NOTE TO USERS

New insights into MKK7 regulation in mammalian cells

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Identification of a regulated site in MKK7 activation loop

Abstract: Eukaryotic cells respond to external stimuli by activating mitogen-activated protein kinase (MAPK) pathways. MAPK signaling pathways include the ERK, p38 and JNK cascades. All MAPK family members are activated by the dual phosphorylation of a tripeptide motif located in their activation loop. JNK is phosphorylated and activated by two upstream MAPKKs, termed MKK4 and MKK7. These kinases are themselves activated through phosphorylation of serine/threonine residues in their activation loop by upstream MAPKKKs. MKK7 is thought to be activated primarily by cytokines while MKK4 is primarily activated by environmental stress. For my M.Sc. project, I have validated and characterized several phospho-MKK7 specific antibodies directed against MKK7 Ser271, Thr275 and Ser277, which are phospho-acceptor residues located in the MKK7 activation loop. By producing MKK7 mutant constructs in which each of the phospho-acceptor sites were substituted with Ala or Asp, I have demonstrated that we succeeded in generating phosphospecific antibodies against each of the phospho-acceptor sites. Of the three sites, only Thr275 is regulated by external stimuli whereas Ser271 and Ser277 appear to be constitutively phosphorylated. Both TNFα and sorbitol treatment induce phosphorylation of the Thr275 residue in MKK7 activation loop; surprisingly, our results show that osmotic stress activates MKK7 more strongly than TNFα. By using RNA interference and immunoprecipitation, we have demonstrated that in endogenously expressed MKK7, Ser271 and Ser277 are constitutively phosphorylated while Thr275 is stimuli-regulated. Together, these studies provide fundamental new insights into MKK7 regulation in mammalian cells.

Identification d'un site régulé dans la boucle d'activation de la protéine MKK7

Résumé : Les cellules eucaryotes répondent aux stimuli externes en activant les voies de signalisation impliquant les protéines kinases activées par des agents mitogènes (MAPK). Ces voies de signalisation incluent les kinases ERK, p38 et JNK. Tous les membres de la famille de protéines MAPK sont activés suite à la phosphorylation d'un motif tri-peptidique situé dans une région de leur structure appelée boucle d'activation. La protéine JNK est phosphorylée et activée par deux autres kinases situées en amont appelées MKK4 et MKK7. Ces dernières sont elles-mêmes activées via la phosphorylation des acides aminés sérine et thréonine dans leur boucle d'activation par d'autres kinases situées en amont (MAPKKK). Il a été rapporté que la protéine MKK7 est activée principalement par des cytokines alors que la protéine MKK4 est principalement activée par des stress environnementaux. Dans le cadre de mon projet de maîtrise, j'ai validé et caractérisé plusieurs anticorps spécifiques de la forme phosphorylée de MKK7. Les anticorps ont été générés de façon à reconnaître spécifiquement la protéine lorsqu'elle est phosphorylée à un des acides aminés suivants : sérine 271, thréonine 275 et sérine 277. Ces sites sont les accepteurs de phosphate situés dans la boucle d'activation de MKK7. En produisant des constructions mutantes dans lesquelles ces sites accepteurs de phosphate ont été substitués avec des alanines ou des acides aspartiques, j'ai pu démontré la validité et la spécificité des anticorps générés. De plus, j'ai pu démontré que parmi ces trois sites, seulement la thréonine 275 est régulée par des stimuli externes alors que la sérine 271 et la sérine 277 semblent être phosphorylées de façon constitutive. Le traitement des cellules avec le sorbitol (stress osmotique) ou avec le facteur alpha de nécrose des tumeurs (TNFα) induit la

phosphorylation de la thréonine 275 dans la boucle d'activation de MKK7. Cependant, à notre surprise, nos résultats démontrent que le stress osmotique active plus fortement MKK7 que le facteur alpha de nécrose des tumeurs (TNFα). De plus, en utilisant de l'ARN interférant spécifiquement avec la transcription de MKK7 , nous avons démontré que dans les protéines MKK7 endogènes, la sérine 271 et la sérine 277 sont phosphorylées de façon constitutive alors que la thréonine 275 est régulée. Ensemble, ces résultats constituent des découvertes fondamentales pour la compréhension de la régulation de la protéine MKK7 dans les cellules de mammifères.

Contribution of Authors

Chapter 2: The JNK signaling pathway

I. Montcalm, S., Forte S., Salehi AH., Barker, PA. (2004) **MKK7 is regulated through constitutive and stimuli-induced phosphorylation of activation loop amino acids.** (In preparation).

As the first author of the above indicated manuscript, I performed phospho-MKK7 antibodies characterization (anti-pSer271, anti-pThr275 and anti-pSer277) and have generated alanine and aspartic acid mutant forms of MKK7 (S271A, T275A, S277A, S271D, T275D, S277D). I performed transient transfections of wild-type MKK7, MKK4 and mutants forms of MKK7 in HEK293T cells followed by treatment of cells with either TNFα or sorbitol in order to assessed phosphorylation status of the three phospho-acceptor sites in MKK7 activation loop (Figure 2 to 5). As well, I performed in vitro transcription/translation followed by in vitro kinase assay of Wt-MKK7 and a kinase dead form of MKK7 (KD-MKK7) in order to determine if phosphorylation observed in MKK7 activation loop was due to MKK7 autophosphorylation (Figure 6). Furthermore, I treated 293T cells with sorbitol in order to verify the phosphorylation status of the three phospho-acceptor sites in activation loop of endogenous MKK7 (Figure 7 and 8).

List of Scientific Abbreviations

AIF	Apoptosis Inducing Factor
Ala	Alanine
AP-1	Activating Protein-1
Apaf-1	Apoptotic Protease Activating Factor-1
ASK1	Apoptosis Signal-regulating Kinase 1
Asp	Aspartic Acid
ATF-2	Activating Transcription Factor 2
ATP	Adenosine Triphosphate
BDNF	Brain-derived Neurotrophic Factor
BH	Bcl-2 Homology
cAMP	cyclic Adenosine Monophosphate
Cdk2	Cyclin-dependent Kinase 2
CNS	Central Nervous System
CRIB	Cdc42/Rac1 Interactive Binding
DD	Death Domain
DISC	Death-inducing Signaling Complex
DLK	Dual Leucine zipper Kinase
DNA	DeoxyriboNucleic Acid
DSPs	Dual Specificity Phosphatases
EGF	Epidermal Growth Factor
ERK	Extracellular signal Regulated Kinase
ES	Embryonic stem cells
FADD	Fas-associated Death Domain
FAP	Fas-associated Phosphatase
GCK	Germinal Center Kinase
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney
IAP	Inhibitor of Apoptosis
IL	Interleukin
JKAP	JNK pathway-Associated Phosphatase
JNK	c-Jun N-terminal Kinase
JSP-1	JNK Stimulatory Phosphatase-1
KCl	Potassium Chloride
kD	Kilodalton
LPA	Lysophosphatidic Acid
NF-κB	Nuclear factor-kappa B
MAGE	Melanoma Antigen
MAPK	Mitogen-activated protein Kinase
MAPKK (MKK)	Mitogen-activated protein Kinase (Kinase)
MAPKKK (MKKK)	Mitogen-activated protein Kinase Kinase (Kinase)
MAPKKKK (MKKKK)	Mitogen-activated protein Kinase Kinase Kinase (Kinase)
MEFs	Mouse Embryonic Fibroblasts
MEKK	MAPK/ERK Kinase Kinase
MKPs	MAPK phosphatases

MLK	Mixed Lineage Kinase
NADE	p75(NTR)-Associated cell Death Executor
NGF	Nerve Growth Factor
NogoR	Nogo Receptor
NRAGE	Neurotrophin Receptor-interacting MAGE Homolog
NRIF	Neurotrophin Receptor-interacting Factor
NT	Neurotrophin
p75NTR	p75 Neurotrophin Receptor
PC12	Pheochromocytoma Cell line 12
PI3-kinase	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PNTL	1,10-phenanthroline
POSH	Plenty of SH3s
PP2Cα	Protein phosphatase 2Cα
PTB	Phosphotyrosine Binding Domain
PTPase	Protein Tyrosine Phosphatase
RhoGDI	Rho GDP dissociation inhibitor
RIP	Receptor-interacting Protein
RNA	RiboNucleic Acid
SAPK	Stress-activated protein Kinase
SCG	Superior Cervical Ganglion
SDS/PAGE	Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis
Ser	Serine
siRNA	Small Interfering Ribonucleic Acid
SKRP1	SAPK pathway-regulating phosphatase 1
Smac/Diablo	Second Mitochondria-derived Activator of Caspase
Thr	Threonine
TNFα	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor Receptor
TPR	Tetratricopeptide Repeat Domain
TRADD	TNFR-associated Death Domain
TRAF	TNFR-associated Factor
Trk	Tropomyosin-related Kinase
Tyr	Tyrosine
UV	Ultraviolet
Wt	Wild Type

Chapter 1

Introduction and Literature Review

Introduction

Cells respond to extracellular stimuli by initiating specific intracellular events, such as the signaling cascade that leads to activation of the mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases. All eukaryotic cells can activate multiple MAPK pathways, which coordinately regulate diverse cellular activities including gene expression, mitosis, differentiation, motility, survival and apoptosis.

The three major distinct MAPK signaling pathways identified in eukaryotic cells include the extracellular signal-regulated kinase (ERKs), the p38 kinases and the c-Jun NH2-terminal kinases (JNKs). MAPKs can be activated by a wide variety of different stimuli, but in general, ERKs are preferentially activated in response to growth factors and phorbol esters, while the JNKs and p38 are more responsive to cellular stress such as ionizing irradiation, heat shock, osmotic imbalance, DNA damage, bacterial product lipopolysaccharide and inflammatory cytokines (Cuenda, 2000;Pearson *et al.*, 2001). Thus, the JNK and the p38 pathways are also known as Stress Activated Protein Kinase (SAPK) pathways. Generally, activation of the ERK forms of MAPK causes survival responses, whereas activation of the SAPKs promotes cell death.

Although each MAPK has unique characteristics, a number of features are shared by the MAPK pathways studied to date. Each MAPK pathway is composed of a set of three evolutionarily conserved, from yeast to humans, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and the activated MAPKK in turn phosphorylates and activates MAPK.

The JNK family of MAPK has been reported to be involved in many forms of neuronal death. Indeed, JNKs have been shown to be involved in glutamate excitotoxicity, NGF withdrawal and p75NTR-induced neuronal apoptosis (Xia *et al*, 1995;Yang *et al*, 1997b; Aloyz *et al*, 1998;Bhakar *et al*, 2003;Kuan *et al*, 2003;). Thus, it appears that the JNK pathway could be a potential target to treat neurodegenerative diseases. However, biological tools needed to understand the regulation of the JNK pathway have been missing. This thesis will discuss the research conducted during my Master's studies in order to design, produce and validate new biological tools necessary to further characterize the JNK pathway. My research mainly focused on the dual-specificity kinase, MKK7, an upstream activator of JNK.

Neurotrophins and Neurotrophins receptors: An overview

The neurotrophins are a family of growth factors that include nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and brain-derived neurotrophic factor (BDNF). These factors are involved in many cell processes such as regulation of survival, differentiation, synaptic activity, axonal and dendritic outgrowth, and apoptosis. The neurotrophins exert their roles by activating two distinct types of cell-surface receptors, the Trk receptors (Trks) which are tyrosine kinase receptors that comprise TrkA, TrkB and TrkC, and p75 receptor (p75NTR), a member of the tumor necrosis factor receptor family (Carter and Lewin, 1997;Kaplan and Miller, 2000;Lee *et al*., 2001;Lewin and Barde, 1996). The Trks are undisputed survival-promoting receptors and are involved in regulation of neuronal growth. The Trks are bound and activated by specific neurotrophins, with TrkA preferentially binding NGF, TrkB preferring BDNF

and NT-4/5, and TrkC interacting with NT-3 (Patapoutian and Reichardt, 2001;Yoon *et al.*, 1998). On the other hand, the role of p75 still remains controversial, in part because it is associated with the promotion of both apoptosis and survival, it binds all neurotrophins with approximately equal affinity (Barker, 1998;Barrett, 2000;Yoon *et al.*, 1998) and it can act alone and in concert with TrkA.

One consistent piece of evidence emerging in the literature is that p75 can induce apoptosis when activated by a neurotrophin (Barrett, 2000;Dechant and Barde, 1997;Kaplan and Miller, 2000). However, when coexpressed with Trk, p75NTR behaves as a Trk co-receptor that enhances or alleviates Trk responses to the various neurotrophins. p75 has been shown to mediate survival by activating NF-κB (Bhakar *et al.*, 1999;Carter *et al.*, 1996), PI3-kinase and Akt (DeFreitas *et al.*, 2001;Descamps *et al.*, 2001;Roux *et al.*, 2001). p75 has also been shown to activate c-jun N-terminal kinase (JNK) (Casaccia-Bonnefil *et al.*, 1996;Yoon *et al.*, 1998), and Caspases (Gu *et al.*, 1999) to mediate apoptosis.

Extrinsic vs Intrinsic Death Pathways

Cellular apoptotic mechanisms occur in two main pathways. In the extrinsic pathway, apoptosis is induced by pro-apoptotic receptors such as TNF receptor superfamily. These receptors assemble a death-inducing signaling complex (DISC) in which TRADD or FADD binds directly to the receptor's death domain (DD), thereby allowing activation and aggregation of Caspase 8 (initiator caspase) and this leads to subsequent activation of the Caspase cascade including Caspases 3, -6, and –7 (Baud and Karin, 2001;Denecker *et al.*, 2001;Strasser *et al.*, 2000). In the intrinsic pathway, pro-

apoptotic « BH3-domain only » proteins, members of the Bcl-2 family are activated and allow Bax and Bak to reduce mitochondrial integrity, causing Cytochrome c, Smac/Diablo and AIF to leave the mitochondria and accumulate in the cytoplasm (Gross *et al.*, 1999;Harris and Johnson, Jr., 2001;Joza *et al.*, 2002;Verhagen *et al.*, 2000). Cytochrome c in association with Apaf-1 facilitates Caspase 9 activation and thereby induces a Caspase cascade that results in cell death (Desagher and Martinou, 2000;Gottlieb, 2000;Kroemer and Reed, 2000;Matsuyama and Reed, 2000).

These pathways are not mutually exclusive because Caspase 8 activation leads to the cleavage and activation of Bid, a « BH3-domain only » protein, which is a member of the Bcl-2 family that results in activation of the mitochondrial death pathway (Joza *et al.*, 2002). Moreover, recent studies have shown that JNK activation by the cytokine TNFα causes Caspase-8-independent processing of Bid into jBid, which then targets mitochondria to trigger selective release of the apoptogenic factor Smac/Diablo. In the cytosol, this factor then binds to and antagonizes IAP1, thereby relieving Caspase-8 from the inhibitory effects of the TRAF2-IAP1 complex (Deng *et al.*, 2003;Wajant *et al.*, 2003).

p75NTR: Atypical member of the TNF receptor family

The p75NTR apoptotic cascade is distinct from that induced by other pro-apoptotic TNF receptor superfamily members. p75NTR is an atypical member of the TNF receptor family due to its propensity to dimerize rather than trimerize. Also, p75 acts as a tyrosine kinase co-receptor and the neurotrophins bound to it are structurally unrelated to the TNF ligands which typically bind TNFR family members. Indeed,

p75NTR binds dimeric and soluble ligands rather than trimeric and membrane-bound ligands. Also, contrary to other TNF receptors family, p75NTR does not bind FADD (P.Barker, unpublished results;Wang *et al.*, 2001). Moreover, Caspase 8 induction does not appear to be involved in p75NTR-mediated apoptosis, but Caspase 9 is activated during p75NTR-mediated killing (P.Barker, unpublished results;Wang *et al.*, 2001;Gu *et al.*, 1999). Taken together, these data are consistent with the hypothesis that the signaling pathways responsible for p75NTR-dependent apoptosis involve DD effectors that result in preferential activation of the mitochondrial (intrinsic) death pathway.

p75NTR interacting proteins

The precise physiological functions of p75NTR are still being defined. Because p75NTR has no intrinsic enzymatic activity, this has led to a search to identify proteins that interact with the receptor's cytosolic domain and mediates its signaling effects. Yeast two hybrid technology has resulted in the identification of several p75NTR interacting proteins that include NRIF1 and NRIF2 (Benzel *et al.*, 2001;Casademunt *et al.*, 1999;Chittka and Chao, 1999), SC-1 (Chittka and Chao, 1999), NADE protein (Mukai *et al.*, 2000), TRAF proteins (Khursigara *et al.*, 1999;Krajewska *et al.*, 1998;Wajant *et al.*, 2001), RIP-2 (Khursigara *et al.*, 2001), FAP PTPase (Irie *et al.*, 1999), RhoA GTPase (Yamashita *et al.*, 1999), and caveolin (Bilderback *et al.*, 1997). Recently, Nogo Receptor (NogoR) and Rho GDP dissociation inhibitor (RhoGDI) have been reported to interact with p75NTR (Wang *et al.*, 2002;Yamashita and Tohyama, 2003).

To date, direct p75NTR interactors have not been linked to specific apoptotic cascades. However, it was demonstrated by our group that NRAGE, a novel member of

the MAGE family, binds to p75NTR under physiological conditions and facilitates p75NTR-dependent cell death (Salehi *et al.*, 2000). The results of additional studies indicated that NRAGE is a potent apoptotic inducer that activates a mitochondrial death pathway involving Cytochrome c release and activation of Caspase-9, Caspase-7 and Caspase-3 through a JNK- and c-Jun-dependent pathway (Salehi *et al.*, 2002).

Despite the identified interacting proteins, the precise signaling events that link p75NTR activation to apoptotic cascades remain uncertain. Several findings suggest that activation of the JNK pathway may play a key role.

NGF withdrawal studies

Pioneering studies from Eugene Johnson's group performed in the late 1980's showed that apoptosis resulting from NGF withdrawal required both transcription and translation (Martin *et al.*, 1988), and more recent studies have shown that the c-Jun amino-terminal kinase (JNK) cascade and the c-Jun mediated transcription play a crucial role in mitochondrial Cytochrome c release, Caspase 9 activation and apoptosis of NGF-deprived sympathetic neurons (Bruckner *et al.*, 2001;Deshmukh *et al.*, 1996;Deshmukh and Johnson, Jr., 1998;Eilers *et al.*, 2001;Harding *et al.*, 2001;Martinou *et al.*, 1999;Putcha *et al.*, 1999). In favor with this model, a recent study has shown that JNK is required for UV-induced activation of the Cytochrome c-mediated death pathway in primary murine embryonic fibroblasts (Tournier *et al.*, 2000). The specific signaling events that lead to activation of the JNK pathway after neurotrophin withdrawal remain uncertain but some signaling events are starting to be elucidated.

In the early signaling events, it seems that apoptosis following NGF withdrawal requires activity of the small GTP-binding proteins Cdc42 and Rac1 and also the activation of the c-jun N-terminal kinase (JNK) pathway (Mota *et al.*, 2001). In fact, it has been shown that following NGF withdrawal, the small GTPases Cdc42 and Rac1 become activated (Bazenet *et al.*, 1998), and bind the Cdc42/Rac1 interactive binding (CRIB) motif of MAPKKKs such as GCK, MEKK1-4 and MLK2-3 (Hirai *et al.*, 1996;Rana *et al.*, 1996;Sakuma *et al.*, 1997;Tibbles *et al.*, 1996). Activated MAPKKK(s) then phosphorylate and activate MKK4/7 (MAPKK family members) (Xu *et al.*, 2001), which phosphorylate and activate specific JNK isoforms (Bruckner *et al.*, 2001;Sakuma *et al.*, 1997). Once phosphorylated, JNKs target AP-1 transcription factors such as c-jun, ATF-2 and Elk-1 which induce transcription (Bruckner *et al.*, 2001). The precise targets of c-Jun for the induction of apoptosis have been the subject of intense interest and recently, « BH3-domain only » family members, Bim and Dp5, have been identified as pro-apoptotic genes induced in a c-Jun-dependent manner in both sympathetic neurons subjected to NGF withdrawal and in cerebellar granule cells deprived of KCl (Harris and Johnson, Jr., 2001;Whitfield *et al.*, 2001).

Many studies are consistent with this model of apoptosis. First, Xu *et al.* (2001) have shown that members of the mixed-lineage kinase (MLK) family (including MLK1, MLK2, MLK3, and dual leucine zipper kinase (DLK)) are expressed in neuronal cells and are likely to act between Rac1/Cdc42 and MKK4/7 in death signaling. The mixed-lineage kinases (MLK) belong to a family of mitogen-activated protein (MAP) kinase kinase kinases. In this study, Xu *et al.* (2001) have shown that overexpression of MLKs effectively induces apoptotic death of cultured neuronal PC12 cells and sympathetic

neurons, while expression of dominant-negative forms of MLKs suppresses death evoked by NGF deprivation or by expression of activated forms of Rac1 and Cdc42. CEP-1347, a chemical compound that inhibits the activity of MLKs, is able to block neuronal death caused by NGF deprivation, and also protects PC12 cells from death induced by overexpression of MLK family members. Dominant-negative forms of MKK4 and –7 and c-Jun also protect against death induced by MLK overexpression, placing MLKs upstream of these kinases.

Mota *et al.* (2001) have studied the role of MLK3 in the induction of apoptosis in sympathetic neurons. They have shown that overexpression of an active form of MLK3 induces activation of the JNK pathway and apoptosis in SCG neurons. In addition, it has been shown that overexpression of kinase dead mutants of MLK3 blocks apoptosis as well as c-Jun phosphorylation induced by NGF deprivation. In their experiment, MLK3 activity was increased 5 hr after NGF withdrawal in both differentiated PC12 cells and SCG neurons, and revealed that MLK3 lies downstream of Cdc42 in the neuronal death pathway. These results suggest that MLK3, or a closely related kinase, is a physiological element of NGF withdrawal-induced activation of the Cdc42-c-Jun pathway and neuronal death.

Some other studies have demonstrated that overexpression of constitutively active forms of Rac1 and Cdc42 (Rac1 V12 [mutated at position 12 to V] and Cdc42 V12) leads to activation of the JNK pathway and to death of Jurkat T lymphocytes, PC12 cells, and sympathetic neurons and that overexpression of dominant-negative mutants of Cdc42 and Rac1 (Cdc42 N17 and Rac N17) in sympathetic neurons prevents elevation of c-Jun and death normally evoked by NGF withdrawal (Bazenet *et al.*, 1998;Chuang *et al.*, 1997).

Moreover, overexpression of Rac1N17 reverses the induction of death by Cdc42 V12, whereas Cdc42 N17 has no effect on Rac1 V12-induced death, suggesting that Cdc42 lies upstream of Rac1 in this pathway (Bazenet *et al.*, 1998).

p75NTR might facilitate NGF withdrawal death

Studies have suggested that p75NTR can facilitate neuronal apoptosis resulting from neurotrophin withdrawal. Indeed, a significant delay in death following NGF-withdrawal was observed in sympathetic neurons derived from $p75NTR^{ExonIII-/-}$ mice when compared to wild-type mice (Bamji *et al.*, 1998). Also, sensory neurons and differentiated PC12 cells become less sensitive to NGF withdrawal-mediated death when p75NTR expression is reduced (Barret and Georgiou, 1996; Barrett and Bartlett, 1994).

In favor of this hypothesis, many signaling effectors activated in p75NTR-mediated death are identical to those activated in response to NGF deprivation. Indeed, Harrington *et al.* (2002) have reported that p75NTR activates Rac GTPase and JNK in an NGF-dependent manner by comparing wild-type and $p75^{-/-}$ oligodendrocytes. They have also shown that N17Rac (dominant-negative form) blocks this JNK activation and subsequent NGF-dependent apoptosis, indicating that activation of Rac GTPase is required for JNK activation and apoptosis induced by p75NTR. They have also showed that p75NTR-mediated Rac activation is modulated by coactivation of Trk. Together these data identify Rac GTPase as one of the key molecules whose activity is critical for cell survival and death in neurotrophin signaling.

Our group has generated adenoviral constructs encoding p75NTR and we have shown that overexpression of p75NTR in PC12 cells induces apoptosis by releasing

Cytochrome c and by activating Caspases 3 and 9 in a JNK-dependent manner (Bhakar *et al.*, 2003). Moreover, p75NTR-dependent apoptosis correlates with an increase in c-jun N-terminal kinase (JNK) activity (Bamji *et al.*, 1998;Casaccia-Bonnefil *et al.*, 1996;Friedman, 2000;Roux *et al.*, 2001), and blockade of the JNK pathway with chemical inhibitors (Friedman, 2000;Yoon *et al.*, 1998) or dominant-negative forms of JNK (Harrington *et al.*, 2002) attenuates p75NTR-dependent death in oligodendrocytes and hippocampal neurons. Our group has also shown that p75-induced activation of JNK leads to oligomerization of the BH3-only protein Bad by post-translational modification (Bhakar *et al.*, 2003). The phosphorylation of Bad by JNK leads to Bax and Bak translocation at the level of the mitochondria and contributes to initiation of apoptosis.

JNK in glutamate neurotoxicity

As described earlier, JNK signaling appears to be an important contributor to NGF-withdrawal and p75NTR-induced apoptosis. However, it is unclear whether JNK signaling has a role in glutamate neurotoxicity. Glutamate is considered as the principle excitatory amino acid in the CNS. It has been shown that sustained exposure to glutamate can destroy retinal neurons (Lucas and Newhouse, 1957). In a subsequent set of pioneering experiments it was established that this toxicity, which was later called excitotoxicity, was not unique to glutamate or to retinal neurons, but was a feature common to the actions of all excitatory amino acids on central neurons (Olney and Sharpe, 1969;Olney *et al.*, 1971). Reinforcing evidences indicate that glutamate neurotoxicity may participate in the pathogenesis of different types of acute injury to the CNS: injuries due to prolonged seizures, compromised blood supply (ischemia),

compromised oxygen supply (hypoxia), glucose deprivation and mechanical trauma (Choi, 1988). Hypoxia-ischemia can occur either globally (cardiac arrest) or focally when a cerebral artery becomes abruptly occluded (stroke). These types of acute injury produce neuronal depolarization, increase synaptic glutamate release, decrease cellular glutamate uptake and cause a net build-up of extracellular glutamate (Hansen, 1985; Hirsh and Gibson, 1984; Drejer *et al.*, 1985; Benveniste *et al.*, 1984). Glutamate then binds to glutamate receptors on post-synaptic neurons and leads to membrane depolarization and opening of the calcium channels which results in an increase in free calcium. Sustained excitotoxic signal often leads to apoptosis and recents studies indicate that glutamate toxicity may involve JNK (Coyle and Puttfarcken, 1993;Schwarzschild *et al.*, 1997).

In accordance with this hypothesis, a recent study has reported that disruption of the gene encoding JNK3 in mice caused the mice to be resistant to the excitotoxic glutamate-receptor agonist kainic acid. Reduction in seizure activity and prevention of hippocampal neuron apoptosis was observed in these mice (Yang *et al.*, 1997b). Moreover, another study has shown that targeted deletion of JNK3 protects mice from brain injury after cerebral ischemia-hypoxia but also reduces the stress-induced JNK activity (Kuan *et al.*, 2003). These results suggest that JNK3 is a potential target for neuroprotection therapies in glutamate-induced neurotoxicity.

Prelude to Chapter 2

Studies on NGF withdrawal and p75NTR-mediated apoptosis have allowed identification of players that contribute to this cellular mechanism. The evidence indicates that the JNK signaling pathway plays a key role since it has been shown to be involved in both death paradigms. Also, as previously described, the JNK signaling pathway seems to be a major contributor to apoptosis induced by glutamate excitotoxicity. Thus, it appears that the JNK pathway could be a potential target to control apoptosis and cure neurodegenerative diseases. However, despite the fact that significant progress has been made in understanding the signaling cascades that lead to JNK activation, key aspects of this pathway remain elusive, specifically the mechanism by which the upstream activators of JNK, MKK4 and MKK7 are activated by phosphorylation.

In order to further characterize the JNK signaling pathway, I sought to determine the molecular mechanisms by which the JNK activators are regulated, with a main focus on MKK7. These investigations which formed the basis of my Master's research are discussed in Chapter 2.

Chapter 2

The JNK signaling pathway

An introduction to the JNK Protein Family

As previously mentioned, the c-Jun N-terminal protein kinases (JNKs) are an evolutionary conserved family of serine/threonine protein kinases. Together with the p38 kinases and extracellular signal-regulated kinases (ERKs), they form the mitogen-activated protein kinases family (Mielke and Herdegen, 2000). The events leading to identification and cloning of JNKs began in 1990 when a mammalian stress-activated protein kinase (SAPK) was discovered after intraperitoneal injection of cycloheximide (a protein synthesis inhibitor) (Kyriakis and Avruch, 1990). In this study, a 54 kDa protein kinase was activated and showed activity toward microtubule-associated protein-2. In parallel studies, identification of activated c-Jun protein kinases following exposure of cells to UV-radiation was demonstrated by affinity purification using the N-terminal transactivation domain of c-Jun. These proteins kinases were 46 and 55 kDa and termed JNKs (Hibi *et al.*, 1993). It was only in 1994 that JNKs and SAPKs were found to be identical (Derijard *et al.*, 1994).

JNKs are encoded by three different genes jnk1, jnk2, and jnk3. Two of these genes, jnk1 and jnk2, are ubiquitously expressed, whereas jnk3 expression is restricted to the brain, testis and heart (Yang *et al.*, 1997b). The transcripts of all three genes (JNK1, JNK2 and JNK3) are alternatively spliced to give rise to a total of ten JNK isoforms. This splicing yields proteins of 46 and 55 kDa, which represent products with or without a C-terminal extension.

The biological functions of JNK MAPKs

There are two categories of studies used to determine JNK MAPK function. The first one is a biochemical approach that focused on the identification of the JNK-specific substrates and the second is to evaluate JNK function involving the alteration in expression of these proteins in mice or Drosophila.

Many substrates are phosphorylated by the JNKs, including the nuclear transcription factors c-Jun, ATF2, Elk-1 and NFAT4 (Gupta *et al.*, 1996). Nuclear translocation of JNKs has been shown to be necessary for JNKs to associate with those substrates (Cavigelli *et al.*, 1995). Even though no functional differences between the splicing variants have been reported, it was shown that the JNK proteins interact differently with ATF-2, Elk-1 and c-Jun (Gupta *et al.*, 1996). ATF-2 heterodimerizes with c-Jun and stimulates expression of the c-Jun gene (Cruzalegui *et al.*, 1999). Elk-1 is involved in the induction of the c-fos gene, whose product forms the AP-1 heterodimer with c-Jun (Treisman, 1995). AP-1 is composed of dimeric complexes formed by members of the Jun and Fos groups of transcription factors. The AP-1 transcription factor mediates immediate-early gene expression in response to the exposure of cells to extracellular stimuli. It has been demonstrated that AP-1 transcription factors are involved in both induction and prevention of apoptosis (Shaulian and Karin, 2002). Evidence for pro-apoptotic functions comes from experiments showing that overexpression of c-Jun or c-Fos induces apoptosis in several cell lines (Bossy-Wetzel *et al.*, 1997;Preston *et al.*, 1996). It has also been shown that the product of the gene encoding FasL promotes apoptosis and that induction of this gene is AP-1 dependent. On the other hand, some experiments suggest that c-Jun protects cells against death after

treatment with UV radiation and this activity is due to suppression of transcription of Fas. Another experiment using hepatocytes derived from c-jun -/- mice that showed massive apoptosis provides another example of anti-apoptotic activity of c-Jun (Hilberg *et al.*, 1993). Also, a recent study has shown that JNK activation of JunD mediates survival signaling by collaborating with NF-κB to increase anti-apoptotic gene expression (Lamb *et al.*, 2003). Finally, a model has been suggested in which induction of AP-1 by JNK results in activation of various genes, such as FasL, Bim, or Bcl3, whose products are either negative or positive regulators of apoptosis (Shaulian and Karin, 2002). Thus, it is the balance between pro-apoptotic and anti-apoptotic target genes that will lead to cell survival or cell death.

Apart from transcription factors, JNK has also been shown to phosphorylate several cytoplasmic substrates. Some of these substrates are important for the physiological function and apoptotic action of JNKs in the nervous system. First, JNKs can phosphorylate tau protein (Reynolds *et al.*, 1997). This suggests that JNKs may be involved in Alzheimers disease. JNKs can also phosphorylate neurofilament heavy chain and the glucocorticoid receptor, resulting in inhibition of transcriptional activation of the receptor (Rogatsky *et al.*, 1998; Giasson *et al.*, 1997). It has also been shown that the JNK signalling pathway activates the tumor suppressor p53 (Fuchs *et al.*, 1998). This pro-apoptotic transcription factor enhances Bax induction by suppressing the action of Bcl-2 proteins. JNKs are also shown to directly antagonize the function of the anti-apoptotic proteins Bcl-2 via a phosphorylation mechanism. However, the site of the JNK-induced phosphorylation of Bcl-2 has also been suggested to be required for its prosurvival function. More specifically, the exact targets of JNK involve in the release of

Cytochrome c from the mitochondria in the intrinsic pathway of apoptosis is not yet defined, but some studies are beginning to provide data that will elucidate this question. One study has demonstrated that following DNA damage, JNK translocates to the mitochondria where it associates and phosphorylates the anti-apoptotic proteins Bcl-Xl (Kharbanda *et al.*, 2000). This phosphorylation enhances Cytochrome c release by suppressing the inhibition of Bcl-Xl proteins on pro-apoptotic proteins of the Bcl-2 family such as Bax. Another recent study has shown that JNK induces the phosphorylation of the BH3-only protein Bad at serine 128. This phosphorylation enhances the apoptotic effect of Bad in primary neurons by affecting the ability of growth factors to inhibit Bad-mediated apoptosis (Donovan *et al.*, 2002). Moreover, our group has recently shown that apoptosis induced by p75NTR overexpression requires JNK-dependent phosphorylation of Bad at serine 128 (Bhakar *et al.*, 2003). However, it has been recently reported that JNK can phosphorylate Bad at threonine 201 to give an anti-apoptotic response in hepatocytes (Yu *et al.*, 2004). Finally, another study has shown that activation of p75NTR induces the JNK-dependent phosphorylation of endogenous BimEL, another BH3-only protein, at serine 65 and contributes to apoptosis (Becker *et al.*, 2004).

Alteration of expression of the JNKs proteins also gives clues about their biological functions. The general results of these experiments support a role of the JNK MAPKs as positive mediators in cell death and their roles in neuronal development. One showed an increase resistance to UV-induced apoptosis in lung carcinoma cell line using a dominant-negative JNK1 mutant (Butterfield *et al.*, 1997). Knockout studies have provided important information on the functions of JNK isoforms, particularly during

development. It was shown that deletion of each individual isoform of JNK has no detrimental effect on the animals. Double knockouts of both JNK1 and JNK3 or JNK2 and JNK3 were also normal. However, JNK1 and JNK2 knockout mice were embryonic lethal and showed abnormal hindbrain development. It was shown that JNK1 and JNK2 are required for regional specific apoptosis during early brain development (Barr and Bogoyevitch, 2001;Kuan *et al.*, 1999). Altered apoptosis and defective neural tube morphogenesis in the absence of both JNK1 and JNK2 was also reported (Sabapathy *et al.*, 1999). These results also suggest that JNK1 and JNK2 are able to functionally complement each other. Finally, mouse embryonic fibroblasts (MEFs) isolated from JNK1 and JNK2 null mice have been shown to be resistant to UV-induced apoptosis due to a failure to activate the mitochondrial-dependent death pathway (Tournier *et al.*, 2000). This study provides another evidence for a pro-apoptotic function of JNK.

Protein Kinase Structure

Protein kinases contain a highly conserved kinase domain. These range from 250 to 300 amino acid residues, corresponding to about 30 kD. In 1988, comparison of the primary structures of 65 protein kinases was performed using alignment of their catalytic domains and clearly demonstrated their overall similarity (Hanks *et al*, 1988). They were not conserved uniformly but, rather, consisted of alternating regions of high and low conservation. Eleven major conserved subdomains (I to XI) were identified.

Because the eukaryotic protein kinases share a conserved catalytic core, crystallographic structure of one kinase provides insights into the entire family. In 1991, the crystal structure of cAMP-dependent protein kinase (PKA) was elucidated by X-ray

diffraction methods and enabled new understanding of the molecular basis for the functioning of a protein kinase (Knighton *et al*, 1991a; Knighton *et al*, 1991b; Taylor *et al*, 1992). Two years later, two additional protein kinase structures, a cell cycle dependent protein kinase (Cdk2) and the mitogen-activated protein kinase ERK2, were solved (De Bondt *et al*, 1993; Zhang *et al*, 1994). Crystal structure of JNK3 has also been recently reported (Xie *et al*., 1998;Scapin *et al*., 2003). Structural comparison between these kinases reveals features that are common to the protein kinase family and that are enzyme-specific (Taylor and Radzio-Andzelm, 1994). Most importantly, this study validated the initial prediction that all protein kinases would have the same general folding of the catalytic core.

Crystal structures studies uncovered the general structure that protein kinases adopt. Kinases are separated into two lobes. The N lobe is a non-catalytic region and the smaller of the two lobes. It is composed of a five-stranded β sheet (subdomains I-IV) and one predominent α helix, called helix αC. The other lobe, called the C lobe is larger and is predominantly helical (subdomains VIA-XI). ATP, which is necessary for providing is γ-phosphate during the phosphorylation of the substrate by the kinase, is bound in a deep cleft between the two lobes. It has been reported that a conserved glycine-rich sequence (GXGXXGXV) found in the first two β strands, as well as an invariant lysine in the third β strand contribute in many ways to protein kinase function mainly by interacting with ATP (Bossemeyer, 1994; Bossemeyer, 1995). Indeed, it has been proposed that the invariant lysine contributes to the correct stereochemical orientation of the triphosphate and the γ-phosphate for catalysis and thus, protein kinases are very sensisitve to mutations of this lysine (Vetrie *et al*, 1993; Bossemeyer, 1993). Another important

characteristic of the kinase structure is a region of the protein called the activation loop. The activation loop is a flexible surface loop and is typically 20-30 residues in length. This loop is found between subdomains VII and VIII (Hanks *et al*, 1988; Cobb and Goldsmith, 1995;Zhang *et al.*, 1995). In most kinases, this loop is phosphorylated when the kinase is active. It was shown that phosphorylation of the activation loop stabilizes it in an open and extended conformation to allow substrate binding (Huse and Kuriyan, 2002). Data from several X-ray structures suggest that the non-phosphorylated activation loop limits access of substrates to the active site (Jeffrey *et al*, 1995; Hubbard, 1997). Indeed, these studies suggest that substrates either cannot gain access or have very limited access to the active site in the dephosphorylated enzyme. Thus, the activation loop serves as a competitor for the substrate when the enzyme is repressed. It should be noted that although many protein kinases are phosphorylated in the activation loop, some are not. In those kinases, a negatively charged residue is substituted for the phosphoamino acid (Johnson *et al*, 1996).

MKK4 and MKK7: Upstream activators of JNK

All MAPK family members are activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) located in their activation loop (Dérijard *et al.*, 1994;Nakielny *et al.*, 1992;Kyriakis *et al.*, 1991;Cobb and Goldsmith, 1995;Canagarajah *et al.*, 1997). This reaction is mediated by dual specificity MAPK kinases (MKKs). The defining motif of all ten JNK isoforms is the amino acid sequence Thr-Pro-Tyr (TPY) (Davis, 2000; Barr and Bogoyevitch, 2001). However, for other MAPK family members, the defining motif is distinct. Indeed, the dual phoshorylation motif for p38 MAPKs is Thr-Gly-Tyr (TGY)

and it is Thr-Glu-Tyr (TEY) for ERKs. The immediate upstream activators of JNK are MKK4 and MKK7. MKK4 was first identified and cloned in 1995 (Lin *et al.*, 1995) while MKK7 was identified in 1997 by five different groups (Tournier *et al.*, 1997;Wu *et al.*, 1997;Lu *et al.*, 1997;Yao *et al.*,1997;Moriguchi *et al.*, 1997).

MKK4 mRNA is widely expressed in murine and human tissues, but is most abundant in brain and skeletal muscle (Sanchez *et al.*, 1994). The distribution and expression of MKK4 during embryonic development and in adult mice has been studied by in situ hybridization and immunohistochemical staining (Carboni *et al.*, 1997). It was shown that mouse MKK4 expression is detected preferentially in most of the central nervous system, in liver and in thymus during early stages of development. MKK4 expression remains at a constant level in the adult brain, but expression in fetal liver and thymus gradually decreases as embryogenesis proceeds. Three MKK4 protein kinase isoforms of about 399 amino acids, with distinct N-terminal regions, have been identified (Davis, 2000).

The mRNA for MKK7 is widely expressed in humans and mice and encodes a 47-kDa protein (419 amino acids), as determined by immunoblotting endogenous MKK7 with an antibody raised against its N terminus (Foltz *et al.*, 1998). Another group has demonstrated that six isoforms of MKK7 are created by alternative splicing of the gene to generate a group of protein kinases with three different N termini (α, β, and γ isoforms) and two different COOH termini (1 and 2 isoforms) (Tournier *et al.*, 1999). It was shown that this N-terminal extension binds directly to the MKK7 substrate, JNK.

MKK4 and MKK7 are dual-specificity kinases because they are each capable of phosphorylating the threonine and tyrosine residues in the TPY motif of JNK. However,

some studies indicate that JNK is activated synergistically by MKK4 and MKK7, with MKK4 phosphorylating preferentially the tyrosine residue (Sanchez *et al.*, 1994) and MKK7 then phosphorylating the threonine residue. Indeed, recent studies have shown that MKK4 preferentially phosphorylates the tyrosine residue and MKK7 the threonine residue of JNK in vitro (Lawler *et al.*, 1998;Fleming *et al.*, 2000). Also, strong support for this activation mechanism has been obtained in vivo from studies of MKK4- and MKK7-gene disruption in ES cells (Wada *et al.*, 2001;Kishimoto *et al.*, 2003). These studies indicate that the tyrosine and threonine residues of JNK are sequentially phosphorylated by MKK4 and MKK7, respectively, in living cells. It was also reported that this activation mechanism of JNK occurs in an all-or-none manner and that the full activation of JNK requires the phosphorylation of both Thr and Tyr (Fleming *et al.*, 2000;Bagowski and Ferrel, 2001;Bagowsky *et al.*, 2003). It is interesting to note that despite the general agreement of this activation mechanism, one study had suggested that MKK7 phosphorylated the threonine first followed by MKK4 phosphorylation of the tyrosine residue in the TPY motif of JNK (Lisnock *et al.*, 2000).

It was previously believed that each of the MAP kinase kinases functions as an activator of a single group of MAP kinases, but it was subsequently shown that MKK4 can phosphorylate and activate both JNK and p38 MAP kinases. On the other hand, it was shown that MKK7 activates and phosphorylates JNK, but not ERK or p38 both in vitro and in vivo (Tournier *et al.*, 1997). Therefore, MKK7 appears to be a specific JNK activator whereas MKK4 may have broader activities (Holland *et al.*, 1997;Tournier *et al.*, 1997;Lu *et al.*, 1997;Moriguchi *et al.*, 1997).

Immunofluorescence analysis and subcellular fractionation studies have demonstrated that MKK4 and MKK7 isoforms are particularly enriched in cytosolic fraction although they are also detected in nuclear compartments of cultured cells (Cuenda, 2000;Tournier *et al.*, 1999). However, it was shown that the presence of MKK7 in the nucleus was not required for JNK activation in vivo (Tournier *et al.*, 1999).

MKK4 and MKK7 deletion studies

To examine the function of MKK4 in vivo, several studies have investigated the effect of targeted disruption of the MKK4 gene. In the first study, targeted disruption of the MKK4 gene causes embryonic death before day 14 (Yang *et al.*, 1997a). These data indicate that the MKK4 gene is required for viability. The embryogenic death in MKK4 (-/-) mice was due to defective liver formation and liver cell apoptosis (Ganiatsas *et al.*, 1998;Nishina *et al.*, 1999). The mechanism that accounts for the defect in liver development observed in MKK4 (-/-) embryos remains to be established. Interestingly, the same phenotype was observed in c-Jun (-/-) mice (Hilberg *et al.*, 1993) but not in JNK1 (-/-), JNK2 (-/-) or JNK1 (-/-) X JNK2 (-/-) mice. However, conflicting results have been obtained since no liver apoptosis was observed in c-jun^{AA}/jun^{AA} mice in which endogenous c-jun alleles were replaced with non-phosphorylatable c-jun (Behrens *et al.*, 1999). Thus, these studies suggest that during hepatogenesis, downstream effectors other than JNK may mediate the cell survival effect of MKK4 and phosphorylation of c-jun might not be required for its anti-apoptotic role.

Targeted gene disruption of MKK7 in mice has shown that this kinase is also required for embryonic development (Dong *et al.*, 2000). The cause of MKK7 (-/-)

embryonic death has been shown to be the result of impaired proliferation of hepatocytes (Wada *et al.*, 2004). These observations indicate that the MKK4 and MKK7 protein kinases serve nonredundant functions during development. Genetic analysis of the MKK4 (Han *et al.* 1998) and MKK7 (Glise *et al.* 1995) genes in Drosophila supports this conclusion.

Since MKK4 (-/-) and MKK7 (-/-) mice are not viable, the functions of these protein kinases have been studied in cells derived from null embryos. The role of MKK4 in JNK activation and regulation of AP-1 transcriptional activity was examined by investigation of culture embryonic stem (ES) cells lacking MKK4 (Yang *et al.*, 1997a). This experiment has shown that disruption of the MKK4 gene blocked JNK activation induced by MEKK1 or by treatment of the cells with anisomycin or heat shock. AP-1 transcriptional activity was also selectively blocked in these ES cells. The AP-1 transcriptional activity was restored by transfection of MKK4 (-/-) cells with an MKK4 expression vector. However, this study has also shown that JNK activation caused by other forms of environmental stress (UV radiation and osmotic shock) was only partially inhibited in these MKK4 (-/-) cells. Because JNK is activated under these conditions, this indicates that either MKK7 can phosphorylate both the Thr and the Tyr under this situation or that there is another kinase capable of phosphorylating the tyrosine residue under these stimuli.

Biochemical experiments to examine whether JNK is indeed phosphorylated at both the threonine and the tyrosine residues in the MKK4 knockout cells have recently been performed. A study using ES cells lacking MKK4 has shown that JNK activation was markedly impaired upon treatment with different stimuli (1,10-phenanthroline

(PNTL), heat shock, lysophosphatidic acid (LPA) or IL-1β) and showed that this defect was accompanied with a decreased level of the Tyr phosphorylation (Wada *et al.*, 2001). In addition, they have shown that MKK4 could rescue the impaired JNK activation in response to UV irradiation and heat shock in MKK4 (-/-) ES cells while MKK7 isoforms α1,γ1 and γ2 could not. Also, these MKK7 isoforms, but not MKK4, could restore JNK activation in response to heat shock, anisomycin and nocodazole in MKK7 (-/-) ES cells. Interestingly, this experiment has reported different contributions of MKK7 isoforms to the synergistic activation of JNK. Moreover, a recent study using stress-induced (osmotic shock, UV irradiation or anisomycin) MKK4 (-/-) or MKK7 (-/-) ES cells has also reported that JNK activation was markedly impaired in both ES cells. More specifically, they have shown a loss of Thr phosphorylation without a marked reduction in Tyr phosphorylation in MKK7 (-/-) ES cells while in MKK4 (-/-) ES cells, Thr phosphorylation of JNK was attenuated in addition to a decreased level of Tyr phosphorylation (Kishimoto *et al.*, 2003). Thus, these studies suggest that MKK4 and MKK7 appear to be indispensable for stress-induced JNK activation in ES cells. These results are in accordance with the synergistic activation mechanism of JNK previously described. It should be noted that another study, using MKK7 (-/-) ES cells, has shown that in response to ultraviolet radiation and other stress signals, JNK activity was greatly reduced in the absence of MKK7 whereas p38 activity was not (Dong *et al.*, 2000). This confirms the specificity of MKK7 toward JNK and the broader specificity of MKK4 for JNK and p38.

Interestingly, Roger Davis's group has shown that simultaneous disruption of the MKK4 and MKK7 genes in MEFs was required to block JNK activation caused by

exposure of cells to environmental stress. In contrast, disruption of the MKK7 gene alone was sufficient to prevent JNK activation caused by proinflammatory cytokines (such as TNFα) thus indicating that MKK4 and MKK7 serve different functions and might be differentially regulated in the JNK signal transduction pathway (Tournier *et al.*, 2001). Another study had already demonstrated that MKK7 was a major JNK activator in the TNFα signaling pathway, using a dominant-negative form of MKK7 (Moriguchi *et al.*, 1997). Thus, even if MKK4 and MKK7 can be activated both by stressful stimuli and proinflammatory cytokines, it has been proposed that MKK7 is primarily activated by cytokines (e.g., TNFα and IL-1) while MKK4 is primarily activated by environmental stress (Finch *et al.*, 2001;Foltz *et al.*, 1998;Lawler *et al.*, 1997;Tournier *et al.*, 2001). However, new studies (including ours) suggest that both MKK4 and MKK7 are equally activated by stress and cytokines. Indeed, one recent study demonstrated, using phospho-MKK4 and phospho-MKK7 specific antibodies, that TNFα was able to activate both MKK4 and MKK7 in 3T3 fibroblasts derived from primary embryonic fibroblasts (Ventura *et al.*, 2004).

As previously described MKKs, such as MKK4 and MKK7, are activated by phosphorylation in their activation loop by MAPKKKs. Several MAPKKKs have been reported to activate the JNK signaling pathway, including MKK4 and MKK7 (Fanger *et al.*, 1997;Ip and Davis, 1998;Davis, 2000). These include members of the MEKK group (MEKK1-5), the mixed-lineage protein kinase group (MLK1-3, DLK and LZK), the ASK group (ASK1 and ASK2), TAK1 and TPL2. However, it should be noted that evidence suggesting that these MAPKKKs play a role in the activation of the JNK signaling pathway came from overexpression studies and in vitro protein kinase assays and thus, it

is not well established whether these MAPKKKs are physiological regulators of the JNK pathway. Moreover, it is still unclear which MAPKKKs are relevant to specific physiological stimuli such as cytokines and environmental stress.

Interestingly, Tournier *et al.* (1999) have examined, by in vitro kinase assays, the abilitites of some of these MAPKKKs to activate different MKK4 and MKK7 isoforms. Their study showed that these isoforms are differentially activated by upstream MAPKKKs. However, this study does not give clues about which residues in the activation loop of these MAPKKs are phosphorylated to contribute to the activity of these proteins. Alignment and mutational studies have allowed identification of these potential residues in MKK7 activation loop. First, mutational studies revealed which residues in MEK1 (a MAPKK involved in ERK1 and ERK2 activation) activation loop are essential for its activation by the MAPKKK Raf-1 and by physiological stimulus such as EGF (Alessi *et al.*, 1994;Zheng and Guan, 1994). Because the activation loop is the most conserved region among the MAPKK family members, this suggests that all members of the family are activated by the phosphorylation of these sites. In favor of this hypothesis, constitutively active or inactive forms of MEK1, MKK3 and MKK6 have been obtained by substitutions of these corresponding sites with charged or uncharged amino acids, respectively (Mansour *et al.*, 1994;Raingeaud *et al.*, 1996). Thus, sequence alignment of MKK7 with MEK1, MKK3 or MKK6 has allowed identification of corresponding residues in MKK7 activation loop. In human, these sites correspond to serine 271 and threonine 275. Because MKKs are activated by Ser/Thr MAPKKKs, serine 277 in MKK7 activation loop is also considered a potential regulatory site despite the fact that corresponding residue in other MKK family members is an aspartic acid. Constitutively

active and inactive forms of MKK7 by mutation of these three sites with glutatmate or alanine, respectively, have also been reported suggesting a key role of these three amino acids in MKK7 regulation (Holland *et al.*, 1997;Holtmann *et al.*, 1999). However, we cannot rule out the possibilities that other residues outside the activation loop are important for the protein regulation, as shown by the negative regulation of MKK4 due to phosphorylation of its serine 78 by Akt (PKB) (Park *et al.*, 2002).

Regulation and Specificity in the JNK pathway

As we know, members of the MAPK signaling pathways are activated by phosphorylation of specific residues in their activation loop by upstream kinases. In addition to these activating kinases, several types of protein phosphatases have been also shown to regulate MAPK pathways by dephosphorylating and inactivating the MAPKs or their upstream kinases. These protein phosphatases include tyrosine-specific phosphatases (Pulido *et al.*, 1998;Saxena *et al.*, 1999), serine/threonine-specific phosphatases (Takekawa *et al.*, 1998;Alessi *et al.*, 1995) and dual specificity phosphatases (DSPs) (Keyse and Emslie, 1992;Keyse, 2000). Most of the DSPs inactivate MAPKs by dephosphorylating the critical phospho-threonine and phospho-tyrosine in the TXY motif. Therefore, they are also termed MAPK phosphatases (MKPs) (Tanoue *et al.*, 1999;Tanoue *et al.*, 2001). Thus, the duration and extend of MAPK activation are governed by the balance between the activities of upstream activating kinases and protein phosphatases. Several of these phosphatases have been shown to regulate the JNK signaling pathway. Of them, protein phosphatase 2Cα (PP2Cα) has been shown to inhibit both the p38 and the JNK pathways (Takekawa *et al.*, 1998) and a

dual specificity phosphatase termed SKRP1 (SAPK pathway-regulating phosphatase 1) has been reported to inhibit the JNK pathway by interacting with MKK7 but not JNK (Zama *et al.*, 2002a). Interestingly, two dual specificity phosphatases termed JSP-1 (JNK stimulatory phosphatase-1) and JKAP (JNK pathway-associated phosphatase) have been shown to activate the JNK signaling pathway (Shen *et al.*, 2001;Chen *et al.*, 2002). Thus, phosphatases can act as positive regulators of the JNK signaling pathway. Interestingly, JKAP has been shown to interact with JNK and MKK7, but not MKK4, in vivo indicating specificity in the phosphatase signaling.

Protein-protein interactions are thought to be critical for the normal functions of the JNK signaling pathway. Thus, signaling specificity may be mediated through the formation of protein complexes. These complexes may involve the interactions between kinase components of the signaling module or interactions with other proteins. The specificity in the JNK signaling pathway is first determined by a docking site. Indeed, JNK (as other MAPK family members) contains a common docking site that is distant from the active site and binds to motifs (D-domain) that are located in interacting proteins, including substrates, MAPKKs and phosphatases (Gupta *et al.*, 1996;Tanoue *et al.*, 2000;Enslen and Davis, 2001;Tanoue *et al.*, 2002;Tanoue and Nishida, 2002;Tanoue and Nishida, 2003;Weston *et al.*, 2002). A docking site in the N-terminus of MKK4 has been reported to mediate high affinity binding to JNK and competes with similar docking sites in JNK substrates (Ho *et al.*, 2003). Moreover, distinct regions within the N termini of MKK7 and DLK have been shown to mediate their binding to the scaffold protein JIP-1 (Mooney and Whitmarsh, 2004). Also, it was reported that no scaffold protein was required for the MEKK2-MKK7-JNK1 tripartite-complex formation, thus showing the

efficiency of physical interactions through docking sites (Cheng *et al.*, 2000). It should be noted that the amino acids that surround the phosphorylation sites in the activation loop form a recognition motif and further increase the specificity in the signaling pathway (Chang and Karin 2001). A second mechanism ensuring MAPK activation has been proposed to depend on sequential physical interactions between members of a given cascade (Xia *et al.*, 1998). Indeed, it has been shown that JNK1 and JNK2 are bound by the N-terminal extension of MKK4, which also interacts with the catalytic domain of MEKK1 and that each interaction is disrupted on activation of the downstream kinase.

As previously mentioned, formation of signaling complexes may involve interactions with other proteins. Scaffold proteins have been proposed to interact with the JNK signaling components to increase their local concentration to create a functional signaling module and to control the specificity of signal transduction. In addition, scaffold complexes have also been proposed to prevent the activation of the JNK module by irrelevant stimuli and provide spatial and temporal control of JNK signaling. Several putative scaffolds have been reported. Of them, β-Arrestin-2 has been shown to contain a docking site that conforms to the D domain consensus sequence (Enslen and Davis, 2001;Weston *et al.*, 2002) and selectively binds JNK3 and ASK1. β-Arrestin-2 scaffold complex includes MKK4, which binds to both JNK3 and ASK1, but MKK4 does not directly interact with β-Arrestin-2 (McDonald *et al.*, 2000). Interestingly, a motif in the carboxyl terminus of β-arrestin2 was shown to be responsible for activation of JNK3 (Miller *et al.*, 2001).

Another putative scaffold for the JNK signaling pathway is the adapter molecule CrkII. This molecule consists of an N-terminal SH2 domain and two SH3 domains

(Kiyokawa *et al.*, 1997). The SH2 domain of CrkII is known to interact with tyrosine-phosphorylated p130Cas and paxillin in focal adhesion while the SH3 domains have been shown to interact with exchange proteins SOS and C3G, DOCK180 and the MAPKKKK HPK1. A common motif present in each of these proteins mediates the interaction with the N-terminal SH3 domain of CrkII. Interestingly, JNK contains a related motif that also binds to the same SH3 domain (Girardin and Yaniv, 2001). Binding of JNK to CrkII enables the recruitment of JNK to protein complexes that can bind the SH2 domain of CrkII. A co-immunoprecipitation study confirms the presence of multiprotein complexes that include the CrkII/p130Cas adapters together with JNK, MKK4 and HPK1. Interestingly, mutation of JNK within the motif mediating binding to the SH3 domain of CrkII prevents JNK activation mediated by Rac1 and epidermal growth factor (EGF) but does not alter JNK activation induced by UV-irradiation. This suggests different regulation in assembly of multiprotein complexes depend on the stimuli.

Filamin, a large actin-binding protein, is another candidate of scaffold protein since it was found to interact with MKK4 (Marti *et al.*, 1997) and with the zinc finger domain of TRAF2, an adapter protein recruited to activated TNF receptors (Leonardi *et al.*, 2000). TRAF2 has been shown to be essential for TNF-stimulated JNK activation (Yeh *et al.*, 1997) and therefore, cells lacking expression of filamin have been shown to be defective in TNFα-stimulated activation of JNK (Marti *et al.*, 1997).

Important scaffold proteins for the JNK-signaling pathway also include the JNK interacting proteins (JIPs). These proteins are encoded by four genes and all appear to function as dimers (Kelkar *et al.*, 2000;Yasuda *et al.*, 1999). JIP1 and JIP2 proteins are structurally similar and contain a PTB domain and a SH3 domain in the C-terminal

region. JIP1 and JIP2 have been shown to bind JNK, MKK7 and members of the MLK family (Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999). Interestingly, both JIP1 and JIP2 have been shown to interact with the phosphatase MKP7 (Willoughby *et al.*, 2003). This indicates that these JIP scaffold complexes include both activating and inhibitory components of the JNK signaling pathway. Moreover, binding studies have demonstrated functional interactions between the proteins bound to JIP1. Indeed, it has been shown that binding of JNK decreases the affinity of JIP1 for MLK (Nihalani *et al.*, 2001) and that the binding of AKT (PKB) to JIP1 decreases the binding of JNK to JIP1 (Kim *et al.*, 2002). In this latter study, it has also been shown that exposure of cells to excitotoxic stress causes the release of AKT from JIP1, increases JNK binding to JIP1 and thus increases JNK activation. In favor of this result, another study has demonstrated that JIP1 is required for excitotoxicity-induced JNK activation in hippocampal neurons (Whitmarsh *et al.*, 2001). JIP3 and JIP4 are structurally distinct from JIP1 and JIP2 because they contain an extended coiled-coil domain (Ito *et al.*, 1999;Kelkar *et al.*, 2000). JIP3 has been reported to bind JNK, MKK7 and members of the MLK family (Kelkar *et al.*, 2000;Matsuura *et al.*, 2002). Interestingly, it should be noted that JIP3 was found to interact with Wt-MKK7 or with MKK7 mutants (constitutively active or inactive forms) similarly (Matsuura *et al.*, 2002). Interactions of JIP3 with MEKK1 and MKK4 (Ito *et al.*, 1999) and JIP4 with MKK4 and MEKK3 (Lee *et al.*, 2002) have also been reported. An important conserved function of the JIP proteins is their ability to interact with the tetratricopeptide repeat (TPR) domain of kinesin light chain. This interaction is mediated by the coiled-coil domain of JIP3 and JIP4 (Bowman *et al.*, 2000) and by the C terminus of JIP1 and JIP2 (Whitmarsh *et al.*, 2001;Verhey *et al.*, 2001). Thus, each of the JIP

proteins can be transported by the microtubule motor protein kinesin (Goldstein, 2001). It has been shown that this interaction with motor proteins accounts for the accumulation of JIPs in the growth cones of developing neurons and the accumulation of JIP1 and JIP2 at synapses in mature neurons (Meyer *et al.*, 1999;Pellet *et al.*, 2000;Setou *et al.*, 2002). It has been proposed that JIP proteins might act as adapter molecules for the transport of cargo by the kinesin motor protein. Interaction of the JNK signaling members with JIP proteins bound to kinesin motor proteins may act to locally regulate JNK activation in response to specific stimuli. Thus, JIP proteins may contribute to the spatial regulation of the JNK signaling module in vivo.

Interestingly, the dual specificity phosphatase SKRP1 has been reported to have a potential scaffold role for the JNK signaling pathway as it has been shown that these phosphatases interact not only with JNK, but also with MKK7 and ASK1 (Zama *et al.*, 2002b). Finally, POSH (plenty of SH3s), a zinc finger protein that contains four SH3 domains has recently been reported as a scaffold for the apoptotic JNK signaling pathway. POSH has been shown to bind Rac1 (Tapon *et al.*, 1998), JNK, MKK4, MKK7 and members of the MLK family (Xu *et al.*, 2003). This study has shown a decrease in JNK activation and apoptosis in NGF-deprived cells using siRNA to reduce the expression of POSH. Thus, POSH is a physiologically important scaffold protein that links activated Rac1 to activation of JNK.

Rationale and Statement of Hypothesis

JNK proteins are important regulators of many forms of neuronal death. Despite the fact that significant progress has been made in understanding the regulation and specificity in the signaling cascade that leads to JNK activation, some elements of this pathway remain elusive, specifically the mechanism by which MKK4 and MKK7, are activated by phosphorylation. Alignment and mutational studies have allowed identification of three potential regulatory amino acids in human MKK7 activation loop (serine 271, threonine 275 and serine 277). Despite the fact that mutation of these sites with charged or uncharged amino acids generate constitutively active and inactive forms of the protein respectively, these results come from overexpression studies and in vitro protein kinase assays. Direct phosphorylation of these sites has never been assessed.

Thus, I proposed that generation of three phospho-specific antibodies directed against each of these potential regulatory sites will allow us to understand the mechanism by which MKK7 is activated by phosphorylation and further characterized the JNK signaling pathway.

Materials and Methods

Cell lines and reagents. Cell culture reagents were obtained from Hyclone. HEK 293 cells were purchased from American Type Culture Collection (ATCC) and maintained in 5% CO_2 at 37°C in Dulbecco's modified Eagles's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 μg/ml penicillin/streptomycin. ATF2 antibody (cat # 9222), c-jun (cat # 9162), phospho-ATF2 (Thr69/71) (cat # 9225), phospho-c-jun (Ser63) (cat # 9261), phospho-SAPK/JNK (Thr183/Tyr185) (clone G9) (cat # 9255), phospho-SEK1/MKK4 (Thr261) (cat # 9151), phospho-MKK7 (Ser271/Thr275) (cat # 4171) were purchased from Cell Signaling Technology; IκBα (cat # sc-371) and JNK1 (cat # sc-474) were purchased from Santa Cruz Biotechnology; JNKK/MKK4 (clone G282-114) (cat # 554105) and c-myc (clone 9E10) (cat # 14851A; for immunoprecipitation) were purchased from BD Biosciences; MKK7/SKK4 (cat # 07399), phospho-MKK7/SKK4 (Thr275) (cat # 36013), phospho-MKK7/SKK4 (Ser271), phospho-MKK7/SKK4 (Ser277) were generous gifts from Upstate Biotechnology. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Recombinant murine TNFα was obtained from R&D Systems and Sorbitol was obtained from Fisher. MKK7 expression plasmids (MKK7-wt, MKK7-S3E (S271, T275, S277 mutated to E), MKK7-S3A (S271, T275, S277 mutated to A) and MKK7-K149M) were generous gifts from Dr. Michael Kracht (Medical School Hannover, Germany). MKK4 expression plasmid (MKK4-wt) was a generous gift from Dr. Lloyd A. Greene (Columbia University, New York). All constructs are Myc epitope-tagged.

Mutagenesis. MKK7 single amino acid mutants; (S271A), (T275A), (S277A), (S271D), (T275D), (S277D) were generated from MKK7-wt expression plasmid using the QuikChange® Site-Directed Mutagenesis kit (Stratagene; cat # 200519). Introduction of the appropriate mutation was confirmed by sequencing (McGill University and Genome Quebec Innovation Centre).

Immunoblotting and immunoprecipitation. Cells were lysed in a buffer containing: (50 mM Tris (pH 8.0), 500 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 50 mM b-glycerophoshate, 20 mM paranitrophenylphosphate (PNPP), 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and protease inhibitors tablets (1 tablet / 10 ml; obtained from Roche). Samples were sonicated for 12 s and were separated by SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane. Membranes were blocked in Tris-buffered saline/Tween (10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20) added with 5% (w/v) BSA solution when using phospho-antibodies or 5% (w/v) dried skim milk powder when using other antibodies. The secondary antibody incubations were carried out in the 5% (w/v) dried skim milk powder solution. Immunoreactive bands were detected using the enhanced chemiluminescence solution (PerkinElmer Life Sciences). For immunoprecipitation experiments, samples were incubated with the appropriate antibody and 50 ul of protein-A Sepharose or protein-G Sepharose (Amersham Pharmacia Biotech) at 4°C for 3 hours. Immunocomplexes were washed with lysis buffer, eluted with sample buffer and analysed by SDS-PAGE as described above.

In vitro translation and In vitro kinase Assay. In vitro transcription/translation experiment was performed using the TNT® SP6 Quick Coupled Transcription/Translation System (Promega; cat # PRL2081). In vitro transcription/translation reactions (15ul/reaction) were diluted 1/5 in H_2O, 5X Kinase Buffer (100mM HEPES (pH 7.4), 100mM beta-glycerol phosphate, 100mM $MgCl_2$, 10mM dithiothreitol, 0.5mM sodium orthovanadate), Mg-ATP Cocktail and were incubated at 30°C for 30 min in a shaking water bath. After the kinase reaction, the volume was adjusted to 1 ml with lysis buffer to perform immunoprecipitation.

RNAi. SMARTPOOL RNAi directed against MKK7 was purchased from Dharmacon RNA Technologies. SMARTPOOL technology combine 4 or more selected highly active siRNA duplexes in a single pool, resulting in even greater probability that the siRNA pool reagent will reduce mRNA to low levels. To validate RNAi, HEK293 cells plated in 24 well plates were transfected with varying concentrations of the MKK7 SMARTPOOL RNAi using Lipofectamine 2000 (Invitrogen). Cell lysates were harvested 48 hours after transfection and MKK7 levels were determined by immunoblotting. 20 pmol MKK7 RNAi was sufficient for >95% MKK7 knockdown (data not shown) and this concentration was used in subsequent experiments.

Transfections. All expression plasmids were introduced into HEK 293T cells on 100-mm plates using the calcium phosphate precipitation method. Cells were treated and harvested 48 hours post-transfection. MKK7 SMARTPOOL RNAi was transfected in HEK 293T cells using Lipofectamine 2000 (Invitrogen) (previously described).

Results

Activation loop comparison and phospho-MKK7 antibodies generation

The activation loop is the most conserved region among the MAPKK family members. To highlight this, we have aligned and compared activation loop of MKK7 with the other members of the MAPKK family. We have included sequences from different species (Figure 1A). In light of these alignments, we confirmed that activation loops are very well conserved between different species of many members of the MAPKK family thus suggesting that these regions are well conserved through evolution. However, we denoted a major difference for one potential regulatory site. Indeed, when we compared the homology of the three potential phospho-acceptor sites in human MKK7 activation loop (serine 271, threonine 275 and serine 277) with other MAPKK members, we noted that serine 277 is replaced with an aspartic acid in other members of the family while serine 271 and threonine 275 are well conserved.

Because we want to further characterize the JNK signaling pathway by understanding the mechanism of activation of MKK7 by phosphorylation, we generated three different phospho-MKK7 specific antibodies by injecting synthetic peptides in rabbits. Each of the polyclonal antibodies was raised against one of the potential regulatory site in human MKK7 activation loop (Figure 1B). Based on our observation from the activation loop alignment (Figure 1A), we expect that antibodies raised against phospho-serine 277 will be MKK7-specific while the other two antibodies should cross-react with other MKK family members.

Phospho-MKK7 antibodies reveal constitutive phosphorylation of Ser271 and Ser277 and regulated phosphorylation of Thr275.

MKK7 and MKK4 proteins have been shown to be activated by the cytokine TNFα and environmental stresses such as osmotic shock (Moriguchi *et al.*, 1997;Foltz *et al.*, 1998;Ventura *et al.*, 2004). We thus, tested the ability of TNFα and sorbitol to activate exogenous Myc-MKK7 (Wt) or Myc-MKK4 (Wt) transiently overexpressed in HEK 293T cells. A Myc-tagged non-phosphorylable form of MKK7 (S3A), in which the three potential regulatory sites were replaced with alanine, was used as a negative control (Figure 2A, lanes 7 to 9). Briefly, Myc-tagged proteins were immunoprecipitated from extracts of cells subjected to no treatment, 50 ng/ml TNFα or 0.5M sorbitol. The phospho-status of these proteins was assessed by Western Blots. As shown in Fig. 2A, the expression of Myc-MKK7 and S3A was constant. As expected using our three phospho-specific antibodies, no immunoreactive bands were detected in the negative control (Figure 2A, lanes 7 to 9). In contrast, immunoreactive bands were obtained in Wt-MKK7 (Figure 2A, lanes 4 to 6). Interestingly, constitutive phosphorylation was observed for serine 271 and serine 277 while threonine 275 appeared to be induced by both stimuli.

We also used our phospho-MKK7 antibodies to assess the phospho status of MKK4 since cross-reactivity was expected based on activation loop sequences alignment. As seen in Fig. 2B, Myc-MKK4 levels were constant. Consistent with our hypothesis (Figure 1A), immunoreactive bands were observed for MKK4 residues corresponding to serine 271 and threonine 275 in MKK7 (serine 257 and threonine 261 in MKK4-See Figure 1A) while no bands were observed for the amino acid corresponding to serine 277

in MKK7 since it is replaced by an aspartic acid in MKK4 (Figure 2B). Interestingly, we also observed constitutive and regulated phosphorylation in MKK4. To confirm the regulated phosphorylation observed in MKK4, we have also used a phospho-specific MKK4 antibody (raised against threonine 261). We observed a similar result as the one obtained with the MKK7 anti-phospho-threonine 275 antibody.

To confirm that the TNFα and sorbitol treatments were effective, we assessed activation of endogenous MKK4 and c-Jun, using phospho-specific antibodies, and IKBα degradation as an indication of TNFα-induced NF-κB signaling (Papa *et al.*, 2004). Figure 2A and 2B show that our system is effectively working since phosphorylation of MKK4 and c-jun was detected in TNFα- or sorbitol-treated cells but not in untreated cells. As expected, we observed IKBα degradation in cells treated with TNFα but not in cells treated with sorbitol.

Regulated phosphorylation on Thr275 is dose and time-dependent.

In order to further assess the phosphorylation induced by TNFα and sorbitol, we next assessed dose-dependent and time-dependent effects of both treatments. Briefly, 293T cells were transiently transfected with Wt-MKK7 or Wt-MKK4 and were subsequently treated 15 minutes with increasing concentrations of TNFα and sorbitol or with a fixed dose of TNFα and sorbitol for various time. Overexpressed proteins were immunoprecipated and endogenous proteins were individually analyzed by Western blots. As shown in Figure 3A, all proteins analyzed were activated at 15 and 30 minutes but we observed a reduction in phosphorylation starting at 60 min. Also, consistent with the ability of c-jun to autoactivate its own transcription (Angel and Karin, 1991),

increasing amount of c-jun proteins were observed at 60 and 120 minutes in TNFα-treated cells. However, this increase was not observed in sorbitol-treated cells. Possible explanations for this difference will be discussed (See Discussion).

Figure 3B shows a dose-dependent effect of both TNFα and sorbitol treatments on JNK and c-jun phosphorylation. As shown on the Western blots, increased phosphorylation of these proteins were observed with the lowest TNFα and sorbitol dose tested (10ng/ml and 100 mM). Figure 3B also shows a dose-dependent MKK4 and MKK7 regulated site phosphorylation with both treatments, starting at 30 ng/ml for TNFα and 300 mM for sorbitol. Figure 3 suggests that the conditions used in Figure 2 (15 minutes treatment with 50 ng/ml TNFα or 500 mM sorbitol) are optimal. Thus, these conditions were maintained for future experiments.

Constitutive phosphorylation is required for induced activity

We were surprised by the results showing regulated phosphorylation at threonine 275 but constitutive phosphorylation on serine 271 and serine 277 in human MKK7. To explore the regulatory relationship between these distinct phosphorylation sites, we generated three MKK7 mutants in which each of these three sites has been individually mutated to alanine while keeping the other two sites unchanged (S271A, T275A, S277A). We then, assessed the effects of these specific mutations on the phosphorylation of the two other phospho-acceptor sites. Here again, overexpressed proteins were immunoprecipitated from extracts of non-treated cells or from cells treated with 50 ng/ml TNFα or 0.5M sorbitol.

As shown in Fig. 4, MKK7 expression level was similar for all constructs. As expected, no phosphorylation of serine 271 was noted in lanes containing MKK7 S271A mutants (lanes 4 to 6). Mutation of threonine 275 or serine 277 to alanine did not affect serine 271 phosphorylation as immunoreactive bands were detected when T275A and S277A mutants were overexpressed (lanes 7 to 12). We also assessed the effects of individual alanine mutations on threonine 275 phosphorylation. Interestingly, we observed that serine 271 or serine 277 mutation affected the regulated phosphorylation of threonine 275 since the intensity of immunoreactive bands was decreased by about ten-fold (compare Wt with both serine mutants), suggesting that constitutive phosphorylation on both serine residues is required for induced phosphorylation of threonine 275.

Figure 4 also shows that mutation of serine 271 does not affect serine 277 phosphorylation but mutation of threonine 275 appears to prevent phosphorylation of serine 277 as no immunoreactive bands were detected. As shown on the Western blots, we have also assessed activation of several endogenous proteins. We observed that TNFα and sorbitol treatments activated endogenous MKK4, JNK, c-jun and ATF-2. Interestingly, we noted that TNFα activated endogenous JNK to a lesser extend than sorbitol but activated c-jun to the same degree. Again, we observed TNFα-mediated IKBα degradation.

Aspartic acid mutations increase regulated phosphorylation

To further explore the regulatory relationship between these distinct phosphorylation sites, we next investigated the effect of mimicking phosphorylation on the regulated phosphorylation sites. It has been reported that substitution of amino acids

with negatively charged amino acids mimics phosphorylation (Holland *et al.*, 1997;Karger *et al.*, 2003). We thus generated three MKK7 mutants in which serine 271, threonine 275 and serine 277 have been individually mutated to aspartic acid while keeping the other two sites unchanged (S271D, T275D, S277D). These mutants, Wt-MKK7 and a negative control (a constitutively active form of MKK7 in which serine 271, threonine 275 and serine 277 have been substituted with glutamate), were transiently overexpressed in 293T cells. Overexpressed proteins were immunoprecipitated as usual after TNFα and sorbitol treatments. Figure 5 shows that phosphorylation of threonine 275 is increased by about two-fold when serine 271 is mutated to aspartic acid and that mutation of serine 277 with aspartic acid increases threonine 275 phosphorylation by more than ten-fold (compare Wt with both serine mutants). Also, as predicted, no phosphorylation of threonine 275 was observed in lanes expressing S3E or T275D (Figure 5, lanes 4-6 and lanes 10-12). No effect on serine 271 phosphorylation was observed when threonine 275 or serine 277 was mutated to aspartic acid and as expected, no serine 271 phosphorylation was detected in lanes expressing S3E and S271D mutants (Figure 5, lanes 4 to 9). Mutation of serine 271 did not affect serine 277 phosphorylation and contrary to the result obtained in Figure 4, mutation of threonine 275 to aspartic acid instead of alanine allowed phosphorylation of serine 277. No serine 277 phosphorylation was observed in lanes expressing S3E and S277D mutants (Figure 5, lanes 4-6 and 13-15). Endogenous proteins activation and IKBα degradation were identical to those in Figure 4. Finally, although the constitutively active form of MKK7 (S3E) has been reported to generate a more robust JNK and c-Jun phosphorylation in vitro when

compared to Wt-MKK7 (Holtmann *et al.*, 1999), this was not observed in our studies (compare pJNK and pJun in Wt-MKK7 and S3E overexpressed lanes 4-6).

Constitutive phosphorylation of Ser271 and Ser277 is due to an upstream regulator.

We next investigated if phosphorylation of the three phospho-acceptor sites in MKK7 activation loop is due to an upstream regulator or to MKK7 autophosphorylation. Wt-MKK7 and a MKK7 kinase dead mutant (KD-MKK7), in which the invariant lysine has been mutated to methionine (K149M), were produced by in vitro transcription/translation reactions and reaction products were subjected to an in vitro kinase assay and were subsequently immunoprecipitated. Myc-Mint and no plasmid were used as positive and negative controls, respectively, for the in vitro transcription/translation reactions. Phospho-status of MKK7 phospho-acceptor sites was then assessed by Western blots. As shown in Figure 6, MKK7 proteins were detected and phosphorylation of serine 271 and serine 277 were observed with both Wt-MKK7 and KD-MKK7 thus suggesting that constitutive phosphorylation of these residues is due to an upstream activator present in the in vitro transcription/translation reaction and not MKK7 autophosphorylation. Absence of threonine 275 phosphorylation suggests that this amino acid is not phosphorylate by MKK7 itself.

Threonine 275 phosphorylation is stimuli-regulated in vivo

Since constitutive and regulated phosphorylation were observed in our overexpression system, we next assessed if MKK7 is similarly regulated in vivo. 293T cells were transfected with non-specific RNAi or with RNAi directed against MKK7 and

were left untreated or treated with sorbitol. The phosphorylation status of serine 271, threonine 275 and serine 277 in endogenous MKK7 was then assessed by Western blots. As shown in Figure 7, endogenous MKK7 levels decreased by almost 100% in cells transfected with the MKK7 specific RNAi but remained constant in untransfected cells or in 293T transfected with the non-specific RNAi. Moreover, Figure 7 shows that in endogenous MKK7, threonine 275 phosphorylation is regulated since no bands were observed in non-treated cells compared with sorbitol-treated cells. Specific MKK7 RNAi confirmed this result and the specificity of the phospho-threonine 275 antibody as no band was observed in sorbitol-treated cells in presence of this RNAi but phosphorylation of threonine was observed in sorbitol-treated cells in presence of the non-specific RNAi. Also, on the contrary to our overexpression model, we could not observe phosphorylation of serine 271 or serine 277 in endogenous MKK7. This might be explained by a weaker affinity of these antibodies compared with phospho-threonine 275 antibody.

Serine 271 and serine 277 are constitutively phosphorylated in vivo

To determine if serine 271 and serine 277 are constitutively phosphorylated in vivo, we next performed immunoprecipitation of endogenous MKK7 in order to enhance and facilitate detection by phospho-serine 271 and phospho-serine 277 antibodies. Immunoprecipitated endogenous MKK7 from untreated or sorbitol-treated 293T was analysed for the phosphorylation status of serine 271 and serine 277 by Western blots. As shown in Figure 8, serine 271 and serine 277 were constitutively phosphorylated in vivo as bands were observed in both non-treated and sorbitol-treated cells.

Chapter 3

Discussion and Closing Remarks

Discussion

Many studies have shown that JNK is a key regulator of several forms of neuronal apoptosis. JNKs have been reported to be involved in glutamate excitotoxicity, NGF withdrawal and p75NTR-induced neuronal apoptosis (Xia *et al*, 1995;Yang *et al*, 1997; Aloyz *et al*, 1998;Bhakar *et al*, 2003;Kuan *et al*, 2003;). However, despite that significant progress made in understanding the signaling cascade and molecular mechanisms that lead to JNK activation, key aspects of this pathway remain elusive. The mechanism by which the upstream activators of JNK, MKK4 and MKK7, are activated by phosphorylation is still unclear. Here we show that MKK7 and MKK4 are regulated through constitutive and stimuli-induced phosphorylation of activation loop amino acids.

MKK4 and MKK7 are activated by constitutive and stimuli-induced phosphorylation of activation loop amino acids.

It is now well established that the JNK signaling pathway is activated by many forms of stimuli such as ionizing irradiation, heat shock, osmotic imbalance, DNA damage, bacterial product lipopolysaccharide and inflammatory cytokines (Cuenda, 2000;Pearson *et al*., 2001). Moreover, it is also well known that JNK activation occurs through sequential phosphorylation of proteins in a kinase cascade which includes MAPKKKs and MAPKKs. MAPKKK phosphorylates and thereby activates MAPKK and activated MAPKK in turn phosphorylates and activates MAPK. It is also well established that JNK and other MAPK family members are activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) located in their activation loop. Studies have demonstrated that JNK is activated synergistically by MKK4 and MKK7,

both in vitro and in vivo, with MKK4 phosphorylating preferentially the tyrosine residue first and MKK7 phosphorylating the threonine residue after the tyrosine phosphorylation. (Sanchez *et al.*, 1994;Lawler *et al.*, 1998;Fleming *et al.*, 2000;Wada *et al.*, 2001;Kishimoto *et al.*, 2003). Consistent with the mechanism of activation of MAPKs, which requires dual phosphorylation of key residues in their activation loop, many groups have shown that MAPKKs require serine/threonine phosphorylation within their activation loop to become activated (Alessi *et al.*, 1994;Zheng and Guan, 1994;Mansour *et al.*, 1994;Raingeaud *et al.*, 1996;Holland *et al.*, 1997;Holtmann *et al.*, 1999).

In accordance with these reports, we demonstrate that MKK7 and MKK4 are phophorylated on serine and threonine residues within their activation loop after TNFα or sorbitol treatments. Interestingly, we reveal that serine 271 and serine 277 in human MKK7 as well as serine 257 in human MKK4 are constitutively phosphorylated while threonine 275 in MKK7 and threonine 261 in MKK4 are stimuli-regulated. Importantly, our results suggest that this phosphorylation mechanism occurs in vivo on endogenous MKK7 (Figures 7 and 8). Our study also demonstrate regulated-phosphorylation of threonine 261 in endogenous MKK4 (Figure 2B-Lysate). To our knowledge, this MKK phosphorylation scheme has not been previously reported.

Through mutational studies we show regulatory relationship between these three distinct sites in MKK7 activation loop. Mutation of threonine 275 or serine 277 to alanine or aspartic acid does not affect serine 271 phosphorylation and mutation of serine 271 to alanine or aspartic acid does not affect serine 277 phosphorylation. On the other hand, mutation of threonine 275 with alanine appears to prevent phosphorylation of serine 277 while mutation of this residue with aspartic acid facilitates serine 277 phosphorylation.

Moreover, our mutational analysis reveals that serine 271 or serine 277 mutation to alanine prevents regulated phosphorylation of threonine 275 while mutation of serine 271 or serine 277 with aspartic acid increases threonine 275 phosphorylation by about two-fold or more than ten-fold respectively. We thus show that constitutive phosphorylation on both serine residues is required for induced phosphorylation of threonine 275.

Differences in the regulatory relationship between these phospho-acceptor sites could be explained by the position of these residues in the activation loop. Indeed, the fact that serine 271 or serine 277 mutation does not influence each others phosphorylation might be because they are six residues apart. On the other hand, the fact that serine 271 or serine 277 mutation affects threonine 275 phosphorylation could also be due to the distance of these residues from threonine 275. Serine 277 aspartic acid mutation appears to have a stronger effect than serine 271 aspartic acid mutation on threonine 275 phosphorylation. Again, this could be explained by the fact that these amino acids are only two residues apart. In addition to amino acid position within the activation loop, we believe that the negatively charged amino acid leads to a conformational change in the activation loop that renders threonine 275 more accessible to upstream regulators than when the regular negatively charged phosphate group is present. Thus, since activation loop is a flexible loop (Zhang *et al.*, 1995), our results suggest that regulation of its conformational structure is very important for proprer amino acid phosphorylation by upstream activators. Despite reports that Wt-MKK7 and MKK7 mutants (constitutively active and inactive forms) interact similarly with JIP3 (Matsuura *et al.*, 2002), it is possible that the difference in phosphorylation levels obtained with

different mutations is the result of changes in the conformational structure of the activation loop that altered docking interactions with other proteins such as MAPKKKs.

We recognize that even if different phosphorylation levels were observed through our mutational analysis, our results do not show a correlation between the phosphorylation status and the kinase activity. Alanine mutants should, in theory, act as dominant suppressors for JNK activation when overexpressed. However, our immublot analysis demonstrates similar endogenous JNK phosphorylation when Wt-MKK7 or MKK7 alanine mutants are overexpressed (Figure 4). This result can be rationalized in a number of ways. First, this result could suggest that a specific pool of endogenous MKK7 is sufficient, with endogenous MKK4, to activate JNK and that this pool is not affected by protein overexpression. Another possibility is that the MKK7 isoform we are using (MKK7β1) cannot act as a dominant suppressor of JNK activation in vivo. On the other hand, since MKK7 preferentially phosphorylates the threonine residue in the TPY motif of JNK, we are not able to confirm that the immunoreactive bands in our Western blot reflect threonine phosphorylation. The phospho-JNK antibody used was raised against the phospho-threonine and the phospho-tyrosine and thus it is possible that the immunoreactive bands reflect more tyrosine phosphorylation due to MKK4 than threonine phosphorylation. Further investigations assessing phosphorylation of the threonine and the tyrosine residue individually, in the TPY motif of JNK, will clarify if MKK7 alanine mutants decrease threonine phosphorylation of JNK and thus act as dominant suppressors. These investigations are currently under investigation by others in our group. The fact that we did not observed increase JNK phosphorylation when the MKK7 constitutively active form mutant (S3E) was overexpressed (Figure 5) can also be

explained by the fact that the immunoreactive bands reflect more tyrosine phosphorylation of JNK since the same phospho-JNK antibody was used. However, if the phospho-JNK antibody reflects also threonine phosphorylation, the fact that Wt-MKK7 and S3E overexpression result in the same amount of JNK phosphorylation in vivo but not in vitro, as S3E gives about 7 times more JNK phosphorylation in vitro than Wt-MKK7, could be explained by the TNFα and sorbitol treatments. In the in vitro experiment, plasmids encoding Wt-MKK7 and S3E were transiently co-transfected with JNK constructs in 293T cells for 48 hours and then activation status was assessed in an in vitro kinase assay (Holtmann *et al.*, 1999). However, this study was performed in the absence of stimuli and this may explain why S3E mutant expression resulted in more JNK activation than Wt-MKK7. We show that TNFα and sorbitol treatments result in MKK7 phosphorylation and thus believe that this phosphorylation result in the activation of MKK7 and thus could explain the JNK phosphorylation obtained from Wt-MKK7 and S3E overexpression. However, as for alanine mutants, further investigations assessing JNK phosphorylation on the threonine and the tyrosine residue individually when aspartic acid mutants are overexpressed will clarify if these MKK7 mutants increase threonine phosphorylation of JNK. These investigations are also currently under investigation by others in our group.

Interestingly, we show that TNFα activates endogenous JNK to a lesser extent than sorbitol but activates c-jun to the same degree (Figure 4 and 5). This suggest that sorbitol is a "broader" stimulus than TNFα. Although the osmosensors are not yet identified, we think that sorbitol globally "stress" the cells and results in the activation and cross talk of many signaling pathways that lead to activation of different JNK

isoforms. On the other hand, we think that TNFα is a more specific activator and thus results in activation of only certain JNK isoforms. We think that these specific JNK isoforms activated by TNFα are responsible for c-jun activation. Thus, among the different JNK isoforms activated by sorbitol, only a subset of these isoforms might contribute to c-jun activation. Another explanation could be that, although sorbitol induces stronger JNK activation than TNFα, the level of JNK activation with either is sufficient to activate all of the cell's c-jun (saturation effect).

Time-dependent effects of TNFα and sorbitol on proteins phosphorylation

Through a time-course experiment, we showed that TNFα and sorbitol induce phosphorylation for up to 30 minutes but that this phosphorylation declines soon after. One could argue that this decrease in phosphorylation levels might be explained by increase in stability and activity of protein phosphatases (Brondello *et al.*, 1999). Alternatively, one could rationalize that this decrease in phosphorylation levels reflect protein targeted degradation after phosphorylation (Musti *et al.*, 1997;Pederson *et al.*, 2001;Israel, 2000). Our immunoblot analysis suggests a role for protein phosphatases as no decrease in total protein levels was noted. Interestingly, of the three phospho-acceptor sites in MKK7, only threonine 275 appears to be targeted by phosphatases as no decrease in serine 271 and serine 277 phosphorylation was observed. Specific threonine residue dephosphorylation by serine/threonine phosphatase has already been reported (Morita *et al.*, 2001). We recognize that even if our results suggest a role for protein phosphatases in the decrease phosphorylation of many proteins, our time-course experiment does not bring any evidence if the dephosphorylation of the different proteins is due to the same

group or different groups of phosphatases. Further investigations addressing these questions are required. Hopefully, these studies will identify the protein phosphatases involved in this process.

Through our time-course experiment, we also reveal that c-jun upregulation does not occur in sorbitol-treated cells. We believe that several reasons can explain this surprising result. As previously mentioned, c-jun heterodimerizes with ATF-2 to regulate its own transcription (Cruzalegui *et al.*, 1999). Thus, it is possible that sorbitol leads to c-jun phosphorylation on residues other than the ones known to activate it (serine 63 and serine 73) and that this phosphorylation result in a decrease affinity of c-jun for ATF2. On the other hand, it is possible that sorbitol increases expression of another transcription factor that selectively binds to c-jun and inhibits c-jun DNA binding activity or that sorbitol increases transcriptional repressors expression. C/EBPalpha has been shown to interact with c-Jun and prevents c-Jun from binding to DNA (Rangatia *et al.*, 2002). In addition, it can be hypothesized that sorbitol treatment increases histone deacetylase activity, which is known to silence gene expression (de Ruijter *et al.*, 2003). Finally, it has been demonstrated that the proteasomal-ubiquitin pathway regulates c-jun proteins degradation (Treier *et al.*, 1994;Jariel-Encontre *et al.*, 1997;Musti *et al.*, 1997). Thus, it is possible that exposure to sorbitol leads to upregulation and stabilisation of the proteins involved in the proteasomal degradation pathway and thus accelerates the rate of c-jun degradation. However, we recognize that stability of other proteins known to be regulated by the proteasomal degradation pathway should be assessed to verify this hypothesis.

MKK7 activation loop amino acids phosphorylation is due to upstream regulator.

Through our in vitro transcription/translation reactions and in vitro kinase assays, we showed that MKK7 activation loop amino acids phosphorylation is due to upstream regulator and not to MKK7 autophosphorylation. The fact that immunoreactive bands were observed for serine 271 and serine 277 in the mutant form of MKK7 that cannot autophosphorylate shows that constitutive phosphorylation of the MKK7 activation loop amino acids is the result of an upstream regulator. Moreover, the fact that immunoreactive bands were observed for these two residues when Wt-MKK7 was produced suggests that the upstream regulator was present in an active form in the in vitro transcription/translation mixture. In addition, the absence of threonine 275 phosphorylation when Wt-MKK7 was allowed to autophosphorylate, demonstrates that this amino acid is not phosphorylated by MKK7 itself. This result suggests that the upstream kinase that usually phosphorylates this residue is not present in the kinase reaction or that this upstream kinase was indeed present but in a non-activated form.

Closing Remarks

Among the MAPK pathways present in eukaryotic cells, the JNK signaling pathway is a major contributor in neuronal apoptosis. Thus, enhancing our knowledge on this pathway will ultimately lead to new therapies for neurodegenerative diseases. While the synergistic activation of JNK by MKK4 and MKK7 has gained acceptance, the molecular mechanisms by which upstream regulators are activated by phosphorylation remain unclear. With the discovery of protein scaffolds, we are beginning to understand the specificity but also the complexity of the JNK signaling pathway (Kelkar *et al.*, 2000;Yasuda *et al.*, 1999;Xu *et al.*, 2003).

The present investigations mainly sought to determine the mechanism by which MKK7 is activated by phosphorylation. To answer this question, we have generated three phospho-MKK7 polyclonal antibodies. Each of the polyclonal antibodies was raised against one of the potential regulatory sites in human MKK7 activation loop. Our results show that TNFα and sorbitol lead to phosphorylation of serine and threonine residues within MKK7 and MKK4 activation loop. Interestingly, we reveal constitutive phosphorylation of serines residues and stimuli-induced phosphorylation of threonine residues. Importantly, our results show that this phosphorylation mechanism occurs in vivo and that these phosphorylation events are the result of upstream MAPKKK regulator.

Our studies bring novel insights into MAPKKs regulation in HEK293T cells and contribute to the understanding of the JNK signaling pathway. Because of the complexity of this signaling pathway, many questions remain to be answered. Further studies are necessary to clarify the details of JNK signaling regulation and this could lead to the

development of new therapies to control JNK activation and cure neurodegenerative diseases.

Figure Legends

Figure 1. Alignment of MKKs activation loop from different species and phospho-MKK7 specific antibodies generation. A. MKKs activation loop sequences from different species were aligned and compared. B. Diagram showing the three synthetic phospho-peptides used to generate phospho-specific MKK7 polyclonal antibodies.

Figure 2. Activation of exogenous MKK7 and MKK4 by TNFα and Sorbitol.

A. HEK 293T cells were left either untransfected or transfected with 5 ug of expression plasmid encoding MKK7-wt or S3A. 48 hours post-transfection, cells were left untreated (0), treated with TNFα (50ng/ml) (T) or with Sorbitol (500mM) (S) for 15 minutes. Cells were lysed and immunoprecipitated with an antibody directed against Myc. Immunocomplexes were immunoblotted for pS271, pT275, pS277, p-MKK4, MKK4 and MKK7. Pre-IP lysates were immunoblotted for p-MKK4, MKK4, p-Jun, Jun and IκBα. B. HEK 293T cells were left either untransfected or transfected with 5 ug of expression plasmid encoding MKK4-wt and were treated and immunoblotted as in A. These experiments were performed twice and yielded identical results.

Figure 3. Dose-dependent and time-dependent effects of TNFα and sorbitol on proteins phosphorylation. A. HEK 293T cells were transfected with 5 ug of expression plasmid encoding MKK7-wt or MKK4-wt. 48 hours post-transfection, cells were left either untreated (0) or treated with TNFα (50ng/ml) or with Sorbitol (500mM) for the indicated times. Cells were lysed and immunoprecipitated with an antibody directed

against Myc. Immunocomplexes were immunoblotted for pS271, pT275, pS277, MKK7, p-MKK4 and MKK4. Pre-IP lysates were immunoblotted for p-JNK, JNK, p-Jun, Jun and IκBα. B. HEK 293T cells were transfected with 5 ug of expression plasmid encoding MKK7-wt or MKK4-wt. 48 hours post-transfection, cells were left either untreated (0) or treated with increasing amount of TNFα or Sorbitol as indicated for 15 minutes. Cells were lysed and immunoprecipitated with an antibody directed against Myc. Immunocomplexes were immunoblotted for pT275, MKK7, p-MKK4 and MKK4. Pre-IP lysates were immunoblotted for p-JNK, JNK, p-Jun, Jun and IκBα. These experiments were performed twice and yielded identical results.

Figure 4. Effects of individual amino acid replacement of phospho-acceptor site in MKK7 activation loop with alanine on the two other phosphorylation sites. HEK 293T cells were left either untransfected or transfected with 5 ug of expression plasmid encoding MKK7-wt or with 8 ug of expression plasmid encoding specific alanine mutants. 48 hours post-transfection, cells were left untreated (0) or treated with TNFα (50ng/ml) (T) or with Sorbitol (500mM) (S) for 15 minutes. Cells were lysed and immunoprecipitated with an antibody directed against Myc. Immunocomplexes were immunoblotted for pS271, pT275, pS277 and MKK7. Pre-IP lysates were immunoblotted for MKK7, p-MKK4, MKK4, p-JNK, JNK, pJun, Jun, pATF2, ATF2 and IκBα. These experiments were performed twice and yielded identical results.

Figure 5. Effects of individual amino acid replacement of phospho-acceptor site in MKK7 activation loop with aspartic acid on the two other phosphorylation sites. HEK 293T cells were left either untransfected or transfected with 5 ug of expression plasmid encoding MKK7-wt or with 8 ug of S3E mutant and expression plasmid encoding specific aspartic acid mutants. 48 hours post-transfection, cells were left untreated (0) or treated with TNFα (50ng/ml) (T) or with Sorbitol (500mM) (S) for 15 minutes. Cells were lysed and immunoprecipitated with an antibody directed against Myc. Immunocomplexes were immunoblotted for pS271, pT275, pS277 and MKK7. Pre-IP lysates were immunoblotted for MKK7, p-MKK4, MKK4, p-JNK, JNK, pJun, Jun, pATF2, ATF2 and IκBα. These experiments were performed twice and yielded identical results.

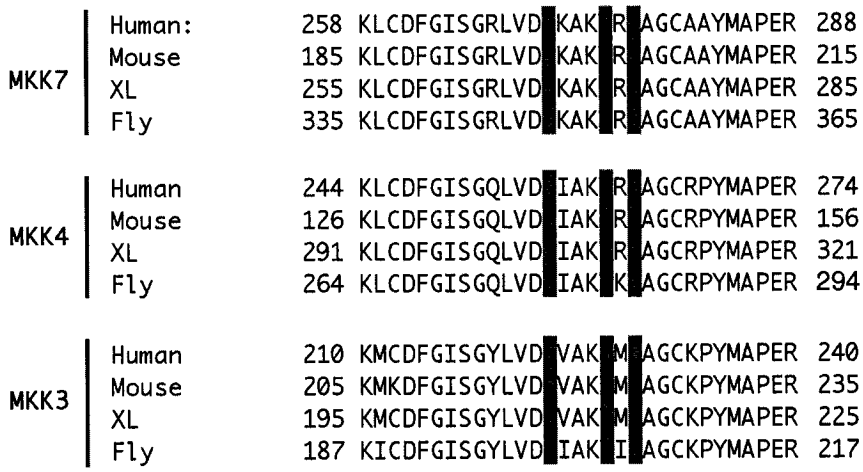
Figure 6. MKK7 phosphorylation is due to an upstream activator. In vitro transcription/translation experiment was performed using no plasmid (negative control), Myc-Mint (positive control), Myc-MKK7 (Wt) and Myc-MKK7-K149M (KD-MKK7). In vitro transcription/translation products were subjected to an in vitro kinase reaction and were immunoprecipitated using an antibody directed against Myc. Immunocomplexes were immunoblotted for pS271, pT275, pS277 and MKK7. These experiments were performed twice and yielded identical results.

Figure 7. Threonine 275 phosphorylation in vivo. HEK 293T cells were left untransfected or transfected with 20pmol of non-specific RNAi or with RNAi directed against MKK7. 48 hours post-transfection cells were left untreated or treated with Sorbitol (500mM) for 15 minutes. Cells were lysed and immunoblotted for pT275 and MKK7.

Figure 8. Serine 271 and serine 277 phosphorylation in vivo. HEK 293T cells, untreated or treated with Sorbitol (500mM) for 15 minutes, were lysed and immunoprecipitated with an antibody directed against MKK7. Immunocomplexes were immunoblotted for pS271, pT275, pS277 and MKK7. These experiments were performed twice and yielded identical results.

Figure 1

A



Human MKK7

B

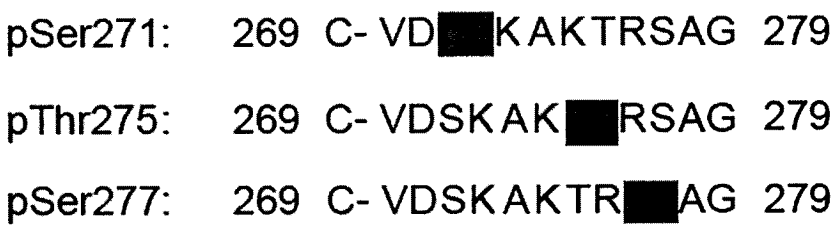
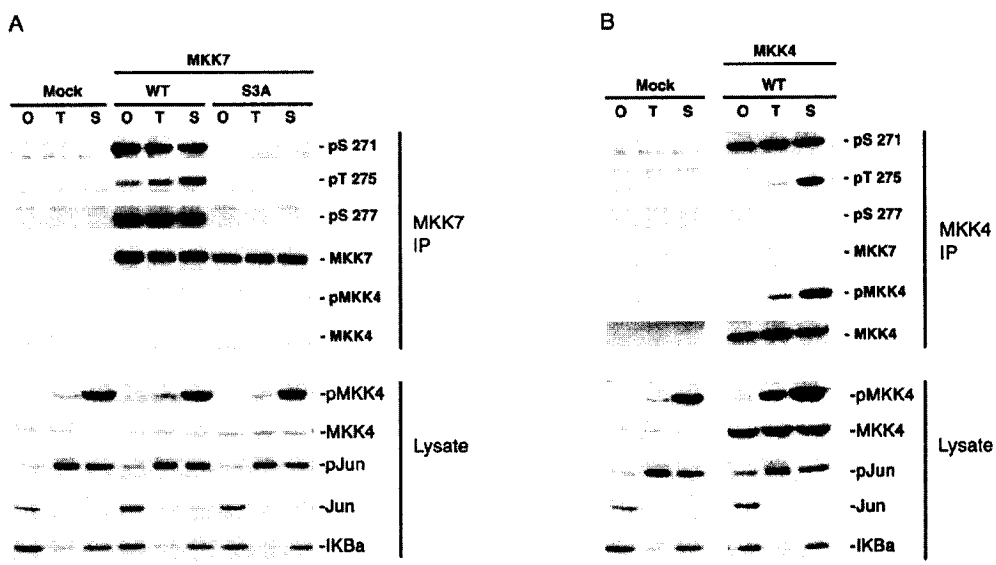


Figure 2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WT
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- pT 275
- pS 277
- MKK7
- pMKK4
- MKK4
MKK7 IP
-pMKK4
-MKK4
-pJun
-Jun
-IKBa
Lysate
B
MKK4
Mock
WT
O T S O T S
- pS 271
- pT 275
- pS 277
- MKK7
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- MKK4
MKK4 IP
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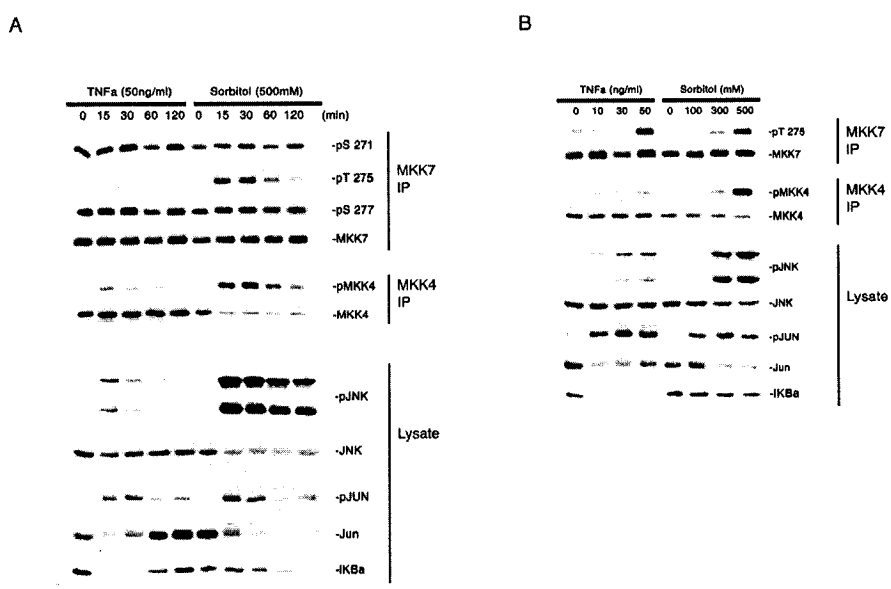
-MKK4
-pJun
-Jun
-IKBa
Lysate

Figure 3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Sorbitol (500mM)
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-pT 275
-pS 277
-MKK7
MKK7 IP
-pMKK4
-MKK4
MKK4 IP
-pJNK
-JNK
-pJUN
-Jun
-IKBa
Lysate
B
TNFa (ng/ml)
Sorbitol (mM)
0 10 30 50 0 100 300 500
-pT 275
-MKK7
MKK7 IP
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-MKK4
MKK4 IP
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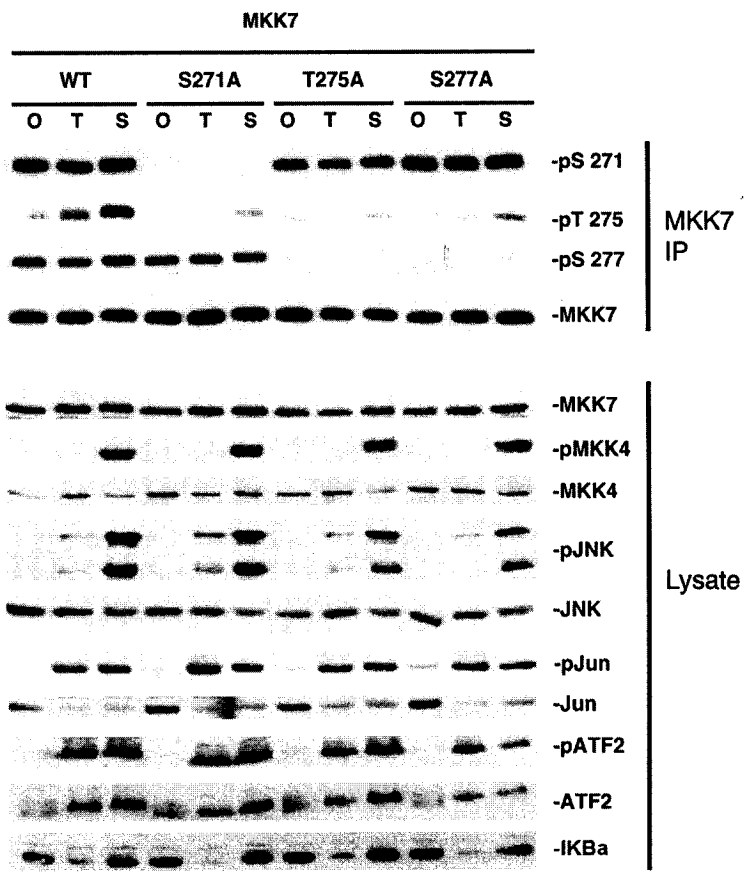
-JNK
-pJUN
-Jun
-IKBa
Lysate

Figure 4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S277A
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MKK7 IP
-MKK7
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-JNK
-pJun
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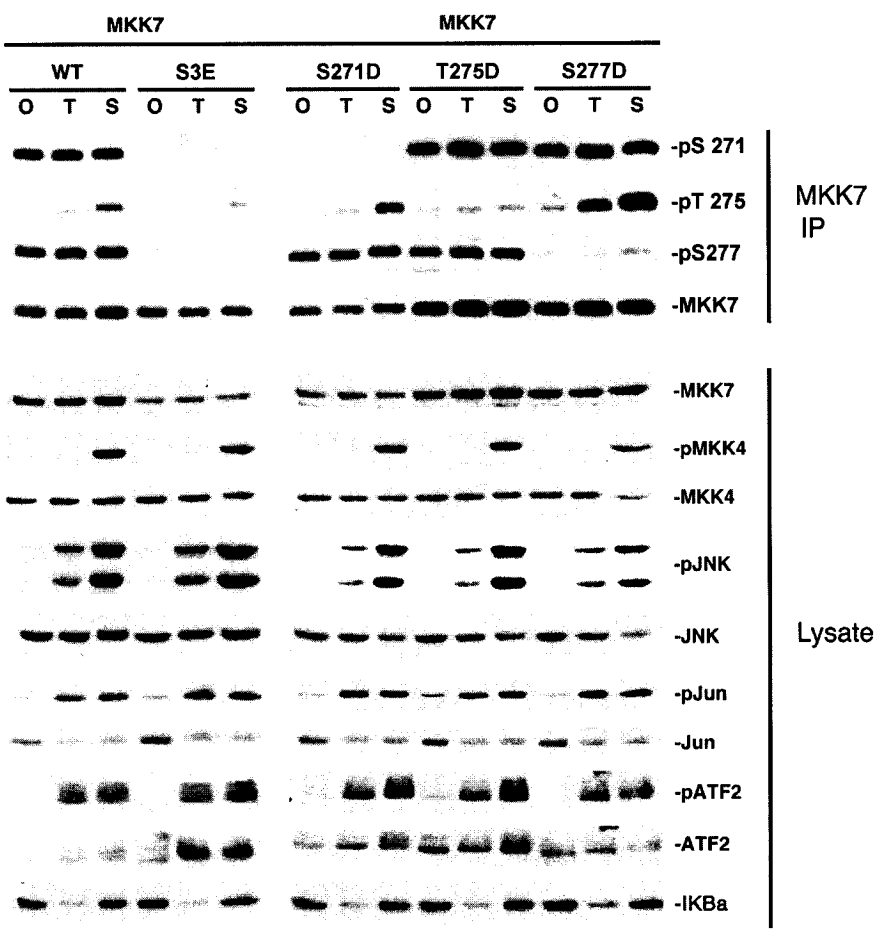
-pATF2
-ATF2
-IKBa
Lysate

Figure 5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WT
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MKK7 IP
-MKK7
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-JNK
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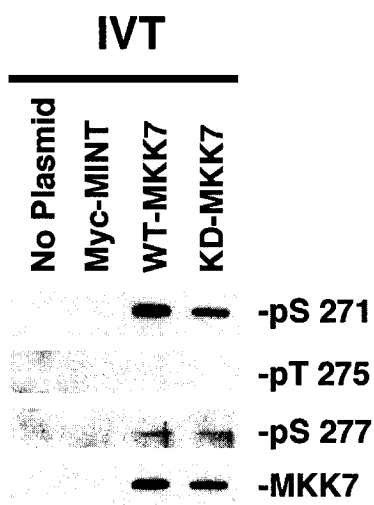
-Jun
-pATF2
-ATF2
-IKBa
Lysate

Figure 6


IVT
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WT-MKK7
KD-MKK7
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-pT 275
-pS 277
-MKK7

Figure 7

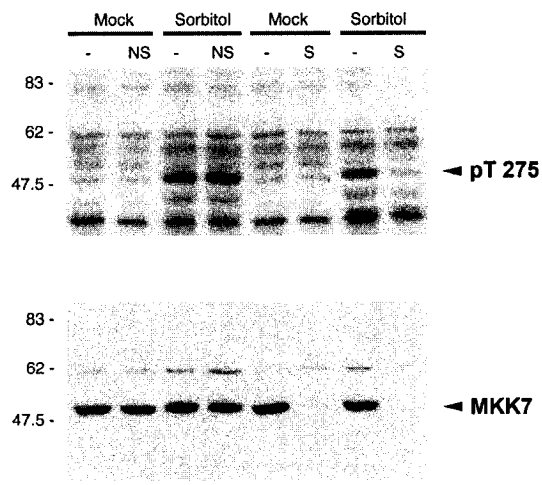
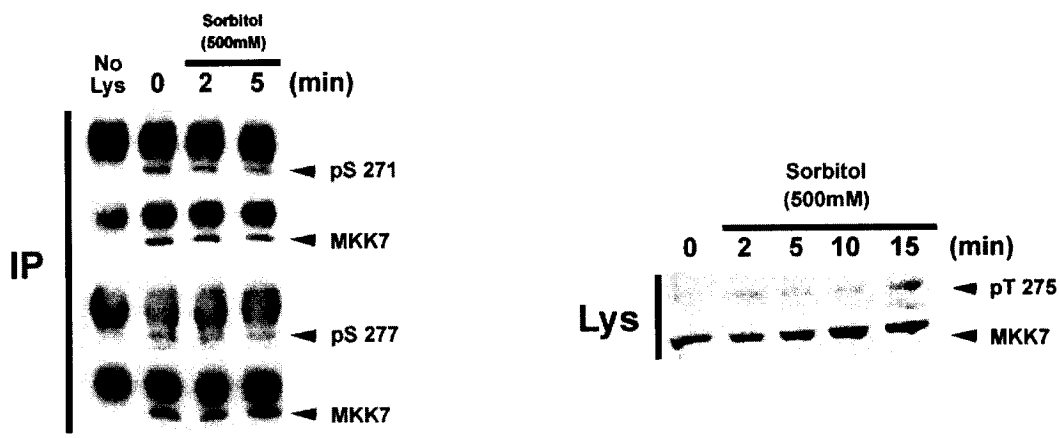

Mock
Sorbitol
Mock
Sorbitol
-
NS
-
NS
-
S
-
S
83 -
62 -
47.5 -
pT 275
83 -
62 -
47.5 -
MKK7

Figure 8


Sorbitol
(500mM)
No
Lys
0
2
5
(min)
IP
pS 271
MKK7
pS 277
MKK7
Sorbitol
(500mM)
0
2
5
10
15
(min)
Lys
pT 275
MKK7

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Appendix

Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

Internal Radioisotope User Permit

Issued by:

The Radiation Safety Committee of the
Montreal Neurological Institute and Hospital
Authorized by the Canadian Nuclear Safety Commission
CNSC Radioisotope Licence Number: 01187-2-08.3 (or any revision thereof)

1. **Radioisotope User Permit Number** : **MNI_002 (Rev.1)**
   Classification : Basic
   Date of Issue : May 1, 2003
   Revision Date : November 18, 2004
   Expiry Date : April 30, 2008

2. Name of Principal Investigator : Barker, Philip

3. **Department** : **CNS**

4. **Location(s) approved by this permit** : **MP038 & BT214 (shared) & BT218 (shared)**

5. **Radioisotopes approved by this permit** : See non-shaded cells in table below.

Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.

approved for use of:	^{3}H	^{14}C	^{32}P	^{35}S	^{125}I
10'000 exemption quantities (E. Q)	10 TBq or 270 Ci		100 MBq or 2.7 mCi	1 TBq or 27 Ci	10 GBq or 270 mCi
your possession limit is:	1 GBq or 27mCi		370 MBq or 10 mCi	370 MBq or 10 mCi	370 MBq or 10 mCi

6. **Personal Dosimeters Required** :YES (for users of P- 32)

7. **Method of Disposal** : All radioactive waste (solid and liquid) must be disposed of through the containers provided by the McIntyre Waste Management Facility (McGill) and brought to the central waste storage cage in the basement of the MNH/I (room 045 B).

8. Special Conditions:

Gloves and lab coats mandatory.
Weekly wipe tests required in areas where radioisotopes are used.
Ring badges required for staff using > 50MBq (~1.5mCi) of ^{32}P.
Monitoring of all work surfaces where ^{32}P is used at the end of the work day.
Radio-iodinations: Must be carried out in a working fume hood. Schedule thyroid monitoring. Use of proper survey equipment during radio-iodine manipulations.

The Radiation Safety Officer, MNH/I: ____ (E.Meyer, RSO, ext. 8927)

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

Protocol #: 4835
Investigator #: 324
Approval End Date: ~~Dec. 31, 2004~~
Facility Committee: MNI

SIGNALLING MECHANISMS OF THE p75 NEUROTROPHIN RECEPTOR

Title: Molecular Mechanisms of Neuronal Differentiation
(must match the title of the funding source application)

EXTENSION = MARCH 31, 2005

☒ New Application ☐ Renewal of Protocol # ____ ☐ Pilot Category *(see section 11)*: D

1. Investigator Data:

Principal Investigator: Dr. Phil A. Barker Phone #: 398-3064
Department: Centre for Neuronal Survival - Montreal Neurological Inst. Fax#: 398-5214
Address: 3801 University St., Montreal, Quebec, H3A 2B4 Email: phil.barker@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Phil Barker	Work #:	398-3064	Emergency #:	830-3243
Name:	Kathleen Dickson	Work #:	398-3212	Emergency #:	792-2125

3. Funding Source:

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status: ☐ Awarded ☒ Pending
Funding period: 2004-2009

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

For Office Use Only

ACTION	✓	DATE
COs	✓	11 97
OB	✓	Jan 26/04
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y):	Apr. 1/04	or ongoing ☐
Expected Date of Completion of Animal Use (d/m/y):	Mar. 31/05	or ongoing ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Date: 1/5/2004

Chair, Facility Animal Care Committee:		Date:
University Veterinarians:		Date: 1/22/04
Chair, Ethics Subcommittee *(as per UACC policy)*:		Date: 22 2004
Approved Animal Use	Beginning: Jan 1, 2004	Ending: Dec. 31, 2004

☒ This protocol has been approved with the modifications noted in Section 13.

EXTENSION = MARCH 31, 2005

November 2002

14 JAN. 2003

TOTAL P.02

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

; should be commenced without prior approval of an application to use biohazardous materials. Submit this application ir, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

ICIPAL INVESTIGATOR: **Dr. Phil A. Barker**

RESS: **Montreal Neurological Institute** TELEPHONE: **398-3064**

3801 University St. FAX NUMBER: **398-5214**

ARTMENT: **Centre for Neuronal Survival** E-MAIL: **phil.barker@mcgill.ca**

JECT TITLE: **Signaling Mechanisms of the p75 neurotrophin receptor**

NDING SOURCE: CIHR * NSERC NIH FQRNT FRSQ

INTERNAL OTHER (specify)

ant No.: **38942** Beginning date: **April 1, 2004** End date: **March 31, 2009**

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cate if this is

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

IFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies e applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety ines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

1): 1 2 3 4 Containment Level

DR. GALLUS J. ENIEDIS
Dept. of Microbiology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

al Investigator or course director: URE date: 11 (day) 12 (month) 03 (year)

erson, Biohazards Committee: date: 11-12-03 (day month year)

ved period: beginning 01 (day) - 04 (month) - 2004 (year) ending 31 (day) - 03 (month) - 2009 (year)

n the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

SEARCH PERSONNEL: (attach additional sheets if preferred)