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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director RICHARD VAIIANCOURT April 05, 02
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ABSTRACT

The cAMP-dependent protein kinase, PKA, mediates diverse cellular processes including proliferation and differentiation, as well as the phosphorylation of proteins involved in cell death and survival such as BAD and GSK-3β. In this work, I demonstrate that PKA phosphorylates the stress-activated protein kinase, MEKK3 in vivo and in vitro. When (His)_6FLAG•MEKK3 was expressed in Sf9 insect cells and purified with Ni-Sepharose, we identified 14-3-3 protein by liquid chromatography and electrospray tandem mass spectrometry (LC-MS) as co-purifying with recombinant MEKK3. The yeast two hybrid system was also utilized to identify 14-3-3 as interacting proteins with MEKK3 as well as Bcl-xL. The interaction between MEKK3 and Bcl-xL was specific for the caspase-cleaved Bcl-xL which suggests a role for MEKK3 in apoptotic signaling pathways. However, the physiological significance of this interaction is unclear since caspase-cleaved Bcl-xL did not act as a MEKK3 substrate or alter MEKK3 kinase activity. Since 14-3-3 proteins have been reported to interact with proteins through phosphoserine, we sequenced (His)_6FLAG•MEKK3 by LC-MS to identify phosphorylated amino acids. Of the tryptic peptides sequenced, two consisted of amino acids 164-174 and 335-349 and serines 166 and 337 were phosphorylated within the respective peptides. Phosphorylation of both serines was localized within two consensus PKA phosphorylation sites, RX(S/T). PKA activators such as serum and forskolin increased phosphorylation of endogenous MEKK3 at Ser166 as well as the preferential recruitment of phospho-MEKK3 with 14-3-3. These results connect the stress-activated pathways regulated by MEKK3 with pathways regulated by PKA.
CHAPTER 1
INTRODUCTION, HYPOTHESES AND AIMS

1.1 INTRODUCTION

The mitogen-activated protein kinase (MAPK) cascades are conserved across multiple species including, humans, yeast, Drosophila melanogaster, Xenopus laevis, and Caenorhabditis elegans. An extracellular stimulus results in the activation of sequential MAPK cascades. Typically, a three-kinase module consisting of a MAP3K, MAP2K, and MAPK modulate the cellular response (Figure 1.1). Ultimately MAPK phosphorylation and activation leads to an intracellular response such as, proliferation, differentiation, or apoptosis (41). Thus far four mammalian MAPKs have been identified as follows: MAPK^{JNK}, MAPK^{ERK}, MAPK^{p38}, and MAPK^{ERK5} [reviewed in (42)].

1.1.1 Organization of MAPK Signaling Pathways

Input into the MAPK module begins with activation of a serine/threonine kinase, generically referred to as a MAP3K, either by a small GTP-binding protein or a MAP4K such as, p21-activated kinase (PAK) (9, 18, 29). Subsequently, MAP2Ks are activated through phosphorylation by one of the MAP3Ks. All MAP2Ks share a common feature; that is, their activation through dual phosphorylation on threonine and tyrosine residues within their activation loop by an upstream MAP3K. Lastly, phosphorylation of MAPKs by MAP2Ks leads to phosphorylation of substrates that often include transcription factors
Figure 1.1. Generalized scheme of a MAPK pathway. The core MAPK module consists of a MAPKKK (MAP3K), MAPKK (MAP2K), and MAPK that transduce an extracellular stimulus through a cell. The sequential phosphorylation by an upstream kinase results in a biological response within the cell.
that contain a proline rich consensus site, Pro-X-Ser/Thr-Pro (4). The phosphorylation of MAPK substrates results in the modulation of gene expression and a variety of biological responses within the cell.

Although many diverse signals exist which activate signal transduction pathways, the families of MAPK and the coordinating MAP3K and MAP2K kinases within the MAPK module lead to a specific biological response (Figure 1.2). For instance, extracellular signal-regulated kinase (ERK) MAPK activity is primarily in response to growth factor stimulation including epidermal growth factor or platelet derived growth factor which leads to cell growth. The ERK pathway is regulated by the MAP2Ks, MEK1 and MEK2 (2). The c-Jun N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK) MAPK is characterized as the stress-activated pathway and responds to cell stresses such as, UV irradiation, heat shock, and reactive oxygen species (ROS). MAP2Ks controlling JNK are MKK4 and MKK7 (12, 28, 36, 39). Similar to JNK, p38 was identified based on the response to cellular stress. However, the p38 pathway is responsive to osmotic and environmental stress and is controlled by the MAP2Ks, MKK3 and MKK6 (22, 31, 33, 37).

1.1.2 Regulation of MAP3Ks

A family of upstream kinases that positively regulate MAP3K activity are the PAKs, which are the mammalian equivalent of the *S. cerevisiae* protein, STE20 that is the upstream kinase of STE11 (Figure 1.3) (23). The expression of PAK3 can partially restore the STE20 null defect in *S. cerevisiae* (6). In mammalian cells, PAK1 and PAK3
Figure 1.2. JNK, ERK, and p38 MAPK cascades. These MAPKs are shown with representative components of their core MAPK module and a specific cell stimulus, MAP2K, MAP3K, and transcription factor. The phosphorylation of transcription factors and potential biological responses are shown for JNK, ERK, and p38, respectively. MAP3Ks are not restricted to act only on the MAP2Ks depicted in this diagram. Cross-talk can occur between MAPK pathways.
can activate p38 MAPK (5), while PAK2 preferentially activates JNK (18), suggesting that the PAK family of serine/threonine kinases function to phosphorylate and activate MAP3Ks. In addition, PAK3 has been shown to directly phosphorylate Raf-1 (26).

Besides activation of MAP3Ks by PAKs, the negative regulation of MAP3Ks by upstream kinases has also been reported. The serine/threonine protein kinase, Akt, is a mediator of cell survival signals. Akt serves as a downstream target of PI3-Kinase and is activated in response to a wide variety of cell survival signals (10). Thus far, members of the Raf family, B-Raf (21) and c-Raf (35, 49) are inactivated by Akt dependent phosphorylation. Additionally, elevation of cAMP levels results in activation of the cyclic nucleotide dependent protein kinase A, PKA, and subsequent inactivation of Raf-1 (30). Thus, negative regulation is another means to control MAP3K pathways.

1.1.3 Cloning of MEKKs

At least 14 members of the MAP3K family have been identified in mammalian cells. MAP3Ks can be characterized into families that include Raf, MEKK, and apoptosis signal-regulated kinase 1 (ASK-1). The first mammalian MAP3Ks were identified by Johnson and colleagues in which they isolated a murine cDNA clone encoding MEKK1, whose translated product was homologous to the yeast protein, STE11, a MAP3K in the yeast pheromone response pathway (27). MEKK1 activates
JNK and ERK as expected by its position in MAPK cascades (46). After the cloning of MEKK1, similar cloning strategies were utilized to isolate additional MAP3Ks, such as, MEKK2 (7), MEKK3 (7, 13), MEKK4 (20, 38) and MEKK5 (40) that is also known as ASK1 (24). MEKKs were identified using a polymerase chain reaction (PCR) based approach and these kinases were identified based on their homology to the yeast protein kinase, Ste11. The function of Ste11 in the yeast pheromone response pathway is to phosphorylate and activate Ste7 (Figure 1.3), however; the functions or upstream regulators of the MEKKs remain uncertain since they were cloned based on homology to Ste11 and not in a stimulus-dependent manner.
Figure 1.3. Mating response pathway of yeast strain, *S. cerevisiae*. The mammalian serine/threonine protein kinases, MEKKs are homologous to Ste11. Pheromone results in the activation of MAP4K, Ste20, and subsequently Ste11 and Ste7, the respective MAP3K and MAP2K components in the yeast pathway. Ultimately, Fus3 and Kss1 MAPK activation results in a mating response.
1.1.4 Characterization of MEKKs

There are currently four known members of the MEKK (Mitogen-Activated Protein (MAP) kinase/Extracellular Signal-Regulated Kinase (ERK) Kinase Kinase) family referred to as MEKK1, 2, 3 and 4. All the MEKK proteins are serine/threonine kinases that are believed to function two steps upstream of the ERK, JNK, and p38 MAP kinases. MEKK3 is a 71 kDa protein that is encoded by 626 amino acids and it shares greater than 77% homology with MEKK2, a 69 kDa protein encoded by 619 amino acids (Figure 1.4). MEKK2 and MEKK3 (69 kDa) are smaller protein kinases in the MEKK family as compared to MEKK1 (196 kDa) and MEKK4 (180 kDa). No identifiable domains besides the carboxy-terminal kinase domain have been identified in MEKK2 and MEKK3. These kinases differ primarily in their amino-terminal or regulatory domains that are only 65% conserved, while the carboxy-terminal kinase domains that are encoded by amino acids 362-619 or 368-626 for MEKK2 and MEKK3 respectively share greater than 94% homology in their catalytic domains (Figure 1.5). The activation of multiple MAPKs, including JNK, ERK, p38 and BMK has been demonstrated through transient expression of MEKK3 in mammalian cells (13, 82, 8) (Figure 1.6). MEKK3 and MEKK2 were initially identified as activators of JNK and ERK MAPKs (7). MEKK3 activates JNK and ERK and is thought to be immediately upstream of the MAP2Ks, JNKK or MEK, however, the direct phosphorylation of these kinases by MEKK3 has not been demonstrated. The human homolog of murine MEKK3 was cloned.
### Figure 1.4. MEKK2 and MEKK3 Amino Acid Alignment

Colons indicate identical amino acids at that position in the sequence. The non-conserved amino acid residues are shown in bold. MEKK2 and MEKK3 are highly homologous within their carboxy-terminal kinase domains.
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Figure 1.5
Figure 1.5. Alignment of the kinase domains of MEKK3 and MEKK2. Roman numerals indicate the 11 conserved subdomains within the catalytic domain. The most conserved amino acid residues within the kinase subdomains are underlined while the non-conserved residues are shown in bold. Asterisk indicates the active-site lysine at amino acids, 389 and 391 in MEKK2 and MEKK3, respectively.
Figure 1.6. Multiple signaling pathways are activated by MEKK3. The activation of ERK, JNK, p38, and BMK MAPK cascades by MEKK3 has been demonstrated. Additionally, the transcription factor, NF-κB is activated by MEKK3. An upstream regulator of MEKK3 and phosphorylation sites have not been identified.
and identified as an activator of JNK and ERK MAPKs (13).

1.1.5 Pathways Regulated by MEKKs

The small GTP-binding proteins, Ras, Rac, Cdc42, and Rho have been identified as upstream components of MEKK signaling pathways (9). The kinase domain of MEKK1 interacts with Ras (116). Furthermore, MEKK and GTP-binding was observed through the physical association between MEKK1 and MEKK4 with Rac and Cdc42 (116). The significance of this interaction was demonstrated in which kinase-inactive MEKK1 or MEKK4 blocked the activation of JNK mediated by these small GTP-binding proteins, however, in similar experiments MEKK2 or MEKK3 did not associate with Rac, Cdc42 or Rho (116). Thus, the upstream MAP4K or small GTP-binding proteins that regulate MEKK2 and MEKK3 remain unknown.

Perhaps the intracellular localization of MEKKs would suggest potential roles for these proteins and their physiological functions. Early attempts to identify the localization of MEKK3 were unsuccessful. MEKK2 and MEKK4 were found to be predominantly Golgi associated proteins while MEKK1 was both nuclear and cytoplasmic (15). Therefore, the identification of MEKK3 localization in the cell may provide some insight into the function of this MAP3K.

Recently, MEKK3 has been identified as the upstream kinase for the MAP2K, MEK5 (8), however, only MEKK3 with a truncation of the first 11 amino acids of the amino-terminal regulatory domain was shown to directly phosphorylate MEK5. These studies were controversial since the full-length MEKK3 did not phosphorylate MEK5.
The newest MAPK, ERK5, is activated in response to oxidative stress, growth factors, and hyperosmotic stress (1, 25). This MAPK contains MEK5 at the level of MAP2K. These studies were the first to identify a MAP2K protein as a substrate of a MEKK3, yet given the number of diverse pathways that are activated by MEKK3, perhaps other protein substrate have not been identified.

Besides MAPK activation by MEKK3 overexpression, the transcription factor, NF-κB is activated by MEKK3 and MEKK2. In fact, the transient overexpression of MEKK2 and MEKK3 can activate the protein kinase, IKK (48). IKK is known to function as an upstream component of the NFκB complex that leads to IKβ phosphorylation. The mechanism for MEKK3 activation of NF-κB was undetermined, but the direct phosphorylation of IKKβ by MEKK3 has now been demonstrated (45).

MEKK3 controls cell cycle progression and cyclin expression (14). An estrogen inducible form of human MEKK3 stably expressed in fibroblasts resulted in the inhibition of cell cycle progression, specifically through arrest of cells in G1 of the cell cycle. Further examination of cells revealed that expression of cyclin D1 was downregulated in response to MEKK3 overexpression. This finding is significant because cyclin D1 plays a critical role in the advancement of cells through G1. Other proteins that regulate cell cycle, including the kinase subunits of cdk2 and cdk4 were also downregulated upon induction of MEKK3 expression. These findings suggest MEKK3 is a critical protein for cell cycle control.

MEKK3 knockout mice were generated to identify physiological functions for MEKK3 (44). Deletion of MEKK3 resulted in embryonic lethality at day 11 due to
impaired blood vessel formation. Although genes affecting blood vessel development such as, Tie-1, and Tie-2 were studied, no changes were observed in expression levels with MEKK3 mutants. MEKK3 also activated the transcription factor, Mef2c (myocyte-specific enhancer factor 2C) that activates the p38 MAPK. Thus, MEKK3 may play a role in cardiovascular development.

A ubiquitous group of proteins that function in signal transduction pathways are the 14-3-3 proteins (3). These proteins have been shown to interact with various protein kinases including Raf-1 (17, 19), c-Bcr, Bcr-Abl (34), and the MEKKs (16), in particular MEKK2 and MEKK3. More recently, the negative regulation of proteins that bind 14-3-3 proteins has been reported. As mentioned earlier, many Akt substrates are negatively regulated as a consequence of Akt phosphorylation. An additional component of their negative regulation is the subsequent binding of 14-3-3 to phosphoserines. In cell death and survival pathways, 14-3-3 proteins bind the Akt substrate and pro-apoptotic protein, BAD, thereby promoting cell survival (11). Additionally, the protein phosphatase, Cdc25 is negatively regulated by 14-3-3 binding (32). A recent function of 14-3-3 includes the prevention of apoptosis through inhibition of MAPK cascades (43). The stress-activated kinase, ASK-1 which promotes apoptosis is found to be inhibited by the association with 14-3-3 (47). Although 14-3-3 proteins have several functions is signal transduction pathways, the function of 14-3-3 binding to members of the MEKK family has not been determined.

1.2 HYPOTHESIS AND AIMS
Given the importance of MAPKs in cell survival and apoptosis, the overall goal of my dissertation research was to further characterize the stress-activated protein kinase, MEKK3, since the activity of MEKK3 is likely to regulate the MAPK family of proteins. Various techniques including the yeast two hybrid and the baculovirus insect cell sytem were utilized to identify and characterize interacting proteins and phosphorylation sites. Other objectives of these studies were to gain a better understanding of the interaction between MEKK3 and 14-3-3, in particular to determine whether phosphorylation modulates kinase activity or 14-3-3 binding. Although MEKK3 and MEKK2 are highly homologous MAP3Ks, it is predicted that the differences in their amino-terminal sequences and phosphorylation sites are determinants of substrate specificity and regulation by upstream kinases or small GTP binding proteins. Since MEKK2 and MEKK3 share similar homology and activate similar MAPK pathways, the purpose of my experiments was to identify and characterize phosphorylation sites and novel proteins that specifically regulate MEKK3.
CHAPTER 2
IDENTIFICATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT WITH MEKK3

2.1 INTRODUCTION

Previously, the only known functions for MEKK3 were the activation of JNK and ERK kinase pathways. In an attempt to elucidate a physiological function for MEKK3, the yeast two-hybrid method was utilized to identify proteins that interact with and potentially regulate MEKK3. This method utilizes two fusion proteins that are expressed simultaneously and their subsequent interaction results in the activation of reporter genes in yeast (52). One of the fusion proteins contains the DNA binding domain, LexA (202 amino acids), which is fused in-frame with the protein of interest. For this project, full-length MEKK3 (626 amino acids) was fused to LexA in the plasmid, pBTM116 and used as bait in the yeast two-hybrid system (Figure 2.1). The other fusion protein was derived from a mouse embryo library generated by random primed cDNA synthesis and size selected for fragments of 400-800 base pairs (bp) inserted into the VP16 plasmid. These cDNA fragments were translated as a fusion protein with VP16, which contains a transcriptional activation domain. A physical interaction between MEKK3 and a protein derived from the cDNA library results in the transcription of reporter genes. LacZ reporter gene activity may be determined by measuring β-galactosidase activity.
Figure 2.1. Overview of Yeast Two-hybrid Screen. Yeast were co-transformed with two plasmids, pBTM116 and pVP16. The bait plasmid, pBTM116 encodes the LexA DNA binding domain fused to full-length MEKK3 and contains the TRP1 gene for selection in yeast. The prey plasmid, VP16, encodes the VP16 activation domain fused to mouse embryo cDNA fragments and contains the LEU2 gene for selection in yeast. The yeast strain contains the LacZ gene for β-galactosidase analysis and the HIS gene for selection on histidine deficient media. β-galactosidase activity was measured to detect the interaction between the LexA•MEKK3 fusion protein and a protein in the cDNA library.
2.2 EXPERIMENTAL PROCEDURES

2.2.1 Yeast Two-hybrid Library Screen

LexA•MEKK3 expressed in the yeast strain, L40 was plated onto YC-UTL media and grown for 48 h at 30°C. YC-UT was inoculated with 6-8 colonies and grown overnight at 30°C in 10 ml YC-UT with shaking at 250 rpm. The following morning, 10 OD units of the yeast culture were transferred into 100 ml YC-UT and grown overnight at 30°C while shaking at 250 rpm. The next day 200 OD units were transferred into 900 ml YPAD and then incubated at room temperature for approximately 3 h or until the OD_{600} reached 0.5. After this incubation, yeast were collected by centrifugation for 6 min at 2,500 rpm and each yeast pellet was resuspended with 125 ml sterile 1X TE (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0) and pelleted by centrifugation for 6 min at 2,500 rpm at room temperature. Pellets were resuspended into a final volume of 20 ml of 100 mM lithium acetate (LiOAc)/0.5X TE. The mouse embryo cDNA library was prepared by adding 0.5 mg mouse embryo library cDNA to 140 ml of 100 mM LiOAc/40%PEG3350/1XTE and 10 mg salmon sperm DNA in a 500 ml flask. The resuspended yeast pellets were added to the 500 ml flask containing the mouse embryo cDNA library and incubated at 30°C for 30 min while shaking at 175 rpm. Yeast were transferred to a 2 L flask and 17.6 ml DMSO was added while swirling gently and this mixture was incubated for 15 min at 42°C with swirling every 2 min. After the DMSO incubation, yeast were briefly chilled on ice following the addition of 400 ml of YPAD,
and then centrifuged at 2,500 rpm for 6 min at room temperature. Each yeast pellet was washed with 125 ml YPA, the media was aspirated, and then yeast were incubated for 1 h at 30°C in 1 L of YPAD (pre-warmed to 30°C) while shaking at 175 rpm. Yeast were collected by centrifugation for 6 min at 2,500 rpm at room temperature and washed with 125 ml of 1X TE followed by centrifugation for 2,500 rpm for 6 min. The yeast pellets were resuspended in a final volume of 1 L of pre-warmed YC-UTL and incubated for 4 h at 30°C to allow recovery from the heat shock step. Following this recovery, yeast were pelleted by centrifugation at 2,500 rpm for 6 min and washed with 125 ml of YC-THULL (without glucose) and collected by centrifugation for 2,500 rpm for 6 min and the wash was repeated once. A final volume of 30 ml of YC-THULL was added to resuspend the pellets and 0.3 ml of this resuspension was cultured on 15 cm YC-THULL plates containing 1 or 5 mM 3-aminotriazole. Yeast transformants were grown at 30°C for 60 h.

2.2.2 Isolation of Yeast DNA

Following the initial two-hybrid screen, yeast DNA was isolated from individual colonies that grew on YC-THULL plates. The purpose of this isolation procedure was to isolate DNA from the bait plasmid that interacted with MEKK3 since transformants on YC-THULL expressed both VP16 and pBTM116 plasmid DNA. Individual colonies were selected from YC-THULL plates and grown in 2 ml YC-THULL for 24 h at 30°C with shaking at 250 rpm. Yeast were collected by centrifugation of 1.5 ml of the culture at 14,000 rpm for 5 sec. The supernatant was discarded except for 100 μl of supernatant
and pellet that were kept to resuspend yeast in 0.2 ml of yeast lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA). Glass beads (0.3 g of 0.5 mm diameter), and 0.2 ml phenol:chloroform:isoamyl alcohol (25:24:1) were added to the supernatant and mixed for 2 min at a setting of 5-6 on the microvortexer. Yeast were centrifuged at 14,000 rpm for 5 min at room temperature. Yeast DNA was precipitated by transferring the supernatant to a 1.5 ml eppendorf tube, adding 1/10 volume of 3 M NaOAc, pH 5.2, 2.5 volumes of 100% ethanol and incubating for 30 min at -80°C. The DNA pellet was isolated by centrifugation for 5 min at 14,000 rpm, washed twice with 1 ml of 70% ethanol, air dried and resuspended with 20 μl of 1X TE. High efficiency competent HB101 bacteria (Promega) were transformed with 5 μl of yeast DNA and grown on M9 plates for 48 h. Colonies were selected from M9 plates and grown in 4 ml of LB (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0) containing ampicillin (AMP) at a concentration of 50 μg/ml overnight at 37°C. Bacterial DNA was isolated from 2 ml of overnight culture using the Wizard DNA isolation kit according to manufacturer’s protocols (Promega). DNA was sequenced using VP16 primer 5'-GAGTTTGAGCAGATGTTT-3' or cotransformed into L40 yeast strain with original bait plasmid, LexA•MEKK3 or LexA•lamin (negative control) for β-galactosidase assay.

2.2.3 Small-scale yeast co-transformation

Yeast were co-transformed with bacterial DNA from the cDNA library protein and LexA•MEKK3 to verify the interaction on YC-THULL plates. A colony of L40 yeast was selected and incubated in 10 ml of YPAD overnight at 30°C while shaking at
250 rpm. The overnight culture was transferred to 40 ml of YPAD and grown an additional 2 h at 30°C while shaking at 250 rpm. Yeast were pelleted by centrifugation at 2,500 rpm for 5 min, resuspended in 40 ml of 1X TE and centrifuged at 2,500 rpm for 5 min. After centrifugation, the 1X TE was aspirated and the pellet was resuspended in 2 ml of 100 mM lithium acetate/0.5X TE and incubated at room temperature for 10 min. During this incubation, 1 µg of bacterial DNA from HB101 DNA transformation described above and 100 µg salmon sperm DNA were dispensed into a 1.5 ml eppendorf tube. Approximately 100 µl of resuspended yeast was added to the DNA and mixed well, followed by the addition of 700 µl of 100 mM lithium acetate/40%PEG3350/1XTE, and the samples were incubated for 30 min at 30°C. Yeast were heat shocked by the addition of 88 µl of DMSO and incubating for 7 min at 42°C. The samples were centrifuged for 10 sec at 14,000 rpm and supernatant was aspirated. The yeast pellet was resuspended in 1 ml of 1X TE and washed twice with 1X TE and supernatant removed after the final wash. The final pellet was resuspended in 20 µl of 1X TE. Yeast were plated on YC-UTL and grown for 48 h before proceeding with liquid β-galactosidase assay.

2.2.4 Liquid β-galactosidase Assay

Yeast colonies were grown overnight in 5 ml YC-UTL at 30°C with shaking at 250 rpm. The overnight yeast cultures were transferred into 50 ml YPAD liquid media and grown approximately 3-5 h until an OD_{600} of 0.5-0.6 was reached. Yeast were collected by centrifugation of 1.5 ml of the culture at 14,000 rpm for 30 sec at room
temperature. The supernatant was aspirated and yeast pellets were washed with 1.5 ml of Z buffer (\(\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}, \ 16.1 \ \text{g/L} ; \ \text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}, \ 5.5\text{g/L} ; \ \text{KCl}, \ 0.75\text{g/L} ; \ \text{MgSO}_4 \cdot 7\text{H}_2\text{O}, \ 0.246\text{g/L}, \ \text{pH} \ 7.0\)). Following centrifugation at 14,000 rpm for 30 sec, yeast pellets were resuspended in 300 \(\mu\text{l}\) of Z buffer. The yeast resuspension (150 \(\mu\text{l}\)) was transferred into a 1.7 ml eppendorf tube and placed in liquid nitrogen until frozen and thawed at 37\(^\circ\text{C}\) prior to performing the assay. The reaction was performed in the presence of 160 \(\mu\text{l}\) of 4 mg/ml o-nitrophenyl \(\beta\)-D-galactopyranoside (ONPG) as a substrate and 700 \(\mu\text{l}\) of Z buffer containing 3.8 mM \(\beta\)-mercaptoethanol at 30\(^\circ\text{C}\) for 15 minutes or until a yellow color developed. The reaction was terminated by the addition of 0.4 ml of 1 M NaCO\(_3\) and the OD\text{420} was recorded. \(\beta\)-galactosidase units were calculated according to the following equation: 

\[
[(1000)(\text{OD}_{420})/(\text{incubation time})(\text{sample volume})(\text{OD}_{600})]
\]

Samples expressing at least five-fold activity above background control were subjected to DNA sequence analysis by the University of Arizona Biotechnology Facility. The nucleotide sequence was then compared to DNA sequences at the National Library of Medicine with the Basic Local Alignment Search Tool (BLAST). This analysis allowed us to identify proteins that interacted with MEKK3.

### 2.2.5 Cloning of Kinase-Inactive, KM-MEKK3 into \text{pBTM116}

Kinase-inactive, KM-MEKK3, was generated by mutating the active site lysine at amino acid 391 to methionine via a three step PCR mutagenesis protocol. The first PCR reaction contained 250 ng of MEKK3/pBlueBacHis 2B as template in a 100 \(\mu\text{l}\) reaction containing final concentrations as follows: 1X Expand Taq buffer (Gibco BRL), 500 \(\mu\text{M}\)
dNTPs, 0.2 μM primers, and 1 μl Expand Taq polymerase. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, 55°C for 3 min, 72°C for 3 min, and a final extension for 10 min at 72°C. Primers for MEKK3 were as follows: sense primer for mutagenesis of lysine 391 to methionine which also included the restriction enzyme site for NheI as underlined, 5'-GGA CGT GAG CTA GCT TCT ATG CAG GTC CAG-3' and antisense primer, 5'-TAC CTA GCA TGA ACA GAT TGA TCT GCC GGG TGT ACT TG-3'. The NheI restriction site was generated to distinguish between wild-type and kinase-inactive MEKK3. The PCR product was resolved by 1% TAE agarose, the gel band was excised and purified using a Geneclean kit (Bio 101) according to manufacturer protocol. 10 μl of purified PCR product was subject to a second PCR reaction in the presence of 250 ng MEKK3/pBlueBac His 2 B, 1X Taq buffer, 500 μM dNTPs, 1 μl Expand Taq DNA polymerase, and 1.5 mM MgCl₂ for 5 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension of 10 min at 72°C. For the final reaction, 0.2 μM of primers, sense, 5'-AGC ACA AAT GGC GAG AAC-3' and antisense primer, 5'-T ACC TAG CAT GAA CAG ATT G-3' that amplified though the Kas1 and Stu1 restriction sites in wild-type MEKK3 were added to the previous PCR reaction for 25 cycles as follows: 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension for 10 min at 72°C. The PCR product was resolved by 1% TAE agarose, the gel band was excised and purified as described above, and digested with the restriction enzymes, Kas1 and Stu1 to generate a fragment of 450 bp that was ligated into MEKK3/pBlueBacHis 2B that was digested with Kas1 and Stu1 to remove the 450 bp fragment containing lysine in the
active site of MEKK3. This ligation reaction generated KM-MEKK3/pBlueBacHis 2B. A restriction digest with the \textit{Nhe} restriction enzyme was performed to confirm the mutation of the active site lysine.

KM-MEKK3/pBlueBacHis 2B was subsequently cut with \textit{SalI} and \textit{BamHI} to excise a 1.3 bp fragment of MEKK3 containing the BamHI site at 976 bp through the end of MEKK3 with SalI at the 3'-end. Next, an 800 bp fragment of MEKK3 was excised from MEKK3/pGEM-T digested with \textit{SalI} and \textit{BamHI}. The plasmid, pBTM116 was digested with \textit{SalI} and ligated with the fragments of MEKK3 generated above to yield the full-length KM-MEKK3 in pBTM116.

2.2.6 Cloning of Full-length Bcl-x\textsubscript{L} into pVP16

Full-length Bcl-x\textsubscript{L} was cloned via RT-PCR from 15 day mouse embryo RNA. cDNA synthesis was performed in a 20 \(\mu\)l reaction containing final concentrations of 5 \(\mu\)g RNA, 1X First Strand buffer (Gibco BRL), 1 mM DTT, 1 \(\mu\)l Oligo(dT) (500\(\mu\)g/ml), and 500 \(\mu\)M dNTPs. The cDNA reaction was incubated at 42°C for 2 min prior to the addition of 1 \(\mu\)l Superscript II (Gibco BRL) and incubated for 50 min at 42°C. The reaction was inactivated by heating for 15 min at 70°C. 2 \(\mu\)l of the mouse embryo cDNA reaction was added as template to a 100 \(\mu\)l PCR reaction containing final concentrations of 1X Deep Vent buffer plus MgSO\textsubscript{4} (New England Biolabs), 0.5 \(\mu\)l Deep Vent Taq polymerase (New England Biolabs), 200 \(\mu\)M dNTPs (Gibco), and 0.5 \(u\)M of each primer (restriction enzyme sites are underlined) sense 5'-GTT GAG CCG CGG CCG CAA TTC ACC ATG TCT-3' (containing an \textit{NotI} restriction site) and antisense 5'-GGC CAC AGG
AAT TCC CGT CAG GAA TCA GCG GTT-3' (containing a EcoRI restriction enzyme site). The PCR conditions were as follows: an initial denaturation step at 99°C for 5 min followed by 40 cycles at 99°C for 1 min, 55°C for 1 min and 75°C for 1 min, and a final extension step at 75°C for 7 min. A subsequent extension step for 10 min at 72°C with 2.5 Units of High Fidelity Taq polymerase was performed to add 3’A overhangs to facilitate cloning into pGEM-T. The PCR reaction yielded a fragment of approximately 603 bp that was cloned into the pGEM-T TA cloning vector (Promega) for sequence analysis. The Bcl-xL fragment was excised from pGEM-T with EcoRI and NotI and subcloned into the plasmid, VP16 digested with NotI and EcoRI to generate Bcl-xL/VP16. DNA was isolated from this Bcl-xL construct and used for transformation in yeast.
2.3 RESULTS

A total of $1.2 \times 10^7$ yeast transformants were screened on 27 YC-THULL plates containing either 1 or 5 mM 3-aminotriazole or 2 plates without 3-amino-triazole. Initial screening on YC-THULL plates yielded approximately 88 colonies that grew under selective conditions. Subsequent isolation of yeast DNA and transformation into HB101 bacteria resulted in 32 samples for a small-scale yeast co-transformation and β-galactosidase analysis. Eleven of these 32 samples expressing β-galactosidase activity above background levels were subject to DNA sequencing for protein identification. The DNA sequence for six of the clones corresponded to three known proteins as follows: two clones corresponded to partial sequences of Bcl-xL, one clone was identified as 14-3-3ε, and the three other samples were a partial match for the sequence of a protein believed to function as a transcriptional repressor. β-galactosidase activity for the interaction between MEKK3 and these three proteins is shown in Figure 2.2. The isolation of Bcl-xL was consistent with a prior yeast two-hybrid screen with only the amino-terminus of MEKK3, which also isolated a partial Bcl-xL sequence (Vaillancourt unpublished data). In addition, the interaction between MEKK3 and 14-3-3ε was previously documented by our laboratory (16).

β-galactosidase assays in yeast have confirmed the specificity of the interaction between MEKK3 and a truncated Bcl-xL (two-hybrid clone, amino acids 38-193). As
shown in Figure 2.3, the interaction between MEKK3 and Bcl-x\textsubscript{L} is specific for the truncated form of Bcl-x\textsubscript{L} since neither wild-type or KM-MEKK3 interacted with the full-length Bcl-x\textsubscript{L} in yeast. The expression of MEKK3 and Bcl-x\textsubscript{L} was confirmed by immunoblotting (data not shown). In addition, both wild-type and kinase-inactive, KM-MEKK3 interact with the truncated Bcl-x\textsubscript{L} suggesting that the kinase activity of MEKK3 is not necessary to mediate the interaction (Figure 2.3). Based on the homology of MEKK2 and MEKK3, β-galactosidase activity was also measured for MEKK2 and Bcl-x\textsubscript{L}. However, neither wild-type or kinase-inactive MEKK2 interacted with truncated Bcl-x\textsubscript{L} as determined by the lack of β-galactosidase activity (Figure 2.4), suggesting that the interaction between MEKK3 and the truncated form of Bcl-x\textsubscript{L} was specific for the MEKK3 kinase.
Figure 2.2. Truncated BclxL, 14-3-3ε and a Transcriptional Repressor Interact with MEKK3 in Yeast. Yeast strain, L40 was co-transformed with LexA•MEKK3 in the expression plasmid pBTM116 and various mouse cDNA library clones expressed in VP16. β-galactosidase activity was measured to determine the interaction between MEKK3 and the truncated Bcl-xL (amino acids 38-193), 14-3-3ε (full-length) or the transcriptional repressor. β-galactosidase units are expressed as the mean and standard deviation of samples run in triplicate for each condition. The specificity of β-galactosidase activity is determined by using VP16 and LexA-lamin as negative controls.
Figure 2.3. Full-length Bcl-x<sub>L</sub> does not interact with MEKK3. Yeast strain, L40 was co-transformed with LexA•MEKK3 (wild-type) or LexA•KM-MEKK3 (kinase-inactive) in the expression plasmid pBTM116 and either full-length (FL) or truncated Bcl-x<sub>L</sub> (amino acids 38-193) (tBcl-x<sub>L</sub>) expressed in VP16. β-galactosidase activity was measured to determine the interaction between MEKK3 and the truncated Bcl-x<sub>L</sub> (amino acids 38-193) or full-length Bcl-x<sub>L</sub> inserted into VP16. Samples were assayed in triplicate for each condition and values are expressed as the mean and standard deviation.
Figure 2.4. Bcl-xL does not interact with MEKK2. Yeast were co-transformed with wild-type or kinase-inactive MEKK2 in pBTM116 with truncated Bcl-xL in VP16. LexA· lamin and VP16 served as negative controls as described earlier. Samples were assayed in triplicate for each condition and β-galactosidase units are expressed as the mean and standard deviation.
2.4 DISCUSSION

Since Bcl-x<sub>L</sub> plays a role in cell survival and cell death pathways, the interaction between MEKK3 and Bcl-x<sub>L</sub> provided the first link between cell survival pathways and a MAP3K. However, due to the nature of the cDNA library, only a partial fragment of Bcl-x<sub>L</sub> protein was identified as interacting with MEKK3. Additional studies to examine the interaction between MEKK3 and full-length Bcl-x<sub>L</sub> failed to show any interaction. It should be noted that the library clone only contained amino acids 38-193 in which the Bcl-2 homology domain, BH4, was missing (Figure 2.5).

The BH4 domain is only found in members of the Bcl-2 family that promote cell survival. This amino-terminal region of Bcl-2 has been shown to be essential for the inhibition of apoptosis (56). Furthermore, proteolytic cleavage of Bcl-2 by caspase-3 eliminates this domain and accelerates cell death (50, 54, 55). Cleavage of Bcl-x<sub>L</sub> has also been associated with a loss of its ability to prevent apoptosis (51,53). Therefore, an interaction between the truncated Bcl-x<sub>L</sub> and MEKK3 would suggest a role in apoptotic rather than survival pathways. This interaction also suggests that the BH4 domain is inhibitory and that the interaction between MEKK3 and Bcl-x<sub>L</sub> occurs after caspase-activation cleaves this domain.

Although MEKK3 and MEKK2 share 77% homology, truncated Bcl-x<sub>L</sub> does not interact with either wild-type or kinase-inactive MEKK2. Both wild-type and kinase-inactive MEKK3 interacted with truncated Bcl-x<sub>L</sub>, which suggests that the interaction does not require the catalytic activity of MEKK3. Since MEKK2 did not interact with
truncated Bcl-x<sub>L</sub>, our studies suggest a unique role for MEKK3 in regulation of cell survival and death pathways involving Bcl-x<sub>L</sub>.

The yeast two-hybrid system was a useful tool that allowed us to identify Bcl-x<sub>L</sub> and 14-3-3 as proteins that interact with MEKK3. These findings were of particular interest since 14-3-3 proteins have been identified as ubiquitous proteins in signal transduction pathways (3). These proteins can function in cell cycle control and bind phospho-serine and phospho-threonine residues (57). 14-3-3 proteins have been characterized as binding partners for other serine/threonine kinases, such as Raf-1 (19). The interaction between MEKK3 and 14-3-3 implies the binding of 14-3-3 to phosphorylated residues in yeast expressed MEKK3. However, it is uncertain whether 14-3-3 acts merely as a scaffolding protein or to modulate MEKK3 signaling pathways.

It should also be noted that a drawback of the yeast two-hybrid system is the potential for false positives, therefore, my next objective was to determine whether MEKK3 and Bcl-x<sub>L</sub> interact in a mammalian expression system. Further studies were also necessary to examine the interaction between the caspase-cleaved Bcl-x<sub>L</sub> protein and MEKK3 since the truncated Bcl-x<sub>L</sub> library protein does not represent a physiologically relevant protein.
Bcl-x<sub>L</sub> MSQSNRELVVDPLSY KLSQKGYWSQFSDV EERTEAPEETEAER ETPSAINGNPSWHLA 60

clone

Bcl-x<sub>S</sub> MSQSNRELVVDPLSY KLSQKGYWSQFSDV EERTEAPEETEAER ETPSAINGNPSWHLA 60


Figure 2.5. Amino Acid Alignment of Full-length Bcl-x<sub>L</sub>, Bcl-x<sub>S</sub> and the cDNA library Bcl-x<sub>L</sub> clone. Bcl-2 family members, Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub> share conserved Bcl-2 homology (BH) domains. The cDNA library clone of Bcl-x<sub>L</sub> is truncated at the amino and carboxy terminus and lacks the BH4 domain.
CHAPTER 3
CHARACTERIZATION OF THE INTERACTION BETWEEN MEKK3 AND BCL-Xₐ

3.1 INTRODUCTION

Programmed cell death or apoptosis is a highly regulated cellular process. Some characteristics of this process include mitochondrial shrinking, caspase activation and DNA fragmentation (59). In response to changes in the mitochondrial membrane, cytochrome c is released and leads to a sequential activation of cysteine proteases, caspases, and ultimately cell death.

Among the proteins that regulate this process is the Bcl-2 family of proteins. These proteins have critical roles in both apoptotic and cell survival signaling (62). Bcl-xₐ, a key anti-apoptotic protein in the Bcl-2 family, blocks the release of cytochrome c from mitochondria thus inhibiting apoptosis (72). When the pro-apoptotic protein, BAD, is not phosphorylated, it exists as a heterodimer with Bcl-xₐ. These heterodimers function as pro-apoptotic complexes (72), however, in response to growth factors and cytokines, BAD becomes phosphorylated, dissociates from Bcl-xₐ and binds 14-3-3. Once 14-3-3 is bound to phosphorylated BAD, it functions to sequester BAD away from Bcl-xₐ and provide an anti-apoptotic signal.

Another Bcl-2 binding protein is Bag-1 (68, 70). In contrast to the apoptotic functions of BAD, Bag-1 is anti-apoptotic. Thus far, Bag-1 has only been characterized
as cooperating with Bcl-2 to suppress apoptosis. However, since Bcl-2 and Bcl-xL share significant homology, Bag-1 likely interacts with and promotes Bcl-xL pathways as well.

Post-translational modifications of members of the Bcl-2 family have been reported (60, 64, 66). In particular, Bcl-2 and Bcl-xL are phosphorylated in response to chemotherapeutic agents and drugs that target microtubules. The upstream kinase that is responsible for these phosphorylation events has not been identified. Since proteins that interacted with MEKK3 in the two hybrid system may act downstream of MEKK3 signaling pathways, recombinant Bcl-xL proteins were tested as potential MEKK3 substrates in vitro. An interaction between MEKK3 and Bcl-xL suggests that MEKK3 is involved in cell death and survival pathways.

Proteins in the Bcl-2 family are also modified through proteolytic cleavage by caspases (50, 51, 55, 71). Bcl-2 is cleaved at Asp31, Asp34, or Asp36. This proteolytic cleavage results in the loss of the amino-terminal BH4 domain and renders Bcl-2 inactive. Bcl-xL has two putative caspase-3 cleavage sites within the amino-terminal domain at amino-acids 34 and 61. Although proteolytic cleavage has been characterized, proteins that interact with these truncated forms of Bcl-xL have not been reported.

In this chapter the interaction between MEKK3 and Bcl-xL was further characterized beyond the yeast two hybrid findings. Furthermore, physiologically relevant Bcl-xL proteins were purified and tested as adaptor proteins in a MEKK3 protein complex or as putative MEKK3 substrates in an in vitro kinase assay. Since MEKK3 and MEKK2 are highly homologous protein kinases, another purpose of these studies was to address any differences between the interaction of these proteins with Bcl-xL.
3.2 EXPERIMENTAL PROCEDURES

3.2.1 Generation of Recombinant Glutathione-S-Transferase (GST) fusion protein constructs

Δ38 BcL-xL (amino acids 38-193, from two-hybrid screen) was excised from pBTM116 with the restriction enzymes, NotI and EcoRI and ligated into pGEX-5X-1 (Pharmacia) digested with the same enzymes. The pGEX-5X-1 plasmid was selected for recombinant protein expression because it contains a GST motif and a Factor Xa cleavage site. DNA was transformed into the E. coli strain, BL21(DE3)pLysS (Novagen) for production of recombinant GST-Δ38 BcL-xL.

Full-length cDNA encoding BcL-xL was constructed using 15 day mouse embryonic RNA as a template. cDNA was produced using Superscript Reverse Transcriptase (Gibco BRL) in a 100 µl PCR reaction containing the following: 1X Deep Vent Taq Polymerase buffer with MgSO₄, 200 µM dNTPs, 0.5 µl Deep Vent Taq, and 0.2 µM primers (sense, 5'-TCT GCT GAA TTC ACC ATG TCT CAG AGC AAC CGG-3' and antisense, 5'-ATT ATG CGG CCG CAA TCA CTT CCG ACT GAA GAG TGA-3'). The underlined nucleotides indicate EcoRI and NotI restriction endonuclease sites, respectively. These restriction sites were generated to facilitate cloning into pGEX-5X-1 for recombinant protein expression. PCR cycling conditions were as follows: an initial denaturation at 99°C for 5 min, followed by 40 cycles of 99°C for 1 min, 55°C for 1 min, 75°C for 1 min, and a final extension for 7 min at 72°C. After PCR cycling, 2.5U Taq polymerase was added for 10 min incubation at 72°C to add 3'
A overhangs required for cloning into the pGEM-T vector (Promega). This PCR product was resolved by 1% TAE agarose and the gel band was excised and purified using the Geneclean kit according to manufacturer's protocol (Bio 101). The purified PCR product of 603 bp was ligated into pGEM-T. Subsequently, Bcl-x<sub>L</sub> was excised from pGEM-T with EcoR1 and Not1 and ligated into pGEX-5X-1 cut with EcoR1 and Not1. Bcl-x<sub>L</sub>/pGEX-5X-1 plasmid DNA was transformed into <i>E. coli</i> strain, BL21(DE3)pLysS for production of recombinant GST-Bcl-x<sub>L</sub>.

Δ34 HA-Bcl-x<sub>L</sub> was constructed using HA-Bcl-x<sub>L</sub>/pcDNA 3.1 as template in a 100 μl PCR reaction containing the following: 1X Buffer, 1.5 mM MgCl<sub>2</sub>, 10% DMSO, 200 μM dNTPs, 0.7 μl High Fidelity Taq Polymerase and 0.5 μM primers (sense, 5'-TTT AGT GAA TTC ACC ATG AAT AGG ACT GAG GCC-3' and antisense, 5'-CTC GAG CGG CCG CCA TCA AGC GTA GTC CGG CAC GTC GTA-3' containing EcoR1 and Not1 sites, respectively). The resulting PCR product was cloned into pGEM-T and excised with EcoR1 and Not1 and ligated into pGEX-5X-1 digested with the same restriction enzymes. Δ61 Bcl-x<sub>L</sub> was constructed using the same approach, however, sense primer for Δ61 Bcl-x<sub>L</sub> was, 5'-TGG GAA TTC ACC ATG AGC CCG GCC GTG AAT GGA-3' and the same antisense primer was used as described above. PCR cycling conditions for both constructs were as follows, an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension at 72°C for 10 min.

Full-length cDNA encoding BAD was constructed using 15 day mouse embryo total RNA as a template. cDNA was produced using Superscript Reverse Transcriptase
(Gibco BRL) in a 100 µl PCR reaction to obtain the full-length BAD in the presence of 10% DMSO, 1 X Taq buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.7 µl High Fidelity Taq polymerase, and 0.5 µM of primers, sense 5'-GCC TCC AGG ATC CAA ATG GGA ACC-3' containing a BamH1 endonuclease site and antisense primer, 5'-TCC GGG ATG TCG ACC AGA AGA TCA CTG-3' containing a SalI endonuclease site. PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 3 min, 58°C for 1 min, 72°C for 1 min, and a final extension for 7 min at 72°C. The resulting PCR product of 605 bp was subcloned into pGEM-T (Promega) and sequenced. Subsequently, BAD was excised from pGEM-T with BamH1 and SalI and ligated into pGEX-5X-1 cut with the same enzymes. The E. coli strain, BL21(DE3)pLysS was transformed with BAD/pGEX-5X-1 plasmid DNA for production of recombinant GST-BAD.

Lamin was excised from pBTM116 provided by Anne Vojtek (69) using EcoRI and SalI endonuclease sites and ligated into pGEX-5X-1 cut with the same enzymes. Lamin/pGEX-5X-1 was transformed into the E. coli strain, BL21(DE3)pLysS for production of recombinant GST-lamin.

All nucleotide sequences were confirmed by automated DNA sequencing at the Arizona Research Labs at the University of Arizona.

3.2.2 Expression and purification of GST fusion proteins

Transformed E. coli were grown on the day before purification in 5 ml of LB-AMP at 37°C while shaking at 250 rpm, then transferred to 50 ml LB-AMP for growth
overnight at 37°C. Immediately following the overnight incubation, the bacterial culture was transferred into 450 ml of LB-AMP (50 μg/ml) and incubated at 37°C with shaking at 250 rpm for 1 h or until the OD₆₀₀ reached 0.5-0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added for a final concentration of 0.2 mM to induce fusion protein expression after the OD₆₀₀ reached the desired value. After inducing protein production for 3 h, bacteria were harvested by centrifugation at 6,000 rpm for 6 min. Bacterial pellets were resuspended in 30 ml of resuspension buffer (1X PBS, 1% Triton X-100, 1% Aprotinin, 100 μg/ml leupeptin, 1 mM PMSF, and 1 mM EDTA) and lysed by sonication for 20 sec interval for 3 times while keeping bacteria on ice. The insoluble protein was removed from the bacterial resuspension by centrifugation at 10,000 rpm for 10 min. The supernatant was collected as the soluble fraction and 15 ml of supernatant was added to 0.4 ml of Glutathione Sepharose 4B beads (Pharmacia) that were pre-washed twice with 10 ml of resuspension buffer. Bacterial suspension and beads were then incubated for at least 1 h at 4°C in 15 ml conical tubes while rotating. The beads were then washed for 4 times with 10 ml of resuspension buffer by centrifugation at 500 rpm and aspirating the supernatant. After the last wash, supernatant was aspirated and 0.4 ml of storage buffer (1X PBS/50%glycerol) was added to the beads. Recombinant fusion proteins bound to glutathione beads were stored at -20°C until use.
3.2.3 Construction of Recombinant (HIS)$_6$FLAG•MEKK3 for Baculovirus Expression in Sf9 Insect Cells

The FLAG epitope (DYKDDDDK) was introduced at the amino-terminus of MEKK3 using a two-step PCR protocol with overlapping primers to construct the epitope. In the first reaction using HA-MEKK3 in pCMV5 as the template, the sense primer was 5'-GAC GAC GAT GAC AAG GAT GAA CAA GAG GCA TTA GAC-3' and the antisense primer was 5'-CGT ATA GGA TCC TTG CCG-3', which overlaps the BamH1 site approximately 580 bp from the start methionine. The product of this PCR reaction was used as a template for the next reaction using the sense primer, containing the SalI endonuclease site, 5'-TGG AGT CGA CTC GAC TAC AAG GAC GAC GAT GAC AAG-3' and the same antisense primer described above. The resulting PCR product was subcloned into pGEM-T and sequenced. The 580 bp FLAG•MEKK3 fragment was excised from pGEM-T with SalI and BamH1. The remaining 1700 bp fragment of MEKK3 was obtained by digesting MEKK3 in pBTM116 with BamH1 and SalI (16). The two portions of MEKK3 were re-ligated into pBTM116 to yield full-length FLAG•MEKK3, which was then re-ligated into pBlueBacHis2 B (Invitrogen) that had been digested with SalI. FLAG•KM-MEKK3/pBlueBacHis2B was prepared as described above using the 580 bp fragment containing the FLAG epitope and ligating with the BamH1/SalI 1700 bp fragment from KM-MEKK3 in pBTM116. For identification purposes, a NheI endonuclease site was engineered into KM-MEKK3 in pBTM116 with the sense primer, 5'-GGA CGT GAG CTA GCT TCT AT*G CAG GTC CAG-3', using the megaprimer method of site-directed mutagenesis (65). The NheI site
was a silent mutation that did not change the amino acid sequence of MEKK3, while the asterisk in the primer sequence denotes the changed nucleotide that creates the mutation. This mutation was introduced to distinguish between the wild type and kinase-inactive nucleotide sequences for MEKK3.

3.2.4 Purification of HexaHistidine Tagged Proteins

Sf9 insect cells were cultured in 500 ml of Grace's complete insect media (Gibco BRL) by shaking at 225 rpm at 27°C until a density of 1 X 10^6 cells/ml was reached. Recombinant (His)_6FLAG•MEKK3 baculovirus was added at a multiplicity of infection (MOI) of 2.5 to insect cells and infection proceeded for 40 h prior to harvest. Cells were collected by centrifugation for 5 min at 6,000 rpm. The supernatant was aspirated and pellets were resuspended in 25 ml of lysis buffer (50 mM NaPO_4, 100 mM KCl, 1% Tween, 1% Aprotinin, 100 μg/ml leupeptin, and 10 mM β-mercaptoethanol) and sonicated three times for 20 sec to shear DNA. Soluble proteins were recovered by centrifugation for 10 min at 10,000 rpm. Supernatants were transferred into 15 ml conical tubes and 12.5 ml of supernatant was incubated with 0.45 ml of Nickel sepharose (Invitrogen) for at least 1 h at 4°C. Beads were collected by centrifugation for 1 min at 500 rpm. Samples were purified by sequential washes (3X) with buffer (50 mM NaPO_4, pH 8.0 or 6.2, 100 mM KCl, 1% Tween, and 10 mM β-mercaptoethanol) to remove non-specific proteins that bound the Nickel resin. After the last wash, beads were transferred into a 1.5 ml eppendorf tube and washed once with 50 mM NaPO_4, supernatant was
aspirated, and 0.45 ml of storage buffer was added. Beads were stored at -20°C until use.

3.2.5 Transient Transfection of HEK 293 cells by Calcium Phosphate

HEK 293 (human embryonic kidney) cells were cultured on 10 cm plates in DMEM containing 10% FBS under 5% CO2 until 60% confluence was reached. Approximately, 0.5 ml of 2X HEPES-buffered saline, (HEPES 10g/L, NaCl 16g/L, KCl 0.74g/L, Na2HPO4 0.25g/L, glucose 2g/L, pH 7.05) was added into a 15 ml conical tube containing 10 μg plasmid DNA. Calcium phosphate precipitates were formed by mixing with a vortex and adding 0.5 ml of 250 mM CaCl2. The HEPES/CaCl2 mixture was incubated at room temperature for 20 min to allow the formation of precipitates. The precipitate solution was added dropwise to a plate of HEK 293 cells in 9 ml of media while swirling gently. Cells were returned to the incubator for 4-5 h. After the 4 h incubation, the media was aspirated and 3 ml of 10% DMSO in 1X PBS was added for 2 min at room temperature. After the DMSO shock, media was aspirated and replaced with 10 ml of DMEM/10% FBS. Cells were placed in the incubator and harvested 48 h post transfection.

3.2.6 GST Precipitation Experiments

Immediately following a 48 h incubation from the calcium phosphate transfection, HEK 293 cellular extracts were prepared by lysis in NP40 buffer (0.2% NP40, 20 mM Tris pH 8.0, 137 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM MgCl2, 1% aprotinin, 5
μg/ml leupeptin, and 1 mM PMSF). Approximately 1 mg of cell extract was incubated with 0.5 μg GST fusion protein bound to Sepharose beads for 4 h at 4°C. Samples were then washed twice with PAN NP40 (10 mM Pipes, pH 7.0, 100 mM NaCl, 1% Aprotinin, and 0.5% NP40) and once with PAN (10 mM Pipes, pH 7.0, 100 mM NaCl, and 1% Aprotinin) and resolved by 8% SDS-PAGE. After transfer to nitrocellulose membrane, the blot was probed with an amino-terminal MEKK3 antibody (described below) followed by goat-anti-rabbit HRP antibody (Zymed). Proteins were visualized by enhanced chemiluminescence followed by autoradiography.

3.2.7 MEKK3 Antibody Production and Purification

Rabbit polyclonal antibody that recognizes MEKK3 was raised against a fusion protein consisting of maltose-binding protein (MBP) and amino acids 10-44 of MEKK3. The following sense, 5'-GCA TTA GAA TTC ATC ATG AAG GAC CTG GTG-3', and antisense, 5'-TTC AAA CAA GCT TCT CTA GTC ACT CTG CCT GTT TGG-3', primers were used in a PCR reaction with HA-MEKK3 in pCMV5 as the template. The underlined and italicized nucleotides indicate restriction endonuclease sites and translation termination sites, respectively. A 105 bp fragment was generated and cloned into the pGEM-T TA cloning vector (Promega). The nucleotide sequence was confirmed by automated DNA sequencing at the Arizona Research Labs at the University of Arizona. The cloned DNA was ligated into the EcoR1 and HindIII endonuclease sites of the pMAL-c2 vector (New England Biolabs) and pRSETB (Invitrogen). The resulting plasmids were transformed into the BL21(DE3)pLysS E. coli strain and expression of the
fusion proteins was induced by IPTG for 3 h at 37°C. Fusion proteins were purified using amylose or nickel conjugated to Sepharose according to the directions provided by the manufacturer. MBP-MEKK3 (10-44) was used as an antigen while the hexahistidine fusion protein, (His)$_6$MEKK3 (10-44), was conjugated to CNBr-activated Sepharose 4B (Amersham Pharmacia) according to manufacturer’s protocol to affinity purify MEKK3 specific antibody.

Given the amino acid similarity between MEKK3 and MEKK2, an affinity column consisting of amino acids 11-44 of MEKK2 was prepared so that MEKK3 antibody that also cross-reacts with MEKK2 could be subtracted from the serum. A hexahistidine MEKK2 fusion protein was prepared as described above. The following sense, 5’-TTG AAT GGA TCC ATG CAA GAT TTG GCT GTC-3’, and antisense, 5’-TTC AAA GCT XAC TCA A ATC ATT CTG TTT TTT-3’, primers were used in a PCR reaction with HA-MEKK2 in pCMV5 as the template. A 102 bp fragment was generated and cloned into the pGEM-T TA cloning vector and the nucleotide sequence was confirmed by automated DNA sequencing. The cloned DNA was ligated into the BamH1 and HindIII endonuclease sites of pRSETA. The resulting plasmid was transformed into the BL21(DE3)pLysS E. coli strain and the expressed protein was purified as described above. (His)$_6$MEKK2 (11-44) was conjugated to CNBr-activated Sepharose 4B to remove MEKK2-specific antibody that was raised against MBP-MEKK3 (10-44).

Rabbit serum (12.5 ml) raised against MBP-MEKK3 (10-44) was applied to a 3 ml affinity column consisting of (His)$_6$MEKK3 (10-44) at ambient temperature. Flow-
through serum was applied twice to the column and then the column was washed with PBS until the OD$_{280}$ returned to baseline level. Bound antibody was eluted with 3.5 M MgCl$_2$ and diluted with 3 volumes of H$_2$O prior to concentrating in 25 x 16 mm dialysis membrane (MWCO 12-14,000) in a tray of Aquacide III (Calbiochem). After 5 h, the volume was reduced to ~5 ml and dialyzed overnight in 4 L of 1X PBS at 4°C.

Subsequently, affinity-purified MEKK3 antibody was passed twice over a 3 ml affinity column consisting of (His)$_6$MEKK2 (11-44) to remove antibody that cross-reacts with MEKK2. Antibody that passed through the column, represented polyclonal antibody that was specific for MEKK3 (data not shown), and was concentrated with Aquacide III (Calbiochem) and dialyzed in 1X PBS as described above. Sodium azide was added for a final concentration of 0.02% to affinity-purified antibody after dialysis. The affinity-purified antibody was stored at 4°C until use.

### 3.2.8 In Vitro Kinase Assays using Recombinant GST Fusion Proteins as Substrates

In vitro kinase assays were performed using immunoprecipitated MEKK3 from Sf9 insect cells in a 50 μl reaction mixture containing 0.5 μg GST fusion protein, 1X PAN, 1X UKB (20 mM Pipes, pH 7.0, 20 mM MnCl$_2$, 20 mM MgCl$_2$, 1% Aprotinin), and 10 μCi [γ-32P]ATP. The reaction was incubated for 20 min at 30°C before stopping with 10 μl of 5X Laemmli sample buffer (50% glycerol, 0.3 M Tris/HCl pH 6.8, 11.5% SDS, 50 mM DTT, and 0.1% Bromophenol Blue) and performing SDS-PAGE. Gel was
fixed in 10% acetic acid/50% methanol for 1 h at room temperature and dried for 2 h at 80°C before autoradiography was performed.
3.3 RESULTS

Recombinant GST-Bcl-x\textsubscript{L} fusion proteins were prepared to determine whether Bcl-x\textsubscript{L} was a substrate for phosphorylation by MEKK3 in an \textit{in vitro} kinase assay or to use in GST precipitation experiments to determine whether an interaction between MEKK3 and Bcl-x\textsubscript{L} occurs in mammalian cells. Initial studies were conducted to determine whether the Bcl-x\textsubscript{L} clone that was obtained in the two hybrid system, Δ38 Bcl-x\textsubscript{L}, interacted with MEKK3. Both the Δ38 Bcl-x\textsubscript{L} and full-length Bcl-x\textsubscript{L} were purified from bacteria and bound to Glutathione Sepharose beads, (Figure 3.1, lanes c and f respectively). HEK 293 cells were transiently transfected with either vector alone, pCMV5 (Figure 3.2, lanes a-d) or HA-MEKK3/pCMV5 (Figure 3.2, lanes e-h) for GST immunoprecipitation experiments. Cellular extracts were prepared 48 h later and incubated with either recombinant GST-lamin, GST-Δ38 Bcl-x\textsubscript{L} or GST-14-3-3\textepsilon bound to glutathione sepharose beads. GST-Δ38 Bcl-x\textsubscript{L} (lane g) and GST-14-3-3\textepsilon (lane h) associated with MEKK3. MEKK3 did not associate with GST-lamin (lane e), showing that the interaction between MEKK3 and the GST-Δ38 Bcl-x\textsubscript{L} proteins was dependent upon Δ38 Bcl-x\textsubscript{L}, not the GST portion of the fusion protein. These data demonstrate that MEKK3 associates with Δ38 Bcl-x\textsubscript{L} and 14-3-3\textepsilon in both yeast and mammalian cells.
Figure 3.1. Production of recombinant GST-Δ38 Bcl-xL and GST-Δ61 Bcl-xL fusion proteins. The E. coli strain, BL21(DE3)pLysS was transformed with Bcl-xL constructs in the plasmid, pGEX-5X-1. Bacteria were grown until an OD_{600} of 0.5 was reached. A fraction of the bacterial samples were collected before IPTG induction (lanes a and d) and samples were taken 3 h after IPTG induction (lanes b and e). Fusion proteins bound to Glutathione Sepharose beads are shown in lanes c and f. Samples were resolved by 10% SDS-PAGE and proteins visualized by Coomasie Blue staining. Low molecular weight proteins standards (Bio-Rad) are shown in the first lane to determine protein size.
Figure 3.2. MEKK3 interacts with Δ38 Bcl-xL and 14-3-3ε. HEK 293 cells were transfected with plasmid DNA encoding either vector, pCMV5 (lanes a-d) or HA-MEKK3/pCMV5 (lanes e-h). Approximately 48 h later, cell extracts were prepared and incubated with recombinant GST fusion proteins as follows: GST-lamin (lanes a and e), GST-Bcl-xL (lanes b and f), GST-Δ38 Bcl-xL (lanes c and g), and GST-14-3-3ε (lanes d and h). Lysates are shown in lanes i and j as immunoblotting controls. Samples were resolved by 10% SDS-PAGE and transferred to nitrocellulose for MEKK3 immunoblotting.
During apoptosis Bcl-x\textsubscript{L} is cleaved by caspases at amino acid 61. Δ61 Bcl-x\textsubscript{L} recombinant fusion protein was generated and tested for its ability to interact with MEKK3. Sf9 insect cells were infected with baculovirus encoding MEKK3, KM-MEKK3 or control protein expression. Sf9 cellular extracts were incubated with either buffer alone (Fig 3.3, lanes g-i) or Δ61 HA-Bcl-x\textsubscript{L} (Figure 3.4, lanes j-l) that was cleaved with factor Xa to remove the GST motif. MEKK3 was immunoprecipitated, the proteins were resolved by SDS-PAGE, and then immunoblotted with antibodies that recognize Bcl-x\textsubscript{L}. Δ61 Bcl-x\textsubscript{L} interacted with wild type MEKK3 and KM-MEKK3 (Figure 3.3, lanes k and l). MEKK3 was not immunoprecipitated in the absence of Δ61 Bcl-x\textsubscript{L} (Figure 3.3, lanes h and i).

Similar MEKK3 immunoprecipitation experiments were also performed with Δ34 Bcl-x\textsubscript{L}, Δ61 Bcl-x\textsubscript{L} and full-length Bcl-x\textsubscript{L}. The purpose of these experiments was to determine whether the MEKK3 interacts with full-length Bcl-x\textsubscript{L} or if this interaction occurs after caspase-cleavage of Bcl-x\textsubscript{L}. As described above, recombinant fusion proteins were combined with Sf9 cellular extracts and MEKK3 was immunoprecipitated. Immunoblotting with the HA monoclonal antibody that recognizes all the truncated Bcl-x\textsubscript{L} proteins demonstrated that MEKK3 interacted with Δ61 Bcl-x\textsubscript{L} (Figure 3.4, lanes f and j), but not the full-length protein or Δ34 Bcl-x\textsubscript{L} (Figure 3.4, lanes 7 and 11).

Given the amino acid similarity between MEKK2 and MEKK3, we analyzed the interaction between Bcl-x\textsubscript{L} and MEKK2 to further characterize the specificity of the MEKK3 and Bcl-x\textsubscript{L} interaction. Sf9 cells were infected with recombinant baculovirus encoding for MEKK2 expression or control plasmid. Extracts from infected Sf9 cells
Figure 3.3
Figure 3.3. MEKK3 interacts with Δ61 Bcl-xL. Sf9 cells were infected with baculovirus that encodes wild type or kinase-inactive MEKK3 expression. Approximately 1 mg of cellular extract was incubated with recombinant fusion protein Δ61 Bcl-xL (lanes j-l) or buffer (lanes g-i). MEKK3 was precipitated with an amino-terminal MEKK3 antibody followed by Protein A. Samples were resolved by 10% SDS-PAGE. After transfer to nitrocellulose, the blot was probed with antibodies to detect MEKK3 (top panels) or Δ61 Bcl-xL (bottom panels). Proteins were visualized by enhanced chemiluminescence and autoradiography. Prior to immunoprecipitation, a fraction of the cell extract plus recombinant protein was removed for immunoblotting (left panels).
Figure 3.4
Figure 3.4. Full-length Bcl-x<sub>L</sub> does not associate with MEKK3. A. Sf9 cells were infected with baculovirus for wild type or kinase-inactive MEKK3 expression. Approximately 1 mg of protein was incubated with recombinant fusion proteins for full-length Bcl-x<sub>L</sub>, Δ34 Bcl-x<sub>L</sub>, or buffer. Buffer without fusion protein was used as a negative control. Δ34 and Δ61 contained the HA epitope. MEKK3 was immunoprecipitated with a rabbit MEKK3 antibody followed by Protein A. Samples were then washed with buffer and resolved by 10% SDS-PAGE. After transfer to nitrocellulose, the blot was probed with rat HA antisera followed by goat-anti-rat HRP antibody for detection of Δ34 and Δ61 Bcl-x<sub>L</sub> HA-tagged constructs. Alternatively, the blot was probed with rabbit Bcl-x<sub>L</sub> antisera and goat-anti-rabbit HRP for detection of full-length Bcl-x<sub>L</sub>. Proteins were visualized by enhanced chemiluminescence and autoradiography. B. Prior to immunoprecipitation a fraction of the cell lysate plus recombinant protein was removed for immunoblotting. Detection of protein bands was performed as described above to confirm the sizes of recombinant Bcl-x<sub>L</sub> fusion proteins.
were incubated with Δ61 Bcl-x\textsubscript{L} fusion protein as described above. MEKK2 was expressed (Figure 3.5, lane d), however, it did not precipitate Δ61 Bcl-x\textsubscript{L} (Figure 3.5, lane b). Thus, in agreement with the yeast two hybrid data, Bcl-x\textsubscript{L} only interacted with MEKK3.

Since phosphorylation of BAD and Bcl-x\textsubscript{L} has been reported to modulate their function, these fusion proteins were tested as potential MEKK3 substrates in an \textit{in vitro} kinase assay. To test whether BAD serves as a substrate for MEKK3, GST-BAD fusion protein was expressed in bacteria (Figure 3.6) and the recombinant protein was used in the kinase reaction. MEKK3 was immunoprecipitated from Sf9 cells infected with recombinant baculovirus and a kinase assay performed with either BAD or Bcl-x\textsubscript{L} fusion proteins as substrates. Autophosphorylation of wild type MEKK3 as a band of approximately 80 kDa was observed (Figure 3.7, lanes b, e, h, and k), while autophosphorylation was not observed with KM-MEKK3 which contains a lysine to methionine point mutation in the active site that eliminates the catalytic activity (Figure 3.6, lanes c, f, j, and l). Recombinant substrates, GST-BAD (Figure 3.7, lane e), GST-Δ38 Bcl-x\textsubscript{L} (lane h), or full-length GST-Bcl-x\textsubscript{L} (lane k), were not phosphorylated by wild type MEKK3 with expected sizes of 46, 43, and 46 kDa respectively.

Given that Δ38 Bcl-x\textsubscript{L} or BAD were not phosphorylated by MEKK3, the next alternative tested was whether full-length Bcl-x\textsubscript{L} or Δ38 Bcl-x\textsubscript{L} could act as a regulator of MEKK3 kinase activity. MEKK3 was immunoprecipitated from Sf9 cells and
Figure 3.5

Immunoprecipitation

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Lysates

- MEKK2
- IgG
- Δ61 Bcl-x<sub>L</sub>

Figure 3.5
Figure 3.5. MEKK2 does not interact with Δ61 Bcl-x\textsubscript{L}. MEKK2 protein was purified from Sf9 cells infected with baculovirus that encodes for MEKK2 expression. Purified, recombinant MEKK2 or control viral protein was incubated with recombinant Δ61 Bcl-x\textsubscript{L} fusion protein. A rabbit polyclonal antibody was used to immunoprecipitate MEKK2 from Sf9 cells that were infected with baculovirus that encodes MEKK2 (lane a) or virus containing no insert cDNA as a control (lane b). Prior to immunoprecipitation, a fraction of the cell lysate plus recombinant protein was removed for immunoblotting (lanes c and d). Samples were then washed with buffer and resolved by 10% SDS-PAGE. After transfer to nitrocellulose, the blot was probed with antisera for detection of Δ61 Bcl-x\textsubscript{L} (bottom half of the blot). The blot was also probed with a rabbit MEKK2 antisera followed by goat-anti-rabbit HRP for MEKK2 detection (top half of the blot). Proteins were visualized by enhanced chemiluminescence and autoradiography.
Figure 3.6. Production of recombinant GST-BAD fusion protein. BAD/pGEX-5X-1 plasmid DNA was transformed into the *E. coli* strain, BL21(DE3)pLYS for production of recombinant fusion protein. Bacteria were grown until an OD$_{600}$ of 0.5 was reached and samples were taken 3 h after induction with IPTG (lane b) and without induction (lane a). GST-BAD bound to Glutathione Sepharose beads is shown in lane c. Samples were resolved by 10% SDS-PAGE and stained with Coomassie Blue for protein visualization. Bio-Rad low molecular weight protein standards are shown in the first lane.
Figure 3.7. MEKK3 does not phosphorylate GST-BAD or GST-Bcl-xL. Sf9 cells were infected with baculovirus containing no insert cDNA (lanes a, d, g, and j), MEKK3 (lanes b, e, h, and k), or KM-MEKK3 (lanes c, f, i, and l). As indicated recombinant GST fusion proteins were added to immunoprecipitated MEKK3 and an *in vitro* kinase assay was performed. SDS-PAGE and autoradiography were performed to detect phosphorylated proteins. Autophosphorylation of MEKK3 was observed in lanes b, e, h, and k.
recombinant Bcl-x<sub>L</sub> was added in an *in vitro* kinase assay in addition to a non-relevant substrate, myelin basic protein, MBP. MEKK3 phosphorylated MBP to the same extent in the presence or absence of Bcl-x<sub>L</sub> (Figure 3.8, lanes d and h). Thus, Bcl-x<sub>L</sub> did not appear to regulate MEKK3 kinase activity. In addition, MEKK3 retained the ability to autophosphorylate in the presence of Bcl-x<sub>L</sub>.

Another alternative hypothesis tested was whether MEKK3 activity towards Bcl-x<sub>L</sub> required another component of the cell survival signaling pathway. Since BAD binds Bcl-x<sub>L</sub>, an *in vitro* kinase assay was performed with the addition of both recombinant BAD and Bcl-x<sub>L</sub>. Despite the presence of recombinant BAD in the kinase reaction, MEKK3 was still unable to phosphorylate either full-length Bcl-x<sub>L</sub> (Figure 3.9, lane f) or caspase-cleaved Bcl-x<sub>L</sub> (data not shown).

Bag-1 is another protein in the Bcl-2 family and it promotes the positive regulation of protein kinases including Raf-1 (70). Therefore, Bag-1 was expressed in bacteria as a recombinant GST fusion protein and prepared as described for Bcl-x<sub>L</sub> and tested for its ability to modulate MEKK3 kinase activity towards Bcl-x<sub>L</sub> proteins. Recombinant Bag-1 and Bcl-x<sub>L</sub> were added to immunoprecipitated MEKK3 from transfected HEK 293 cells for an *in vitro* kinase assay. Similar to previous experiments, MEKK3 autophosphorylation was observed, however, Bcl-x<sub>L</sub> or Δ38 Bcl-x<sub>L</sub> (Figure 3.10, lanes c and e) were not phosphorylated by MEKK3 in the presence of Bag-1. Thus, the addition of Bag-1 did not promote the phosphorylation of Bcl-x<sub>L</sub> by MEKK3.
Figure 3.8. MEKK3 does not phosphorylate caspase-cleaved Bcl-xL. Sf9 insect cells were infected with baculovirus as a source of recombinant MEKK3. Cellular extracts were prepared 40 h later and MEKK3 was immunoprecipitated using MEKK3 antibody and Protein A Sepharose. MEKK3 was incubated with either no substrate (lanes a and e), Δ61 Bcl-xL (lane b), myelin basic protein (MBP) (lanes c and g), MBP and Δ61 (lane d), full-length Bcl-xL (lane f), or MBP and full-length Bcl-xL (lane h) and in vitro kinase assay performed. Samples were resolved by SDS-PAGE and autoradiography performed to visualize phosphorylated proteins.
Figure 3.9. Recombinant BAD does not modulate MEKK3 phosphorylation of Bcl-xL. HEK 293 cells were transfected with expression plasmids for vector, pCMV5, or HA-MEKK3/pCMV5. Cellular lysates were prepared 48 h later and MEKK3 immunoprecipitated with a rabbit MEKK3 antibody and Protein A Sepharose. Immunoprecipitates were incubated with Bcl-xL (lanes a and d), BAD (lanes b and e) or BAD and Bcl-xL (lanes c and f) and in vitro kinase assay performed. Samples were resolved by 10% SDS-PAGE and autoradiography performed.
Figure 3.10
Figure 3.10. GST-Bag-1 does not affect MEKK3 kinase activity. A. Preparation of GST-Bag-1 fusion protein. Recombinant protein was purified as described earlier. Bacterial samples were harvested at pre-induction (lane a), 3 h post IPTG induction (lane b) and purified GST-Bag-1 bound to glutathione sepharose beads (lane c). Samples were resolved by 10% SDS-PAGE and proteins visualized by Coomasie Blue staining. B. Recombinant Bag-1 fusion protein does not promote MEKK3 phosphorylation of Bcl-x<sub>L</sub>. Sf9 insect cells were infected with baculovirus for MEKK3 expression. MEKK3 was precipitated with MEKK3 antibody and Protein A Sepharose. Immunoprecipitated MEKK3 was incubated with either no substrate (lane a), Δ38 Bcl-x<sub>L</sub> (lane b), Δ38 Bcl-x<sub>L</sub> and Bag-1 (lane c), Bcl-x<sub>L</sub> (lane d), or Bcl-x<sub>L</sub> and Bag-1 (lane e) and an in vitro kinase assay performed. SDS-PAGE was performed and phosphorylated proteins detected by autoradiography.
3.4 DISCUSSION

The results from GST precipitation experiments in mammalian and Sf9 insect cells were consistent with those obtained using the yeast two hybrid system in which only truncated Bcl-x\textsubscript{L} interacted with MEKK3 and KM-MEK3. In fact, both the recombinant protein representative of the yeast two hybrid clone, Δ38 Bcl-x\textsubscript{L} and the caspase-cleaved protein, Δ61 Bcl-x\textsubscript{L} interacted with MEKK3. However, the closely related protein kinase, MEKK2 did not interact with Δ61 Bcl-x\textsubscript{L}. These results suggest that the MEKK3 and truncated Bcl-x\textsubscript{L} interaction is unique and not shared with this closely related MAP3K. Experiments using either mammalian or insect cell systems also confirmed that the interaction between MEKK3 and Bcl-x\textsubscript{L} is dependent upon the loss of the amino-terminal, BH4 domain of Bcl-x\textsubscript{L} since the full-length protein did not interact in any system tested. Additionally, both wild type and kinase-inactive MEKK3 interacted with the truncated forms of Bcl-x\textsubscript{L} which demonstrates that the interaction is not dependent upon the catalytic activity of MEKK3.

During the course of these studies, the Bcl-x\textsubscript{L} cleavage site was identified as the aspartic acid at amino acid 61 (53). Ultimately, the interaction between MEKK3 occurred with the caspase-cleaved Δ61 Bcl-x\textsubscript{L} and not the full-length Bcl-x\textsubscript{L}. These results suggest the interaction between MEKK3 and Bcl-x\textsubscript{L} after the induction of apoptosis and a role for MEKK3 in cell death signaling pathways.
Treatment of cells with anticancer drugs such as, etoposide, results in phosphorylation of Bcl-2 family members (60). It was hypothesized that a possible consequence of the MEKK3 and tBcl-xL interaction might be phosphorylation. Therefore, Bcl-xL fusion proteins were tested as potential MEKK3 substrates since the substrates for MEKK3 remain uncertain. However, despite their physical interaction, experiments to determine whether Bcl-xL served as a MEKK3 substrate were unsuccessful. Neither the full-length or truncated forms of Bcl-xL were phosphorylated by MEKK3.

Since the downstream substrate for MEKK3 remains elusive perhaps Bcl-xL serves to modulate MEKK3 kinase activity towards its downstream substrate. To test this hypothesis, Bcl-xL was added in addition to the non-specific kinase substrate, MBP in an in vitro kinase assay with MEKK3. The presence of full-length Bcl-xL or caspase-cleaved Bcl-xL did not affect MEKK3 activity towards MBP.

The phosphorylation of BAD on serine is one of the regulatory steps that is necessary for apoptotic and anti-apoptotic signals (74). Since Bcl-xL binds BAD, another alternative hypothesis tested was whether MEKK3 activity towards Bcl-xL required this component of the cell survival signaling pathway or whether MEKK3 is responsible for BAD phosphorylation and its inactivation. In vitro kinase assays were performed with the addition of both recombinant GST-BAD and GST-Bcl-xL to determine if BAD was a putative MEKK3 substrate. Despite adding BAD to a kinase reaction, MEKK3 was still unable to phosphorylate either full-length or caspase-cleaved Bcl-xL. Similiar to the kinase assay with Bcl-xL, autophosphorylation of MEKK3 was
observed. These results indicated that BAD was not the substrate for MEKK3 since the phosphorylation of GST-BAD was not detected and that BAD does not modulate MEKK3 kinase activity. At the time of these experiments, the upstream kinase that phosphorylates BAD was unknown. However, Akt has since been identified as the upstream kinase for BAD phosphorylation at serine 136 (58).

Another Bcl-2 binding protein that promotes cell survival is Bag-1 (68). One function of Bag-1 in cells is the activation of kinase activity for the MAP3K, Raf-1 (70). Since Raf-1 and MEKK3 function at the level of MAP3K, it was hypothesized that Bag-1 might serve as an accessory protein necessary to promote Bcl-xL phosphorylation by MEKK3. However, the addition of Bag-1, as a GST fusion protein, did not promote phosphorylation of truncated or full-length Bcl-xL by MEKK3.

Recently, the identification of scaffolding proteins regulating MAPK activation have been characterized. JNK-interacting protein (JIP) serves as an adaptor for a protein complex including JNK1/2, M KK7 and the MAP3K, MLK1 (42, 73). JIP can also serve as a scaffolding protein within the Dual Leucine Zipper-Bearing Kinase signaling pathway (DLK) (63). In fact, the recruitment of JNK disrupts the association of unphosphorylated DLK bound to JIP and leads to DLK dimerization and phosphorylation. MEK1 binding partner (MP-1) has been identified as an adaptor protein that anchors MEK1 and ERK1 leading to activation of this signaling cascade (67).

Similar to the MAPKs, scaffolding proteins are conserved across multiple species. Adaptor proteins have been identified in yeast. Ste5 is an adaptor protein in S. cerevisiae, which anchors Ste11 (the yeast MEKK3 homolog), Ste7, and FUS3 that are
the required components of a pheromone mating response pathway (61). Perhaps MEKK3 cannot phosphorylate Bcl-x<sub>L</sub> in the mammalian systems tested due to the absence of an essential adaptor molecule that recruits the components of the MAPK module.

These experiments demonstrated a physical association between the stress-activated protein kinase, MEKK3 and the caspase-cleaved product of Bcl-x<sub>L</sub>. These experiments confirmed that MEKK3 interacts with cleaved Bcl-x<sub>L</sub> in yeast and mammalian systems, as well as, 14-3-3 proteins. Further studies with caspase-cleaved Bcl-x<sub>L</sub> were not conducted since this form of Bcl-x<sub>L</sub> could not regulate MEKK3 kinase activity nor could it serve as a substrate of MEKK3. Thus, the physiological significance of the interaction between MEKK3 and the caspase-cleaved form of Bcl-x<sub>L</sub> remains unknown. However, these studies suggest a role for MEKK3 in cell death and survival pathways involving Bcl-x<sub>L</sub> that may become clearer as more components of these pathways are identified.
CHAPTER 4

IN VIVO AND IN VITRO PHOSPHORYLATION OF MEKK3

4.1 INTRODUCTION

Although much research has focused on the identification of MAPK cascades that are activated by MAP3Ks, the direct phosphorylation and identification of phosphorylation sites of mammalian STE11 homologues, such as the MEKKs, has not been shown. To identify the putative phosphorylation sites and an upstream regulator of MEKK3, we generated a recombinant baculovirus for MEKK3 expression in Sf9 insect cells. This system has been previously used to identify phosphorylation sites of the protein kinase, Raf (98). In this chapter, we utilized liquid chromatography and tandem mass spectrometry (LC-MS) to analyze the recombinant MEKK3 expressed in Sf9 insect cells.

A diverse group of protein kinases referred to as AGC kinases phosphorylate substrates on serine and threonine residues that are located close to arginine or lysine residues (76, 93, 105). AGC kinases include the cyclic nucleotide dependent protein kinases, PKA, PKG, and PKC. Additional AGC family members include: p70 ribosomal S6 Kinase, p90RSK, and Akt. The serine/threonine kinase, Akt, is a critical protein for cell survival pathways. Akt activation in response to growth factors and cytokines is well documented and linked to the inactivation of pathways downstream of its substrates. Akt substrates include a diverse group of proteins including the pro-apoptotic protein, BAD
(58), the transcriptional factor, Forkhead (77), and glycogen synthase kinase (GSK-3β) (80). Although these proteins function in diverse roles in the cell, all are negatively regulated by Akt and share the consensus site, RXRXXS/T for Akt phosphorylation.

Negative regulation is also a common feature of other AGC substrates including phosphorylation of BAD by PKA at Ser155 (96), Ser112 of BAD by p90RSK (103), and Ser621 of Raf-1 by PKA (97). In general, the peptide sequence, RXXS/T which is found in MEKK3 at Ser166 and Ser337 is considered as the AGC consensus phosphorylation site. In this chapter, studies providing the identification and characterization of in vivo phosphorylation sites for MEKK3 will be described as well as regulation of these sites by upstream kinases including the AGC kinases.
4.2 EXPERIMENTAL PROCEDURES

4.2.1 In Gel Tryptic Digestion

Stained proteins were excised from the gel with a razor blade and washed as described previously (79). The gel was cut into 1-mm cubes and placed in an Eppendorf tube. Gel pieces were washed sequentially for 15 minutes each with 50% acetonitrile, 100% acetonitrile, and 0.1 M ammonium bicarbonate. An equal volume of acetonitrile was added to the ammonium bicarbonate solution and then the gel pieces were dried under vacuum for 15 minutes. Cysteines were reduced by adding 10 mM DTT in 0.1 M ammonium bicarbonate to the dried gel pieces for 45 minutes at 56°C. The DTT solution was removed and 55 mM iodoacetamide in 0.1 M ammonium bicarbonate was added for 30 minutes at ambient temperature to alkylate cysteines. The alkylating solution was removed by aspiration and the gel pieces washed in 0.1 M ammonium bicarbonate. An equal volume of acetonitrile was added to the gel pieces for 15 minutes and then the sample was dried under vacuum to dry the gel pieces.

To the dried gel pieces was added 40 μl of 0.1 μg/μl of sequence grade trypsin. Once the gel pieces became hydrated, an additional aliquot of trypsin was added and incubated overnight at 37°C. Tryptic peptides were recovered by adding 10 μl 2% TFA and collecting the supernatant in a clean Eppendorf tube. The gel pieces were incubated with 70 μl of 0.1% TFA and sonicated for 30 minutes. The sonication procedure was
repeated twice, washing once with 30% acetonitrile and 0.1% TFA and once with 60% acetonitrile and 0.1% TFA. The supernatants were collected and pooled into one tube, reducing the volume under vacuum and storing at -20°C until HPLC analysis.

4.2.2 Mass Spectrometry

HPLC-MS was performed on tryptic digests with a Finnigan-MAT LCQ instrument equipped with a Spectrasystem TSP P4000 HPLC and AS3000 auto sampler. Automated acquisition of MS-MS spectra was performed by data-dependent scanning with the Excalibur™ software (Finnigan MAT, San Jose, CA). Total run time was set to 40 minutes. Peptides were analyzed on a Vydac Proteins and Peptides C18 microbore column (Vydac, Hesperia, CA) eluted with ACN/water/HCOOH/TFA (1/99/0.1/0.0085, v/v/v/v) for 3 min, which then was programmed to 85% acetonitrile over 27 min and held for 10 min. A flow rate of 0.1 ml/min was achieved with a splitter tee upstream of a Microm Magic Flow splitter box. Data dependent scanning was performed with a default charge state of 2, an isolation width of 3.0 amu, an activation amplitude of 40.0%, an activation time of 30.0 ms, and a required minimum signal of 75000 counts. Global dependent data settings were an exclusion mass width of 3.0 amu, a reject mass width of 3.0 amu, with dynamic exclusion enabled, a repeat count of 1.0, a repeat duration 1.0, an exclusion duration of 5.0 min, and an exclusion mass width of 3.0 amu. The scan event series included one full scan with mass range 300 to 2000 dalton, followed by three dependent MS-MS scans of the most intense ion. Capillary temperature, sheath gas pressure, and auxiliary gas pressure were 200°C, 84 PSI, and 32 PSI, respectively.
4.2.3 Peptide Sequencing and Identification

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to purify and sequence tryptic peptides derived from proteins prepared by electrophoresis (83). Eluting peptides were ionized by electrospray ionization, detected, and specific peptide ions were automatically selected and fragmented by a triple quadrupole mass spectrometer. The mass spectrometer alternates between MS mode for peptide mass identification and MS/MS mode for peptide characterization and sequencing. Selected peptides were fragmented by collision-induced dissociation (CID) to generate tandem mass spectrum, which contain the sequence information for a single peptide. For the identification of proteins, individual CID mass spectra were compared by the computer program SEQUEST (108) to predicted spectra from protein sequence databases. Identification of a peptide, by default, allowed for identification of the protein from the electrophoresis gel.

4.2.4 Generation of MEKK3 Serine 166 Phospho-antibody

Peptides were synthesized corresponding to amino acids 159-171 of MEKK3, CEPRSRHLSVSSQN or CEPRSRHL(pS)VSSQN where pS denotes a phospho-serine (New England Peptide, Inc.). Peptides were dissolved at 1 mg/ml in PBS and the pH was adjusted to 7.5 with 0.2 N NaOH for conjugation to KLH (keyhole limpet hemocyanin). 5 mg peptide was combined with 5 mg KLH and incubated at 25°C for 2 h. Conjugated peptide was dialyzed overnight in PBS. Protein concentration was determined and
peptides aliquoted into 250 or 500 μg for injection into New Zealand white rabbits (Covance, Denver, PA).

The non-phosphopeptide, CEPRSRHLSVSSQN was dissolved in 50 mM Tris, pH 8.5 and 5 mM EDTA. Sulfolink coupling gel (Pierce #20401) was brought to room temperature and approximately 5 ml of gel slurry (2.5 ml column volume) was packed into column (Econo columns, Fisher Scientific). The column was equilibrated with 6 volumes of 50 mM Tris, pH 8.0 and 5 mM EDTA prior to addition of 4 mg of peptide. Gel and peptide were incubated for 15 min at room temperature without mixing. Subsequently peptide and column mixture were incubated for 30 min at room temperature without mixing. Column was washed with 3 volumes of 50 mM Tris, pH 8.5 and 5 mM EDTA. 50 mM cysteine (prepared in 50 mM Tris, pH 8.5 and 5 mM EDTA) was added to block nonspecific sites on the gel by applying 1 ml of cysteine for every 1 ml of gel and this reaction proceeded for 15 mins with no mixing and 30 mins mixing. Column was washed with 16 volumes of 1 M NaCl and 16 volumes of 0.05% sodium azide/PBS. Column was stored at 4°C prior to use.

Phosphospecific antibody was purified via a 2 step affinity column preparation. Approximately 7 ml rabbit antiserum was applied through a 0.45 μM filter. A 1 ml Protein A Sepharose column was washed with PBS until a baseline was established. Antiserum was applied twice and column subsequently washed with PBS until baseline established again. MEKK3 antibody was eluted with 3.5 M MgCl2 and immediately diluted 4 fold with H2O. Antibody was concentrated in dialysis tubing with Aquacide III (Calbiochem) and dialyzed overnight in PBS. Antibody was applied to non-phospho-MEKK3Ser166
affinity column and the flow-thru fraction was collected as putative phospho-MEKK3\(^{\text{Ser166}}\) antibody. Bound antibody was eluted as non-phospho-MEKK3\(^{\text{Ser166}}\) antibody with 3.5 M MgCl\(_2\) and diluted with H\(_2\)O. The antibody was dialyzed in PBS and stored at 4°C as described above until use.

4.2.5 Generation of MEKK3 Serine 337 Phospho-antibody

This antibody was prepared as described for phospho-MEKK3\(^{\text{Ser166}}\) described above. However, peptides were synthesized corresponding to amino acids 328-342 of MEKK3, CLDPRGRLRSADSEN or CLDPRGRLR(pS)ADSEN where pS denotes a phospho-serine (New England Peptide, Inc.).

4.2.6 PKA In Vitro Kinase Assay

NIH3T3 cells were deprived of serum in 0.1% BSA, 100 units/ml penicillin and 100 μg/ml streptomycin in DMEM for 48 h prior to stimulation with 50 μM forskolin or 10 μM H89. Cellular extracts were prepared by lysis in buffer (20 mM Tris pH 7.6, 0.5% NP40, 0.25 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 2 mM Na\(_3\)VO\(_4\), 0.25 μg/ml leupeptin, 1 mM DTT, and 1 μg/ml aprotinin). Extracts were cleared of cellular debris by centrifugation at 14,000 rpm for 5 min at 4°C. The resulting supernatant was incubated for 90 min at 4°C with 1 μg of anti-PKA antibody (Santa Cruz, sc-903). Immune complexes were isolated with Protein A bound to Sepharose for 90 min at 4°C. The immunoprecipitates were then washed twice with 1 ml of PAN NP40 [10 mM Pipes, pH 7.0, 20 μg/ml aprotinin, and 100 mM NaCl (PAN), and 0.5% NP40] and once with
PAN, collecting the immunoprecipitates at 2,000 rpm in a microcentrifuge for 1 min. Subsequently, an in vitro kinase assay was performed using 0.5 μg MBP-MEKK3 (91) as substrate in the presence of 10 μCi [γ-32P]ATP for 20 min at 30°C. Alternatively, MEKK3 was expressed in HEK293 cells and cellular extracts prepared as described above. FLAGM2-Agarose was used to isolate MEKK3 and an in vitro kinase assay was performed with 5U of PKA catalytic subunit (Promega) in the presence of 10 μCi [γ-32P]ATP for 20 min at 30°C.

4.2.7 Preparation of (His)6FLAG•MEKK3 constructs for expression

FLAG•KM-MEKK3/pBlueBacHis2B was used as a template to generate recombinant FLAG•KM-MEKK3 with serine to alanine point mutations within the consensus PKA motifs. (S166A), (S337A), and (S166/337A) FLAG•KM-MEKK3 were constructed by site-directed mutagenesis using a two-step PCR protocol (65). The following sense primer, overlapping the KasI endonuclease site of MEKK3, 5'- CGT GGG CGC CTA CGG GCC GCG GGA ACA GTG AG-3' and antisense 5'-TAC CTA GCA TGA ACA GAT TGA TCT GCC GGG TGT ACT TG-3' primer were used in a first PCR reaction for mutating serine 337 to alanine. This PCR product was used for a second PCR reaction with sense primer 5'-AGC ACA AAT GGC GAG AAC-3' and antisense primer 5'-T ACC TAG CAT GAA CAG ATT G-3'. This PCR product was subcloned into pGEM-T (Promega) and sequenced, then the 350 bp KasI/StuI fragment containing the S337A mutation was ligated into FLAG•KM-MEKK3/pBlueBacHis2B cleaved with KasI/StuI. For construction of the serine 166 to alanine mutant, the first
sense PCR primer, whose underlined sequence annealed to the pBlueBacHis2B vector upstream of the \textit{Bam}H1 site in the polylinker, was 5'-CA ATC TGT TCA TGC TAG GTA GGA CAG CAA ATG GGT CGG-3' and antisense 5'-CTG GGA GCT CAC AGC* CAG GTG-3'. The second PCR reaction primers were sense 5'-CA ATC TGT TCA TGC TAG GTA-3' and antisense overlapping the \textit{Bam}H1 endonuclease site of MEKK3, 5'-CGT ATA GGA TCC TTG CCG-3'. The resulting 600 bp product was subcloned into pGEM-T and sequenced. The 600 bp MEKK3 fragment was excised from pGEM-T using the \textit{Bam}H1 site in the polylinker of pBlueBacHis2B and the \textit{Bam}H1 site of MEKK3. FLAG•KM-MEKK3/pBlueBacHis2B was cleaved with \textit{Bam}H1 and religated with the 600 bp fragment containing the S166A mutation. To generate the double mutant, (S166/337A) FLAG•KM-MEKK3, the cDNA that encodes the single mutant (S337A) FLAG•KM-MEKK3 was cleaved with \textit{Bam}H1 and religated with the 600 bp fragment containing S166A that was also cleaved with \textit{Bam}H1. Baculovirus encoding (His)$_6$FLAG•MEKK3 and the various point mutants were produced according to the directions provided by the manufacturer. Once the baculovirus encoding the constructs was obtained, recombinant proteins were typically obtained from a 500 ml culture of Sf9 insect cells, infected at an MOI of 5 and a density of 1 x 10$^6$ cells/ml for 36 h, and then purified with Ni-Sepharose as described by the manufacturer. Recombinant (His)$_6$FLAG•KM-MEKK3 and the serine to alanine point mutants that were used as substrates in kinase assays were incubated with 1% Empigen BB in PBS while bound to Ni-Sepharose for 1 h to remove co-purifying proteins. The detergent was removed by washing twice with 25 volumes of 50 mM NaPO$_4$, pH 8, 100 mM KCl, 0.1% Tween 20,
10 mM β-mercaptoethanol, and twice with the same buffer at pH 6.2. Recombinant proteins were eluted from the Ni-Sepharose with 1 ml of 250 mM imidazole in the pH 6.2 buffer, repeating a total of three times. The eluted protein was dialyzed in 1 liter of 10 mM Hepes, pH 7.2, 1 mM EDTA, 14.3 mM β-mercaptoethanol, 0.025% Triton X-100, and 1 μg/ml aprotinin for 1 h and then dialyzed in 500 ml of the same buffer containing 50% glycerol. Recombinant proteins were stored at -20°C.

The cDNAs encoding (His)_6FLAG•MEKK3 and the various point mutants were excised from pBlueBacHis2B with SalI, generating blunt ends with Klenow, and ligating into pcDNA3.1 His A (Invitrogen) cleaved with EcoRV.

4.2.8 Precipitation of proteins with GST•14-3-3ε

NIH 3T3 cells were serum-starved for 48 h and cell extracts were prepared as described above. Approximately 350 μg of cellular lysate was incubated with recombinant GST•lamin or GST•14-3-3ε fusion proteins bound to Sepharose beads for 4 h at 4°C. Samples were then washed twice with PAN-NP40 and once with PAN, collecting the precipitated proteins at 2,000 rpm in a microcentrifuge for 1 minute, then resolving the proteins by 8% SDS-PAGE. After transfer to nitrocellulose, the membrane was probed with an amino-terminal antibody that recognizes total MEKK3 or phospho-MEKK3^{Ser166} followed by goat-anti-rabbit HRP antibody (Zymed).

Alternatively, COS-7 cells were transiently transfected using DEAE-dextran with 2 μg FLAG-MEKK3 constructs in pcDNA3.1 His A. COS-7 cell extracts were prepared 48 h post transfection in lysis buffer as described above. Approximately 1 mg of cellular
lysate was incubated with recombinant GST•lamin or GST•14-3-3ε fusion proteins bound to Sepharose beads for 4 h at 4°C. Samples were then washed twice with PAN-NP40 [10 mM Pipes, pH 7.0, 20 µg/ml aprotinin, and 100 mM NaCl (PAN), and 0.5% NP40] and once with PAN, collecting the precipitated proteins at 2,000 rpm in a microcentrifuge for 1 minute, then resolving the proteins by 8% SDS-PAGE. After transfer to nitrocellulose, the membrane was probed with phospho-MEKK3Ser166 antisera followed by goat-anti-rabbit HRP antibody (Zymed).

4.2.9 MEKK3 immunoprecipitation and phospho-immunoblotting

NIH3T3 cells were deprived of serum for 48 h prior to harvest. Prior to harvest cells were treated with 50 µM forskolin for 15 min (Alomone labs), 10 µM H89 (Calbiochem) for 1 h, 100 nM wortmannin (Sigma) for 30 min, 100 nM rapamycin (Calbiochem) for 30 min, 50 µM PD98059 (Calbiochem) for 30 min, or 5 ng/ml TNFα (R&D systems). Cellular extracts were prepared by lysis in buffer as described above. Extracts were cleared of cellular debris by centrifugation at 14,000 rpm for 5 min at 4°C. The resulting supernatant was incubated for 90 min at 4°C with MEKK3 amino-terminal antibody conjugated to Protein A for 4 h at 4°C. The immunoprecipitates were then washed twice with 1 ml of PAN NP40 and once with PAN, collecting the immunoprecipitates at 2,000 rpm in a microcentrifuge for 1 min. Subsequently, 8% SDS-PAGE was performed and the nitrocellulose membrane was probed for phospho-MEKK3Ser166 or total MEKK3. Lysates were also immunoblotted for phospho-AktSer473 (Cell Signalling), p70 (Cell Signalling), phospho-p70 (Cell Signalling), phospho-
ERK1/ERK2 (Cell signaling), or Akt (Santa Cruz, sc-8312), ERK1 (Santa Cruz, sc-93-G), or ERK2 (Santa Cruz, sc-154-G).
4.3 RESULTS

In order to gain insight as to the physiological function of MEKK3, we chose to identify proteins that interact with MEKK3. We postulated that the identity of such proteins, as long as their function was known, would provide clues as to the role of MEKK3 in the cell. To accomplish our objective, we prepared baculovirus that encodes wild type or kinase-inactive (His)$_6$FLAG•MEKK3, so that we could affinity purify interacting proteins from cell extracts by using recombinant MEKK3 as the bait. The FLAG monoclonal antibody and the hexahistidine epitopes were engineered into the amino-terminus of MEKK3 to purify the recombinant protein in two steps. However, we found that the FLAG epitope did not greatly enhance the purity of the recombinant protein (data not shown). Therefore, recombinant (His)$_6$FLAG•MEKK3, bound to Ni-Sepharose, was used in these studies.

Recombinant wild type and kinase-inactive (His)$_6$FLAG•MEKK3 were expressed and purified from Sf9 insect cells with Ni-Sepharose, then resolved by SDS-PAGE and the gel was stained to assess the purity of the recombinant proteins. A group of proteins between 28 and 40 kDa co-purified with both (His)$_6$FLAG•MEKK3 and (His)$_6$FLAG•KM-MEKK3. However, it appeared that within this group of proteins, a 36 kDa protein co-purified with (His)$_6$FLAG•MEKK3 and not (His)$_6$FLAG•KM-MEKK3 (Figure 4.1A). We used electrospray ionization and tandem mass spectrometry to identify the protein that appeared to selectively interact with (His)$_6$FLAG•MEKK3 and
not (His)$_6$FLAG•KM-MEK3. However, rather than use Coomassie Blue to stain the proteins as shown in figure 1A, (His)$_6$FLAG•MEKK3 was resolved by SDS-PAGE and stained with imidazole-zinc salts as described previously [data not shown, (79)]. We have found that this staining procedure is as sensitive as silver staining (unpublished observation, Adams and Vaillancourt). The major advantage of this staining method is that it does not require acid fixation of proteins, which may adversely affect in-gel tryptic digestion of proteins.

The 36 kDa protein that co-purified with (His)$_6$FLAG•MEKK3 was excised from the gel, then an in-gel tryptic digestion was performed. Tryptic peptides were separated by capillary reverse phase chromatography and analyzed by electrospray ionization and tandem mass spectrometry (LC-MS). From this analysis, peptides with a mass unit between 300 and 2000 daltons were automatically selected for collision-induced dissociation. The resulting tandem mass spectra were screened for peptides by the SEQUEST computer program. Of the tryptic peptides that were sequenced from the 36 kDa protein, SEQUEST identified two peptides derived from Sf9 insect cell 14-3-3 protein isoforms (Figure 4.1B). The sequence for one of the peptides matched the 14-3-3$\varepsilon$ isoform from amino acids 143 to 153, while the other peptide sequence matched that of the 14-3-3 isoform derived from soybean. This peptide is likely derived from a unique 14-3-3 isoform expressed in Sf9 insect cells and not yet cloned.

To further confirm our results obtained by LC-MS, we used immunoblotting as an independent method to identify 14-3-3 proteins as interacting with (His)$_6$FLAG•MEKK3.
Figure 4.1
Figure 4.1. 14-3-3 co-purifies with (His)$_6$FLAG•MEKK3. A. Recombinant (His)$_6$FLAG•MEKK3 and (His)$_6$FLAG•KM-MEKK3 were expressed and purified from Sf9 insect cells with Ni-Sepharose batch affinity chromatography and then 10 µg was resolved by SDS-PAGE and stained with Coomassie blue. The polypeptide with a molecular mass of ~50 kDa is a proteolytic fragment of MEKK3 that corresponds to the amino-terminus, as determined by immunoblotting with antibody that recognizes the amino-terminus of MEKK3 and by LC-MS (data not shown). B. Tryptic peptides obtained by LC-MS analysis of the 36 kDa protein determined that the co-purifying protein from Sf9 cells was 14-3-3. Two tryptic peptides were obtained and the sequence (left column) is shown with the corresponding accession numbers (middle column). C. Recombinant (His)$_6$FLAG•MEKK3 and (His)$_6$FLAG•KM-MEKK3 were resolved by SDS-PAGE and immunoblotted with antibody that recognizes MEKK3 (top panel) or 14-3-3 (lower panel) after treatment with (lanes b and d) or without (lanes a and c) 1% Empigen BB to remove co-purifying proteins.
Wild type and kinase-inactive \((\text{His})_6\text{FLAG} \bullet \text{MEKK3}\) were purified from Sf9 insect cells with Ni-Sepharose, then a fraction of the preparation was resolved by SDS-PAGE and the lower portion of the gel was immunoblotted with antibody specific to amino acids 3-21 of 14-3-3\(\beta\). This peptide sequence is highly conserved among 14-3-3 isoforms across species (3) and this antibody cross-reacts with multiple 14-3-3 isoforms. We detected two distinct polypeptides that were immunoreactive with the 14-3-3\(\beta\) antibody (Figure 4.1C, lane a), which was consistent with two different 14-3-3 isoforms detected by LC-MS. In addition, the presence of 14-3-3 isoforms was only detected with \((\text{His})_6\text{FLAG} \bullet \text{MEKK3}\), and not the kinase-inactive protein that contains a lysine to methionine point mutation (compare lanes a and c). Moreover, we found that KM-MEKK3 when fused to LexA does not interact with a fusion protein consisting of VP16 and 14-3-3 as measured by \(\beta\)-galactosidase activity in the yeast two hybrid system (data not shown), while wild type MEKK3 interacted with 14-3-3 in similar experiments (16). Our data from Sf9 insect cells, as well as yeast, suggest that the autophosphorylation activity of MEKK3 contributes a phosphorylated amino acid that is important for the interaction with 14-3-3.

The binding of 14-3-3 proteins with other proteins is typically associated with the presence of a phosphoserine consensus motif (102, 107). For example, phosphorylation of keratin 18 at serine 33 is essential for binding with 14-3-3 protein (92). It has been reported that the interaction between keratin 18 and 14-3-3 mobilizes keratin from the cytoskeletal to the cytosolic compartment of the cell (94). Thus, the binding of 14-3-3
proteins to MEKK3 may localize MEKK3 in the cytosolic compartment, and therefore may be a mechanism to compartmentalize MEKK3 kinase activity within the cell.

Mobilization of keratins from cellular compartments, due to phosphorylation and binding of 14-3-3 proteins, is important for cell cycle progression and such studies have utilized the zwitterionic detergent, Empigen BB, to characterize this process (92, 94). We utilized this detergent as a tool to determine whether we could further purify recombinant (His)_6FLAG•MEKK3 from 14-3-3 proteins derived from Sf9 insect cells. (His)_6FLAG•MEKK3 bound to Ni-Sepharose was incubated with 1% Empigen BB for one hour. We found that this procedure removed all detectable 14-3-3 proteins that co-purified with (His)_6FLAG•MEKK3 (Figure 1C, lanes b and d), without affecting the ionic interaction between the hexahistidine epitope on MEKK3 and Ni-Sepharose. Based on this observation, we have incorporated this purification step into our protocol for purifying (His)_6FLAG•MEKK3 from co-purifying proteins in insect cells.

The observation that 14-3-3 proteins co-purify with (His)_6FLAG•MEKK3 and the knowledge that 14-3-3 proteins bind to phosphoserine motifs suggested that (His)_6FLAG•MEKK3 expressed in Sf9 cells was phosphorylated on serine. Thus, our objective was to identify phosphorylated amino acids on (His)_6FLAG•MEKK3 by using LC-MS as a detection method. (His)_6FLAG•MEKK3, as shown in figure 4.1A, was excised from a gel and treated with trypsin. Tryptic peptides were separated by capillary reverse phase chromatography and analyzed by electrospray ionization and tandem mass spectrometry. The resulting tandem mass spectra were screened for phosphopeptides by
the SEQUEST computer program with user-defined parameters set to detect phosphorylation of serine, threonine, and tyrosine. Eight tryptic peptides derived from (His)$_6$FLAG•MEKK3 were detected by the SEQUEST program (data not shown) and phosphoserine was found in two of those peptides. The MS/MS spectra were analyzed manually and by using the scoring algorithm for spectral analysis (SALSA) (87) to verify the position of phosphoserine within each of the peptides. The phosphopeptides consisted of amino acids 164-174 and 335-349, with elution times of 19.7 and 21.1 minutes, respectively. Serines 166 and 337 were identified as phosphorylated within these peptides (Figures 4.2 and 4.3). It should be emphasized that LC-MS analysis of peptides derived from (His)$_6$FLAG•KM-MEKK3 indicated that approximately 50% of the recombinant protein obtained from Sf9 insect cells was not phosphorylated at Ser166 and Ser337.

The MS/MS spectrum for the phosphopeptide, $^{16}$HLpSVSSQNPG$^{174}$, with a $m/z$ ratio of 631.5 for the $[M + 2H]^{2+}$ ion is shown in figure 2. Neutral loss of the doubly charged phosphate, which has a mass of 49 dalton, from the parent ion produced the most abundant product ion with a $m/z$ of 582 $[M + 2H - H_3PO_4]^{2+}$. However, neutral loss of phosphate from the parent ion only indicates the presence of phosphate on the peptide. Phosphorylation of Ser166 was confirmed by observation of the $b_3$ fragment ion, 418 $m/z$, which is a mass that is consistent with a peptide sequence of HLpS. Neutral loss of phosphate from the HLpS peptide would produce a fragment ion of 98 dalton less than the parent ion of 418 $m/z$. Accordingly, a fragment ion with a $m/z$ of 320 $[b_3 - H_3PO_4]$ was consistent with neutral loss of phosphate from HLpS. Additional $b$ series ions were
detected and identified as \([b_7 - H_3PO_4]\) of 721 m/z, \(b_7\) of 819 m/z, \([b_8 - H_3PO_4]\) of 835 m/z, and \(b_8\) of 933 m/z. These b ions demonstrate phosphorylation of the peptide but do not unequivocally identify the phosphorylated amino acid. Of the b series ions, only the \(b_3\) ion proves that Ser166 is phosphorylated. Conversely, the \(y_7\) and \(y_8\) fragment ions of 745 and 845 m/z, respectively, indicate that phosphate was not present on Ser168 or Ser169. A m/z of 745 was a mass consistent for an unphosphorylated peptide composed of amino acids SSQNPGR. Similarly, a m/z of 845 was consistent with an unphosphorylated peptide consisting of VSSQNPGR. In contrast, the \(y_9\) fragment ion of 1011 m/z was consistent with phosphorylation at Ser166 and a peptide sequence of pSVSSQNPGR. As further confirmation of phosphorylation at Ser166, an additional fragment ion of 380 m/z was observed which is consistent with the mass to charge ratio of an internal peptide fragment consisting of \(^{165}\text{LpSV}^{167}\).
Figure 4.2. Tandem mass spectra of phosphopeptide 164-174 of (His)_6FLAG•MEKK3. Tryptic peptides derived from (His)_6FLAG•KM-MEKK3 were separated by liquid chromatography on a C_{18} microbore column and peptides were detected with a LCQ mass spectrometer. Eluting peptides were ionized by electrospray ionization and specific peptide ions were automatically selected and fragmented by a triple quadrupole mass spectrometer. The mass spectrometer alternates between MS mode for peptide mass identification and MS/MS mode for peptide characterization and sequencing. Selected peptides were fragmented by collision-induced dissociation (CID) to generate tandem mass spectrum, which contain the sequence information for a single peptide. For the identification of proteins, individual CID mass spectra were compared by the computer program Sequest (108) to predicted spectra from protein sequence databases. Identification of a peptide, HlpSVSSQNPG{r}^{174} (inset), allowed for identification of Ser166 as phosphorylated within the (His)_6FLAG•MEKK3 sequence. The b_3 and y_8 ion fragments provide the most compelling evidence for phosphorylation at Ser166.
An additional phosphopeptide from the liquid chromatography column corresponded to amino acids $^{335}$LRpSADSENALTQER$^{349}$ of (His)$_6$FLAG•MEKK3 (Figure 4.3). The peptide had a mass of 1770, while the doubly charged ion species had a $[M + 2H]^{2+}$ of 885 and neutral loss of doubly charged phosphate $[M + 2H - H_3PO_4]^{2+}$ produced a fragment ion of 836 $m/z$, which was the most abundant ion in the spectrum. Fragment ions of the b and y series of the tandem mass spectrum indicated that Ser337, and not Ser340 or Thr345, was phosphorylated within this peptide. The $y_{12}$ and $y_{13}$ fragment ions were the most diagnostic ions that demonstrate unambiguous phosphorylation of Ser337. The $m/z$ of 1332 for $y_{12}$ and 1499 for $y_{13}$ show a difference of 167 daltons, which is consistent with the mass of phosphoserine. If Ser337 were not phosphorylated, the $y_{13}$ fragment ion would have increased by only 87, which is the mass of unmodified serine, relative to the $y_{12}$ ion. However, a mass increase of 167 in the $y_{13}$ fragment ion provides strong evidence for phosphorylation of Ser337. Additionally, fragment ions $b_4$ and $b_5$, 508 and 623 $m/z$, respectively, correspond with peptide fragments LRpSA and LRpSAD. Similarly, fragmentation of the $b_6$ ion of 710 $m/z$ corresponds to peptide fragment LRpSADS, which confirms phosphorylation of Ser337 and not Ser340. The $y_5$ and $y_{10}$ ion fragments of 632 and 1146 $m/z$, respectively, correspond to peptides $^{345}$TVQER$^{349}$ and $^{340}$SENALTQER$^{349}$, which also confirm that Thr345 and Ser340 were not phosphorylated. In addition, the $y_8$ and $y_9$ fragment ions of 930 and 1059 $m/z$ further confirmed that Thr345 was not phosphorylated within the
Figure 4.3. Tandem mass spectra of phosphopeptide 335-349 of (His)$_6$FLAG•MEKK3. Tryptic peptides derived from (His)$_6$FLAG•KM-MEKK3 were separated and analyzed by LC-MS as described in figure 1. Identification of a peptide, $^{335}$LR$pSADSENALTvQR^{349}$ (inset), allowed for identification of Ser337 as phosphorylated within the (His)$_6$FLAG•MEKK3 sequence. The $y_5$, $y_{10}$, $y_{12}$, and $y_{13}$ fragment ions provide the most compelling evidence for phosphorylation at Ser337, and not Ser340 or Thr345.
tryptic peptide. Thus, of the three possible Ser/Thr phosphorylation sites in residues 335 to 349, only Ser337 was modified by phosphate.

We identified two phosphopeptides in (His)_6FLAG•MEKK3 that were localized within the consensus phosphorylation site, (RXXS/T) for AGC kinases. Examples of such kinases include, PKA, p70 S6 kinase, p90RSK, and Akt (76, 93, 105). Endogenous PKA was immunoprecipitated from NIH3T3 cells and utilized for an in vitro kinase assay with a fusion protein consisting of maltose binding protein (MBP), and the amino terminus of MEKK3 [amino acids 4-361, (16)]. As shown in Figure 4.4A, activated PKA phosphorylates the MBP-MEKK3 fusion protein 2 fold (lane e) over basal levels (lane d) and was reduced to basal levels by pre-treatment with the PKA inhibitor, H89 (lane f).

Since we were unable to express full-length MEKK3 as a fusion protein with MBP, we used HEK 293 cells to express (His)_6FLAG•KM-MEKK3 and the serine to alanine mutant (S166A). These proteins were used as substrates to determine whether serine 166 was phosphorylated by PKA. After transfection, HEK 293 cells were deprived of serum for 16 h and then MEKK3 was immunoprecipitated with FLAG M2 Agarose and used as a substrate for the catalytic subunit of PKA. Proteins were resolved by SDS-PAGE and detected by autoradiography. In the absence of PKA, there was no detectable phosphorylation of (His)_6FLAG•KM-MEKK3 (Figure 4.4B, lane c) indicating that kinase-inactive MEKK3 was unable to autophosphorylate or that no other kinases precipitated with MEKK3. We found that (His)_6FLAG•KM-MEKK3 (Figure 4.4B, lane
Figure 4.4
Figure 4.4. MEKK3 is phosphorylated in vitro by PKA. A. PKA was immunoprecipitated from NIH3T3 cells and an in vitro kinase assay was performed with (lanes d-f) or without (lanes a-c) MBP-MEKK3. B. (His)$_6$FLAG•KM-MEKK3 or (His)$_6$FLAG•KM-MEKK3 (S166A) were transiently transfected into HEK 293 cells. Cells were serum-starved 16 h prior to harvest and MEKK3 was immunoprecipitated with FLAG M2-Agarose and then used as substrate with the PKA catalytic subunit (lanes b, d, and f) for an in vitro kinase assay with [$\gamma$-$^{32}$P]ATP. The kinase reaction was terminated with Laemmli sample buffer and the proteins were resolved by SDS-PAGE and the autoradiogram of the gel is shown.
d) was phosphorylated by PKA and that mutation of serine 166 reduced PKA-dependent phosphorylation by 50% (lane f). Western blotting was performed and indicated similar amounts of MEKK3 recombinant proteins were added for the *in vitro* kinase assay (data not shown). Thus, Ser166 of MEKK3 is phosphorylated by PKA suggesting that MEKK3 is a downstream substrate of PKA. In addition, these data strongly suggest that infection of Sf9 insect cells results in PKA-dependent phosphorylation of \((\text{His})_6\text{FLAG•MEKK3}\) at Ser166, while the enzyme that phosphorylates Ser337 remains to be determined.

We generated phospho-specific antibodies to measure phosphorylation at either Ser166 or Ser337. To test the specificity of the phospho-antibodies, Sf9 insect cells were infected with baculovirus encoding MEKK3 serine to alanine point mutants and harvested for immunoblotting at 40 h post-infection. As shown in Figure 4.5A, phospho-MEKK3<sup>Ser166</sup> antibody recognizes KM-MEKK3 (lane a) or wild-type MEKK3 (lane b), but not the S166A (lane c) point mutant. Similarly, the phospho-MEKK3<sup>Ser337</sup> antibody recognized phosphorylation of KM-MEKK3 and wild-type MEKK3 (Figure 4.5B, lanes a and b, respectively), while no phosphorylation was detected at Ser337 with the mutant, S337A (lane d). The expression of all MEKK3 constructs was verified by immunoblotting with an amino-terminal MEKK3 antibody (Figure 4.5C). These data demonstrate that the phospho-antibodies are specific for phosphorylation at Ser166 or Ser337 of MEKK3 and confirm our LC-MS analysis of MEKK3 phosphorylation when expressed in Sf9 insect cells.
Figure 4.5. Generation of phospho-specific antibodies against Ser166 and Ser337 of MEKK3. Sf9 insect cells were infected with recombinant baculovirus for the indicated (His)_6FLAG•MEKK3 constructs: KM-MEKK3 (lane a), MEKK3 (lane b), S166A MEKK3 (lane c), and S337A MEKK3 (lane d). Cell lysates were prepared 40 h later and 8% SDS-PAGE was performed. Approximately 30 μg of cell lysates was immunoblotted for Ser166 (panel A), Ser337 (panel B) or total MEKK3 (panel C). Results are representative of three separate experiments.
The association of 14-3-3 proteins with phosphorylated PKA substrates such as BAD and the kinase, Raf-1, is an important mechanism to regulate the activity of the phosphorylated proteins. The phosphorylation status of MEKK3 may play a role in mediating an interaction with 14-3-3 proteins; therefore we examined the binding of endogenous MEKK3, from NIH3T3 cells, to 14-3-3 in response to PKA activators. Cell extracts were prepared and incubated with GST fusion proteins, consisting of GST•14-3-3ε (16) and GST•lamin (69), bound to Sepharose. Proteins that precipitated with the GST fusion proteins were resolved by SDS-PAGE and immunoblotted with an antibody that recognizes phospho-MEKK3Ser166 or the amino-terminus of MEKK3. We found that GST•14-3-3ε precipitated MEKK3 (Figure 4.6, lanes a-e), which was consistent with our results in which 14-3-3 proteins co-purified with (His)6FLAG•MEKK3 expressed in Sf9 insect cells (Figure 4.1A). These results are also consistent with our previous data obtained using MEKK3 and the yeast two-hybrid system (16). Upon PKA activation with forskolin, a 5.8 fold increase in phospho-MEKK3Ser166 binding to 14-3-3 was observed over basal levels (Figure 4.6, lane d). Pre-treatment for 60 min with the PKA inhibitor, H89, resulted in a 52% reduction in this binding (lane e). Serum treatment also produced a 6.9 fold increase in MEKK3pSer166 bound to 14-3-3 (Figure 6, lane b) and was reduced by 29% with H89 pre-treatment (Figure 4.6, lane c). MEKK3 did not bind GST-lamin (Figure 4.6, lanes f-j). Our results demonstrate that factors in serum promote phosphorylation of Ser166. Furthermore, factors that regulate PKA, such as forskolin, promote phosphorylation of Ser166 to a similar extent as serum.
Fold Induction

Figure 4.6

Treatment

a b c d e f g h i j

FSK FSK+H89 FBS FBS+H89 basal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold Induction</th>
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<tbody>
<tr>
<td>Basal</td>
<td>0</td>
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<tr>
<td>FBS</td>
<td>1</td>
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<tr>
<td>FSK</td>
<td>2</td>
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<tr>
<td>FBS+H89</td>
<td>3</td>
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<td>FSK+H89</td>
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MMP3

Phospho-MEK3

FSK FSK+H89 FBS FBS+H89 basal

GST-Lamin

CST-14-3-3
Figure 4.6. Phosphorylation of Serine 166 of MEKK3 is not required for 14-3-3ε binding. NIH3T3 cells were serum-starved 48 h prior to treatment with 50 μM forskolin for 10 min (lanes d and i), 10% FBS for 15 min (lanes b and g), or 10 μM H89 pretreatment for 1h prior to forskolin or serum treatment (lanes e, j, c, and h). NIH3T3 cell extracts were prepared 48 h post serum starving and incubated with recombinant GST•14-3-3ε (lanes a-e) or GST•lamin (lanes f-j) fusion proteins bound to Sepharose beads for 4 h at 4°C. Samples were washed and then resolved by 8% SDS-PAGE. After transfer to nitrocellulose, the membrane was probed with phospho-MEKK3Ser166 (upper panel) or amino-terminal MEKK3 antisera (lower panel) followed by goat-anti-rabbit HP antibody. Results are representative of three separate experiments. Quantitation by Scion Image is shown below immunoblot.
Interestingly, phosphorylation of Ser337 was not mediated by PKA as forskolin treatment did not increase MEKK3 phosphorylation at Ser337 (data not shown). It was also observed that reprobing the immunoblot from the GST-14-3-3 immunoprecipitation with an amino-terminal MEKK3 antibody showed the binding of MEKK3 with 14-3-3 under basal conditions (Figure 4.6, bottom panel, lane a). Therefore, phosphorylation at Ser166 was not essential for the interaction between MEKK3 and 14-3-3, but phosphorylation at Ser166 appears to increase the amount of phospho-MEKK3 bound to 14-3-3.

To further characterize the binding of MEKK3 and 14-3-3, the MEKK3 point mutants were expressed in Cos-7 cells and incubated with GST•14-3-3ε and GST•lamin bound to Sepharose as described above. However, mutation of the PKA phosphorylation site at Ser166 was not sufficient to eliminate 14-3-3 binding to (S166A) FLAG•MEKK3 (Figure 4.7, lane f). In addition, mutation of Ser337 (S337A) or serines 166 and 337 to alanine (S166/337A) failed to prevent the interaction with GST•14-3-3ε (Figure 4.7, lanes h and j). Using a different approach in which we immunoprecipitated 14-3-3 protein, we also found that transfected MEKK3 and the serine to alanine point mutants co-precipitated with 14-3-3 (data not shown). It should be noted that the serine to alanine mutants had the same autophosphorylation activity suggesting that the mutations did not affect the protein conformation and that Ser166 and Ser337 are not the major autophosphorylation sites (data not shown). These results suggest that phosphorylation of Ser166 and 337 is not required for interaction with 14-3-3 proteins or
Figure 4.7. Recombinant MEKK3 point mutants interact with 14-3-3ε. COS-7 cells were transiently transfected using DEAE-dextran with 2 μg FLAG-MEKK3 constructs in pcDNA3.1 His A. COS-7 cell extracts were prepared 48 h post transfection and incubated with recombinant GST•lamin (lanes a, c, e, g, and i) or GST•14-3-3ε (lanes b, d, f, h, and j) fusion proteins bound to Sepharose beads for 4 h at 4°C. Samples were washed and resolved by SDS-PAGE as described above. After transfer to nitrocellulose, the membrane was probed with MEKK3 antisera followed by goat-anti-rabbit HRP antibody. Results are representative of three separate experiments.
autophosphorylation activity. Moreover, these results suggest that additional phosphorylation sites, such as the predicted autophosphorylation site in the activation loop between subdomains VII and VIII of the kinase domain (91), exist within MEKK3 and likely provide a scaffold for 14-3-3 proteins.

In addition to the 14-3-3 precipitation experiments, the phosphorylation status of MEKK3 at Ser166 was characterized in NIH3T3 cells by immunoprecipitation of MEKK3 and immunoblotting for phospho-MEKK3\textsuperscript{Ser166} expression following PKA activation or inhibition. As shown in figure 4.8, treatment with forskolin (lanes b) increased phosphorylation at Ser166 by 2-fold and was abolished by pre-treatment with H89 (lane c). Treatment with serum also increased phosphorylation at Ser166 in MEKK3 by 3.4 fold (lane e) and was not reduced by wortmannin pre-treatment (lane f). These results are consistent with the 14-3-3 precipitation experiments in which phosphorylation at Ser166 of MEKK3 is PKA-dependent.

Other members of the AGC kinase family besides PKA recognize the phosphorylation motif identified at Ser166 of MEKK3. Therefore, to address whether phosphorylation at this site was mediated by additional kinases, phospho-MEKK3\textsuperscript{Ser166} immunoblotting was performed after stimulation of the relevant kinase pathways. It was observed that serum treatment resulted in the phosphorylation of MEKK3 at Ser166 and Ser337 (Figure 4.9A, lane b). However, MEKK3 phosphorylation was still observed with MEK1 inhibition by PD98059 (Figure 4.9A, lane c). As a control we analyzed ERK1 and ERK2 phosphorylation to confirm that the MEK1 pathway was inhibited by
Figure 4.8

Phospho-MEKK3$^{\text{Ser166}}$

MEKK3

Fold Induction

Treatment

basal  FSK  FSK+H89  FSK+W  FBS  FBS+W

bar graphs showing fold induction
Figure 4.8. PKA-Dependent Phosphorylation of Serine 166 of MEKK3. NIH3T3 cells cellular extracts were prepared 48 h post serum starving and MEKK3 was immunoprecipitated using the amino-terminal MEKK3 rabbit antibody conjugated to Protein A. Cells were treated for 15 min with 50 μM forskolin (lane b) or pretreated with 10 μM H89 for 1 h prior to forskolin treatment (lane c). Immunoprecipitates were immunoblotted for phospho-MEKK3<sup>Ser166</sup> (upper panel) or MEKK3 amino-terminal antibody (lower panel) to verify immunoprecipitation. Quantitation for MEKK3<sup>Ser166</sup> was performed using Scion image and is depicted below immunoblot. Results are representative of three separate experiments.
PD98059 (Figure 4.9C, lane c). Thus, MEKK3 phosphorylation at Ser166 is not dependent upon activation of p90RSK which is downstream of ERK. Similar experiments were performed to characterize the role of p70 S6 kinase or Akt on Ser166 and Ser337 phosphorylation of MEKK3. Pre-treatment of NIH3T3 cells with rapamycin or wortmannin inhibited serum-dependent phosphorylation of p70 S6 kinase and Akt (Figure 4.10C and 4.11C, lane c, respectively), while MEKK3 was still phosphorylated at Ser166 and Ser337 (Figure 4.10A and 4.11A, lane c). Immunoblotting was also performed with the respective non-phospho-antibodies to confirm equal protein levels for ERK1 and ERK2, p70 or Akt (Figures 4.9D, 4.10D, and 4.11D, lanes a-c) or MEKK3 (Figures 4.9B, 4.10B, 4.11D, lanes a-c). Thus, our data demonstrate that p90RSK, p70 S6 kinase and Akt are not involved in the phosphorylation of MEKK3 at Ser166 or Ser337.

In terms of NFκB signaling MEKK3 was recently reported to be phosphorylated in a TNF-dependent manner (45). Therefore, we utilized the phospho-specific MEKK3 antibodies to examine whether TNF treatment caused a stimulus dependent phosphorylation at Ser166. Stimulation of NIH3T3 cells with TNFα (Figure 4.12, lane c) resulted in a 2 fold increase in MEKK3 phosphorylation at Ser166 over basal levels (lane a). This TNF-dependent phosphorylation at Ser166 presents a non AGC kinase dependent pathway resulting in MEKK3 phosphorylation for future studies.
Figure 4.9. Phosphorylation of Serines 166 and 337 are p90RSK Independent.

NIH3T3 cells were serum starved for 48 h prior to harvesting. Cells were treated with 10% FBS for 15 min (lane b) or 50 μM PD98059 for 30 min prior to FBS (lane c). MEKK3 was immunoprecipitated and immunoblotting was performed for phospho-MEKK3Ser166 and phospho-MEKK3Ser337 (A) or MEKK3 (B). Lysates were immunoblotted for phospho-ERK1/2 (C) or for ERK1/2 (D).
Figure 4.10. **Phosphorylation of Serines 166 and 337 are p70S6 Kinase Independent.** NIH3T3 cellular lysates were prepared at 48 h after serum starvation. Cells were treated with 10% FBS for 15 min (lane b) or 100 nM rapamycin for 30 min prior to FBS (lane c). MEKK3 was immunoprecipitated and immunoblotting for phospho-MEKK3<sup>Ser166</sup> and phospho-MEKK3<sup>Ser337</sup> (A) and MEKK3 (B) was performed or lysates were immunoblotted phospho-p70<sup>Thr389</sup> (C) or p70 (D) detection.
Figure 4.11. Phosphorylation of Serine 166 is Akt Independent. NIH3T3 cells were serum starved as in figure 6. Cells were treated with 10% FBS for 15 min (lane b) or 100 nM wortmannin for 30 min prior to FBS (lane c). MEKK3 immunoprecipitates were immunoblotted for phospho-MEKK3<sup>Ser166</sup> and phospho-MEKK3<sup>Ser337</sup> (A) and MEKK3 (B) or cellular lysates were probed for phospho-Akt<sup>Ser473</sup> (C) or Akt (D).
Figure 4.12. TNF Dependent Phosphorylation of MEKK3 at Ser166. NIH3T3 cellular extracts were prepared 48 h after serum starve treatment (lane a), 10% FBS for 10 min (lane b), and 5 ng/ml TNFα (lane c). MEKK3 was immunoprecipitated with an amino-terminal antibody and Protein A sepharose. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted for phospho-MEKK3<sup>Ser166</sup> (top panel) or MEKK3 (bottom panel).
4.4 DISCUSSION

Since proteins that act upstream of MEKK3 had not been identified and are likely regulators of MEKK3, our goal was to identify proteins that regulate MEKK3 by co-precipitating proteins with recombinant (His)$_6$FLAG-MEKK3 that was bound to Ni-Sepharose and then identifying the proteins by LC-MS as described previously (83). Using this approach, we demonstrate that 14-3-3 proteins from Sf9 insect cells interact with MEKK3, which is consistent with our previous results using the yeast two-hybrid system in which we show that 14-3-3ε interacts with MEKK3 (16).

Unequivocal agreement on the amino acid sequence of a consensus 14-3-3 binding site is difficult, as researchers have shown that 14-3-3 proteins recognize somewhat different motifs (57, 102, 107). However, it is generally accepted that phosphoserine is an essential component of the motif. It is clear that 14-3-3 proteins interact with amino acid sequence motifs on proteins that do not match the proposed 14-3-3 consensus motifs. For example, Cbl interacts with τ and ζ 14-3-3 isoforms (95), but lacks the consensus motif described by others. Similarly, inspection of the MEKK3 amino acid sequence indicates that it does not contain the consensus 14-3-3 binding motifs described by these groups (57, 102, 107). Nonetheless, we demonstrate that 14-3-3 interacts with MEKK3, with a preferential interaction with phospho-MEKK$^{3\text{Ser166}}$ upon PKA activation. This
interaction is consistent with previous studies and the results from the yeast two hybrid system performed in our laboratory (16).

The interaction between recombinant MEKK3 and 14-3-3 proteins suggested that MEKK3 is phosphorylated on serine, since phosphoserine is the essential component of all proposed 14-3-3 recognition motifs (95, 57, 102, 107). A unique feature of the baculoviral expression system is that protein modifications such as phosphorylation occur in insect cells. Examples include phosphorylation of Src (100) and simian virus 40 large T antigen (90). In combination with the sensitivity of electrospary ionization mass spectrometry technology to sequence peptides and identify amino acid modifications such as phosphate incorporation, we were able to identify tryptic phosphopeptides derived from recombinant MEKK3 expressed in Sf9 cells. Not only were we able to identify tryptic peptides, but we precisely localized phosphorylated amino acids of MEKK3. Phosphopeptides obtained from MEKK3 were consistent with phosphorylation of Ser166 (HLpSVSSQNPG) and Ser337 (LRpSADSENALTQER) and further analysis of adjacent amino acids in the MEKK3 sequence revealed these serine modifications were localized within the consensus PKA phosphorylation motif, RXX(S/T), in which serine or threonine is phosphorylated. Analysis of these recombinant proteins revealed that these are not autophosphorylation sites of MEKK3 as point mutants exhibited the same autophosphorylation activity as wild-type.

The baculovirus/Sf9 insect cell expression system has been a useful tool for investigators to elucidate signaling mechanisms due to its high homology with signaling pathways of other organisms. For example, the baculovirus p35 protein inhibits caspases
and therefore inhibits apoptosis in insect cells during the infection process (78), and inhibits apoptosis when ectopically expressed in Drosophila (89) and nematodes (104). These results provide strong evidence that infection of insect cells with baculovirus activates cell survival signal transduction pathways, much like insulin and IGF-1 in mammalian cells (75). Moreover, infection of insect cells with baculovirus that encodes Akt results in phosphorylation of Akt at PDK-dependent sites \([i.e., \text{Thr}308 \text{ and Ser}473, (84)]\). Expression of recombinant Raf-1 in Sf9 also results in phosphorylation at serines (98) including serine 621, which is negatively regulated by PKA (103). Similarly, our results strongly suggest that Sf9 insect cells contain a homolog to mammalian PKA that phosphorylates MEKK3 at Ser166.

The AGC kinases phosphorylate substrates on serine and threonine residues that are located close to arginine or lysine residues. Although Ser166 and Ser337 match the consensus site for multiple AGC kinases, our studies demonstrate that p90RSK, p70 S6 kinase, and Akt pathways are not responsible for phosphorylation of these sites. In studies thus far, phosphorylation at Ser166 is mediated solely by PKA while the kinase that phosphorylates Ser337 is the subject of ongoing experiments. Mutation of Ser166 to alanine reduced phosphorylation of MEKK3 by PKA, however, this mutation did not abolish phosphorylation. This observation raises the question as to whether other PKA sites exist within MEKK3.

We demonstrated that recombinant MEKK3 is phosphorylated in Sf9 insect cells. In addition, our experiments utilizing GST-14-3-3ε to precipitate MEKK3 demonstrated the PKA dependent phosphorylation at Ser166 and 14-3-3 binding in mammalian cells.
However, phosphorylation at Ser166 was not a requirement for 14-3-3 binding, which suggests that other phosphorylation sites, or conformational changes, exist within MEKK3 to mediate this interaction.

Serine phosphorylation of BAD promotes its association with 14-3-3 and serves to prevent BAD mediated apoptosis by inhibition of BAD and Bcl-xL heterodimer formation (81). Similarly, Akt-dependent phosphorylation of the Forkhead transcription factor (FKHL1) promotes its association with 14-3-3 and sequesters the FKHL1/14-3-3 protein complex in the cytoplasm thus preventing gene transcription (77). PKA phosphorylates MEKK3 at Ser166, yet this phosphorylation site is not required for the interaction with 14-3-3. Perhaps the interaction between 14-3-3 and MEKK3 is essential for the intracellular localization of MEKK3, much like the keratins. As indicated earlier, the interaction between keratin 18 and 14-3-3 mobilizes keratin from the cytoskeletal to the cytosolic compartment of the cell (94). Thus, the binding of 14-3-3 proteins to MEKK3 may localize MEKK3 to the cytosolic compartment, providing a mechanism to localize MEKK3 kinase activity to a cellular compartment.

More recently, it has been suggested that the primary role of 14-3-3 proteins is to prevent apoptosis, partly though regulation of MAP kinases (106). We have shown that PKA is an upstream kinase that phosphorylates MEKK3 at Ser166. Given the role of PKA in anti-apoptotic pathways, phosphorylation of MEKK3 by PKA suggests a role for MEKK3 in cell survival and that phosphorylated MEKK3 may function as an anti-apoptotic protein, much like BAD. In studies thus far, PKA phosphorylation of its substrates results in decreased activity, for proteins including, BAD (88, 96), GSK-3β
(85), CREB (86) and Raf-1 (97, 101). However, while Bcl-xL is the binding partner for BAD under pro-apoptotic conditions (58, 74), the binding partners for phosphorylated MEKK3, besides 14-3-3, are unknown. Transient, over-expression of MEKK3 results in the activation of many downstream signaling pathways and proteins within these pathways may provide clues as to which protein selectively interacts with phosphorylated MEKK3. For example, MEKK3 can activate the JNK (13), ERK (13), p38 (82), BMK1 [Big Mitogen-activated Protein Kinase], (8), and the NF-κB signaling pathways (48). In addition, inducible expression of the catalytic domain of MEKK3 arrests cell cycle progression though p38 (14), while recent data provides evidence that MEKK3 is involved in early embryonic cardiovascular development and these effects are mediated through a p38 MAPK cascade by the transcription factor, MEF2C (44). It was previously noted that MEKK3 over-expression results in inhibition of cell proliferation and cyclin D1 down regulation (14). Recently, studies have shown that cyclin D1 expression is inhibited by forskolin in airway smooth muscle cells (99). Given the role of PKA in these processes, perhaps the mechanism for these effects is, in part, due to PKA dependent MEKK3 phosphorylation. Since multiple MAPK signaling pathways are regulated by MEKK3, there are many candidate proteins to assess as binding partners for MEKK3 that is phosphorylated at Ser166. Future studies will determine which signaling pathways are activated or inactivated in response to PKA-dependent phosphorylation of MEKK3 at Ser166.

The finding that MEKK3 is phosphorylated by TNF at Ser166 provides a stimulus dependent phosphorylation of MEKK3 not mediated by AGC kinases. Given the
recent identification of TNF mediated NF-κB activation by MEKK3 (45), our studies present the first clue as to the phosphorylation site regulating this downstream pathway. Further studies are required to determine whether Ser337 phosphorylation is also mediated by TNF and whether MEKK3 point mutants inhibit NF-κB activation.
CHAPTER 5
REGULATION OF DOWNSTREAM SIGNALING PATHWAYS BY MEKK3

5.1 INTRODUCTION

In the previous chapter, we utilized the baculovirus insect cell system for expression of recombinant \((\text{His})_6\text{FLAG•MEKK3}\) and identified the phosphorylation of MEKK3 at Ser166 and Ser337. However, the physiological significance or effect of these phosphorylation sites on downstream signaling pathways was not determined. As noted earlier, the phosphorylation of proteins by AGC kinases often results in negative regulation of their activity. Since MAPK activation has been shown through MEKK3 overexpression (13,82,8), serine to alanine point mutations at Ser166 and Ser337 of MEKK3 were evaluated for their effect on MAPK activity.

Besides regulation of kinase activity, phosphorylation of AGC kinase substrates can localize proteins into different cellular compartments. For instance, phosphorylation of the Forkhead transcription factor by Akt prevents its translocation into the nucleus (77). MEKK3 localization in the cell has not been reported, therefore, another objective of this chapter was to characterize MEKK3 by immunoblotting and immunofluorescence. We were particularly interested in MEKK3 localization in response to stimuli that result in phosphorylation at Ser166 or Ser337.
5.2 EXPERIMENTAL PROCEDURES

5.2.1 DEAE-Dextran Transfection of Cos-7 Cells

Cos-7 cells were cultured on 10 cm plates until a density of 60% confluency was reached for transient transfection using DEAE-dextran. Plasmid DNA was combined with 40 µl solution C, 200 µl DEAE-dextran solution, and 35 µl chloroquine solution. Cells were washed twice with PBS, 4 ml NuSerum (Becton Dickinson) was added, and the DNA/dextran mixture was applied to cells and incubated for 4 h. After this incubation, cells were washed once with 3 ml of PBS and 3 ml 10% DMSO/DMEM was added for 2 min at room temperature. Following the DMSO incubation, cells were washed with 4 ml of PBS and then incubated for 48 h in 10 ml of DMEM/5%FBS/5%calf serum before harvest.

5.2.2 JNK and ERK In Vitro Kinase Assays

Cos-7 cells were co-transfected by the DEAE-dextran method as described above with 4 µg of the serine to alanine point mutants of MEKK3, and 2 µg HA-JNK1/SRα3 or HA-ERK2/pcDNA3 plasmid DNA. Cell extracts were prepared 48 h post transfection with NP40 lysis buffer (described in chapter 4). HA-JNK1 and HA-ERK1 were immunoprecipitated with the 12CA5 monoclonal antibody (Roche) and Protein A sepharose, washed twice with PAN NP40 and once with PAN prior to the in vitro kinase reaction. An in vitro kinase assay was performed in the presence of 1X UKB, PAN, 10 µCi [γ-32P]ATP, and 0.5 µg GST-c-jun (111) or GST-Elk (New England Biolabs), for 20
min at 30°C, respectively, and then phosphorylated proteins were resolved by 8% SDS-PAGE and visualized by autoradiography.

5.2.3 NF-κB luciferase activity

Cos-7 cells were co-transfected by the DEAE-dextran method with 4 μg of the serine to alanine point mutants of MEKK3 and 2 μg NF-κB luciferase reporter plasmid. At 48 h post transfection cellular extracts were prepared using 1X Passive lysis buffer (Promega). Samples for each transfection condition were normalized for protein concentration prior to reading on luminometer (Turner Designs, TD-20/20). Luminometer counts were determined by transferring 20 μl of cell extract into a luminometer tube containing 100 μl LAR II (Promega) and mixing by pipetting 2 or 3 times. Tubes were placed in luminometer and readings recorded.

5.2.4 Isolation of cytoplasmic and nuclear extracts

NIH3T3 cells were cultured on 15 cm plates and serum-starved (0.1 % BSA, DMEM pH 7.4, 100 units/ml penicillin and 100 μg/ml streptomycin) for 48 h prior to harvest. Cells were washed twice with 15 ml of PBS and scraped off the plate with 1 ml PBS into 1.7 ml eppendorf tubes. Samples were subject to centrifugation at 2,000 rpm for 1 min and media was aspirated followed by another wash with 1 ml of PBS and centrifugation at 14,000 rpm for 1 min. The supernatant was removed after the PBS wash and cytosolic extracts were prepared by the addition of 0.3 ml buffer A (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF,
0.2 mM NaF, 0.2 mM Na_3VO_4, 0.3 mg/ml leupeptin) and incubating at 4°C for 15 min. Samples were mixed by vortexing at a setting of 5-6 for 15 sec after the addition of 25 μl Buffer B (10% NP-40) and collected by centrifugation at 14,000 rpm for 1 min. After this centrifugation, the supernatant was collected as the cytosolic fraction and the pellet was resuspended in 100 μl of buffer C (50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 10% glycerol, 0.2 mM NaF, 0.2 mM Na_3VO_4). The nuclear extract was collected by centrifugation at 4°C for 5 min. Cytoplasmic and nuclear extracts were resolved by SDS-PAGE and immunoblotted with an amino-terminal MEKK3 antibody (described in chapter 4) or PARP antibody (Santa Cruz).

5.2.5 Immunofluorescent labeling of NIH3T3 Cells

NIH3T3 cells were grown on 22 mm glass coverslips (VWR) in 10 cm tissue culture plates to 60% confluence and then serum starved for 16 h in DMEM as described above prior to harvest. Cells were transferred into 6 well dishes (VWR) for drug treatment and subsequent procedures. After the drug treatment, cells were washed twice with PBS and fixed with 2 ml of 70% methanol/30% acetone for 30 min at -20°C. After fixing, cells were washed twice with PBS and incubated in antibody dilution buffer (2% BSA/0.3%Tween20/TBS) for 60 min at room temperature to block non-specific binding. Following block in BSA/TTBS cells were incubated with MEKK3^{Ser166} peptide antibody at a dilution of 1:250 in 1% BSA/TTBS overnight while rotating at 4°C. After the primary antibody incubation, cells were washed twice with PBS and Alexa secondary
antibody (Molecular Probes) was diluted 1:250 in 1% BSA/TTBS. Alexa secondary antibody was incubated with the cells overnight at 4°C. Cells were washed twice with PBS and mounted cell side down with 50 µl of p-phenylenediamine (Sigma) to slides and sealed with cytoseal (VWR).
5.3 RESULTS

MEKK3 and the serine to alanine mutants were transfected into COS-7 cells along with HA-tagged JNK1 in order to assess whether phosphorylation of MEKK3 at Ser166 and 337 affected the ability of MEKK3 to activate downstream MAP kinase pathways. JNK1 was immunoprecipitated and its kinase activity was measured by the phosphorylation of GST-c-jun in the presence or absence of MEKK3. In cells expressing either (S166A), (S337A), or (S166/337A) MEKK3 showed no increase or decrease in HA-JNK1 (Figure 5.1, lanes c, d, and e, respectively) activity as compared to wild-type MEKK3 transfection (lane b). Thus MEKK3 phosphorylation at Ser166 and Ser337 is not required for activation of the JNK kinase pathway.

The MEKK3 point mutants were also tested for their effects on the ERK kinase pathway. ERK2 kinase activity was determined by the phosphoroylation of the transcription factor, GST-Elk. As shown in Figure 5.2A, in cells expressing either (S166A), (S337A), or (S166/337A) MEKK3 showed no increase or decrease in HA-ERK1 activity (lanes c, d, and e, respectively). Autoradiography yielded a band of approximately 45 kDa for ERK, 49 kDa for GST-Elk, and another band of 78 kDa (lanes b-e). This band of 78 kDa was only visualized in cells co-transfected with MEKK3 and this correlated with the size of MEKK3 expression in cell lysates (Figure 5.2, panel B).
Figure 5.1. Serines 166 and 337 of MEKK3 are not required for JNK activity. COS-7 cells were co-transfected with 2 μg HA-JNK1 in pSRα3 and either 4 μg pcDNA 3.1 His A (lane a) or 4 μg of FLAG-MEKK3 constructs in pcDNA 3.1 His A as follows: FLAG-MEKK3 (lane b), (S166A) FLAG-MEKK3 (lane c), (S337A) FLAG-MEKK3 (lane d), (S166/337A) FLAG-MEKK3 (lane e). Cellular lysates were prepared 48 h post transfection and immunoprecipitation of HA-JNK1 was performed using the 12CA5 monoclonal antibody. An *in vitro* kinase was performed to determine JNK1 activity by phosphorylation of GST-c-jun fusion protein and an autoradiogram is shown in Panel A. Lysates were immunoblotted for MEKK3 (Panel B) or HA-JNK1 (Panel C) to verify transfection.
Figure 5.2
Figure 5.2. Serines 166 and 337 of MEKK3 are not required for ERK activity. COS-7 cells were transfected as described in the legend to Figure 5.1, however 2 µg of HA-ERK2 in pcDNA3 was transfected in these experiments. COS-7 cellular extracts were prepared 48 h post transfection. Lysates were subject to immunoprecipitation using the 12CA5 monoclonal antibody and subsequently an *in vitro* kinase assay was performed using GST-Elk (New England Biolabs) as a substrate to determine ERK kinase activity. An autoradiogram is shown in Panel A. Lysates were immunoblotted for MEKK3 (Panel B) or HA-ERK2 (Panel C) to verify transfection.
Since cells were co-transfected with ERK2 and MEKK3, the appearance of a 78 kDa protein band in the ERK kinase assay was suspected to be MEKK3.

Further studies were conducted in which ERK2 was immunoprecipitated and MEKK3 immunoblotting was performed instead of autoradiography. For these experiments, cells were treated with serum prior to ERK2 co-precipitation since we demonstrated that serum treatment results in phosphorylation of MEKK3 at Ser166 (Chapter 4). The association between MEKK3 and ERK2 occurred under basal conditions and with serum treatment (Figure 5.3, lanes c and d). The co-precipitation of ERK2 and MEKK3 was also observed with S166A, S337A and S166/337A (data not shown). These experiments demonstrate that the interaction of MEKK3 and ERK is not dependent on phosphorylation of MEKK3 at Ser166 or Ser337.

To further characterize the interaction between MEKK3 and ERK2 an in vitro kinase assay was performed with the kinaseinactive, KM-ERK2. Co-precipitation of MEKK3 was observed with ERK2 (Figure 5.4, lanes c and d), however, MEKK3 did not co-precipitate with KM-ERK2 (lanes a and b). Immunoblotting was performed to verify transfection of ERK and MEKK3 (data not shown). Therefore, the catalytic activity of ERK is required for its association with MEKK3.

Previous studies established the activation of the transcription factor, NF-κB through MEKK3 overexpression (48). In addition to MAPK activation by the serine to alanine point mutants of MEKK3, these constructs were characterized for their ability to activate NF-κB. MEKK3 and the point mutants were able to activate a NF-κB luciferase reporter gene (Figure 5.5). There were no changes in the relative luciferase activity
Figure 5.3. MEKK3 and ERK co-precipitate. (His)_6FLAG•MEKK3/pcDNA 3.1 His A and HA-ERK2/pcDNA3 plasmid DNA were co-transfected using DEAE-dextran into Cos-7 cells. HA-ERK2 was immunoprecipitated using the 12CA5 monoclonal antibody and proteins were resolved by 8% SDS-PAGE. Proteins were transferred to nitrocellulose for immunoblotting with the amino-terminal MEKK3 antibody (top panel) or the 12CA5 monoclonal antibody for ERK2 (bottom panel). Proteins were visualized by enhanced chemiluminescence.
Figure 5.4. MEKK3 and ERK Co-precipitation is dependent upon the catalytic activity of ERK. MEKK3 and KM-ERK2 (lanes c and d) or ERK2 (lanes a and b) were co-transfected into Cos-7 cells. ERK2 was immunoprecipitated as described above. Following immunoprecipitation, an *in vitro* kinase assay was performed. Proteins were resolved by SDS-PAGE and autoradiography was performed to detect phosphorylated proteins.
**Figure 5.5.** Serines 166 and 337 of MEKK3 are not required for NF-κB activation.

NF-κB luciferase activity was measured after co-transfection of plasmid DNA encoding vector, pcDNA3.1 HisA, wild-type MEKK3, S166A, S337, or S166/337A and NF-κB-luciferase. Luciferase activity was measured 48 h after transfection.
between wild-type MEKK3 and S166A, S337A, or S166/337A. These data demonstrate that phosphorylation of Ser166 of MEKK3, as well as phosphorylation of Ser337, is not necessary for activation of either JNK or ERK MAP kinases or the NF-κB signaling pathway.

Since MAPK pathways were not modulated by the phosphorylation status of MEKK3, we next determined whether the subcellular localization of MEKK3 might be affected by phosphorylation at Ser166. Cytoplasmic and nuclear fractions were prepared from NIH3T3 cells under various treatment conditions. As shown in Figure 5.6, MEKK3 was found in cytosolic (lane a) and nuclear fractions under basal conditions (lane d). Upon PKA activation with forskolin (lanes b and e) or inhibition of PKA with H89 (lanes c and f), MEKK3 was still found in both fractions without any significant change in the total amount of MEKK3. To confirm the isolation of nuclear fractions, cell extracts were immunoblotted for the nuclear protein, PARP. As shown in Figure 5.6, PARP was only detected in nuclear lysates, lanes d-f. Thus, immunoblotting demonstrated that MEKK3 was localized within cytoplasmic and nuclear compartments of the cell.

MEKK3 was also visualized by immunofluorescence in NIH3T3 cells. The purpose of these experiments was to verify the immunoblotting results in which MEKK3 expression was detected in nuclear and cytoplasmic extracts. NIH3T3 cells were treated with known stimuli to phosphorylate MEKK3 at Ser166. Immunofluorescent labeling with the MEKK3^{Ser166} antibody demonstrated punctate staining of nuclei under basal
conditions (Figure 5.7, panel A). The staining of nuclei was more pronounced upon forskolin and serum (panels B and E)
Figure 5.6. MEKK3 is localized in nuclear and cytoplasmic cellular compartments.

Cytoplasmic and nuclear extracts were prepared from NIH3T3 cells after 48 h serum-starve treatment. Cells were treated for 15 min with 50 µM forskolin (lanes b and e) or 50 µM forskolin after 1h pre-treatment with 10 µM H89 (lanes c and f). Extracts were resolved by 8% SDS-PAGE and transferred to nitrocellulose for immunoblotting with an amino-terminal MEKK3 antibody (Panel A) or PARP (Panel B). Proteins were visualized by enhanced chemiluminescence.
Figure 5.7. Immunofluorescent localization of MEKK3 in NIH3T3 cells. Endogenous MEKK3 in NIH3T3 cells was labeled with MEKK3$^{\text{Ser166}}$ antibody after 16 h serum starve (A), 50 μM forskolin for 15 min (B), 10 μM H89 pretreatment for 1 h followed by 50 μM forskolin, 10 μM H89 for 1 h (D) and 10% FBS (E). MEKK3 was visualized by conventional epifluorescence microscopy using an Olympus IX70 microscope and a 60x oil immersion objective.
treatment, while H89 pretreatment (C) diminished the staining observed with forskolin. Therefore, both immunoblotting and immunofluorescence confirmed the expression of MEKK3 in nuclear compartments.

The finding that MEKK3 was localized in the nucleus prompted experiments to re-examine the co-precipitation of MEKK3 and ERK under conditions known to modulate phosphorylation at Ser166 of MEKK3. NIH3T3 cells were utilized to examine whether endogenous ERK and MEKK3 associate in a stimulus dependent manner. Cytoplasmic and nuclear extracts were prepared as described earlier, MEKK3 was immunoprecipitated, and immunoblotting for ERK1 and ERK 2 was performed. ERK2 co-precipitated with MEKK3 under basal conditions in cytoplasmic (Figure 5.8, lane b) and nuclear (lane g) fractions. This co-precipitation was not altered by serum (lanes e and j) or forskolin (c and h) treatment. In addition, inhibition of ERK activity with PD98059 (lanes f and k) did not alter the association between MEKK3 and ERK.
**Figure 5.8.** Endogenous MEKK3 and ERK co-precipitate in NIH3T3 cells. Cytoplasmic (lanes b-f) and nuclear extracts (lanes g-k) were prepared from NIH3T3 cells after 48 h serum withdrawal and treatment conditions as follows: 50 μM forskolin for 15 min, 50 μM forskolin for 15 min after pre-treatment with 10 μM H89 for 1 h, 10% FBS for 10 min, 10% FBS for 15 min after 30 min pre-treatment with 50 μM PD98059. MEKK3 was immunoprecipitated using an amino-terminal MEKK3 antibody. Proteins were resolved by SDS-PAGE and immunoblotting performed for MEKK3 (Panel A) or ERK1 and ERK2 (Panel B). NIH lysate from 10% FBS treatment is shown in lane a, to indicate the position of MEKK3 and ERK1 and ERK2.
5.4 DISCUSSION

Although MEKK3 has been previously characterized as an activator of MAPK cascades through transient over-expression (8, 13), our studies are the first to demonstrate a physical interaction between a MAPK, ERK and MEKK3. Furthermore, this interaction was dependent upon the catalytic activity of ERK. The co-precipitation of ERK and MEKK3 also raises the question as to whether the interaction between these proteins is direct or modulated through another protein. Further studies are also required to elucidate whether the corresponding MAP2K, MEK1, is found in a complex with ERK and MEKK3. Interestingly, although over-expression of wild-type and point mutants of MEKK3 activated JNK, MEKK3 was not observed as a co-purifying protein after JNK immunoprecipitation and autoradiography. Thus, the interaction between a MAPK, and MEKK3 appears to be specific for ERK.

Immunofluorescence and immunoblotting demonstrated the nuclear localization of MEKK3. The studies are the first to describe the intracellular localization of MEKK3 and it suggests that MEKK3 specific substrates may be localized in the nucleus. ERK translocation to the nucleus is required for activity towards its substrates including the transcription factor, Elk. PKA is known to exert its effects on nuclear localized proteins including CREB (110). Therefore, the identification of MEKK3 in the nuclear compartment of the cell was consistent with the interaction with ERK and phosphorylation by PKA at Ser166 (chapter 4). However, mutation of MEKK3 serines
166 and 337 did not alter the activation of ERK which suggests that ERK alters MEKK3 activity towards its substrates.

14-3-3 proteins mediate the translocation of Cdc25 (109), Forkhead transcription factor (77), and telomerase (112) into different cellular compartments. The increased association of phospho-MEKK3 and 14-3-3 after MEKK3 serine 166 phosphorylation suggests that 14-3-3 moves MEKK3 into different cellular compartments or produces a conformational change for substrate binding. Future studies will address whether nuclear localization is correlated with a change in MEKK3 kinase activity and whether 14-3-3 proteins co-localize with MEKK3.

MEKK3 was found in nuclei by immunofluorescence and immunoblotting under basal conditions. However, the immunofluorescent labeling of nuclei was more pronounced upon stimulation with serum or forskolin which suggests a confirmational change in MEKK3 thereby making the MEKK3 peptide epitope more accessible to the antibody. Perhaps this change in protein folding is required to make MEKK3 more accessible to downstream substrates and promote 14-3-3 binding. Since MEKK3 substrates remain uncertain, the finding that MEKK3 is localized in the nuclear compartment implies that substrates might be nuclear proteins.
6.1 SUMMARY AND CONCLUSIONS

In this dissertation I have identified and characterized in vivo phosphorylation sites for the stress-activated protein kinase, MEKK3 at Ser166 and Ser337. The phosphorylation of MEKK3 on these residues matches the consensus site for AGC kinases. Phospho-specific antibodies for Ser166 and Ser337 were generated to characterize these phosphorylation sites. Through extensive studies with pharmacological inhibitors of AGC kinases, the PKA dependent phosphorylation at Ser166 was demonstrated. In addition, phosphorylation at Ser166 and Ser337 were characterized as independent of Akt, p70S6K, and p90RSK. While the phosphorylation of Ser166 is understood in part, the upstream kinase for Ser337 has not been identified.

The protein kinases MEKK2 and MEKK3 share 77% overall homology whereas their amino-terminal regulatory domains are only 65% homologous. The consensus AGC kinase sites are located within the amino-terminal domain of MEKK3 and are not found in the corresponding sequence for MEKK2. Thus, our studies suggest PKA dependent phosphorylation is a unique mechanism for MEKK3 regulation.

Besides the identification and characterization of in vivo phosphorylation sites for MEKK3, another goal was to identify proteins that interact with MEKK3. In the yeast two hybrid system 14-3-3, Bcl-xL, and a transcriptional repressor interacted with MEKK3. Further studies with Bcl-xL showed an interaction between MEKK3 and the
caspase-cleaved Bcl-xL whereas the full-length Bcl-xL did not interact. These results suggest a role for MEKK3 in apoptotic signaling pathways. This interaction was specific for MEKK3 as the closely related MEKK2 did not interact with caspase-cleaved Bcl-xL. However, the physiological significance of the interaction between MEKK3 and caspase-cleaved Bcl-xL remains unknown since Bcl-xL did not alter MEKK3 kinase activity or act as a substrate.

14-3-3 proteins co-purified with MEKK3 in both the yeast two-hybrid and baculovirus insect systems. However, the role of 14-3-3 in MEKK3 regulation remains elusive as serine-to-alanine point mutants of MEKK3 retained the ability to bind 14-3-3. Given the interaction between MEKK3 and 14-3-3, it is possible that 14-3-3 functions as a scaffolding protein for the interaction of MEKK3 and ERK. In addition, 14-3-3 proteins may be required to localize MEKK3 with its downstream substrates.

The activation of MAPKs by MEKK3 has been reported, however, the mechanism for this effect is undetermined. During the course of studies to identify MAPK pathways regulated serine-to-alanine point mutants of MEKK3, it was observed that MEKK3 co-precipitated with ERK. Our studies which demonstrated an interaction between ERK and MEKK3 may provide an explanation for the mechanism of ERK activation by MEKK3.

Our immunofluorescence and immunoblotting experiments established that MEKK3 is localized in the nuclear compartment of the cell. This localization suggests new roles for MEKK3 in cell growth and proliferation pathways controlled by ERK and PKA. MEKK3 downstream substrates remain uncertain; therefore, these findings
indicate that the nuclear fraction of the cell needs to be analyzed for MEKK3 substrates and other interacting proteins.
6.1 FUTURE STUDIES

This research was the first to identify an upstream kinase that phosphorylated the MAP3K, MEKK3 at Ser166 in a stimulus dependent manner. Although PKA-dependent phosphorylation was identified for Ser166, the kinase that regulates Ser337 has not been identified. Other AGC kinases remain to be tested as putative regulators of MEKK3 phosphorylation at Ser337.

In addition to PKA dependent phosphorylation of MEKK3, we demonstrated that TNF dependent phosphorylation of MEKK3 occurs at Ser166. TNF dependent activation of NF-κB and direct phosphorylation of IKK by MEKK3 has been reported (45). Although the NF-κB signaling pathway was evaluated with the MEKK3 point mutants through transient over-expression, the effect of TNF stimulation on the ability of endogenous MEKK3 to phosphorylate IKK and intracellular localization has not been examined.

The downstream effects of MEKK3 phosphorylation at Ser166 and Ser337 also remain uncertain. Although the MAPK pathways were evaluated with MEKK3 point mutants, no effects on either JNK or ERK were observed with transient over-expression. We demonstrated the nuclear localization of MEKK3, however, the effect of MEKK3 kinase activity towards the non-specific kinase substrate, MBP, has not been evaluated with immunoprecipitated MEKK3 from nuclear extracts after phosphorylation at Ser166 or Ser337.
Another remaining question is to identify autophosphorylation sites within MEKK3. Ser166 and Ser337 MEKK3 point mutants retained the ability to autophosphorylate. This suggests these sites are not required for autophosphorylation. With the advantage of newer proteomic techniques, future studies will address other phosphorylation sites within MEKK3 to elucidate the role for this protein kinase in signal transduction pathways.

MAPK signaling pathways have been of particular interest due to their modulation of apoptotic and cell survival pathways (113). The identification of protein kinases and their substrates presents an opportunity for pharmacological inhibitors in the treatment of diseases. In fact, the tyrosine kinase inhibitor drug, STI571, is now used for the treatment leukemia (114). With the completion of the human genome project it has been predicted that protein kinases comprise 6% of all genes (115). Consequently, further characterization of protein kinase signaling pathways will be essential for drug discovery and the treatment of diseases.
APPENDIX A

YEAST TWO-HYBRID MEDIA AND PLATES

10X Dropout Solution

1 mg/ml Arginine Monohydrochloride
1 mg/ml Cysteine Hydrochloride
1 mg/ml Threonine
0.5 mg/ml Aspartic Acid
0.5 mg/ml Isoleucine
0.5 mg/ml Methionine
0.5 mg/ml Phenylalanine
0.5 mg/ml Proline
0.5 mg/ml Serine
0.5 mg/ml Valine

Stock solutions

10 mg/ml Leucine
10 mg/ml Histidine
25 mg/ml Lysine Monohydrate

YC-THULL-IL

100 ml of 10X Dropout Solution
100 mg Adenine
50 mg Tyrosine
5 g Ammonium sulfate
1.2 g Yeast Nitrogen Base [without amino acids and (NH₄)₂SO₄]
6 g NaOH

Bring to 900 ml with H₂O, add 20 g Agar per liter (if preparing plates), autoclave, let cool and then add 100 ml of 20% glucose.

YC-UTL-IL

100 ml of 10X Dropout Solution
100 mg Adenine
50 mg Tyrosine
4 ml of 25 mg/ml Lysine
5 ml of 10 mg/ml Histidine
5 g Ammonium sulfate
1.2 g Yeast Nitrogen Base [without amino acids and (NH₄)₂SO₄]
6 g NaOH

Bring to 900 ml with H₂O, add 20 g Agar per liter (if preparing plates), autoclave, let cool and then add 100 ml of 20% glucose.

**VC-UT-1L**

100 ml of 10X Dropout Solution
100 mg Adenine
50 mg Tyrosine
4 ml of 25 mg/ml Lysine
5 ml of 10 mg/ml Histidine
10 ml of 10 mg/ml Leucine
5 g Ammonium sulfate
1.2 g Yeast Nitrogen Base [without amino acids and (NH₄)₂SO₄]
6 g NaOH

Bring to 900 ml with H₂O, add 20 g Agar per liter (if preparing plates), autoclave, let cool and then add 100 ml of 20% glucose.

**YPA-1L**

10 g Yeast extract
20 g Peptone
0.1 Adenine

Bring to 900 ml with H₂O, autoclave.

**YPAD-1L**

Prepare YPA as described above, add 100 ml of 20% glucose

**5X M9 Media**

30 g anhydrous Na₂HPO₄
15 g KH₂PO₄
5 g NH₄Cl
2.5 g NaCl
QS to 1 L and autoclave.

**M9 Plates**

783 ml H₂O
15 g Agar
Autoclave, then cool to 48-55°C and add:

200 ml 5X M9 media
1 ml 50 mg/ml ampicillin
4 ml 10 mg/ml L-proline
1 ml 1M thiamine-HCl (337 mg/ml)
10 ml 20% glucose
1 ml 1 M MgSO₄
APPENDIX B

DEAE-DEXTRAN TRANSFECTION SOLUTIONS

**Solution A**
8 g NaCl
0.38 g KCl
0.2 g Na$_2$HPO$_4$
3 g Tris base
Adjust pH to 7.4 and volume to 100 ml, filter sterilize prior to use.

**Solution B**
1.5 g CaCl$_2$$\cdot$2H$_2$O
1.0 g MgCl$_2$$\cdot$6H$_2$O
Adjust volume to 100 ml and filter sterilize prior to use.

**Solution C**
10 ml solution A
1 ml solution B
89 ml H$_2$O

**DEAE-dextran solution**
10 mg/ml DEAE-Dextran (MW 500,000) in solution C

**Chloroquine Disphosphate salt**
52 mg/10 ml solution C
Store at 4°C in the dark
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