase, c-Mos (Castro et al., 2001; Watanabe et al., 1989), and contain high levels of active MEK kinase, MEK and MAPK (Takenaka et al., 1997).

Second, M-phase arrested Xenopus egg extracts were subjected to different fractionation approaches in order to isolate mitotic MEK kinase activity. MEK kinases activate the down-stream MEKs by phosphorylating them at Serine and Threonine residues within the activation loop (Zheng and Guan, 1994). Thus, monitoring phosphorylation status of these residues in recombinant MEK following its incubation in vitro with aliquots of samples was used as a screening tool to detect the presence of endogenous MEK kinase activity and visualize its redistribution among different fractions. The outcome of this part of the study was the development of a purification scheme leading to an enrichment of mitotic MEK kinase activity.

Up-scaled quantities of the M-phase arrested extracts were subjected to the developed purification protocol. The final fractions containing active mitotic MEK kinase were analyzed for their composition by Western blotting and Silver staining. Loss-of-function approach was undertaken to validate involvement of the identified candidate MEK kinase into mitotic activation of the MAPK cascade.
Results

Preparation of extracts from parthenogenetically activated Xenopus eggs, which are devoid of a germ-cell specific MEK kinase, c-Mos

It is well established that activation of the MAPK pathway during Xenopus oocyte maturation is directed by a germ-cell specific MEK kinase, c-Mos (Castro et al., 2001). A temporal increase in intracellular calcium concentration induced by egg fertilization triggers degradation of c-Mos (Nishizawa et al., 1993; Watanabe et al., 1989) and inactivation of MAPK signaling (Ferrell et al., 1991), which contribute to the transition to the embryonic cell cycling (Castro et al., 2001). As shown in Figure 7, fertilization of mature Xenopus oocytes in vitro led to efficient degradation of c-Mos protein. By 50 min, c-Mos was undetectable by immunoblot analysis with anti-c-Mos antibodies.

To recapitulate proper transition from CSF arrest to embryonic cycling in my experimental system, the time required for c-Mos degradation following Ca^{2+} ionophore treatment was determined. To do this, mature Xenopus oocytes were parthenogenetically activated with Ca^{2+} ionophore A23187, collected at 10 min intervals and analyzed for the presence of c-Mos protein by Western blotting. As shown in Figure 7, Ca^{2+} ionophore activation of Xenopus oocytes led to a complete disappearance of c-Mos within 40-50 min after activation. Importantly, the first mitotic cleavage, observed by 80 min post-activation, occurred in the absence of c-Mos. Therefore, I concluded that the activated eggs must be incubated at room temperature for 50-60 min before processing into extracts. Using this protocol, I was able to consistently prepare Xenopus egg extracts that
are devoid of detectable amounts of c-Mos protein and recapitulate \textit{in vitro} early embryonic cell cycles.

Figure 7. c-Mos is degraded at similar times for \textit{in vitro} fertilized (upper panel) or \text{Ca}^{2+} ionophore activated (lower panel) \textit{Xenopus} eggs and undetectable during mitosis
Mature oocytes were fertilized or activated with \text{Ca}^{2+} ionophore, collected at the indicated time points and lysed. Lysates were separated by SDS-PAGE and immunoblotted with c-Mos antibodies. Note that the first embryonic cleavage occurred by 80 min in fertilized eggs. Equal loading was verified by Coomassie staining.
Development of a protocol for purification of mitotic MEK kinase activity

S-phase extracts were prepared from activated *Xenopus* eggs using the protocol described above. Then, S-phase extracts were cycled into a stable M-phase state by the addition of non-degradable cyclin B. Due to the absence of growth factor control, MAPK activation is restricted to M-phase in *Xenopus* egg extracts (Fig. 8). Note that *Xenopus* oocytes and early embryos contain only one isoform of ERKs, ERK2 (Zaitsevskaya and Cooper, 1992). Since it is the first identified *Xenopus*’s MAPK (Gotoh et al., 1991a), it is traditionally referred as to p42 MAPK.

A biochemical assay for detecting MEK kinase activity in *Xenopus* egg extracts was developed in Dr Guadagno laboratory. Aliquots of extracts were diluted in the kinase buffer and incubated *in vitro* in the presence of recombinant unactive GST-MEK. The products of the reaction were separated by SDS-PAGE and phosphorylation of GST-MEK at the activation segment (Ser-217/Ser-221) was studied by Western blotting with phospho-MEK specific antibodies. Despite the absence of c-Mos, MEK kinase activity was strongly detected in M-phase egg extracts compared to S-phase egg extracts (Fig. 8) implying that some other MEK kinase responsible for activation of the MAPK pathway at M-phase. Several MEK kinases have been shown to directly activate MEK1/2. This includes Raf family members (Hagemann and Rapp, 1999), MLK3 (Hartkamp et al., 1999), MEKKs 1-3, c-Mos, andTpl-2 (Fanger et al., 1997; Lewis et al., 1998).

Unfortunately, at the time when this project was initiated, research tools (antibodies, siRNA technique, etc), which would enable me to specifically target a certain MEK kinase in order to elicit its effect on mitotic activation of the MAPK pathway, were
Figure 8. M-phase arrested *Xenopus* egg extracts contain active MAPK and MEK kinase activity

Equal amounts of S- and M-phase *Xenopus* egg extracts were subjected to an *in vitro* MEK kinase assay with recombinant GST-MEK as a substrate. Phospho-MEK antibodies were used to analyze phosphorylation of GST-MEK. Activation of endogenous MAPK was analyzed by phospho-MAPK Western blotting. Equal sample loading was confirmed by immunoblotting for MAPK protein (data not shown).

not readily available. Therefore, to facilitate identification of the mitotic MEK kinase, I decided to purify MEK kinase activity from M-phase arrested *Xenopus* egg extracts. In many regards, *Xenopus* egg extracts represent an ideal model system for purifying and identifying the MEK kinase responsible for mitotic activation of the MAPK cascade.

First, *Xenopus* eggs provide a rich source of components of the MAPK cascade.

Secondly, activation of the MAPK cascade is restricted to mitosis in *Xenopus* egg extracts that autonomously undergo synchronous cell cycles of S- and M-phases (Guadagno and Ferrell, 1998; Takenaka et al., 1997). Finally, the *Xenopus* egg extracts are amenable to biochemical manipulations not feasible with tissue culture cells.
As a first purification step, the crude M-phase arrested *Xenopus* egg extracts were separated by ultracentrifugation (100,000 g 1.5 hr, twice) into cytosolic and membrane fractions. By applying equivalent amounts of crude and cytosolic fractions to an *in vitro* MEK kinase assay, I showed that the majority of the total M-phase MEK kinase activity is present in the cytosolic fraction (Table 1). This suggests that the mitotic MEK kinase activity is cytosolic and not membrane-bound.

As a second purification step, I fractionated the M-phase cytosol by ammonium sulfate (AS) precipitation. The M-phase cytosolic fraction was supplemented with an appropriate volume of EB buffer containing 50% ammonium sulfate to reach a final 25% salt saturation. After this, samples were rotated for 1.5 hr at 4°C and precipitated proteins were pelleted by a high-speed centrifugation (10,000 g 15 min at 4°C). The protein pellet was resuspended and proteins remaining in the supernatant were transferred to a new tube and subjected to sequential 50% and 75% AS precipitations as described above. Finally, to monitor the redistribution of the MEK kinase activity among different fractions, equal amounts of protein from all three AS cuts (0-25%, 25-50% and 50-75% saturation) as well as the final supernatant were subjected to an *in vitro* MEK kinase assay. It was determined that the majority (>90%) of the mitotic MEK kinase activity from the cytosolic fraction was precipitated in the 0-25% AS cut (data not shown). Thus, mitotic MEK kinase activity from cytosolic fraction of M-phase arrested *Xenopus* egg extracts can be precipitated by low AS saturation, which provides a convenient purification step.
To narrow down further AS concentration necessary for precipitation of the MEK kinase activity, I performed the following AS cuts with M-phase cytosol: 0-10%, 10-15%, 15-20%, 20-25% and 25-30%. The precipitated protein fractions were resuspended and subjected to an *in vitro* MEK kinase assay. As shown in Figure 9, the majority (>90%) of MEK kinase activity was precipitated in the 15-20% AS cut. Note, that the 20% ammonium sulfate saturation precipitated only 10% of the total proteins from the original M-phase cytosol fraction (Fig. 9). Thus, 20% ammonium sulfate saturation can be used as an efficient step in purification of the mitotic MEK kinase activity.

Next, a chromatography approach was utilized to further purify the MEK kinase activity contained within the 20% AS cut. Several chromatography exchangers were screened for the ability to efficiently separate MEK kinase activity. The following chromatography columns proved not to be useful in purifying the MEK kinase activity: a Mono S HR 5/5 column (a strong cation exchanger), a HiTrap DEAE Sepharose column (a weak anion exchanger), a Butyl Sepharose 4FF column (a hydrophobic interaction exchanger, aliphatic interactions), a Phenyl Sepharose 6FF column (a hydrophobic interaction exchanger, aromatic interactions).

Fortunately, I was able to show that anionic exchange columns were effective for purifying the MEK kinase activity. As shown in Figure 10, I was able to separate mitotic MEK kinase activity by using a HiTrap Q Sepharose HP column (a strong anion exchanger). Specifically, by using a straight gradient of 0 – 1 M NaCl, the MEK kinase activity eluted at a range of 19-36% salt saturation. To further focus the elution point for the MEK kinase activity and eliminate contamination of the MEK kinase active fractions
Figure 9. 20% ammonium sulfate saturation precipitates MEK kinase activity from M-phase arrested *Xenopus* egg extracts

Cytosolic fraction of M-phase arrested *Xenopus* egg extracts was sequentially subjected to protein precipitation with increasing concentration of ammonium sulfate. Precipitated proteins were recovered and subjected to an *in vitro* MEK kinase assay. Protein concentration of the obtained fractions was determined by using Bio-Rad Protein Assay kit. Note that previously it was shown that concentrations of ammonium sulfate higher that 25% do not precipitate MEK kinase activity from M-phase cytosolic fraction.
Figure 10. Partial purification of mitotic MEK kinase activity from 20% ammonium sulfate precipitated fraction by using HiTrap Q Sepharose HP anion-exchange chromatography (0 – 1.0 M NaCl elution gradient)

1.5 mg of 20% ammonium sulfate precipitated fraction resuspended in buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl2) was applied to a 5 ml HiTrap Q Sepharose HP column (Amersham Biosciences). Proteins were eluted with a straight gradient of 1 M NaCl (buffer B: 50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 M NaCl). Aliquots of eluted fractions were subjected to an in vitro MEK kinase assay. Note that MEK kinase activity was eluted at a 0.19 – 0.36 M NaCl range.
with irrelevant proteins with similar desalting properties, I decided to increase the elution resolution in the range of 0 – 30% of salt saturation. To do this, proteins bound to a HiTrap Q Sepharose HP column were eluted with a step-wise gradient of 1 M NaCl: 0 – 30% saturation (4 column volumes) and 30 – 100% saturation (1 column volume). This significantly increased the efficiency of purification (Fig. 11). The MEK kinase activity, eluted at 0.22 M NaCl, was concentrated in a fewer number of fractions and contained less total protein.

Another anion-exchange column (Mono Q HR) also turned out to be a promising tool for purification of mitotic MEK kinase activity. By applying the 20% AS precipitated fraction to a Mono Q HR 5/5 column and eluting bound proteins with a straight gradient of 0 – 1 M NaCl, I was able to collect mitotic MEK kinase activity in six discrete 0.5 ml fractions eluted at a 0.36 – 0.51 M NaCl (Fig. 12). Mono Q resin is composed of finer beaded particles than HiTrap Q Sepharose and thus is suitable for a more refine protein separation. Therefore, I decided to use a Mono Q column as a purification step following separation on HiTrap Q Sepharose column. Indeed, application of MEK kinase active fractions eluted from a HiTrap Q Sepharose column to a Mono Q column further purified the MEK kinase activity (not shown). To increase the efficiency of separation of eluted proteins on a Mono Q column, I modified the elution conditions to a step-wise gradient of 1 M NaCl: 0 – 30% (3 column volumes), 30 – 50% (15 column volumes) and 50 – 100% (2 column volumes).
Figure 11. Partial purification of mitotic MEK kinase activity from 20% ammonium sulfate precipitated fraction by using HiTrap Q Sepharose HP anion-exchange chromatography (0 – 0.3 – 1.0 M NaCl elution gradient)

3.0 mg of 20% ammonium sulfate precipitated fraction resuspended in buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl2) was applied to a 5 ml HiTrap Q Sepharose HP column (Amersham Biosciences). Proteins were eluted with a step-wise gradient of 1 M NaCl (buffer B: 50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 M NaCl). Aliquots of eluted fractions were subjected to an in vitro MEK kinase assay. Note that MEK kinase activity was eluted at a 0.22 M NaCl range.
Figure 12. Partial purification of mitotic MEK kinase activity from 20% ammonium sulfate precipitated fraction by using MonoQ anion-exchange chromatography

1.9 mg of 20% ammonium sulfate precipitated fraction resuspended in buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl2) was applied to a 1 ml Mono Q HR column (Amersham Biosciences). Proteins were eluted with a straight gradient of 1 M NaCl (buffer B: 50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 M NaCl). Aliquots of eluted fractions were subjected to an *in vitro* MEK kinase assay. Note that MEK kinase activity was eluted at a 0.36 – 0.51 M NaCl range.
Thus, by subjecting M-phase arrested *Xenopus* egg extracts to different purification approaches and using an *in vitro* MEK kinase assay as a screening tool, I worked out several efficient purification techniques, which can be combined in one purification protocol to separate mitotic MEK kinase activity.

*Purification of MEK kinase activity from M-phase arrested extracts*

Based on results described in the previous section, I designed a four-step purification scheme to purify MEK kinase activity present in M-phase arrested *Xenopus* egg extracts (Fig. 13A). Crude S-phase extracts prepared from parthenogenetically activated *Xenopus* eggs were cycled into a stable M-phase by the addition of non-degradable recombinant cyclin B. The M-phase state of the extracts was confirmed by analyzing an *in vitro* histone H1 kinase activity and/or observing the nuclear envelope breakdown and chromatin condensation under fluorescent microscopy. Then, the crude M-phase extracts were separated into cytosolic and membrane fractions by ultracentrifugation. The cytosolic fraction, containing nearly all of the MEK kinase activity (Table 1), was separated by ammonium sulfate precipitation. Approximately 90% of the MEK kinase activity was precipitated in the 0-20% ammonium sulfate cut and purified further by anion exchange chromatography on HiTrap Q Sepharose and Mono Q columns. Collectively, these purification steps enriched MEK kinase activity by ~260-fold (Table 1). At the final purification step, a single protein peak eluted from the Mono Q column (Fig. 13B) contained MEK kinase activity as assessed by both phosphorylation
Figure 13. Purification of MEK kinase activity from M-phase arrested *Xenopus* egg extracts
A. Scheme for purification of an M-phase MEK kinase activity. B. Mono Q elution profile over a three-step 0 - 1.0 M NaCl gradient. C, Mono Q fractions #10-16 contain an M-phase MEK kinase activity. MEK kinase activity of Mono Q fractions # 4-60 was measured in an *in vitro* MEK kinase assay. MEK kinase activity was not detected in the flow through volume (not shown).
**Table 1. Purification of MEK kinase activity from M-phase arrested *Xenopus* egg extracts**

<table>
<thead>
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<th>Step</th>
<th>Total protein, mg</th>
<th>Total activity, a.u.*</th>
<th>Specific activity, a.u./mg</th>
<th>Yield, %</th>
<th>Fold purification</th>
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<tr>
<td>Crude M-phase extract</td>
<td>1156.8</td>
<td>246.2</td>
<td>0.21</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cytosol fraction</td>
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<td>260.0</td>
<td>0.86</td>
<td>100</td>
<td>4.1</td>
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<td>20% AS cut</td>
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<td>236.8</td>
<td>8.46</td>
<td>91.1</td>
<td>40.3</td>
</tr>
<tr>
<td>HiTrap Q Sepharose</td>
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<td>105.75</td>
<td>27.83</td>
<td>40.7</td>
<td>132.5</td>
</tr>
<tr>
<td>Mono Q</td>
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<td>22.05</td>
<td>54.44</td>
<td>8.5</td>
<td>259.2</td>
</tr>
</tbody>
</table>

* a.u. - arbitrary units; fractions from each purification step were subjected to an *in vitro* MEK kinase assay, the levels of GST-MEK phosphorylation were determined by Western blotting and quantified by using ImageQuant software.

of recombinant MEK (Fig. 13C) and activation of the MAPK cascade in an *in vitro* linked kinase assay (Fig. 14). Therefore, my results suggest that the kinase activity purified from M-phase arrested *Xenopus* egg extracts represents a *bona fide* MEK kinase. This protocol was applied at least three times and consistently led to an enrichment of the mitotic MEK kinase activity.
Figure 14. Final Mono Q fractions contain M-phase MEK kinase activity
Equal aliquots of Mono Q fractions # 8 – 18 were subjected to an in vitro linked kinase assay as described in Materials and Methods chapter. Briefly, aliquots of Mono Q fractions # 8 – 18 were incubated in vitro with recombinant unactive GST-MEK and GST-ERK. Next, the reaction mix was supplemented with myelin basic protein (MBP) as a substrate for ERK and incubation was continued. The levels of ERK activation in the linked kinase reaction were determined by the levels of MBP phosphorylation. To assess contribution of endogenous MAPK in the purified fractions to MBP phosphorylation, the aliquots of the fractions were incubated with MBP alone, without addition of GST-MEK and GST-ERK. The levels of MEK kinase activity were represented as a difference between MBP phosphorylation in the cascade kinase reaction and MBP phosphorylation by the fractions itself. The levels of MBP phosphorylation were visualized by autoradiography and quantified by using ImageQuant software.
**B-Raf is enriched at the final stage of the mitotic MEK kinase purification**

Silver staining analysis of the final MEK kinase active fractions revealed several protein bands. An example of one of the active fractions is shown (Fig. 15, lane 1). A prominent protein band between 90-100 kDa correlated with M-phase MEK kinase activity throughout the progress of purification (see Fig. 45) and migrated at a similar molecular weight range as B-Raf. Therefore, I used a polyclonal antibody raised against a highly conserved N-terminal portion of *Human* B-Raf (Fig. 16) to assess whether the protein band might represent B-Raf. As shown in Figure 15 (lanes 5 and 6), this antibody readily recognized *Xenopus* B-Raf in crude S- and M-phase egg extracts as a 95 kDa doublet.

When a purified fraction containing mitotic MEK kinase activity was immunoblotted with the B-Raf antibody, B-Raf was strongly detected (Fig. 15, lane 2 versus lane 6). On the other hand, neither c-Mos nor Raf-1 were detected by Western blotting (Fig. 15, lanes 3 and 4). Similar results were observed for other fractions containing partially purified MEK kinase activity. Thus, I conclude that B-Raf, not Raf-1 or c-Mos, is enriched during purification of the mitotic MEK kinase activity.
Figure 15. B-Raf is enriched at the final stage of the mitotic MEK kinase purification
Mono Q fraction #12 (see Fig.11) was separated on 8% SDS-PAGE and analyzed by either silver staining (lane 1) or Western blotting with anti-B-Raf (lane 2), anti-Raf-1 (lane 3), or anti-c-Mos (lane 4) antibodies. Similar results were obtained with fraction #11. Equal amounts of total protein from crude S-phase (lane 5), M-phase (lanes 6, 7, 8), and CSF extracts (lane 9) were immunoblotted for B-Raf (lanes 5, 6), Raf-1 (lane 7), and c-Mos (lanes 8, 9). Note that the asterisk indicates recombinant GST-cyclin B recognized by Santa Cruz B-Raf polyclonal antibodies, raised against a GST-conjugated B-Raf peptide.
Figure 16. Alignment of the amino acid sequences of *Xenopus* (AAZ06667) and *Human* (P15056) B-Raf proteins using MegAlign software.

sc9002 antibody epitope comprising an N-terminal region of *Human* B-Raf is shown in red dashed line.
Depletion of B-Raf, but not Raf-1, blocks activation of MAPK at mitosis

Based on the purification results, I hypothesized that B-Raf might be required for activation of the MEK/MAPK pathway at mitosis. To test this directly, endogenous B-Raf was quantitatively removed (~99%) from S-phase extracts by two rounds of immunodepletion (Fig. 17A) and the depleted extracts were cycled into mitosis by the addition of recombinant non-degradable cyclin B. At indicated time points, aliquots of egg extracts were collected to assess MAPK activation. Entry into mitosis was monitored by nuclear envelope breakdown and chromatin condensation, which in control and B-Raf-depleted extracts occurred at 20 min after cyclin B addition. Following mitotic entry, MAPK became activated at 30 min in control extracts (Fig. 17B, first and second lanes). In contrast, mitotic MAPK activation was strongly inhibited in B-Raf depleted extracts (Fig. 17B, third lane). The addition of recombinant B-Raf protein to B-Raf-depleted extracts was sufficient to restore MAPK activation at levels similar to control extracts (Fig. 17B, fourth lane). Thus, I conclude that B-Raf is essential for activation of the MAPK pathway during mitosis in *Xenopus* egg extracts.

Raf-1, another member of Raf MEK kinase family, is a classical activator of the MAPK cascade after mitogenic stimulation. Interestingly, studies in mammalian cells have implicated a possible role for Raf-1 in G2/M progression (Laird et al., 1995; Pathan et al., 1996; Ziogas et al., 1998). Since Raf-1 is expressed in *Xenopus* egg extracts (Fig. 18A, first lane), I decided to test whether activation of MAPK at mitosis in *Xenopus* egg extracts depends on Raf-1. In order to do this, Raf-1 was removed from extracts by immunodepletion (Fig. 18A). After this, the Raf-1 depleted extracts were driven into
Figure 17. B-Raf is required for activation of MAPK at mitosis
A. B-Raf protein levels in S-phase extracts after mock- and B-Raf-depletion, and adding back of recombinant His-tagged B-Raf.  B. MAPK activation at mitosis is blocked in B-Raf-depleted extracts and restored after addition of recombinant B-Raf. Mock- and B-Raf-depleted extracts were driven into mitosis with non-degradable cyclin B and assessed for MAPK activation by phospho-MAPK Western blotting at indicated times.
Figure 18. Raf-1 is not required for mitotic activation of MAPK pathway
A. Raf-1 protein levels in S-phase extracts after mock- and Raf-1-immunodepletion.  B. Raf-1 is not required for activation of MAPK during mitosis.  Mock- and Raf-1-depleted extracts were driven into mitosis with non-degradable cyclin B.  1-ml aliquots of the egg extract were collected at the indicated times to monitor MAPK activation by phospho-MAPK immunoblotting.  Equal loading of samples was confirmed by Ponceau S staining of membranes.
M-phase by the addition of recombinant non-degradable cyclin B. Aliquots of the extracts were taken at the indicated times, and time-course of MAPK activation was studied by phospho-MAPK Western blot. As it shown on Figure 18B, depletion of endogenous Raf-1 protein had no effect on mitotic activation of the MAPK pathway. Thus, I conclude that Raf-1 is not required for mitotic activation of MAPK signaling in *Xenopus* egg extracts.

**Conclusions**

The primary aim of this study was to identify the MEK kinase responsible for mitotic activation of the MEK/MAPK pathway in *Xenopus* egg extracts. To address this, I developed a procedure for preparation of M-phase arrested *Xenopus* egg extracts devoid of a meiotic MEK kinase, c-Mos. By using these extracts as a starting material, I developed a purification protocol leading to an enrichment of the mitotic MEK kinase activity. Fractionation of M-phase arrested *Xenopus* egg extracts led to the purification of a single peak of MEK kinase activity that was identified by Western analysis as B-Raf. Neither c-Mos nor Raf-1 were detected in the purified MEK kinase active fraction. The results from my immunodepletion experiments definitively show that B-Raf is critical for activating the MAPK pathway during mitosis in *Xenopus* egg extracts. In contrast, the related Raf family member, Raf-1, is not required for M-phase activation of the MAPK pathway. Thus, I conclude that B-Raf is the major MEK kinase responsible for activation of the MAPK signaling at mitosis (Fig. 19)
Figure 19. B-Raf activates the MEK/MAPK cascade at mitosis
Chapter Three

Characterization of B-Raf at Mitosis

Introduction

In Chapter Two, I demonstrated that B-Raf functions as a critical activator of the MAPK pathway during mitosis. Presently, little is known about the cell cycle regulation of B-Raf at M-phase. In this Chapter I propose to characterize the mitotic regulation of B-Raf activity using the cell-free system of *Xenopus* egg extracts. Specifically, data describing possible cell cycle dependent changes in B-Raf activity, posttranslational modification by phosphorylation and protein interactions with its down-stream target, MEK, will be presented.

Results

*B-Raf activity is elevated during mitosis*

To examine the regulation of the B-Raf/MEK/MAPK cascade at mitosis, I decided to characterize B-Raf activity in *Xenopus* egg extracts. Based on the requirement for B-Raf in activation of the MAPK cascade during mitosis, I predicted that B-Raf activity would be highest during M-phase. To measure B-Raf activity, similar amounts
of B-Raf protein were immunoprecipitated from S- and M-phase arrested egg extracts and subjected to an *in vitro* linked kinase assay. The data showed that B-Raf activity is markedly elevated (4-6 fold) in M-phase arrested extracts compared to S-phase extracts (Fig. 20A). A modest amount of B-Raf activity detected in S-phase extracts likely represents its high basal activity due to both constitutive phosphorylation and the presence of a phospho-mimicking aspartic acid residue in the positive regulatory N-region (Mason et al., 1999). Controls that either lack recombinant MEK and ERK or measure background kinase activity associated with rabbit IgG complexes demonstrated the specificity of the *in vitro* cascade reaction.

Next, I examined B-Raf activity in *Xenopus* egg cycling extracts that naturally oscillate between S- and M-phases. First, extracts prepared from activated *Xenopus* eggs were incubated at room temperature to initiate cycling. At 10-min intervals, equal aliquots of cycling extracts were collected, snap-frozen on dry ice and stored at -80°C. Extracts cycling between S- and M-phase was monitored by observing nuclear envelope breakdown/chromatin condensation and measuring Cdk1/cyclin B activity in an *in vitro* histone H1 kinase assay. To measure B-Raf activity throughout the cell cycle, equal amounts of B-Raf protein were immunoprecipitated from *Xenopus* egg cycling extracts, washed several times and subjected to an *in vitro* linked kinase assay. I consistently observed a fluctuation of B-Raf activity during the cell cycle peaking highest during M-phase (Fig. 20B). Thus, my data shows that B-Raf is activated in an M-phase dependent manner in *Xenopus* egg extracts.
Figure 20. B-Raf activity is elevated during mitosis

A. B-Raf is activated in M-phase arrested extracts. B-Raf immunoprecipitates from S- and M-phase egg extracts were subjected to an *in vitro* linked kinase. Equal loading of immunoprecipitated B-Raf per kinase reaction was confirmed by Western analysis.

B. B-Raf activity is up-regulated during mitosis in *Xenopus* cycling extracts. B-Raf was immunoprecipitated from aliquots of cycling egg extract at indicated times and subjected to an *in vitro* linked kinase assay. *In vitro* histone H1 kinase activity of the same aliquots was measured in parallel as a marker for Cdk1/cyclin B activation and mitotic entry. Activation of MAPK was studied by phospho-MAPK Western blotting.
In a recent report, high amounts of B-Raf activity were detected in *Xenopus* egg extracts but the authors were unable to measure differences in B-Raf activity between S- and M-phases (Yue et al., 2006). I speculate that the negative data could be caused by functional limitations of the two-component *in vitro* MEK kinase assay used in their study to measure B-Raf activity. I utilized an *in vitro* B-Raf/MEK/ERK/MBP linked kinase assay, which allows greater signal amplification, and used at least 5-fold more recombinant MEK. Thus, saturation of the reaction by purified active B-Raf complexes is less likely to occur under my assay conditions. Therefore, I conclude that my cascade kinase reaction is more sensitive and reliable for detecting changes in B-Raf activity.

To rule out the possibility that a MEK kinase associated with B-Raf might contribute to the activation of ERK in the linked kinase reaction, I decided to compare *in vitro* kinase activities of catalytically inactive and wild type B-Raf immunoprecipitates from S- and M-phases. First, a site-directed technique was used to create a kinase inactive mutant of *Xenopus* B-Raf by substituting Lysine residue in the ATP-binding site to Methionine (Guan et al., 2000). Next, wild type and kinase dead recombinant myc-B-Raf proteins were expressed in *Xenopus* CSF extracts and introduced for incubation in S- and M-phase arrested extracts (see Materials and Methods for details). Finally, the recombinant myc-B-Raf proteins were recovered by myc-tag immunoprecipitation and subjected to an *in vitro* linked kinase assay as described above. My results show that the catalytically inactive B-Raf immunoprecipitates do not display any MEK kinase activity in an *in vitro* linked kinase assay compared to wild type B-Raf (Fig. 21). This result
Figure 21. Kinase-dead B-Raf immuno-complexes from S- and M-phase arrested extracts do not possess MEK kinase activity

Kinase-dead (KD) and wild-type (WT) myc-B-Raf were expressed in *Xenopus* egg extracts as described. Aliquots containing the recombinant myc-B-Raf were introduced in a 1:20 ratio to S-phase arrested extracts, which after one-hour incubation at room temperature were driven into M-phase with non-degradable GST-cyclin B. Myc-B-Raf proteins were purified by means of myc-tag antibodies and subjected to an *in vitro* linked kinase assay. Loading of immunopurified myc-B-Raf per kinase reaction was verified by myc-tag Western blotting. Cdk1/cyclin B activity of the corresponding extracts was measured in an *in vitro* histone H1 kinase assay (not shown).
clearly demonstrates that the activation of the MAPK cascade by B-Raf immunoprecipitates in the \textit{in vitro} linked kinase assay reflects B-Raf activity.

I would like to point out that my results implicating B-Raf at mitosis conflict with a recent study suggesting that c-Mos might regulate M-phase activation of the MAPK cascade in \textit{Xenopus} egg extracts (Yue and Ferrell, 2004). I speculate that the main reason for these conflicting results stem from differences in how the egg extracts were prepared. In contrast to Yue and Ferrell’s study, I do not detect any c-Mos protein in my egg extract preparations due to allowing the activated eggs incubating at room temperature for 50 min prior to processing them into extracts (see Fig. 7). Moreover, I do not detect any MEK kinase activity associated with c-Mos immuno-complexes isolated from M-phase arrested \textit{Xenopus} egg extracts (Fig. 22). Therefore, I am unable to assess any biological significance for c-Mos in my egg extract system. The absence of c-Mos in my egg extracts closely mimics its disappearance shortly following fertilization that was demonstrated in earlier studies (Nishizawa et al., 1993; Watanabe et al., 1991; Watanabe et al., 1989) and in this study (Fig. 7). Furthermore, the proposal that c-Mos could trigger the transient activation of the MAPK pathway at mitosis does not comply with its well-established role as a cytostatic factor during oocyte maturation (Castro et al., 2001) or from studies showing that micro-injection of even small amounts of c-Mos can mediate a metaphase arrest in cleaving \textit{Xenopus} embryos (Sagata et al., 1989). Finally, gene knockout studies in mice argue against an essential role for c-Mos at mitosis in somatic tissues since mos (-/-) mice are viable without any tissue abnormalities (Colledge et al., 1994; Hashimoto et al., 1994). On the other hand, homozygous knockouts for B-Raf are
embryonically lethal (Wojnowski et al., 2000; Wojnowski et al., 1997). Thus, I propose that B-Raf, rather than c-Mos, is the mitotic MEK kinase that plays an essential role during mitosis in activating the MAPK cascade.

Figure 22. c-Mos immunoprecipitates do not possess an M-phase MEK kinase activity
Santa Cruz and Abcam c-Mos antibodies were used to immunoprecipitate MEK kinase activity from M-phase arrested extracts (c-Mos immunoprecipitates 1 and 2 respectively). The immunoprecipitates were washed and subjected to an *in vitro* linked kinase assay with recombinant unactive MEK and ERK. Since M-phase arrested extracts have undetectable levels of c-Mos, Coomassie staining for IgGs was used as a loading control. Note that equivalent amounts of B-Raf antibodies immunoprecipitate significantly higher levels of mitotic MEK kinase activity.
B-Raf associates with MEK in Xenopus egg extracts

Next, I examined whether M-phase activation of B-Raf correlates with its ability to associate with MEK, the direct target of B-Raf. To do this, co-immunoprecipitation assays were performed for B-Raf and MEK. B-Raf or MEK complexes were immunopurified from S- and M-phase Xenopus egg extracts and subjected to Western analysis for detection of both MEK and B-Raf. As shown in Figure 23, MEK and B-Raf were found in a complex in both S- and M-phase egg extracts suggesting that their association is independent of B-Raf activation at mitosis.

Figure 23. B-Raf associates with MEK in Xenopus egg extracts
Aliquots of S- and M-phase arrested extracts were incubated with B-Raf, MEK, or rabbit IgG (mock control) antibodies. Protein complexes purified on protein A beads were subjected to Western analysis for both B-Raf and MEK.
B-Raf does not associate with Raf-1 in mitotic Xenopus egg extracts

Recent work suggested that B-Raf/Raf-1 heterodimerization following mitogen stimulation is an important mechanism that ensures proper signaling via the Raf-MEK-ERK cascade (Rushworth et al., 2006). However, my data indicate that Raf-1, unlike B-Raf, is not involved in the regulation of the MAPK pathway during mitosis in Xenopus egg extracts. Indeed, Raf-1 was not purified as a mitotic MEK kinase from Xenopus egg extracts (Fig. 15) and its immunodepletion did not affect MAPK activation at mitosis (Fig. 18). These results suggest that B-Raf and Raf-1 are not implicated in the same signaling cascades in Xenopus egg extracts. To obtain a direct evidence for this, I performed B-Raf/Raf-1 co-immunoprecipitation to analyze B-Raf/Raf-1 interactions. Equal amounts of M-phase arrested extracts were subjected to B-Raf, Raf-1 or mock immunoprecipitation. Antibody complexes recovered on protein A beads were washed and analyzed for the presence of Raf-1 protein by Western blotting. As shown in Figure 24A, anti-Raf-1 antibody readily immunoprecipitated Raf-1 from M-phase arrested extracts. Contrary, B-Raf immunoprecipitates did not show immunoreactivity with Raf-1 antibodies, indicating that mitotic B-Raf does not interact with Raf-1 in Xenopus egg extracts. Interestingly, analysis of Raf-1 and MEK interaction in Xenopus egg extracts showed that Raf-1 immunoprecipitates from S- and M-phase extracts failed to co-purify MEK, whereas B-Raf antibodies reproducibly co-immunoprecipitated MEK (Fig. 24B and Fig. 23). This further supports that Raf-1 is uncoupled from MAPK signaling during mitosis as shown in this (Fig. 18) and previous studies (Laird et al., 1999; Ziogas et al., 1998).
Figure 24. Raf-1 is not co-immunoprecipitated with B-Raf and MEK from *Xenopus* egg extracts
A. Aliquots of M-phase arrested extracts were incubated with Raf-1, B-Raf antibodies or rabbit IgG (mock control). Protein complexes purified on protein A beads were subjected to Western analysis for Raf-1. B. Aliquots of S- and M-phase arrested extracts were incubated with B-Raf or Raf-1 antibodies or rabbit IgG (mock control). Protein complexes purified on protein A beads were subjected to Western analysis for B-Raf/MEK and Raf-1/MEK co-immunoprecipitation.
B-Raf undergoes hyperphosphorylation during mitosis

As revealed by Western blotting, B-Raf undergoes a prominent electrophoretic shift in M-phase arrested egg extracts (Fig. 25A) and during mitosis in cycling egg extracts (Fig. 25B, 60-70 and 140 min). It is well established that phosphorylation comprises one of the major mechanism of post-translational regulation of B-Raf activity (Chong et al., 2003). Therefore, I tested whether the electrophoretic shift of B-Raf during mitosis is due to phosphorylation. To do this, B-Raf immunocomplexes isolated from S- and M-phase egg extracts were subjected to an in vitro treatment with lambda protein phosphatase. After completion of the reaction, proteins were separated by 8% SDS-PAGE and changes in B-Raf electrophoretic mobility were analyzed by Western blotting. The results show that phosphatase treatment progressively eliminated the shift of B-Raf isolated from M-phase extracts (Fig. 25C) demonstrating that it stemmed from phosphorylation. B-Raf isolated from S-phase egg extracts was also sensitive to phosphatase treatment since it is constitutively phosphorylated in the positive regulatory N-region (Mason et al., 1999). Therefore, I conclude that Xenopus B-Raf is constitutively phosphorylated in S-phase extracts and becomes hyperphosphorylated during mitosis.

Mitotic activation of B-Raf stems from phosphorylation

Is mitotic phosphorylation of B-Raf important for B-Raf activation? To answer this question, I studied how dephosphorylation affects activity of mitotic B-Raf. Similar to the above described experiment, equal amounts of S- and M-phase immunoprecipitated
Figure 25. B-Raf undergoes hyperphosphorylation during mitosis
A. The electrophoretic mobility of B-Raf shifts up at M-phase. Equal aliquots of S- and M- phase arrested extracts were separated by 8% SDS-PAGE and subjected to immunoblot analysis. Cdk1 activity was measured in an *in vitro* histone H1 kinase assay. Coomassie blue staining was used to confirm equivalent loading of histone H1. B. B-Raf undergoes an electrophoretic mobility shift during mitosis in *Xenopus* egg cycling extracts. Aliquots of cycling egg extracts were collected at indicated times over two cell cycles and immunoblotted for B-Raf or MEK (loading control). C. B-Raf hypershift stems from phosphorylation. B-Raf immunoprecipitated from S- or M-phase egg extracts was treated with lambda protein phosphatase (lambda-PP) for indicated times, separated by SDS PAGE, and subjected to immunoblot analysis.
B-Raf were subjected to an in vitro dephosphorylation with lambda protein phosphatase. After the completion of the reaction, B-Raf immunoprecipitates were washed with copious amounts of buffer containing phosphatase inhibitors (25 mM NaF and 10 mM Na$_3$VO$_4$) and B-Raf activity was measured in an in vitro linked kinase assay. The results showed that dephosphorylation of mitotic B-Raf abolished its kinase activity (Fig. 26). Besides this, the basal activity of B-Raf isolated from S-phase extracts was also sensitive to phosphatase treatment. Thus, this data demonstrates that phosphorylation is critical for regulating M-phase specific activation of B-Raf.

![Figure 26. Mitotic activation of B-Raf stems from phosphorylation](image)

B-Raf immunopurified from S- or M-phase extracts was treated with lambda protein phosphatase (lambda-PP) and subjected to immunoblot analysis (top panel) or an in vitro linked kinase assay (bottom panel).
Xenopus B-Raf is not phosphorylated at the conserved Threonine-633 and Serine-636 residues during mitosis

Phosphorylation of Threonine-599 and Serine-602 located within an activation segment of Human B-Raf is critical for B-Raf activation, specifically for Ras-dependent induction of B-Raf activity (Zhang and Guan, 2000). Since these phosphorylation sites are conserved to Xenopus B-Raf (Threonine-633 and Serine-636, respectively, see Fig. 16), I asked whether phosphorylation at the indicated sites occurs during mitosis. To answer this question, equal amounts of S- and M-phase extracts were probed with anti-B-Raf phospho-Threonine-599 and phospho-Serine-602 antibodies. The results showed that Xenopus 95 kDa B-Raf is not phosphorylated at the indicated residues neither in S- nor in M-phases (Fig. 27). Interestingly, the small B-Raf isoform (~68 kDa) present in Xenopus egg extracts and previously described in human cells (Moodie et al., 1994; Oshima et al., 1991; Sithanandam et al., 1990) is highly phosphorylated in an M-phase dependent manner at the conserved Threonine-633 and Serine-363 within the activation segment. While beyond the scope of this study, it would be extremely interesting to determine functions and regulation of the smaller 68 kDa B-Raf isoform at mitosis by developing B-Raf antibodies capable of immunoprecipitation of the smaller B-Raf isoform.
Figure 27. *Xenopus* 95 kDa B-Raf is not phosphorylated at the conserved Threonine-633 and Serine-636 residues during mitosis

Aliquots of S- and M-phase extracts were separated by 8% SDS-PAGE and immunoblotted for phospho-Thr-599/Ser-602 B-Raf (1), 68 kDa B-Raf (2) and 95 kDa B-Raf (3). Note that Threonine-599 and Serine-602 residues in the activation segment of *Human* B-Raf correspond to Threonine-633 and Serine-636 in *Xenopus* B-Raf (see Fig. 16).
Conclusions

In this Chapter, I provide evidence that B-Raf is regulated in an M-phase dependent manner in *Xenopus* egg extracts. First of all, I show that consistent with its requirement in mitotic activation of the MAPK pathway, B-Raf activity is remarkably and transiently increased during mitosis. Furthermore, *Xenopus* B-Raf undergoes an M-phase specific hyperphosphorylation. Importantly, mitotic phosphorylation of B-Raf is critical for its activation during M-phase. Thus, I speculate that up-stream kinase(s) target B-Raf at mitosis to allow for the activation of the B-Raf/MEK/MAPK pathway. Interestingly, 95 kDa *Xenopus* B-Raf is not phosphorylated at the conserved Threonine-633 and Serine-363 within the activation loop during mitosis, contrary to its Ras-dependent activation following mitogenic stimulation (Zhang and Guan, 2000). This indicates that the mechanism of B-Raf activation at mitosis is principally different from that described for B-Raf during the cell cycle entry, which occurs in response to mitogen stimulation via activated Ras (Roovers and Assoian, 2000). Finally, I demonstrate that B-Raf does not form heterodimers with Raf-1 and associates with endogenous MEK, its direct target, validating that B-Raf functions as a MEK kinase in *Xenopus* egg extracts.
Chapter Four

Regulation of Mitotic B-Raf by Cdk1/cyclin B

Introduction

In this chapter I propose to analyze the timing of Cdk1/cyclin B and B-Raf activation in *Xenopus* egg extracts that cycle from S-phase to mitosis as well as a requirement of Cdk1/cyclin B activity in activation of B-Raf in mitotic extracts. Second, Cdk1/cyclin B association with B-Raf in mitotic *Xenopus* egg extracts will be characterized based on data obtained in co-immunoprecipitation studies. Lastly, I will present data that demonstrate that B-Raf is phosphorylated by Cdk1/cyclin B and that this phosphorylation is important for activation of B-Raf and the MAPK cascade during mitosis in *Xenopus* egg extracts.

Results

*Cdk1/cyclin B triggers activation of B-Raf in Xenopus egg extracts*

From my analysis of temporal activation of Cdk1/cyclin B and B-Raf in *Xenopus* egg cycling extracts, I show that B-Raf activation during mitosis follows Cdk1/cyclin B activity (Fig. 20B). To study the biochemical relationship between Cdk1/cyclin B and B-
Raf, I asked whether Cdk1/cyclin B could trigger B-Raf activity in *Xenopus* egg extracts cycled into a stable mitotic state. Specifically, after the addition of recombinant non-degradable GST-cyclin B to S-phase arrested extracts, I collected aliquots of extract at 10 min intervals. Cdk1/cyclin B activity was measured by an *in vitro* histone H1 kinase assay. In parallel, B-Raf was immunoprecipitated from 5 µl of the same time-point aliquots and subjected to an *in vitro* linked kinase assay to determine its activity. The data shown in Figure 28A demonstrates that activation of B-Raf follows Cdk1/cyclin B activation suggesting that Cdk1/cyclin B directly or indirectly controls mitotic activation of B-Raf.

Is Cdk1/cyclin B activity necessary for B-Raf activity during mitosis? To answer this question, I treated M-phase arrested extracts with Flavopiridol, a Cdk specific inhibitor (Shapiro, 2004) and then analyzed B-Raf activity as outlined above. Following inhibition of Cdk1/cyclin B, I observed a reduction in activity of mitotic B-Raf (Fig. 28B). This indicates that the enzymatic activity of Cdk1/cyclin B complexes is involved in the control of B-Raf activation during mitosis. Therefore, the data presented here strengthens the idea that Cdk1/cyclin B serves as a direct or indirect activator of mitotic B-Raf.

* B-Raf associates with active Cdk1/cyclin B complexes during mitosis

My data suggest that M-phase specific activation of B-Raf could be under control of Cdk1/cyclin B. Interestingly, Cdk1, recombinant GST-cyclin B, and B-Raf were all detected in the final step of the mitotic MEK kinase purification, described in the Chapter
Figure 28. B-Raf activity in M-phase arrested *Xenopus* egg extracts depends on Cdk1/cyclin B activity

A. Time-course of Cdk1/cyclin B and B-Raf activation in *Xenopus* egg extracts after GST-cyclin B addition. Cdk1/cyclin B activity is necessary for B-Raf activation during mitosis. Cdk1 activity in M-phase arrested extracts was inhibited with Flavopiridol. B-Raf was immuno-purified and its activity was measured in an *in vitro* histone H1 kinase assay. Equal loading of B-Raf immunoprecipitates per kinase assay was confirmed by B-Raf Western blotting. Cdk1/cyclin B activity was measured in *in vitro* histone H1 kinase assay.

B. **Figure 28.** B-Raf activity in M-phase arrested *Xenopus* egg extracts depends on Cdk1/cyclin B activity

A. Time-course of Cdk1/cyclin B and B-Raf activation in *Xenopus* egg extracts after GST-cyclin B addition. Cdk1/cyclin B activity is necessary for B-Raf activation during mitosis. Cdk1 activity in M-phase arrested extracts was inhibited with Flavopiridol. B-Raf was immuno-purified and its activity was measured in an *in vitro* histone H1 kinase assay. Equal loading of B-Raf immunoprecipitates per kinase assay was confirmed by B-Raf Western blotting. Cdk1/cyclin B activity was measured in *in vitro* histone H1 kinase assay.

B. **Figure 28.** B-Raf activity in M-phase arrested *Xenopus* egg extracts depends on Cdk1/cyclin B activity

A. Time-course of Cdk1/cyclin B and B-Raf activation in *Xenopus* egg extracts after GST-cyclin B addition. Cdk1/cyclin B activity is necessary for B-Raf activation during mitosis. Cdk1 activity in M-phase arrested extracts was inhibited with Flavopiridol. B-Raf was immuno-purified and its activity was measured in an *in vitro* histone H1 kinase assay. Equal loading of B-Raf immunoprecipitates per kinase assay was confirmed by B-Raf Western blotting. Cdk1/cyclin B activity was measured in *in vitro* histone H1 kinase assay.
Two (Fig. 29A). To determine whether Cdk1/GST-cyclin B and B-Raf were co-purified independently or within a large multi-protein complex, a glutathione beads pull-down from S- and M-phase extracts was performed. As shown in Fig. 29B, both Cdk1 and B-Raf were readily detectable in GST-cyclin B complexes isolated from M-phase arrested extracts. In contrast, glutathione beads pull-down from S-phase extracts, which do not contain GST-conjugated cyclin B, failed to precipitate either B-Raf or Cdk1. These studies were extended into *Xenopus* egg cycling extracts to examine endogenous Cdk1/cyclin B and B-Raf interactions during the embryonic cell cycle. Cyclin B immunoprecipitates from time-course aliquots of cycling extract were analyzed by Western blotting for both B-Raf and Cdk1 protein or subjected to an *in vitro* H1 kinase assays to measure Cdk1 activity. Cell cycle progression of the extracts was assessed by nuclear formation, nuclear envelope breakdown and chromatin condensation. The results clearly show that B-Raf associates with Cdk1/cyclin B complexes during mitosis and this interaction parallels the activation of Cdk1 (Fig. 29C). Analogous experiments were performed by using Cdk1 antibody. Cdk1 immunocomplexes were purified from time-course aliquots of cycling extracts and analyzed for the presence of B-Raf and Cdk1 by Western blotting as well as for Cdk1/cyclin B activity by applying them to an *in vitro* H1 kinase assay. Similar to the results obtained with Cyclin B immunoprecipitates, I observed an increase in B-Raf co-immunoprecipitation at the beginning of mitosis, which overlapped with activation of Cdk1/cyclin B complexes (Fig. 29D). Thus, these data reveal that B-Raf directly associates with active Cdk1/cyclin B at mitosis.
Figure 29. B-Raf associates with active Cdk1/cyclin B complexes during mitosis
A. MonoQ column fraction containing partially purified mitotic MEK kinase activity was analyzed by B-Raf, cyclin B, and Cdk1 Western blotting. B. Cdk1/GST-cyclin B complexes were purified from M-phase arrested extracts by using Glutathione beads. C. Cyclin B immuno-complexes were precipitated from aliquots of cycling extracts and subjected to B-Raf and Cdk1 Western blotting, and an in vitro H1 kinase assay to measure an associated Cdk1 activity. D. Cdk1 immuno-complexes were precipitated from aliquots of cycling extracts and subjected to B-Raf and Cdk1 Western blotting, and an in vitro H1 kinase assay.
Cdk1/cyclin B (and MAPK) directly phosphorylates B-Raf in M-phase arrested Xenopus egg extracts

B-Raf directly associates with active Cdk1/cyclin B complexes and its activation occurs downstream of and depends on active Cdk1/cyclin B. All these data suggest that B-Raf could be a direct target of Cdk1/cyclin B. To clarify this, I decided to study whether phosphorylation of B-Raf in mitotic Xenopus egg extracts depends on Cdk1/cyclin B. Specifically, I analyzed the status of B-Raf phosphorylation in M-phase arrested extracts where Cdk1/cyclin B activity was selectively blocked by Flavopiridol treatment. Under these conditions, I observed a minor reduction in B-Raf’s mitotic electrophoretic mobility (Fig. 30, third lane). To obtain further evidence that Cdk1/cyclin B phosphorylate B-Raf at mitosis, I decided to immunopurify B-Raf from M-phase arrested extracts and subject it to Western blot analysis with phospho-Cdk substrate antibodies. It is well established that active Cdkks phosphorylate their substrates at the Serine/Threonine residue within the conserved Serine/Threonine-Proline-X-Lysine/Leucine sequence (Nigg, 1991). I decided to take advantage of commercially available phospho-antibodies, which recognize both Cdk and MAPK phosphorylated substrates at the consensus sequences S*XK/L and PXS*P respectively. Since these antibodies may recognize both Cdk and MAPK phosphorylation, I prepared M-phase extracts inhibited either for Cdk1/cyclin B (treated with Flavopiridol) or MAPK (treated with U0126, a MEK inhibitor) as well as M-phase extract where both kinases were inhibited by the treatment with both inhibitors. As shown in Figure 30, phosphorylation of B-Raf at the PXS*P/S*PXK/L sites is dramatically increased from S- to M-phase.
Figure 30. Cdk1/cyclin B (and MAPK) directly phosphorylates B-Raf in M-phase arrested Xenopus egg extracts

Extracts were treated with Flavopiridol and/or U0126 to inhibit Cdk1/cyclin B and MEK correspondently. B-Raf phosphorylation was analyzed by monitoring B-Raf shift and reactivity of B-Raf immunoprecipitates with phospho-Cdk/MAPK substrate antibodies (PXS*P/S*PXK/L).
(fourth lane). Importantly, inhibition of Cdk1/cyclin B led to a reduction in B-Raf’s phosphorylation (Fig. 30, forth lane). This indicates that Cdk1/cyclin B directly phosphorylates B-Raf at Cdns consensus phospho-site(s) during mitosis. Interestingly, data presented in Figure 30, fourth lane, demonstrates also that MAPK as well directly targets B-Raf at mitosis. Studies on MAPK’s involvement in the regulation of mitotic B-Raf are presented in Chapter Five.

*Cdk1/cyclin B directly phosphorylates Xenopus B-Raf in vitro at a conserved residue Serine-144*

*Xenopus* B-Raf contains two conserved SPXK sequences, which represent potential Cdk consensus sites (Fig. 31). Interestingly, the first N-proximal site most closely resembles a sequence of a Cdk1/cyclin B preferential phosphorylation site, which was characterized as of K/R-S/T-P-Polar amino acid-K/L (Moreno and Nurse, 1990). Based on the results presented in the previous section, Serine residues within these two sites (namely, Serine-144 and Serine-328) are possible targets for Cdk1/cyclin B phosphorylation. To address this directly, I prepared recombinant non-phosphorylatable *Xenopus* myc-tagged B-Raf mutants. Briefly, *Xenopus* B-raf coding sequence, cloned in the Guadagno lab, was sub-cloned into modified pGEM vector and subjected to site-directed mutagenesis to produce Ser144Ala, Ser-328-Ala and Ser-144-Ala/Ser-328-Ala mutants. Corresponding mRNAs were synthesized by an in vitro transcription reaction and translated in CSF *Xenopus* egg extracts as described under the Materials and Methods. The mutant as well as non-mutated myc-B-Raf proteins were purified by the
Figure 31. Conserved putative Cdk1/cyclin B phosphorylation sites in *Xenopus* B-Raf protein
means of myc-tag antibodies and used as substrates for an in vitro phosphorylation by active recombinant Cdk1/cyclin B in the presence of radioactive ATP. As shown in Figure 32, non-mutated myc-B-Raf was readily radiolabeled by Cdk1/cyclin B, demonstrating that B-Raf can be directly phosphorylated by Cdk1/cyclin B. Importantly, the only Ser-328-Ala mutant displayed a phosphorylation by Cdk1/cyclin B (Fig. 32). This data indicates that B-Raf’s Serine-144 is the main target of Cdk1/cyclin B phosphorylation in vitro. This is the first evidence that B-Raf can be directly phosphorylated by Cdk1/cyclin B in vitro and that this phosphorylation occurs preferentially at the conserved Serine-144 residue within the Cdk consensus sequence. Note that to reduce the possibility of irrelevant myc-B-Raf radiolabeling because of B-Raf autophosphorylation activity, all recombinant myc-B-Raf proteins used in this assay were kinase dead (KD) due to mutation in the ATP binding domain.

Ser-144-Ala B-Raf mutant is not activated in an M-phase dependent manner

Data described in the previous section implies that Cdk1/cyclin B mediated phosphorylation at Serine-144 may regulate mitotic activation of B-Raf in Xenopus egg extracts. If this prediction is true, than the M-phase specific activation of non-phosphoralatable Ser-144-Ala myc-B-Raf mutant should be blocked. To test this, I directly measured activities of wild type (WT) and Ser-144-Ala myc-B-Raf proteins in S- and M-phase arrested extracts. Briefly, the recombinant WT and mutant myc-B-Raf proteins were expressed in Xenopus CSF extracts. Aliquots of the extracts containing the recombinant myc-tag B-Raf were introduced into S-phase arrested extracts. After 1 hr
Figure 32. Cdk1/cyclin B directly phosphorylates Xenopus B-Raf in vitro at a conserved Serine-144

WT and mutant myc-B-Raf proteins were expressed in Xenopus egg extracts, purified by means of myc-tag antibodies and subjected to an in vitro phosphorylation by active recombinant Cdk1/cyclin B. Phosphorylation of myc-B-Raf proteins was visualized by autoradiography. Myt-tag Western blotting was performed as a loading control.
incubation the extracts were driven into M-phase by the addition of recombinant non-degradable cyclin B. myc-B-Raf proteins were purified from S- and M-phase extracts with myc-tag antibodies and subjected to an in vitro linked kinase assay to assess their activities. As expected, wild type myc-B-Raf underwent a 3-4 fold activation at M-phase (Fig. 33). Importantly, the mutant non-phosphorylatable Ser-144-Ala myc-B-Raf was not activated and its activity remained essentially the same from S- to M-phase (Fig. 33). This shows that Ser-144-Ala B-Raf mutant is not responsive to an M-phase specific activation.

Ser-144-Ala B-Raf mutant does not activate MAPK cascade during mitosis

Is phosphorylation at Serine-144 important for B-Raf’s functioning as a mitotic MEK kinase? To answer this, I studied whether the mutant Ser-144-Ala myc-B-Raf can rescue mitotic activation of the MEK/MAPK cascade in B-Raf depleted extracts. Endogenous B-Raf was immunodepleted form S-phase arrested extracts as described earlier (see Chapter Two). Then recombinant wild type or Ser-144-Ala myc-B-Raf proteins were introduced into B-Raf depleted extracts (Fig. 34A) and the extracts were driven into M-phase by the addition of recombinant non-degradable cyclin B. 1.0 µl aliquots were collected every 20 min and analyzed for MAPK activation by phospho-MAPK Western blotting. As expected, depletion of endogenous B-Raf abrogated activation of MAPK in M-phase arrested extracts (Fig. 34B, second lane). The addition of recombinant wild type myc-B-Raf reconstituted mitotic activation of MAPK at levels similar to those observed in control (non-depleted) extracts (Fig. 34B, first and third
Figure 33. Ser-144-Ala B-Raf mutant is not activated in an M-phase dependent manner

Wild type (WT) and Ser-144-Ala (S144A) myc-B-Raf proteins were incubated in S- and M-phase arrested B-Raf depleted extracts, immunopurified by means of myc-tag antibodies and subjected to an in vitro linked kinase assay.
Ser-144-Ala myc-B-Raf mutant does not activate MAPK cascade during mitosis

A. Levels of B-Raf protein in mock and B-Raf depleted Xenopus egg extracts, and in B-Raf depleted extracts supplemented with recombinant wild-type or Ser-144-Ala mutated myc-B-Raf. B. Ser-144-Ala myc B-Raf mutant, unlike wild-type myc B-Raf, does not rescue MAPK activation in M-phase B-Raf-depleted extracts. Mock and B-Raf depleted extracts, and B-Raf depleted extracts supplemented with recombinant wild-type or Ser-144-Ala myc-B-Raf were driven into mitosis with recombinant non-degradable cyclin B. At the indicated times aliquots were collected and MAPK activation was assessed by phospho-MAPK Western blotting.
Importantly, the addition of equivalent amount of non-phosphorylatable Ser-144-Ala myc-B-Raf mutant failed to rescue MAPK activation at mitosis in B-Raf depleted extracts (Fig. 34B, fourth lane). Thus, phosphorylation of Serine-144 residue within the Cdk phosphorylation sequence contributes to activation of B-Raf, and enables B-Raf to function as an M-phase MEK kinase.

*Ser-144-Ala B-Raf mutant exerts a dominant-negative effect on MAPK activation during mitosis*

Since Ser-144-Ala myc-B-Raf does not activate MAPK cascade at mitosis in B-Raf depleted extracts, I decided to study whether this mutant can compete with endogenous B-Raf and interfere with mitotic activation of MAPK in non-depleted extracts. Similar amounts of wild type and Ser-144-Ala myc-B-Raf proteins (Fig. 35, first lane), equivalent to the levels of endogenous B-Raf, were introduced into S- and M-phase arrested *Xenopus* egg extracts. Following 1.0 hr incubation, extracts were analyzed for the levels of MAPK activation by phospho-MAPK Western blotting. As shown in Figure 35 (second lane), MAPK phosphorylation in M-phase extracts supplemented with Ser-144-Ala myc-B-Raf mutant was significantly reduced compared to M-phase extracts supplemented with wild type myc-B-Raf. Note that activation of Cdk1/cyclin B resembled a normal pattern in both extracts (Fig. 35, third lane). This data reconfirms the importance of phosphorylation at the Ser-144 for B-Raf’s ability to function as an M-phase MEK kinase. Furthermore, it suggests that the Ser-144-Ala myc-B-Raf mutant can
Figure 35. Ser-144-Ala B-Raf mutant blocks MAPK activation in M-phase extracts
Equal amounts of wild-type and mutant Ser-144-Ala myc-B-Raf proteins were incubated in S- or M-phase arrested Xenopus egg extracts. MAPK activation was assessed by phospho-MAPK Western blotting. Note that addition of myc-B-Raf proteins did not affect activation of Cdk1/cyclin B.
be utilized as a potential research tool to selectively inhibit MAPK signaling at mitosis in *Xenopus* egg extracts and, possibly, tissue cell cultures.

**Conclusions**

The data presented in this Chapter reveals a novel mechanism of regulation for the B-Raf/MEK/MAPK cascade. For the first time, I demonstrate that B-Raf serves as a direct link between the mitotic cell cycle machinery and the MAPK pathway. Indeed, Cdk1/cyclin B associates and phosphorylates B-Raf at mitosis in *Xenopus* egg extracts. Data from an *in vitro* kinase reaction show that active Cdk1/cyclin B directly phosphorylates B-Raf at its conserved Serine-144 residue. Biochemical analysis demonstrated that phosphorylation of B-Raf at this residue is critical for the M-phase dependent activation of B-Raf as well as a functionality of B-Raf as a mitotic MEK kinase. In conclusion, I identify a new B-Raf regulatory site and provide insights into the mechanism that regulates the activation of the MAPK pathway during mitosis (Fig. 36).
Figure 36. Cdk1/cyclin B directly phosphorylates B-Raf at Serine-144 to trigger the B-Raf/MEK/MAPK cascade.
Chapter Five

Negative Feedback Regulation of Mitotic B-Raf by MAPK

Introduction

The data presented in the previous chapters indicate that during mitosis B-Raf can be regulated by MAPK. First of all, I repeatedly observed that the pronounced B-Raf’s hypershift during mitosis coincides with MAPK activation (see Fig. 20B and Fig. 30). A similar correlation was recently reported in activated Human B lymphocytes (Brummer et al., 2003). More importantly, the authors showed that Human B-Raf is directly phosphorylated by MAPK at the conserved C-terminal sequence and that this causes B-Raf hypershift (Brummer et al., 2003). Since the C-terminal MAPK phosphorylation site is conserved in Xenopus B-Raf, it is important to elucidate whether the same mechanism operates during mitosis. Indeed, blocking of MAPK activation in M-phase arrested Xenopus egg extracts by pretreatment with a MEK inhibitor U0126 reduces the levels of B-Raf’s mitotic hyperphosphorylation (see Fig. 30). In addition, the treatment with a MEK inhibitor U0126 reduces B-Raf’s reactivity with Cdk/MAPK phospho-substrate antibodies (see Fig. 30). Thus, the goal of the studies presented in this Chapter is to further explore the MAPK-dependent phosphorylation of B-Raf in Xenopus egg extracts
during mitosis and characterize its role in regulating activity of mitotic B-Raf.

Specifically, data that describe interactions between endogenous B-Raf and MAPK during M-phase and phosphorylation of *Xenopus* B-Raf by MAPK will be presented. Finally, by observing changes in B-Raf activity under conditions when the MAPK-mediated feedback is blocked or enhanced, the effect of this MAPK phosphorylation on B-Raf activity will be characterized.

**Results**

*Mitotic hyperphosphorylation of B-Raf depends on active MAPK*

B-Raf was previously reported to undergo an electrophoretic mobility shift during B lymphocyte activation through feedback phosphorylation by ERK (Brummer et al., 2003). Therefore, I asked whether B-Raf’s hyper-shift in mitotic *Xenopus* egg extracts is dependent on MAPK. To answer this, *Xenopus* egg extracts were treated with a MEK-specific inhibitor U0126 prior to cycling them into mitosis. The immunoblot analysis confirmed inhibition of MAPK, which led to blocking M-phase specific hyperphosphorylation of B-Raf (Fig. 37A, see as well Fig. 30). Similar results were observed in *Xenopus* cycling extracts treated with the MEK inhibitor U0126 (Fig. 37B). Note that the MEK inhibitor U0126 does not affect Cdk1 activation in *Xenopus* egg extracts, as assessed both by microscopic analysis of nuclear envelope breakdown/chromatin condensation and by measuring Cdk1 activity in an *in vitro* H1
kinase assay (see Fig. 30). Thus, MAPK feedback substantially contributes to the mitotic hypershift of B-Raf.

Figure 37. Mitotic hyperphosphorylation of B-Raf depends on active MAPK

A. Mitotic hyper-shift of B-Raf is blocked in the presence of the MEK inhibitor U0126. S-phase extracts were cycled into M-phase with recombinant GST-cyclin B in the presence of 50 nM U0126 or DMSO. Equal aliquots of the egg extracts were separated by 8% SDS-PAGE and analyzed by B-Raf and phospho-MAPK Western blotting.

B. Treatment of Xenopus cycling egg extracts with U0126 blocks hyper-shift of B-Raf during mitosis. Aliquots of cycling extracts were separated by 8% SDS-PAGE and analyzed by Western blotting.
Mitotic B-Raf associates with MAPK

If MAPK directly phosphorylates B-Raf at mitosis, then it might be possible to detect the association between these two proteins. To test this possibility, equivalent amounts of MAPK were immunoprecipitated from S- and M-phase egg extracts and subjected to Western analysis with B-Raf antibodies. The levels of B-Raf co-immunoprecipitated with MAPK were much higher in the MAPK complexes isolated from M-phase arrested extracts compared to S-phase extracts (Fig. 38A).

To analyze dynamics of B-Raf/MAPK interaction during mitosis, I studied how B-Raf/MAPK association is changing in Xenopus egg cycling extracts. MAPK was immunoprecipitated from frozen time-course aliquots and obtained immunoprecipitates were subjected to B-Raf Western blotting. Interestingly, I detected a two-phase B-Raf/MAPK association throughout mitotic progression (Fig. 38B). First, B-Raf was associated with MAPK at the on-set of mitosis, which overlapped with Cdk1/cyclin B activation. Secondly, B-Raf was bound to MAPK during middle-late mitosis, which coincided with robust MAPK activity. Since I repeatedly observe the appearance of B-Raf’s mitotic hyper-shift during middle-late stages of mitosis (Fig. 20B, Fig. 25B), it is tempting to speculate that during the second phase of B-Raf/MAPK interaction, MAPK may exert a direct B-Raf phosphorylation. Taken together, my data show that B-Raf becomes associated with MAPK in egg extracts specifically during M-phase.
Figure 38. Mitotic B-Raf associates with MAPK

A. B-Raf associates with MAPK in M-phase arrested extracts. MAPK and control immunoprecipitates from S- and M-phase extracts were separated on 10% SDS-PAGE and analyzed by B-Raf and MAPK Western blotting. B. B-Raf associates with MAPK twice throughout mitosis in cycling extracts. MAPK immunoprecipitates from aliquots of cycling extracts collected at the indicated time-points were separated on 10% SDS-PAGE and analyzed by B-Raf and MAPK Western blotting. Cdk1 activity of the corresponding aliquots was assessed in an in vitro histone H1 kinase assay.
MAPK directly phosphorylates Xenopus B-Raf in vitro and causes B-Raf’s electrophoretic mobility shift

The association of MAPK with B-Raf suggests that it might contribute directly to B-Raf hyperphosphorylation. To study this, I performed an in vitro kinase assay using endogenous Xenopus B-Raf isolated from S-phase extracts as a potential substrate for active recombinant ERK2. The results show that active ERK2 readily phosphorylated B-Raf in vitro (Fig. 39A). Moreover, phosphorylation of B-Raf by ERK2 caused a reduction in the electrophoretic mobility of B-Raf (Fig. 39B, forth and fifth lanes). Therefore, I conclude that MAPK can directly phosphorylate Xenopus B-Raf and contribute to B-Raf’s mitotic shift.

MAPK directly phosphorylates Xenopus B-Raf in vitro at the conserved C-terminal SPKTP motif

Human B-Raf is phosphorylated directly by ERK2 at both MAPK consensus S/TP sites within the C-terminal SPKTP motif (Brummer et al., 2003). However, the effect of this phosphorylation on B-Raf activity has not been described. Since the SPKTP motif is conserved between Human and Xenopus B-Raf (Fig. 40A), I asked whether the same phosphorylation sites in Xenopus B-Raf protein are targeted by MAPK. To do this, I utilized a site-directed mutagenesis technique and introduced alanine residues at the two phospho-acceptor sites (serine and threonine) within the C-terminal SPKTP motif to created a B-Raf non-phosphorylatable APKAP mutant. mRNAs of wild type (WT) and mutant (MT) myc-tagged Xenopus B-Raf were translated in CSF-arrested
Figure 39. MAPK directly phosphorylates *Xenopus* B-Raf *in vitro* and causes B-Raf’s electrophoretic mobility shift

A. MAPK directly phosphorylate immunopurified B-Raf *in vitro*. B-Raf was immunoprecipitated from S-phase arrested *Xenopus* egg extracts and subjected to an *in vitro* phosphorylation by active recombinant ERK2 in the presence of radioactive ATP. Radiolabeling of B-Raf protein was visualized by autoradiography. B. Phosphorylation of B-Raf by MAPK causes B-Raf hypershift. B-Raf was immunopurified from S-phase arrested extracts and incubated *in vitro* with active recombinant ERK2 in the presence or absence of ATP. Reaction mixer was separated by 8% SDS-PAGE and B-Raf was analyzed by B-Raf immunoblotting.
extracts (see Materials and Methods for details). Then, the recombinant myc-tagged B-Raf proteins were re-isolated by immunoprecipitation with myc-tag antibodies and subjected to an in vitro phosphorylation by active recombinant ERK2 in the presence of radioactive ATP. As shown in Figure 40B, radio-labeling of the *Xenopus* myc-tagged B-Raf mutant in the presence of active ERK2 was strongly suppressed compared to wild type myc-B-Raf demonstrating that these conserved sites are targeted by ERK2.

*C-terminal APKAP B-Raf mutant displays a reduced MAPK-dependent shift at mitosis*

To study whether MAPK-mediated phosphorylation at B-Raf’s C-terminal SPKTP motif occurs during mitosis, I analyzed the phosphorylation status of the C-terminal B-Raf mutant in M-phase arrested *Xenopus* egg extracts. The non-phosphorylatable myc-B-Raf APKAP mutant introduced to M-phase arrested extracts. After 1.0 hr incubation, the electrophoretic mobility of mutant B-Raf was analyzed by 8% SDS-PAGE. The data showed that the mitotic hypershift of B-Raf (APKAP) mutant was markedly reduced compared to that of wild type B-Raf (Fig. 40C, third and fourth lanes) suggesting that these MAPK sites are phosphorylated in *Xenopus* M-phase egg extracts. Interestingly, the electrophoretic mobility of the B-Raf (APKAP) mutant was slightly increased following inhibition of MAPK with a MEK inhibitor U0126 (Fig. 40C, third and sixth lanes). This indicates that perhaps some other B-Raf site(s) are phosphorylated in an MAPK-dependent manner during mitosis.

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Figure 40. MAPK directly phosphorylates *Xenopus* B-Raf at the conserved C-terminal SPKTP motif

A. Amino acid alignment of the C-terminal *Human* (P15056) and *Xenopus* (AAZ06667) B-Raf regions using MegAlign software. Putative MAPK binding and phosphorylation sites are indicated. B. The conserved C-terminal SPKTP motif in *Xenopus* B-Raf is phosphorylated by ERK2. Recombinant full-length wild type (WT) and the C-terminal APKAP mutant (MT) myc-B-Raf proteins were expressed in *Xenopus* egg extracts as described, immunopurified by myc-epitope antibodies and subjected to an *in vitro* kinase reaction with recombinant active Erk2 and gamma-32P-ATP. Phosphorylation of myc-B-Raf was detected by autoradiography (top panel). Levels of myc-B-Raf per reaction were estimated by Western analysis using myc epitope antibodies (bottom panel). C. APKAP Mutant (MT) B-Raf displays a reduced M-phase electrophoretic mobility. Recombinant wild type (WT) or APKAP mutant (MT) myc-B-Raf proteins were incubated in S-, M-phase extracts, or M-phase extracts pre-treated with U0126. Phosphorylation status of the recombinant B-Raf proteins was analyzed by Western analysis.
C-terminal APKAP B-Raf mutant possesses an elevated MEK kinase activity

Currently, it is unknown whether the MAPK-mediated feedback phosphorylation of B-Raf at the conserved C-terminal SPKTP motif plays a role in regulating its kinase activity. Therefore, I analyzed the activity of the myc-tagged B-Raf (APKAP) mutant in *Xenopus* egg extracts. Myc-tagged B-Raf wild type and (APKAP) mutant proteins were incubated in either S- or M-phase egg extracts, re-isolated by means of myc-tag antibodies, and subjected to an *in vitro* linked kinase assay. As expected, wild type B-Raf kinase activity was 2-4-fold higher in M-phase egg extracts than in S-phase extracts (Fig. 41, bars 1 and 3). Interestingly, the kinase activity of the non-phosphorylatable B-Raf APKAP mutant isolated from M-phase egg extracts was 2-3 fold higher than activity of wild type B-Raf (Fig. 41, bars 3 and 4). This suggests that phosphorylation of the C-terminal SPKTP motif renders a negative effect on B-Raf activity. Importantly, the C-terminal APKAP B-Raf mutant was still activated in an M-phase specific manner (Fig. 41, bars 2 and 4). This implies that the C-terminal mutation does not interfere with the ability of the mutant B-Raf to respond to activation mitotic signaling inputs. I observed as well that the C-terminal APKAP B-Raf mutant possesses a higher kinase activity in S-phase extracts compared to wild type B-Raf (Fig. 41, bars 1 and 2). Since it is missing the MAPK phosphorylation sites, which potentially are involved in negative feed back regulation, this B-Raf mutant may be more resistant to inactivation and, thus, display an elevated kinase activity at S-phase. Collectively, these results show that MAPK phosphorylates B-Raf at the C-terminal SPKTP sequence and suggest that feedback phosphorylation at these sites negatively regulates B-Raf during mitosis.
Figure 41. Phosphorylation-defective C-terminal APKAP B-Raf mutant possesses an elevated MEK kinase activity

*Xenopus* wild-type (WT) or APKAP mutant (MT) full-length myc-B-Raf proteins were incubated either in S- or M-phase extracts, immunopurified by means of myc-epitope antibodies and subjected to an *in vitro* linked kinase assay. Levels of myc-B-Raf per reaction were estimated by Western blotting.
Inhibition of MAPK activity with a MEK inhibitor U0126 activates B-Raf in M-phase arrested extracts

If MAPK feedback negatively regulates B-Raf activity in Xenopus mitotic egg extracts, then I predict that blocking MAPK activity would lead to an increase in B-Raf activity at mitosis. To test this prediction, I pretreated Xenopus egg extracts with a MEK specific inhibitor U0126 before driving them into M-phase. Inhibition of MEK blocks activation of MAPK and, thus, eliminates the MAPK-mediated feedback loop. Then endogenous B-Raf was immunopurified from control (treated with DMSO) and U0126 treated extracts and subjected to an in vitro linked kinase assay. The results show that B-Raf activity is enhanced in the absence of MAPK feedback compared to non-treated extracts (Fig. 42).

Over-activation of MAPK with constitutively active recombinant MEK inhibits B-Raf activity in M-phase arrested extracts

Likewise, I tested whether B-Raf activity would decrease under conditions of constant MAPK feedback. Indeed, the addition of a constitutively active recombinant MEK to M-phase arrested egg extracts maintained high levels of MAPK activity and led to a substantial decrease in B-Raf activity (Fig. 43). Together, these results implicate MAPK in a feedback loop, which phosphorylates the C-terminal SPKTP sequence of B-Raf to negatively regulate its activity.
Figure 42. Inhibition of MAPK activity with a MEK inhibitor U0126 activates B-Raf in M-phase arrested extracts

To inhibit MAPK activation during mitosis, S-phase arrested extracts were supplemented with a MEK inhibitor, U0126, concomitantly with driving them into M-phase with recombinant non-degradable cyclin B. After 1.0 hr incubation at room temperature extracts were quick frozen. MAPK activation was assessed by phospho-MAPK Western blotting and activity of B-Raf was measured in an in vitro linked kinase assay. Loading of immunopurified B-Raf per kinase reaction was verified by B-Raf Western blotting. Note that the addition of the MEK inhibitor did not affect Cdk1/cyclin B activity (not shown).
Figure 43. Over-activation of MAPK with constitutively active recombinant MEK inhibits B-Raf activity in M-phase arrested extracts
To over-activate MAPK during mitosis, M-phase arrested extracts were supplemented with recombinant constitutively active MEK. After 1.0 hr incubation at room temperature extracts were quick frozen. MAPK activation was assessed by phospho-MAPK Western blotting and activity of B-Raf was measured in an in vitro linked kinase assay. Loading of immunopurified B-Raf per kinase reaction was verified by B-Raf Western blotting. Note that the addition of constitutively active MEK did not affect Cdk1/cyclin B activity.
Conclusions

The experiments presented in this Chapter demonstrate that mitotic B-Raf in Xenopus egg extracts undergoes a direct regulation by MAPK. First, B-Raf associates with MAPK specifically during M-phase and its mitotic hyperphosphorylation depends on active MAPK. Secondly, active MAPK can directly phosphorylate Xenopus B-Raf in vitro and induce its electrophoretic mobility shift at levels similar to that of mitotic B-Raf. Interestingly, the in vitro phosphorylation of B-Raf by MAPK as well as MAPK-dependent phosphorylation of B-Raf in mitotic extracts occurs predominantly at the C-terminal conserved SPKTP motif. Elimination of this phosphorylation site enhances activity of mitotic B-Raf. Further, inhibition or over-activation of MAPK in M-phase arrested Xenopus egg extracts oppositely affects B-Raf activity: enhances or diminishes it, respectively. These results together demonstrate that the MAPK-mediated feedback phosphorylation at the C-terminus negatively regulates B-Raf activity during mitosis. Thus, I propose the negative MAPK-mediated feedback loop contributes to down-regulation of the B-Raf/MEK/MAPK at the late mitosis (Fig. 44).
Figure 44. MAPK directly phosphorylates mitotic B-Raf at Serine-784 and Threonine-787 to inhibit B-Raf activity
Chapter Six

Characterization of large multi-protein complexes containing active mitotic B-Raf

Introduction

B-Raf is known to be regulated at the level of protein-protein interaction. Identification of proteins interacting with B-Raf under different biological contexts is important for deciphering how B-Raf regulates diverse cellular processes. The majority of known B-Raf interacting proteins were identified by co-immunoprecipitation technique or the yeast two-hybrid system (Berruti, 2000; Papin et al., 1996). On the other hand, some studies report that B-Raf can be purified within large multi-protein complexes (Jaiswal et al., 1996; Mizutani et al., 2001; Mizutani et al., 1998). This implies that regulation of B-Raf by protein-protein assembly may be much more complex than currently understood. Proteomics is a powerful approach for analyzing multi-protein complexes. The goal of research presented on this Chapter is to characterize the partially purified large molecular weight multi-protein complexes that contain active mitotic B-Raf. In addition, proteomics data obtained by using mass spectrometry analysis will be presented to describe the candidate components of the mitotic B-Raf complexes.
Results

Purification of mitotic MEK kinase activity as B-Raf containing large multi-protein complexes

As described in Chapter Two, I have developed a purification protocol (see Fig.13A), which leads to an enrichment of MEK kinase activity present in M-phase arrested Xenopus egg extracts. Briefly, crude Xenopus M-phase egg extracts were subjected to two rounds of ultracentrifugation (100,000 g for 1.5 hr) to obtain the cytosolic fraction. The M-phase cytosol was fractionated by ammonium sulfate precipitation. A 0-20% ammonium sulfate cut, containing 90% of the MEK kinase activity, was sequentially subjected to separation on two anion-exchange columns, HiTrap Q Sepharose HP and Mono Q HR 5/5. During this purification two observations relevant to this Chapter were made. First, the MEK kinase activity elutes as a single peak from the final Mono Q column (Fig. 13B), which may imply that the eluted proteins are purified within one large complex. Secondly, Silver stain analysis of equal amounts of protein (5 µg) at each of the purification step detected several protein bands, some of which are increased in intensity at each successive purification step (Fig. 45) indicating that they may be associated with the purified MEK kinase activity. Western analysis shows that B-Raf (95 kDa) is enriched in the final purified fraction (see Fig. 15, second lane) and most likely represents one on these protein bands. Thus, there are at least two indirect observations indicating that the partially purified mitotic MEK kinase activity may be represented by multi-protein complex(s) containing B-Raf. To obtain a direct
Figure 45. Protein profiles of mitotic MEK kinase active fraction throughout progression of the M-phase MEK kinase purification

Equal amounts (5 µg) of MEK kinase active fractions were separated by 8% SDS-PAGE and their protein compositions were visualized by Silver staining.
evidence whether these individual proteins co-purified separately or together in a complex at the final (Mono Q column) stage, I ran 5 µg of proteins of the purified MEK kinase active fraction on a 5-20% gradient native polyacrylamide gel under non-denaturing conditions to allow protein complexes to remain together. After completion of the run, I visualized a pattern of protein separation by Silver staining. A single strong broad band was detected (with a few weak minor bands below) suggesting that most of the proteins in the mitotic MEK kinase active fraction are together in a large complex (Fig. 46, first lane). Is B-Raf a part of this large protein complex? To answer this, Western analysis was performed on the same sample. The results show that the majority of B-Raf resided in the major higher molecular weight band (Fig. 46, second lane). Collectively, these results suggest that the purified mitotic MEK kinase activity represents large molecular weight complex(s), which include B-Raf and several other unknown proteins.

Identification of potential components of large multi-protein complexes containing active mitotic B-Raf

I postulate that components of the purified mitotic MEK kinase active complex(s) might serve a role in regulating B-Raf activation or function during mitosis. Therefore, it is important to identify these proteins. Fortuitously, I determined by Western analysis that both Cdk1 and cyclin B are present in the purified MEK kinase active fraction (see Fig. 29A) and show that they associate with B-Raf at mitosis in Xenopus egg extracts (see Fig. 29B, C and D). To further characterize this protein complex, I used a proteomics...
Figure 46. Mitotic MEK kinase activity purified from M-phase arrested *Xenopus* egg extracts resembles a large B-Raf-containing protein complex. Final MEK kinase active fraction was run on a gradient non-denaturing PAGE and analyzed by Silver staining and B-Raf Western blotting.
approach to identify the other proteins purified with the mitotic MEK kinase activity. To
do this, I performed a large-scale purification of the MEK kinase activity from *Xenopus*
immitotic egg extracts using an established four-step protocol (Fig. 13A). 50 µg of purified
MEK kinase activity was separated by SDS PAGE and stained with colloidal blue
(Invitrogen). Fourteen protein bands detected by colloidal blue were carefully excised
from the gel and pooled into three groups for Mass spectrometric (MS) analysis.
Sequence analysis of trypsin-digested peptides was performed at the Harvard
Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray
tandem mass spectrometry on a Finnigan quadrupole ion trap mass spectrometer. The
individual MS/MS peptide spectra were then correlated with known protein sequences.
In addition to B-Raf, Cdk1, and cyclin B, over 25 new proteins were identified (Table 2).

Many of the proteins identified in the purified MEK kinase active complex have
established roles in actin cytoskeleton regulation (actin, filamin, talin 2, gelsolin),
receptor-mediated endocytosis (Adaptin, Epsin1, beta-adaptin 1 subunit, clathrin heavy
chain, HABP1, and AP-2), nuclear transport and nuclear pore complex formation
(CAS/Cse1, Nup93, importin beta 3, Nup 155), mitotic regulation (CAS/Cse1, APACD)
and chromatin-related functions (RPA, ATR). The proteosome activator alpha subunit
PA28, previously shown to be a B-Raf-specific interacting protein (Kalmes et al., 1998),
was identified by MS as a potential component of the B-Raf containing mitotic MEK
kinase active protein complex(s). The identification of PA28 proteasome subunit
supports the validity of my approach for discovering novel proteins that comprise a large
multiprotein complex containing B-Raf.
Table 2. Proteins identified by Mass spectrometry in the final mitotic MEK kinase active fraction

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<th>Name</th>
<th>MW</th>
<th>MS/MS spectra</th>
<th>Comments</th>
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<td><strong>Cytoskeletal proteins</strong></td>
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<tr>
<td>Beta-Actin</td>
<td>42 kD</td>
<td>53</td>
<td>Structural component of cytoskeleton</td>
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<td>Nup93</td>
<td>93 kD</td>
<td>35</td>
<td>Nucleoporin, a nuclear pore complex protein</td>
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<td>Filamin</td>
<td>280 kD</td>
<td>28</td>
<td>Non-muscle actin-binding scaffold protein</td>
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<td>Gelsolin</td>
<td>86 kD</td>
<td>17</td>
<td>Actin-binding de-polymerizing factor</td>
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<td>Nup155</td>
<td>155 kD</td>
<td>14</td>
<td>Nuclear pore complex protein</td>
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<td>Talin 2</td>
<td>220 kD</td>
<td>2</td>
<td>Adaptor between integrin and actin cytoskeleton</td>
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<td><strong>Cellular Trafficking proteins</strong></td>
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<td>Adaptin</td>
<td>90 kD</td>
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<td>Mediator of intracellular Golgi trafficking</td>
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<td>Importin beta 3</td>
<td>120 kD</td>
<td>37</td>
<td>Mediator of nuclear transport</td>
</tr>
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<td>CAS/CSE1</td>
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<td>27</td>
<td>Regulator of nuclear/cytosolic reshuffling of importin alpha</td>
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<tr>
<td>AP-2</td>
<td>48 kD</td>
<td>9</td>
<td>Adaptor protein required for uptake of cargo proteins</td>
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<tr>
<td>Beta-Adaptin 1</td>
<td>105 kD</td>
<td>7</td>
<td>A subunit of AP-1 complex involved in clathrin assembly in Golgi</td>
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<td>Clathrin, heavy chain</td>
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<td>Structural component of Golgi trafficking vesicles</td>
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<td>HABP1</td>
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<td>Nucleus/mitochondria and extracellular trafficking agent</td>
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<td>Tom1</td>
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<td>2</td>
<td>Sorts ubiquitinated proteins into multivesicular bodies</td>
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<td>SNAP</td>
<td>32 kD</td>
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<td>Golgi protein</td>
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<tr>
<td>Epsin1</td>
<td>90 kD</td>
<td>2</td>
<td>Regulator of endocytosis</td>
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Table 2. Proteins identified by Mass spectrometry in purified mitotic MEK kinase active fraction (continued)

<table>
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<td><strong>Mitotic proteins</strong></td>
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<td>Apacd</td>
<td>29 kD</td>
<td>10</td>
<td>Microtubule dynamics regulator; localizes to centrosomes and midbody</td>
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<td>Epsin1</td>
<td>90 kD</td>
<td>2</td>
<td>Endocytosis; Cdk1/cyclin B substrate during mitosis</td>
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<td><strong>DNA Metabolism proteins</strong></td>
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<td>Replication Factor A</td>
<td>70 kD</td>
<td>22</td>
<td>Assists T-antigen in initiating the replication system</td>
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<tr>
<td>Wellcome CRC pSK</td>
<td>4</td>
<td>Nucleotide metabolism</td>
<td></td>
</tr>
<tr>
<td>ATR</td>
<td>292 kD</td>
<td>3</td>
<td>DNA damage checkpoint regulator</td>
</tr>
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<td><strong>Protein Stability Regulatory factors</strong></td>
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<td>CCt8</td>
<td>60 kD</td>
<td>66</td>
<td>Subunit of the Cct chaperonin complex</td>
</tr>
<tr>
<td>HSP70</td>
<td>70 kD</td>
<td>24</td>
<td>Chaperone protein</td>
</tr>
<tr>
<td>PA-28-beta</td>
<td>28 kD</td>
<td>3</td>
<td>Proteosome activator subunit</td>
</tr>
<tr>
<td>Tom1</td>
<td>54 kD</td>
<td>2</td>
<td>Sorts ubiquitinated proteins into multivesicular bodies</td>
</tr>
<tr>
<td><strong>Intracellular Metabolism Proteins</strong></td>
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<td>Ndrg20</td>
<td>40 kD</td>
<td>16</td>
<td>Alpha/beta hydrolase</td>
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<td>Aldolase</td>
<td>40 kD</td>
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<td>Glucose metabolism</td>
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<tr>
<td>Cn2</td>
<td>52 kD</td>
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<td>Cytosolic dipeptidase</td>
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Described above approaches for characterization of mitotic MEK kinase active complex(s) do not provide an estimation of its molecular weight. To get some insights into the size of the B-Raf containing mitotic MEK kinase active complex(s), I used gel filtration chromatography, which allows for the separation of proteins based on their sizes under native conditions. First, the retention capacity of the column in use (Superdex 200 HR 10/30) was pre-calibrated with a mix of proteins of known molecular weights. After this, 2.6 mg of 20% ammonium sulfate precipitated fraction containing B-Raf activity was applied to the column and separated under the same native conditions. Aliquots of the collected fractions were subjected to an in vitro MEK kinase assay and B-Raf Western blotting to analyze size-wise distribution of the mitotic MEK kinase activity and B-Raf protein respectively. As shown in Figure 47, both mitotic MEK kinase activity and B-Raf were located in a higher 400-700 kD molecular weight range of fractions (#28-38). This data is consistent with the idea that active mitotic B-Raf exists in large protein complexes. Interestingly, B-Raf was also detected in middle-range molecular weight fractions (90-300 kD), but total MEK kinase activity of these fractions was significantly lower. Since mitotic MEK kinase activity in Xenopus egg extracts is represented by B-Raf (Fig. 17), this may indicate that active mitotic B-Raf functions in high molecular weight protein complexes.
Figure 47. Active mitotic B-Raf is purified by gel filtration as a large 700-400 kDa protein complex
2.6 mg of 20% ammonium sulfate precipitated fraction was separated on a Superdex 200 HR 10/30 gel filtration column. Collected fractions were analyzed for levels of MEK kinase activity by an in vitro MEK kinase assay and for the presence of B-Raf by Western blotting. To determine molecular sizes of the eluted proteins the column retention capacity was calibrated with a mix of proteins with known molecular weights.
If protein-protein interactions are important for B-Raf signaling at mitosis in *Xenopus* egg extracts, than I would speculate that formation of these higher molecular B-Raf containing protein complex(s) should be dynamic throughout the cell cycle. To obtain some preliminary evidence for this, I decided to compare protein profiles co-immunopurified with B-Raf antibodies from S-phase (inactive B-Raf) and M-phase (active B-Raf) *Xenopus* egg extracts. Equal amounts of B-Raf protein were immunoprecipitated from S and M-phase arrested *Xenopus* egg extracts, separated by a 10% SDS PAGE, and co-immunoprecipitated proteins were visualized by Silver staining. As shown in Figure 48, there is a significant difference in a set of proteins, which interact with B-Raf at S- and M-phases. Specifically, 120 kD and 45 kD proteins associated with B-Raf preferentially during S-phase, whereas 65 kD and 77 kD proteins interacted exclusively with mitotic B-Raf. This preliminary data indicates that assembly of B-Raf complexes changes from S- to M-phases.

**Conclusions**

In this Chapter, I show that mitotic MEK kinase activity present in *Xenopus* egg extracts is purified as B-Raf containing multi-protein complex(s). Gel filtration estimates the size of these active mitotic B-Raf protein complex(s) to be in the range of 400-700 kDa. Protein composition of B-Raf immunoprecipitated complexes in *Xenopus* egg extracts changes from S- to M-phase, indicating that formation of B-Raf protein associations is controlled in the cell cycle dependent manner and may be important for
Figure 48. Protein profile co-immunoprecipitated with B-Raf is changing from S- to M-phases
B-Raf immuno-complexes precipitated from equal amounts of S- and M-phase arrested extracts were washed and separated on 10% SDS-PAGE. Proteins co-purified with B-Raf antibodies were visualized by Silver staining.
regulation of B-Raf functions. To generate a broader view of regulation of B-Raf during M-phase, proteins potentially comprising the mitotic B-Raf containing MEK kinase active protein complex(s) were identified by mass spectrometry. These include 25 proteins with known functions involved in actin cytoskeleton regulation, receptor-mediated endocytosis, nuclear/cytosol trafficking and nuclear pore complex formation, mitotic regulation and proteins with chromatin-related functions. Future studies are necessary to validate interaction of these proteins within B-Raf containing multi-protein complex(s) and disclose their involvement in regulation of B-Raf functions.
Chapter Seven

General Discussion

A novel role for B-Raf in cell cycle regulation

The central discovery of this dissertation is the demonstration that B-Raf is the major MEK kinase required for activation of the MAPK pathway at mitosis. This work provides the first evidence that B-Raf functions at mitosis and expands our understanding of B-Raf’s involvement in the regulation of the cell cycle. In particular, it is proposed that B-Raf signaling controls progression through the cell cycle at two different stages: during the G1/S transition and at mitosis.

MAPK signaling has several roles at mitosis, including regulation of the G2/M transition (Liu et al., 2004; Wright et al., 1999), control of the spindle formation (Horne and Guadagno, 2003; Zhang et al., 2005), maintenance of the spindle assembly arrest (Chau and Shibuya, 1999; Minshull et al., 1994), regulation of Golgi fragmentation (Aebersold et al., 2004; Shaul and Seger, 2006) and the duration of mitosis (Guadagno and Ferrell, 1998; Roberts et al., 2002). I speculate that at least some of these MAPK functions at mitosis are regulated by B-Raf. Indeed, a number of recent studies by the Guadagno laboratory have extended my work of B-Raf to human somatic cells. First, B-
Raf was shown to localize to the mitotic apparatus in HFF (human foreskin fibroblasts) cells in a pattern similar to one observed for active MEK and ERK (Shapiro et al., 1998; Willard and Crouch, 2001; Zecevic et al., 1998). Specifically, it was shown that B-Raf localizes to the centrosomes throughout mitosis and spindle microtubule and kinetochores during metaphase. Further, phosphorylated (Thr-599 and Ser-602) B-Raf was detected in the areas of condensed chromatin during prophase, at the centrosomes, kinetochores and chromosomes during metaphase, at the spindle midzone during late anaphase and at the midbody during cytokinesis (Borysova MK, Guadagno TM, 2006, manuscript submitted). Loss-of-function studies showed that reduction of endogenous B-Raf by RNA interference treatment led to the accumulation of abnormal mitotic spindles (Borysova MK, Guadagno TM, 2006, manuscript submitted). Thus, these data support the idea that B-Raf directs MAPK signaling during mitosis in somatic cells to regulate the mitotic spindle.

In addition, B-Raf may as well be implicated in the regulation of mitotic spindle checkpoint signaling. Specifically, it was shown that expression of the human mitotic checkpoint kinase Mps1 (Weiss and Winey, 1996) is dependent on B-Raf/MEK/ERK signaling (Cui Y, Guadagno TM, manuscript in preparation). Thus, Mps1 may be an important target of B-Raf signaling during mitosis to regulate the spindle checkpoint. Further studies are required to explore the possible implication of B-Raf in the mechanism of spindle checkpoint.

Based on the data presented in my dissertation, I speculate that mitotic B-Raf is not involved in the regulation of Golgi fragmentation during mitosis. In the experimental
system used in my studies the vast majority of mitotic MEK kinase (B-Raf) activity was detected in the cytosol fraction void of membranes. Thus, it is unlikely that mitotic B-Raf localizes to Golgi apparatus. However, this was not specifically addressed in my experiments. A recent study proposed that Raf-1 activates Golgi-associated pools of MEK during mitosis thereby regulating Golgi fragmentation at the mitotic onset (Colanzi et al., 2003). However, this conclusion is based on the data obtained by inhibition of Raf-1 activity with the autoinhibitory domain of Raf-1 (Bruder et al., 1992) or Raf kinase inhibitory protein (RKIP) (Yeung et al., 2000), both of which may interfere with activation of the other members of the Raf family, A-Raf and B-Raf. Thus, the involvement of a particular Raf family member in the regulation of Golgi fragmentation during mitosis is not conclusively defined yet.

The potential implication of mitotic B-Raf in the regulation of the G2/M transition, which is shown to be dependent on MAPK signaling in somatic cells, is discussed in the next section (B-Raf directly links Cdk1/cyclin B and MAPK signaling during mitosis).

Identifying a role for B-Raf at mitosis further expands our understanding of the prominent role that B-Raf has in mediating cell proliferation during embryogenesis. Gene knockout of B-Raf in mice demonstrated that B-Raf is critical for proper fetal development (Wojnowski et al., 2000; Wojnowski et al., 1997). It was suggested that B-Raf’s involvement in embryogenesis was attributed to its pro-survival functions (Wojnowski et al., 2000; Wojnowski et al., 1997). However, my new data indicates that
regulation of cell division during early embryogenesis could be another important role for B-Raf.

B-Raf is an important oncogene which is mutated and activated in 8% of all human tumors and in nearly 70% of melanomas (Davies et al., 2002). Activating mutations in the B-Raf gene lead to constitutive B-Raf signaling and elevated ERK activity. It is tempting to speculate that constitutive B-Raf signaling could promote mitotic errors that contribute to B-Raf-driven tumorigenesis. In fact, deregulation of B-Raf’s mitotic functions may provoke the formation of aberrant mitotic structures, which can lead to genomic instability.

In summary, identification of B-Raf as the mitotic MEK kinase is a novel finding, which expands our comprehensive understanding of B-Raf’s involvement in the regulation of the cell cycle. Further exploration of potential mitotic roles for B-Raf will shed insights into the mechanisms of B-Raf’s biological functions, such as its involvement in embryogenesis, cell proliferation and oncogenesis.

**B-Raf directly links Cdk1/cyclin B and MAPK signaling during mitosis**

The data in my dissertation demonstrate that Cdk1/cyclin B, a major regulator of mitotic entry, directly phosphorylates B-Raf and that this phosphorylation contributes to the activation of B-Raf at mitosis. Several lines of evidence support this novel mechanism as a regulator of mitotic B-Raf activity. First, activation of endogenous B-Raf in *Xenopus* egg extracts occurs subsequent to the activation of Cdk1/cyclin B (Fig.
dependent manner (Fig. 29) and undergoes a Cdk-dependent phosphorylation at some of its Cdk-phosphorylation consensus site(s) (Fig. 30). Third, active Cdk1/cyclin B directly phosphorylates in vitro recombinant *Xenopus* B-Raf at its conserved Serine-144 residue (Fig. 32). Finally, a non-phosphorylatable Ser-144-Ala B-Raf mutant does not undergo activation in an M-phase dependent manner (Fig. 33) and does not activate the MAPK pathway at mitosis (Fig. 34); furthermore, the mutant exerts a dominant-negative effect by blocking mitotic activation of MAPK in *Xenopus* egg extracts (Fig. 35). Together, these data suggest that Cdk1/cyclin B directly phosphorylates and activates mitotic B-Raf.

A role for Cdk1/cyclin as an activator of B-Raf opens new perspectives for understanding how B-Raf is regulated during the cell cycle. The mechanism of B-Raf activation at M-phase appears to be distinct from its activation mechanism during cell cycle entry (Roovers and Assoian, 2000). During cell cycle initiation extracellular signals trigger activation of B-Raf. Binding of mitogen proteins to the appropriate growth-factor receptors induces activation of Ras, which in turn recruits B-Raf to the plasma membrane and induces its activation. At mitosis an intrinsic mechanism involving the mitotic machinery leads to B-Raf activation. B-Raf accommodates mitotic signals from Cdk1/cyclin B that promote activation of the MAPK pathway. Based on what is known about B-Raf regulation by phosphorylation, it can be proposed that B-Raf is receptive to two distinctive regulatory phosphorylations. Specifically, following extracellular mitogenic stimulation B-Raf undergoes a Ras dependent phosphorylation at
its Threonine-599 and Serine-602 residues within the activation loop (Zhang and Guan, 2000). Interestingly, my data show that mitotic 95 kDa B-Raf is not phosphorylated at these sites in mitotic Xenopus egg extracts (Fig. 27). However, the activation of mitotic B-Raf in the Xenopus egg extract system requires phosphorylation at its Cdk/cyclin B phosphorylation site. Further analysis in somatic cells will need to determine whether the same Serine-144 site is phosphorylated at mitosis. This could be analyzed by developing phospho-site specific antibodies and performing immunofluorescent studies. Thus, during cell cycle entry and mitosis B-Raf can be subjected to two different sets of phosphorylation, both of which contribute to B-Raf’s activation.

Future studies are necessary to elucidate the molecular basis for how phosphorylation at the Serine-144 residue induces B-Raf into an active state. According to the existing dogma, activation of Raf kinases results from conformational changes that open the C-terminal kinase domain from the inhibitory N-terminal regulatory domain (Kolch, 2000). Therefore, I speculate that the presence of a negative charge at the Serine-144 residue can promote the aforementioned intramolecular changes necessary for B-Raf activation, similar to other known regulatory phosphorylations. It should be noted that if this prediction is correct, this would be the first identified site in the N-terminal portion of B-Raf protein that undergoes a positive regulatory phosphorylation. Alternatively, my data do not exclude that the phosphorylation of B-Raf at the Ser-144 residue creates a binding site for a B-Raf interactor, which in turn mediates B-Raf activation.

The proposed biochemical mechanism of B-Raf activation by a direct Cdk1/cyclin B phosphorylation helps explain how MAPK signaling and the cell cycle regulatory
machinery are coordinated during M-phase. From the present study, it appears that activation of the MAPK pathway during M-phase occurs down-stream of Cdk1/cyclin B and that B-Raf serves a direct link that integrates activation of MAPK signaling to the initiation of M-phase. Therefore, it can be proposed that activation of mitotic B-Raf is coordinated to the completion of DNA replication and occurs after commitment to mitosis.

The role for B-Raf as a link between Cdk1/cyclin B and the MAPK pathway was described by using the *Xenopus* egg extracts, which represent the early embryonic cell cycle. It is likely that a similar mechanism coordinates Cdk1 and MAPK activities during the somatic cell cycle. Circumstantial evidence indicates that B-Raf may be under the regulation of Cdk1/cyclin B in tissue culture cells. Indeed, both B-Raf and active Cdk1/cyclin B localize to the same mitotic structures. Similar to B-Raf, at the early M-phase Cdk1/cyclin B is found at the centrosomes (Jackman et al., 2003) and at the mitotic spindle and condensed chromatin at metaphase (Huo et al., 2005; Lee et al., 2003; Stiffler et al., 1999).

In conclusion, I demonstrate that Cdk1/cyclin B phosphorylation of B-Raf is required for its activation at mitosis. The M-phase activation of B-Raf via Cdk1/cyclin B phosphorylation provides a novel mechanism of B-Raf regulation. It expands our understanding of how B-Raf signaling is controlled throughout the cell cycle and begins to elucidate how activation of the MAPK cascade is coordinated with the mitotic machinery.
B-Raf, but not Raf-1, regulates MAPK activation at mitosis

The studies herein provide evidence that the role of mitotic MEK kinase is specific to B-Raf within the Raf kinase family. My data demonstrate that another member of the Raf family, Raf-1, does not display features of a MEK kinase involved in the regulation of MAPK signaling during mitosis. In fact, immunodepletion of Raf-1, unlike B-Raf, from *Xenopus* egg extracts does not affect MAPK activation at M-phase, indicating that that the presence of Raf-1 is irrelevant for triggering the MAPK pathway during mitosis (this study and Yue and Ferrell, 2004). This is consistent with the previously published data showing that activation of Raf-1 in nocodazole-arrested mitotic cells does not lead to activation of the down-stream components of the MAPK cascade (Laird et al., 1999; Ziogas et al., 1998). The present study provides further evidence that Raf-1 is uncoupled from mitotic MAPK signaling. Co-immunoprecipitation analyses showed that Raf-1 does not associate with its classical down-stream target, MEK, in *Xenopus* egg extracts, whereas the B-Raf/MEK interactions were reproducibly detected. Next, B-Raf, but not Raf-1, was enriched during purification of the mitotic MEK kinase activity, demonstrating that B-Raf is the major MEK kinase present in M-phase arrested *Xenopus* egg extracts. Finally, a recent study demonstrated that down-regulation of B-Raf, but not Raf-1, via RNA interference (RNAi) abrogates normal spindle formation in somatic cells (Borysova MK, Guadagno TM, 2006, manuscript submitted). Together, these data demonstrate that B-Raf, but not Raf-1, functions as the mitotic MEK kinase.
Recently it has been proposed that formation of B-Raf/Raf-1 heterodimers is important for activation of the MAPK pathway following mitogenic stimulation (Weber et al., 2001). However, I did not detect interactions between B-Raf and Raf-1 in mitotic *Xenopus* egg extracts. Therefore, I conclude that Raf-1 is not involved in B-Raf mediated activation of the MAPK cascade at M-phase.

Several biochemical mechanisms may ensure the specificity of B-Raf versus Raf-1 as the mitotic MEK kinase. As mentioned above, specificity can be controlled by a mechanism that favors the preferential association of mitotic MEK with B-Raf versus Raf-1. Secondly, Raf-1, unlike B-Raf, may be unreceptive to mitotic up-stream regulatory inputs necessary to trigger its activation as a MEK kinase. My work revealed that phosphorylation of *Xenopus* B-Raf at its Cdk1/cyclin B phosphorylation site (Serine-144 residue) is critical for activation of B-Raf and for B-Raf’s capacity to trigger the MAPK cascade at M-phase. Interestingly, this Cdk1/cyclin B phosphorylation site is conserved only among B-Raf members of the Raf kinase family (Fig. 49). Thus, Raf-1 may not be implicated into the Cdk1/cyclin B – MAPK pathway due to the absence of this regulatory site. The Serine-144 residue, targeted by Cdk1/cyclin B, is located in the N-proximal portion of B-Raf, which does not resemble homology to other members of the Raf family. It is intriguing that regulation of this domain may enable B-Raf’s specific functions. This idea is supported as well by another study which showed that the N-terminal domain of B-Raf is critical for B-Raf, but not Raf-1, specific interaction with the alpha-subunit of the 11S proteasome regulator (Kalmes et al., 1998). Association of the 11S regulator with the 20S proteasome core complex affects the specificity of the
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**Figure 49. The N-terminal Cdk1/cyclin B phosphorylation site is conserved among B-Raf members of Raf kinase family**

The N-terminal portion of amino acid sequences of *Xenopus* B-Raf (AAZ06667), *Human* B-Raf (P15056), *Mouse* B-Raf (CAB81555), *Chicken* B-Raf (Q04982), *Zebrafish* B-Raf (BAD16728), *Human* Raf-1 (P04049), *Mouse* Raf-1 (NP084056), *Chicken* Raf-1 (CA30069), *Xenopus* Raf-1 (P09560), *Zebrafish* Raf-1 (BAD34647), *Human* A-Raf (TVHUAF) and *Mouse* A-Raf (P04627) were aligned by using MegAlign software. The Cdk1/cyclin B phosphorylation site is shown in red frame; the N-terminal portion of CR1 is shaded in blue.
ubiquitin directed proteolysis (Dick et al., 1996; Groettrup et al., 1996). However, whether B-Raf regulates the 11S proteasome subunit is not clear since the 11S alpha-subunit is not phosphorylated by B-Raf (Kalmes et al., 1998).

Notably, it has already been reported that B-Raf and Raf-1 can respond differently to up-stream signaling inputs. A small GTP-ase, Rap1, activates B-Raf, but does not activate Raf-1 (Bos et al., 2001; Houslay and Kolch, 2000; York et al., 1998). The mechanism of B-Raf activation by Cdk1/cyclin B proposed herein further highlights the regulatory differences within the Raf kinase family.

It appears that throughout the cell cycle, Raf-1’s capacity to activate the MAPK pathway is triggered only following mitogenic stimulation by growth factors. Since cells undergoing mitosis do not respond to extracellular stimulation by growth factors, it is proposed that during G2/M the signaling cascade from growth-factor receptors to MAPK via Raf-1 is blocked by Cdk1/cyclin B activity (Dangi and Shapiro, 2005). Specifically, it was shown that Raf-1 and its up-stream components of the mitogen-dependent pathway, Sos-1 and Grb2, associate with Cdk1 in nocodazole-arrested mitotic cells and undergo a Cdk1-dependent phosphorylation. Importantly, Cdk1/cyclin B activity inhibits activation of MAPK in growth-factor treated nocodazole-arrested cells as well as in nocodazole-arrested cells expressing constitutively active H-Ras or Raf-1 (Dangi and Shapiro, 2005).

In conclusion, my results provide evidence that the role of mitotic MEK kinase is specific to B-Raf within the Raf family. Unlike B-Raf, Raf-1 is not required for MAPK activation in mitotic *Xenopus* egg extracts. Further, Raf-1 was neither purified as the M-
phase MEK kinase or shown to associate with MEK. I propose that the B-Raf specific N-terminal domain may enable B-Raf to be receptive for mitotic regulatory inputs via Cdk1/cyclin B. Uncoupling Raf-1 from association with MEK and a Cdk1/cyclin B mediated inhibition of the growth-factor receptor pathway may as well ensure B-Raf’s specificity in functioning as the mitotic MEK kinase.

**Mitotic B-Raf undergoes a negative feedback regulation by MAPK**

Feedback loop phosphorylation is broadly implicated in the regulation of MAPK signaling. The guanine nucleotide exchange factor, Sos-1, is phosphorylated by MAPK following EGF receptor stimulation (Corbalan-Garcia et al., 1996; Waters et al., 1996). This phosphorylation is thought to represent a negative regulatory feedback loop that desensitizes the receptor-mediated signaling by inhibiting Sos-1 interactions with adaptor proteins, such as Grb2 (Corbalan-Garcia et al., 1996; Waters et al., 1996). The phosphorylation feedback loop operates within the MAPK module itself. MEK was shown to exert a positive feedback regulation on Raf-1 (Zimmermann et al., 1997). Raf-1 was demonstrated to undergo both negative (Dougherty et al., 2005) and positive (Balan et al., 2006) feedback regulations by MAPK.

The results of my dissertation demonstrated that B-Raf undergoes negative feedback regulation by MAPK during mitosis. My data showed that the hyperphosphorylation of mitotic B-Raf is primarily caused by a MAPK-mediated phosphorylation at Serine784 and Threonine787 residues within the conserved C-
terminal SPKTP motif of B-Raf. A similar situation was reported for B-Raf in activated human B lymphocytes (Brummer et al., 2003). Furthermore, a B-Raf mutant that mimics constitutive phosphorylation at the C-terminal MAPK phosphorylation sites is characterized by a decreased efficiency in promoting differentiation of PC12 cells (Brummer et al., 2003), whereas a non-phosphorylatable B-Raf mutant enhances PC12 differentiation (Rushworth et al., 2006). This suggests that phosphorylation of the C-terminal SPKTP motif negatively affects B-Raf biological functions.

My study is the first to address the effect of C-terminal phosphorylation on B-Raf kinase activity. I showed that kinase activity of a non-phosphorylatable B-Raf mutant following incubation in M-phase extracts is significantly higher compared to wild-type B-Raf. Further, inhibition and over-activation of endogenous MAPK in M-phase arrested Xenopus egg extracts enhances or diminishes B-Raf activity, respectively. Together, my data support the proposal that B-Raf activity during mitosis is negatively regulated by a feedback loop mediated by MAPK phosphorylation.

The mechanism by which phosphorylation of the C-terminal sites down-regulates activity of B-Raf requires further studies. It was proposed that MAPK-mediated phosphorylation at the Threonine residue within the C-terminal phosphorylation site abrogates B-Raf signaling by promoting disassociation of B-Raf/Raf-1 heterodimers (Rushworth et al., 2006). However, it is unlikely that the same mechanism operates during mitosis, since B-Raf/Raf-1 heterodimers do not form in M-phase arrested Xenopus egg extracts. The C-terminal MAPK phosphorylation sites are located within close proximity to a 14-3-3 binding site (Brummer et al., 2003). Thus, it is possible that the
MAPK phosphorylation modulates B-Raf association with 14-3-3 protein and thereby induces down-regulation of its activity.

Why is a B-Raf activity in M-phase arrested *Xenopus* egg extracts elevated over S-phase levels despite its hyperphosphorylation and the presence of active MAPK? I speculate that mitotic B-Raf in these extracts is represented by two different species: active B-Raf, which is not phosphorylated at the C-terminus, and inactive hyperphosphorylated B-Raf. Another explanation is that the MAPK-mediated feedback phosphorylation of B-Raf is not sufficient to completely inhibit B-Raf activity and needs to be combined with other regulatory events, for instance inactivation of Cdk1/cyclin B.

Analysis of temporal fluctuations of Cdk1/cyclin B, B-Raf and MAPK activities and B-Raf hyperphosphorylation in cycling *Xenopus* egg extracts shows that inactivation and hyperphosphorylation of B-Raf during mitosis overlaps with inactivation of Cdk1/cyclin B and activation of MAPK (Fig. 20). Based on these data, I speculate that the negative feedback phosphorylation of B-Raf by MAPK occurs at the middle-to-late mitosis and contributes to inactivation of B-Raf at times of cyclin B degradation and inactivation of B-Raf’s activator, Cdk1/cyclin B. It is plausible to suggest that this mechanism of B-Raf inhibition via a MAPK-mediated feedback loop ensures a transient activation of the B-Raf/MEK/MAPK pathway necessary to accommodate M-phase progression and exit from mitosis.

In summary, mitotic B-Raf undergoes feedback phosphorylation via activated MAPK that appears to negatively regulate B-Raf activity. I postulate that this mechanism ensures a transient activation of the B-Raf/MEK/MAPK pathway during mitosis.
Together, my work describes a comprehensive mechanism for the regulation of B-Raf activity during mitosis. I propose that activation of Cdk1/cyclin B at the M-phase onset directly triggers activation of B-Raf, perhaps at the spindle apparatus, which directs activation of the MAPK cascade. At the latter stages of mitosis, probably after cyclin B degradation and inactivation of Cdk1, MAPK feeds back to B-Raf to down-regulate its activity (Fig. 50). Together, these regulatory mechanisms control transient activation of the B-Raf/MEK/MAPK cascade during mitosis.

Mitotic B-Raf functions in multi-protein complexes

Active B-Raf was reportedly purified within large protein complexes (Jaiswal et al., 1996; Mizutani et al., 2001; Mizutani et al., 1998). Specifically, purification of a Ras-dependent Raf-1 activator from a rat brain homogenate led to isolation of a 400 kDa B-Raf containing protein complex (Jaiswal et al., 1996; Mizutani et al., 2001; Mizutani et al., 1998) and B-Raf from PC12 cell lysates was isolated within large (>300 kDa) protein complexes (Jaiswal et al., 1996; Mizutani et al., 2001; Mizutani et al., 1998). The work from my dissertation suggests that active mitotic B-Raf exists as well in large multi-protein complexes. Purification of M-phase MEK kinase activity from M-phase arrested Xenopus egg extracts resulted in a single peak of MEK kinase activity eluted from a Mono Q chromatography column. Separation of the final MEK kinase active fraction under native non-denaturizing conditions showed that B-Raf was present in a large protein complex. Analysis of the protein composition of this complex by SDS-
**Figure 50. Proposed mechanism for regulation of the B-Raf/MEK/MAPK cascade at mitosis**

During S-phases of embryonic cell cycling B-Raf is inactive and associated with MEK. At the on-set of M-phase active Cdk1/cyclin B associates with B-Raf and directly phosphorylates it at Serine-144. This contributes to activation of B-Raf and triggers the B-Raf/MEK/MAPK cascade. At later stages of M-phase active MAPK exerts a feed-back phosphorylation of B-Raf at its C-terminal Serine-784 and Threonine-787, which down-regulates B-Raf activity and probably leads to turn-down of mitotic MAPK signaling. Activation states and events are shown in red, inhibitory ones – in blue.
PAGE revealed that it consists of many proteins, many of which were enriched throughout the purification. By using gel filtration, it was estimated that the molecular weight range of the active mitotic B-Raf containing complex is between 400-700 kDa.

Preliminary studies demonstrate that a set of proteins that associate with B-Raf immunoprecipitates in *Xenopus* egg extracts differs slightly from S- to M-phases (Fig. 48). Thus, the formation of B-Raf complexes in *Xenopus* egg extracts seems to be dynamic and may be an important mechanism that controls B-Raf’s regulation and/or proper functioning at M-phase. The biochemical analysis of the purified B-Raf complex described several proteins that may associate with B-Raf to regulate its activity and signaling during mitosis. My work demonstrated that B-Raf associates with active Cdk1/cyclin B complexes and MAPK in an M-phase dependent manner. Interestingly, association between Cdk1/cyclin B and B-Raf was detected at the beginning of mitosis in *Xenopus* egg extracts, whereas B-Raf’s association with MAPK was visualized twice throughout mitosis, at the beginning and during middle-late M-phase. Besides this, it was shown that B-Raf forms complexes with MEK both at S- and M-phases.

Additionally, over 25 proteins were identified as potential components of the B-Raf containing protein complexes (Table 2). Several of the identified proteins have already been linked to the regulation or functions of the MAPK cascade. For instance, beta-actin was identified as one of the predominant components of the purified mitotic MEK kinase active complexes. It has been shown that B-Raf (Pritchard et al., 2004), MEK (Pawlak and Helfman, 2002) and MAPK (Barros and Marshall, 2005) are involved in the regulation of actin cytoskeleton functions. Future studies are necessary to elucidate
whether the B-Raf/MEK/MAPK dependent reorganization of actin cytoskeleton occurs at M-phase. This can be addressed by studying how reduction in B-Raf, MEK and MAPK levels following specific siRNA treatments affects the actin cytoskeleton structures in mitotic cells.

Some of the identified candidate proteins are known substrates and/or interactors of B-Raf, MEK, MAPK or Cdk1/cyclin B. Specifically, PA-28-beta, a proteasome activator subunit, is shown to be a B-Raf interactor (Kalmes et al., 1998). CAS/CSE1, a protein essential for proper chromosome segregation in yeast (Brinkmann et al., 1995; Xiao et al., 1993), which localizes to mitotic spindle in somatic cells (Scherf et al., 1996), is thought to be a non-traditional substrate for MEK1 (Scherf et al., 1998). HABP1, a regulator of intracellular traffic, is a substrate for MAPK (Majumdar et al., 2002). Finally, Epsin1, a protein that regulates endocytosis (Rosse et al., 2003) and replication factor A (Dutta and Stillman, 1992) are substrates for Cdk1/cyclin B.

Other proteins identified by Mass spectrometry are linked to MAPK signaling. It is proposed that importin 3 beta is involved in the intracellular translocation of active ERKs following injury of neurons (Perlson et al., 2005). Filamin A, an actin-binding protein, has been implicated in the regulation of MAPK activation following stimulation of extracellular receptors (He et al., 2003; Scott et al., 2006). ATR’s nuclear translocation upon DNA damage was shown to be regulated by ERK (Wu et al., 2006). Future studies are necessary to validate association of the identified proteins within the mitotic B-Raf complexes and directly assess their involvement in the regulation of B-Raf activity or functions at mitosis.
In conclusion, the data presented in this study demonstrate that mitotic active B-Raf exists in large protein complexes. Identification of proteins associated with a large molecular weight complex with B-Raf during M-phase will aid in deciphering the mitotic mechanisms of B-Raf regulation and function. This work provides some insights into the nature of B-Raf protein-protein interactions that may contribute to B-Raf signaling during mitosis. I showed that mitotic B-Raf directly associates with Cdk1/cyclin B, MEK and MAPK. Additionally, a proteomics approach was utilized to identify other candidate B-Raf interactors at mitosis. Over 25 proteins, which are structural or regulatory components of the cytoskeleton and cellular trafficking or implicated in mitosis, DNA metabolism or regulation of protein stability, were identified. Some of these proteins have been already linked to MAPK or Cdk1/cyclin B signaling. This fact encourages the use of the candidate protein list as a good starting point for future investigations that elucidate the regulation and functions of the mitotic B-Raf complexes.

**Future research directions**

Identification of B-Raf as a mitotic MEK kinase is a novel finding that opens a broad spectrum of new research interests. I propose three main research directions for future studies of B-Raf signaling at mitosis. First is the elucidation of biochemical mechanisms that regulate mitotic B-Raf. Second is defining B-Raf’s roles during mitosis. And third is investigating the effects of oncogenic B-Raf at mitosis.
There are several aspects of B-Raf regulation at mitosis that can be addressed in future studies. In this dissertation I show that phosphorylation is the major mode of regulation of mitotic B-Raf. Further studies are required to completely decipher how phosphorylation regulates B-Raf at M-phase. Specifically, it will be interesting to elucidate the mechanism by which phosphorylation of the Cdk1/cyclin B phosphorylation site (Serine-151 residue in *Human* B-Raf) contributes to activation of B-Raf, as well as a mechanism of inhibitory phosphorylation at the C-terminal MAPK phosphorylation sites (Serine-750 and Threonine 753 residues in *Human* B-Raf). This can be accomplished by analyzing activities of corresponding phospho-mimicking and non-phosphorylatable mutants as well as their associations with regulatory and effector proteins of the MAPK cascade at mitosis. Analysis of the B-Raf tertiary structure by X-ray crystallography and virtual molecular 3D modeling could provide detailed information how phosphorylations of these sites affect B-Raf’s conformation. Since Cdk1/cyclin B and MAPK phosphorylations oppositely affect activity of mitotic B-Raf, it will be interesting to understand how these two phosphorylation events are coordinated throughout mitosis.

To address this, phospho-specific antibodies need to be prepared and then used to analyze a time-course of B-Raf phosphorylation throughout mitosis. Furthermore, it needs to be addressed whether mitotic B-Raf undergoes regulatory phosphorylation at other sites. A broad analysis of B-Raf phospho-modifications can be performed by phospho-microsequencing of B-Raf immunopurified from mitotic *Xenopus* egg extracts. Finally, on the flip side, the phosphatases that dephosphorylate B-Raf’s regulatory phospho-sites will be important to study. This can be done by analyzing B-Raf activity and
phosphorylation during mitosis following treatment with specific phosphatase inhibitors or in *in vitro* phosphatase assays.

Analysis of the protein complexes that form with B-Raf during mitosis is another important area of biochemical studies. The proteomics data provided in this study can be used as a good starting point for this research. Co-immunoprecipitation studies and biochemical purification approaches could be used to validate interaction of the identified proteins with B-Raf at mitosis. Validation of particular B-Raf mitotic interactors will refine and focus future studies of B-Raf regulation and functions at mitosis.

B-Raf is known to undergo alternative splicing, which can modulate its activity (Papin et al., 1998). My data indicate that a small 68 kDa isoform of B-Raf is phosphorylated within the activation loop in M-phase arrested extracts, indicating that the 68 kDa B-Raf may function during mitosis along with the 95 kDa B-Raf studied in this work. Interestingly, my data showed that immunodepletion of 95 kDa B-Raf from *Xenopus* egg extracts does not affect the levels of 68 kDa (not shown) suggesting that the smaller B-Raf isoform is not required for activation of the MAPK cascade at mitosis. However, the contribution of 68 kDa B-Raf in MAPK activation in mitotic extracts was not addressed directly. Thus, it still needs to be determined whether 68 kDa B-Raf regulates MAPK-independent functions at mitosis or cooperates with 95 kDa B-Raf in the regulation of mitotic MAPK signaling. It would be very interesting to explore this possibility further in future studies by raising antibodies that can immunoprecipitate the smaller B-Raf isoform.
It needs to be addressed whether B-Raf protein levels are regulated in an M-phase dependent manner. By using Western analysis I have reproducibly observed an enrichment of B-Raf during mitosis and decrease of its levels at the subsequent S-phase (Fig. 20B, 25B, 37B). This implies that B-Raf protein levels may be up-regulated during M-phase and down-regulated at the mitotic exit. The possibility that B-Raf expression is regulated in an M-phase dependent manner needs to be addressed directly in the future studies. Studying stability of B-Raf throughout the cell cycle will be used to elucidate this question. However, B-Raf protein does not possess classical D- or KEN-box sequences for ubiquitination, which targets mitotic proteins for proteasome-directed degradation (Pfleger and Kirschner, 2000). Thus, it is unlikely that mitotic B-Raf undergoes APC-directed proteolysis.

Recent work revealed that B-Raf localizes to mitotic structures, such as the mitotic spindle, centrosomes, condensed chromatin, kinetochores and midbody (Borysova MK, Guadagno TM, 2006, manuscript submitted). Proper intracellular localization of B-Raf can play an important role in ensuring specificity of B-Raf’s regulation and functions during mitosis. Thus, studying biochemical mechanisms that control intracellular distribution of mitotic B-Raf will contribute to understanding regulation of B-Raf signaling. To study this, the domains necessary for B-Raf’s localization to certain sub-cellular structures need to be defined. This can be accomplished by analyzing sub-cellular localization of different B-Raf truncated mutants.

The results of my dissertation research suggest that B-Raf can be activated twice throughout the cell cycle: at the G1/S following mitogenic stimulation and during
mitosis. To generate a comprehensive picture of B-Raf’s regulation during cell cycle, it would be interesting to elucidate how these two different B-Raf regulatory mechanisms are coordinated. This can be accomplished by studying a time-course of B-Raf’s phosphorylation at the Cdk1/cyclin B-dependent and Ras-dependent residues throughout the cell cycle in a synchronized population of tissue culture cells.

Therefore, several aspects of B-Raf regulation during mitosis require further study. These include further exploration of phosphorylation and protein complex formation as modes of regulation of mitotic B-Raf, elucidation whether B-Raf protein levels are regulated in an M-phase dependent manner and whether alternative isoforms of B-Raf are involved at mitosis and, finally, the characterization of mechanisms of B-Raf intracellular localization.

The second direction for future research is studying B-Raf’s mitotic functions. First of all, it should be addressed whether the known MAPK’s functions at mitosis are mediated by B-Raf. A recent study demonstrated that B-Raf localizes to the mitotic apparatus and is required for proper spindle formation in somatic cells (Borysova MK, Guadagno TM, 2006, manuscript submitted), a mitotic role which already has been defined for MAPK (Horne and Guadagno, 2003). To further understand the mechanism by which B-Raf regulates the mitotic spindle, it will be necessary to identify what substrates are targeted by the B-Raf/MEK/MAPK cascade to promote proper formation of the mitotic spindle. Among other MAPK functions that may be mediated by B-Raf are the mitotic spindle checkpoint, regulation of M-phase entry, and Golgi fragmentation. B-Raf loss-of-function and gain-of-function studies will reveal whether B-Raf is linked to
some of the abovementioned mitotic events. There is a possibility as well that B-Raf may have mitotic roles, which are not dependent on MAPK. Tissue cell lines and the cell-free system of *Xenopus* egg extracts could be utilized to explore B-Raf’s functions during mitosis.

The third research direction is to study whether disregulation of B-Raf’s mitotic functions can provoke tumorigenesis. Recently B-Raf was recognized as a prominent oncogene, which is mutated and activated in a large proportion of human cancers, particularly, melanoma (Davies et al., 2002). Since I have shown that B-Raf activates MAPK signaling at mitosis and is implicated in the regulation of the mitotic spindle in somatic cells (Borysova MK, Guadagno TM, 2006, manuscript submitted), it is plausible to hypothesize that disregulation of B-Raf mitotic functions may result from expression of the B-Raf oncogene. This may lead to improper chromosome segregation and genomic instability, thereby provoking tumorigenesis. Analysis of mitotic abnormalities and the rate of accumulated chromosomal aberrations in somatic cell lines ectopically expressing the oncogenic form of B-Raf will clarify its role in tumorigenesis.

In conclusion, my dissertation research describes B-Raf as the mitotic MEK kinase critical for activation of the MAPK cascade. I demonstrate that B-Raf activation at mitosis is coupled to the mitotic regulatory machinery, namely Cdk1/cyclin B. In addition, recent studies have demonstrated a role for B-Raf in regulating the mitotic spindle in human somatic cells. Therefore, I propose that B-Raf is an essential regulator of mitosis. Future research is necessary to define and characterize B-Raf’s regulation and functions at mitosis in normal cells and in cancer.
Chapter Eight

Materials and Methods

Preparation of Xenopus egg extracts

S-phase arrested Xenopus egg extracts were prepared essentially as described (Chen et al., 1998). Briefly, mature Xenopus oocytes were dejellied in 2% L-cysteine solution (pH 7.8), washed in MMR buffer (5 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM EDTA) and parthenogenetically activated with the Ca²⁺ ionophore A23187 (0.2 µg/ml) for 2.5 minutes. After Ca²⁺ ionophore withdrawal, the activated eggs were incubated in egg lysis buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 50 µg/ml cyclohexamide) for another 50-60 min at room temperature before processing into extracts by a high-speed centrifugation (10, 000 g 15 min at 4°C). This method closely mimics the degradation of c-Mos, a meiosis specific MEK kinase, which is observed following fertilization (Chen et al., 1998; Nishizawa et al., 1993; Watanabe et al., 1991; Watanabe et al., 1989). M-phase arrested extracts were prepared by supplementing crude S-phase egg extracts with recombinant non-degradable sea urchin Δ90 cyclin B1 (75-100 nM final) and incubating at room temperature for 60 min. Expression and purification of GST-Δ90 cyclin B was performed as described (Horne and Guadagno, 2003). Note that
all preparations of M-phase extracts were checked for the activation of MAPK and the absence of c-Mos protein by immunoblot analysis.

Cycling extracts were prepared in a procedure similar to described above, except that eggs activated with the calcium ionophore A23187 were incubated in XB buffer (10 mM HEPES, pH 7.7, 50 mM sucrose, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂) for 45 min before processing them into extracts. Cytostatic factor (CSF) arrested *Xenopus* egg extracts were prepared as described (Murray, 1991).

*Studying c-Mos degradation in fertilized and Ca²⁺ ionophore activated Xenopus oocytes*

Mature *Xenopus* oocytes were fertilized with sperms freshly isolated from male *Xenopus* frogs (Heasman et al., 1991) or activated with Ca²⁺ ionophore A23187 as mentioned above. Fertilized and Ca²⁺ ionophore activated eggs were incubated at room temperature in 0.25X MMR solution. (1.25 mM HEPES, pH 7.8, 25 mM NaCl, 0.5 mM KCl, 0.25 mM MgCl₂, 0.5 mM CaCl₂, 0.025 mM EDTA). 10 eggs were collected at 10-minute intervals and frozen on dry ice. Frozen eggs were thawed on ice and resuspended in 100 µl of extraction buffer (20 mM HEPES, pH 7.2, 0.25 M sucrose, 0.1 M NaCl, 50 mM β-glycerol phosphate, 2.5 mM MgCl₂, 1 mM Na₃VO₄, 25 mM NaF) supplemented with 10 µg/ml of each leupeptin, pepstatin, and aprotinin. Lysates were subjected to centrifugation for 12 min at 4°C in an eppendorf centrifuge. The levels of c-Mos protein in the clarified extracts were visualized by Western analysis with c-Mos antibodies.
**Immunoblot analysis**

Primary antibodies used include: mouse monoclonal anti-phospho (T202/Y204) ERK (1:2000), rabbit polyclonal anti-phospho (S217/S221) MEK (1:1000), and mouse monoclonal myc-tag (1:1000) were purchased from Cell Signaling; rabbit polyclonal anti-B-Raf (sc9002; 0.02 µg/ml), anti-Raf-1 (0.4 µg/ml), anti-c-Mos (0.4 µg/ml), anti-ERK2 (0.2 µg/ml) and anti-phospho (T599/S602) B-Raf (0.2 µg/ml) were purchased from Santa Cruz; mouse monoclonal anti-Cdk1 (0.2 µg/ml) was purchased from Calbiochem, rabbit anti-MEK (1:1000) was prepared by Zymed Laboratories (South San Francisco, CA) against an N-terminal 16 amino acid sequence of *Xenopus* MEK1, rabbit anti-MAPK (1:1000) were provided by Jim Ferrel (Stanford University) and rabbit anti-cyclin B were provided by James Maller (University of Colorado).

Secondary antibodies included species-specific alkaline phosphatase-conjugated anti-mouse (Jackson ImmunoResearch Laboratories) and anti-rabbit (Sigma) IgG that were detected with the CDP-Star chemiluminescence substrate (Roche Diagnostic).

**Purification of mitotic MEK kinase activity from Xenopus egg extracts**

Crude M-phase arrested extracts prepared as described above were ultracentrifugated twice at 100,000 g for 1.5 hr to isolate cytosolic fraction. Mitotic cytosol fraction was diluted in ice-cold egg lysis buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT) supplemented with 10 µg/ml of each leupeptin, pepstatin, and aprotinin and mixed 3:2 with the same buffer containing 50% ammonium sulfate to reach final 20% saturation. Samples were rotated for 2.0 hrs at 4°C
and precipitated proteins were pelleted by a high-speed centrifugation (10,000g 15 min at 4°C). The pellet was resuspended in buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl_2) supplemented with 25 mM NaF and 1 mM Na_3 VO_4, and 10 µg/ml of each pepstatin, leupeptin and chymostatin, and subjected to chromatography separation by using FPLC-AKTA (Amersham Biosciences). First, the pellet was applied to a 10-ml HiTrap Q Sepharose HP column (Amersham Biosciences). The proteins were eluted with a step-wise NaCl gradient in buffer A: 0-0.3 M (40 ml), 0.3-1 M (10 ml). Fractions that contained MEK kinase activity (0.17-0.25 M NaCl) were pooled together and applied to a Mono Q HR 5/5 column (Amersham Biosciences). The proteins were eluted with a step-wise NaCl gradient in buffer A: 0-0.35 M (3 ml), 0.35-0.50 M (15 ml), 0.5-1 M (3 ml). Fractions containing MEK kinase activity (0.37-0.42 M NaCl) were collected and analyzed for protein composition by Western blotting and Silver Staining with GelCode SilverSNAP Stain Kit (Pierce). All purification steps were performed at 4°C and assayed for MEK kinase activity.

**MEK kinase assays**

To measure MEK kinase activity, samples were incubated in 30 μl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl_2, 0.1 mM ATP, 25 mM NaF, 1 mM Na_3 VO_4, 1 mM DTT) containing 0.5 µg of recombinant unactive GST-MEK1 (Upstate) for 20 min at 25°C. The kinase reaction was stopped with SDS sample buffer and reaction products were separated by SDS-PAGE. Phosphorylation of recombinant GST-
MEK1 at serine residues 217 and 221 was analyzed by immunoblotting with phospho-
S217/S221 MEK antibodies (Cell Signaling).

Alternatively, MEK kinase activity was measured in an in vitro linked kinase
assay as described (Guan et al., 2000). Briefly, aliquots of purified fractions or protein A
beads immunocomplexes were incubated with 1.0 µg of recombinant unactive GST-
MEK1 (Upstate) in 25 µl of reaction buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 25
mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, 5 mM NaF, 1 mM Na₃VO₄, 0.1 mM
ATP) for 20 min at room temperature. The reaction mix was briefly centrifugated to
pellet immunocomplexes. Next, 20 µl of the supernatant was mixed with 10 µl of the
reaction buffer containing 9.0 µg of recombinant unactive GST-ERK and reaction was
continued for another 15 min. Finally, 3 µl aliquot of the reaction was mixed with 30 µl
of reaction buffer containing 50 µg of MBP and 5 µCi of ³²Pγ-ATP and incubated for
another 10 min at room temperature. The kinase reaction was stopped with SDS sample
buffer, separated on 15% SDS-PAGE, and transferred to a PVDF membrane. The levels
of MBP phosphorylation was visualized by autoradiography and quantified by using
ImageQuant software.

**In vitro histone H1 kinase assay**

To measure Cdk1/cyclin B activity, 1 µl aliquots of *Xenopus* egg extracts were
incubated in 50 µl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM
EGTA, 2 mM DTT, 0.1 mM ATP, 10 µM PKI, 0.01% Brij 35) containing 20 µg H1
protein (Upstate) and 2 µCi ³²Pγ-ATP for 20 min at 30°C. Reactions were stopped by the
addition of SDS sample buffer and heating at 95°C for 5 min. Reaction products were separated by 10% SDS PAGE and phosphorylation levels of histone H1 were determined by autoradiography.

**Immunodepletion**

To immunodeplete B-Raf from S-phase extracts, 50 µl of extracts were incubated under gently rotation for 1-1.5 hr at 4°C with 15 µg of rabbit polyclonal B-Raf antibodies (Santa Cruz) pre-bound to 5 µl of protein A sepharose 4B Fast Flow beads (Sigma). B-Raf immuno-complexes were pelleted by a quick centrifugation, and a second round of immunodepletion was performed. As a control, extracts were mock depleted in parallel using an equivalent amount of rabbit IgG (Sigma). In a similar procedure, Raf-1 was immunodepleted from S-phase extracts with 5 µg of rabbit polyclonal Raf-1 antibodies (Santa Cruz).

**Immunoprecipitation**

B-Raf immunoprecipitations were performed by adding 4 µg of rabbit polyclonal B-Raf antibodies (Santa Cruz) to 10 µl of *Xenopus* egg extracts diluted in 40 µl of buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 25 mM NaF, 1 mM Na₃VO₄) supplemented with 10 µg/ml of each pepstatin, leupeptin and chymostatin. To immunoprecipitate c-Mos-associated MEK kinase activity from M-phase arrested extracts, 5 µg of either Santa Cruz c-Mos antibodies or Abcam c-Mos antibodies were added to 10 µl of extracts diluted in 40 µl of buffer A. Following a 2 hr incubation on ice, immunocomplexes were
recovered on protein A agarose beads (Sigma), washed twice with the same buffer containing 0.1% Triton X-100, and 3 times with buffer A alone. Washed B-Raf immune complexes were used directly in *in vitro* linked kinase or phosphatase assays or resuspended in SDS sample buffer and analyzed by immunoblotting.

*Co-immunoprecipitation analysis*

For co-immunoprecipitation studies 20 µl of *Xenopus* egg extracts were mixed with 6.5 µg of anti-B-Raf (Santa Cruz), 6.5 µg of anti-Raf-1 (Santa Cruz), 4 µg of anti-MEK (customer-designed, Zymed Laboratories), 4 µg of anti-MAPK, 4 µg of anti-Cdk1 (Calbiochem) or 6 µg of anti-cyclin B1. Extracts were diluted 1:1 in EB buffer (80 mM β-glycerol phosphate, pH 7.3, 15 mM MgCl₂, 20 mM EGTA, 25 mM NaF, 1 mM Na₃VO₄) supplemented with 0.1% Triton X-100 and 10 µg/ml of each pepstatin, leupeptin and chymostatin. The immune complexes were recovered on protein A agarose beads (Sigma) and washed three times with EB buffer containing 0.1% Triton X-100.

*Generation of wild-type and mutant *Xenopus* B-Raf constructs*

A *Xenopus* B-Raf cDNA clone was obtained from ATCC (Image Clone I.D. 6860469). DNA sequencing analysis, performed at H. Lee Moffitt Cancer Center DNA facility, confirmed a full-length *Xenopus* cDNA containing an open reading frame of 803 amino acids. The sequence data of *Xenopus* B-Raf has been submitted to the Genbank database under accession N° DQ097958. The B-raf coding region was excised at Hind III sites and sub-cloned into a modified pGEM transcription vector (kindly provided by
Dr. Rey-Huei Chen from the University of Cornell) down-stream of an N-terminal myc-tag.

Mutant constructs were generated by using the QuikChange site-directed mutagenesis kit (Stratagene). The following myc-B-Raf mutants were prepared and verified by DNA sequencing (H. Lee Moffitt Cancer Center DNA facility): Ser-144-Ala, Ser-329-Ala, Ser-144-Ala/Ser-329-Ala, Ser-784-Ala/Thr-787-Ala, Lys-517-Met, Lys-517-Met/Ser-144-Ala, Lys-517-Met/Ser-329-Ala, Lys-517-Met/Ser-144-Ala/Ser-329-Ala.

Expression of recombinant myc-B-Raf proteins in CSF Xenopus egg extracts

Expression of wild-type and mutant B-Raf proteins was performed similarly as described (Sharp-Baker and Chen, 2001). Briefly, wild type and mutant myc-B-raf transcripts were generated using the mMESSAGE mMACHINE T7 transcription kit (Ambion). 4 µg of purified mRNA transcripts were introduced into 21 µl of Xenopus CSF-arrested egg extracts supplemented with 2.5 µl of rabbit reticulocyte lysate (Ambion). Protein expression was carried out at 23°C for 5 hr with gentle mixing every 15 min. Expression of recombinant proteins was confirmed by Western blotting with myc epitope antibodies. In order to reproduce the S-phase phosphorylation status of the recombinant B-Raf proteins, aliquots of translated WT or mutant myc-B-Raf proteins were introduced into S-phase arrested extracts in a 1:20 ratio and incubated for 30-60 min at room temperature. To induce M-phase phosphorylation of the recombinant proteins, aliquots of S-phase extracts containing myc-B-Raf protein were driven into M-phase by
adding recombinant non-degradable cyclin B. Western blot analysis revealed that recombinant myc-B-Raf proteins undergo dephosphorylation during incubation in S-phase extracts and acquire hyperphosphorylation during incubation in M-phase extracts.

**Purification of recombinant myc-B-Raf proteins from Xenopus egg extracts**

Myc-B-Raf recombinant proteins were purified by immunoprecipitation using myc-tag antibodies. Aliquots of egg extracts containing myc-B-Raf were diluted 1:20 in buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl₂, 10% glycerol, 5 mM EGTA, 1 mM DTT, 1 mM Na₂VO₄, 5 mM NaF, 0.1% Triton X-100 and 10 µg/ml of each pepstatin, leupeptin and chymostatin) and incubated on ice with myc-tag monoclonal antibodies (Cell Signaling) for 2 hrs. Protein A Sepharose beads were added and incubation was continued for another 12-16 hrs with gentle inversion at 4ºC. Immuno-complexes were washed twice with the same buffer and twice with the reaction buffer (25 mM HEPES, pH 7.5, 25 mM β-glycerophosphate, 10 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 1 mM Na₂VO₄, 5 mM NaF) before applying to *in vitro* kinase assays.

**Phosphatase Treatment**

B-Raf immunoprecipitates from S- and M-phase arrested extracts were incubated with 50 units of recombinant lambda protein phosphatase (Upstate Biotech) in 50 µl of phosphatase buffer (50 mM HEPES, pH 7.5, 0.1 % BSA, 100 µM EDTA, 2 mM MnCl₂, and 5 mM DTT) for 30 min at 37ºC. To stop reactions, precipitates were washed with
copious amount of the buffer containing phosphatase inhibitors (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 25 mM NaF, 1 mM Na₃VO₄).

**In vitro Cdk1/cyclin B kinase assay**

Myc-B-Raf IPs were incubated with 100 units of recombinant active Cdk1/cyclin B (New England Biolabs) in 30 µl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.1 mM ATP, 0.01% Brij 35) with radioactive 
³²PγATP (5 µCi/reaction) for 30 min at 30°C. Reactions were stopped by the addition of SDS sample buffer and heating at 95°C for 5 min.

**In vitro ERK2 kinase assay**

Endogenous B-Raf or myc-B-Raf immunoprecipitates were incubated with 20 units of recombinant active ERK2 (New England Biolabs) in 30 µl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.1 mM ATP, 0.01% Brij 35) with or without radioactive 
³²PγATP (5 µCi/reaction) for 30 min at 30°C. Reactions were stopped by the addition of SDS sample buffer and heating at 95°C for 5 min.

**Native gel electrophoresis and transfer**

Samples were run in a 4-25% gradient gel (BioRad) in SDS-free buffer (250 mM Tris, 200 mM glycine) for 12-14 hrs at 4°C. Transferring of separated proteins to PVDF
membrane was performed in the transfer buffer supplemented with 0.5% SDS (20 mM Tris, 150 mM glycine, 0.5% SDS).

Gel filtration

Size exclusion chromatography was performed at 4°C using Superdex 200 HP 10/30 column (bed volume ~24 ml; Amersham Biosciences) with a FPLC-AKTA (Amersham Biosciences) purification system in the buffer containing 50 mM HEPES, pH 7.5, 10 mM MgCl₂ and 10 μg/ml of each pepstatin, leupeptin and chymostatin. The flow rate was 0.25 ml/min and the sample volume was 100 μl.
References


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About the Author

Sergiy I. Borysov was born in Ukraine. He received a Master’s Degree in Biochemistry (1998) from Kiev National Taras Shevchenko University. While being a student of this University, Sergiy was a recipient of the International Soros Support for Educational Sciences Grant (1995 and 1997) and a winner of All-Ukrainian Students Contests in Biology (1996, 1997).

Sergiy Borysov entered an Interdisciplinary Ph.D. program in Cellular and Molecular Biology at the University of South Florida in January 2001. In June 2001 he affiliated with the Department of Biochemistry and Molecular Biology. While in the Ph.D. program, Mr. Borysov received an Outstanding Graduate Student Award from the College of Medicine (2005). Sergiy Borysov was a recipient of the American Heart Association Pre-Doctoral Fellowship (2003-2005). His findings have been presented at the regional and international scientific meetings and were published in the Journal of Biological Chemistry in June 2006.